# AN ECOLOGICAL STUDY ON SEED BORNE FUNGI AND MYCOTOXINS IN MAIZE (ZEA MAYS L.)

ABSTRACT

**Chrysanthemum Massar**

**THESIS**

Submitted in Fulfilment of the Requirement of the Degree of

**Doctor of Philosophy**

To

**DEPARTMENT OF BOTANY**
**SCHOOL OF LIFE SCIENCES**
NORTH - EASTERN HILL UNIVERSITY
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## ABSTRACT

Seasonal study on seed borne mycoflora of two local maize varieties, i.e. local white and local yellow was carried out using three techniques of isolations, viz. blotter method, agar plate method and dilution plate method. A total of 37 fungal species belonging to 14 genera were isolated from both the varieties. Agar plate method and blotter method harboured more number of fungal species than the dilution plate method. The number of fungal species was gradually decreased in winter months compared to wet summer and autumn months. The field fungi were replaced by the storage fungi during colder months during storage of the seeds. The variation in fungal species on two varieties was also observed. Local yellow variety yielded maximum number of fungi (33) than local white variety (22). Species of *Aspergillus*, *Fusarium* and *Penicillium* were dominant in both the varieties during the whole period of investigation. The percentage frequency of occurrence of *Alternaria* spp., *Chaetomium* spp., *Cladosporium* spp., *Drechlera* spp., *Helminthosporium sacchari*, *Mucor* spp., *Rhizopus* spp., *Trichoderma* spp. and *Verticillium albo-atrum* decreased along with the progress of storage time and were gradually replaced by the species of *Aspergillus*, *Fusarium* and *Penicillium*.

Two types of storage conditions (shaded and smoked) commonly practiced by the tribal people of this region were selected to study the fungal flora of maize seeds. None of the storage practices were completely safe from fungal contamination. The invasion on seeds by

*Fusarium* spp. was observed in both the storage conditions.

Under shaded storage condition, *Fusarium moniliforme* was recorded with highest frequency (13.3%) and *Aspergillus niger* and *Penicillium rubrum* with least frequency (0.8%) of seeds on local white variety by agar plate method. Under smoked storage condition, *Fusarium solani* was recorded at the highest frequency and *Rhizopus* sp. and *Penicillium rubrum* with lowest frequency. Seeds of local yellow variety also harboured *Fusarium* sp. with highest frequency under shaded condition of storage, whereas the lowest frequency was by *Penicillium brefeldianum* and *Trichoderma harzianum* as isolated by agar plate method. Similarly, in smoked condition, *Fusarium* sp. was recorded to have a high frequency of occurrence of 22.1% and 20% and the minimum frequency was recorded by *Penicillium brefeldianum* (1.2%) and *Cladosporium herbarum* (0.3%) by agar plate and blotter methods respectively.

Positive correlation was observed between the chitin content and the fungal population. Fungal infection of maize seeds was maximum (100%) during wet summer and autumn seasons and minimum (40%) during winter season. However, trend of chitin contents in maize seeds during wet summer and autumn seasons differed from each other, even though they had the same percentage of seed infection. Autumn season revealed higher chitin contents (328.2 µg/g in local white variety and 440.4 µg/g in local yellow variety) than wet summer season (246 µg/g in local white variety and 250.9 µg/g in local yellow variety).

Lowest chitin content (126 μg/g in local white variety and 128.5 μg/g in local yellow variety) in seeds was observed during winter season in both the maize varieties.

The percentage of seed infection and chitin contents were higher in local yellow variety than the local white variety in all seasons.

Toxigenic strains of *Aspergillus flavus, A. ochraceous, Fusarium moniliforme* and *Penicillium citrinum* were isolated from both the varieties of maize which contaminated the seeds at varying degree. All the seed samples were positive to BGYF test and aflatoxin was detected in the seed samples of both the varieties under natural conditions. Other mycotoxins like ochratoxins, zearalenone and citrinin were also detected. Aflatoxin $B_1$ was the most common mycotoxin produced by *A. flavus*. Its maximum production was 1.40 ppm in local yellow variety of maize during winter season. Local white variety of maize seeds contained aflatoxin $B_1$, $B_2$ and $G_1$, whereas local yellow variety had aflatoxin $G_2$ in addition to these aflatoxins.

In local white variety, out of 30 isolates of *A. flavus*, 6 isolates were toxigenic and yielded aflatoxin $B_1$ and $G_1$ during winter season. In wet summer season, out of 46 isolates, 22 were toxigenic and yielded aflatoxin $B_1$ and $G_1$ whereas in autumn season only 16 isolates out of 50 yielded aflatoxin $B_1$ and $B_2$. Maximum concentration of mycotoxins was obtained during wet summer season followed by autumn and winter season. Under storage conditions, aflatoxin $B_1$ was detected in higher quantity in shaded storage conditions than in

smoked conditions. The qualitative and quantitative variation between different mycotoxins on two varieties of maize seeds was observed in different seasons.

It was observed that temperature and moisture were the most important factors governing the growth of *A. flavus* on seeds of two maize varieties. Maximum production of aflatoxin was observed at 30°C and at a relative humidity of 95 to 100%. *A. flavus* did not invade any samples of corn stored below 15% moisture contents of seeds.

Significant variations in aflatoxins were observed between the physical factors i.e. moisture content, temperature and relative humidity on both the maize varieties.

The relative deterioration of seed quality after infecting by three dominant seed borne fungi., ;viz. *Aspergillus flavus, Fusarium moniliforme* and *Penicillium citrium* was determined in terms of the nutritive value of maize seeds, dry weight, sugars and protein contents. It was observed that the dry weight of the seeds was reduced due to fungal invasion in both the varieties. Insignificant variation in the loss of seed dry weight due to fungal infection was recorded in local white variety while *A. flavus* caused maximum loss in the seed dry weight in local yellow variety after 180 days of infection. Fungal infested seeds exhibited a depletion in total sugars and non-reducing sugars with corresponding increase in reducing sugars. A continuous and gradual depletion of protein contents of the seeds due to fungal infestation was noticed after 180 days of fungal

invasion.

Effects of aflatoxin $B_1$ on spore germination and mycelial growth of *A. flavus*, *F. moniliforme* and *P. citrinum* was studied at different temperatures and at different time interval of incubation period. The various concentrations of aflatoxins used in the present study were either stimulatory or inhibitory to spore germination and mycelial growth of different fungi. However, the stimulatory/inhibitory capability of aflatoxin $B_1$ varied with specific fungal species. Aflatoxin $B_1$ was stimulatory to the growth of *A. flavus*, whereas growth of *F. moniliforme* and *P. citrinum* was inhibited by aflatoxin $B_1$. The maximum inhibition was observed at 1 ppm concentration of aflatoxins $B_1$.

Aflatoxin $B_1$ has also caused inhibition in the seed germination of both the maize varieties. Maximum inhibition was recorded at 1ppm concentration. The seeds of local white variety were more affected than local yellow variety. Similar trend on inhibition of seed germination was observed with zearelenone. However, in case of citrinin, maximum inhibition of seed germination was exhibited by local white variety than local white one.

Reduction of seedling growth was observed in all the treated samples compared with untreated ones. Maximum inhibition of seedling growth was observed at 1 ppm concentrated aflatoxin $B_1$ treatment whereas the minimum was observed at 0.1 ppm concentration. The pattern of inhibition of seedling growth due to zearalenone and

citrinin was also similar.

Significant variation in the reduction of weight of animals was observed between pure aflatoxin $B_1$ fed animals and the crude extract fed ones. No liver tumor was observed in any test animals though some of them showed abnormally light color of liver. Loss of hairs from the skin of mice was observed due to aflatoxin $B_1$ contaminated feeding. In take of 60 ppb aflatoxin $B_1$ by some of the mice of 3 weeks old was lethal.

It was observed that extracts of different plant parts of *Centella asiatica* inhibited the growth of *A. flavus* by 51.16% and aflatoxin $B_1$ and *A. sativum* were also inhibitory to the growth of *A. flavus* and aflatoxin $B_1$ production. The least inhibitory effect was observed with the extracts of *Thuja orientalis*. The inhibition of fungal biomass and aflatoxin $B_1$ production followed a specific trend, *C. asiatica* > *A. cepa* > *A. sativum* > *T. orientalis*.

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# CERTIFICATE

I certify that the thesis entitles "An ecological study on seed borne fungi and mycotoxins in maize (*Zea mays* L.)" submitted by Mrs. Chrysanthemum Massar for the Degree of Doctor of Philosophy of the North Eastern Hill University, Shillong, embodies the record of original investigation carried out by her under my supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D Degree. This work has not been submitted for any Degree of any other University.

Signature of the supervisor

G. D. Sharma

( G. D. Sharma)

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# A C K N O W L E D G E M E N T S

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(C.Massar)

*To*

*my*

*Late Father*

# *General Introduction*

# GENERAL INTRODUCTION

Maize (*Zea mays* L.) is one of the most important cereal crops of the world. It is grown from a latitude 50°N which marks the northern limit of the growing areas in Europe and Asia to about 40°S in South America. It can widely fit in different conditions of temperature, moisture, length of frost-free season, and other environmental conditions. With its world average yield of 27-28 q/ha, maize ranks first among cereals and is followed by rice, wheat and millets. India's position is fifth in terms of total cultivated area and eleventh with regards to the production (Leonard and Martin, 1963).

The name maize is derived from the South American Indian Arawak name "mahiz". Maize is also known by the name "Indian Corn" and in America simply as "corn". In India, maize was introduced from America at the beginning of 17th century.

India is a major producer of maize in Asia and its greater part of geographical area is suitable for growing the crop. It is an important cereal which occupies the second place after paddy cultivation. Maize is used mainly as a staple human food, a feed for livestock and as a raw material for many industrial products. Both the area and production of maize in India have steadily increased during the past three decades. During 1985-1986, maize cultivation was done in 5.8 million hectares, with a production of 6.85 million tonnes. But in comparison to the world average yield, India's average

is too low, which is only 11.72 q/ha.

About 90% global food crops are propagated through seeds (Neergaard, 1977). Therefore, it is amply clear that the success of global food requirement for the increasing population greatly depends on the good quality of seed of high yielding and disease resistance varieties.

The increase in maize production in the country was the result of various factors, one of the most important being the creating and development of an organised seed industry to support agricultural development. Since then the seed industry has developed rapidly. In spite of this, only about 20% of the required seeds for cereals and pulses are produced and marketed through the organised public and private seed sectors. The balance comes from traditional sources such as the part of the produce saved by the farmers as seeds for sowing. Agricultural production can be improved if small and marginal farmers get quality seeds of good varieties and other vital input.

Seeds carry a wide range of microorganisms externally or internally which become active at the advent of favourable conditions and cause extensive damage to the seeds and seedlings. Amongst these microorganisms, fungi play a significant role in determining the quality and longevity of the seeds (Christensen and Lopez, 1963). Therefore, in order to improve the agricultural products, seed health testing and use of healthy seeds are of great importance.

The different types of maize diseases recorded in India are : seed-rot and seedling blight, foliar diseases, downy mildews, stalk rots and wilts, rusts, smuts and cob and ear rots. Other than these, moulds cause extensive damage to maize seeds during storage. The association of mycoflora with seeds in natural conditions is a common feature. These moulds may invade the seeds either in fields, storage and transit. Some of the important field fungi that invade the corn seeds belong to *hyphomycetes*, (Bilgrami *et al.*, 1980). Certain species of *Aspergillus* and *Penicillium* also infect the seeds during storage (Mislivec and Tuite, 1970). More than 150 species of fungi have been reported from cereal grains (Christensen and Kaufman, 1969), most of which have been isolated from surface sterilized seeds. A wide range of them are associated with corn kernels (Tuite, 1961; Dhanraj and Mathur, 1965; Hesseltine *et al.*, 1976; Siradhana *et al.*, 1978).

Payak *et al.* (1978) reported the seed rot and seedling diseases of corn caused by the species of *Fusarium*, *Rhizoctonia*, *Aspergillus*, *Pythium* and *Cephalosporium*. Fungal infected seeds showed meagre germination, pre-emergence seed rot, post emergence wilting and ultimately seedling collapse. Ear rot due to *Fusarium* sp. causes extensive damage to the crop.

The invasion of the various fungi to the seeds greatly depend on environmental factors like temperature, moisture content, relative humidity, pH of the substrate and damage of seeds by insects, birds,

rats and transit breakage (Bilgrami *et al.*, 1980; Qasem and Christensen, 1958; Lutey and Christensen, 1963; Nautiyal and Chatterjee, 1981; Singh, 1980). Besides, factors like harvesting and handling of equipments also have great influence on the growth of seed borne fungi (Sauer, 1986).

Seed borne fungi cause damage to the seeds in various ways. In case of severe infections the quality of seed gets deteriorated during storage, in field and at the time of harvest (Marsh and Payne, 1984; Hesseltine and Bothast, 1977). The degree of fungal invasion of corn tissue is mainly determined by analyzing the chitin as glucosamine (Donald and Mirocha, 1977; Hepper, 1977; Bethlenfalvay *et al.*, 1981).

Moulds produce several biologically active chemical compounds, majority of which are channelized to human and animal systems through various types of foods. Some of these contaminants are highly toxic (Anon., 1976; Moreau, 1969). The production of toxins in food commodities by moulds has been known since biblical times but their role in inciting disease syndrome was realized only when "ergotism" was caused by the consumption of barley and rye infected with *Claviceps purpurea* (Barger, 1931). These contaminants/chemicals are known as "mycotoxicoses". The word "mycotoxin" is derived from Greek "mykes" (fungus) and the Latin "toxicum" (poison) and is used to designate such secondary fungal metabolites which cause pathological abnormalities in man and warm blooded animals (Uraguchi and Yamazaki, 1978). "Of the innumerable diseases that affect man and domestic

animals, the mycotoxicoses are perhaps the most unfamiliar and least investigated." The dictum, "The Neglected Disease" was born but the situation changed drastically in 1960, when a disease of unknown etiology, termed as "Turkey X Disease" appeared in England (Blount, 1961). Investigations into this disease resulted to the discovery of aflatoxins as the causative agents.

The capability of fungi to produce mycotoxin is restricted to few species. Most of these are the members of *Aspergillus* group. *A. flavus* and *A. parasiticus* are the main aflatoxin producers. All the strains of *A. flavus* do not produce aflatoxins.

Sterigmatocystin has been characterized as a metabolite of *A. versicolor*. Van der Watt and Purchase (1970) have included sterigmatocystin as a carcinogen in the list of mycotoxins causing a high incidence of hepatomas in the Bantus of South Africa.

Ochratoxins have been isolated from *A. ochraceous* which invaded stored wheat (Christensen, 1962) and red and black pepper (Christensen *et al.*, 1967). The fungus has also been isolated from cereals and legume products (Scott, 1965). Van Walbeek et *al.* (1969) have shown that ochratoxins can also be produced by *Penicillium viridicatum*.

The mycotoxin, patulin produced by *P. urticae* reduced seed germination and seedling growth of wheat (Norstadt and McCalla, 1969). It has also been demonstrated to be a carcinogen (Dickens and Jones, 1965).

Yellow colored rice imported by Japan from Thailand in 1951 was

contaminated with *P. citrinum* which produced citrinin. Subsequent studies revealed that the fungus was distributed over the rice producing areas in the world, as it was detected in the rice imported to Japan from Burma, Italy, Egypt, United States of America, and Red China (Tsunoda, 1953a, b; Hirayama, 1954).

Zearalenone, or F-2 toxin is produced by *Fusarium graminearum*. The estrogenic lactone was assayed from naturally infected corn ears in Indiana. It caused estrogenic syndrome in swine (Caldwell and Tuite, 1974).

Studies on seed mycoflora have revealed that the mycotoxicoses are not restricted to any geographical or climatic region. Cereal grains, peanuts and cotton seeds appear to be the most important food and feed substances that may be contaminated with mycotoxins. However, probably no edible substances can be regarded as absolutely safe from possible mycotoxin contamination. Most of the cereal and other food stuffs contains moulds, which may appear harmless but some of them can be pathogenic or toxigenic and may be the causal agent of severe diseases.

Mycotoxin production can occur in the field *(Bilgrami et al.*, 1981), during pre and post harvests, storage and shipment. Factors governing mould growth and production of various mycotoxins are not completely understood although moisture, temperature and humidity probably play an important role (Lillehoj *et al.*, 1973), but $CO_2$, $O_2$, seed quality and fungal community can also lead to mycotoxin

contamination in foods and feeds (Hesseltine, 1976).

Bilgrami *et al.*, (1980) recorded changes in the qualitative and quantitative pattern of amino acids, organic acids, sugars, starch, protein, nitrogen and caloric value of maize seeds due to *Aspergillus parasiticus* infestation. Recent studies also revealed the deterioration of seeds by storage fungi (Kumari and Karan, 1981; Vijayalakshmi and Rao, 1985; Maheshwari and Mathur, 1985; Dube *et al.*, 1988).

A number of fungal species especially with reference to seed borne fungi were found to secrete exotoxins causing reduction in seed germination (Dwivedi and Singh, 1973; Tripathi, 1974). The culture filtrates of different seed fungi were known to produce either stimulatory or inhibitory effect on seed germination (Vidhyasekaran *et al.*, 1970; Mridha and Anwar, 1980; Singh and Gupta, 1984). Reduction in seed germination due to seed borne fungi had been attributed to the secretion of phytotoxins (Anwar and Mridha, 1987).

Mycotoxicoses continue to be a major health hazard to animals due to fungal contaminated foodstuffs. Pathological changes have been noted in the kidney, heart and liver in birds by the consumption of *A. flavus* mixed foodstuff (Baruah *et al.*, 1980; Bilgrami *et al.*, 1986).

The problem associated with mycotoxins is complex and requires to develop a sensitive method for screening foodstuffs for toxins. Some work on the control of mycotoxins in cereals has been carried out

in laboratory condition (Hesseltine, 1973; Bilgrami *et al.*, 1980; Sharma *et al.*, 1981) but detailed studies are lacking in field condition.

The agroclimatic conditions of the north eastern region are suitable for maize cultivation and about 94.2 thousand hectares of land is under maize cultivation. The sowing and harvesting periods of maize (April-August) coincides with rainy season. The region being a hilly one, three types of agricultural systems, i.e. shifting agriculture (jhum), valley land and terrace land are practiced for maize cultivation. Shifting agriculture is extensively used by the tribal people of the region.

In Meghalaya, maize is grown on hill slopes at higher altitude (Plate-1.A) and valleys at lower altitude (Plate-1.B). Two local varieties of maize, the white and yellow varieties (Plate-3.A) are mainly grown by the tribals in this state for human and animal consumption. Primitive practices for storage of corn seeds are prevailed in this region. The socio-economic conditions of the inhabitants of the region do not allow them to store their seeds in modern storage conditions. After harvesting, the maize cobs are either stored with husk or without husk in open (Verandah) (Plate 2.A) or in smoked conditions (Top of chimney place) (Plate 2.B). The stored seeds are consumed by human beings, domesticated animals and are sown for the next cultivation season. Tribal people are not aware of any seed infection and toxin contamination. They consume both infected

**PLATE** – 1.A. Maize cultivation at higher altitudes.
**PLATE** – 1.B. Maize cultivation at lower altitudes.

1. A

1. B

PLATE - 2.A. Maize seeds in shaded storage condition.
PLATE - 2.B. Maize seeds in smoked storage condition.

2. A

2. B

and uninfected seeds by themselves and also feed the same to their domestic animals. Therefore, systematic survey of seed borne fungi and mycotoxins production of maize and their consequent effects on seed health and animal consumption under different ecological conditions with special reference to Meghalaya is of urgent necessity.

The present investigation was, therefore, aimed to study the mycotoxin production in maize seeds by seed borne fungi taking the following experimental aspects :

- Population of fungi and their biomass on the seeds under storage and in field conditions.
- Elaboration of mycotoxins by toxigenic strains of seed borne fungi under storage and field conditions.
- Effect of certain physical factors (temperature, moisture and humidity) on the growth and production of mycotoxin viz. aflatoxin in seed borne fungi.
- Effect of seed borne fungi on quality of maize seeds (in terms of dry weight, sugar and protein).
- Effect of aflatoxin $B_1$ on growth of seed borne fungi, corn seed germination and seedling growth.
- Effect of aflatoxin $B_1$ on test animal (Albino mice).
- Control of mycotoxin (Aflatoxin $B_1$) production by plant extracts.

# *Review of Literature*

# REVIEW OF LITERATURE

Prior to the understanding of the seed borne diseases, the agriculturists were unaware of the causal agents, mode of infection and the magnitude of loss of seeds by the microorganisms. Later on with the advent of scientific knowledge and the invention of modern equipments to study the plant diseases, this field of seed biology has attracted the attention of mycologists and plant pathologists.

Micheli (1729) was the first to collect spores of fungi and described many new genera and illustrated their reproductive structures. Kuhn (1858) published the first authenticative work on diseases of cultivated crops.

The importance of seeds in meeting the world food supply cannot be ignored. About 90% of food crops are mainly propagated by seeds. Seed health is necessary for assuring the better seed germination and growth of healthy seedlings. Seeds are also important carrier of certain fungal pathogens producing mycotoxins which adversely affect the health of those who consume them. In recent past, much emphasis has been laid down on the control of mycotoxin producing fungi associated with various seeds under different environmental conditions.

Seeds also play an important role in carrying many important pathogens from one country to another. An account of the economically important pathogens intercepted on imported seeds and seedling materials is available (Nath and Lambat, 1971). Fungal transmission

on seed is accomplished normally in three ways such as - pathogen mixed with seeds, pathogens externally present on seed and pathogens present internally in the seed (Khare and Sinha, 1983).

Seed borne fungi may affect the grains either as saprophytic or as parasitic ones. Their association determines the seed viability, nutritional value and colouration and production of mycotoxins toxic to man and animals (Christensen and Kernhemp, 1936; Bottomley, 1950; Tsunoda, 1952; Semeniuk, 1954; Joffe, 1960).

Christensen and Kaufmann( 1965) reported common saprophytic field fungi, *Alternaria tenuis*, *Cladosporium herbarum*, *Curvularia* spp., *Epicoccum purpurescens*, *Fusarium* spp. and pathogenic ones as *F. moniliforme* and *Verticillum albo-atrum* (Malone and Muskett, 1964). The pathogenic fungi were found to reduce seed germination and caused diseases of the seedlings (Noble and Richardson, 1968).

It was observed that most of the storage fungi usually do not attack the seed before harvest (Christensen, 1971). They may be present on the seed in low percentage and thus provide the inoculum for storage fungi (Qasem and Christensen, 1958).

The water content of the seed plays an important role on the growth of fungi during storage condition. Christensen and Lopez (1963) had determined the low limits of moisture content which permitted the growth of fungi on stored cereals such as wheat, barley and maize.

Clarke *et al.* (1967, 1969) had recognised various phases in succession of mycoflora in stored seeds. In the first phase, the predominant fungi were *Alternaria*, *Cladosporium* and *Fusarium*. These fungi decrease during the second phase and are replaced in the third phase either by yeast or by *Penicillium* sp. In the fourth phase, typical storage fungi occur, and by spontaneous heating of the grain, the growth of the fungi of the first phase is promoted such as *Absidia* spp., *Mucor* spp.. Certain temperatures like 30°C - 35°C favoured the growth of *Aspergillus fumigatus* and 40°C was favourable for *Humicola lenuginosa* (Mulinge and Chesters, 1970).

Humidity and temperature of the storage practice also have great influence on the invasion of fungi on seeds (Lopez and Christensen, 1967; Mehan and Chohan, 1974).

Ecology of Seed mycoflora :

The invasion of seeds by fungi is mainly by spore producing structures like sclerotia, acervuli, pycnidia, perithecia and mycelia outside or inside the seed. The amount of inoculum needed to cause damage on the seed varies with the kind of crop and the pathogen involved (Suryanarayana, 1978). The association of fungi with the seeds may be divided into 3 categories : (1) Infested - when the pathogens are on the surface of the seeds; (2) Infected - when the pathogens lie within the seed tissues and (3) Concomitant - contamination - when seed material is mixed with bits of infected crop tissues. Therefore, practically all parts of seeds are found to

contain various types of microorganisms. Seeds which ever look healthy may contain rich fungal flora (Padmanabhan, 1949).

The association of fungi with various seeds has been reported by many workers from all over the world (Christensen and Gordon, 1948; Christensen, 1951; Ibrahim and Farag, 1965; Fause and Christensen, 1966; Flanigan, 1969, 1970; EL Shafie and Webster, 1981; Gill *et al.*, 1983; Ahmad *et al.*, 1985; Mazen *et al.*, 1990).

Noble and Richardson (1968) have given a comprehensive list of fungi associated with different seeds. Much of the work of seed mycoflora has been made on the cereals and more than 150 fungal species have been listed from them (Christensen and Kaufmann, 1969). Reports on seed mycoflora of various crops in India have also been made (Kadian and Suryanarayan, 1971; Khare *et al.*, 1972; Singh and Chohan, 1973, 1974; Sarma *et al.*, 1980; Joshi and Gupta, 1980; Srivastava and Gupta, 1981, 1987; Bhale and Khare, 1982; Kanapathipillai, 1982; Prasad *et al.*, 1984, 1987; Singh and Singh, 1985; Kunwar, 1989; Jayaweera *et al.*, 1988; Singh, 1991).

The occurrence of fungi on seeds depends on survivorship of fungal propagules under extreme dry conditions and on the carriers. Ecologically, two distinct groups of fungi, i.e. hydrophillic and xerophyllic ones have been recognised. Neergaard (1975) has compiled a list of hosts and pathogens under International comparative Seed Health Testing Schemes and presented it in 14th International Workshop on Seed pathology.

Seed mycoflora of maize

Podhradszky (1950) reported heavy losses of maize seeds due to *Fusarium moniliforme*. Certain species of *Penicillium* have been found to be associated with both freshly harvested and stored corn kernels (Mislivec and Tuite, 1970).

Different methods were used to study the mycoflora of maize grains during different storage practices (Cahagnier and Poison, 1974). They found that none of the storage methods prevented the development of microorganisms. It all depends on the type of storage chosen that can determine the composition of mycoflora. Aulakh *et al.* (1976) reported that *Aspergillus flavus*, *A. niger*, *F. moniliforme* and *Penicillium* spp. were responsible for causing seed-rot and seedling infections on maize. Certain species of *Alternaria* , *Aspergillus*, *Drechlera*, *Fusarium*, *Nigrospora*, *Penicillium*, *Rhizopus* and *Trichothecium* were dominant on corn from Rajasthan (Bhat and Krishnamachary, 1977). The occurrence of fungi varied from sample to sample of rice seeds (Vaidehi and Rama Rao, 1978). They found that *Aspergillus* and *Penicillium* were the most dominant genera of seed fungi.

Seed borne fungi have been classified by Pelhate (1981) as field fungi (*Fusarium* spp. and *Epicoccum purpurescens*), intermediate fungi (*Cladosporium* spp. *Mucor* spp. and *Trichoderma* spp.) and storage fungi (*Penicillium* spp.) which has been confirmed by various reports on seed mycoflora of maize from other parts of the world (Ilag, 1973; Kanauja,

1974; Singh and Ji, 1980; Christensen 1980; Kim *et al.*, 1984).

*A. flavus* group has commonly been isolated from maize seeds (Zad and Ale-Agha, 1985). Bedi and Jhooty (1986) found different degree of resistance by different cultivars of maize to *A. flavus* grown in Punjab. Mclean and Berjak (1987) indicated that the distinction between field and storage fungi may not be clear-cut, as the latter may also gain access to the seed prior to harvest. The succession of fungal species during storage of seed was dependent on the primary colonizing propagules at the time of harvesting. Inter and intra specific interaction and competition, in addition to seed moisture content was a significant factor in determining the progressive succession of mycoflora (Lillehoj and Zuber, 1988).

Wicklow (1988) studied patterns of fungal association within maize kernels and isolated 11 fungi from surface-disinfected maize grains. *F. moniliforme* was dominant fungi followed by *A flavus* and *A niger*. Succession of microflora on maize grain has also been investigated by Banerjee *et al.* (1988). They found that bacteria and yeast were dominant during the early stages of grain development while filamentous fungi such as *Cladosporium* spp., *Aspergillus* spp., *F. moniliforme*, *Penicillium* spp., and *Trichothecium roseum* dominated the matured grain.

Fungal Biomass in Seed tissue:

Chitin is a constituent of the cell walls of the fungi and can be used as a measure of their growth. Measurement of chitin is an aid

in plant tissue, where other techniques give poor information on the growth of fungi. Golubchuk *et al.* (1960) have correlated the chitin content in 5 varieties of wheat with varying degrees of fungus invasion to the deterioration of wheat during storage conditions. However, they concluded that the analytical procedure needed improvement. Arima and Uozumi (1967) successfully used chitin analysis as a measure of mycelial weight in koji using the method of Ride and Drysdale (1971, 1972). They have suggested that the alkaline method of hydrolysis was better than acid ones resulting in the formation of chitosan. Donald and Mirocha (1977) have shown that chitin analysis was relatively a rapid method to measure fungus invasion of seed.

Carballas and Acea (1988) have also correlated the microbial population significantly with biomass as determined by fumigation method. They attributed the metabolic activity of the biomass to quality and quantity of organic matter which, nevertheless, controlled microbial population size. However, studies on the measurement of fungal biomass in seed tissue has not yet gained the momentum. Therefore, it is required to modify the method available to measure the quantitative changes in fungi, which could latter be related to their metabolic activity.

Mycotoxins in seeds :

Mycotoxins, the toxic metabolites of fungal origin, have been in existence since the earliest of times. An outbreak of "Turkey - X"

disease occurred in England in 1960, due to an aflatoxin contaminated peanut meal used in the feed. This resulted in the loss of millions of young turkey poults (Asplin and Carnaghan, 1961).

Cereal grains are the most important feed and food source that occasionally are contaminated with mycotoxins (Hesseltine, 1974). They are produced on cereals both in the field and in storage conditions. Mycotoxins include aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ as well as aflatoxins $M_1$ and $M_2$, ochratoxins A and B, penicillic acid, patulin, ergot, zearalenone, citrinin, T-2, tenuazonic acid, kojic acid and sterigmatocystin. Of these mycotoxins, aflatoxins, patulin, penicillic acid and sterigmatocystin are carcinogens.

Some studies on the production of mycotoxins in grains in India have been carried out (Gangopadhyay and Chakroborti, 1981; Devi and Polasa, 1982; Bilgrami *et al.*, 1981). Reddy (1983) reported that the occurrence of mycotoxin in maize was (63.33%) followed by Sorghum (60.00%), broken rice (56.25%), ragi (40.54%) and parboiled rice (33.33%). Aflatoxin $B_1$ was reported from all the commodities tested except the parboiled rice. T-2 toxin and ochratoxin-A were noticed in sorghum and broken rice. Citrinin could be isolated only from the parboiled rice.

*A. terreus* produced patulin and terreic acid, and *A. ochraceous*, produced ochratoxin (Surekha and Reddy, 1987). *A. flavus* produced aflatoxins, cyclopiazonic acid and sterigmatocystin and *A. parasiticus* only aflatoxins (Pandey *et al.*, 1989).

Singh (1984) was of the opinion that dry fruits and spices were good sources of mycotoxin development. Aflatoxins, ochratoxin, citrinin and zearalenone were elaborated on various dry fruits.

Zearalenone and aflatoxins have been detected in maize seeds damaged by *Heliothis* sp. of insects and later on infected by the fungi (Blaney *et al.*, 1986). Infected ears of corn have been found to contain mycotoxins which were identified as zearalenone, deoxynivalenol (Vomitoxin) and 15-acetyldeoxynivalenol (Abbas *et al.*, 1988).

Sargeant *et al.*, (1961) for the first time reported aflatoxin production by *A. flavus* grown on sterile peanuts and in Czapek's solutions. Mycotoxins are chemically unrelated fungal metabolites characterised by their ability to induce a variety of toxic responses in man and animals when food or feed containing them is consumed. Of these mycotoxins, only aflatoxins have attracted great attention all over the world (Wogan, 1965).

Hesseltine *et al.*, (1966) have reported that the agricultural commodities; sorghum, peanuts, corn, wheat and rice not only supported growth of *A. flavus* strains, but also produced aflatoxin. They discovered that aflatoxin was produced in these commodities after their long period of storage, under conditions conducive to mold growth. Very little of the toxin was isolated in soybeans because they are poor substrate, even though they support the growth of *A. flavus*. Later, Hesseltine (1967) discovered that the source of the

toxin in the peanut meal was the fungus, *A. flavus* Link ex Fries and he named this toxin as aflatoxin. The toxin is now known to be composed of several closely related compounds. The genus *Aspergillus* is divided into a number of sections, composed of closely related species and *Aspergillus flavus - oryzae* section constitutes one of these. Its members are characterised by a greenish yellow spore color and fail to produce ascospores.

After the coining of the term aflatoxin, various workers have reported aflatoxin production by various isolates of *A. flavus* (Diener and Davis, 1969; Maggon *et al.*, 1969; Detroy *et al.*, 1971; Mehan and Chohan, 1973; Lillehoj *et al.*, 1973; Shotwell, 1977).

Ears of yellow and white dent corn inoculated with *A. flavus* and *A. parasiticus* were found to contain aflatoxin (Rambo *et al.*, 1974). Production of aflatoxin by these cultures exhibited through the conidia stored in the laboratory condition (Boller and Schroeder, 1974). Hesseltine *et al* (1974) have reported that the growth of *A. flavus* was closely paralleled with the aflatoxin contents in the corn seeds. Presence of aflatoxins was also confirmed by the presence of trifluoroacetic acid (Stack and Pohland, 1975).

Rambo *et al.* (1975) observed bright greenish yellow (BGYF) and blue white (BWF) fluorescences in corn seeds infected with *A. flavus* and *A. parasiticus*. The incidence of these fluorescences varied with each isolate in relation to storage conditions. It was noticed that the damage of corn ear caused by insects like corn borers, earworms

and mites, stimulated the growth of *A. flavus* (Fennell *et al.*, 1975; Lillehoj *et al.*, 1976a , b).

Prakash and Siradhana (1978) have reported the production of aflatoxin $B_1$ by *A. flavus* on grains of Ganga - 5 maize hybrid.

Bilgrami *et al.*, (1980) reported aflatoxin production by few isolates of *A. flavus* in standing maize crops. They grouped them into three categories, viz., high, moderate and low toxin producers.

A survey carried out in Tamil Nadu on the production of aflatoxins in feed has revealed that most of foods and feeds harboured the mycotoxin producing fungi (Neelakantan *et al.*, 1981).

Screening of different varieties of maize for the production of aflatoxins by *A. parasiticus* has been done (Bilgrami *et al.*, 1982; Misra and Sinha, 1982). Aflatoxins $B_1$ was detected in four varieties of sorghum during the kharif season (Bhadraiah and Ramarao, 1984). The toxin has also been reported in coconut products from Andhra Pradesh (Kumari *et al.*, 1984). Mall and Chauhan (1984); Mehan and McDonald (1984) have also analysed aflatoxins from the kernels of groundnut infected by *A. flavus*.

Singh and Bedi (1985) studied the toxin producing potential of the most commonly grown varieties of bread wheat and duram wheat. They concluded that none of these varieties were immune to aflatoxin production. Similar reports on the elaboration of aflatoxin in a wide range of food commodities are available (Shotwell and Hesseltine, 1983; Lillehoj *et al.*, 1983; Jeswal, 1986; Wallin, 1986).

Castor *et al.*, (1987) found that harvest of maize in January appeared to yield more aflatoxin-free grains than those harvested in July or October. Production of aflatoxin was also reported from the cotton seeds in Arizona (Lee *et al.*, 1987). They observed that carpel walls of green balls after flowering were punctured to stimulate damage of sucking insects and *A. flavus* was dusted on wound sites during insect infestation process. They suggested that control of insects can lower the aflatoxin potential. Reduction of aflatoxin contamination in some crops has been observed in the plants resistant to the insect feeding (McMillian *et al.*, 1987).

Aflatoxin $B_1$ was detected in 7 out of 17 isolates growing on stored paddy seeds in three different storehouses in Tamil Nadu (Sundaram *et al.*, 1988). Aflatoxin contamination would be limited by harvesting maize as early as possible and any comparison of lines for resistance of aflatoxin accumulation should be done during the linear phase of accumulation (Payne *et al* ., 1988).

Production of aflatoxins on maize in Brazil has also been reported (Sabino *et al.*, 1989). Aflatoxin $B_1$ was detected from 10-900 $\mu$g/kg. Presence of mycotoxins in selected staple foods and feeds in India has been studied (Ramakrishna *et al.*, 1990). Aflatoxins were observed in 45 samples of corn, 4 samples of sorghum and 12 samples of mixed animal feeds with their varying concentrations.

Dwivedi *et al.*, (1990) have also observed the elaboration of aflatoxin in millets by different fungi during storage of the seeds.

Mycotoxin production on different cultivars and lines of broad bean (*Vicia faba* L.) seeds has been studied (El-Kady *et al.* 1991). Aflatoxin contamination in oil cakes has been reported (Kolhe *et al.*, 1993).

Cole *et al.* (1973). isolated a toxin, moniliformin from *F. moniliforme* from southern leaf blight-damaged corn seed. *F. moniliforme* var. *subglutinans* and *F. graminearum* are also known to produce moniliformin (Marasas *et al.*, 1979).

Zearalenone produced by *F. graminearum*, was assayed from infected corn seeds which caused estrogenic syndrome in swine (Caldwell and Tuite, 1974). *F. culmorum* (*F. roseum* 'culmorum') and *F. equiseti* (*F. roseum* 'gibbosum') produced zearalenol in a diastereomeric mixture of alpha and beta isomers (Bottalico *et al.*, 1984). Vinas *et al.* (1985) found that production of zearalenone was rare in nature. Production of zearalenone and trichothecene by some *Fusarium* species associated with Canadian grains has been reported (Neish *et al.*, 1982). Other mycotoxins like deoxynivalenol and 15-acetyldeoxynivalenol produced by this fungus have also been reported (Miller *et al.*, 1983; Neish *et al.*, 1983).

Drysidale (1984) isolated lycomarasmin, fusaric acid, zearalenone, enniatins and naphthazarins from the cultures of different species of *Fusarium*. The fusarial toxins were found associated with maize stalk rot (Logrieco and Bottalico, 1986) which were translocated from stalks to cobs, but not to kernels of uninfected ears.

Deoxynivalenol, diacetoxyscirpenol, HT-2 and T-2 toxin have been reported from wheat infected with *Fusarium* species (Abramson *et al.*, 1987). A number of mycotoxins produced by *Fusarium* species have been reported (Blaney and Dodman, 1988; Mirocha *et al.*, 1989; Hussein *et al.*, 1989).

Chemotaxonomic features have been used to identify different species of the genus *Fusarium*. Isolates of Liseola section produced moniliformin while Discolor section produced zearalenones (Zearalenone and Zearalenols) and trichothecenes type A. The chemotaxonomy was also useful for morphotaxonomic interpretation (Altomare *et al.*, 1989).

Baath *et al.*, (1990) studied the *Fusarium* infestation of silage maize plants. The growth of fusarial mycelium was better between pericarp and aleurone layers and on the scutellum. It also colonized plumule and radicle of endosperm and embryo (Chelkowski *et al.*, 1990).

Perkowski *et al.*, (1990) reported deoxynivalenol and 3-acetyldeoxynivalenol mycotoxins in cereal grains.

Yellowish colored rice imported by Japan from Thailand in 1951 was contaminated with *Penicillium citrinum*. Subsequent studies revealed that this fungus was widely occurring in paddy producing areas of Burma, Italy, Egypt, the United States, and in Red China (Tsunoda, 1953a, b; Hirayama, 1954). The fungus usually grew in stored rice, especially in polished ones and caused a yellowish color on its surface. The colored portions produced fluorescence under UV

radiation (Tsunoda, 1954).

Clinical tests show that citrinin increases urinary output when given orally to rats. Inulin and p-aminohippuric acid tests indicated that renal water reabsorption was inhibited by the citrinin pigment. Histological examination of the kidney of rats revealed turbid swelling of the tubules (Sakai, 1955). The mycotoxin, citrinin was known to occur naturally in cereals (Hesseltine, 1974).

Patulin, a metabolite of *A. clavatus* and *P. patulum* has been found to be toxic to animals (Anonymous, 1952). Forgacs *et al.*, (1954) demonstrated that *A. clavatus* and *A. chevalieri* were responsible for an outbreak of chronic toxicity in calves in USA who consumed the contaminated feed. Moreau and Moreau (1960a, b) reported another outbreak in dairy cattle when farmers in France fed their cows with *A. clavatus* contaminated forage seedlings.

The phytotoxicity of patulin has been demonstrated by Norstadt and McCalla (1969). It was shown that stubble mulching of fields supported extensive growth of *P. urticae*, which produced patulin, causing reduced wheat seed germination and seedling growth. Indeed, wheat seed and seedlings have been shown to be sensitive to as little as 20 ppm concentration of patulin (Ellis and McCalla, 1970). Therefore, patulin is considered as a mycotoxin of potential concern in foods (Stott and Bullerman, 1975). Frank *et al.* (1977) and Neelakantan *et al.* (1982) have isolated patulin from fruits, nuts and vegetables infected with green mould.

Penicillic acid was first reported from cultures of *P. puberulum* (Alsberg and Black, 1913). It has since been found to be a metabolite of a variety of *Penicillium* and *Aspergillus* species. Penicillic acid is toxic to mammals (Murnaghan, 1946) and has also been found to be carcinogenic to rats by subcutaneous injection (Dickens and Jones, 1961). Penicillic acid has been found to be produced in large quantities by certain species of *Penicillium*, viz., *P. puberulum*, *P. martensii* and *P. cyclopium*. Corn blight or blue eye disease of corn caused by these species is quite common when corn seeds are stored at low temperature (Ciegler and Kurtzman, 1970a). The rate of production of penicillic acid depended upon the strain, substrate and incubation temperature (Ciegler and Kurtzman, 1970b).

Thorpe and Johnson (1974) screened the maize samples in USA and observed that penicillic acid was produced by 7 samples out of 20 ranging from 5 to 231 mg/kg. They also reported that the range of penicillic acid in dried bean samples varied from 11 to 179 mg/kg.

Sterigmatocystin has been characterised as a metabolite of *A. versicolor*. This toxin has structural similarities with aflatoxins (Bullock *et al.*, 1962). It was also extracted from the strains of *A. nidulans* growing on peanut and fodder plants. Because of the widespread occurrence of these moulds and the large amount of the compounds elaborated, sterigmatocystin might be as disconcorting as aflatoxins in food, despite its lower toxicity and carcinogenicity (Holzapfel *et al.*, 1966).

Van der Watt and Purchase (1970) have included sterigmatocystin as a carcinogen in the list of mycotoxins causing a high incidence of hepatomas in the Bantus of South Africa. Engelbrecht (1971) has studied the toxic effects of sterigmatocystin on a primary cell culture. These include progressive cell degeneration, inhibition of mitosis and micellar fragmentation.

Rabie *et al.* (1976) have reported production of sterigmatocystin by *A. versicolor* and *Bipolaris sarokiniana* in semi-synthetic liquid and solid media. Also other new species of *Aspergillus* were found to produce sterigmatocystin (Rabie *et al.*, 1977).

The amount of sterigmatocystin in the milled rice or rice bran varies from 3.8 to 4.3 ppm. *A. versicolor* usually occurred around germ tubes as a entangled mycelial mass, but it was rarely found in the major part of the endosperm, unless the grain was cracked (Takahashi *et al.*, 1984). This toxin was detected in feed associated with acute clinical symptoms of bloody diarrhoea and death in dairy cattle in the United States (Vesonder and Horn, 1985).

Ochratoxins have been isolated from *A. ochraceous* which has been found to invade and multiply in stored wheat (Christensen, 1962). The toxigenic fungi have also been isolated from other cereals and legume products (Scott, 1965), red and black pepper (Christensen *et al.*, 1967).

Purchase *et al.* (1967) have apprehended that the high incidence of hepatic cancer among the Bantus of South Africa may be the result

of the toxic metabolites of these moulds. Van Walbeek *et al.*, (1969) have shown that ochratoxins can also be produced by *P. viridicatum*. They occur in a variety of agricultural commodities and have been demonstrated to be toxigenic to ducklings and rats (Patterson and Allcroft, 1970).

Choudry *et al.* (1971) have studied ochratoxin's toxicity in hens. They found that the presence of ochratoxin at 1 ppm in the diet of white leghorn pullets resulted in delayed sexual maturity and lowered rate of egg production. Damage of liver and kidney due to this toxin was similar to the aflatoxin injury. Hesseltine *et al.* (1972) have reported the Aspergilli as ochratoxin producers. The ochratoxin content in sclerotia collected from grains infected with *A. ochraceous* was significantly higher than that produced on synthetic medium (Paster *et al.*, 1986). However, toxins were detected either alone or with citrinin as co-contaminant (Sinha and Prasad, 1994).

Effect of physical factors on seed fungi and mycotoxin production:

Fungi are major cause of deterioration of corn seeds in commercial storage conditions. Warm and humid weather favours the growth of moulds and mycotoxin production (Koehler, 1938; Semenuik and Gilman, 1944).

Milner and Geddes (1946) studied the relation between moisture contents, mould growth and respiration of grain during storage conditions. Chemical deterioration of seeds at different temperatures has also been investigated (Milner *et al.*, 1947).

The influence of temperature, humidity and oxygen concentration on mould growth and biochemical changes in stored yellow corn have been reported (Bottomley *et al.*, 1950). They (1952) further studied that fat acidity, non-reducing sugars and mould flora of stored yellow corn were greatly influenced by aeration, time and seed moisture content. *Alternaria*, *Helminthosporium* and *Fusarium* invaded the kernels before harvest and required a relatively high moisture content for their growth (Delprado and Christensen, 1952; Tuite and Christensen, 1955).

The invasion of seed during storage by the fungi caused high reduction in wheat seed germination (Papvizas and Christensen, 1957). Qasem and Christensen (1958) observed moderate to heavy infection by storage fungi in 159 samples of corn stored for 6-9 months in commercial bins. Degree of invasion by fungi was proportional to the decrease in percentage seed germination and increase in percentage of discolored or damaged germ tubes or germs. Low temperature during seed storage condition was an effective preventing measure to the fungi tested.

Storage of seeds at low moisture content (14%) and low temperature (20°C) for 24 weeks resulted in the death of field fungi like *Helminthosporium*, *Fusarium* and *Alternaria* (Lutey and Christensen, 1963). Christensen (1964) reported better seed germination in corn, wheat and barley free from storage fungi.

Rough rice was invaded by storage fungi at moisture contents of 13.4-13.8% resulting in slight reduction in seed germination (Christensen and Lopez, 1965). Lopez and Christensen (1967) found that *A. flavus* was able to grow on stored corn at 18.5% and above moisture contents and at a temperature of 35°C, besides *A. ochraceous* and *A. candidus*.

Temperature played an important role on the production of aflatoxin by A. flavus (Schindler *et al.*, 1967; Sorenson *et al.*, 1967). The optimum temperature range for both aflatoxin $B_1$ and $G_1$ was 25-30°C, although *A. flavus* was capable of growing over a wide range of temperature from 6-35°C (Diener and Davis, 1969).

Invasion by storage fungi and decrease in seed germination were directly proportional to moisture content and the time of storage (Christensen, 1970).

Boller and Schroeder (1974) studied the influence of relative humidity on production of aflatoxin in rice by *A. parasiticus*. They observed that invasion and colonisation of rice by *A.parasiticus* increased with increase in humidity at 30°C.

Freshly harvested high moist corn was found to have bright greenish-yellow fluorescence of aflatoxins in insect damaged parts (Lillehoj *et al.*, 1975).Hesseltine (1976) was of the opinion that physical factors were major source of the mycotoxin production in foods and feeds. Invasion of toxigenic moulds was affected by the amount of spore inoculum in the field, stresses on the plant,

invertebrate infections, damage by other fungi, plant resistance, mechanical damage, mineral nutrition of plant and temperature. During harvest of the crop, the grains were exposed to mechanical damage and mould inoculum. After harvest, growth of moulds depends on moisture level of the grain, temperature and humidity, rapidity of drying, aeration, microbial inoculum, insects, mixing of grains, chaff and dirt, chemical treatment, internal infection and accidental rewetting of the grain.

Factors like incubation period, temperature, humidity and light affected the formation of aflatoxin $B_1$ by *A. flavus* on maize hybrid (Prakash and Siradhana, 1978).

Infection and production, of aflatoxin $B_1$ by *A. flavus* were measured in a short season, mid season and full season cultivars at three geographically diverse locations in North Carolina (Jones *et al.*, 1981). They observed significant reduction in aflatoxin with early planting and harvesting of the crop.

Nandi *et al.* (1982) studied deterioration of some oil seeds at different temperatures and relative humidities in storage conditions. *A. glaucus*, *A. candidus*, *Penicillium* sp. and *A. flavus* were dominant at 15.5, 16.5, 18.5 and 19% seed moisture of paddy during storage condition respectively (Jayaraman and Kalyanasundaram, 1989; Mian and Fakir, 1989).

Biochemical changes in seed after fungal infection :

The association of fungi with seed under poor storage conditions results in the deterioration of the seed. They bring about certain changes leading to the reduction on their quality and quantity. The role of fungi in seed deterioration has been studied by a number of workers (Christensen, 1957; Qasem and Christensen, 1960; Fields and King, 1962; Gilmann, 1969; Lalithakumari *et al.*, 1971; Bilgrami *et al.*, 1976; Pathak, 1985; Sinha, 1985; Betts *et al.*, 1988).

Aflatoxin producing strain of *A. parasiticus* was reported to cause loss in caloric value of maize seeds (Bilgrami *et al.*, 1979). Biochemical changes have also been observed in fruits inoculated or contaminated with species of aflatoxin producing asperqilli (Singh and Sinha,1982, 1983; Sinha and Singh, 1984, 1987). Similar observations have also been reported in dry fruits like coconut, almond, cashewnut, walnut and makhana (Bilgrami *et al.*, 1983).

Physiological and biochemical changes in mould infected maize seeds and seedlings have been observed (Arinze and Sokirko, 1986).

(I) Changes in protein :

Protein constitute a complex nitrogen source for fungal nutrition. The ability to utilize the organic source of nitrogen depends upon the fungus to convert proteins into simpler amino acids. Changes in protein contents due to fungal infection have been studied during pathogenesis (Kitani *et al.*, 1970; Vidhyasekaran and Durairaj, 1971; Vidhyasekaran *et al.*, 1973; Cherry *et al.*, 1972, 1974, 1975;

Sharma and Wahab, 1975; Prasad *et al.*, 1976; Jamaluddin *et al.*, 1977). Decline in the total nitrogen has been observed (Bilgrami *et al.*, 1980).

Bilgrami *et al.* (1981) have reported significant changes in the quantity and quality of amino acids in maize seeds due to mould infestation.

Reports have demonstrated that the continuous decline in protein and soluble nitrogen occurs during pathogenesis (Roy and Choubey, 1984; Maheshwari and Mathur, 1985; Maheshwari *et al.*, 1985; Sinha, 1985; Pathak, 1985).

Increase in the production of toxins by the species of *Aspergillus* and *Penicillium* was correlated to the level of protein in barley (Haggblom and Ghosh, 1985).

Lalrawna (1987) revealed a continuous fall in the protein contents of the paddy seeds due to fungal infestations. The rate of protein disappearance depended upon the species of fungi and the variety of paddy.

(II) Changes in Sugars:

An increase in sugar contents due to fungal invasion in seeds has been reported (Grewitz and Durbin, 1960; Swamy, 1964). However, Strech and Capellini (1965) and Dayal and Joshi (1968) noticed a decline in reducing and non-reducing sugars in infested plant tissues. Vidhyasekaran (1968) reported an increase in reducing sugar contents of paddy seeds infected with various fungi.

Quantitative changes in sugars have been observed in orange fruits (Singh and Tandon, 1970), mosambi fruits (Srivastava and Tandon, 1970), groundnut foliage (Narain & Addy, 1970), groundnut seeds (Singh *et al.*, 1974), mung seeds (Prasad *et al.*, 1976; Bilgrami *et al.*, 1978), arhar seeds (Sinha and Prasad, 1977), apple fruits (Thind *et al.*, 1977) and Khesari seeds (Sinha and Prasad, 1978) after fungal infection.

Prasad (1983) reported that root, stem and leaf of diseased plant contain less amount of sucrose, glucose and fructose than the healthy plants. Reduction in total sugars due to fungal infestation has also been noticed by other workers (Reddy *et al.*, 1983; Singh and Sinha, 1983). Lobia seeds infested by *A. flavus* showed an increase in reducing sugars and a decrease in non-reducing sugars (Maheshwari and Mathur, 1985; Maheshwari *et al.*, 1985).

Lalrawna (1987) showed that fungal infestation resulted in the fall of total sugar contents while slight increase in the reducing sugars and a simultaneous fall in the non-reducing sugars was noted in all the paddy varieties. There was no consistency with regard to the extent of reduction and period of maximum activity during storage period of the grains.

Aflatoxins and seed germination :

Seed borne fungi produce metabolites which may be hazardous for the health of seed as well as for consumers. Metabolites of fungal origin reduce seed germination and sprouting (Papavizas and

Christensen, 1960; Fields and King, 1962; Humphrey-Jones and Waid, 1963; Christensen and Kaufmann, 1965; Lalithakumari *et al.*, 1972; Dwivedi and Singh 1973; Dublish and Pande, 1976; Rai and Singh, 1977; Pandey *et al.*, 1982).

Crisan (1973) reported the effect of aflatoxin on germination and growth of lettuce. Rati and Ramalingam (1974) recorded four types of infection on seeds, i.e., seed rot, non-emergence of cotyledons, cotyledonary infection and plumule infection.

Brodnik (1975) reported that the toxic products of *F. graminearum* and *F. moniliforme* have influence on the seed germination and embryo growth in maize. Similar observations have also been made by Cebrat *et al.* (1981), Katouli and Marchant (1981), Kulik and Scheen (1982), Naik *et al.* (1982), Charya and Reddy (1982), Kim *et al.* (1984), Vasantha *et al.* (1987) and Mahalinga *et al.* (1988).

Srivastava and Mishra (1971) reported the effect of exudates of *A. niger*, *A. flavus*, *P. humicola* and *F. oxysporum* on the radicle growth of wheat and barley. They noticed that radicle growth was retarded by varying concentrations of all fungal exudates. Effect of aflatoxin on the delay of seed germination and seedling emergence in various crops has been reported (Bhale *et al.*, 1982).

Llewellyn *et al.* (1984) observed the adverse influence of aflatoxins on germination, root elongation and uptake of zinc by *Glycine max.* Jeswal (1987) reported that aflatoxin $B_1$ was inhibitory to maize seed germination, however, its low concentration enhanced the

shoot length. Similar effects of aflatoxin $B_1$ on amylase of maize seed have also been reported (Chatterjee, 1988).

A number of seed borne fungi have toxic effects on seed germination and seedling growth (Prasad and Hiremath, 1983; Singh and Gupta, 1984; Ghewande *et al.*, 1984; Singh *et al.*, 1985).

Penicillic acid produced by *P. cyclopium* and *P. canescens* showed phytotoxicity during corn seed germination (Keromnes and Thouvenot, 1985). Bedi and Chohan (1986) screened weed seeds for aflatoxins and their role in the seed germination of different crop plants. They observed the chlorosis and albinism of the primary leaves in aflatoxin $B_1$ treated seeds.

Mycotoxins produced by *Fusarium sporotrichiella* (Zearalenone) and *Dendrodochium toxicum* (rorridine A and verrucarine A) exerted toxic effect on wheat and maize root cells. Toxin concentration of 10 µg/ml caused depolarization of maize root hair membranes (Pavlovkin *et al.*, 1986).

Seed germination of *Vigna mungo* L., *Lens culnaris*, *Corehorus capsularis* L. and *Brassica rapa* responded differently to the culture filtrates of *A. niger*, *A. flavus*, *F. semitectum*, *F. moniliforme* and *Curvularia lunata* (Anwar and Mridha, 1987).

Kunwar and Mehrotra (1988) also observed the inhibitory effects of culture filtrates of seed storage fungi on the germination and sprouting of wheat grains.

Effect of aflatoxins on the animals :

Growth of moulds on cereals and foodstuffs often appears quite harmless but they may sometimes be pathogenic or toxigenic. In 1961, groundnut meal contaminated with aflatoxin was responsible for the large scale death of turkey poults and ducklings (Allcroft *et al.*, 1961). The incidence highlighted the implications of mycotoxins contaminated food in foodstuffs, particularly of aflatoxin which was also found to affect other species (Allcroft *et al.*, 1962).

Aflatoxins mainly affect the functioning and physiology of liver, kidney, spleen, colon, lung and skin of various animals and human beings (Newberne *et al.*, 1964; Butler, 1974; Wogan, 1974; Krishnamachary *et al.*, 1975, b, c; Singh and Chauhan, 1983; Ranjan, 1985; Bilgrami *et al.*, 1986).

Linderfelser *et al.*, (1976) observed that male and female mice fed with aflatoxin contaminated corn accumulated high level of lipid in the liver.

In case of poultry, serious pathological changes were noted in the kidney, heart and liver when crude toxin preparations from *A. flavus* mixed with foodstuff was given (Baruah *et al.*, 1980). The liver showed necrosis and pittings. Histological study of liver showed haemorrhage, infiltration of lymphocytes, pyknosis and degeneration of hepatocytes. Kidney sections revealed swelling of tubules, haemorrhage, degeneration of basement membrane and glomeruli and infiltration of lymphocytes (Singh, 1984).

Control of mycotoxins :

Prevention of mycotoxins from getting into food and feeds involves several distinct lines of defect: (i) prevention of mould growth, (ii) detection methods, (iii) removal of the toxin if present and (iv) identification of new possible mycotoxin threats (Hesseltine, 1973). According to him, the first study involved the effects of insecticide treatment of sterile and unsterile wheat on the formation of aflatoxin and ochratoxin. The second study was surveyed on incidence of ochratoxin, aflatoxin and zearalenone in white and yellow corn. The third one was on the number of species of *Aspergillus* and *Penicillium* that produced ochratoxin and penicillic acid respectively. The fourth one dealt with the effect of ammonia treatment on the internal mould flora of corn.

The extracts of *Allium sativum* inhibited the growth of fungi (Tansey and Appleton; 1975). The root, bulb and leaf of *A. cepa* and *A. sativum* were all inhibitory to fungal growth (Agrawal, 1978; Singh *et al.*, 1979). The growth of aflatoxin producing fungi like *A. flavus* and *A. parasiticus* was inhibited by extract of *A. cepa* (Sharma *et al.*, 1979).

Inhibition of aflatoxin production has also been reported by some other plant extracts (Bilgrami *et al.*, 1979). They observed that the aqueous extracts of *Arnebia nobalis*, *Nicotiana plumbaginifolia*, *Ricinus communis* and *Cocculus hirsutus* inhibited the aflatoxin production to varying degrees. Root and stem extracts of *N.*

*plumbaginifolia* and *T. communis* inhibited the aflatoxin production by 34.62 and 45.46% in maize and rice respectively.

Misra *et al.* (1980) had screened 14 homeopathic drugs inhibitory to aflatoxin production and growth of *A. parasiticus.* Some wild medicinal plant extracts were also found to inhibit the aflatoxin production and growth of *A. flavus in vitro* condition (Bilgrami *et al.*, 1980). Toxin production was prevented by O-Vanillin in some cereals and oil seeds (Bilgrami *et al.*, 1982).

Daniels (1983) reported that *Fusarium moniliforme* can be eliminated from corn, if they were pretreated in distilled water for 4 h at 18-22°C and subsequently incubated at 60°c for 5 minutes.

Light and heat factors were also observed to inhibit the production of aflatoxins in dry fruits and spices (Bilgrami *et al.*, 1984). The extracts of certain tree barks (Sinha and Singh, 1984) and some chlorophyllous plants were also found to check the growth of seed fungi and mycotoxin production (Sinha, 1985). Production of aflatoxin on wheat grains and groundnut seeds was inhibited by the extracts of rhizome of *Adiantum* and leaf of *Thuja orientalis* (Singh and Sinha, 1985).

Some pesticides have been reported to control the growth of *Fusarium* spp. and production of zearalenone (Draughon and Churchville, 1985).

The control of aflatoxin by chemicals other than pesticides has also been reported (Buchanan *et al.*, 1986). Thioglycerol inhibited the

growth of *A.parasiticus* and aflatoxin production while thioral stimulated *A.flavus* (Umechuruba, 1986).

Reduction in growth of *A.flavus* by solar heating during sun drying (Hussein *et al.*, 1986) and production of aflatoxin in corn by irrigation and tillage (Payne *et al.*, 1986) has been observed.

Girisham and Reddy (1987) observed toxic effect of different volatile substances on the seed mycoflora of pearl millet (*Pennisetum typhoides*). Chemicals like dimedone and potassium metabisulfite suppressed the aflatoxin biogenesis (Sharma *et al.*, 1988).

Sudheer *et al.* (1989) observed significant inhibitory effect on *Trichoconiella padwickii* in paddy by certain plant extracts.

# *Materials and Methods*

# MATERIALS AND METHODS

Selection of sites :

Selection of study sites was done after collecting the information on areas under intensive maize (*Zea mays* L.) cultivation (Government of Meghalaya). Four sites selected for the present study were situated in Nongkrem, Upper Shillong, Mylliem and Laitjem, Khasi Hills, Meghalaya. They were situated at an altitude of a range of 50 - 1950 m, longitude 89°45′ and 92°45′ E and latitude 25° and 26°10′N (Fig. 1.A) respectively. Two local varieties of maize (*Zea mays* L.) viz. Local white and Local yellow were mainly grown in all the study sites during summer/rainy season, while the seeds were stored in the winter season.

Climate of the study sites :

Based on the meteorological conditions of the region, the year may be broadly classified in summer/rainy (wet summer), autumn and winter seasons (Fig. 1.B).

(1) Summer/Rainy (wet summer) season - It starts from June and ends in September. The average rainfall, maximum and minimum temperature of the season was recorded as 493.25 mm, 21.35°C and 15.8° C respectively.

(2) Autumn season - It starts from September and lasts upto November. This period is characterised by low temperature and less rainfall. The average rainfall and maximum and minimum temperature was recorded as 230.3mm, 20.13°C and 9.67°C respectively.

**Figure 1.A.** Map showing the study sites (1-Nongkrem; 2-Upper Shillong; 3-Mylliem; 4-Laitjem) in the East Khasi Hills of Meghalaya (India).

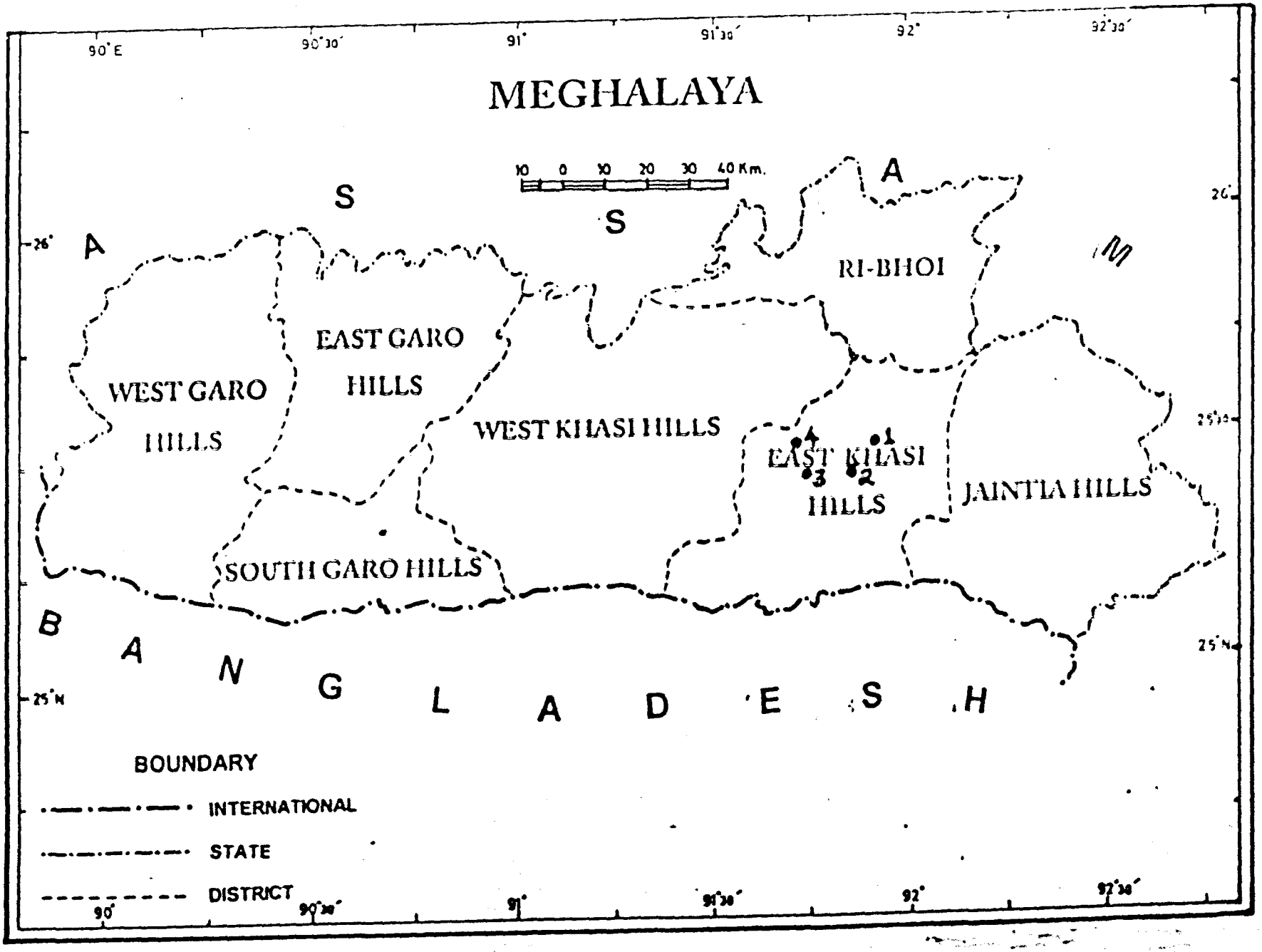
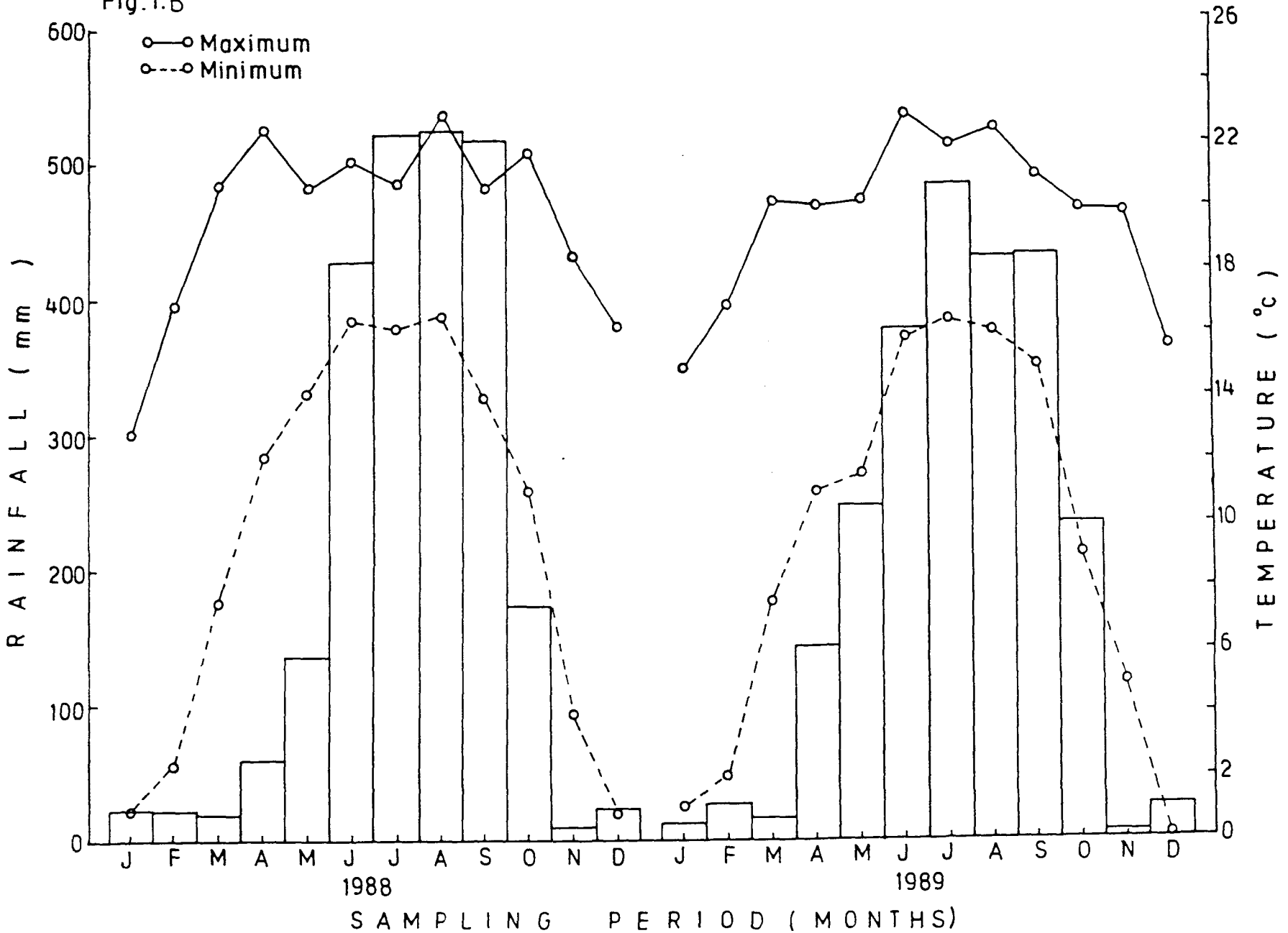

MEGHALAYA
90° E
90°30'
91°
91°30'
92°
92°30'
10 0 10 20 30 40 Km.
S
S
A
A
M
RI-BHOI
EAST GARO HILLS
WEST GARO HILLS
WEST KHASI HILLS
EAST KHASI HILLS
JAINTIA HILLS
SOUTH GARO HILLS
1
2
3
4
B A N G L A D E S H
BOUNDARY
INTERNATIONAL
STATE
DISTRICT

Fig I.A.

**Figure 1.B.** Meteorological data of study sites for maximum (-o-) and minimum (-o-) temperatures (°c) and rainfall (mm) for the period of investigation (January 1988 to December 1989).

Fig.1.B


Maximum
Minimum
RAINFALL ( mm )
TEMPERATURE ( °c )
600
500
400
300
200
100
0
26
22
18
14
10
6
2
0
J F M A M J J A S O N D
1988
J F M A M J J A S O N D
1989
SAMPLING PERIOD ( MONTHS)

(3) Winter season - It extends from November to February. It is characterised with low temperature and less rainfall. The average rainfall, maximum and minimum temperature was recorded as 18.15 mm, 16.02°C and 2.07°C respectively.

Brief background of maize cultivation in the State:

The maize is grown on the slope of hills of Meghalaya during wet summer season when temperature and humidity are high. The sowing period varies from March to May and the cobs are harvested from the month of July to September. Unlike other places of India, tribal inhabitants of this area consume only raw, roasted and boiled seeds. The cobs are stored either in shaded place or in interior warm places i.e. above chimney place (Plates 2.A & 2.B). The inhabitants in interior locality never use any specific storage container for the cobs. However, in villages near to urban settlement, employ gunny bags and tins to store the cobs. Seeds are never separated from the cobs either to store them for germination or for consumption. Therefore, the climatic condition and storage practices of maize in the state makes interesting to investigate fungal infection and mycotoxin production.

Study materials

Two varieties of locally cultivated maize seeds, viz., local white and local yellow, hereupon designated as LW and LY respectively were collected from different study sites in the Khasi hills district of Meghalaya. These two varieties were mainly cultivated and consumed

by the local tribal population, therefore, they were selected for the present study. They vary in their nutritive constituents, taste, size and colour of seeds.

Sampling of materials :

Maize cobs were collected from the bulk stored without husk either in shaded condition (hanged in verandah) or in smoked condition (hanged above the fire place), from standing crop and agricultural fields maintained by the farmers in their respective houses and fields respectively. A composite sampling of each single variety was procured from same locality and the same farmers and fields throughout the study period.

Collection of corn seeds was done from the bulk storage following the procedures of International Rules for Seed Testing (ISTA, 1966, 1976). Several individual samples were randomly collected from different parts of the bulk (top, middle, bottom and sides). The primary samples drawn from different parts of the bulk were then mixed to make a composite sample which was reduced to the desired quantity.

The final sample, technically called the sub-sample was transferred into a sterilized polythene bag and sealed.

Similar procedure was followed to collect the samples of corn seeds from the fields as was done in case of the storage conditions. Care was taken to avoid external contamination by cleaning the hands and other instruments with 90% ethyl alcohol.

Samples of different maize varieties were brought to the laboratory. Selection of seeds for plating purpose and determination of their moisture contents was done. The seed lots were kept in oven at 60°C for 48 h. Subsequently, the samples were stored at room temperature till the extraction of mycotoxin was completed. The time lapse between sample collection and toxin extraction was kept as minimum as possible. Care was taken to avoid fungal contamination, invasion my mites and rats before extraction.

Survey of mycotoxin producing seed borne fungi was done for two years from storage and field conditions. For each year sampling was done in three seasons, i.e. winter, wet summer and autumn when the crop was in storage, field and harvest conditions respectively.

Determination of moisture content of the grains :

Moisture contents of maize grains was determined by "Oven dried method" (Agrawal, 1986).

5 g of the seed sample was dried in an oven at 80°C for 24 h and reweighed. The process was repeated till a constant weight was obtained. Three replicates were maintained for each sample. The loss in weight was calculated. The percentage moisture content of the seed was calculated by using the following formula:

$$\text{Moisture content (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

Where $W_1$ = Initial weight of fresh seeds

$W_2$ = Weight of oven dried seeds.

1. Isolation of seed borne fungi :

Isolation of seed borne fungi from both varieties of maize was carried out for two years (1988 and 1989) in three seasons (wet summer, winter, and autumn) each year. The data of each season was expressed as an average value of three months. Working samples for periodical screening were drawn aseptically from the stock samples in the laboratory.

The following methods were followed for the isolation of fungi from maize seeds throughout the period of investigation.

(i) Standard blotter method - Standard blotter method of International Seed Testing Association (ISTA, 1966) as modified by Limonard (1966) was followed to isolate the seed fungi. Three sterilized Petri dish (9.5 cm diameter) layered with sterilized cotton and filter paper were moistened with sterile distilled water, so that little surplus of water was left on the surface of the paper. One hundred seeds were randomly selected from each of the variety and 10 of them were plated equidistantly in each Petri dish. Plating of seeds was done in a Laminar Flow Chamber. Petri dishes were incubated at 20±1°C for three days. Thereafter, they were transferred for 12h to another incubator at 10°C and subsequently incubated at 25±1°C for another 7 days with 12 h photoperiod. The plates were observed under stereobinocular microscope.

Infected seeds were counted and the percentage frequency of occurrence of different fungi was calculated using the formula :

$$\text{Frequency of fungal species (\%)} = \frac{\text{Total no. of colonies of individual species}}{\text{Total no. of colonies of all species}} \times 100$$

(ii) Agar plate method - The International Rule for Seed Testing (ISTA, 1966) was followed to isolate the seed on nutrient agar medium. Potato dextrose agar medium was sterilized, poured aseptically into plates and allowed to cool. One hundred seeds of maize were randomly selected from each variety and 10 of them were spaced out in a petri dish containing PDA medium, using a sterilized forcep. Pouring and plating of medium were carried out in Laminar Flow Chamber. Thereafter, plates were incubated at 25±1°C with 12 h photoperiod for 7 days in a incubator. Fungal colonies on infected seeds were counted and the percentage relative abundance of each fungus was determined.

(iii) Dilution plate method - One g of seed sample was added to 99 ml of sterile distilled water and shaken for 30 minutes on an electrical shaker. Ten mililitre solution from this dilution was aseptically transferred to 90 ml of sterile distilled water to get a dilution of 1 : 1000. One mililitre suspension from the final dilution (1:1000) was inoculated on Potato Dextrose Agar (PDA) and Nutrient Agar medium in Petri plate. Petri dishes were rotated gently in a swirling motion to dispense the inoculum uniformly over the surface of the medium. Five replicates were maintained for each sample. The whole process of inoculation was carried out in Laminar Flow Chamber. The plates were incubated at 25±1°C for 5 d in an incubator.

1.2 Identification and pure culture of fungal isolates :

Fungi isolated from the seeds were identified following the keys of Gilman (1956), Subramanian (1971), Barnett and Hunter (1972).

Pure cultures of fungi isolated from maize seeds by different techniques were done by single spore technique. The cultures were maintained on Czapek's Dox Agar and Potato Dextrose Agar (PDA) media at 4°C.

1.3 Interpretation of results :

The percentage frequency of occurrence of individual fungi was calculated using the formula -

$$\% = \frac{\text{Total no. of individual species of fungus}}{\text{Total no. of all the species of fungi}} \times 100$$

1.4. Fungal Biomass in maize seeds :

Chitin digestion method was used to determine the fungal biomass in maize seeds (Ride and Drysdale, 1972).

100 g infected seeds used for isolation of seed mycoflora were dried at 80°C for 72 h and stored at room temperature in tightly capped glass bottles until used. The extraction and analysis of chitin was carried out as follows :

Sample preparation for hydrolysis of chitin to chitosan :

(i) 50 g of seed sample was ground in an electric grinder for 1.5 mins.

(ii) 0.2 g of the finely ground flour was placed into a 30 ml plastic autoclavable centrifuge tube.

(iii) 5 ml of KOH (120 g KOH in 100 ml $H_2O$) was added to it.

(iv) The test-tube and contents were sterilized at 15 psi in autoclaved for 15 mins and then cooled to 0°C.

(v) Ice cold 70% ethanol (8 ml) was added to each tube and subsequently layered with 1 ml celite (1 g/20 ml 70% ethanol); this facilitated the precipitation of chitosan.

(vi) The tubes were then centrifuged (12,000 rpm) for 10 mins at 0°C and the supernatent was decanted.

(vii) The pellet was resuspended by adding 8 ml of ice cold 40% ethanol to each tube and stirred thoroughly.

(viii) Step (vi) was repeated.

(ix) The pellet was resuspended in distilled water (8 ml) and the pH adjusted to 2.0±0.2.

(x) Step (vi) was repeated.

Colorimetric assay for chitosan:

(xi) 1 and 1.5 ml of $KHSO_4$ (5% W/V) and 1.5 ml $NaNO_2$ were added to each tube and the total volume was brought to 4.5 ml with distilled water. The precipitate was stirred into solution.

(xii) A 1.5 ml aliquot was withdrawn from each tube and placed into separate test tube containing 0.5 ml $NH_4SO_3NH_2$ (12% W/V). The tubes were thoroughly shaken.

(xiii) 1.5 ml 3-methyl-2-benzothiozolone hydrazone (MBTH; 0.5% W/V) was added and mixed.

(xiv) A known concentration series of glucosamine-HCL (Sigma) in the range of 10,20,30,40,50,100 and 150 µg per tube (total volume equals 3 ml) was prepared. Water was substituted for glucosamine-HCL in the blank.

(xv) The tubes containing the experimental samples and the standard solution were heated in a hot water bath (90°C) for 4 mins., removed and cooled in an ice bath.

(xvi) 1.5 ml FeCL$_3$ (0.5% W/V) was added to each tube and allowed to stand for 30 mins. at room temperature; alternatively, the mixture was filtered.

(xvii) The absorbance of the solution was read at 650 nm in a Beckman spectrophotometer (USA). The chitin content was estimated from the standard curve of glucosamine HCL and represented as µg/g dry weight of seed.

2. Production of mycotoxins in maize seeds :

2.1 Seeds Samples :

Samples of 500 g each of maize seeds representing the two local varieties of maize, viz. local white and local yellow were screened for mycotoxins.

2.2 Observation of maize seeds under ultra violet light for Toxin :

Bright greenish yellow fluorescence (BGYF) was considered as a tentative test for distinguishing the aflatoxin contaminated seeds from the healthy ones (Fennel *et al.*, 1973). Based on this principle, all the collected seeds were observed under ultra violet light (360

nm). Samples giving typical blue green yellow fluorescence (BGYF) were selected for the extraction of aflatoxins.

2.3 Extraction of mycotoxins from maize grains :

Seeds giving BGYF fluorescence were selected for chemical extraction of mycotoxins (Thomas *et al.*, 1975). 50 g of dried seeds were powdered and blended with 250 ml methanol: water (60: 40 V/V) for 15 min. The extract was filtered through Whatmann No. 1 filter paper. 125 ml of the filtrate was taken into a separating funnel (500 ml). To it, 30 ml of saturated NaCl and 50 ml of n-hexane were added. The funnel was shaken vigorously and the lower methanolic layer was collected while upper hexane layer was discarded. 40 ml of chloroform was added to the funnel and shaken again. The layers were allowed to separate. The lowest chloroform layer was obtained into a flask contained 5 g of cupric carbonate and agitated. Cupric carbonate was allowed to settle down and chloroform was decanted through Whatmann No. 42 filter paper passing through the bed of anhydrous $Na_2SO_4$. The chloroform extract was evaporated to dryness and residue was dissolved in 1 ml of chloroform and reserved for Thin Layer Chromatography (TLC) analysis.

2.4 Screening of fungal isolates for their mycotoxin producing ability :

Aflatoxin elaborating potential of different isolates of *Aspergillus* was tested in the Rice-flour liquid medium (Misra and Sinha, 1979). Production of zearalenone by the isolates of *Fusarium*

*moniliforme*, citrinin by *Penicillium citrinum* and ochratoxin by *A. ochraceous* were tested on Czapek's Dox liquid medium (Raper and Thom, 1949).

2.5 Extraction of mycotoxins from culture filtrates :

The method of Jones (1972) was followed to extract the mycotoxins from the culture filtrates of different fungi. 25 ml of the liquid medium (Rice flour for aflatoxin and Czapek's Dox for the other mycotoxins) was taken in a 250 ml Erlenmayer flask and was sterilized at 15 psi for 15 min. The medium was allowed to cool, 0.5 ml spore suspension of the test fungi was inoculated in the medium and incubated in BOD incubator at 25±1°C for 7 d. During the incubation period, cultures were shaken at an interval of 24 h. 10 ml of methanol was added in the flask in order to kill the spores inside the flask. The liquid culture was filtered through Whatmann No. 1 filter paper and the amount of filtrate was transferred to a 500 ml separating funnel. Before filtration, pinch of anhydrous $Na_2SO_4$ was put on the filter paper to absorb the extra moisture. 10 ml of chloroform was added two times to the filtrate in the separating funnel. The lower layer of 10 ml chloroform was collected in a test tube. The chloroform extract was evaporated to dryness and residue was dissolved in 1 ml of chloroform and reserved for Thin Layer Chromatographic (TLC) analysis.

2.6 Qualitative analysis of mycotoxins :

Qualitative analysis of mycotoxin was done by thin layer chromatography (TLC) method. To prepare the TLC plates, uniform clean glass plates (20 cm x 20 cm) were used. 36 g of silica gel G was mixed with 72 ml distilled water to form a slurry which was immediately used for coating of 6 plates (uniform size, 0.5 nm thick) with the help of applicator. The plates were dried at room temperature and activated in an oven at 120 °C for 1 h. The plates were cooled before use.50 $\mu$l of chloroform extract of various mycotoxins was spotted on TLC plate along with mycotoxin standard.

For detection of aflatoxin solvent system comprising toluene : isoamyl alcohol : methanol (90:32:2 V/V/V) was used which gave better resolution of different aflatoxins (Reddy *et al.*, 1970).

For detection of other mycotoxins, two solvent systems i.e., toluene : ethyl acetate : formic acid, 6:3:1 V/V/V (TEF) and benzene : methanol : acetic acid, 24 : 2 : 1 V/V/V (BMA) were used (Scott *et al.*, 1970).

The plates were developed in a chromatography chamber. TLC plates were air dried and examined under long (360 nm) and short (260 nm) wavelength UV lamp in the UV chamber. The spots giving fluorescence were marked and noted for their colour.

2.7 Quantitative estimation of mycotoxins :

Quantitative estimation of mycotoxins was done by spectrophotometric method (Nabney and Nesbitt, 1965). The spots of

mycotoxins on TLC plates were scrapped and extracted in 5 ml cold methanol separately. They were then centrifuged at 3000 rpm for 15 min. The ultra violet absorption spectrum of the methanolic solution was recorded in Beckman spectrophotometer and the amount of mycotoxins in the sample was calculated by the following formula :

$$\text{The amount of mycotoxin } (\mu g/ml) = \frac{D \times M \times 10^6}{E \times L \times 1000}$$

Where D = Optical density

M = Molecular weight

E = Molar extinction coefficient

L = Path length (1 cm cell was used)

Table 1 : Molecular weight (M) and molar extinction coefficient (E) of various mycotoxins at respective wave lengths (Nabney and Nesbitt, 1965).

| Mycotoxins | Wavelength (nm) | Molecular weight (M) | Molar extinction coefficient (E) |
|---|---|---|---|
| Aflatoxins | | | |
| $B_1$ | 360 | 312 | 22,000 |
| $B_2$ | 362 | 314 | 23,400 |
| $G_1$ | 360 | 328 | 18,700 |
| $G_2$ | 362 | 330 | 21,000 |
| Citrinin | 322 | 259 | 16,100 |
| Zearalenone | 236 | 318 | 29,700 |
| | 274 | 318 | 13,909 |
| Ochratoxin | 316 | 318 | 6,020 |

2.8 Chemical confirmation of mycotoxins:

Chemical confirmation of aflatoxin $B_1$ was done by Trifluoroacetic acid (TFA) as suggested by Stack and Pohland (1975). After reaction with TFA, aflatoxin $B_1$ appeared as a blue fluorescence spot at $R_f$ about 1/4th to that of aflatoxin $B_1$ on TLC plate. In some cases where the amount of aflatoxin $B_1$ was very low, the spot was sprayed with 25% sulphuric acid, which changed the colour of fluorescence from blue to yellow.

Presence of ochratoxin on TLC plate was confirmed by treating the spot with ammonia fumes which changed blue green fluorescence to deep blue colour (Davis *et al.*, 1969).

Citrinin was confirmed by spraying TLC plate with freshly prepared mixture of 0.5 ml p-anisaldehyde in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of concentrated sulphuric acid and then by heating TLC plate at 130°C for 10 min. This changed yellow streak of citrinin to yellowish-green under long wave UV light (Scott *et al.*, 1970).

Zearalenone was confirmed by spraying TLC plate with acidic p-anisaldehyde solution (Scott *et al.*, 1970) by which greenish - blue fluorescence turned into faint brown (in visible light) and faint yellow ( in long wave UV - light).

3. Experiment on the effect of physical factors like seed moisture, air temperature and air humidity on the production of mycotoxins by the seed borne fungi was carried out *in vivo*.

3.1 Seed moisture - Eight moisture levels i.e., 15, 20, 25, 30, 35, 40% wet wt of freshly harvested maize seeds were maintained as suggested by Harris and Lindbald (1978).

Ten replicates of 500 g seeds from each moisture level were taken in sterilized polythylene bags and sealed air tight. Ten samples from each moisture level were stored at 25±1°C. Assessment of mycotoxins was done after 10,20 and 30dof moisture content maintenance.

3.2 Air temperature - 500 g seeds with 25% moisture level were stored in polythylene bag at 5, 15, 25, 30, 35 and 40°C. Qualitative and quantitative estimation of mycotoxin production was carried out at 30d interval as described earlier.

3.3 Air humidity - Ten replicates of 500 g seeds were kept at room temperature (15°C - 25°C) in dessicators at 75, 80, 85, 90, 95 and 100% relative humidity (RH) using different salt solution (Boller and Schroeder, 1974). The seeds were taken in wide mouth containers covering with wiremesh to facilitate easy aeration. The mycotoxin production was estimated at 10, 20, and 30th day of storage.

4. Effect of certain seed borne fungi on the quality of seeds :

4.1 Seed samples - Seed of two varieties of maize, viz. local white and local yellow were used as the seed samples.

4.2 Fungal species - Three dominant seed borne toxin producing fungi isolated earlier from maize seeds, viz. *A. flavus*, *F. moniliforme* and *P. citrinum* were used as the test fungi.

The pure cultures of the test fungi which were maintained in the culture tube slant were transferred and multiplied in Petri dishes containing potato dextrose agar medium. Spore suspensions of 10 days old cultures of the test fungi were prepared. The concentration of the spores was adjusted to approximately 4 x $10^4$ spores/ml with the help of haemocytometer.

150 g seeds were taken for each treatment. The seeds were surface sterilized in 0.1% mercuric chloride solution for 3 mins. followed by several washings in sterile water. Extra water from the seeds was removed by sterilized blotting papers and transferred to oven at 60°C for 24 h.

Thereafter, they were transferred to a 100 ml Erlenmeyer flask and inoculated with 5 ml of the spore suspension of each test fungus separately. The flasks were shaken vigorously to get a homogenised spore load over seeds. All the three fungi were tested separately on both the varieties of maize seeds.

Each inoculated sample was transferred to a cloth bag and stored in a dessicator at 96% relative humidity maintained by a solution of sulphuric acid (CMI, 1974).

Another set of surface sterilized seeds was stored under a similar conditions without any fungal inoculation and was treated as control. Both sets were stored at room temperature for a period of six months at the intervals of 60 days.

At the end of each storage period, the infested seeds were thoroughly washed under running tap water to remove fungal mycelium from the seed surfaces. Subsequently the seeds were dried at 60°C for 48 h and powdered for further studies.

Periodical analysis of reducing and non-reducing sugars, proteins and dry weight of seeds was done in fungal treated and untreated seeds.

4.3 Determination on dry weight of seeds :

100 seeds were randomly selected and dried in hot air oven at 100°C for 24 h and then placed in a dessicator charged with calcium chloride for about 30 min and weighed. The dry weight of the control seeds was compared with that of the test fungus treated ones at a regular intervals of 60 days and it was expressed as the percentage change over the control using the formula :

$$\text{Loss of dry weight (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

Where $W_1$ = Dry weight of the control seeds

$W_2$ = Dry weight of the treated seeds

Three replicates were maintained for each sample.

4.4 Estimation of total sugars :

Total sugar contents of maize seeds were estimated by the method of Bilgrami *et al.* (1979). To 0.2 g dried and powdered seed sample, 25 ml of 80% ethanol was added and stirred thoroughly. After 5 minutes, it was centrifuged and the supernatent was collected.

Extraction was repeated by adding 30 ml of hot 80% ethanol to the residue and centrifuged at 2000 rpm. The extracts were combined and ethanol was evaporated in an evaporating disc. The residue left at the bottom was dissolved in 5 ml distilled water and used for the determination of the total sugar.

To 2 ml of the solution 0.14 ml of 80% aqueous phenol was added followed by the rapid addition of 5 ml conc. $H_2SO_4$. After thorough mixing, the solution was allowed to stand for 30 min. The optical density of the yellow solution was measured at 490 nm in a Spectrophotometer (Hitachi). Standard curve of glucose was prepared by the same procedure. Four replicates were used for each treatment and sugar contents were determined from the standard known concentrations expressed as mg/g of the sample.

4.5 Estimation of reducing and non-reducing sugars :

Reducing sugar content of maize seeds was estimated as described by Peach and Tracey (1955). 0.3 g of the seeds was ground to powder and blended with 1.5 ml glass distilled water in a glass homogenizer. To this 0.2 ml of 0.3 N barium hydroxide solution was added followed by 0.2 ml of 5% zinc sulphate solution and thoroughly mixed. The entire content was centrifuged. To 1 ml of the supernatent, 1 ml of alkaline copper reagent (prepared by dissolving 4 g of $CuSO_4 . 5H_2O$ , 24 g anhydrous $Na_2CO_3$, 16 g Na-K-tartrate in 1 litre water) was added. The mixture was heated on boiling water bath for 15 min and cooled. To this 1 ml of arsenomolybdate reagent (prepared by dissolving 25 g

ammonium molybdate in 450 ml of distilled water and adding 3 g of $Na_2HSO_4$. $7H_2O$) dissolved in 25 ml of water, mixed and placed in an incubator at 37°C for 24 h) was added and left to stand for few minutes till the effervescence ceased. The blue colour was diluted with glass distilled water upto 10 ml and the optical density was read at 510 nm. The optical density was compared with the standard glucose solution. The reducing sugar content was expressed as mg/g of the sample.

Non-reducing sugar was calculated by subtracting the value of reducing sugar from the total sugar contents of seeds.

4.6 Estimation of proteins:

Protein was estimated by following the method of Lowry *et al.* (1951). Powdered seed samples were suspended in 1 ml of 1 N NaOH at 100°C for 4.5 min. 5 ml of reagent C (Alkaline Copper reagent - prepared by mixing 50 ml of reagent A (Alkaline sodium carbonate solution - 2% $Na_2CO_3$ in 0.1 N NaOH) and 1 ml of reagent B (Copper sulphate - sodium potassium tartrate solution - prepared by 0.5% $CUSO_4.5H_2O$ in 1% sodium potassium tartrate; freshly prepared) was added to it and the mixture was allowed to stand at room temperature for 10 min. 0.5 ml of Folin-Ciocalteau reagent (prepared by taking a mixture containing 100 g of sodium tungstate, 25 g of disodium molybdate, 700 ml of water, 50 ml of 85% $H_3PO_4$ and 100 ml of conc HCL in a 1 litre flask. The mixture was refluxed gently for 10 h. 150g of lithium sulphate, 50 ml of $H_2O$ and a few drops of bromine water

were added. The mixture was boiled for 15 min. without condenser to remove the excess of bromine. It was then cooled, diluted to 1 litre and filtered. The reagent has no greenish tint, which was diluted with equal volume of water and poured in to the tube immediately. After 30 min., the absorbance of the solution was measured at 750 nm. Standard curve for protein was prepared by the same procedure. Three replicates were used. The amount of protein in the samples was calculated with a standard curve prepared using bovine serum albumin [prepared by dissolving 25 mg of bovine serum albumin in distilled water and was made up to 100 ml in a standard flask. 1.0 ml of this solution contained 25 $\mu$g of albumin (10 mg/100 ml of .5 N NaOH)]. The results were expressed as mg/g of the sample.

5. Effect of aflatoxin $B_1$ on the growth of certain seed borne fungi, seed germination and seedling growth :

5.1 Seed samples - Seeds of the two varieties of maize were used for the experiment.

5.2 Fungal species - The fungi isolated from maize seeds, i.e. *A. flavus*, *F. moniliforme* and *P. citrinum* were used as the test fungi.

5.3 Toxin tested - Aflatoxin $B_1$ was used.

5.4.1 Fungal spore germination - Effect of aflatoxin $B_1$ on the germination of spores of test fungus was studied by hanging drop method. Single spore cultures of the test fungus were grown on PDA medium. Spores were collected from 10 days old fungal cultures and suspended in various concentrations of aflatoxin $B_1$ viz. 0.1 ppm, 0.5

ppm & 1 ppm were prepared. Number of spores was adjusted to about 20-25 spores per microscopic field under 150 x magnification. A drop of the spore suspension was placed on clean micro-cover glass and subsequently placed on a cavity slide in an inverted position, so that the spores are hanging in the cavity. Grease was applied on the edge of the cavity to prevent the displacement of the micro-cover glass and humidity in cavity. Three replicates were maintained for each sample. The slides were then kept in a sterile Petri dish containing 10 ml of sterile water to maintain the humidity of the chamber. The whole set was incubated at 5, 15, 20, 25 and 30°C. Control sets were also maintained for each fungus using sterile distilled water as control.

Observation for the germination of spores was made at 2 h, 5 h, 10 h and 24 h of incubation. Number of germinated spores was counted from ten microscopic fields and the percentage of germination was calculated. The percentage inhibition/ stimulation of spores germination was calculated.

$$G_1(\%) = \frac{G_1 - G_2}{G_1} \times 100$$

Where $G_1$ = Inhibition/Stimulation of spore germination (%)

$G_1$ = Spore germination in distiled water (control)

$G_2$ = Spore germination in aflatoxin

5.4.2 Effect of aflatoxinz $B_1$ on mycelial growth :

The effect of aflatoxin $B_1$ on the mycelial growth of different test fungi was tested at different concentrations, by poisoned food

technique. Single spore culture of the test fungus was maintained on Potato Dextrose Agar (PDA). Ten days old cultures were grown on PDA medium.

To prepare aflatoxin $B_1$-100 ml of sterilized potato dextrose agar medium was cooled to 45°C in sterile conical flask and mixed with different concentrations of the aflatoxin (0.1 ppm, 0.5 ppm and 1 ppm). Thereafter, these were poured into sterile Petri dishes, allowed to cool and solidify. Three replicates were used for each concentration of the mycotoxin. Control set comprised only PDA medium without any aflatoxin for each fungus.

Fungal discs (5 mm) were punched with steilized cork border from 7 d old pure cultures of the test fungi. One disc in three replicates of each fungus was inoculated separately on to a nutrient agar medium with different concentrations of aflatoxin $B_1$ and incubated at 25±1°C for 7 d Colony diameter was measured at 24 h intervals. Inhibition on mycelial growth was calculated as follows :

$$M_i = \frac{M_c - M_t}{M_c} \times 100$$

Where $M_i$ = Inhibition of mycelial growth

$M_c$ = Colony diameter on control medium

$M_t$ = Colony diameter on aflatoxin treated medium

5.4.3 Seed germination and seedling growth :

One hundred healthy seeds of both the maize varieties viz. Local White (LW) and Local Yellow (LY) were surface sterilised with 0·1%

mercuric chloride solution for 10 minutes and thoroughly washed with sterilised distilled water. Thereafter, they were soaked in 10 ml of 0.1, 0.5, ppm and 1 ppm toxin concentrations for 24 h in 250 ml conical flasks separately. The seeds were then plated at the rate of 10 seeds per Petri dish containing sterilized moist filter paper. The sterilised distilled water served as control. Seeds were incubated at room temperature. The percentage germination of the seeds was recorded after 5 d of incubation. The inhibition of germination of seeds treated with mycotoxin was calculated as follows :

$$S_i = \frac{S_c - S_t}{S_c} \times 100$$

Where $S_i$ = Seed germination (%)

$S_c$ = Germination of seeds in control set

$S_t$ = Germination of seeds in aflatoxin treated sets

Another set of 100 germinated seeds with uniform shoot (1 cm) and root (3 cm) length was selected and transferred to freshly prepared moist chambers. 5 ml of different concentrations of mycotoxins mentioned above was applied on alternate day in three replicates separately and the seedlings (root and shoot) were measured periodically from 5 d to 15 d. Inhibition/promotion of seedling growth was determined by comparing the data with distilled water treated seedlings (control).

6. Effect of aflatoxin on animal :

6.1 Test animal - Healthy albino mice was used as test animal. They were obtained from the Department of Genetics, Veterinary college, Khanapara, Gauhati, Assam.

6.2 Test aflatoxin $B_1$-Pure aflatoxin $B_1$ was obtained from Sigma Chemical Company, U.S.A. Crude aflatoxin $B_1$ extracted from *A. flavus* on local varieties of maize.

8.3 Feeding effects - The albino mice of different age groups, i.e. 3, 6 and 9 weeks old were selected. Half of each age group was male and another half female. The animals were divided according to age and sex into groups of 10. These were kept in separate cages at a constant temperature (25°C) and humidity (60%) under hygienic conditions. In first set of treatment, mice weighing 10-15 g were fed with pure aflatoxin $B_1$ with 0.25 ml of 60 ppb of the solution by incubating an hour before feeding the regular meal. Similarly, in second set of treatment, the same number of mice having the same age and weights were fed with crude aflatoxin $B_1$ isolated from *A. flavus*. In third set of treatment, mice were fed with sterilized maize seeds without any mycotoxins which served as control. Standard sterilized maize seeds were mixed with the toxin and supplied to all the treated mice regularly at meal time.

Observations of the change in weight of mice were recorded for 6 months. Other parameters like effect on liver, skin and rate of mortality of mice were also recorded.

After 7 months, the mice were sacrificed and postmortem examinationswere done for the histopathological study on liver.

The infected parts of the skin were fixed in 10% formalin and 5 micron thick vertical section were cut and stained with haematoxylin stain before microscopic examination.

The rate of mortality was determined using the following formula:

$$\text{Mortality (\%)} = \frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100$$

7. Control of mycotoxin (aflatoxin) by plant extracts :

Dominant plant species used for medicinal purpose by the tribals were selected for the present investigation.

Plant extracts tested - Bulb extracts of *Allium cepa* L. and *A. sativum* L.; leaf extracts of *Thuja orientalis* L. and *Centella asiatica* (Linn) Urban were tested for the control of mycotoxins as suggested by Bilgrami *et al.* (1980).

Aqueous extracts (2:10 W/V) of different plant parts were prepared in glass homogenizer. The homogenized liquid was filtered and centrifuged at 3000 rpm. The supernatent was used as test extract. 1 ml of the test extract was mixed with 24 ml of the liquid rice-flour medium (Misra and Sinha, 1979) in a 250 ml Erlenmeyer flask. For control 1 ml of distilled water was mixed with 24 ml medium and each was run in triplicate. The flasks were sterilized and inoculated with 0.5 ml spore suspension of *A. flavus*. The flasks were incubated at 28±1°C for 10 days and manually shaken twice a day.

On the 11th day of incubation, the fungal mass was filtered through Whatman No.1 filter paper. Mycelial mat was dried at 60°C for two successive days and average mycelial dry weight was determined for the growth of the fungus. Percentage inhibition was calculated by the following formula :

$$\text{Percentage inhibition} = \frac{\text{Control-Treated}}{\text{Control}} \times 100$$

The filtrate was used for the extraction and estimation of aflatoxin $B_1$

## Appendix - I

### List of the media and their constituents used for the Isolation of seed fungi

1.3 Following nutrient media were used during the period of investigation.

(1) Potato Dextrose Agar Medium (Lucy and Bridgmon, 1962).

| | | |
|---|---|---|
| Potato | - | 200 g |
| Dextrose | - | 20 g |
| Agar | - | 20 g |
| Water | - | 1000 ml |

Potatoes were scrubbed clean and cut into small pieces. 200 g of sliced potato was taken and rinsed rapidly in running water, boiled for 1 h, until it became soft. It was then filtered through muslin cloth, the potato pieces were mashed and squeezed. Agar was added to the filtrate and dissolved, dextrose was added and stirred to dissolve. Total volume of the mixture was made upto 1 lit. by adding distilled water and sterilized at 15 psi in autoclave.

(2) Water Agar Medium

| | | |
|---|---|---|
| Agar | - | 15 g |
| Water | - | 1000 ml |

Agar was dissolved in 1000 ml of tap water and sterilized in autoclave at 15 psi for 15 minutes.

(3) Czapek's Dox Agar Medium (Raper and Thom, 1949).

| | | |
|---|---|---|
| $NaNO_3$ | - | 3.0 g |
| $KH_2PO_4$ | - | 1.0 g |
| $MgSO_4.7H_2O$ | - | 0.5 g |
| KCL | - | 0.5 g |
| $FeSO_4.7H_2O$ | - | 0.01 g |
| Surcrose | - | 30.0 g |
| Agar | - | 20.0 g |
| Distilled water | - | 1000 ml |

Sucrose was added prior to final sterilization. Czapek-Dox contains 3.0 g of $NaNO_3$ per liter. 2 g yeast extract per liter may be added.

(4) Peptone-Dextrose-Rose Bengal Agar Medium (Martin, 1950)

| | | |
|---|---|---|
| Agar | - | 20.0 g |
| $KH_2PO_4$ | - | 1.0 g |
| $MgSO_4.7H_2O$ | - | 0.5 g |
| Peptone | - | 5.0 g |
| Dextrose | - | 10.0 g |
| Rose Bengal(%) | - | 3.3 ml |
| Distilled water | - | 1000 ml |
| Streptomycin | - | 30.0 mg (after autoclaving) |

(5) Yeast extract Agar Medium (YES medium), Davis *et al.*, 1972.

| | | |
|---|---|---|
| Yeast extract | - | 4.0 g |
| Malt extract | - | 10.0 g |
| Dextrose | - | 4.0 g |
| Agar | - | 15.0 g |
| Distilled water | - | 1000 ml |
| Tetracycline | - | 30 µg/ml. |

Tetracylcine was added ater sterilization of medium, when it was cooled to 40°C.

(6) Rice-flour liquid Medium (Misra and Sinha, 1979).

| | | |
|---|---|---|
| Rice-flour (Basmati 370) | - | 40 g |
| Sucrose | - | 30 g |
| Yeast extrat | - | 1 g |
| Distilled water | - | 1000 ml |

Rice flour was boiled in 500 ml distilled water for 15 minutes and filtered through fine muslin cloth. Sucrose and yeast extract were added to it and the constituents were thoroughly dissolved. The volume was subsequently raised to 1 litre with distilled water.

(7) SMKY Liquid Medium (Diener and Davis, 1966)

| | | |
|---|---|---|
| Sucrose | - | 200. g |
| $MgSO_4.7H_2O$ | - | 0.5 g |
| $KNO_3$ | - | 30 g |
| Yeast Extract | - | 7 g |
| Distilled water | - | 1000 ml |

8. Czapek's Dox Liquid Medium (Raper & Thom, 1949)

| | | |
|---|---|---|
| $NaNO_3$ | - | 3.0 g |
| $KH_2PO_4$ | - | 1.0 g |
| $MgSO_4.7H_2O$ | - | 0.5 g |
| KCL | - | 0.5 g |
| $FeSO_4.7H_2O$ | - | 0.01 g |
| Sucrose | - | 30.0 g |
| Distilled water | - | 1000 ml |

Statistical analysis has been done wherever necessary following the method of Misra and Misra (1983).

# *Results*

# I. Ecology of seed borne fungi

1. Survey of seed borne fungi:

Table 1.1 and Figure 1.1 depicted the incidence of fungal species on maize seeds in three different seasons, viz. winter, wet summer and autumn, using three methods of isolation. A total of 37 fungal species were isolated in all the seasons. 28 fungal species were isolated in winter, 29 species in summer season and 32 fungal species in autumn. For wet summer and autumn seasons the maize crop was in field and the dominant seed borne fungi recorded were *Alternaria* spp., *Aspergillus* spp., *Cladosporium* spp., *Curvularia lunata*, *Drechlera maydis*, *Fusarium* spp., *Helminthosporium sacchari*, *Mucor* spp., *Penicillium* spp., *Rhizopus* spp., *Trichoderma* spp., *Verticillium albo-atrum*, etc. Most of the field fungi were replaced by the storage fungi in the winter season like *Aspergillus candidus*, *A. flavus*, *A. niger*, *A. ochraceous*, *A. parasiticus*, *A. terreus*, *A. versicolor*, *Fusarium moniliforme*, *F. oxysporum*, *F. solani*, *Penicillium* spp., etc. (Plate-4.A) Some fungal species like *Aspergillus* spp., *Fusarium* spp., *Mucor* spp., *Penicillium* spp., *Verticillium albo-atrum* were common in all the three seasons, though their percentage of occurrence varied considerably.

The total number of fungal species was more in local yellow variety than in local white ones (Fig 1.1). Fig 1.1 also showed that fungal species were maximum during wet summer season followed by autumn and winter on local yellow variety whereas, their number was maximum in autumn followed by wet summer and winter on local white

variety.

2. Different methods of isolation:

Table 1.2, 1.3 and Figure 1.2 depicted the occurrence of fungal species during different seasons by three methods, viz. agar plate method, blotter method and dilution plate method. A total number of 37 species of seed borne fungi belonging to 14 genera was isolated from two varieties of maize in different seasons.

Local white variety harboured 19 species belonging to 8 genera as isolated by agar plate method, 18 fungi belonging to 8 genera by blotter method and 12 species belonging to 5 genera by dilution plate method during winter season. In wet summer season, 20 fungi belonging to 11 genera were isolated by agar plate method, 19 species belonging to 12 genera by blotter method and 15 species belonging to 8 genera by dilution plate method. During autumn season 19 fungal species belonging to 10 genera were isolated by agar plate method, 20 species belonging to 9 genera were isolated by blotter method and 15 fungal species belonging to 7 genera by dilution plate method. Local yellow variety of maize harboured 18 fungal species belonging to 7 genera as isolated by agar plate method, 22 species belonging to 9 genera by blotter method and 13 species belonging to 5 genera by dilution plate method during winter season. In wet summer season, 25 fungal species belonging to 12 genera by agar plate method, 27 species belonging to 13 genera by blotter method and 16 species belonging to 7 genera appeared in the dilution plate method. During autumn season, 24 fungal

species belonging to 11 genera were isolated by agar plate method, 21 species belonging to 9 genera by blotter method and 14 species belonging to 7 genera by dilution plate technique.

*Aspergillus candidus*, *A. flavus*, *A. niger*, *A. versicolor*, *Fusarium moniliforme*, *F. oxysporum*, *F. solani*, *F.* sp., *Helminthosporium sacchari*, *Mucor* sp., *Penicillium brefeldianum*, *P. nigricans*, *P. rubrum*, *P.* sp., *Rhizopus nigricans*, *R.* sp., *Trichoderma harzianum*, *T. viride*, *T.* sp., *Verticillium albo-atrum* and sterile mycelia were isolated by all the three techniques. *Alternaria alternata*, *A. tenuis*, *Aspergillus parasiticus*, *Chaetomium globosum*, *Cladosporium herbarum*, *Cladosporium* sp., *Curvularia lunata*, *Drechlera maydis*, *Mucor hiemalis*, *Penicillium chrysogenum* were detected only by agar plate and blotter methods whereas *Aspergillus ochraceous*, *A. terreus* and *Chaetomium* sp. were isolated only by blotter method. *Penicillium citrinum* and *Trichoderma koningii* were detected by agar plate and dilution plate methods but could not be detected on the blotter method during the whole period of investigation.

From the Tables 1.2, 1.3 and Figure 1.2, it was evident that agar plate and blotter methods were more suitable to isolate the seed borne fungi then dilution plate method. The blotter method allowed more fungi to grow and reproduce compared to the other two methods. Dilution plate method mostly favoured the seed surface mycoflora which were saprophytic in nature like *Aspergillus* sp., *Fusarium* sp., *Mucor*

sp., *Penicillium* sp., *Rhizopus* sp., *Trichoderma* sp. and *Verticillium* sp..

Maximum fungi were observed in wet summer season, followed by autumn and winter in both the varieties. The number of fungal species was more in local yellow variety than in white one (Fig 1.2).

3. Variation of fungal species between the two varieties of maize:

The incidence of fungal species on the two varieties of maize seeds, viz. local white and local yellow varieties was shown in Table 1.2, 1.3 and Figure 1.2. Between the two varieties, local yellow yielded maximum number of fungal species than local white variety. Thirty three seed fungi belonging to 14 genera were isolated from the local yellow variety of maize whereas 22 species belonging to 12 genera appeared on local white variety. *Fusarium* spp., *Mucor* sp., *Penicillium* spp., *Rhizopus* spp., *Trichoderma* spp. and *Verticillium albo-atrum* were dominant on both the maize varieties. *Chaetomium* sp., *Cladosporium* sp., *Drechlera maydis*, *Helminthosporium sacchari* and sterile mycelia were less frequent on both the varieties. Fungal species, viz. *Alternaria tenuis* and *Mucor hiemalis* were isolated only from local white variety. The fungal species which rarely occurred in local yellow only were *Alternaria alternata*, *Aspergillus ochraceous*, *A. parasiticus*, *A. terreus*, *A. versicolor*, *Chaetomium globosum*, *Cladosporium herbarum*, *Curvulara lunata*, *Penicillium citrinum*, *P. nigricans*, *Rhizopus nigricans*, *Stachybotrys atra*, *Trichoderma koningii* and *T. viride*.

4. Quantitative assessment of fungal species:

From local white maize variety, agar plate method yielded 19, 20 and 19 fungal species during winter, wet summer and autumn respectively, blotter method harboured 18, 19 and 20 species respectively and dilution plate method supported 12, 15 and 15 fungi respectively (Table 1.4). In case of the local yellow variety, agar plate method yielded 18, 25 and 24 fungal species during winter, wet summer and autumn respectively, blotter method yielded 22, 27 and 21 species respectively and 13, 16 and 14 species by dilution plate method respectively (Table 1.5).

Thirty seven fungal species were isolated from both the varieties. *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., *Trichoderma harzianum* and *Verticillium albo-atrum* occurred in all the seasons. The percentage frequency of *Fusarium* spp. was maximum followed by *Aspergillus* spp. and *Penicillium* spp. (Fig 1.3). *Curvularia* sp. and *Stachybotrys atra* occurred with least frequency and were restricted to local yellow variety.

The changes in fungal population of two local varieties of maize seeds during different seasons (winter, wet summer and autumn) is presented in Table 1.6. From the table it is clear that *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., *Rhizopus* sp. dominated the surface fungal flora of maize seeds.

5. Comparative studies between two different storage practices in respect to the incidence of fungi on two local varieties of maize seeds:

Table 1.6 depicted the incidence of seed fungi on two local varieties stored in two storage conditions as isolated by agar plate, blotter and dilution plate methods during winter season.

In both the maize varieties, a total number of 26 fungal species belonging to 10 genera was isolated (Fig. 1.4). Dominant fungal genera were *Aspergillus*, *Fusarium* and *Penicillium*. In local white variety, (Plate-3.B) a total number of 17 fungal species was isolated from both shaded and smoked storage conditions. They were *Aspergillus candidus*, *A. flavus*, *A. niger*, *Fusarium moniliforme*, *F. oxysporum*, *F. solani*, *F.* sp, *Mucor hiemalis*, *Mucor* sp., *Penicillium brefeldianum*, *P. chrysogenum*, *P. rubrum*, *P.* sp., *Rhizopus* sp., *Trichoderma harzianum*, *T.* sp., and sterile mycelia, whereas in local yellow variety, (Plate-3.C) a total number of 20 and 19 fungal species were isolated from shaded and smoked storage conditions respectively. They were *Aspergillus candidus*, *A. flavus*, *A. niger*, *A. ochraceous*, *A. parasiticus*, *A. terreus*, *A. versicolor*, *Chaetomium globosum*, *Cladosporium herbarum*, *Fusarium moniliforme*, *F. oxysporum*, *F. solani*, *F.* sp., *Mucor* sp., *Penicillium brefeldianum*, *P. chrysogenum*, *P. citrinum*, *P. nigricans*, *P.* sp., *Rhizopus* sp., *Stachybotrys atra*, *Trichoderma harzianum*, and sterile mycelia.

As a result, local yellow variety was found to have more number of fungal species than the local white variety.

The percentage frequency occurrence of different fungi on maize seeds of different storage conditions as determined by the three methods of isolation is presented in Table 1.8. From the table, it is evident that the maize seeds of two different storage conditions were frequently associated with a good number of fungi. Amongst which *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. were the most dominant fungi.

In shaded condition, local white variety recorded *Fusarium moniliforme* to have the highest frequency of 13.3% and the lowest of 0.8% in *Aspergillus niger* and *Penicillium rubrum* as isolated by agar plate method. Blotter method and dilution plate method recorded *Fusarium* sp. to have the maximum frequency of 10.9% and 25%, minimum by *Aspergillus niger* and *Rhizopus* sp. of 0.8% and 1.2% respectively. Smoked condition of storage recorded *Fusarium solani* as to have the highest frequency of 12.9%, 13,8% and 18% while the lowest was observed in *Rhizopus* sp. (2%), *Penicillium rubrum* (2.3%) and *Rhizopus* sp. (1.4%) as isolated by agar plate, blotter and dilution plate methods respectively.

Local yellow variety recorded in shaded condition of storage, *Fusarium* sp. to have the highest frequency of 19.9% whereas the lowest were shared by *Penicillium brefeldianum* (20%) and *Trichoderma harzianum* (2.0%) as isolated by agar plate method. Blotter method also

recorded *Fusarium* sp. as having the highest frequency (19.0%) while *F. moniliforme*, the lowest of 0.1%. *Fusarium solani* was recorded to have the maximum frequency of 20% and *Rhizopus* sp., the lowest (3.4%) as isolated by dilution plate method.

Similarly, in smoked condition, *Fusarium* sp. was recorded to have a high frequency of occurrence of 22.1% and 20% and the minimum frequency was recorded by *Penicillium brefeldianum* (1.2%) and *Cladosporium herbarum* (0.3%) by agar plate and blotter methods respectively. Dilution plate method recorded *Fusarium solani*, the highest frequency of occurrence (11.2%) and lowest, *Aspergillus niger* (3.4%).

It can also be observed from Table 1.7 that in both storage conditions, Fusarium spp. has the highest frequency of occurrence other than the other fungal species occurred on maize seeds.

Figure 1.4 shows that there was not much differences in the total no. of fungal species isolated by the methods between the two storage conditions. Both show almost equal number of fungal species even though their frequency of occurrence may vary.

6. Seasonal variation in seed infection and chitin contents in two local maize varieties:

Table 1.8 depicted the percentage of seed infection and the amount of chitin content on the two local varieties of maize seeds. Fungal infection was maximum (100%) during wet summer and autumn seasons and minimum (40%) during winter season. However, trend of

chitin contents in maize seeds during wet summer and autumn seasons differed from each other, even though they had the same percentage of seed infection. Autumn season revealed much higher (328.2 µg/g in local white variety and 440.4 µg/g in local yellow variety) amount than wet summer season (246 µg/g in local white variety and 250.9 µg/g in local yellow variety). Lowest chitin content (126 µg/g in local white variety and 128.5 µg/g in local yellow variety) in seeds was observed during winter season in both the maize varieties.

Figure 1.5 showed that the percentage of seed infection and chitin contents were observed to be higher in local yellow variety than the local white variety in all seasons.

During winter season, the percentage of seed infection as well as amount of chitin content was found to be much less than other seasons. Seed infections(%) in wet summer and autumn were observed to be equal but amount of chitin content was observed to be more in autumn than wet summer.

**Table 1.1** : Distribution of seed borne fungi in different seasons.

| Fungi | Seasons | | |
|---|---|---|---|
| | Winter | Wet Summer | Autumn |
| *Alternaria alternata* (Fries) Keissler | – | – | – |
| | (–) | (+) | (+) |
| *A. tenuis* Nees | – | + | + |
| | (–) | (–) | (–) |
| *Aspergillus candidus* Link ex Fries | + | + | + |
| | (+) | (+) | (+) |
| *A. flavus* Link ex Fries | + | + | + |
| | (+) | (+) | (+) |
| *A. niger* Van Tieghem | + | – | + |
| | (+) | (–) | (+) |
| *A. ochraceous* Wilhelm | – | – | – |
| | (+) | (+) | (+) |
| *A. parasiticus* Speare | – | – | – |
| | (+) | (+) | (+) |
| *A. terreus* Thom | – | – | – |
| | (+) | (–) | (–) |
| *A. versicolor* (Vuillemin) Tiraboschi | – | – | – |
| | (+) | (+) | (+) |
| *Chaetomium globosum* Kunze | – | – | – |
| | (+) | (–) | (+) |
| *Chaetomium* sp. | – | + | + |
| | (–) | (+) | (–) |
| *Cladosporium herbarum* Link ex Fries | – | – | – |
| | (+) | (+) | (+) |
| *Cladosporium sp.* | – | + | + |
| | (–) | (+) | (–) |
| *Curvularia lunata* (Wakker) Boedijn | – | – | – |
| | (–) | (+) | (–) |
| *Drechlera maydis* (Nisikado) Subram & Jain | – | + | – |
| | (–) | (+) | (–) |
| *Fusarium moniliforme* Sheldon | + | + | + |
| | (+) | (+) | (+) |
| *F. oxysporum* Schl. ex Fries | + | + | + |
| | (+) | (+) | (+) |
| *F. solani* (Mart.) Sacc | + | + | + |
| | (+) | (+) | (+) |
| *F.* sp . | + | + | + |
| | (+) | (+) | (+) |
| *Helminthosporium sacchari* Butler | – | + | + |
| | (–) | (+) | (+) |
| *Mucor hiemalis* Wehmer | + | – | + |
| | (–) | (–) | (–) |
| *Mucor* Sp. | + | + | + |
| | (+) | (+) | (+) |

Contd.

**Table 1.1**

| Fungi | Seasons | | | |
|---|---|---|---|---|
| | Winter | Wet | Summer | Autumn |
| *Penicillium brefeldianum* Dodge | + | + | + | |
| | (+) | (+) | (+) | |
| *P. chrysogenum* Thom | + | + | + | |
| | (+) | (+) | (+) | |
| *P. citrinum* Thom | − | − | − | |
| | (+) | (−) | (+) | |
| *P. nigricans* Bain. ex Thom | − | − | − | |
| | (+) | (+) | (+) | |
| *P. rubrum* Stoll | + | + | + | |
| | (−) | (+) | (+) | |
| *P.* sp. | + | + | + | |
| | (+) | (+) | (+) | |
| *Rhizopus nigricans* Ehrenberg | − | − | − | |
| | (−) | (+) | (+) | |
| *R.* sp. | − | − | − | |
| | (+) | (+) | (+) | |
| *Stachybotrys atra* Corda | − | − | − | |
| | (+) | (−) | (−) | |
| *Trichoderma harzianum* Rifai | + | + | + | |
| | (+) | (+) | (+) | |
| *T. koningii* Oud | − | − | − | |
| | (−) | (−) | (+) | |
| *T. viride* Pers ex Fries | − | − | − | |
| | (−) | (+) | (−) | |
| *T.* sp. | + | + | + | |
| | (−) | (−) | (−) | |
| *Verticillium albo-atrum* Rienke & Berth | − | + | + | |
| | (−) | (+) | (+) | |
| Mycelial sterilia | + | + | + | |
| | (+) | (+) | (+) | |

+ = Present  Signs in parentheses represent Local yellow variety and in open Local white variety.

− = Absent

**Table 1.2 :** Incidence of seed fungi on local white variety (LW) of maize during different seasons.

| Fungi | Winter | | | Wet summer | | | Autumn | | |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | D | A | B | D | A | B | D |
| *Alternaria tenuis* | − | − | − | + | + | − | + | − | − |
| *Aspergillus candidus* | + | + | + | + | + | + | + | + | + |
| *A. flavus* | + | + | + | + | + | + | + | + | + |
| *A. niger* | + | + | + | − | − | − | + | + | + |
| *Chaetomium* sp. | − | − | − | − | + | − | − | + | − |
| *Cladosporium* sp. | − | − | − | + | + | − | + | − | − |
| *Drechlera maydis* | − | − | − | + | + | − | − | − | − |
| *Fusaruim moniliforme* | + | + | + | + | + | + | + | + | + |
| *F. oxysporum* | + | + | − | + | + | + | − | + | − |
| *F. solani* | + | + | + | + | + | + | + | + | + |
| *F.* sp. | + | + | + | + | + | + | + | + | + |
| *Helminthosporium sacchari* | − | − | − | + | + | + | + | + | − |
| *Mucor hiemalis* | + | + | − | − | − | − | + | + | − |
| *Mucor* sp. | + | + | − | + | + | + | + | + | + |
| *Penicillium brefeldianum* | + | + | + | + | − | − | + | + | + |
| *P. chrysogenum* | + | + | − | + | + | − | − | + | − |
| *P. rubrum* | + | + | + | + | + | + | + | + | + |
| *P.* sp. | + | + | + | + | + | + | + | + | + |
| *Rhizopus* sp. | + | + | + | + | + | + | + | + | + |
| *Trichoderma harzianum* | + | + | − | + | + | + | + | + | + |
| *T.* sp. | + | − | − | + | − | + | + | + | + |
| *Verticillium albo-atrum* | − | − | − | + | + | + | + | + | + |
| Mycelial sterilia | + | + | + | + | + | + | + | + | + |

A = Agar plate method
B = Blotter method
D = Dilution plate method

+ = present
− = absent

**Table 1.3 :** Incidence of seed fungi on local yellow variety (LY) of maize during different seasons.

| Fungi | Winter | | | Wet summer | | | Autumn | | |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | D | A | B | D | A | B | D |
| *Alternaria alternata* | – | – | – | + | + | – | + | – | – |
| *Aspergillus candidus* | + | + | + | + | + | + | + | + | + |
| *A. flavus* | + | + | + | + | + | + | + | + | + |
| *A. niger* | + | + | + | – | – | – | + | + | + |
| *A. ochraceous* | – | + | – | – | + | – | – | + | – |
| *A. parasiticus* | + | + | – | – | + | – | – | + | – |
| *A. terreus* | – | + | – | – | – | – | – | – | – |
| *A. versicolor* | + | + | + | + | + | – | + | + | – |
| *Chaetomium globosum* | – | + | – | – | – | – | + | + | – |
| *Chaetomium* sp. | – | – | – | – | + | – | – | – | – |
| *Cladosporium herbarum* | – | + | – | + | + | – | + | – | – |
| *Cladosporium sp.* | – | – | – | + | + | – | – | – | – |
| *Curvularia lunata* | – | – | – | + | + | – | – | – | – |
| *Drechlera maydis* | – | – | – | + | + | – | – | – | – |
| *Fusarium moniliforme* | + | + | + | + | + | + | + | + | + |
| *F. oxysporum* | + | + | – | + | + | + | + | + | – |
| *F. solani* | + | + | + | + | + | + | + | + | + |
| *F. sp.* | + | + | + | + | + | + | + | + | + |
| *Helminthosporium sacchari* | – | – | – | + | + | – | + | + | – |
| *Mucor* sp. | – | + | – | + | + | + | + | + | + |
| *Penicillium brefeldianum* | + | + | – | + | – | – | + | + | – |
| *P. chrysogenum* | + | + | – | + | + | – | – | – | – |
| *P. citrinum* | – | – | + | – | – | – | + | – | – |
| *P. nigricans* | + | + | + | + | + | + | + | + | + |

Contd.

**Table 1.3**

| Fungi | Winter | | | Wet summer | | | Autumn | | |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | D | A | B | D | A | B | D |
| *P. rubrum* | − | − | − | + | + | + | + | − | + |
| *P.* sp. | + | + | + | + | + | + | + | + | + |
| *Rhizopus nigricans* | − | − | − | + | + | + | + | + | − |
| *R.* sp. | − | − | + | + | + | + | + | + | + |
| *Stachybotrys atra* | + | − | − | − | − | − | − | − | − |
| *Trichoderma harzianum* | + | + | − | + | + | + | + | + | − |
| *T. koningii* | − | − | − | − | − | − | + | − | + |
| *T. viride* | − | − | − | + | + | + | − | − | − |
| *Verticillium albo-atrum* | − | − | − | + | + | + | + | + | + |
| Mycelial sterilia | + | + | + | + | + | + | + | + | + |

A = Agar plate method
B = Blotter method
D = Dilution plate method

+ = present
− = absent

Table 1.4 : Seasonal variation in the frequency of occurrence (%) of seed fungi on local white variety of maize.

| Fungi | Winter | | | Wet summer | | | Autumn | | |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | D | A | B | D | A | B | D |
| *Alternaria tenuis* | - | - | - | 4.7 | 7.7 | - | 4.9 | - | - |
| *Aspergillus candidus* | 3.5 | 4.2 | 14.9 | 3.3 | 3.5 | 16.2 | 5.2 | 10.0 | 10.2 |
| *A. flavus* | 6.2 | 5.9 | 6.9 | 2.5 | 4.7 | 8.1 | 10.0 | 15.2 | 7.5 |
| *A. niger* | 1.2 | 2.3 | 7.8 | - | - | - | 4.2 | 5.7 | 9.6 |
| *Chaetomium* sp. | - | - | - | - | 2.1 | - | - | 4.2 | - |
| *Cladosporium* sp. | - | - | - | 1.9 | 1.2 | - | 2.5 | - | - |
| *Drechlera maydis* | - | - | - | 1.5 | 5.2 | - | - | - | - |
| *Fusarium moniliforme* | 9.5 | 10.3 | 16.7 | 14.5 | 6.9 | 14.2 | 8.2 | 8.7 | 12.2 |
| *F. oxysporum* | 10.5 | 6.9 | - | 2.5 | 5.1 | 8.1 | - | 2.1 | - |
| *F. solani* | 11.5 | 10.9 | 17.9 | 11.2 | 7.9 | 13.1 | 10.2 | 10.0 | 9.3 |
| *F.* sp. | 9.0 | 10.7 | 19.8 | 7.5 | 8.8 | 15.3 | 9.3 | 8.2 | 8.9 |
| *Helminthosporium sacchari* | - | - | - | 4.9 | 9.2 | 0.1 | 5.7 | 2.2 | - |
| *Mucor hiemalis* | 5.2 | 4.7 | - | - | - | - | 5.2 | 2.9 | - |
| *Mucor* sp. | 2.1 | 5.9 | - | 6.1 | 4.5 | 0.7 | 4.7 | 4.2 | 7.8 |
| *Penicillium brefeldianum* | 5.3 | 6.9 | 4.4 | 4.1 | - | - | 3.3 | 0.2 | 6.9 |
| *P. chrysogenum* | 6.7 | 6.1 | - | 9.1 | 5.9 | - | - | 1.3 | - |
| *P. rubrum* | 1.2 | 2.1 | 3.7 | 0.5 | 6.8 | 2.7 | 5.2 | 5.4 | 5.3 |
| *P.* sp. | 6.7 | 7.3 | 2.1 | 2.1 | 7.9 | 3.2 | 4.1 | 3.0 | 7.3 |
| *Rhizopus* sp. | 3.3 | 3.5 | 1.3 | 2.9 | 4.5 | 0.8 | 3.0 | 2.1 | 6.0 |
| *Trichoderma harzianum* | 3.9 | 2.2 | - | 6.8 | 1.2 | 1.3 | 2.9 | 2.1 | 5.4 |
| *T.* sp. | 4.1 | - | - | 5.8 | - | 5.7 | 0.7 | 0.4 | 0.8 |
| *Verticillium albo-atrum* | - | - | - | 6.1 | 4.1 | 5.9 | 4.3 | 6.7 | 1.3 |
| Mycelial sterilia | 4.3 | 1.7 | 0.8 | 2.5 | 3.9 | 4.4 | 5.5 | 5.6 | 0.2 |

A = Agar plate method  - = absent
B = Blotter method
D = Dilution plate method

Table 1.5 : Seasonal changes in frequency of occurrence (%) of seed fungi on local yellow variety of maize.

| Fungi | Winter | | | Wet summer | | | Autumn | | |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | D | A | B | D | A | B | D |
| *Alternaria alternata* | - | - | - | 2.3 | 5.0 | - | 2.0 | - | - |
| *Aspergillus candidus* | 8.0 | 5.4 | 9.9 | 3.1 | 2.3 | 10.3 | 4.0 | 4.6 | 14.7 |
| *A. flavus* | 4.0 | 8.1 | 8.7 | 2.3 | 3.4 | 9.5 | 9.6 | 10.5 | 8.9 |
| *A. niger* | 4.0 | 2.0 | 6.8 | - | - | - | 2.4 | 4.0 | 5.6 |
| *A. ochraceous* | - | 3.4 | - | - | 2.4 | - | - | 3.0 | - |
| *A. parasiticus* | 12.0 | 5.3 | - | - | 1.2 | - | - | 4.0 | - |
| *A. terreus* | - | 8.0 | - | - | - | - | - | - | - |
| *A. versicolor* | 4.0 | 5.5 | 6.9 | 1.5 | 2.1 | - | 8.0 | 4.0 | - |
| *Chaetomium globosum* | - | 2.7 | - | - | - | - | 4.0 | 2.0 | - |
| *Chaetomium* sp. | - | - | - | - | 1.1 | - | - | - | - |
| *Cladosporium herbarum* | - | 0.4 | - | 2.7 | 3.5 | - | 2.0 | - | - |
| *Cladosporium* sp. | - | - | - | 0.4 | 0.4 | - | - | - | - |
| *Curvularia lunata* | - | - | - | 0.6 | 3.4 | - | - | - | - |
| *Drechlera maydis* | - | - | - | 1.1 | 4.7 | - | - | - | - |
| *Fusarium moniliforme* | 8.0 | 0.6 | 9.8 | 4.1 | 5.7 | 13.3 | 6.0 | 6.0 | 9.7 |
| *F. oxysporum* | 10.0 | 0.8 | - | 2.2 | 4.4 | 7.7 | 6.0 | 1.0 | - |
| *F. solani* | 4.0 | 10.0 | 15.6 | 6.5 | 8.6 | 10.5 | 5.6 | 16.0 | 8.9 |
| *F.* sp. | 21.0 | 17.0 | 12.4 | 8.3 | 7.7 | 10.8 | 8.0 | 5.0 | 11.7 |
| *Helminthosporium sacchari* | - | - | - | 4.3 | 5.7 | - | 4.4 | 5.4 | - |
| *Mucor* sp. | - | 1.1 | - | 9.5 | 3.7 | 2.7 | 4.0 | 6.0 | 6.8 |
| *Penicillium brefeldianum* | 1.6 | 2.0 | - | 3.3 | - | - | 2.7 | 6.5 | - |
| *P. chrysogenum* | 3.4 | 2.0 | - | 0.3 | 4.0 | - | - | - | - |
| *P. citrinum* | - | - | 5.3 | - | - | - | 2.1 | - | - |
| *P. nigricans* | 4.0 | 3.0 | 7.6 | 4.2 | 2.2 | 4.5 | 1.3 | 4.4 | 5.3 |
| *P. rubrum* | - | - | - | 2.2 | 5.2 | 5.3 | 3.5 | - | 4.9 |
| *P.* sp. | 5.0 | 2.7 | 6.7 | 1.4 | 6.2 | 6.9 | 3.3 | 6.0 | 7.9 |

Contd.

Table 1.5

| Fungi | Winter | | | Wet summer | | | Autumn | | |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | D | A | B | D | A | B | D |
| *Rhizopus nigricans* | - | - | - | 6.6 | 3.1 | 4.7 | 4.0 | 2.4 | - |
| *R.* sp. | - | - | 3.4 | 3.3 | 1.2 | 5.7 | 2.3 | 1.3 | 5.7 |
| *Stachybotrys atra* | 3.0 | - | - | - | - | - | - | - | - |
| *Trichoderma harzianum* | 2.0 | 2.7 | - | 7.5 | 0.1 | 2.2 | 3.3 | 1.4 | - |
| *T. koningii* | - | - | - | - | - | - | 3.5 | - | 4.2 |
| *T. viride* | - | - | - | 6.8 | 0.3 | 3.4 | - | - | - |
| *Verticillium albo-atrum* | - | - | - | 5.9 | 5.6 | 1.7 | 6.0 | 3.7 | 4.1 |
| Mycelial sterilia | 2.0 | 5.0 | 3.8 | 8.9 | 4.5 | 0.5 | 4.1 | 2.1 | 1.9 |

A = Agar plate method
B = Blotter method
D = Dilution plate method

- = absent

Table 1.6 : Number of fungal species ($x10^3$/g) on maize seeds in different seasons.

| Fungi | Local White variety (LW) | | | Local Yellow Variety (LY) | | |
|---|---|---|---|---|---|---|
| | Seasons | | | | | |
| | Winter | Wet Summer | Autumn | Winter | Wet Summer | Autumn |
| *Aspergillus candidus* | 0.9 | 0.2 | 0.2 | 0.9 | 0.3 | 0.7 |
| *A. flavus* | 0.9 | 0.1 | 0.5 | 0.7 | 0.5 | 0.9 |
| *A. niger* | 0.8 | - | 0.6 | 0.8 | - | 0.6 |
| *A. versicolor* | - | - | - | 0.9 | - | - |
| *Fusarium moniliforme* | 0.7 | 0.2 | 0.2 | 0.8 | 0.3 | 0.7 |
| *F. oxysporum* | - | 0.1 | - | - | 0.7 | - |
| *F. solani* | 0.9 | 0.1 | 0.3 | 0.6 | 0.5 | 0.9 |
| *F.* sp. | 0.8 | 0.3 | 0.9 | 0.4 | 0.8 | 0.7 |
| *Helminthosporium sacchari* | - | 0.1 | - | - | - | - |
| *Mucor* sp. | - | 0.7 | 0.8 | - | 0.7 | 0.8 |
| *Penicillium brefeldianum* | 0.4 | - | 0.9 | - | - | - |
| *P. citrinum* | - | - | - | 0.3 | - | - |
| *P. nigricans* | - | - | - | 0.6 | 0.5 | 0.3 |
| *P. rubrum* | 0.7 | 0.7 | 0.3 | - | 0.3 | 0.9 |
| *P.* sp. | 0.1 | 0.2 | 0.3 | 0.7 | 0.9 | 0.9 |
| *Rhizopus nigricans* | - | - | - | - | 0.7 | - |
| *R.* sp. | 0.3 | 0.8 | 0.1 | 0.4 | 0.7 | 0.7 |
| *Trichoderma harzianum* | - | 0.3 | 0.4 | - | 0.2 | - |
| *T. koningii* | - | - | - | - | - | 0.2 |
| *T. viride* | - | - | - | - | 0.4 | - |
| *T.* sp. | - | 0.7 | 0.8 | - | - | - |
| *Verticillium albo-atrum* | 0.5 | 0.9 | 0.3 | 0.1 | 0.7 | 0.1 |
| Mycelial sterilia | 0.8 | 0.4 | 0.2 | 0.8 | 0.5 | 0.9 |

- = absent

Table 1.6 : Incidence of seed fungi on two varieties of maize under different storage conditions.

| Fungi | Local White variety (LW) | | | | | | Local Yellow Variety (LY) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Shaded condition | | | Smoked condition | | | Shaded condition | | | Smoked condition | | |
| | A | B | D | A | B | D | A | B | D | A | B | D |
| *Aspergillus candidus* | + | + | + | + | + | + | + | + | + | + | + | + |
| *A. flavus* | + | + | + | + | + | + | + | + | + | + | + | + |
| *A. niger* | + | + | + | + | + | + | + | + | + | + | + | + |
| *A. ochraceous* | - | - | - | - | - | - | - | + | - | - | + | - |
| *A. parasiticus* | - | - | - | - | - | - | + | - | - | + | + | - |
| *A. terreus* | - | - | - | - | - | - | - | + | - | - | - | - |
| *A. versicolor* | - | - | - | - | - | - | + | + | + | + | + | + |
| *Chaetomium globosum* | - | - | - | - | - | - | - | + | - | - | + | - |
| *Cladosporium herbarum* | - | - | - | - | - | - | - | + | - | - | + | - |
| *Fusarium moniliforae* | + | + | + | + | + | + | + | + | + | + | + | + |
| *F. oxysporum* | + | + | - | + | + | - | + | + | - | + | + | - |
| *F. solani* | + | + | + | + | + | + | + | + | + | + | + | + |
| *F. sp.* | + | + | + | + | + | + | + | + | + | + | + | + |
| *Mucor hiemalis* | + | + | - | + | + | - | - | - | - | - | - | - |
| *Mucor sp.* | + | + | - | + | + | - | - | + | - | - | - | - |
| *Penicillium brefeldianum* | + | + | + | + | + | + | + | + | - | + | + | - |
| *P. chrysogenun* | + | + | - | + | + | - | + | + | - | + | + | - |
| *P. citrinum* | - | - | - | - | - | - | - | - | + | - | - | + |
| *P. nigricans* | - | - | - | - | - | - | - | - | - | + | + | + |
| *P. rubrum* | + | + | + | + | + | + | - | - | - | - | - | - |
| *P. sp.* | + | + | + | + | + | + | + | + | + | + | + | + |
| *Rhizopus sp.* | + | + | + | + | + | + | - | - | + | - | - | - |

Table 1.6

| Fungi | Local White variety (LW) | | | | | | Local Yellow Variety (LY) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Shaded condition | | | Smoked condition | | | Shaded condition | | | Smoked condition | | |
| | A | B | D | A | B | D | A | B | D | A | B | D |
| *Stachybotrys atra* | - | - | - | - | - | - | - | - | - | + | - | - |
| *Trichoderma harzianum* | + | + | - | + | + | - | + | + | - | - | + | - |
| *T.* sp. | + | - | - | + | - | - | - | - | - | - | - | - |
| *Mycelial sterilia* | + | + | + | + | + | + | + | + | + | + | + | + |

A = Agar plate method
B = Blotter method
D = Dilution plate method

+ = present
- = absent

Table 1.7 : Frequency of seed fungi on two varieties of maize under different storage conditions.

| Fungi | Local White variety (LW) | | | | | | Local Yellow Variety (LY) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Shaded condition | | | Smoked condition | | | Shaded condition | | | Smoked condition | | |
| | A | B | D | A | B | D | A | B | D | A | B | D |
| *Aspergillus candidus* | 3.0 | 4.0 | 12.4 | 4.0 | 4.4 | 17.4 | 7.4 | 5.1 | 9. 8 | 8.6 | 5.7 | 10.0 |
| *A. flavus* | 3.4 | 4.3 | 8.6 | 9.0 | 7.5 | 5.2 | 2.7 | 8.2 | 7.4 | 5.3 | 8.0 | 10.0 |
| *A. niger* | 0.8 | 0.8 | 8.0 | 1.6 | 3.8 | 7.6 | 3.3 | 2.1 | 10.0 | 4.7 | 1.9 | 3.4 |
| *A. ochraceous* | - | - | - | - | - | - | - | 1.7 | - | - | 1.7 | - |
| *A. parasiticus* | - | - | - | - | - | - | 9.9 | - | - | 14.1 | 5.3 | - |
| *A. terreus* | - | - | - | - | - | - | - | 8.0 | - | - | - | - |
| *A. versicolor* | - | - | - | - | - | - | 3.5 | 6.2 | 3.8 | 4.5 | 4.8 | 10.0 |
| *Chaetomium globosum* | - | - | - | - | - | - | - | 2.9 | - | - | 2.5 | - |
| *Cladosporium herbarum* | - | - | - | - | - | - | - | 0.5 | - | - | 0.3 | - |
| *Fusarium moniliforme* | 13.3 | 10.0 | 16.9 | 5.7 | 10.6 | 16.5 | 9.1 | 0.1 | 11.0 | 6.9 | 1.1 | 8.6 |
| *F. oxysporum* | 10.7 | 9.8 | - | 10.3 | 4.0 | - | 10.0 | 0.9 | - | 10.0 | 0.7 | - |
| *F. solani* | 10.1 | 8.7 | 17.8 | 12.9 | 13.8 | 18.0 | 6.7 | 18.8 | 20.0 | 1.3 | 16.2 | 11.2 |
| *F.* sp. | 13.3 | 10.9 | 25.0 | 4.7 | 10.5 | 14.6 | 19.9 | 19.0 | 16.0 | 22.1 | 20.0 | 8.8 |
| *Mucor hiemalis* | 4.8 | 4.9 | - | 6.2 | 4.5 | - | - | - | - | - | - | - |
| *Mucor* sp. | 0.9 | 6.2 | - | 3.2 | 5.6 | - | - | 1.1 | - | - | - | - |
| *Penicillium brefeldianum* | 4.1 | 7.9 | 4.6 | 6.3 | 5.9 | 4.2 | 2.0 | 1.4 | - | 1.2 | 2.6 | - |
| *P. chrysogenun* | 6.2 | 5.9 | - | 6.5 | 6.3 | - | 4.0 | 1.9 | - | 2.8 | 2.1 | - |
| *P. citrinum* | - | - | - | - | - | - | - | - | 6.0 | - | - | 4.6 |
| *P. nigricans* | - | - | - | - | - | - | - | - | - | 4.0 | 3.0 | 7.6 |
| *P. rubrum* | 0.8 | 1.9 | 4.0 | 1.6 | 2.3 | 3.4 | - | - | - | - | - | - |
| *P.* sp. | 6.5 | 8.2 | 2.2 | 6.2 | 6.4 | 2.0 | 6.7 | 2.8 | 6.4 | 3.3 | 2.6 | 7.0 |
| *Rhizopus* sp. | 2.0 | 4.0 | 1.2 | 4.6 | 3.0 | 1.4 | - | - | 3.4 | - | - | - |

Table 1.7

| Fungi | Local White variety (LW) | | | | | | Local Yellow Variety (LY) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Shaded condition | | | Smoked condition | | | Shaded condition | | | Smoked condition | | |
| | A | B | D | A | B | D | A | B | D | A | B | D |
| *Stachybotrys atra* | - | - | - | - | - | - | - | - | - | 3.0 | - | - |
| *Trichoderma harzianum* | 4.5 | 1.8 | - | 3.3 | 2.6 | - | 2.0 | 2.9 | - | - | 2.5 | - |
| *T.* sp. | 3.8 | - | - | 4.4 | - | - | - | - | - | - | - | - |
| Mycelial sterilia | 4.2 | 1.7 | 0.9 | 4.4 | 1.7 | 0.7 | 2.0 | 5.0 | 3.9 | 2.0 | 5.0 | 3.7 |

A = Agar plate method
B = Blotter method
D = Dilution plate method

+ = present
- = absent

Table 1.8 : Seasonal variation in fungal populatiuon ($x10^3$ per g) on maize seeds under different storage conditions.

| Fungi | Local White variety (LW) | | | | | | Local Yellow Variety (LY) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Shaded condition | | | Smoked condition | | | Shaded condition | | | Smoked condition | | |
| | A | B | D | A | B | D | A | B | D | A | B | D |
| *Aspergillus candidus* | 0.4 | | | 0.4 | | | 0.8 | | | 0.1 | | |
| *A. flavus* | 0.6 | | | 0.2 | | | 0.4 | | | 0.1 | | |
| *A. niger* | 0.8 | | | 0.6 | | | 0.1 | | | 0.4 | | |
| *A. versicolor* | - | | | - | | | 0.8 | | | 0.1 | | |
| *Fusarium moniliforme* | 0.9 | | | 0.5 | | | 0.1 | | | 0.6 | | |
| *F. solani* | 0.8 | | | 0.8 | | | 0.2 | | | 0.2 | | |
| *F. sp.* | 0.5 | | | 0.6 | | | 0.6 | | | 0.8 | | |
| *Penicillium brefeldianum* | 0.6 | | | 0.2 | | | | | | - | | |
| *P. citrinum* | - | | | - | | | 0.6 | | | 0.6 | | |
| *P. nigricans* | - | | | - | | | - | | | 0.6 | | |
| *P. rubrum* | 0.4 | | | 0.4 | | | - | | | - | | |
| *P. sp.* | 0.2 | | | 0.2 | | | 0.4 | | | 0.7 | | |
| *Verticillium albo-atrum* | 0.5 | | | 0.5 | | | 0.5 | | | 0.2 | | |
| Mycelial sterilia | 0.9 | | | 0.7 | | | 0.9 | | | 0.7 | | |

- = absent

**Table 1.8** : Seasonal variation in seed infection and chitin content (ug/g) on two maize varieties.

| Fungi | Local White variety (LW) | | Local Yellow Variety (LY) | |
|---|---|---|---|---|
| | Infection(%) | Chitin content(μg/g) | Infection(%) | Chitin content(μg/g) |
| Winter | 40 | 126 ±16.2 | 50 | 128.5 ±21.0 |
| Wet Summer | 100 | 246 ±38.2 | 100 | 250.9 ±27.5 |
| Autumn | 100 | 328.2 ±54.5 | 100 | 440.4 ±45.0 |

**Figure 1.1.** Seasonal variation of fungal species on seeds of local white (LW) and local yellow (LY) varieties of maize.

Fig. 1.1.

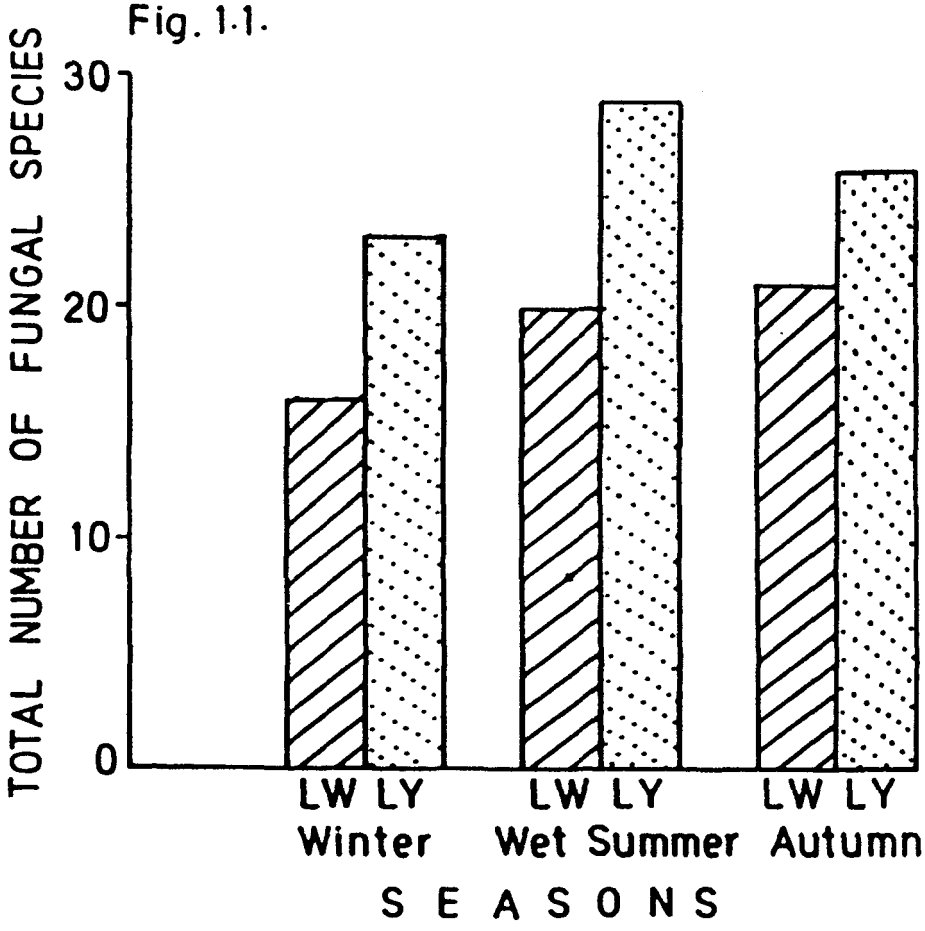

TOTAL NUMBER OF FUNGAL SPECIES
30
20
10
0
LW LY
Winter
LW LY
Wet Summer
LW LY
Autumn
S E A S O N S

**Figure 1.2.** Seasonal variation of fungal species on seeds of local white (LW) and local yellow (LY) varieties of maize using different methods.

Fig. 1.2.

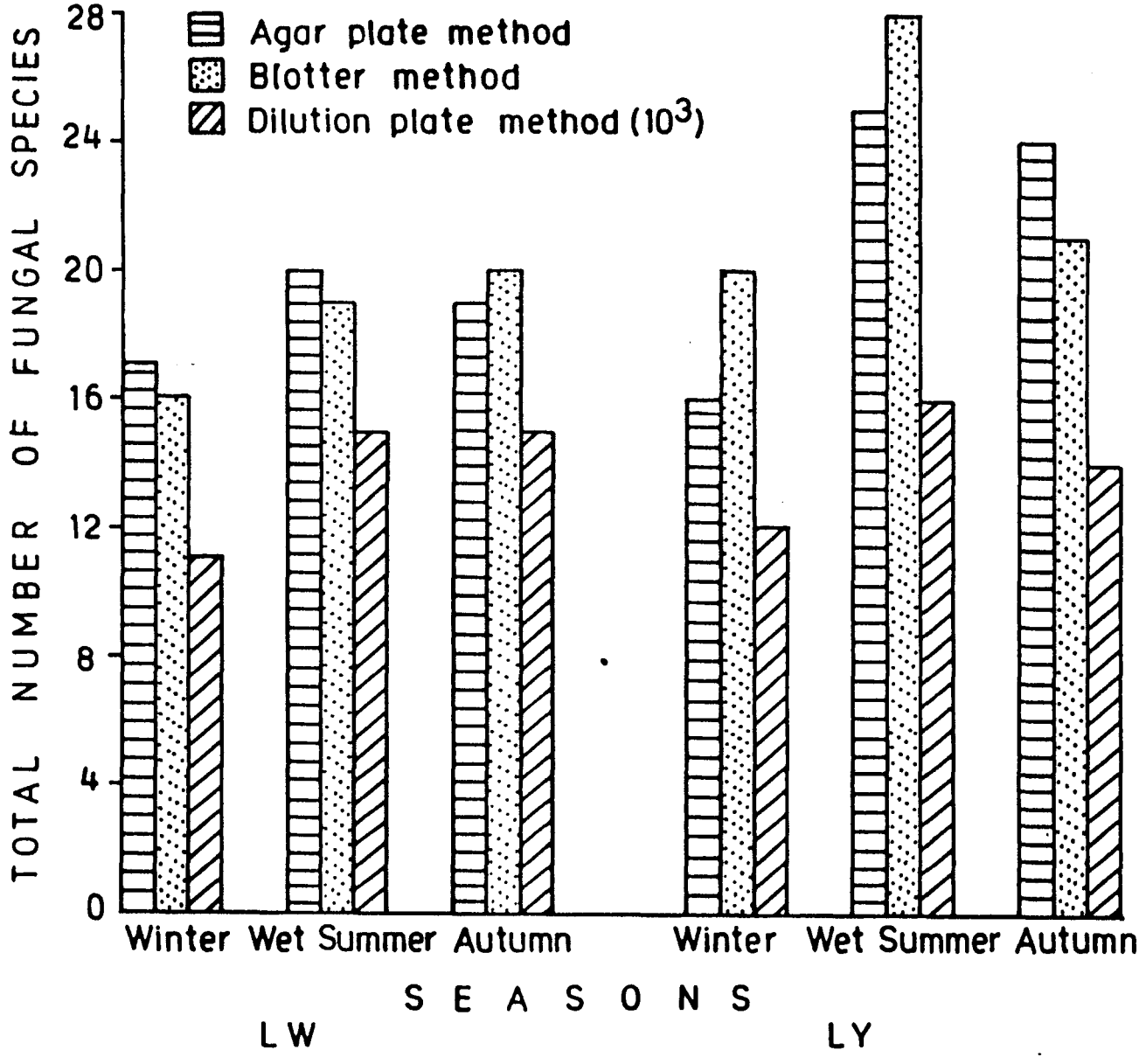

Agar plate method
Blotter method
Dilution plate method ($10^3$)
TOTAL NUMBER OF FUNGAL SPECIES
28
24
20
16
12
8
4
0
Winter
Wet Summer
Autumn
Winter
Wet Summer
Autumn
SEASONS
LW
LY

**Figure 1.3.** Seasonal changes in average frequency (%) of seed borne fungi on local white (LW) and local yellow (LY) varieties of maize.

Fig. 1.3.

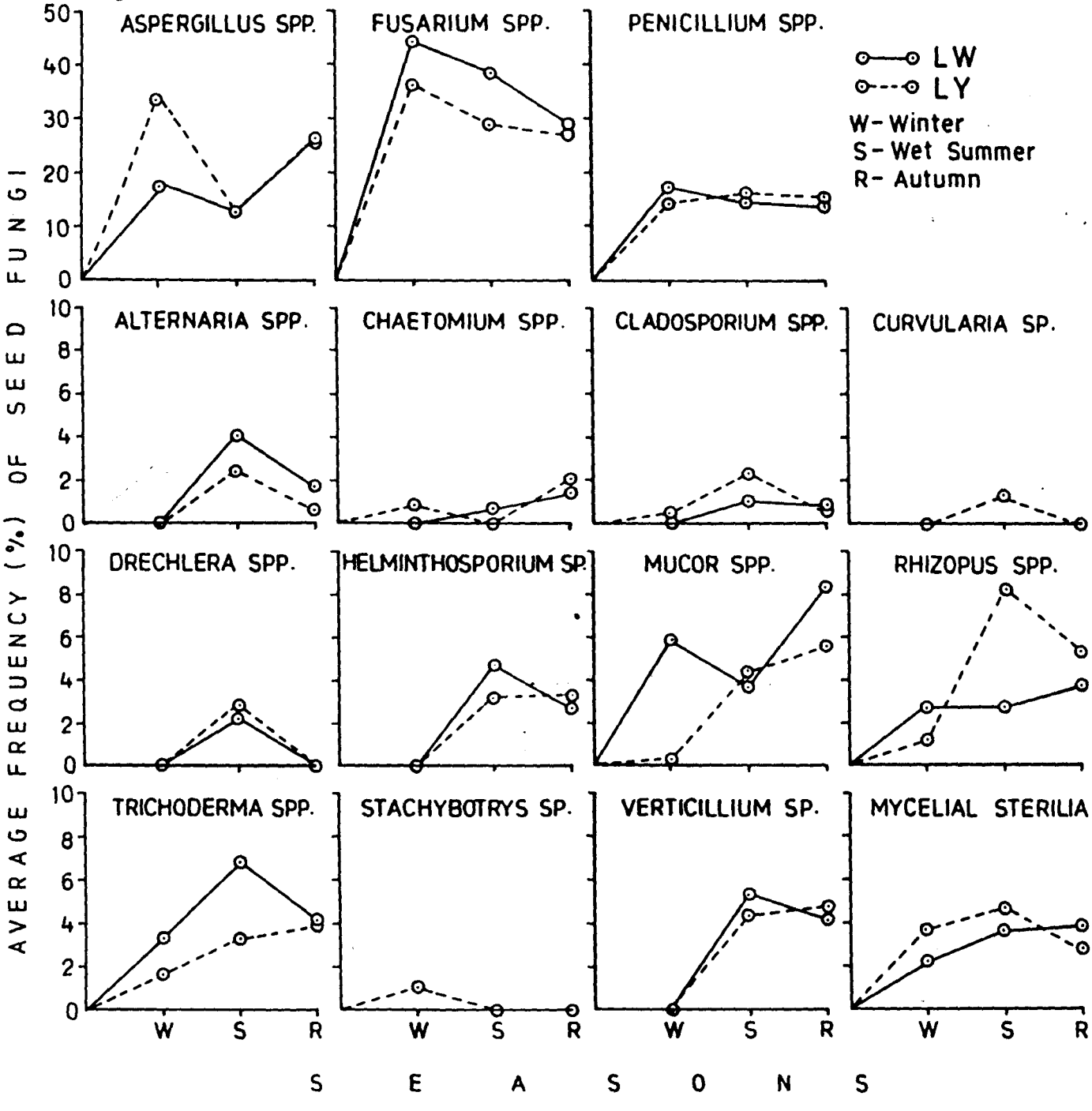

ASPERGILLUS SPP.
FUSARIUM SPP.
PENICILLIUM SPP.
LW
LY
W- Winter
S- Wet Summer
R- Autumn
ALTERNARIA SPP.
CHAETOMIUM SPP.
CLADOSPORIUM SPP.
CURVULARIA SP.
DRECHLERA SPP.
HELMINTHOSPORIUM SP.
MUCOR SPP.
RHIZOPUS SPP.
TRICHODERMA SPP.
STACHYBOTRYS SP.
VERTICILLIUM SP.
MYCELIAL STERILIA
AVERAGE FREQUENCY (%) OF SEED FUNGI
0
2
4
6
8
10
20
30
40
50
W
S
R
SEASONS

**Figure 1.4.** Population of seed borne fungi on local white (LW) and Local yellow (LY) maize varieties under two storage conditions, i.e. shaded (oc) and smoked (smc) conditions.

Fig.14.

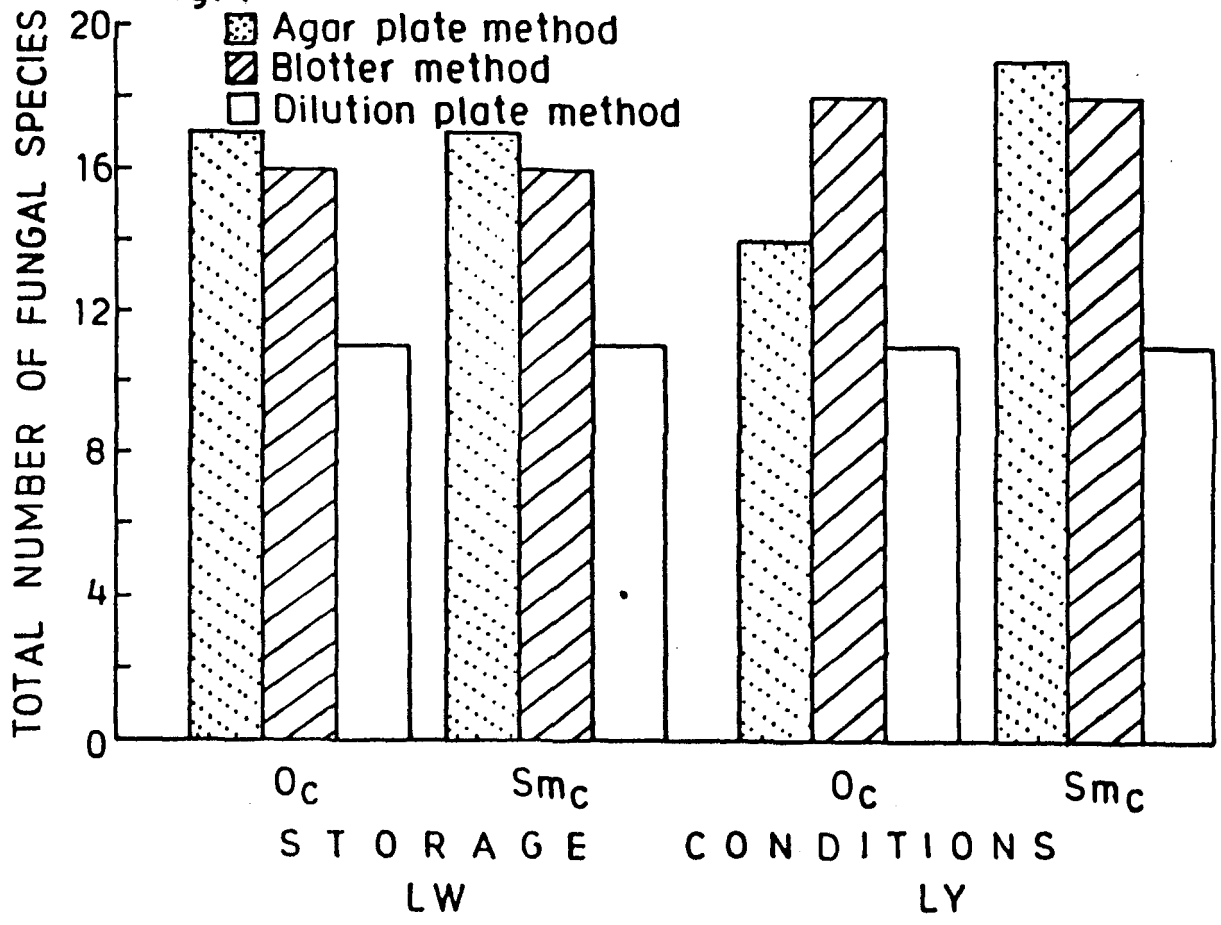

Agar plate method
Blotter method
Dilution plate method
TOTAL NUMBER OF FUNGAL SPECIES
20
16
12
8
4
0
Oc
Smc
Oc
Smc
STORAGE CONDITIONS
LW
LY

**Figure 1.5.** Seasonal variation in fungal seed infection and chitin contents on local white (LW) and local Yellow (LY) varieties of maize.

Fig. 1.5.

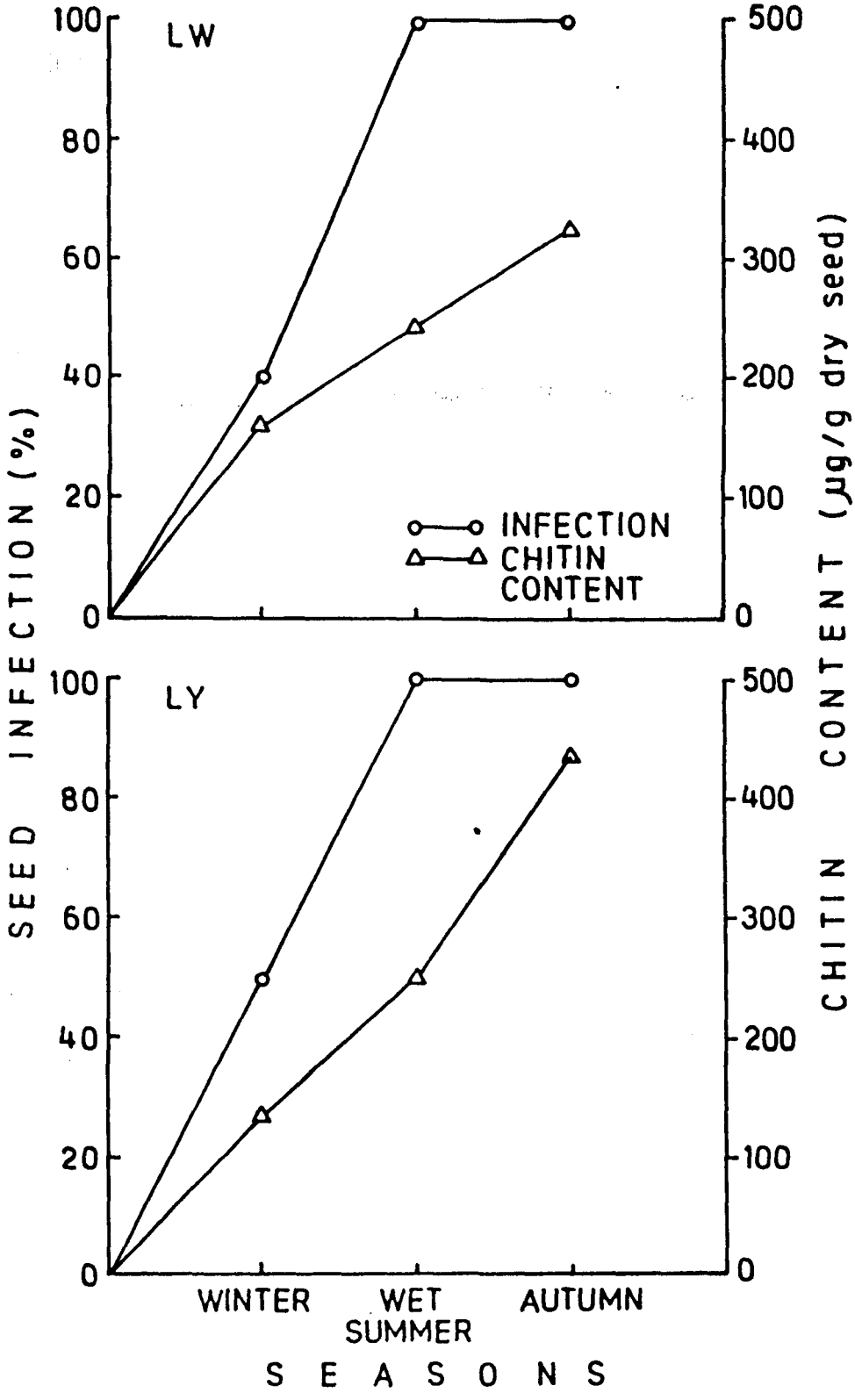

LW
LY
INFECTION
CHITIN CONTENT
SEED INFECTION (%)
CHITIN CONTENT (µg/g dry seed)
0
20
40
60
80
100
100
200
300
400
500
WINTER
WET SUMMER
AUTUMN
SEASONS

PLATE – 3.A. Local white and local yellow varieties of maize seeds.
PLATE – 3.B. Infected maize cobs after storage is local white variety.
PLATE – 3.C. Infected maize cobs after storage in local yellow variety.

3.A

3.B

3.C

## II. Screening of maize seeds for mycotoxins

Two varieties of maize seeds, viz. Local white and Local yellow varieties were tested for the toxigenic strains of *Aspergillus flavus*, *A. ochraceous*, *Fusarium moniliforme*, *Penicillium citrinum* and detection of aflatoxin, ochratoxin, zearalenone and citrinin elaboration respectively in their natural as well as in the laboratory conditions.

The percentage moisture contents of the seeds at different seasons (winter, wet summer and autumn) was recorded to be in the range of 5-15% during winter, 15-25% in wet summer and 25-40% in autumn season.

On bright greenish-yellow fluorescence (BGYF) test, all the samples understudy exhibited a positive fluorescence. The intensity of fluorescence spots was rather faint in some of the samples.

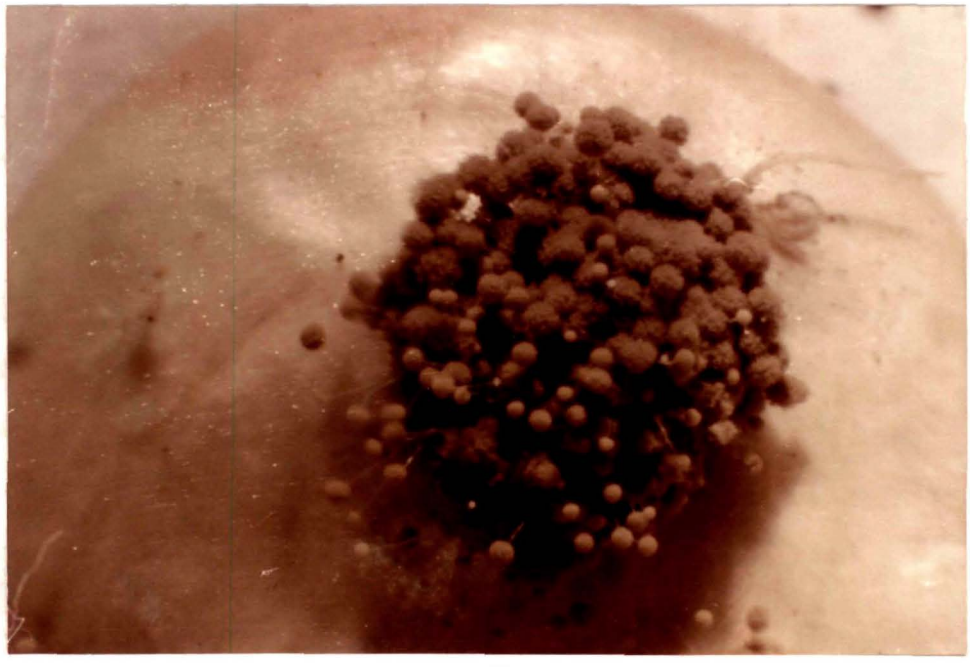
Figure 2.1.A showed that the highest concentration of aflatoxin was observed during wet summer season and the least was during winter. Production of zearalenone, citrinin and ochratoxin was maximum during autumn and minimum in winter season (Fig 2.1.B,C,D). Local yellow variety induced maximum production of mycotoxins than the local white variety of maize in all the seasons (Fig. 2.1.A,B,C,D).

Statistical analysis (two way analysis of variance) showed that there were non-significant (NS) variations between seasons whereas significant (S) variations were observed between the mycotoxins

**PLATE** – 4.A. Some fungi associated with maize seeds during storage.

**PLATE** – 4.B. A toxigenic strain of *Aspergillus flavus* producing aflatoxin $B_1$.

4.A



4.B

(P>0.001) as shown in Table 2.9.

Table 2.1 depicted the percentage occurrence of *A. flavus* in both local varieties of maize seeds in different seasons. It was highest during autumn, followed by wet summer and winter. Local yellow variety supported higher percentage occurrence of *A. flavus* than the local white variety. Table 2.2 depicted the seasonal variation in aflatoxins and has revealed that maximum amount of aflatoxin $B_1$(0.27 ppm) and aflatoxin $G_1$(0.05 ppm) was in winter months in local white variety. In wet summer months, only aflatoxin $B_1$(0.9 ppm) was detected, whereas in autumn, aflatoxin $B_1$(0.4 ppm) and $B_2$(0.1 ppm) were detected. In local yellow variety, aflatoxin $B_1$(1.4 ppm) was detected during winter. In wet summer season, aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ were detected with an amount of 1.3 ppm, 0.2 ppm, 0.4 ppm and 0.2 ppm respectively. In autumn season, only aflatoxin $B_1$ and $G_1$ were isolated with a concentration of 1.0 ppm and 0.02 ppm respectively.

Aflatoxin $B_1$ (Plate-4.8) was found to be the most common one amongst all the aflatoxins, even though its concentration varies from variety to variety and from season to season. The maximum production of aflatoxin $B_1$ (1.44 ppm) was detected in winter in local yellow variety and lowest (0.27 ppm) was analysed in winter in local white variety. Aflatoxin $B_2$ and $G_1$ were detected in both the varieties whereas aflatoxin $G_2$ was produced only in local yellow variety during wet summer season.

The results have indicated that local white variety contained only aflatoxin $B_1$, $B_2$ and $G_1$, whereas local yellow variety have all the four types of aflatoxins, viz. aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$.

The percentage occurrence of *Fusarium moniliforme* and production of zearelenone in maize seeds in different seasons has been compiled in Table 2.3. The table showed that the occurrence of *F. moniliforme* as well as the production of zearalenone were maximum during autumn season followed by wet summer and winter in both the maize varieties.

Similarly, occurrence of *Penicillium citrinum* and *Aspergillus ochraceous*, production of citrinin and ochratoxin on maize seeds in different seasons as shown in Table 2.4 and 2.5 were found to have the maximum occurrence of *P. citrinum* and *A. ochraceous* as well as the production of citrinin and ochratoxin during autumn season followed by wet summer and winter seasons in both varieties respectively.

It is clear from Table 2.6 that 30 isolates of A. flavus were isolated from different samples of local white maize seeds of which 6 isolates were found to be toxigenic and yielded aflatoxin $B_1$ and $G_1$ (0.32 ppm and 0.11 ppm) respectively during winter season. During wet summer season, 46 isolates were obtained out of which 22 were toxigenic and yielded aflatoxin $B_1$ and $G_1$ (1.1 ppm and 0.6 ppm) respectively whereas in autumn season only 16 isolates out of 50 isolates were found to be toxigenic yielding aflatoxin $B_1$ and $B_2$ at the concentrations of 0.7 ppm and 0.5 ppm respectively.

Table 2.6 has indicated that zearalenone was also detected in local white variety of maize. Out of 52 isolates of *F. moniliforme* screened, only 21 isolates were found to be toxigenic with a concentration of 0.2 ppm during winter season, followed by wet summer season whereby 10 isolates were toxigenic out of 35 isolates isolated and having a an amount of 1.4 ppm. The least number of isolates which were toxigenic had been detected during autumn season where only 2 isolates out of 26 isolates were found to be toxigenic producing 0.9 ppm mycotoxin.

The total amount of all the mycotoxins detected in different seasons in local white variety was presented in Figure 2.2. It has been clear from the figure that maximum concentration of mycotoxins was obtained during wet summer season followed by autumn and winter seasons.

Similarly, Table 2.7 has shown that 32 isolates of *A. flavus* were isolated from local yellow variety out of which 10 isolates were toxigenic and yielded only aflatoxin $B_1$ with a concentration of 1.52 ppm during winter season. The other mycotoxins detected during winter season were ochratoxin (0.4 ppm), zearalenone (1.5 ppm) and citrinin (0.1 ppm). In wet summer season, aflatoxin $B_1$ and $G_1$ were detected at concentration of 2.52 ppm and 1.72 ppm respectively. ochratoxin (0.9 ppm) and zearalenone (2.8 ppm) were also produced while citrinin was not detected during this period.

Fig.2.3 showed the maximum amount of mycotoxins during wet summer season followed by autumn and winter seasons.

Statistical analysis (two way analysis of variance) showed significant variations between seasons in local white variety ($P>0.05$) and local yellow variety ($P>0.01$) and also between fungal species in both varieties ($P>0.01$) as shown in Table 2.9.

It is clear from Table 2.8 that aflatoxins were produced more in shaded condition than in smoked storage condition. Aflatoxin $B_1$ was more (1.74 ppm) in local yellow variety than in local white variety (0.52 ppm) under shaded storage condition. Aflatoxin $G_1$ was rarely detected under shaded condition (0.24 ppm). Under smoked condition, the concentration of aflatoxin $B_1$ was 0.35 ppm in local yellow variety and 0.14 ppm in local white variety.

Statistical analysis (two way analysis of variance) showed significant variations between storage conditions between varieties ($P>0.05$) as shown in Table 2.10.

**Table 2.1** : Occurrence (%) of *A. flavus* on local white (LW) and local yellow (LY) varieties of maize seeds in different seasons.

| Maize varieties | Winter | Wet Summer | Autumn |
|---|---|---|---|
| LW | 16 | 25 | 32 |
| LY | 20 | 28 | 40 |

**Table 2.2 :** Production of aflatoxins by *Aspergillus flavus* on maize seeds in different seasons.

| Maize varieties | Amount of aflatoxins (ppm) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Winter | | | | Wet Summer | | | | Autumn | | | |
| | $B_1$ | $B_2$ | $G_1$ | $G_2$ | $B_1$ | $B_2$ | $G_1$ | $G_2$ | $B_1$ | $B_2$ | $G_1$ | $G_2$ |
| LW | 0.27 | 0.0 | 0.05 | 0.0 | 0.9 | 0.0 | 0.0 | 0.0 | 0.4 | 0.1 | 0.0 | 0.0 |
| LY | 1.4 | 0.0 | 0.0 | 0.0 | 1.3 | 0.2 | 0.4 | 0.2 | 1.0 | 0.0 | 0.02 | 0.0 |

**Table 2.3 :** Occurrence and production of zearalenone by *Fusarium moniliforme* on maize seeds in different seasons.

| Maize Varieties | Occurrence (%) | | | Amount of Zearalenone (ppm) | | |
|---|---|---|---|---|---|---|
| | Winter | Wet Summer | Autumn | Winter | Wet Summer | Autumn |
| LW | 18 | 32 | 34 | 0.7 | 1.2 | 1.36 |
| LY | 24 | 36 | 42 | 1.94 | 2.5 | 2.75 |

**Table 2.4 :** Occurrence and production of citrinin by *Penicillium citrinum* on maize seeds in different seasons.

| Maize Varieties | Occurrence (%) | | | Amount of citrinin (ppm) | | |
|---|---|---|---|---|---|---|
| | Winter | Wet Summer | Autumn | Winter | Wet Summer | Autumn |
| LW | 10 | 16 | 22 | 0.29 | 0.53 | 0.68 |
| LY | 14 | 18 | 24 | 0.36 | 1.2 | 1.25 |

Table 2.5 : Occurrence and production of ochratoxin by *Aspergillus ochraceous* on maize seeds in different seasons.

| Maize Varieties | Occurrence (%) | | | Amount of ochratoxin (ppm) | | |
|---|---|---|---|---|---|---|
| | Winter Wet | Summer | Autumn | Winter Wet | Summer | Autumn |
| LW | 12 | 15 | 17 | 0.32 | 0.6 | 0.75 |
| LY | 14 | 18 | 20 | 0.35 | 1.35 | 1.9 |

Table 2.6 : Efficiency of mycotoxins production on local white variety (LW) produced by two fungi in different seasons.

| Fungi | Winter | | | Wet Summer | | | Autumn | | |
|---|---|---|---|---|---|---|---|---|---|
| | No. of Isolates | Type of Mycotoxin | Amount of Mycotoxin (ppm) | No. of Isolates | Type of Mycotoxin | Amount of Mycotoxin (ppm) | No. of Isolates | Type of Mycotoxin | Amount of Mycotoxin (ppm) |
| *Aspergillus flavus* | 30 | $AfB_1$ | 0.32 | 46 | $AfB_1$ | 1.1 | 50 | $AfB_1$ | 0.7 |
| | (6) | $AfG_1$ | 0.11 | (22) | $AfG_1$ | 0.6 | (16) | $AfB_2$ | 0.5 |
| *Fusarium moniliforme* | 52 | Zea | 0.2 | 35 | Zea | 1.4 | 26 | Zea | 0.9 |
| | (21) | | | (10) | | | (2) | | |

Figures in open and in parentheses represent non toxigenic and toxigenic strains of fungal species respectively.

$AfB_1$ = Aflatoxin $B_1$ ; $AfB_2$ = Alfatoxin $B_2$ ; $AfG_1$ = Aflatoxin $G_1$ ; Zea = Zearalenone.

Table 2.7 : Efficiency of mycotoxins production on local Yellow variety (LY) produced by various fungi in different seasons.

| Fungi | Winter | | | Wet Summer | | | Autumn | | |
|---|---|---|---|---|---|---|---|---|---|
| | No. of Isolates | Type of Mycotoxin | Amount of Mycotoxin (ppm) | No. of Isolates | Type of Mycotoxin | Amount of Mycotoxin (ppm) | No. of Isolates | Type of Mycotoxin | Amount of Mycotoxin (ppm) |
| *Aspergillus flavus* | 32 | $AfB_1$ | 1.52 | 38 | $AfB_1$ | 2.52 | 20 | $AfB_1$ | 1.9 |
| | (10) | | | (15) | $AfG_1$ | 1.72 | (9) | $AfG_1$ | 0.5 |
| *A. ochraceous* | 8 | Ochra | 0.4 | 12 | Ochra | 0.9 | 14 | Ochra | 0.5 |
| | (3) | | | (6) | | | (5) | | |
| *Fusarium moniliforme* | 30 | Zea | 1.5 | 10 | Zea | 2.8 | 14 | Zea | 2.2 |
| | (6) | | | (4) | | | (5) | | |
| *Penicillium citrinum* | 12 | Cit | 0.1 | 12 | not | 0.0 | 6 | not | 0.0 |
| | (5) | | | (0) | detected | | (0) | detected | |

Figures in open and in parentheses represent nontoxigenic and toxigenic strains of fungal species respectively.

$AfB_1$ = Aflatoxin $B_1$ ; $AfG_1$ = Aflatoxin $G_1$ ; Ochra = Ochratoxin ; Zea = Zearalenone; Cit = Citrinin.

Table 2.8 : Production of aflatoxins by seed fungi in shaded and smoked storage conditions.

| Maize varieties | Type and amount of aflatoxins (ppm) | | |
|---|---|---|---|
| | Shaded condition | | Smoked condition |
| | $B_1$ | $G_1$ | $B_1$ |
| LW | 0.52 | 0.24 | 0.14 |
| LY | 1.74 | 0.0 | 0.35 |

**Table 2.9** : Analysis of variance between production of mycotoxins and different seasons using 'F' test in both maize varieties.

| Variations | 'F' Value | |
|---|---|---|
| | Local white | Local yellow |
| Due to season | 16.3486 | 32.61 |
| Due to mycotoxins | 8.848312*** | 18.1248 |

*** = Significant at 0.001 level

**Table 2.10** : Analysis of variance between production of mycotoxins fungal species and different seasons using 'F' test in both the maize varieties.

| Variations | 'F' Value | |
|---|---|---|
| | Local white | Local yellow |
| Due to season | 3.540354* | 6.798604** |
| Due to fungal speceis | 6.190444** | 4.808384** |

** = Significant at 0.01 level
* = Significant at 0.05 level

**Table 2.11:** Analysis of variance in production of aflatoxins between different storage practises in both local varieties using 'F' test.

| Variations | 'F' Value |
|---|---|
| Due to storage condition | 1.044563* |
| Due to mycotoxins | 1.841234* |

* = Significant at 0.05 level

**Figure 2.1.** Seasonal variation in mycotoxins concentration (ppm) on seeds of local white (LW) and local yellow (LY) varieties of maize.

Fig. 2.1.

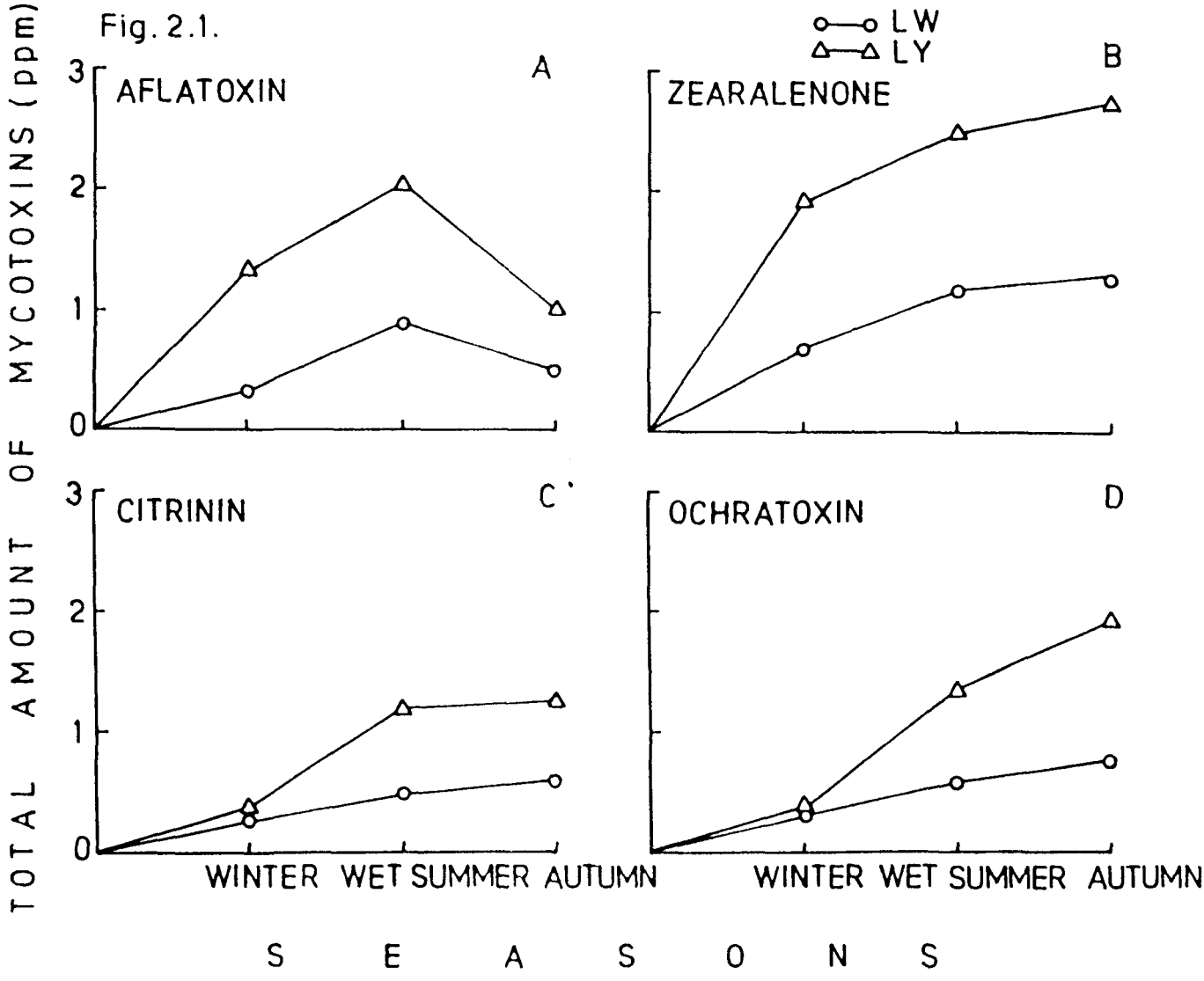

LW
LY
A
AFLATOXIN
B
ZEARALENONE
C
CITRININ
D
OCHRATOXIN
3
2
1
0
TOTAL AMOUNT OF MYCOTOXINS (ppm)
WINTER WET SUMMER AUTUMN
WINTER WET SUMMER AUTUMN
S E A S O N S

**Figure 2.2.** Seasonal production of mycotoxins by different isolates of toxigenic fungi on seeds of local white variety of maize.

Fig. 2.2.

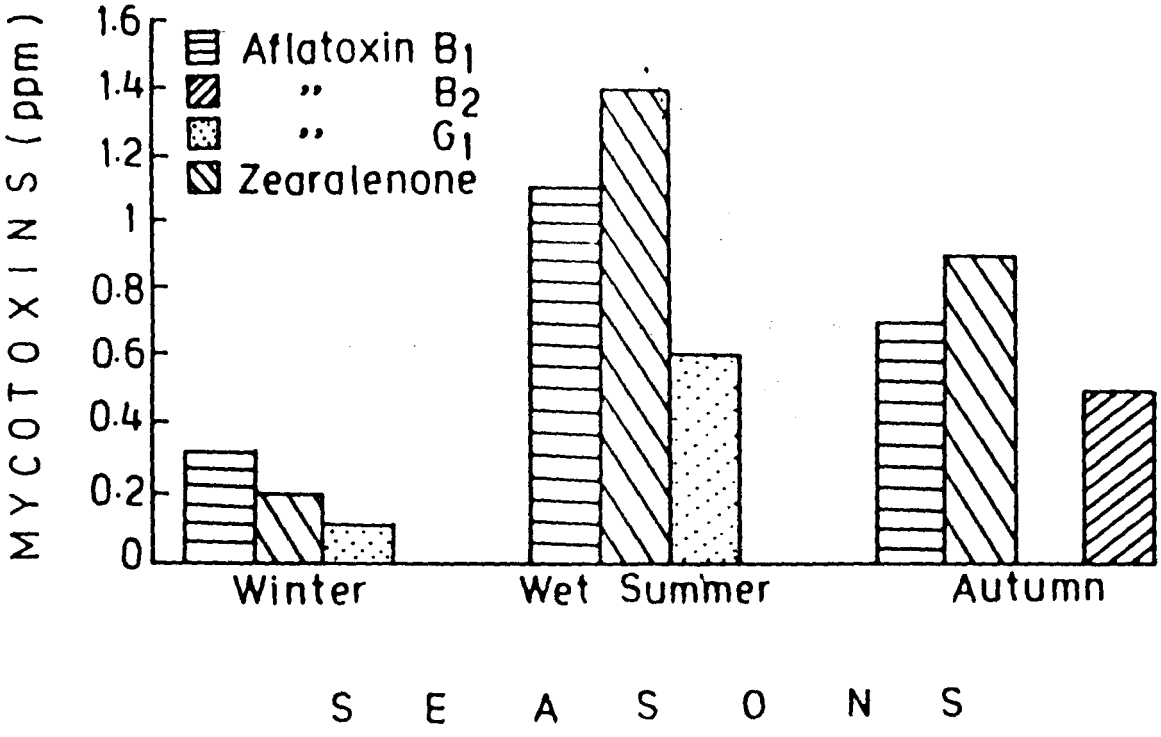
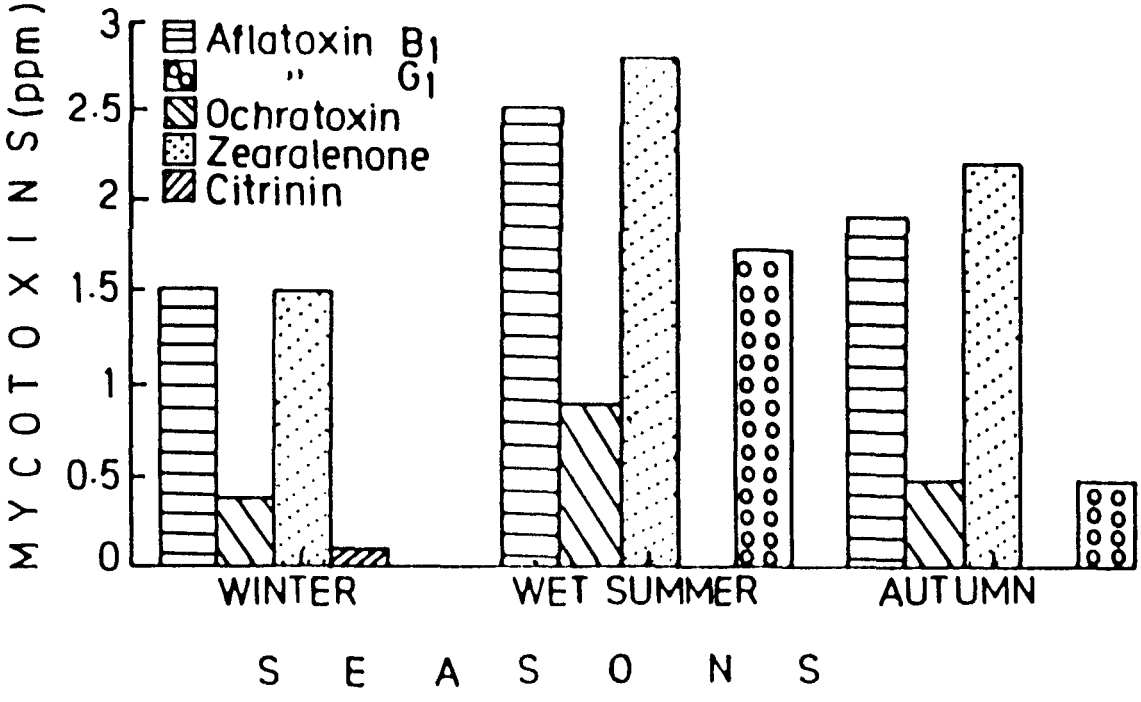

MYCOTOXINS (ppm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Aflatoxin B1
" B2
" G1
Zearalenone
Winter
Wet Summer
Autumn
SEASONS

**Figure 2.3.** Seasonal production of mycotoxins by different isolates of toxigenic fungi on seeds of local yellow variety of maize.

Fig. 2.3.


MYCOTOXINS(ppm)
3
2.5
2
1.5
1
0.5
0
Aflatoxin B1
" G1
Ochratoxin
Zearalenone
Citrinin
WINTER
WET SUMMER
AUTUMN
SEASONS

## III. Effect of physical factors on the production of aflatoxins

1. Effect of moisture content:

The effect of seed moisture contents on the production of aflatoxins is depicted in Figure 3.1. It was clear from the figure that in local white variety, the highest production of aflatoxins was at 35% seed moisture content on the 20th day of incubation period while the lowest was at 40% seed moisture content on the 10th and 30th day of incubation, while it was maximum at 25% seed moisture contents on the 20th day of incubation. Production of aflatoxin was minimum at 15% seed moisture content on the 30th day of incubation.

In general, the production of aflatoxins increased upto 20th day of incubation, thereafter it declined at all the moisture levels.

2. Effect of temperature:

Production of aflatoxins at different temperatures is depicted in Figure 3.2. It was evident that maximum production of aflatoxins was at 30°C on the 20th day of incubation in local white variety while the minimum was observed at 15°C on the 30th day of incubation. Production of aflatoxin was maximum at 30°C in local yellow variety on the 20th day of incubation and lowest on the 10th day at 15°C.

The trend of aflatoxin production was almost similar in both maize varieties (Fig.3.2). The most suitable temperature for the production aflatoxins were 25°C and 30°C. The higher (40°C) and lower (15°C) temperatures did not favour the production of aflatoxin.

Production of aflatoxin increased steadily upto 20th day of incubation and declined thereafter at all the temperatures.

3. Effect of relative humidity:

The variation in aflatoxin due to relative humidity was observed (Fig.3.3). In local white variety, the maximum aflatoxin was produced at highest humidity (100%) on the 20th day of incubation while the minimum was observed at 75% relative humidity on the 10th day of incubation. The trend of aflatoxin production was also similar in local yellow variety (Fig.3.3).

Fig.3.3 also shows similar results on the effect of incubation period on the production of aflatoxin, where the production in maize seeds by seed fungi increased upto 20th day of incubation which decreased thereafter at all the relative humidities tested.

4. Statistical analysis:

Analysis of variance showed that there was non-significant variation in local white variety but significant variation ($P>0.001$) in local yellow variety between incubation periods. Between moisture contents, temperatures and relative humidities, significant variations were observed in both varieties. Table 3.1 showed that in local white variety, significant variation between moisture contents and temperatures ($P>0.05$), relative humidities ($P>0.01$) and in local yellow variety significant variations between moisture contents, temperatures and relative humidities ($P>0.01$) were observed.

**Table 3.1** Analysis of variance on the production of aflatoxins in both local varieties due to moisure content, temperature and relative humidity at different incubation period using 'F' test

| Variations | 'F' Value | |
|---|---|---|
| | Local White | Local Yellow |
| Due to incubation period | 12.86337 | 8.861668*** |
| Due to moisture content | 3.43014* | 5.600821** |
| Due to temperature | 3.709693* | 4.97565** |
| Due to relative humidity | 5.028502** | 4.990427** |

*** = Significant at 0.001 level

** = Significant at 0.01 level

* = Significant at 0.05 level

**Figure 3.1.** Effect of moisture contents(%) of seeds on the production of aflatoxins on seeds of local white (LW) and local yellow (LY) varieties of maize.

Fig. 3.1.

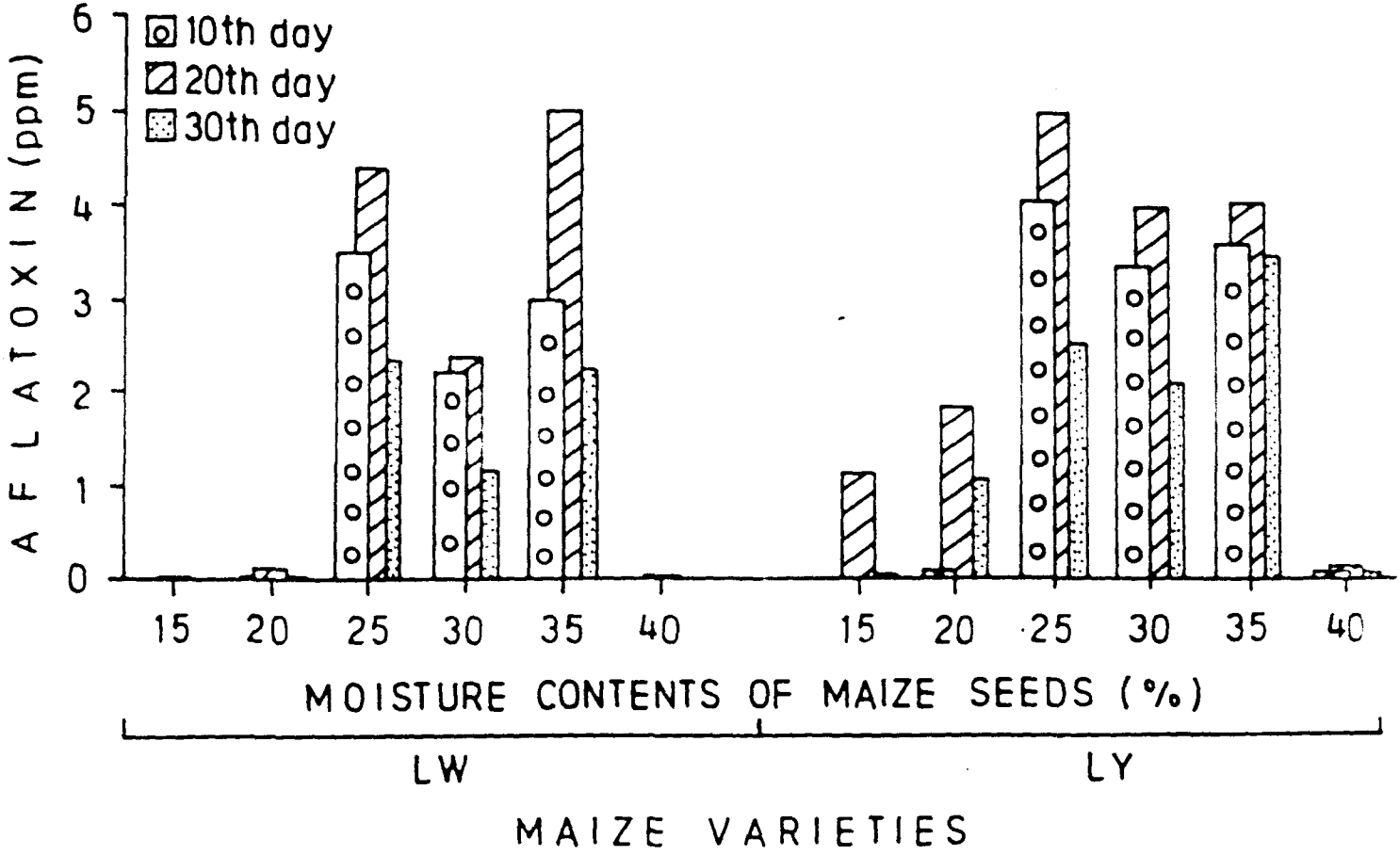

10th day
20th day
30th day
AFLATOXIN (ppm)
0
1
2
3
4
5
6
15
20
25
30
35
40
15
20
25
30
35
40
MOISTURE CONTENTS OF MAIZE SEEDS (%)
LW
LY
MAIZE VARIETIES

**Figure 3.2.** Effect of temperatures (°c) on the production of aflatoxin on seeds of local white (LW) and local yellow (LY) varieties of maize.

Fig. 3.2.

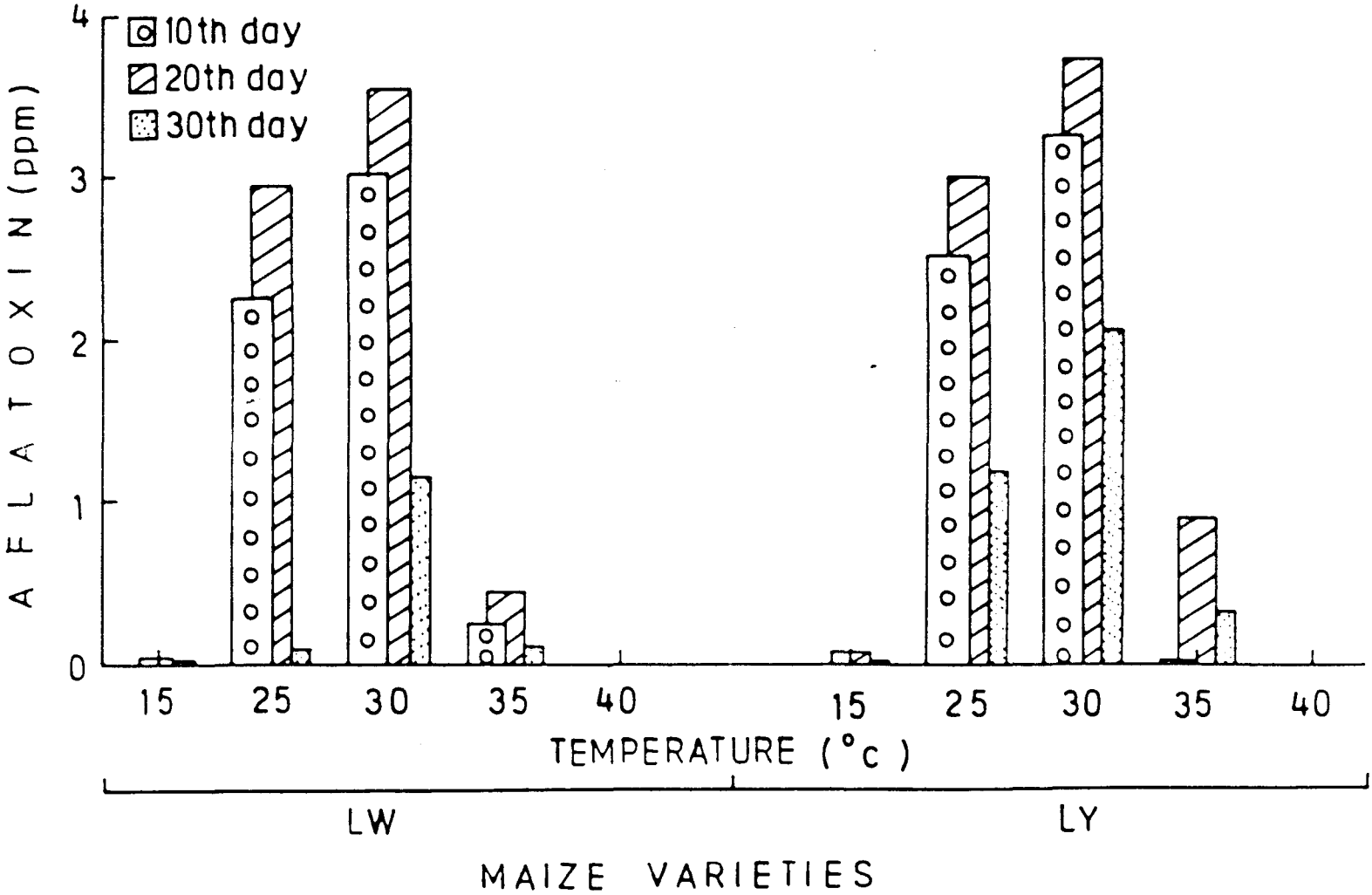
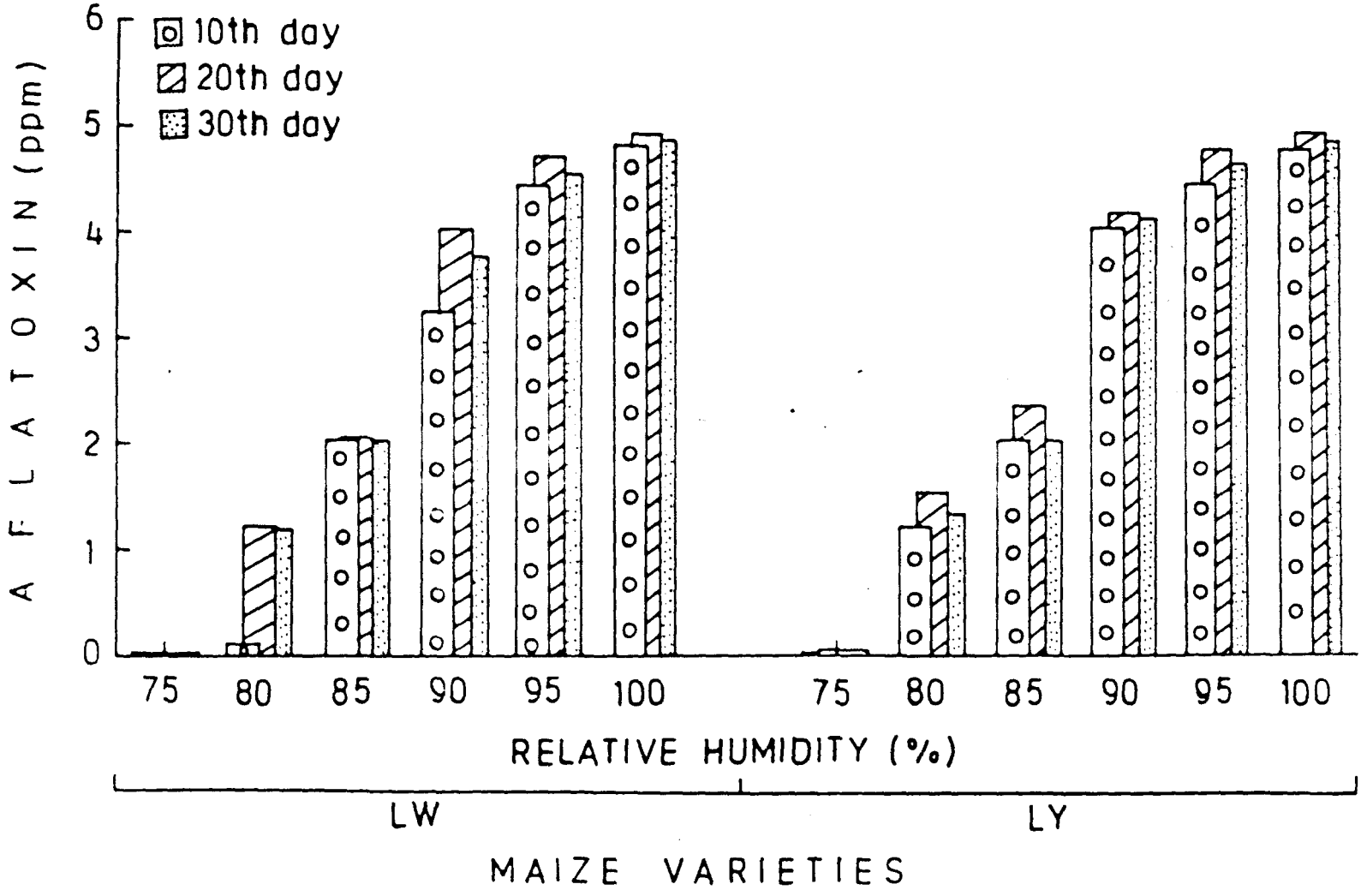

10th day
20th day
30th day
AFLATOXIN (ppm)
0
1
2
3
4
15
25
30
35
40
15
25
30
35
40
TEMPERATURE (°c)
LW
LY
MAIZE VARIETIES

**Figure 3.3.** Effect of different relative humidities (%) on the production of aflatoxins on local white (LW) and local yellow (LY) varieties of maize.

Fig. 3.3.


10th day
20th day
30th day
AFLATOXIN (ppm)
0
1
2
3
4
5
6
75
80
85
90
95
100
75
80
85
90
95
100
RELATIVE HUMIDITY (%)
LW
LY
MAIZE VARIETIES

## IV. Effect of toxigenic fungi on nutritive value of maize seeds

1. Loss in dry weight :

The change in seed dry weight of infected and uninfected seeds was taken at an interval of 60 days for a period of 180 days is presented in Table 4.1. Results have shown that there was a gradual fall of seed dry weight during the incubation period of 180 days in both the treated and control samples. However, the loss in dry weight in the treated samples was significantly more pronounced in comparison to control ones.

Fig.4.1.A has indicated that *Penicillium citrinum* in local white variety has reduced maximum seed dry weight (3.03%) followed by *Fusarium moniliforme* (2.16%) and *Aspergillus flavus* (1.92%) on the 60th day of incubation period whereas *F. moniliforme* was recorded to have the highest inhibition on the seeds with 4.25% followed by *P. citrinum* with 3% and then *A. flavus* with 2.87% on the 120th day incubation period. On the 180th day of incubation, *F. moniliforme* was found to have the maximum inhibition on the seeds with 4.67% followed by *A. flavus* with 4.04% and P. citrinum with 3.4% while in local yellow variety *A. flavus* caused maximum reduction (0.71%) on the 60th day, on the 120th day (2.87%) and on the 180th day (4.04%) of incubation. *F. moniliforme* and *P. citrinum* caused less loss.

Statistical analysis (two way analysis of variance) in dry weight of seeds showed that there were non-significant (NS) variations between the incubation period for both varieties whereas significant

(S) variations were observed between the fungal species (P>0.01) for both varieties as shown in Table 4.5.

2. Changes in sugar content:

The infected and healthy seeds were periodically assessed for total sugar, reducing sugar and non-reducing sugar contents. On 0 day assessment, the sugar content of both healthy and diseased seeds was same which on incubation for 180 days changed considerably in the diseased seeds while that of the healthy seeds did not reveal much change (Table 4.2).

Fig.4.1.B depicted the loss of sugar contents due to *A. flavus*, *F. moniliforme* and *P. citrinum* was more on local white variety than local yellow ones. The loss increased with the increase in incubation period. Amongst the three species, *A. flavus* was found to be the most effective on reducing the total sugar content of seeds in both the local varieties.

Statistical analysis (two way analysis of variance) of the variation in sugar contents of maize seeds due to fungal infestation showed that there was significant variations (P>0.001) between the incubation periods for both varieties and between the fungal species (P>0.001 and P> 0.05 for local white and local yellow respectively) as shown in Table 4.6.

Stimulatory effect of fungal infections on the reducing sugar of seeds and inhibitory effect on the non-reducing ones was noticed (Fig.4.2).

Statistical analysis (ANOVA) of the data on reducing sugar contents of maize seeds due to fungal infestation showed a significant variation due to incubation period ($P>0.001$) for both the varieties and between the fungal species ($P>0.01$) for both the varieties.

Similarly, reduction in non-reducing sugar contents of maize seeds was significant between the incubation period ($P>0.001$) and between the fungal species ($P>0.001$ and $P>0.01$ for local white and local yellow respectively) as shown in Table 4.7.

3. Changes in the protein contents :

Changes in the protein contents of both the local varieties of maize seeds due to fungal infestation at different periods of incubation is depicted in Table 4.4 and Fig.4.3

An initial protein content of 3.24 mg/g and 3.68 mg/g was recorded in local white and local yellow varieties respectively. In case of healthy seeds, the protein value showed slight change at the end of incubation periods whereas the infested seeds exhibited a gradual decrease with an increase in period of incubation. The inhibition of protein content due to various fungal species after 60 days of incubation ranged from 1.23% to 2.77% in local white variety and 1.35% to 2.44% in local yellow ones. After 120 days of incubation it was 1.85% to 3.4% in local white variety and 2.18% to 3.82% in local yellow ones. Finally, after 180 days of incubation, the inhibition (%) in protein content was 1.56% to 3.44% in local white variety and 2.49% to 3.32% in local yellow variety.

No specificity has been observed, all the fungal species tested equally affected the protein content of the seeds in both varieties (Fig.4.3).

Analysis of data (ANOVA) exhibited that variation in protein content of maize seeds due to fungal infestation was insignificant between the incubation periods and significant between the fungal species ($P>0.001$ and $P>0.01$ for local white and local yellow respectively).

**Table 4.1** : Effect of toxigenic fungi on the dry weight of maize seeds.

| Toxigenic fungi | Dry Weight of seeds (g) | | | |
|---|---|---|---|---|
| | 0 day | 60th day | 120th day | 180th day |
| *Aspergillus flavus* | 16.24 | 15.83 | 15.54 | 15.20 |
| | (18.35) | (18.12) | (17.90) | (17.60) |
| *Fusarium moniliforme* | 16.24 | 15.79 | 15.32 | 15.10 |
| | (18.35) | (18.20) | (18.00) | (17.90) |
| *Penicillium citrinum* | 16.24 | 15.65 | 15.52 | 15.30 |
| | (18.35) | (18.21) | (18.10) | (18.04) |
| Control | 16.24 | 16.14 | 16.00 | 15.84 |
| | (18.35) | (18.25) | (18.15) | (18.10) |

Open figures = LW; In parentheses = LY.

**Table 4.2 :** Effect of toxigenic fungi on total sugar contents of maize seeds.

| Toxigenic fungi | Sugar content (mg/g of seed) | | | |
|---|---|---|---|---|
| | 0 day | 60th day | 120th day | 180th day |
| *Aspergillus flavus* | 2.84 | 2.46 | 2.21 | 2.10 |
| | (2.92) | (2.85) | (2.79) | (2.75) |
| *Fusarium moniliforme* | 2.84 | 2.53 | 2.34 | 2.25 |
| | (2.92) | (2.89) | (2.83) | (2.81) |
| *Penicillium citrinum* | 2.84 | 2.72 | 2.52 | 2.44 |
| | (2.92) | (2.82) | (2.76) | (2.75) |
| Control | 2.84 | 2.83 | 2.83 | 2.82 |
| | (2.92) | (2.90) | (2.91) | (2.90) |

Open figures = LW; In parentheses = LY.

**Table 4.3 : Effect of toxigenic fungi on reducing and non-reducing sugar contents of maize seeds.**

| Toxigenic fungi | Sugar content (mg/g of seed) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 day | | 60th day | | 120th Day | | 180th day | |
| | R | NR | R | NR | R | NR | R | NR |
| *Aspergillus flavus* | 1.52 | 1.32 | 1.56 | 0.90 | 1.59 | 0.62 | 1.64 | 0.46 |
| | (1.73) | (1.19) | (1.82) | (1.03) | (1.85) | (0.94) | (1.96) | (0.79) |
| *Fusarium moniliforme* | 1.52 | 1.32 | 1.58 | 0.95 | 1.61 | 0.73 | 1.67 | 0.58 |
| | (1.73) | (1.19) | (1.76) | (1.13) | (1.79) | (1.04) | (1.82) | (0.99) |
| *Penicillium citrinum* | 1.52 | 1.32 | 1.60 | 1.12 | 1.62 | 0.90 | 1.64 | 0.80 |
| | (1.73) | (1.19) | (1.79) | (1.03) | (1.82) | (0.94) | (1.86) | (0.89) |
| Control | 1.52 | 1.32 | 1.53 | 1.30 | 1.53 | 1.30 | 1.54 | 1.28 |
| | (1.73) | (1.19) | (1.74) | (1.16) | (1.74) | (1.17) | (1.75) | (1.15) |

Open figures - LW; In parentheses - LY.

R = Reducing sugar; NR = Non-reducing sugar.

**Table 4.4** : Effect of toxigenic fungi on protein content of maize seeds.

| Toxigenic fungi | Protein content (mg/g) of seed | | | |
|---|---|---|---|---|
| | 0 day | 60th day | 120th day | 180th day |
| *Aspergillus flavus* | 3.24 | 3.15 | 3.12 | 3.08 |
| | (3.68) | (3.63) | (3.56) | (3.51) |
| *Fusarium moniliforme* | 3.24 | 3.20 | 3.17 | 3.14 |
| | (3.68) | (3.59) | (3.52) | (3.49) |
| *Penicillium citrinum* | 3.24 | 3.16 | 3.12 | 3.09 |
| | (3.68) | (3.61) | (3.58) | (3.52) |
| Control | 3.24 | 3.24 | 3.23 | 3.19 |
| | (3.68) | (3.68) | (3.66) | (3.61) |

Open figures = LW; In parentheses = LY.

**Table 4.5** : Analysis of variance in dry weight of seeds due to fungal species at different incubation period in both maize varieties using 'F' test.

| Variations | 'F' Value | |
|---|---|---|
| | Local white | Local yellow |
| Due to incubation period | 22.45338 | 15.7193 |
| Due to fungal infection | 6.270097* | 4.017544** |

* = Significant at 0.05 level
** = Significant at 0.01 level

**Table 4.6** : Analysis of variance in total sugar content of seeds by fungal species and different incubation period in both maize varieties using 'F' test.

| Variations | 'F' Value | |
|---|---|---|
| | Local white | Local yellow |
| Due to incubation period | 7.675327*** | 9.279475*** |
| Due to fungal infection | 7.094234*** | 6.117904** |

*** = Significant at 0.001 level
** = Significant at 0.01 level

**Table 4.7:** Analysis of variance in reducing and non-reducing sugar content of seeds by fungal species and different incubation periods in both maize varieties using 'F' test.

| Variations | 'F' Value | | | |
|---|---|---|---|---|
| | Local white | | Local yellow | |
| | Reducing | Non-reducing | Reducing | Non-reducing |
| Due to incubation period | 11.16279*** | 8.738828*** | 7.121442*** | 8.795959*** |
| Due to fungal infection | 5.751938** | 6.902977*** | 5.293169** | 5.790233** |

*** = Significant at 0.001 level

** = Significant at 0.01 level

**Table 4.8 :** Analysis of variance in protein content of seeds by fungal species at different incubation period in both maize varieties using 'F' test.

| Variations | 'F' Value | |
|---|---|---|
| | Local white | Local yellow |
| Due to incubation period | 15.61517 | 21.1816 |
| Due to fungal infection | 8.578651*** | 7.298206** |

*** = Significant at 0.001 level

** = Significant at 0.01 level

**Figure 4.1.B.** Reduction (%) of total sugar contents of local white (LW) and local yellow (LY) maize varieties by toxigenic fungi.

**Figure 4.1.A.** Reduction (%) of dry weight of seeds of local white (LW) and local yellow (LY) varieties of maize by toxigenic fungi.

Fig. 4.1.

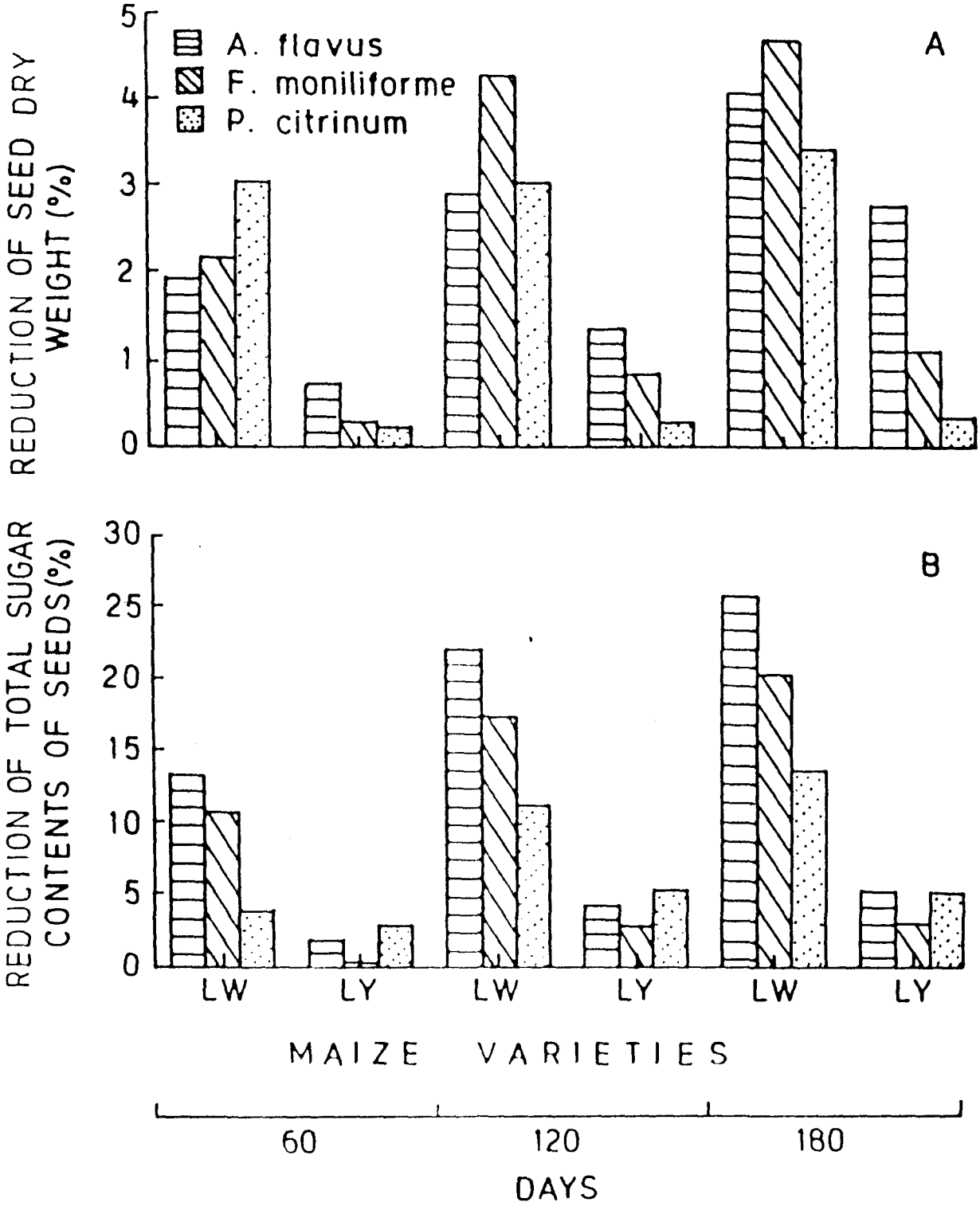

A. flavus
F. moniliforme
P. citrinum
A
B
REDUCTION OF SEED DRY WEIGHT (%)
REDUCTION OF TOTAL SUGAR CONTENTS OF SEEDS (%)
0
1
2
3
4
5
0
5
10
15
20
25
30
LW
LY
LW
LY
LW
LY
MAIZE VARIETIES
60
120
180
DAYS

**Figure 4.2.** Stimulation (%) of reducing (R) and inhibition (%) of non-reducing (NR) sugar contents of local white (LW) and local yellow (LY) maize varieties by toxigenic fungi on 60th day (A), 120th day (B) and 180th day (C).

Fig. 4.2.

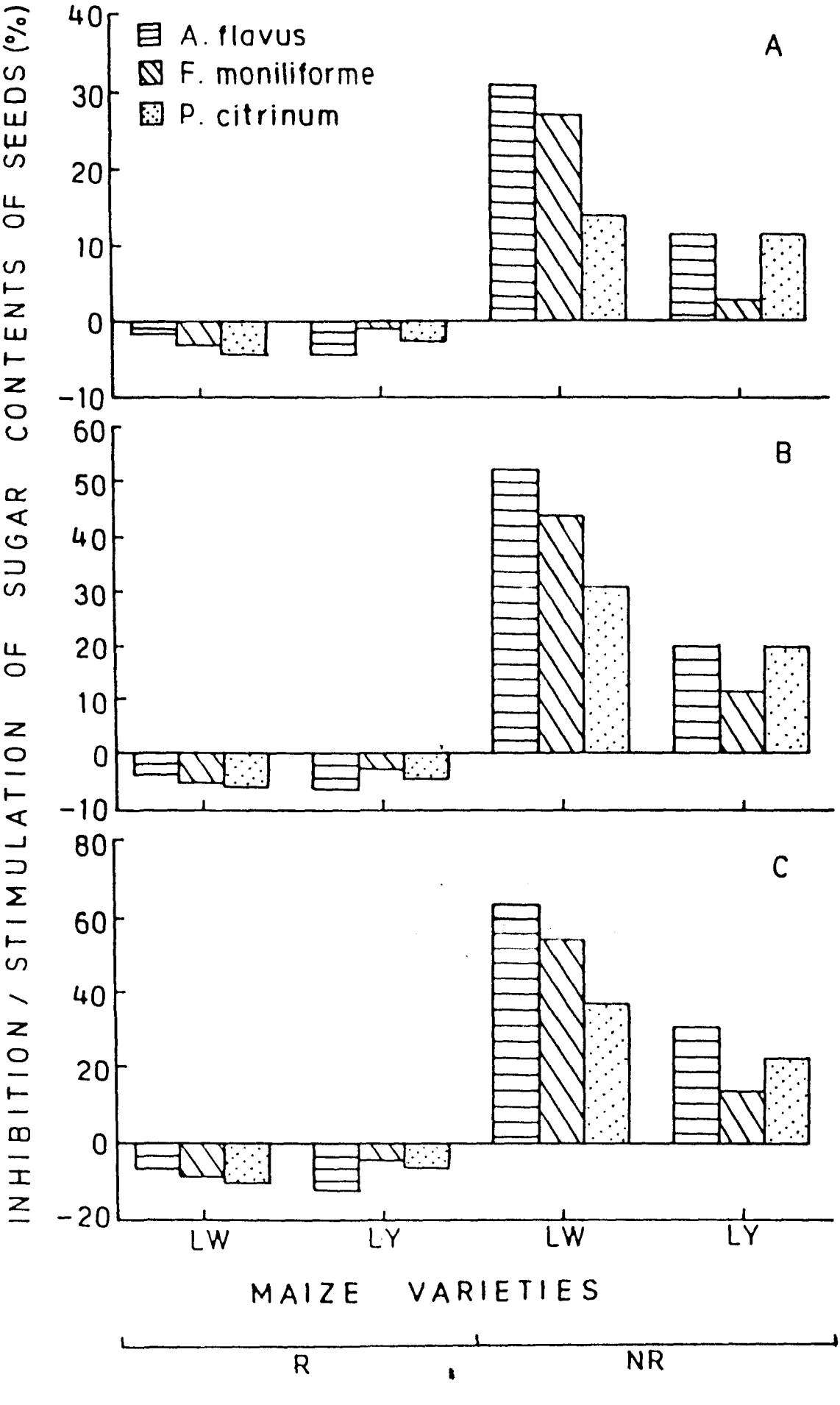
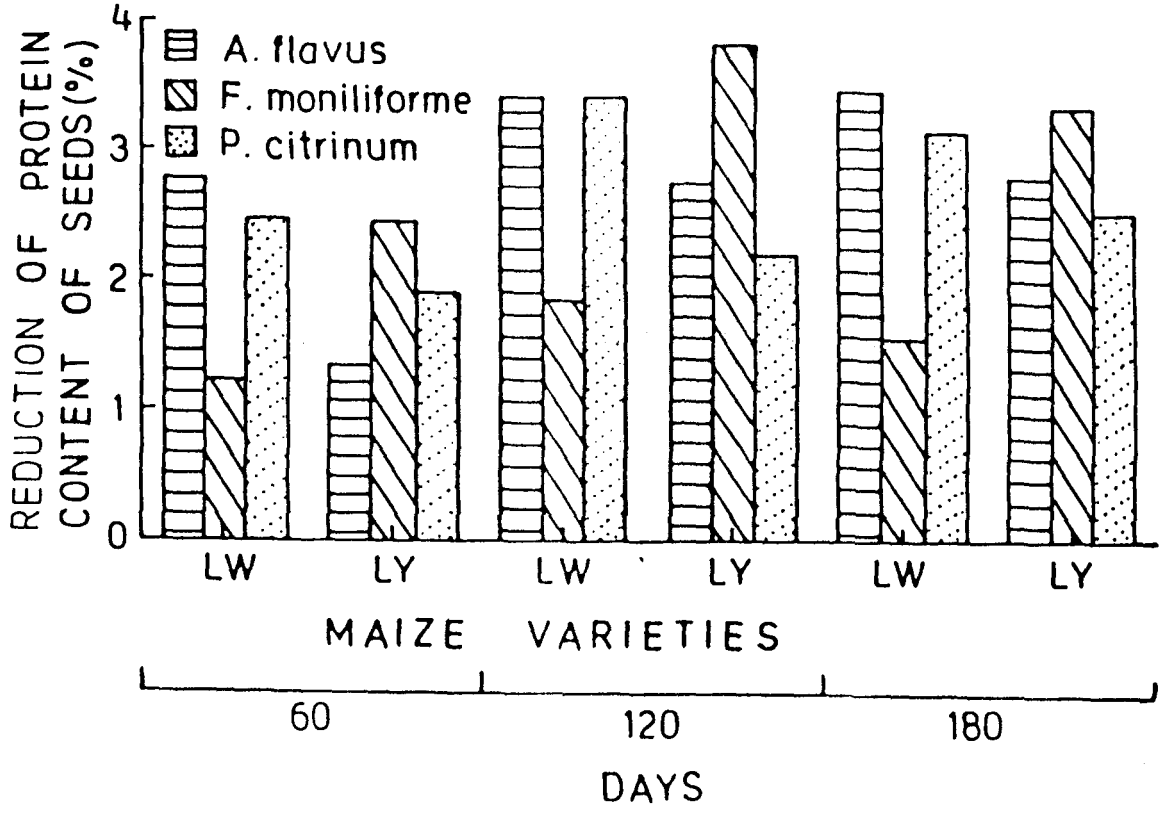

A. flavus
F. moniliforme
P. citrinum
A
B
C
INHIBITION / STIMULATION OF SUGAR CONTENTS OF SEEDS (%)
40
30
20
10
0
-10
60
50
40
30
20
10
0
-10
80
60
40
20
0
-20
LW
LY
LW
LY
MAIZE VARIETIES
R
NR

**Figure 4.3.** Reduction (%) of protein contents of local white (LW) and local yellow (LY) maize varieties by toxigenic fungi.

Fig. 4.3.


REDUCTION OF PROTEIN CONTENT OF SEEDS (%)
4
3
2
1
0
A. flavus
F. moniliforme
P. citrinum
LW
LY
LW
LY
LW
LY
MAIZE VARIETIES
60
120
180
DAYS

## V. Effect of aflatoxin B1 on spore germination and mycelial growth, mycotoxins on seed germination and seedling growth of maize

1. Spore germination :

Different concentrations (0.1 ppm, 0.5 ppm and 1 ppm) of aflatoxin $B_1$ were tested against three seed borne fungi, viz. *Aspergillus flavus*, *Fusarium moniliforme* and *Penicillium citrinum* at different temperatures (15°C, 20°C, 25°C and 30°C) and length of incubation (2h, 5h, 10h and 24h) for fungal spore germination (Table 5.1 and Figures 5.1, 5.2 and 5.3).

Aflatoxin $B_1$ exhibited a stimulatory effect on spore germination of *A. flavus* at the different temperatures (Fig.5.1) and an inhibitory effect on *F. moniliforme* and *P. citrinum* spore germination respectively (Fig.5.2 and 5.3). The degrees of stimulation/inhibition, however varied for different fungi in various treatments.

Analysis of data (ANOVA) of the variation in spore germination of toxigenic fungi due to aflatoxin $B_1$ showed that there was significant variations between temperatures ($P>0.01$, $P>0.05$, $P>0.01$) for *A. flavus*, *F. moniliforme* and *P. citrinum* respectively and between concentrations ($P>0.01$) for *A. flavus* and ($P>0.05$) for *F. moniliforme* and *P. citrinum* as shown in Table 5.5.

Table 5.6 showed the significant variations between temperatures ($P>0.05$) in all concentrations of aflatoxin $B_1$. Between fungal species significant variations ($P>0.05$ and $P>0.01$) for 0.1 ppm and 0.5 ppm

respectively whereas non-significant variation at 1.0 ppm concentration has been noted (Table 5.6).

2. Colony growth :

The test fungi were grown on PDA nutrient medium incorporated with different amount of the aflatoxin $B_1$ to get a series of concentrations. The control sets were maintained on pure PDA medium. The difference of the colony diameter between the control set and the treated was taken as the effect of aflatoxin $B_1$ on the mycelial growth of the fungus.

Table 5.2 depicted that the different concentrations (0.1 ppm, 0.5 ppm and 1.0 ppm) of aflatoxin $B_1$ increased the colony growth of *A. flavus* except at 144h of incubation where the growth in control and treated is equal. Similar results were observed with 0.1 ppm concentration of aflatoxin $B_1$ on the colony growth after 24h, 72h and 120h of incubation periods.

Table 5.2 also depicted that all the different concentrations (0.1 ppm, 0.5 ppm and 1.0 ppm) of aflatoxin $B_1$ caused reduction on the colony growth of *F. moniliforme* and *P. citrinum*. It has been observed that even at 144h of incubation period the colony growth of *F. moniliforme* and *P. citrinum* were much reduced when compared to that of the respective controls.

Fig.5.4.A depicted that 0.1 ppm, 0.5 ppm and 1 ppm concentrations of aflatoxin $B_1$ stimulatory effect on growth of A. flavus except at 144h of incubation where no stimulatory/inhibitory effect was

observed. Similar results were observed with 0.1 ppm concentration after 24h, 72h and 120h of incubation periods. Maximum stimulation was observed after 48h of incubation at 1.0 ppm aflatoxin added nutrient medium.

All the concentrations (0.1 ppm, 0.5 ppm and 1.0 ppm) of aflatoxin $B_1$ were inhibitory on the colony growth of F. moniliforme (Fig.5.4.B) and P. citrinum (Fig.5.4.C). Maximum inhibition was observed at 1.0 ppm aflatoxin $B_1$ in both the fungi. It was also observed that the lower concentration of aflatoxins were less inhibitory.

Significant variation on growth of toxigenic fungal species by aflatoxin $B_1$ has been noted (Table 5.7). Between incubation period ($P>0.001$, $P>0.01$ and $P>0.05$) for *A. flavus*, *F. moniliforme* and *P. citrinum* respectively). Similarly between concentration, significant variation ($P>0.001$) for all species has been noted.

Table 5.8 showed the analysis of data of the different concentrations of aflatoxin $B_1$ on growth of toxigenic fungi. Between incubation period in-significant variation at 0.1 ppm has been noted but significant variations ($P>0.001$) at 0.5 ppm and 1.0 ppm has been noted. Between fungal species, there was no significant variation.

3. Seed germination and seedling growth :

Response of two maize varieties to different mycotoxins, i.e. aflatoxin $B_1$, zearalenone and citrinin on seed germination and seedling growth was depicted in Tables (5.3 and 5.4) and Figures (5.5,

5.6 and 5.7).

Maximum inhibition was recorded at 1 ppm of aflatoxin $B_1$ in both the maize varieties. The seeds of local white variety were more affected than local yellow variety (Fig.5.5.A). Similar trend on inhibition of seed germination was observed with zearalenone (Fig.5.5.B). However, in case of citrinin maximum inhibition on seed germination was exhibited by local yellow variety than local white one.

Reduction of seedling growth was observed in all the treated samples compared with untreated ones (Table 5.3). The table showed that on the 5th day of incubation, the maximum shoot growth (1.68 cm) was observed with 0.1 ppm of citrinin and root growth (5.36 cm) with 0.1 ppm of aflatoxin $B_1$; minimum shoot growth (1.15 cm) and root growth (3.82 cm) were with 1.0 ppm of zearalenone. On the 10th day, the maximum shoot growth (5.62 cm) and root growth (6.65 cm) were observed with 0.1 ppm of citrinin and minimum with 1 ppm of citrinin while on the 15th day, their growth was maximum on 0.1 ppm of aflatoxin $B_1$ and minimum on 1 ppm of citrinin on shoot growth and zearalenone of root growth.

Fig.5.6.A depicted the inhibition (%) on seedling growth of maize in local white variety by aflatoxin $B_1$. Maximum inhibition of seedling growth was observed with aflatoxin $B_1$ (1 ppm) whereas the minimum was observed with 0.1 ppm concentration. The pattern of zearalenone and citrinin was also similar (Fig.5.6.B and C).

Table 5.4 depicted the effect of different concentrations (0.1 ppm, 0.5 ppm and 1.0 ppm) of mycotoxins, viz. aflatoxin $B_1$, Zearalenone and citrinin on seedling growth of local yellow variety of maize. Reduction on seedling growth was observed in all the treated samples when compared with the control. On the 5th day of incubation, the maximum shoot length (1.59 cm) and root length (4.98 cm) were observed with 0.1 ppm of citrinin and zearelenone respectively, whereas minimum shoot length (3.56 cm) and root length (2.43 cm) were observed with 1.0 ppm of zearalenone. On the 10th day of incubation, the maximum shoot length (5.02 cm) and root length (11.09 cm) was observed with 0.1 ppm of citrinin and aflatoxin $B_1$ respectively. Minimum shoot length (2.43 cm ) and root length (5.24 cm) were observed with 1.0 ppm of zearalenone. On the 15th day of incubation, maximum shoot length (6.49 cm) and root length (13.56 cm) were observed with 0.1 ppm of citrinin and zearalenone respectively where minimum shoot length (2.85 cm) and root length (7.99 cm) were observed with 1 ppm of citrinin and zearalenone respectively.

Fig.5.7.A depicted the inhibition (%) on seedling growth of maize by aflatoxin $B_1$. Amongst all the concentrations of aflatoxin $B_1$ tested, 1 ppm was observed to have the maximum effect and 0.1 ppm to have the minimum effect on the seedling growth.

Fig.5.7.B showed the inhibition (%) on seedling growth of maize by zearelenone, similar results were observed, where 1 ppm have the highest inhibition whereas 0.1 ppm have the lowest inhibition.

Fig.5.7.C depicted the effect of citrinin on seedling growth of maize. Maximum inhibition was observed with 1 ppm concentration and minimum with 0.1 ppm.

Statistical analysis (ANOVA) exhibited insignificant variations due to incubation period except in local white variety, citrinin on shoot length, zearalenone on root length and in local yellow variety, zearalenone and citrinin on shoot length and zearalenone on root length exhibited significant variations ($P>0.001$). Between concentration significant variation has been noted as shown in Table 5.9.

Table 5.10 exhibited insignificant variation between incubation period except in local yellow variety with 1.0 ppm on shoot length where significant variation has been noted ($P>0.001$). Between mycotoxins significant variation has been noted at different levels.

**Table 5.1 : Effect of aflatoxin $B_1$ on spore germination of toxigenic fungi at different temperatures.**

| Toxigenic Fungi | Concentration of aflatoxin $B_1$ (ppm) | Spore gemination (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 20°C | | | | 25°C | | | | 30°C | | | |
| | | 2h | 5h | 10h | 24h | 2h | 5h | 10h | 24h | 2h | 5h | 10h | 24h |
| | 0.1 | 12 | 16 | 25 | 52 | 8 | 13 | 32 | 62 | 10 | 22 | 34 | 70 |
| *Aspergillus flavus* | 0.5 | 13 | 17 | 26 | 75 | 9 | 15 | 47 | 65 | 12 | 25 | 50 | 80 |
| | 1.0 | 15 | 18 | 35 | 80 | 10 | 16 | 54 | 90 | 14 | 30 | 56 | 90 |
| Control | 0.0 | 10 | 15 | 24 | 50 | 6 | 12 | 30 | 60 | 8 | 20 | 30 | 80 |
| | 0.1 | - | 35 | 44 | 47 | - | 42 | 62 | 70 | - | - | - | - |
| *Fusarium moniliforme* | 0.5 | - | 22 | 20 | 25 | - | 40 | 60 | 64 | - | - | - | - |
| | 1.0 | - | 00 | 00 | 00 | - | 00 | 00 | 00 | - | - | - | - |
| Control | 0.0 | - | 40 | 60 | 70 | - | 56 | 75 | 100 | - | - | - | - |
| | 0.1 | - | 8 | 10 | 45 | - | 20 | 26 | 40 | - | - | - | - |
| *Penicillium citrinum* | 0 .5 | - | 4 | 5 | 20 | - | 00 | 00 | 26 | - | - | - | - |
| | 1.0 | - | - | - | - | - | 00 | 00 | 00 | - | - | - | - |
| Control | 0.0 | - | 10 | 12 | 50 | - | 28 | 42 | 100 | - | - | - | - |

- = No observation

**Table 5.2 : Effect of aflatoxin $B_1$ on the growth of toxigenic fungi.**

| Toxigenic fungi | Concentration of aflatoxin $B_1$ (ppm) | Mean radial expansion (in mm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 24h | 48h | 72h | 96h | 120h | 144h |
| *Aspergillus flavus* | 0.1 | 0.8 | 1.1 | 2.5 | 2.8 | 4.3 | 4.5 |
| | 0.5 | 0.9 | 1.5 | 2.7 | 3.0 | 4.5 | 4.5 |
| | 1.0 | 1.0 | 1.6 | 3.0 | 3.2 | 4.5 | 4.5 |
| Control | 0.0 | 0.8 | 1.0 | 2.5 | 2.7 | 4.3 | 4.5 |
| *Fusarium moniliforme* | 0.1 | 0.5 | 0.7 | 1.5 | 1.9 | 2.5 | 3.0 |
| | 0.5 | 0.3 | 0.7 | 1.4 | 1.6 | 2.0 | 2.4 |
| | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.5 |
| Control | 0.0 | 0.7 | 0.9 | 1.8 | 2.2 | 3.0 | 4.0 |
| *Penicillium citrinum* | 0.1 | 0.6 | 1.0 | 1.9 | 2.5 | 3.5 | 4.0 |
| | 0.5 | 0.5 | 0.6 | 1.2 | 1.9 | 2.2 | 2.4 |
| | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Control | 0.0 | 0.8 | 1.2 | 2.2 | 2.8 | 3.9 | 4.3 |

Table 5.3 : Effect of mycotoxins on seedling growth of local white variety of maize.

| Concentration of mycotoxins (ppm) | Seedling growth (cm) | | | | | |
|---|---|---|---|---|---|---|
| | 5th day | | 10th day | | 15th day | |
| | Shoot | Root | Shoot | Root | Shoot | Root |
| *Aflatoxin* $B_1$ | | | | | | |
| 0.1 | 1.58±0.04 | 5.36±0.17 | 5.08±0.15 | 12.17±0.12 | 6.76±0.19 | 14.48±0.11 |
| 0.5 | 1.34±0.02 | 4.60±0.11 | 4.48±0.08 | 10.14±0.16 | 5.44±0.09 | 12.64±0.16 |
| 1.0 | 1.24±0.02 | 4.47±0.05 | 3.96±0.11 | 8.78±0.09 | 4.17±0.06 | 11.65±0.09 |
| *Zearalenone* | | | | | | |
| 0.1 | 1.60±0.04 | 5.16±0.11 | 4.58±0.08 | 11.19±0.15 | 6.21±0.10 | 13.77±0.15 |
| 0.5 | 1.33±0.02 | 5.04±0.05 | 2.85±0.06 | 7.91±0.09 | 4.45±0.09 | 9.71±0.06 |
| 1.0 | 1.15±0.02 | 3.82±0.07 | 2.84±0.05 | 7.11±0.10 | 4.15±0.07 | 8.11±0.10 |
| *Citrinin* | | | | | | |
| 0.1 | 1.68±0.04 | 5.16±0.12 | 5.62±0.10 | 9.67±0.12 | 6.65±0.17 | 13.48±0.11 |
| 0.5 | 1.35±0.02 | 4.74±0.09 | 4.04±0.08 | 9.24±0.15 | 5.37±0.09 | 11.64±0.16 |
| 1.0 | 1.27±0.03 | 4.69±0.07 | 2.84±0.09 | 6.65±0.09 | 3.11±0.03 | 10.65±0.09 |
| Control 0.0 | 1.75±0.03 | 6.46±0.10 | 6.75±0.16 | 14.08±0.34 | 9.60±0.23 | 15.72±0.07 |

**Table 5.4 :** Effect of mycotoxins on seedling growth on local yellow variety of maize.

| Concentration of mycotoxins (ppm) | Seedling growth (cm) | | | | | |
|---|---|---|---|---|---|---|
| | 5th day | | 10th day | | 15th day | |
| | Shoot | Root | Shoot | Root | Shoot | Root |
| *Aflatoxin* $B_1$ | | | | | | |
| 0.1 | 1.45±0.05 | 5.12±0.07 | 4.96±0.12 | 11.09±0.09 | 6.54±0.17 | 14.02±0.13 |
| 0.5 | 1.21±0.02 | 4.34±0.10 | 3.95±0.23 | 9.83±0.14 | 5.02±0.07 | 12.25±0.08 |
| 1.0 | 1.19±0.01 | 4.21±0.03 | 3.86±0.15 | 8.35±0.07 | 3.96±0.02 | 10.95±0.04 |
| *Zearalenone* | | | | | | |
| 0.1 | 1.55±0.04 | 4.98±0.09 | 3.83±0.09 | 11.02±0.09 | 6.04±0.12 | 13.56±0.14 |
| 0.5 | 1.47±0.03 | 4.73±0.03 | 2.54±0.12 | 6.82±0.05 | 3.95±0.14 | 9.47±0.10 |
| 1.0 | 1.39±0.02 | 3.56±0.05 | 2.43±0.10 | 5.24±0.15 | 3.48±0.03 | 7.99±0.08 |
| *Citrinin* | | | | | | |
| 0.1 | 1.59±0.03 | 4.95±0.12 | 5.02±0.14 | 9.08±0.03 | 6.49±0.15 | 12.84±0.17 |
| 0.5 | 1.28±0.04 | 4.68±0.07 | 3.92±0.09 | 8.75±0.16 | 4.98±0.08 | 11.52±0.09 |
| 1.0 | 1.09±0.02 | 4.43±0.04 | 2.63±0.02 | 6.24±0.07 | 2.85±0.01 | 9.25±0.03 |
| Control 0.0 | 1.62±0.02 | 6.30±0.04 | 6.42±0.12 | 13.96±0.25 | 8.54±0.32 | 15.55±0.14 |

**Table 5.5** :Analysis of variance between spore germination of toxigenic fungi due to aflatoxin $B_1$ at different tempera tures using 'F' test

| Variations | 'F' Value | | |
|---|---|---|---|
| | *Aspergillus flavus* | *Fusarium moniliforme* | *Penicillium citrium* |
| Due to Temperature | 9.028** | 3.48043* | 8.221** |
| Due to Concentrations | 9.23617** | 2.661771* | 4.477582* |

** = Significant at 0.01 level
* = Significant at 0.05 level

**Table 5.6** : Analysis of variance between spore germination due to fungal species at different temperatures using 'F' test.

| Variations | 'F' Value | | |
|---|---|---|---|
| | 0.1 ppm | 0.5 ppm | 1.0 ppm |
| Due to temperature | 1.785685* | 1.109506* | 0.9999* |
| Due to fungal species | 1.64102* | 6.593483** | 676.0016 |

** = Significant at 0.01 level
* = significant at 0.05 level

**Table 5.7** : Analysis of variance on growth of toxigenic fungi by aflatoxin $B_1$ due to incubation period using 'F' test.

| Variations | 'F' Value | | |
|---|---|---|---|
| | *Aspergillus flavus* | *Fusarium moniliforme* | *Penicillium citrium* |
| Due to incubation period | 4.698343*** | 4.001** | 1.041089* |
| Due to concentrations | 5.627521*** | 5.251924*** | 4.993311*** |

*** = Significant at 0.001 level
** = Significant at 0.01 level
* = Significant at 0.05 level

**Table 5.8** : Analysis of Variance on aflatoxin $B_1$ due to fungal species and due to incubation period using 'F' test

| Variations | 'F' Value | | |
|---|---|---|---|
| | 0.1 ppm | 0.5 ppm | 1.0 ppm |
| Due to incubation period | 9.984443 | 4.610482*** | 4.661273*** |
| Due to fungal species | 17.86785 | 20.66411 | 20.08961 |

*** = Significant at 0.001 level

Table 5.9 : Analysis of variance between seedling growth of both maize varieties and aflatoxin $B_1$ at different incubation period using 'F' test.

| Variations | 'F' Value | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Local White | | | | | | Local Yellow | | | | | |
| | Shoot | | | Root | | | Shoot | | | Root | | |
| | Aflatoxin | Zearalenone | Citrinin | Aflatoxin | Zearalenone | Citrinin | Aflatoxin | Zearalenone | Citrinin | Aflatoxin | Zearalenone | Citrinin |
| Due to incubation period | 41.65615 | 39.38534 | 14.91203*** | 119.6211 | 17.44547*** | 51.7637 | 34.90136 | 16.34512*** | 15.22697*** | 134.3707 | 14.17845*** | 42.95143 |
| Due to concentration | 4.192567* | 7.34033** | 5.296657* | 10.06182** | 6.871423** | 4.640901* | 3.839235* | 3.899368 | 5.663162** | 10.63247** | 7.589193** | 5.789413** |

*** = Significant at 0.001 level
** = Significant at 0.01 level
* = Significant at 0.05 level

Table 5.10 : Analysis of variance between seedling growth of both maize varieties and aflatoxin $B_1$ due to fungal species at different incubation period using 'F' test.

| Variations | 'F' Value | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Local White | | | | | | Local Yellow | | | | | |
| | Shoot | | | Root | | | Shoot | | | Root | | |
| | 0.1 ppm | 0.5 ppm | 1.0 ppm | 0.1 ppm | 0.5 ppm | 1.0 ppm | 0.1 ppm | 0.5 ppm | 1.0 ppm | 0.1 ppm | 0.5 ppm | 1.0 ppm |
| *Due to incubation period* | 258.68 | 59.31 | 27.47 | 162.00 | 40.61 | 26.32 | 147.77 | 35.09 | 19.85*** | 180.41 | 34.66 | 36.67 |
| Due to concentration | 2.82* | 3.55* | 1.93* | 3.15* | 2.32* | 2.96* | 2.37* | 2.24* | 2.55* | 3.46* | 2.80* | 6.48** |

*** = Significant at 0.001 level
** = Significant at 0.01 level
* = Significant at 0.05 level

**Figure 5.1.** Stimulation (%) of spore germination of *Aspergillus flavus* by aflatoxin $B_1$ at different temperatures.

Fig. 5.1.

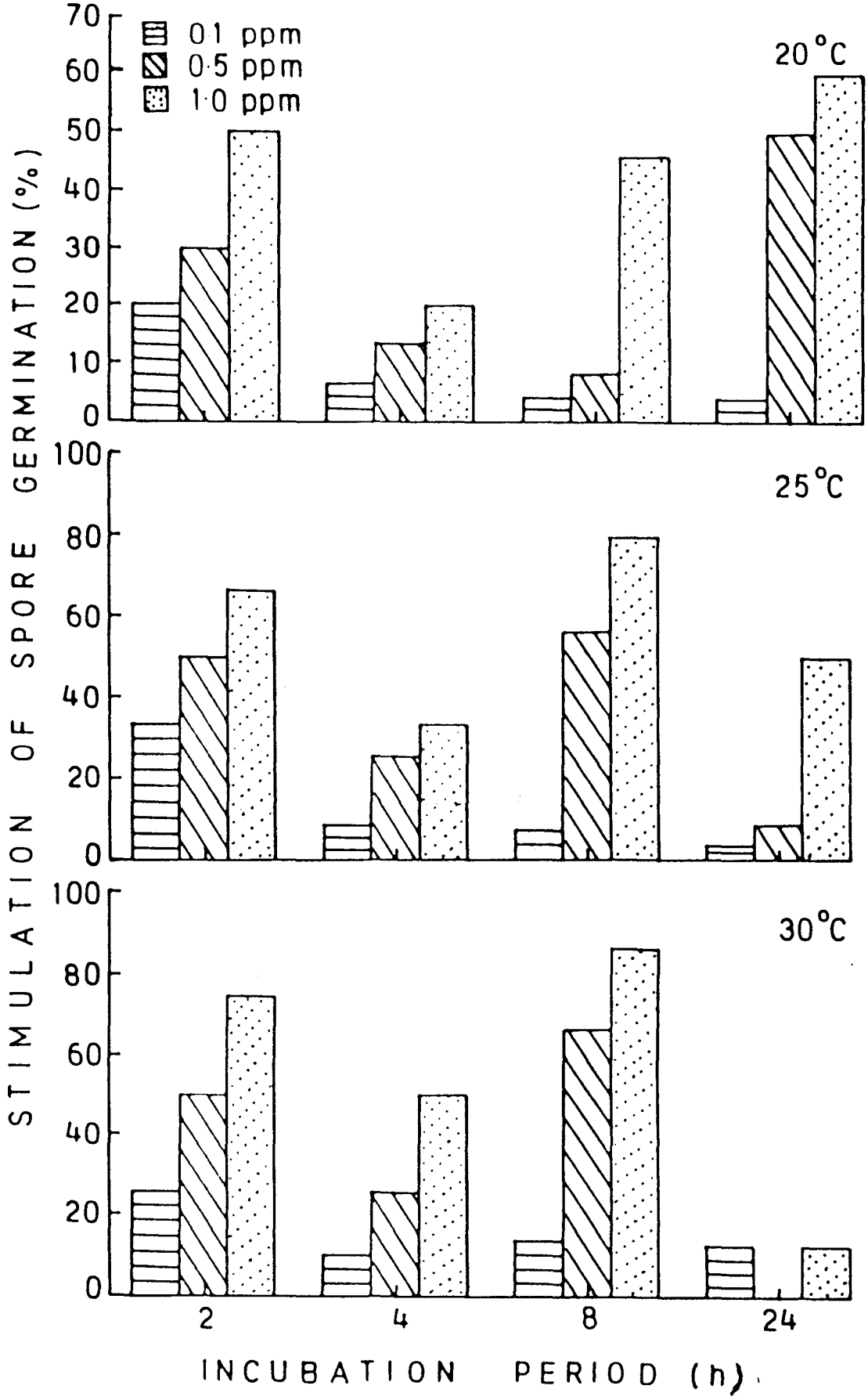

0·1 ppm
0·5 ppm
1·0 ppm
20°C
25°C
30°C
STIMULATION OF SPORE GERMINATION (%)
0
10
20
30
40
50
60
70
80
100
2
4
8
24
INCUBATION PERIOD (h)

**Figure 5.2.** Inhibition (%) of spore germination of *Fusarium moniliforme* by aflatoxin $B_1$ at different temperatures.

Fig. 5.2.

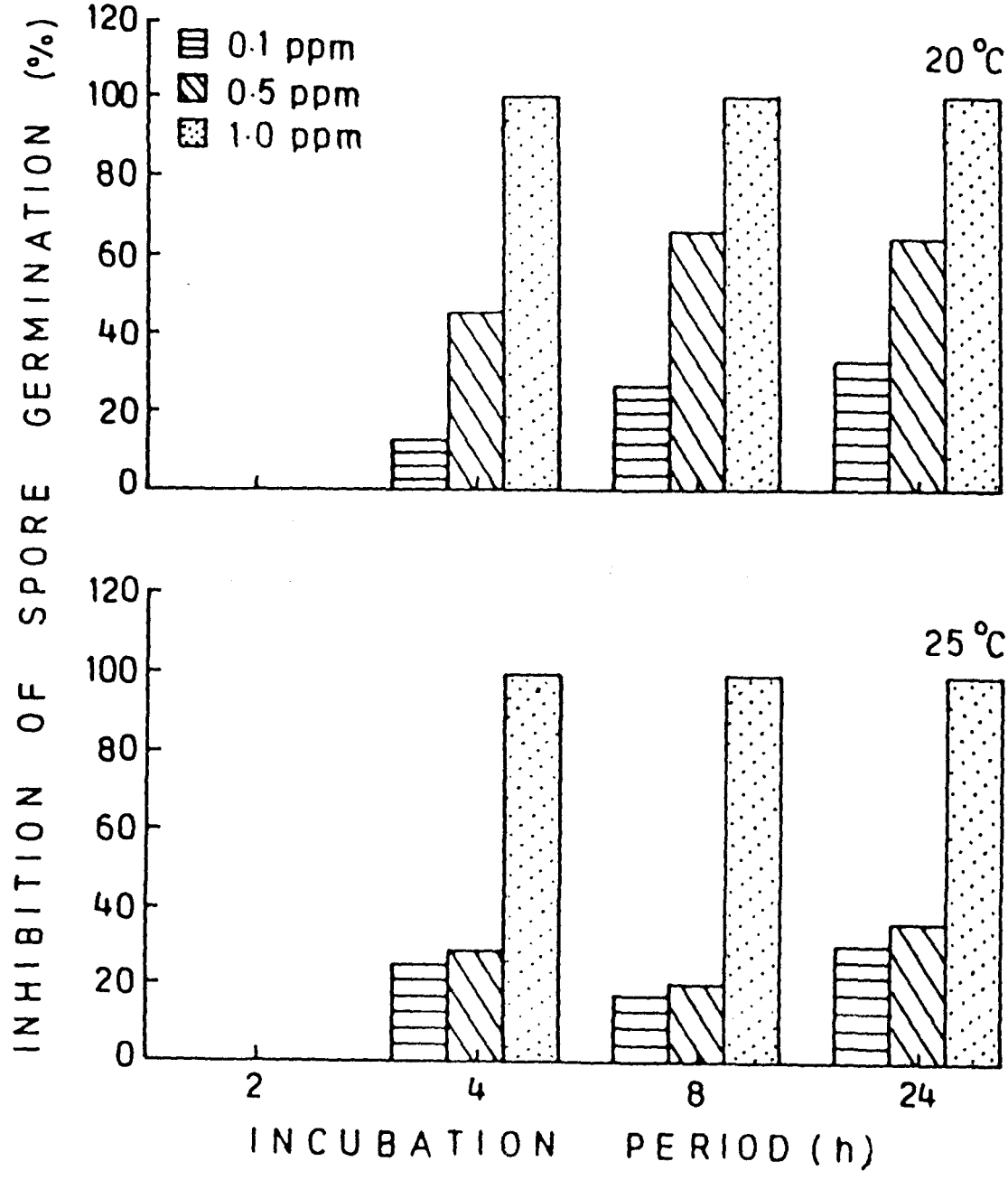

INHIBITION OF SPORE GERMINATION (%)
120
100
80
60
40
20
0
0·1 ppm
0·5 ppm
1·0 ppm
20 °C
25 °C
2
4
8
24
INCUBATION PERIOD (h)

**Figure 5.3.** Inhibition (%) of spore germination of *Penicillium citrinum* by aflatoxin $B_1$ at different temperatures.

Fig. 5.3.

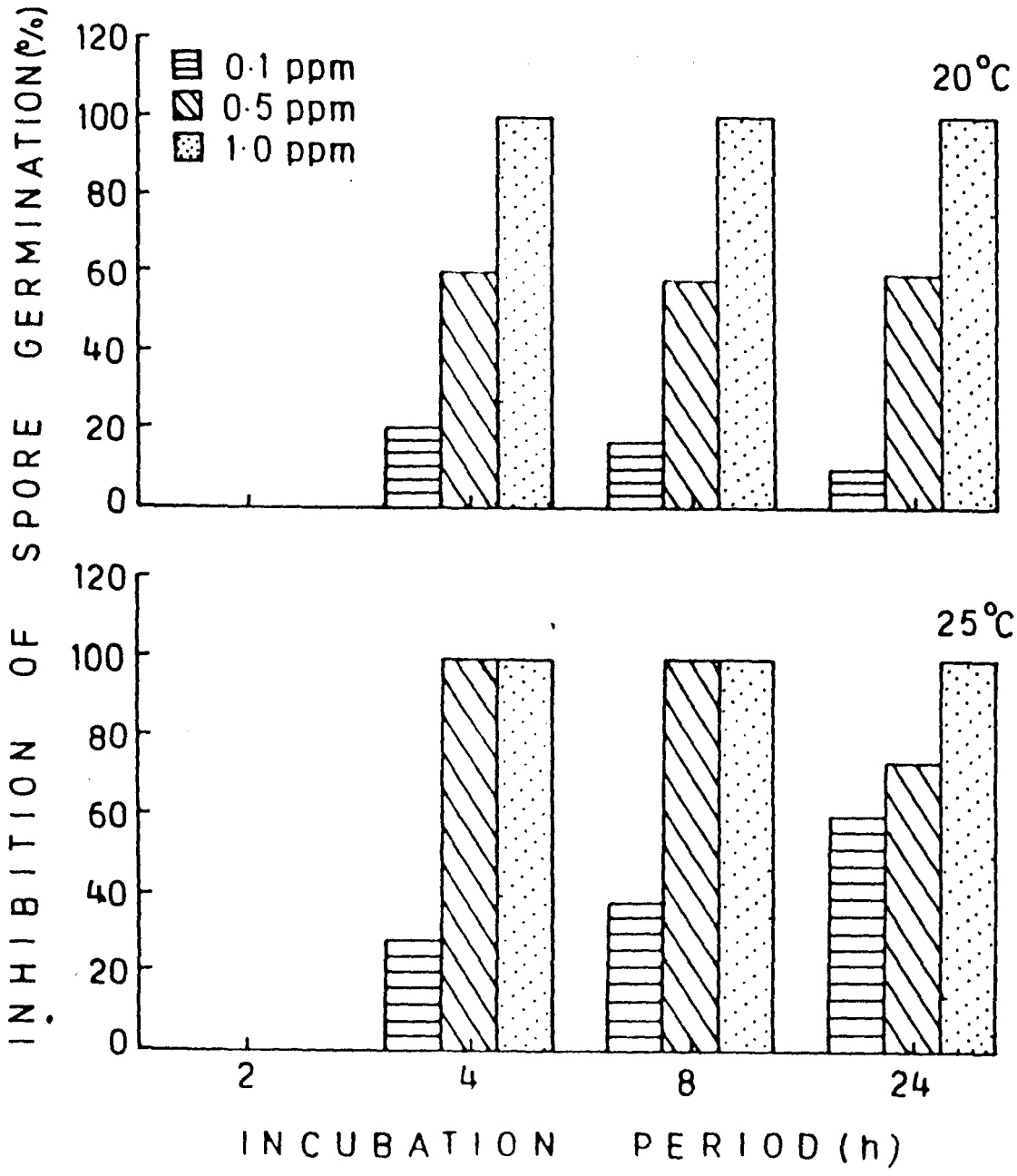

INHIBITION OF SPORE GERMINATION(%)
120
100
80
60
40
20
0
0·1 ppm
0·5 ppm
1·0 ppm
20°C
25°C
2
4
8
24
INCUBATION PERIOD (h)

**Figure 5.4.** Stimulation (%) of growth of *Aspergillus flavus* (A) and inhibition (%) of growth of *Fusarium moniliforme* (B) and *Penicillium citrinum* (C).

Fig. 5.4.

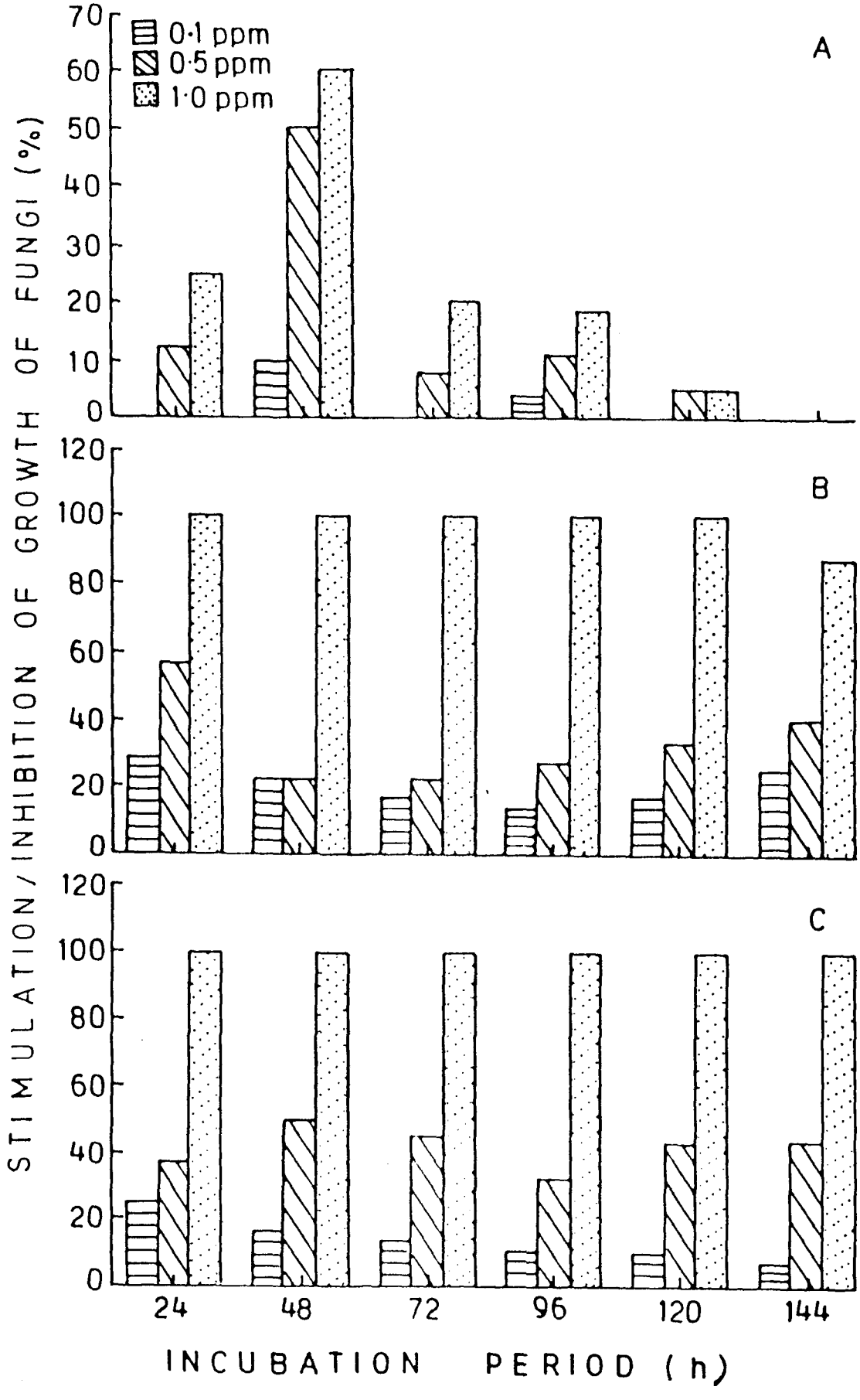

0·1 ppm
0·5 ppm
1·0 ppm
A
B
C
70
60
50
40
30
20
10
0
120
100
80
60
40
20
0
24
48
72
96
120
144
STIMULATION / INHIBITION OF GROWTH OF FUNGI (%)
INCUBATION PERIOD (h)

**Figure 5.5.** Inhibition (%) of seed germination of local white (LW) and local yellow (LY) varieties of maize by aflatoxin $B_1$ (A), zearelenone (B) and citrinin (C).

Fig. 5.5.

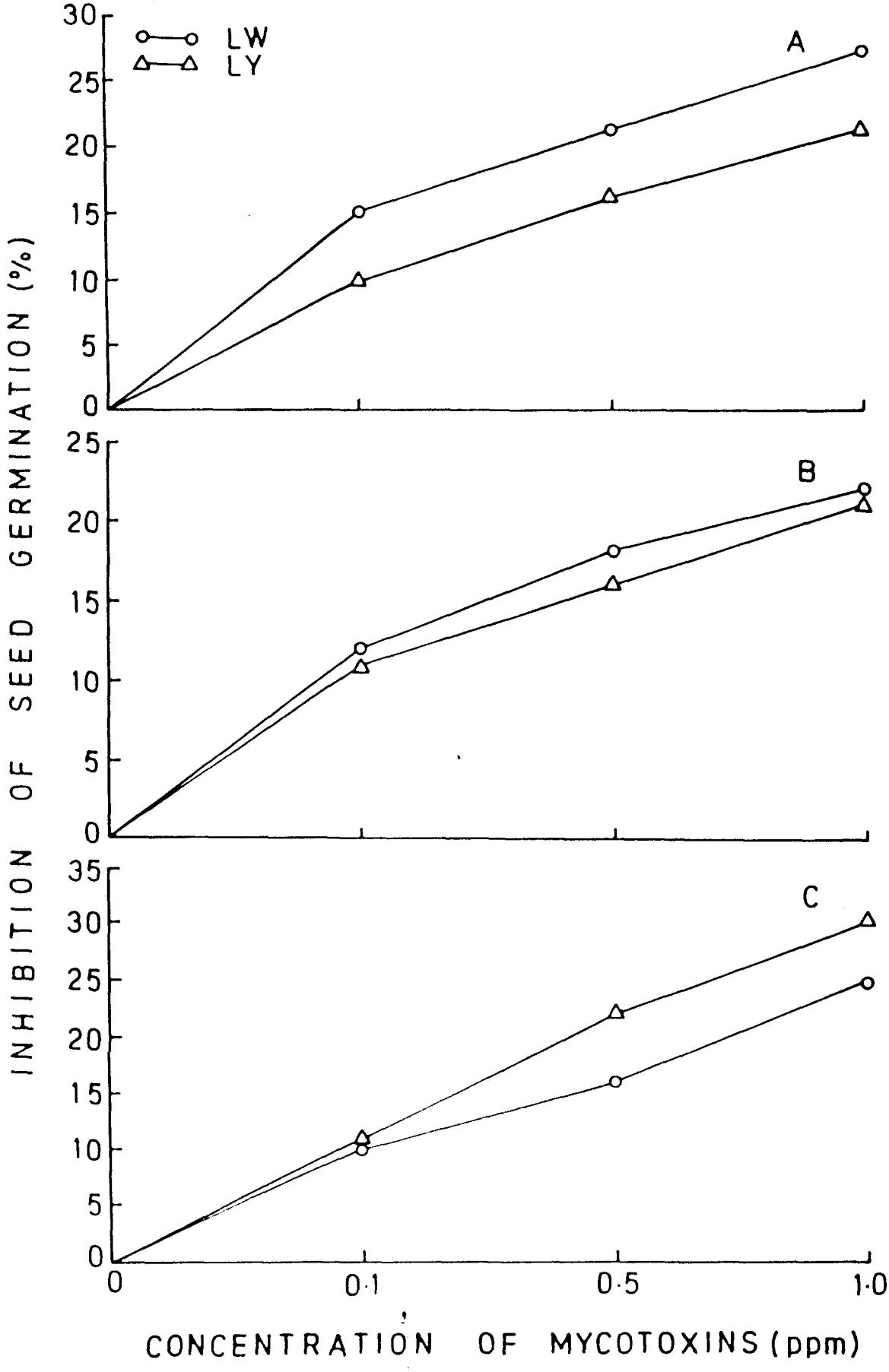

LW
LY
A
B
C
INHIBITION OF SEED GERMINATION (%)
CONCENTRATION OF MYCOTOXINS (ppm)
0
0·1
0·5
1·0

**Figure 5.6.** Effect of mycotoxins on seedling growth of local white variety of maize by aflatoxin $B_1$ (A) zearalenone (B) and citrinin (C).

Fig. 5.6.

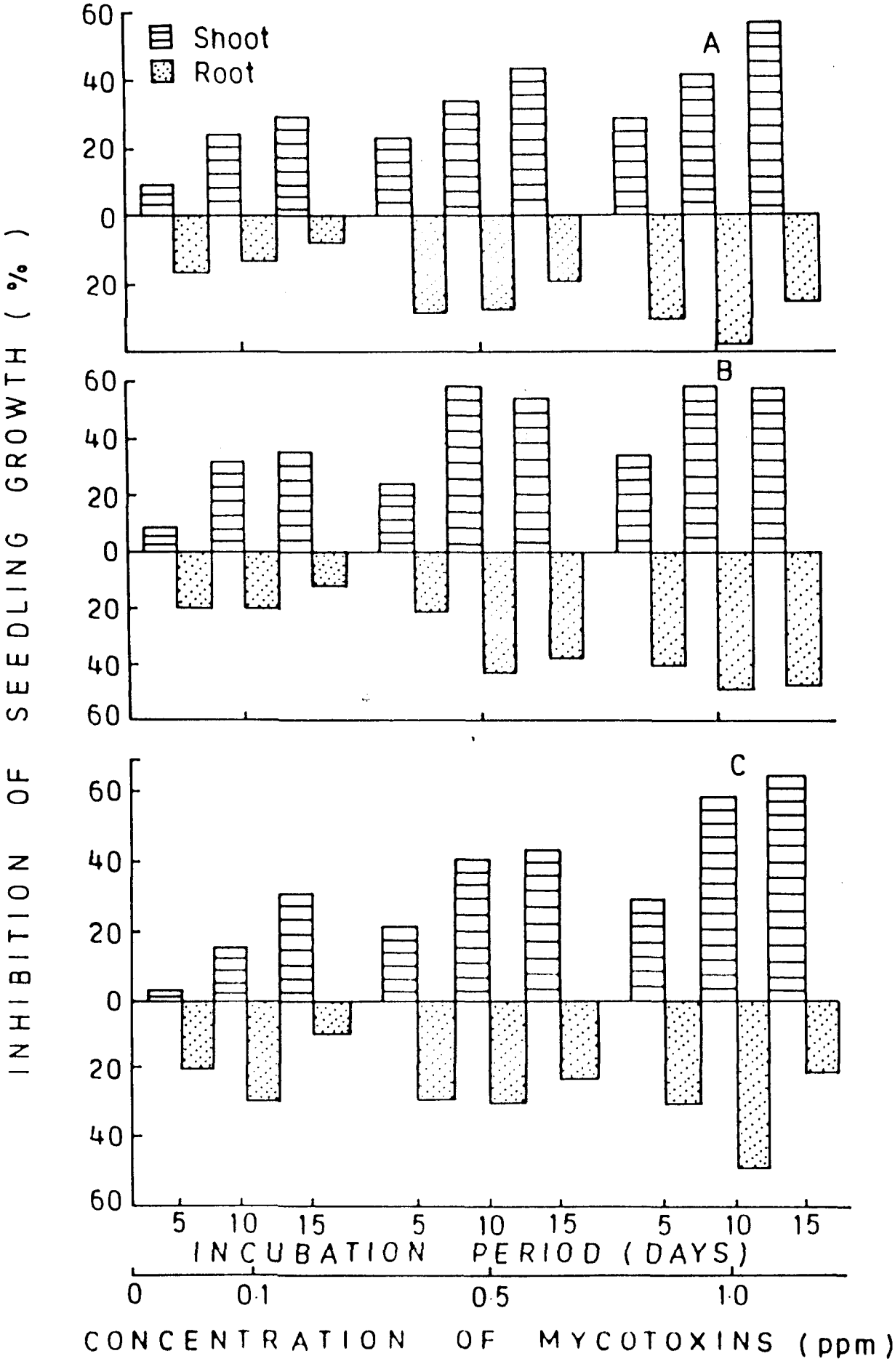
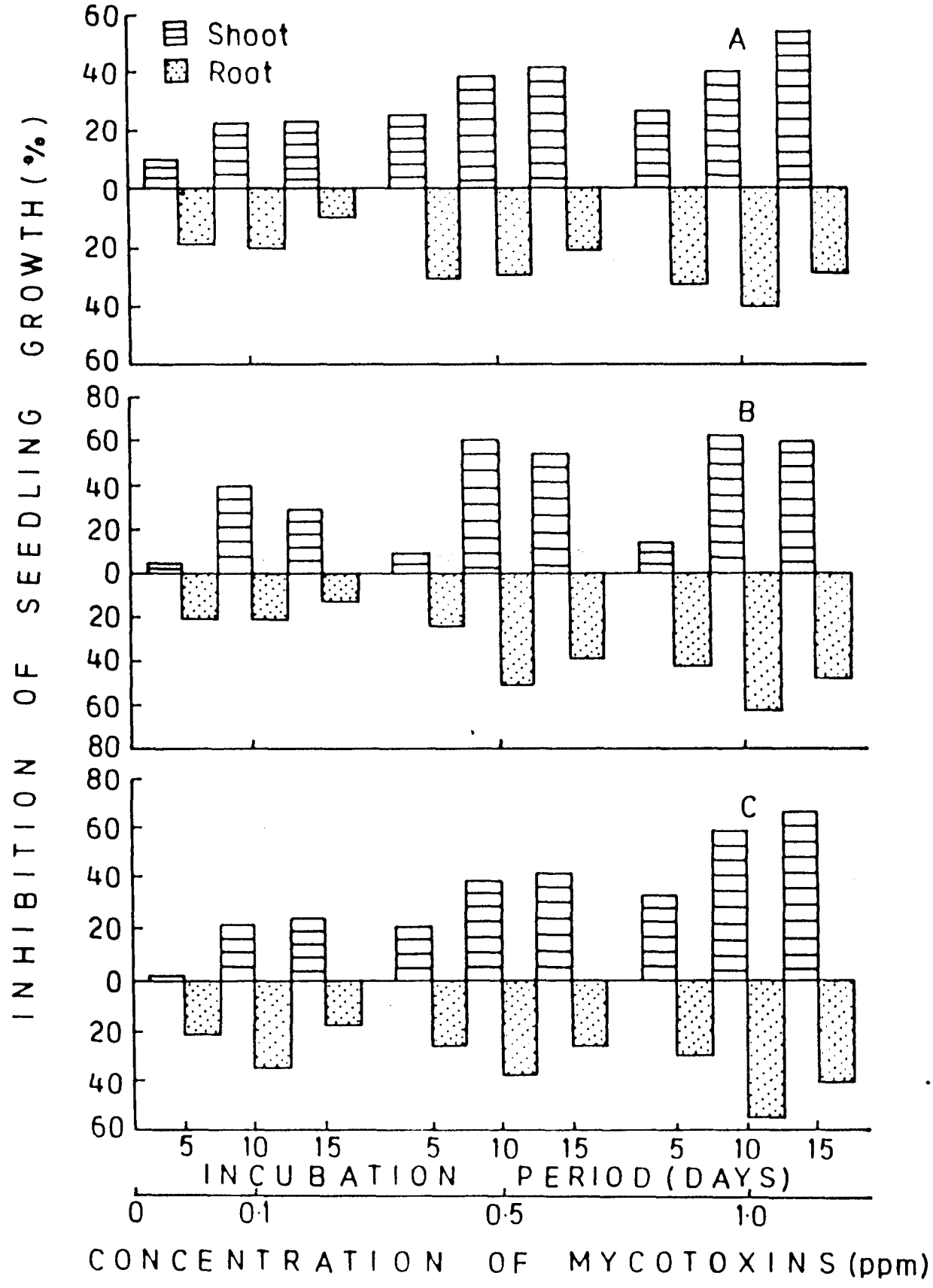

Shoot
Root
A
B
C
60
40
20
0
20
40
60
INHIBITION OF SEEDLING GROWTH ( % )
5 10 15 5 10 15 5 10 15
INCUBATION PERIOD ( DAYS )
0 0·1 0·5 1·0
CONCENTRATION OF MYCOTOXINS ( ppm )

**Figure 5.7.** Effect of mycotoxins on seedling growth of local yellow variety of maize by aflatoxin $B_1$ (A), zearalenone (B) and citrinin (C).

Fig. 5.7.


Shoot
Root
A
B
C
60
40
20
0
20
40
60
80
INHIBITION OF SEEDLING GROWTH (%)
5 10 15 5 10 15 5 10 15
INCUBATION PERIOD (DAYS)
0 0·1 0·5 1·0
CONCENTRATION OF MYCOTOXINS (ppm)

## VI. Effect of aflatoxin B1 on albino mice

1. Effect on the weight :

The mice fed with pure aflatoxin $B_1$ lost more weight than those fed with crude one. Mice of all the age groups were sensitive to aflatoxin feeding and showed negative growth. The young mice have shown the sign of recovery from the aflatoxin contaminated feeding and the older mice were worst affected. The response of male and female mice was different to different feedings of aflatoxin contaminated food. Female mice were affected more than the male ones (Table 6).

2. Effect on the liver :

No liver tumors were observed in any test animal, but some of the mice treated with pure and crude aflatoxin $B_1$ showed abnormally light in color of liver in all three age groups of both the sexes. This abnormal light in color of liver was more pronounced in those mice treated with pure aflatoxin $B_1$ than crude one.

3. Effect on the skin :

Loss of hairs, particularly on the posterior dorsal surface of the body was observed in some of the animals treated with pure and crude aflatoxin $B_1$, but not in control ones. The loss of hairs from skin was more pronounced in the animals treated with pure aflatoxin $B_1$ than the crude form of aflatoxin $B_1$. Vertical sections of the skin showed patches of non-differentiated, deeply stained cell masses in the dermis of mice treated with pure and crude aflatoxin $B_1$.

4. Effect on the rate of mortality :

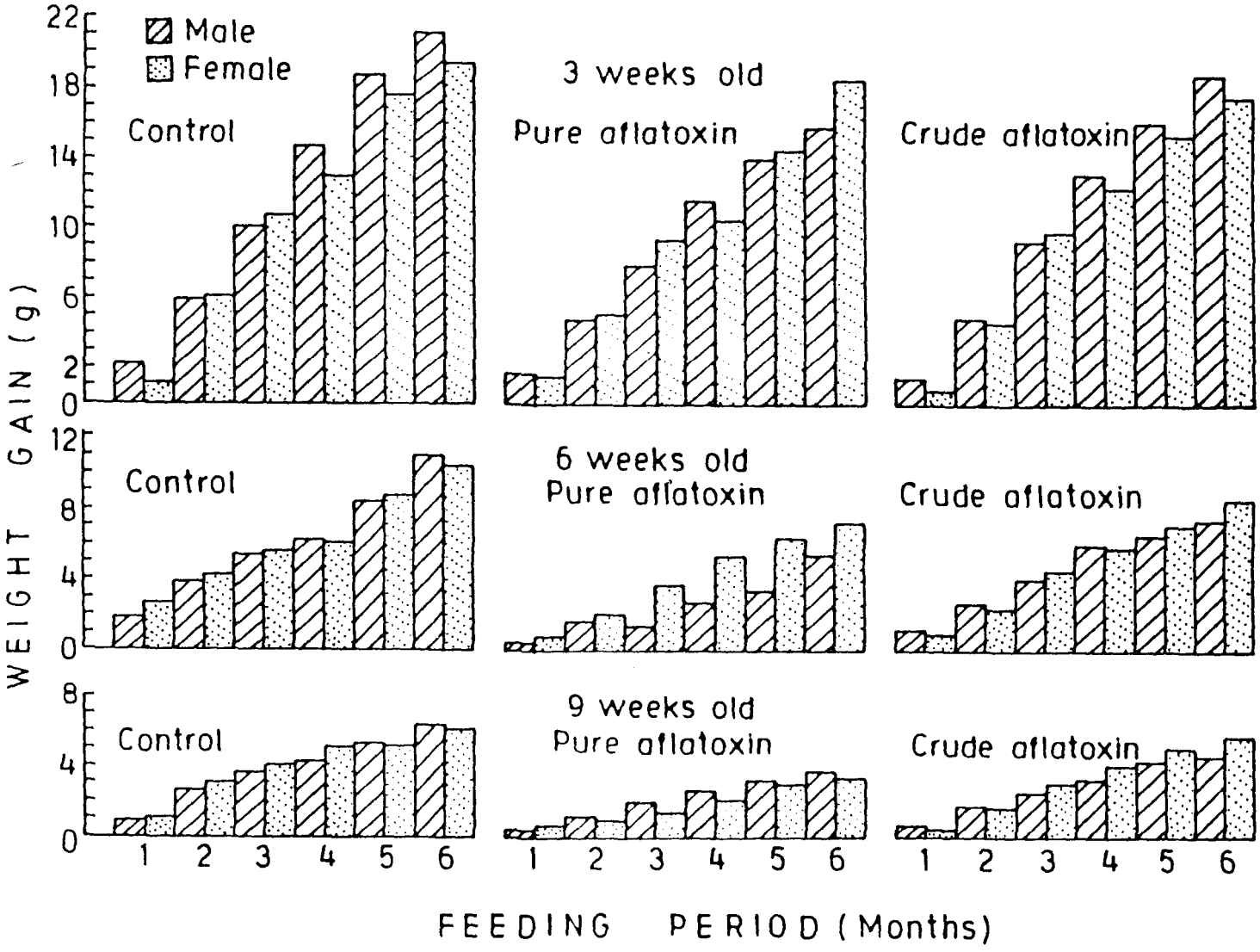
In the present study, it has been observed that the rate of mortality was affected only with the mice treated with pure aflatoxin $B_1$ in the 3 weeks old age group. Out of 10 mice taken, 1 male and 2 females died between 6-7 months feeding period. Mice of 6 and 9 weeks old survived till the time of sacrifice, that is, after 7 months feeding period.

**Table 6** : Effect of feeding mice with aflatoxin $B_1$ on their weight.

| Aflatoxins $B_1$ | Initial age (week) | Sex | Average initial wt. in grams | Average final weight post feeding (Months) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 | 6 |
| Pure aflatoxin $B_1$ | 3 | M | 14.84 | 16.59 | 19.80 | 22.72 | 26.40 | 28.80 | 30.50 |
| | | F | 13.30 | 14.80 | 18.32 | 22.50 | 23.64 | 27.80 | 31.70 |
| | 6 | M | 14.90 | 15.20 | 16.53 | 16.24 | 17.42 | 18.24 | 20.10 |
| | | F | 13.80 | 14.52 | 15.81 | 17.51 | 18.92 | 20.22 | 20.90 |
| | 9 | M | 15.00 | 15.32 | 16.02 | 16.85 | 17.49 | 18.02 | 18.75 |
| | | F | 14.00 | 14.52 | 14.82 | 15.22 | 16.02 | 16.88 | 17.42 |
| Crude aflatoxin $B_1$ | 3 | M | 14.90 | 16.35 | 19.88 | 24.04 | 27.90 | 30.82 | 33.52 |
| | | F | 13.62 | 14.52 | 18.43 | 23.02 | 25.90 | 28.70 | 30.95 |
| | 6 | M | 15.00 | 16.02 | 17.62 | 19.02 | 20.82 | 21.50 | 22.15 |
| | | F | 14.50 | 15.47 | 16.74 | 18.82 | 20.24 | 21.50 | 22.92 |
| | 9 | M | 14.90 | 15.52 | 16.62 | 17.42 | 18.02 | 18.92 | 19.54 |
| | | F | 13.50 | 14.02 | 15.20 | 16.42 | 17.23 | 18.41 | 18.92 |
| Control | 3 | M | 15.02 | 17.09 | 20.90 | 25.02 | 29.82 | 33.90 | 36.34 |
| | | F | 13.90 | 15.00 | 19.90 | 24.52 | 26.80 | 30.98 | 33.68 |
| | 6 | M | 14.90 | 16.71 | 18.71 | 20.11 | 21.01 | 23.21 | 25.71 |
| | | F | 14.00 | 16.60 | 18.10 | 19.30 | 20.10 | 22.60 | 24.20 |
| | 9 | M | 14.50 | 15.40 | 16.82 | 17.90 | 18.51 | 19.70 | 20.60 |
| | | F | 14.01 | 15.00 | 16.30 | 17.50 | 18.30 | 19.20 | 20.02 |

**Figure 6.** Effect of aflatoxin $B_1$ on the changes in the weights of albino mice.

Fig. 6.


Male
Female
3 weeks old
Control
Pure aflatoxin
Crude aflatoxin
6 weeks old
Control
Pure aflatoxin
Crude aflatoxin
9 weeks old
Control
Pure aflatoxin
Crude aflatoxin
WEIGHT GAIN (g)
FEEDING PERIOD (Months)

## VII. Effect of plant extracts on the growth of *Aspergillus flavus* and aflatoxin production :

Aqueous extracts of plants tested had decreasing effect on the production of fungal biomass compared to the untreated fungi (Table 7). Maximum reduction in fungal biomass was observed with the extracts of *Centella asiatica* and least was recorded with *Thuja orientalis*. *Allium cepa* and *A. sativum* also decreased the growth of *A. flavus*.

The growth of fungi coincided with the production of aflatoxin $B_1$. Production of aflatoxin by *A. flavus* was inhibited by the extracts of all the plants but maximum effect was noticed in *Centella asiatica* treatment, while minimum was in *Thuja orientalis* (Fig.7)

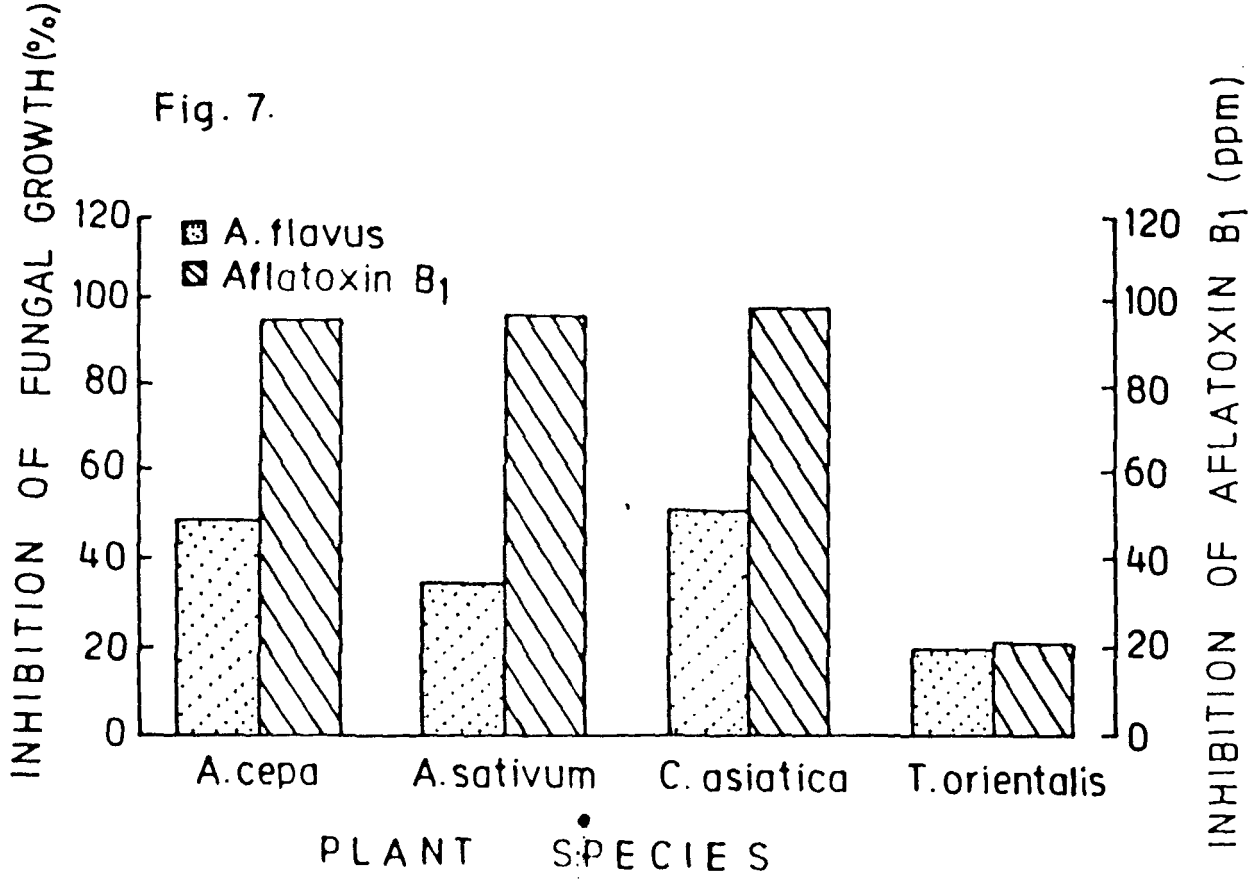
Trend of inhibition of fungal biomass and aflatoxin was *C. asiatica* > *A. cepa* > *A. sativum* > *T. orientalis*.

**Table 7 :** Effect of plant extracts on the growth of *Aspergillus flavus* and aflatoxin production.

| Test species | Plant part used | Fungal biomass (mg/flask) | Amount of aflatoxin $B_1$ |
|---|---|---|---|
| *Alluim cepa* | bulb | 328 | 0.05 |
| *A.sativum* | bulb | 424 | 0.04 |
| *Centella asiatica* | leaf | 315 | 0.02 |
| *Thuja orientalis* | leaf | 515 | 0.85 |
| Control | - | 645 | 1.08 |

**Figure 7.** Inhibition (%) on growth of *Aspergillus flavus* and aflatoxin production by *Allium cepa*, *A. sativum*, *Centella asiatica* and *Thuja orientalis*.

Fig. 7.


INHIBITION OF FUNGAL GROWTH(%)
INHIBITION OF AFLATOXIN B1 (ppm)
A. flavus
Aflatoxin B1
120
100
80
60
40
20
0
A. cepa
A. sativum
C. asiatica
T. orientalis
PLANT SPECIES

# *General Discussion*

Amongst the various techniques used for the isolation of seed borne fungi, blotter method was found to be the most suitable one than agar plate method and dilution plate methods. In terms of number of fungi isolated, blotter method was followed by agar plate method and dilution plate method (de Tempe, 1962; Agarwal *et al.*, 1972; Singh *et al.*, 1973; Lima *et al.*, 1982; Jorgensen, 1982 and Paiva *et al.*, 1984). In the agar plate method certain fast growing fungi, e.g. *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., *Rhizopus* spp. and some sterile mycelia dominated the plates suppressing the growth of slow growing fungi as suggested by Aulakh *et al.* (1976). Therefore, agar plate method may not give correct picture of species composition. However, Gill *et al.* (1983) have observed that PDA method was better than blotter method which was in accordance with some of our findings (Fig.1.2. & Fig.1.4).

Neergaard and Saad (1962) concluded that the blotter method and the agar plate method are both valuable and supplementary to each other. Further these methods have been selected as standard procedures as per International rules for Seed Testing (ISTA, 1966) by International Seed Testing Association after an extensive comparative seed health testing schemes. In the present investigation too, it has been revealed that both agar plate method and blotter methods were necessary for the complete isolation of seed mycoflora. The dilution plate method was found to be useful for the detection of surface mycoflora which are saprophytic species of *Alternaria*, *Aspergillus*,

*Helminthosporium*, *Mucor*, *Fusarium*, *Penicillium*, *Rhizopus*, *Trichoderma* and *Verticillium*. These were mostly field fungi whose frequency of occurrence gradually decreased along with storage time.

Distinct seasonal variations of seed mycoflora was observed during wet summer, autumn and winter seasons of the year. There was a gradual fall in the number of fungal species on the seeds during storage period which increased from wet summer to autumn seasons. Decline in the number of fungi during storage has earlier been observed (Reddy and Reddy, 1983; Larcher *et al.*, 1984; Shree, 1984; Vijayalakshmi and Rao, 1985). It is evident from the Table 1.1 that there was a reduction in the incidence of field fungi and an increase in the storage fungi during the winter season. The storage fungi were mainly *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp.. The reduction of field fungi and increase of storage fungi during storage has also been reported (Schroeder and Sorensen, 1961; Christensen, 1969 and Nandi *et al.*, 1982). The reduction in the incidence of field fungi during storage might be due to the inactivation of inoculum by ageing as suggested by Neergaard (1977). The results of the present investigation have shown that some of the fungal species were encountered in all the seasons (Table 1.1). These fungi have indicated their broad ecological amplitude adopting themselves to different nutrients available during storage period and varying environmental conditions. Those which were restricted to a particular season reflected greater sensitivity to the storage condition and can be categorised as with

narrow ecological amplitude. Fungi of rare occurrence reflected their more demanding nature for specific nutrients and environmental conditions (Bilgrami *et al.*, 1979). The frequent occurrence of some species in all the seasons and reduced frequency or absence of some other species of fungi may be due to unfavourable storage conditions or lack of competitive ability (Gilman and Semeniuk, 1948; Campbell, 1962).

Qualitative variation of fungal species was observed in both the local varieties of maize during different seasons. Altogether 37 fungal species belonging to 14 genera were isolated from the two varieties of maize seeds, of which a maximum of 33 fungal species were isolated from the seeds of the local yellow variety and 22 from local white variety (Table 1.2 and Table 1.3). The variation in the number and type of fungi associated with different types of seeds is well advocated. The species composition of fungi differed due to physico-chemical nature of the seeds, agricultural operations, storage conditions, climatological conditions of the locality during harvesting and sampling (Neergaard, 1970). Vaidehi and Ramarao (1978) found that the fungi vary from sample to sample. Of the 20 species isolated, species belonging to *Aspergillus* and *Penicillium* were the most dominant which substantiated the present findings.

Qualitative assessment of fungal species on maize seeds revealed that species of *Aspergillus*, *Fusarium*, and *Rhizopus* were dominant in both the varieties of maize. *Alternaria* spp., *Chaetomuim* spp.,

*Cladosporium* spp., *Drechlera* spp., *Helminthosporium sacchari*, *Mucor* spp., *Trichoderma* spp., *Verticillium albo-atrum* mycelial sterilia were also recorded from both the varieties though with a less frequency of occurrence (Table 1.4 and Table 1.5). Subdaiah *et al.* (1982) studied the seed mycoflora of maize of local and hybrid varieties from Mysore (India). They also observed the variation in species composition on different cultivars.

A comparative study between two types of storage conditions (shaded and smoked) with reference to the quality of seeds and incidence of fungi revealed the corresponding changes in the moisture contents of the seeds with the changes of relative humidity in the surrounding atmosphere.

Moisture contents of the seeds and temperature of the storage conditions were the primary factors determining the development of storage fungi (Neergaard, 1977; Nandi *et al.*, 1982; Reddy and Reddy, 1983; Malda *et al.*, 1985). The rise and fall of percentage moisture contents of the seeds accompanied by the corresponding rise and fall of the fungal population. Higher percentage of moisture content of the seeds was noted in shaded condition than the smoked ones. The maize seeds of both the storage conditions were frequently associated with a good number of fungi. Amongst which *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. were the most dominant fungi. There was not much difference in the total number of fungal species isolated from the storage conditions (Fig. 1.5) though their frequency of occurrence

differed.

The chitin content and fungal populations of maize seeds showed a positive correlation. A method of measuring the degree of fungal invasion of stored maize seeds was developed using the analysis of chitin, measured as glucosamine, as a criterion. The decline in chitin contents during winter months was related to low moisture contents of the seeds and temperature of storage conditions. Whereas, in the other seasons, where the moisture and temperature were higher, the seed infection as well as the chitin contents were also higher. This revealed that chitin content may alternatively be used as a reliable method to study the fungal invasion of seeds (Donald and Mirocha, 1977; Carballas and Acea, 1988).

Mycotoxin contamination of food stuffs is posing a grave threat to public health due to their potent toxic nature and fairly common occurrence. The range of agricultural and industrial commodities potentially affected by mycotoxic fungi is extremely wide and possibly no edible substance can be regarded as absolutely safe from the mycotoxin contamination. Presence of toxigenic strain of the concerned mould, availability of suitable substrates and congenial climatic conditions are the three major factors which contribute to mycotoxin production under natural conditions. The problem of mycotoxin contamination of foods and feeds is world wide but it is more frequent in those areas of the world where climatic and agricultural conditions are most suitable to mould growth.

Indian climatic conditions coupled with socio-economic backwardness offer excellent conditions for mycotoxin production. Hot and humid climate, which is ideal for mould growth, is prevalent in most parts of India, particularly during the monsoon season. The consumption of mycotoxin contaminated food often becomes indispensable due to acute food shortage and poverty.

Maize (Zea mays L.) is an excellent substrate for mould growth and mycotoxin production. Maize is one of the cereal crop of Meghalaya and is used as a stable food in many regions of this state. The maize seeds are sown in the spring season, i.e., month of March and harvested in the autumn season, i.e., August-September.

In the present investigation, it has been observed that mycotoxins, viz. aflatoxin, zearalenone, ochratoxin and citrinin were commonly associated with maize seeds. The seeds which were contaminated with aflatoxin showed positive response to BGYF test. While screening the seeds for mycotoxins elaboration in nature, it was found that all the seed samples had the presence of mycotoxins (Fig.2.1).

In local white variety, aflatoxin $B_1$, $B_2$ and $G_1$ were detected whereas local yellow variety has all the four types of aflatoxins, viz; aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$ at different seasons. It was clear from Table 2.2 that local yellow variety contained more amount of aflatoxins than the local white variety. This was due to the substrate and external seed morphology of seed which was known to play an

important role in determining the extend of aflatoxin elaboration in the grains (Diener and Davis, 1969). The present investigation was also supported by Devi and Polasa (1982) who examined the maize samples used as poultry feed for mold isolation and toxin production. They found that 28% of the maize samples were contaminated with three mycotoxins and among these the aflatoxin $B_1$, $B_2$ and $G_1$ were found to be above the tolerance level, i.e., 34, 40 and 105 ppb respectively. There were other reports on mycotoxins which had also the supported the present findings (Bilgrami *et al.*, 1982; Sabino *et al.*, 1989; Kolhe *et al.*, 1993).

Natural contamination of aflatoxin is known to be greatly influenced by the environmental factors (Davis and Diener, 1970; Boller and Schroeder, 1974). Oxygen availability, temperature, moisture content, type and nature of the fungal strains are known to play a dominant role in determining the extend of aflatoxin elaboration in the grains (Detroy, *et al.* 1971).

The other mycotoxin detected was zearalenone produced by *Fusaruim moniliforme*. The moderate temperature and humid conditions supported the growth of the mold on seeds under field as well as storage conditions. This was supported by Hesseltine (1976) who reported that temperature and humidity play an important role in the production of mycotoxins. Caldwell and Tuite (1974) have also detected zearalenone by *Fusaruim* sp. from infected corn but reported to be rare in nature (Vinas *et al.*, 1985). The fusarial toxins were mostly associated with

maize stalk rot (Logrieco and Bottalico, 1986) and a number of mycotoxins producing *Fusaruim* species have been reported (Blaney and Dodman, 1988; Mirocha *et al.*, 1989; Hussein *et al.*, 1989).

Citrinin produced by *Penicillium citrinum* was isolated from infected maize seeds when temperature was low. This mycotoxin was known to occur naturally in cereals (Hesseltine, 1974).

Ochratoxin produced by *Aspergillus ochraceous* on maize seeds was isolated and has been found to invade and multiply during storage conditions (Christensen, 1962). It was detected in both the maize varieties (Fig.2.1). The toxigenic fungi besides maize seeds have also been isolated from other cereals and legume products (Scott, 1965), red and black pepper (Christensen *et al.*, 1967), Aspergilli as ochratoxin producers have also been reported (Hesseltine *et al.*, 1972).

At lower altitude, certain mycotoxigenic fungi, viz. *Aspergillus flavus* group producing aflatoxins, *A. ochraceous* producing ochratoxin-A and *Penicillium spp.* producing citrinin are commonly reported during monsoon season in cereal flour. However, toxins were detected either alone or with citrinin as co-contaminant (Sinha and Prasad, 1994).

While studying the mycotoxin producing potentiality of *A. flavus*, *F. moniliforme* and *P. citrinum* in vitro conditions, it was observed that some of the isolates screened at different seasons were found to contain aflatoxin $B_1$, $B_2$ and $G_1$ but not aflatoxin $G_2$ in local white variety (Table 2.6) whereas in local yellow variety, only aflatoxin

$B_1$ and $G_1$ were detected, aflatoxin $B_2$ and $G_2$ were not found (Table 2.7). Aflatoxin $B_1$ was invariably produced by all the toxigenic isolates of *A. flavus*, however, frequency of production of aflatoxins other than $B_1$ was comparatively lower. Variation in the potentiality of isolates of *A. flavus* in producing aflatoxins was probably due to the variations in their genome (Singh, 1988).

Though all types of foods can harbour toxigenic strains of *Aspergillus* which can produce aflatoxins under suitable conditions but surface area of the substrate and availability of oxygen to a great extent affect aflatoxin production (Diener and Davis, 1969). The degree of toxin concentration also depends upon the substrate on which the fungus grows. In the present investigation, aflatoxin $B_1$ and $G_1$ were detected in both maize varieties. Aflatoxin $B_1$ was produced in the range of 0.32-2.52 ppm. The amount of aflatoxin $B_1$ was more in local yellow variety than on local white variety. This may be due to the armoured condition of the maize grains which perhaps interferes with the penetration of the invading fungus and subsequent production of toxins (Jay, 1978).

Variation in ability to produce the aflatoxins was observed in different isolates of *A. flavus* obtained from different drug yielding plants, however, no physiological or genetical characterisation of these isolates was done. It seems that potentiality to produce the type of aflatoxin and their quantity is genetically determined as was observed by Roy *et al* (1988) that out of 50 isolates of *A. flavus*, 12

produced aflatoxin $B_1$, 7 produced aflatoxin $B_1$ and $B_2$ and 2 produced aflatoxin $B_1$ and $G_1$. The aflatoxin producing ability of different isolates ranged from 1.118 ppm to 4.85 ppm. The variation in the aflatoxin production by different isolates of *A. flavus* may also be due to the variation in the toxin producing potentiality of different strains.

Zearalenone was detected from the isolates in local white variety. Out of 52 isolates screened, 21 isolates were toxigenic and produced 0.2 ppm during winter season, followed by wet summer season whereby 10 isolates were toxigenic out of 35 isolates isolated and produced an amount of 1.4 ppm. Autumn season has the least number of isolates which were toxigenic, only 2 isolates out of 26 isolates producing 0.9 ppm. The variation in amount and toxigenic isolates in different seasons was related to their occurrence and climatic conditions. However, all the strains of *Fusaruim moniliforme* were not equally toxigenic (Bilgrami *et al.*, 1980).

Other mycotoxins isolated from different isolates of seed borne fungi were ochratoxin and citrinin which were detected only in local yellow variety which was more suitable for their growth than local white variety. Varietal variation in the mycotoxin producing ability of fungi has been correlated to their physico-chemical properties of seeds (Gangopadhyay and Chakraborti, 1981; Devi and Polasa, 1982; Anjana, 1984; Blaney *et al.*, 1986; Abbas *et al.*, 1988).

Variation in the production of aflatoxins between two storage

conditions was recorded. Seeds stored under shaded condition were having much higher concentration than those under smoked condition. Aflatoxin $B_1$ was detected in both storage conditions whereas aflatoxin $G_1$ was detected only in shaded ones. This was due to the corresponding changes in the percentage moisture content of the seeds with changes of relative humidity in the surrounding atmosphere. These factors were reported as the primary factors determining the production of aflatoxins in stored paddy (Sundaram *et al.*, 1988).

Maize seeds contained a wide range of nutritive substances including carbohydrates, lipids, proteins, minerals and trace elements and, therefore, served as an excellent substrate for the growth of *A. flavus* and production of aflatoxin. The growth of fungi and aflatoxin elaboration, however, depended to a great extent on environmental conditions. Moisture, temperature and relative humidity were the most important factors, which exerted a decisive influence on toxin elaboration by the moulds on maize seeds (Davis and Diener, 1970; Boller and Schroeder, 1974).

The result have indicated that aflatoxins and others mycotoxins can be produced at wide range of moisture levels, and relative humidity (Figs. 3.1, 3,2 & 3.3). The maize varieties selected in the present investigation have indicated that they favoured production of aflatoxins at warm temperature (30° C) and in more humid (100%) conditions as was suggested by Hesseltine (1976).

It was observed that when optimum conditions of moisture, temperature and relative humidity were available, the aflatoxin elaboration was also determined for a longer period (Abbas *et al*, 1988). In the present study, aflatoxin production by *A. flavus* in two local varieties of maize seeds was also estimated at different incubation periods under similar moisture level, temperature and relative humidity (Figs 3.1, 3.2 & 3.3). The concentration of toxins continued to rise with the increase of incubation periods in both maize varieties till it reached the maximum by the 20th day. In the later phases of incubation (30th day) there was a decline in the aflatoxin concentration. This decline may be due to the age of fungal spores, older spores required a longer period to germinate and consequently to develop mycelium and to form toxin. Over long periods of grain storage under conditions that inhibit mold growth, viable microbial inoculum will gradually decrease because of the death of spores. Even in optimum conditions, some time is required for germination of spore of mycelium, penetration of the substrate and toxin production (Christensen and Kaufmann, 1972). Similar results on the effect of incubation period on mycotoxin production have earlier been reported by Bilgrami *et al.* (1980).

Significant variations in aflatoxins were observed between the physical factors, that is, moisture content, temperature and relative humidity with the mycotoxins on both the maize varieties (Table 3.4). This has indicated that the toxigenic strains were adopted to wide

range of climate but certainly required optimum set of moisture, temperature or relative humidity to elaborate maximum amount of aflatoxin (Hesseltine, 1976).

Fungi are major cause of spoilage of stored grains and probably rank second only to the insects ( Christensen and Kaufmann, 1969). In the present investigation, it has been observed that there was a continuous and significantly gradual decline in the dry weight of maize seeds due to fungal infestation in storage ( Fig. 4.1 .A and table 4.5). The loss in weight may be attributed to the hydrolyzing effect of fungal organisms as evidenced by starch depletion as suggested by Vidhyasekaran and Govindaswamy (1968), Vidhyasekaran *et al.* (1973) and Bilgrami *et al.* (1979) on rice, Vidyasekaran and Kandaswamy (1972) on mung, Sinha and Prasad (1977) on arhar, Anahosur and Patil (1983) on sorghum.

Tables 4.2, 4.3 and Figures 4.1.B, 4.2 showed that fungal infestation resulted in the fall of total sugar contents while slight increase in the reducing sugars and a simultaneous fall in the non-reducing sugars in both the maize varieties due to their utilization by the heterotrophs as suggested by Maheshwari and Mathur (1985) and Maheswari *et al*, (1985). There was no consistency with regard to the extent of reduction and period of maximum activity during storage time, which indicated the variation in fungal invasion and their growth as influenced by the changes under natural storage conditions. Earlier reports of Vidhyasekaran (1968), Nema (1983), Prasad (1983)

and Balakrishnan and Nair (1983) had also supported the present findings. The fall in sugar content has also been noticed by Reddy *et al.*, (1983); Singh and Sinha (1983), however our results are in contradiction with the findings of Grewitz and Durbin(1960), Swamy(1964) and Prasad *et al*, (1976) who recorded on increase in sugar contents due to fungal infestation, which may be attributed to the variation in fungal species colonizing the seeds.

A review of Table 4.4 and Figure 4.3 revealed that there was a continuous fall in the protein contents of the seeds due to fungal infestation; the rate of fall in the protein level varied with the maize varieties and with the fungal organisms. The variation in the rate of utilization of seed nitrogen by different fungi due to their ability to assimilate the growth rate ( Prasad *et al.*, 1976; Jamaluddin *et al.*, 1977; Bilgrami *et al.*,1979; 1985). It was evident from the Figure 4.3 that slow reduction in the protein contents at the initial stage has reflected the low fungal activity in the beginning of their growth as suggested by Jamaluddin *et al.* (1977). Sharma (1981) has reported maximum loss in protein contents at later stage of fungal invasion of seeds due to *Aspergillus flavus* as was observed in the present findings. The variation in the protein reduction by various fungi might be due to their capability to secrete protein hydrolysing enzymes ( Roy and Choubey, 1984).

The breakdown of the host protein by fungi to simpler components for the synthesis of their own proteins may also be related to their

growth rate ( Chang and Willets, 1973; Singh *et al.*, 1974b) which may ultimately results in an increase in the soluble nitrogen.

Aflatoxin $B_1$ has great effect on spore germination and mycelial growth of seed borne fungi. It is amply clear from the tabulated results that the different concentrations of aflatoxin $B_1$ used in the present investigation exhibited a stimulatory influence on spore germination and mycelial growth of *Aspergillus flavus* (Figures 5.1 & 5.4. A respectively). This stimulatory effect may be due to exudation of toxin favouring fungal species and inhibiting the other species.

Inhibitory effect of aflatoxin $B_1$ on spore germination and mycelial growth of *F. moniliforme* ( Figure 5.2 & 5.4 B) and *P. citrium* ( Figure 5.3 & 5.4. C) respectively at different temperatures has also been observed, which may be due to the competitive interaction between the exudates exudated by the fungal species tested with the aflatoxin $B_1$.

Spore germination occurred at 20°c-30°c in case of *A. flavus* and 20°c-25°c in case of *F. moniliforme* and *P.citrinum* (Table 5.1). A temperature of 15°c was found to be low and 30°c more than optimum for spore germination of *F. moniliforme* and *P. citrinum* except A. flavus where spores could germinate even at 30°C. Bhargava (1970) and Kapoor and Singh (1977) have indicated that optimum temperature for fungal spore germination lies between 25°C to 30°C which supported our findings.

Seed borne fungi produce metabolites which may be hazardous for the health of seed as well as for consumers. Metabolites of fungal origin reduce seed germination and sprouting (Christensen and Kaufmann, 1965; Lalithakumari *et al.*, 1972; Pandey *et al.*, 1982). These metabolites may also sometimes appear as toxic products and influence seed germination and embryo growth in maize (Brodnik, 1975).

It is evident from the results (Figure 5.5) that the mycotoxins, viz. aflatoxin $B_1$, zearalenone and citrinin isolated earlier had adverse effect on seed germination of maize and inhibited the seedling growth of both maize varieties (Figures 5.6 and 5.7). These findings were supported by Jeswal (1987). The inhibition in seed germination and seedling growth may be due to metabolites secreted by the test fungi (Curtis, 1958). Christensen and Kaufmann (1965) and Tripathi (1974) have reported that various fungi secrete exotoxins causing reduced seed germination and plant growth.

All the three mycotoxins tested has similar inhibitory effects on the seed germination and seedling growth of both the maize varieties indicating the similarity in their mode of action. Inhibition due to aflatoxin $B_1$ on maize seed germination due to the inhibition enzyme synthesis during respiration is known (Chatterjee, (1988). Statistical analysis of significant variations between mycotoxins on inhibiting the seedling growth may be correlated to their amounts.

Significant effects on the reduction of weight of animals was observed with pure aflatoxin $B_1$ feeding than those of the animals fed with crude toxin. This has indicated that production of aflatoxins in natural conditions may be less and less effective than the pure toxin (Austwick and Ayert, 1963). Singh and Chauhan (1983) have observed the biochemical alterations in albino rats fed with *Aspergillus* infested maize diets, however metabolic pathway of toxic action is not worked out. No liver tumor was observed in any test animals though some of the mice treated with pure and crude aflatoxin $B_1$ showed abnormal light colour of liver. These findings agreed with Lindenfelser *et al.* (1976) and Daradhiyar (1980) who failed to demonstrate any tumor in white mice. This may be due to the congestion of blood vessels in the epithelial cells of the liver (Baruah *et al.*, 1980). Variation in toxicity by different types of aflatoxins may be accounted to their molecular structure and amount in infected maize seed which may cause mild disease symptoms in mice and if continued, can lead to serious pathological disorders of kidney, liver, lungs and even carcinoma (Baruah *et al.*, 1980).

Loss of hairs from skin of mice was observed due to aflatoxin $B_1$ contaminated feedings. This was due to the presence of thick-walled capsular structures, which were fully keratinised epidermal cells, forming "Horn-pearl". These were noted at different stages of development in both epidermis and dermis (Bilgrami *et al.* 1986).

The part of the skin which was very badly affected showed patches

of non-differentiated, deeply stained cell masses in the dermis of mice (Newberne *et al.*, Bilgrami *et al.*, 1986). This may be due to the atrophic changes in the sebaceous and sweat glands of the skin of mice (Bilgrami *et al.*, 1986).

In the present investigation, 0.25 ml of 60 ppb of aflatoxin $B_1$ was found to be lethal only to some of the mice of 3 weeks old, mortality at the end of 6-7 months after treatment. The susceptibility of aflatoxins also depends on the sex, age and health of the animal. Young animals are generally more sensitive to aflatoxins (Hesseltine, 1967).

Toxin inhibiting property of the plant extracts do not seem to be directly related to the growth of the fungus, which was luxuriant in many cases, although the plant extracts were inhibitory to toxin production (Table 7 and Figure 7). On the other hand, Singh *et al.* (1979) and Sharma *et al.* (1979) reported that extracts of *Allium cepa* and *A. sativum* inhibited growth of certain fungi which produced aflatoxin. Inhibition of aflatoxin production has also been supported by Bilgrami *et al* (1979). Some wild medicinal extracts were also found to inhibit the aflatoxin production and growth of *A. flavus in vitro* condition (Bilgrami *et al.*, 1980). Leaves of *Centella asiatica* and *Thuja orientalis*, inhibited production of aflatoxins on wheat grains and groundnut seeds (Sinha, 1985; Singh and Sinha, 1985). This was due to medicinal and antimicrobial properties which has long been known and the treatment of fungal infections has been shown to depend

on the presence of fungi toxic chemicals in the plant tissue (Fawcett and Spencer, 1970). Some recent studies have also shown that sap of a vide range of angiosperms have antimicrobial property (Tripathi and Dixit, 1977; Misra and Dixit, 1978). In the present study, although the active principle of plant extracts have not been specifically identified, it is likely that more than one chemical may be responsible for the inhibition of aflatoxin synthesis.

Certain antibiotics have been reported to influence the production of aflatoxins by *Aspergillus flavus* isolates (Kumar and Singh, 1991).

# *Summary*

Seasonal study on seed borne mycoflora of two local maize varieties, i.e. local white and local yellow was carried out using three techniques of isolations, viz. blotter method, agar plate method and dilution plate method. A total of 37 fungal species belonging to 14 genera were isolated from both the varieties. Agar plate method and blotter method harboured more number of fungal species than the dilution plate method. The number of fungal species was gradually decreased in winter months compared to wet summer and autumn months. The field fungi were replaced by the storage fungi during colder months during storage of the seeds. The variation in fungal species on two varieties was also observed. Local yellow variety yielded maximum number of fungi (33) than local white variety (22). Species of *Aspergillus*, *Fusarium* and *Penicillium* were dominant in both the varieties during the whole period of investigation. The percentage frequency of occurrence of *Alternaria* spp., *Chaetomium* spp., *Cladosporium* spp., *Drechlera* spp., *Helminthosporium sacchari*, *Mucor* spp., *Rhizopus* spp., *Trichoderma* spp. and *Verticillium albo-atrum* decreased along with the progress of storage time and were gradually replaced by the species of *Aspergillus*, *Fusarium* and *Penicillium*.

Two types of storage conditions (shaded and smoked) commonly practiced by the tribal people of this region were selected to study the fungal flora of maize seeds. None of the storage practices were completely safe from fungal contamination. The invasion on seeds by *Fusarium* spp. was observed in both the storage conditions.

Under shaded storage condition, *Fusarium moniliforme* was recorded with highest frequency (13.3%) and *Aspergillus niger* and *Penicillium rubrum* with least frequency (0.8%) of seeds on local white variety by agar plate method. Under smoked storage condition, *Fusarium solani* was recorded at the highest frequency and *Rhizopus* sp. and *Penicillium rubrum* with lowest frequency. Seeds of local yellow variety also harboured *Fusarium* sp. with highest frequency under shaded condition of storage, whereas the lowest frequency was by *Penicillium brefeldianum* and *Trichoderma harzianum* as isolated by agar plate method. Similarly, in smoked condition, *Fusarium* sp. was recorded to have a high frequency of occurrence of 22.1% and 20% and the minimum frequency was recorded by *Penicillium brefeldianum* (1.2%) and *Cladosporium herbarum* (0.3%) by agar plate and blotter methods respectively.

Positive correlation was observed between the chitin content and the fungal population. Fungal infection of maize seeds was maximum (100%) during wet summer and autumn seasons and minimum (40%) during winter season. However, trend of chitin contents in maize seeds during wet summer and autumn seasons differed from each other, even though they had the same percentage of seed infection. Autumn season revealed higher chitin contents (328.2 µg/g in local white variety and 440.4 µg/g in local yellow variety) than wet summer season (246 µg/g in local white variety and 250.9 µg/g in local yellow variety). Lowest chitin content (126 µg/g in local white variety and 128.5 µg/g

in local yellow variety) in seeds was observed during winter season in both the maize varieties.

The percentage of seed infection and chitin contents were higher in local yellow variety than the local white variety in all seasons.

Toxigenic strains of *Aspergillus flavus*, *A. ochraceous*, *Fusarium moniliforme* and *Penicillium citrinum* were isolated from both the varieties of maize which contaminated the seeds at varying degree. All the seed samples were positive to BGYF test and aflatoxin was detected in the seed samples of both the varieties under natural conditions. Other mycotoxins like ochratoxins, zearalenone and citrinin were also detected. Aflatoxin $B_1$ was the most common mycotoxin produced by *A. flavus*. Its maximum production was 1.40 ppm in local yellow variety of maize during winter season. Local white variety of maize seeds contained aflatoxin $B_1$, $B_2$ and $G_1$, whereas local yellow variety had aflatoxin $G_2$ in addition to these aflatoxins.

In local white variety, out of 30 isolates of *A. flavus*, 6 isolates were toxigenic and yielded aflatoxin $B_1$ and $G_1$ during winter season. In wet summer season, out of 46 isolates, 22 were toxigenic and yielded aflatoxin $B_1$ and $G_1$ whereas in autumn season only 16 isolates out of 50 yielded aflatoxin $B_1$ and $B_2$. Maximum concentration of mycotoxins was obtained during wet summer season followed by autumn and winter season. Under storage conditions, aflatoxin $B_1$ was detected in higher quantity in shaded storage conditions than in smoked conditions. The qualitative and quantitative variation between

different mycotoxins on two varieties of maize seeds was observed in different seasons.

It was observed that temperature and moisture were the most important factors governing the growth of *A. flavus* on seeds of two maize varieties. Maximum production of aflatoxin was observed at 30°C and at a relative humidity of 95 to 100%. *A. flavus* did not invade any samples of corn stored below 15% moisture contents of seeds.

Significant variations in aflatoxins were observed between the physical factors i.e. moisture content, temperature and relative humidity on both the maize varieties.

The relative deterioration of seed quality after infecting by three dominant seed borne fungi., ;viz. *Aspergillus flavus, Fusarium moniliforme* and *Penicillium citrium* was determined in terms of the nutritive value of maize seeds, dry weight, sugars and protein contents. It was observed that the dry weight of the seeds was reduced due to fungal invasion in both the varieties. Insignificant variation in the loss of seed dry weight due to fungal infection was recorded in local white variety while *A. flavus* caused maximum loss in the seed dry weight in local yellow variety after 180 days of infection. Fungal infested seeds exhibited a depletion in total sugars and non-reducing sugars with corresponding increase in reducing sugars. A continuous and gradual depletion of protein contents of the seeds due to fungal infestation was noticed after 180 days of fungal invasion.

Effects of aflatoxin $B_1$ on spore germination and mycelial growth of *A. flavus*, *F. moniliforme* and *P. citrinum* was studied at different temperatures and at different time interval of incubation period. The various concentrations of aflatoxins used in the present study were either stimulatory or inhibitory to spore germination and mycelial growth of different fungi. However, the stimulatory/inhibitory capability of aflatoxin $B_1$ varied with specific fungal species. Aflatoxin $B_1$ was stimulatory to the growth of *A. flavus*, whereas growth of *F. moniliforme* and *P. citrinum* was inhibited by aflatoxin $B_1$. The maximum inhibition was observed at 1 ppm concentration of aflatoxins $B_1$.

Aflatoxin $B_1$ has also caused inhibition in the seed germination of both the maize varieties. Maximum inhibition was recorded at 1ppm concentration. The seeds of local white variety were more affected than local yellow variety. Similar trend on inhibition of seed germination was observed with zearalenone. However, in case of citrinin, maximum inhibition of seed germination was exhibited by local white variety than local white one.

Reduction of seedling growth was observed in all the treated samples compared with untreated ones. Maximum inhibition of seedling growth was observed at 1 ppm concentrated aflatoxin $B_1$ treatment whereas the minimum was observed at 0.1 ppm concentration. The pattern of inhibition of seedling growth due to zearalenone and citrinin was also similar.

Significant variation in the reduction of weight of animals was observed between pure aflatoxin $B_1$ fed animals and the crude extract fed ones. No liver tumor was observed in any test animals though some of them showed abnormally light color of liver. Loss of hairs from the skin of mice was observed due to aflatoxin $B_1$ contaminated feeding. In take of 60 ppb aflatoxin $B_1$ by some of the mice of 3 weeks old was lethal.

It was observed that extracts of different plant parts of *Centella asiatica* inhibited the growth of *A. flavus* by 51.16% and aflatoxin $B_1$ and *A. sativum* were also inhibitory to the growth of *A. flavus* and aflatoxin $B_1$ production. The least inhibitory effect was observed with the extracts of *Thuja orientalis*. The inhibition of fungal biomass and aflatoxin $B_1$ production followed a specific trend, *C. asiatica* > *A. cepa* > *A. sativum* > *T. orientalis*.

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