MYCOTOXIGENIC FUNGI IN SPICES;MOLECULAR METHODS OF DETECTION AND CONTROL

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by
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DECLARATION

I here by declare that the thesis entitled **"MY COTOXIGENIC FUNGI IN SPICES; MOLECULAR METHODS OF DETECTION AND CONTROL"** submitted to the **University of Mysore** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **MICROBIOLOGY,** is the result of research work carried out by me under the guidance of **Dr. E. RATI RAO**, Scientist, Department of Human Resource Development, Central Food Technological Research Institute (CFTRI), Mysore-570020, India, during the period 2002-2005.

I further declare that the results presented In this thesis have not been submitted for the award of any other Degree or Fellowship.

Date: 15/9/2005

Place: Mysore **(SHADANAIKA)**

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CERTIFICATE

This is to certify that the thesis entitled "**MYCOTOXIGENIC FUNGI IN SPICES, MOLECULAR METHODS OF DETECTION AND CONTROL**" submitted by Mr. Shadanaika, for the award of the degree of **DOCTOR OF PHILOSOPHY** in **MICROBIOLOGY** to the **UNIVERSITY OF MYSORE** is the result of research work carried out by him in the department of Human Resource Development, CFTRI, Mysore-20, under my guidance during the period 2002-2005.

(Dr. E. RATI RAO)
GUIDE

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Shadanaika

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LIST OF ABBREVIATION

ADM	-	Aspergillus Differential Medium
AFB_1 or B_1	-	Aflatoxin B_1
AFB_2 or B_2	-	Aflatoxin B_2
AFG_1 or G_1	-	Aflatoxin G_1
AFG_2 or G_2	-	Aflatoxin G_2
A O A C	-	Association of Official Analytical Chemists
CFTRI	-	Central Food Technological Research Institute
Conc.	-	Concentration
CAC	-	Codex Alimentarius Commission
CCFAC	-	Codex Committee on Food Additives and Contaminants
^{60}Co	-	Radio active cobalt
^{137}Cs	-	Radio active cesium
D	-	Dilution of sample
DAS	-	Diacetoxiscerpinol
E F S A	-	European Food Safety Authority
E U	-	European Union
EDTA	-	Ethylene Diamine Tetra Acetic acid
ELISA	-	Enzyme Linked Immunosorbent Assay
FAO	-	Food and Agriculture Organization
gm	-	grams
HPLC	-	High Performance Liquid Chromatography
h	-	Hour

IAC	-	Immuno affinity column
IARC	-	International Agency for Research on Cancer
JECFA	-	Joint Expert Committee on Food Additives
L	-	Litre
min	-	Minute
µg	-	Microgram
µl	-	Microlitre
ml	-	Milliliter
ng	-	Nanogram
OTA	-	Ochratoxin A
PCR	-	Polymerase Chain Reaction
pH	-	Potentiality of Hydrogen Ion
ppm	-	Parts per million
ppb	-	Parts per billion
PDA	-	Potato Dextrose Agar
PPSFT	-	Plantation Products, Spices and Flavoring Technology
nm	-	Nanometer
RIA	-	Radio-immuno Assay
R_f	-	Retention factor
RTH	-	Room temperature and humidity
sec	-	Second
std	-	Standard
T-2	-	T-2 toxin

TLC	-	Thin Layer Chromatography
vol	-	Volume
WHO	-	World Health Organization

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I N T R O D U C T I O N

Introduction

Safety and security have generally remained basic human needs in today's changing world. Ensuring the safety of food has been a major focus of international and national action bodies over the years. Both microbiological and chemical hazards are of concern. Among chemical hazards, the contamination of food and feed by naturally occurring mycotoxins produced by certain fungi, have been recently characterized as significant sources of food-borne illnesses by the World Health Organization (WHO). In several parts of the world, mycotoxins currently represent a major food safety issue. The knowledge on serious effects of mycotoxins on humans and animals has led many countries to establish regulations on mycotoxins in food and feed in the last decades to safeguard the health of humans, as well as the economical interests of producers and traders. The first limits for mycotoxins were set in the late 1960s for the aflatoxins. The most recent comprehensive review on mycotoxins was published by FAO in 1997 based on an international inquiry carried out in 1994 and 1995. A relevant international inquiry was carried out in 2002 and 2003, yielding much detailed information. Moulds cause various degree of deterioration and decomposition of foods. They can invade and grow on virtually any type of food at any time. Field crops such as grains, nuts, beans, tomatoes, apples and spices are invaded both in the field before harvesting and during storage. They also grow on processed foods and food mixtures. Nearly 250 – 300 fungal species are reported to be associated with seeds in a variety of ways. Food and Agricultural organization (FAO) estimated that about 5 % of all harvested grains are lost before consumption. In the global scenario, spices form an important group virtually indispensable in the culinary art all over the world. India contributes to about 30 % of world trade in spices, which is important to the national economy. Information on mycoflora of Indian spices is limited. The extent of contamination and the nature of mycoflora may vary with geographical condition, post-harvest practices such as drying and storage methods and also the trade practices. Several food borne moulds may also be a potential hazard to human or animal health because of their ability to produce toxic metabolites known as mycotoxins. Mycotoxins are toxic substances produced by fungi growing on grain, feed, or food in the field or in storage. Most mycotoxins are stable compounds that are not destroyed during food processing or home cooking. Even though the generating organisms may not survive food preparation, the preformed toxin may still

be present. Certain food borne moulds may also be a hazard because of their ability to elicit allergic reactions or even cause infection. Mycotoxins may be detrimental to the health of both animals and humans. Although thousands of molds are capable of growing on stored grain, only a few molds belonging to *Aspergillus, Fusarium and Penicillium* are considered to be important producers of mycotoxins. The genus Aspergillus has several species capable of producing mycotoxins. Aspergillus is a large genus with more than 100 species which are of very common occurrence in the environment, principally in soils and decaying vegetation, but a number of species are also closely associated with human foods, particularly cereals and nuts. Many species are xerophilic, and are capable of spoiling foods only just above safe moisture limits. The most significant mycotoxigenic species are *A. flavus, A. parasiticus* and *A.nomius* which produce aflatoxins, *A. ochraceus*, which produce ochratoxin, and *A. versicolor*, which produce sterigmatocystin. *Aspergillus flavus* is of ubiquitous occurrence in nature. *Aspergillus flavus* only produces B_1 and B_2 while *Aspergillus parasiticus* produces these same metabolites along with G_1 and G_2 (Mclean, Dutton 1995). *A. flavus* and *A. parasiticus* are closely related to *A. oryzae* and *A.sojae*, species which are important in the manufacture of fermented foods in Asia, but which do not produce aflatoxins. For obvious reasons, accurate differentiation of these four species is important. A recent study has concluded that the texture of conidial walls is the most reliable differentiating feature, walls of *A. flavus* conidia are usually smooth to finely roughened, while those of *A. parasiticus* are definitely rough. A variety of other characters are also of taxonomic value. *A. flavus* isolates usually make only B aflatoxins and less than 50% of isolates are toxigenic, while *A. parasiticus* isolates make G as well as B aflatoxins, and are invariably toxigenic (Klich and Pitt, 1988b). The symptoms of acute aflatoxin poisoning were studied following the hepatitis outbreak in India by Krishnamachari et al (1975). This disease outbreak was characterized by jaundice, rapidly developing ascites and portal hypertension, with the implication of a food borne toxin involving the liver. However, the ingestion of aflatoxins at the low levels necessary to induce liver cancer is totally asymptomatic and has a very long induction period as well. In India a major outbreaks due to mycotoxins in human described during the last four decades include; (i) aflatoxic hepatitis due to consumption of maize contaminated with aflatoxin; (ii) enteroergotism due to consumption of pearl millet contaminated with ergot produced by the species of Claviceps ; (iii) deoxynivalenol mycotoxicosis due to consumption of wheat contaminated with deoxynivalenol (DON) and (iv) fumonisin mycotoxicosis due to consumption of maize and sorghum contaminated with fumonisins. Mycotoxins DON and fumonisins are produced by the species of Fusarium. Consumption

of rain-damaged mould affected staples containing high levels of mycotoxins has been implicated in the occurrence of these outbreaks. Several of these outbreaks proved evidence for cause and effect relationship between ingestion of mycotoxins and acute disease outbreak. The discovery of fumonisin mycotoxins pose new threats as they are believed to play a role in esophageal cancer in man in endemic regions of South Africa. In India, outbreak of this disease had affected 1325 people in Deccan Plateau in southern India. Unlike other food commodities, spices are also susceptible to contamination or infection due to mycotoxigenic fungi. Spices are natural and tropical aromatic vegetable substances used in various forms, as additives to impart flavor, aroma and color to foods and beverages and also as medicines, as preservatives or antioxidants or as vegetables to flavor foods. The spices and condiments sub-committee of international organization for standardization (ISO) has defined spices and condiments as, “natural vegetable products, or mixtures thereof, without any extraneous matter as are used for flavoring, seasoning and imparting aroma to foods, the term spices applies to the product either in the whole form or in the ground form”. Spices besides seasoning, adding flavor and aroma to foods and beverages, are used in medicines, perfumery, cosmetics and in several other industries. Spices are well known as appetizers and are considered essential in the culinary art all over the world. Spices vary in shape, size, color, flavor and texture etc. According to the International Organization for Standardization (ISO), there is no clear cut division between spices and condiments and as such they have been clubbed together (Pruthi 1992). There are about 86 spices and condiments. Spice essential oils are the volatile aromatic extracts prepared by steam distillation of ground spices (Murphy et al, 1978, Pruthi 1993, Annamali 1994). India is the land of spices and it accounts for about 35 % of the global trade. Out of these pepper, ginger, clove, cinnamon, cassia, mace(nutmeg), pemento, allspice and cardamom alone contributed 90 % of the total world trade. About 80-85 % of spices are sold as whole, unground state and the rest are marketed as grounded spices or in mixes and as spices essential oils and oleoresins (Husain 1996). Spices are known as appetizers and are considered essential in the culinary art all over the world. Some of them also possess antioxidant properties, while others are used as preservatives in foods like pickles and chutneys. Since antiquity, spices have been in use forming one of the important food items in our diet. About 70 spices grown in different parts of the world, 52 spices which are cultivated India are included under the purview of Spices Board, Government of India. Spices such as pepper, cardamom, chillies, ginger and turmeric are considered to be major spices based on volume of production, trade and foreign exchange earnings in the Indian context. Spices

along with gold, silk and precious stones were the principle articles of trade for several centuries. In India, chillies, ginger, turmeric, cardamom and pepper are considered as major spices. In India, there is a considerable volume of international trade in spices. During the past three years i.e. 2001-02, 2002-03 and 2003-04, India exported spices worth of 407.85 million dollars (Rs.194054.88 lakhs), 431.45 million dollars (Rs.208671.02 lakhs) and 415.15 million Dollars (Rs 190508.50 lakhs) respectively. India exported 246566.32 metric tones of spices worth of Rs 190508.50 during the year 2003-2004. Amongst them, chilli contributed 81500.00 metric tones worth of Rs 35511.25, ginger and turmeric contributed 5000.00 and 34500.00 tones worth of Rs 2340.50 and 12751.88 respectively. Spice oils, oleoresins and other value added spice products which constitute new foreign exchange earners have added further new dimension. The export trade of spices has flourished only through continued efforts directed towards increasing spice production, ensuring their quality and competitiveness, development of relevant technologies to upgrade the traditional processing technique, manufacture of sophisticated convenience products to suit the fast changing needs of foreign markets and creating a strong technological base to build up the industry in the country.

Chillies by far constitute the largest and most widely cultivated food commodity among the spices grown in India. Incidentally, India is also the largest producer of chillies in the world. During the year 2003-04, chillies worth of 77.39 million dollars (Rs. 35511.25 lakhs) has been exported. India is the largest producer of dry ginger in the world. Other major ginger producing countries are Australia, China, Jamaica, Nigeria and Taiwan. During the year 2003-04, ginger worth of 5.10 million dollars (Rs. 2340.50 lakhs) has been exported. India is also one of the leading turmeric producers in the world, besides China, Jamaica, Indonesia, Srilanka and Taiwan. During the year 2003-04, turmeric worth of 27.79 million dollars (Rs. 12751.88 lakhs) has been exported (Spices Board, Govt. of India, Statistics, 2004).

During the last four decades, the occurrence of mycotoxins, especially aflatoxins in agricultural commodities has been viewed with great concern, mainly because of their potential health hazards including cancer. The mycotoxin contamination in some spices is suspected for two reasons: (1) many spices are cultivated and processed in warm tropical areas, where conditions favor fungal growth, (2) most of the spice producing countries are developing countries where the sanitary and storage practices are not satisfactory. Though Indian spices are highly regarded for their aroma and flavor and often labeled as pure spices, in recent years, the importing countries have been showing an increasing concern for their microbiological quality. In fact, there were occasional reports

on the natural occurrence of mycotoxigenic fungi and mycotoxins, i.e. aflatoxin, in spices in India and elsewhere. Furthermore, the aflatoxin contamination in ginger and chilli exported from India to U.S.A and Japan has been reported (Trucksess and Stoloff, 1980, Maeda, 1983). By the end of 2003, approximately 100 countries had developed specific limits for mycotoxins in foodstuffs and feedstuffs, and the number continues to grow. A number of publications focusing on limits and regulations for mycotoxins exist (Schuller *et al.*, 1983; Stoloff *et al.*, 1991; Van Egmond, 1991; Van Egmond and Dekker, 1995; Rosner, 1998; Van Egmond, 1999). The authorities in the U.S and European Union (EU) are strict about the presence of aflatoxin in chillies. Tolerence limit for chillies fixed by EU is 10 ppb, while it is 15 ppb in U.S. Mandatory testing of export quality chillies by Indian spices board has been implemented (IFI, 2004). Besides, economic consequences of mould and mycotoxin contamination in spices, the health risk should be viewed with serious concern as the best lots are exported and what is retained for internal market and consumption is usually of poor quality.

The conventional method for the detection of mycotoxigenic fungi are the culture methods, which are time consuming and require expertise in fungal taxonomy. Taxonomic classification can be simplified by the use of selective media, but the time required is not reduced. These problems can be reduced by adopting PCR which can reduce the detection time from several days to several hours. The diagnostic PCR approach for detecting mycotoxigenic fungi is an indirect method. Through the DNA of the organism can be amplified by PCR and detected by gel electrophoresis, it does not distinguish between dead and living mycelium. This fact is however advantageous in the case of mycotoxigenic fungi, as mycotoxins are usually very stable. A positive PCR can therefore, be taken as an indication that the sample potentially contains mycotoxigenic fungi and the sample needs to be further analyzed for mycotoxins. Conventional morphological methods for detecting aflatoxigenic fungi cannot distinguish between aflatoxin producing and non-producing fungi. Molecular approach by PCR is able to distinguish between both the types. The spices infested with aflatoxigenic fungi can be identified by the PCR approach.

As part of control measures, studies on inhibition of mycotoxigenic fungi and mycotoxins in food commodities have been conducted world wide. Since the very first report of mycotoxicisis in animals, different workers have tried different methods by adopting various control measures for mycotoxigenic fungi. Plant varieties developed through breeding that restrict toxin production were developed as preventive measures. Control of fungi through fungicide and Irradiation has been developed. Indiscriminate use of fungicide cause soil pollution while irradiation technique has always

been restricted as the facility is limited. In the recent past the antimicrobial properties of some plant constituents are being exploited on protecting man and animals from moulds and their mycotoxins. Extracts of herbs and spices are increasingly of interest in the food industry because they retard oxidative degradation of lipids (Murcia et al., 1996).

The global effort to protect our environment from pollution, there has been significant changes in plant protection strategies. Use of chemicals such as fungicides which proved to be toxic, carcinogenic and teratogenic to human beings have been minimized through out the world. In this backdrop, studies on control measures using spices especially the spices oils amongst plant extracts became the new field which offer many opportunity to evolve suitable strategies which are safe in view of lack of residual effect.

However, authentic information on mycotoxins in spices and also systematic studies on the magnitude of the problem of potential fungal contamination, detection of mycotoxigenic fungi in spices through rapid and molecular methods, prevention of fungi through scientific storage practices and control of mycotoxigenic fungi are limited. Thus, the problem of major spices such as chilli, ginger and turmeric has been chosen for the present study with the following objectives **:**

- Studies on the natural occurrence of mycotoxigenic fungi and mycotoxins in chilli, ginger and turmeric.
- Evaluation of substrate specificity of chilli, ginger and turmeric for the growth and elaboration of mycotoxigenic fungi.
- Determination of critical moisture level in spices through sorption studies.
- Detection of aflatoxigenic fungi by molecular method (PCR).
- Studies on the control of mycotoxigenic fungi through the application of natural spice oils and by irradiation.

REVIEW OF LITERATURE

Contents

2.1 Introduction

India is predominantly an agrarian country with nearly three fourths of the people dependent on agriculture and rural economy. The most outstanding achievement of Indian agriculture since independence is the phenomenal growth of food grains output. During the last three decades, Indian agriculture has experienced a revolutionary breakthrough in food grain production leading the country from deficit and import arena to the positive situation of self-sufficiency. Some of the states in the country have come across the unprecedented draught of the century for the successive years which has caused tremendous hardship to the people and also loss of production of food grains in those states. Nearly seventy percent of the total production of food grains in India is retained at farm level where the unscientific and faulty storage conditions would enhance the chances of fungal attack, are in practice. Moreover, India being a tropical country, it's climatic conditions having high temperatures and relative humidity, incessant rains and floods in addition to the existing poor drying and storage facilities, facilitate the growth and mycotoxin elaboration by mycotoxigenic moulds. The fungi produce a large number of mycotoxins in food grains and their products. Mycotoxins are considered to be among the most insignificant food contaminants with regard to their impact on public health, food security and the national economy of many countries, particularly the developing ones. Because of their potent toxic nature and their fairly common occurrence under natural conditions, mycotoxins have attracted world - wide attention in the recent years. Moulds and mycotoxins which have insignificant impact upon health of human and animals, and productivity, are currently considered to be of world-wide importance (Miller, 1994). Mycotoxins are an important problem as evidenced by occasional outbreaks of human mycotoxicoses and the role of aflatoxins in liver cancer in West Africa and fumonisins in oesophageal cancer in South Africa are well established (Shephard, 2004).

2.2 Mycotoxigenic Fungi

The mycotoxigenic fungi involved with the human food chain belong mainly to three genera: *Aspergillus, Fusarium and Penicillium.* While *Fusarium* species are destructive plant pathogens producing mycotoxins before, or immediately after harvesting, *Penicillium* and *Aspergillus* species are more commonly found as contaminants of commodities and foods during drying and subsequent storage. There are other molds belonging to the genera *Alternaria, Chaetomium, Cladosporium, Curvularia, Myrothecium, Stachybotrytes,* and *Trichoderma* capable of producing mycotoxins. Three genera of fungi *Aspergillus*, *Penicillium* and *Fusarium* (*Gibberella*) are the ones involved most frequently in cases of mycotoxin contamination in corn, grains, soybeans, spices and the groundnuts.

There are about 160 fungi being recorded capable of producing about 182 mycotoxins and other metabolites (Betina, 1984, 1989; Frisvad and Thrane, 1987). The large number of species represent a broad spectrum of various ecological types capable of utilizing a wide variety of substrates. The aflatoxin-producing moulds occur widely, in temperate, sub-tropical and tropical climates, throughout the world; and the aflatoxins may be produced, both before and after harvest, on many foods and feeds especially oilseeds, edible nuts and cereals (Coker, 1997). *Aspergillus* species can almost infect anything and everything which exists in nature. Way back, Thomas (1978) compiled the data on the deteriorative role of *Aspergillus* species on stored produce which include cereal grains, cotton seeds, soybeans, sunflower seeds, peppercorns, nuts, dried peas, dried fruits, copra, coffee beans, cocoa beans, raw sugar, molasses, bread, butter, cheese, margarine, jams and meat. Lubulwa and Davis (1994) have studied economic losses attributable to the occurrence of aflatoxin only, in maize and groundnuts, in Southeast Asian countries (Thailand, Indonesia and the Philippines).The genus *Aspergillus* belongs to a class of fungi known as the Hypomycetes, which belongs to the subdivision Deuteromycotina. The Hypomycetes consist of organisms with conidia produced directly on mycelia or on single or clustered conidiophores (Beuchat, 1987). *Aspergillus* species are frequently isolated from soil, vegetation, air, corn, peanut and certain substrates (Raper & Fennell, 1965; Hudson, 1969; Horn et al. 1995). *Aspergillus flavus* is of ubiquitous occurrence in nature. *Aspergillus flavus* only produces B_1 and B_2 while *Aspergillus parasiticus* produces these same metabolites along with G_1 and G_2 (Mclean, Dutton 1995). These two species are different and can be distinguished by their colony appearance, microscopic features and can be identified using special culture media. Even though *A.flavus* and *A.parasiticus* are closely related according to their DNA homologies, they are not vegetatively compatible and are, therefore genetically isolated from one another (Neal, Eaton, and Judah 1998). Since the discovery of aflatoxins, it has become the most widely reported foodborne fungus, reflecting its economic and medical importance, and ease of recognition, as well as its universal occurrence. *A. parasiticus* is less common in tropical countries; it is also obscured by the tendency of misidentification of *A. parasiticus* as *A. flavus*. *Aspergillus flavus* and *A. parasiticus* were classified by Raper and Fennell (1965) in what was termed the "*Aspergillus flavus* group", an incorrect terminology now replaced by the correct term "Aspergillus Section Flavi" (Gems et al., 1985). *A. flavus* and *A. parasiticus* are closely related to *A. oryzae* and *A. sojae*, species which are important in the manufacture of fermented foods in Asia, but which do not produce aflatoxins. For obvious reasons, accurate differentiation of these four species is important. A recent study has concluded that the

texture of conidial walls is the most reliable differentiating feature, walls of *A. flavus* conidia are usually smooth to finely roughened, while those of *A. parasiticus* are definitely rough. A variety of other characters are also of taxonomic value. *A. flavus* isolates usually make only B aflatoxins and less than 50% of isolates are toxigenic, while *A. parasiticus* isolates make G as well as B aflatoxins, and are invariably toxigenic (Klich and Pitt, 1988b).

2.3 Mycotoxins

The name mycotoxin is combined word of Greek for fungus 'mykes' and the Latin for 'toxicum' meaning poison. Mycotoxins are secondary metabolites produced by certain filamentous fungi, which can be produced in foods as a result of fungal growth. They are low molecular weight compounds, non-antigenic and diverse group of compounds ranging from polypeptides, alkaloids, benzoquinones, xanthones, coumarins, terpenes, and steroid derivatives. Mycotoxins, particularly Aflatoxins pose a significant threat to both human and animal health because they are not only toxigenic, but also carcinogenic, mutagenic and teratogenic (Hsieh, 1986; Chu, 1997). Mycotoxigenic fungi and mycotoxins, their source and health effects are presented in the table -1. Agricultural commodities which become prone to infection by mycotoxigenic fungi and elaboration of mycotoxins at different stages are presented in the table-2. The problem of mycotoxins has been in existence since the dawn of civilization. The reference to the poisoning of animals by “kede millet” in Kautilya’s Arthashastra (300 B.C) is perhaps the earliest record of mycotoxicosis (Shamasastry, 1960). Although gangrenous ergotism was recorded in the 9th and 10th centuries, it was only later that the ergots of rye were recognized as fungal in origin (Bove, 1970; Beardall and Miller, 1994). German and French scientists attributed it to rye infected by *Claviceps purpurea* (Groger, 1972). In 1913, Alsberg and Black attempted systematic biochemical investigation on toxins of certain moulds isolated from cornmeal. In fact, the Japanese worker, Sakaki (1891), could be considered pre-eminent in the field of mycotoxicology since his first report on toxic effects of rice moulds appeared in the same year.

Association of moulds, its toxins and food commodities has been known since Biblical times but their role in inciting disease syndrome was realized only when it was discovered that "ergotism" was caused due to consumption of barley and rye infected with *Claviceps purpurea.* Cardiac beriberi caused by *Penicillium citreoviride* was recorded in Japan due to consumption of contaminated rice popularly known as Red Mould Diseases. Mycotoxins in yellowed rice have been suspected since 1899 in Japan (Kinosita and Shikata, 1965). Later the toxic effect was attributed to the fungus, *Penicillium citreoviridae* (Uraguchi, 1947). The outbreak of the moldy corn toxicosis in 1952 and 1957

Table – 1. Mycotoxigenic fungi, mycotoxins, source and their health effects.

Fungi	Mycotoxin	Source	Health Effects
Aspergillus flavus and *A. parasiticus*	Aflatoxin B_1, B_2, G_1,G_2 & M_1	Nuts, maize, figs, spices, Milk	Liver cancer and damage. Immune suppression
A.ochraceus and *Penicillium verrucosum*	Ochratoxin A	Wheat, barley, maize, beer, dried fruits, wine, spices, coffee, chocolate, grape juice	Kidney damage and kidney cancer. Immune suppression
Penicillium expansum	Patulin	Apples, fruit products	Possible cancer
Fusarium moniliforme	Fumonisin B1, B2, B3	Maize and maize products	Cancer in rats. brain decay in horses. Lung congestion in pigs? oesophageal cancer
Fusarium graminearum	Deoxynivalenol	Cereals	Damage to digestive tract, bone marrow, spleen, reproductive organs. skin problems. vomiting and feed refusal
Fusarium graminearum	Zearalenone	Cereals	Oestrogenic effects. Interference with conception, ovulation, implantation, fetal development.

Vicam, USA (2001)

Table.2. Mycotoxins on some food commodities

Commodity	Factors	Potential mycotoxins
Cereals	Pre-harvest fungal infection	Deoxynivalenol, T-2 toxin, nivalenol, zearalenone, fumonisins,
Maize and peanuts	Pre-harvest fungal infection	Aflatoxins
Maize and sorghum	Pre-harvest fungal infection	Fumonisins
Stored cereals, nuts and spices	Damp storage conditions	Aflatoxins and ochratoxin
Fruit juice	Mould growth on fruit	Patulin
Dairy products	Animal consumption of mould contaminated feeds	Aflatoxin M_1, Cyclopiazonic acid, Ochratoxin, Patulin, citrinin.
Meat and eggs	Animal consumption of mould contaminated feeds	Ochratoxin, cyclopiazonic acid, citromycetin, roquefortine, fumonisins
Oilseeds	Pre-harvest fungal infection	Tenuazonic acid, alternariol.

Source: (Prelusky, 1994; Smith et al., 1994; Bullerman, 1995; Smith and Thakur, 1995; Webley et al., 1997).

in U.S.A. was later found to be due to the toxigenic fungi viz *Aspergillus flavus* and *Penicillium rubrum* (Forgacs, 1965). In 1964, Dodd observed that facial eczema in sheep in New Zealand was caused by the toxin sporodesmin elaborated by the mould *Pithomyces chartarum.*

The Codex Alimentarius Commission (CAC), supported by FAO and WHO, aims to facilitate world trade and protect the health of consumers through the development of international standards for foods and feeds. Currently 168 countries are members of Codex Alimentarius. Within the CAC, the Codex Committee on Food Additives and Contaminants (CCFAC) derives maximum limits (standards) for additives and contaminants in food, which are decisive in trade conflicts. The CCFAC develops standards based on a procedure that follows the principles of risk analysis as far as possible, according to rules and methods laid down in the Codex Procedural Manual as well as the Codex General Standard for Contaminants and Toxins in Food.

2.3.1 Aflatoxins

The aflatoxins are the most carcinogenic natural substances known and can be found in various food commodities (Diener et al., 1987). Aflatoxins consist of a group of approximately 20 related fungal metabolites, although only aflatoxins B_1, B_2, G_1 and G_2 are normally found in foods. They are produced by at least three species of *Aspergillus, A. flavus*, *A. parasiticus* and *A. nominus* (Bennet,1988), and can occur in a wide range of important raw food commodities, including cereals, nuts, spices, figs and dried fruit. *Aspergillus* species are known to be xerophilic, and capable of spoiling foods only just above safe moisture limits. Toxigenic strains of *Aspergillus flavus* and *A.parasiticus* grow at temperature ranging from 10-12^{o} C to 42-43^{o}C, with an optimum in between 32-33^{0} C (Pitt and Hocking, 1997). Aflatoxins are both acutely and chronically toxic. Aflatoxin B_1 is one of the most potent hepato-carcinogens known, and hence the long-term chronic exposure to extremely low levels of aflatoxins in the diet is an important consideration for human health. The induction of cancer by aflatoxins has been extensively studied. Aflatoxin B_1, aflatoxin M_1, and aflatoxin G_1 have been shown to cause various types of cancer in different animal species. However, in 1988, the International Agency for Research on Cancer (IARC) placed only aflatoxin B_1 on the list of human carcinogens. Historically, the aflatoxins were discovered as a consequence to the death of 100,000 of turkey poults, ducklings and chicks in England in 1960. The cause was found to be due to feed containing Brazilian peanuts, which was infested heavily with *A. flavus* (Austwick, 1978). Although the aflatoxins are the major toxins associated with this mycotoxicosis, another mycotoxin - cyclopiazonic acid has been implicated in the etiology of Turkey X disease (Bradburn *et al.*, 1994). The implicating

poultry feed produced by Old Cake Mills, Ltd company in London, on intensive analysis revealed a series of UV fluorescent compounds on thin layer chromatography which was identified as aflatoxins (Rustom 1997). This meal, termed Rosetti meal (from the name of the ship in which it was imported), proved to be both toxic and carcinogenic. The ground nut was found to be contaminated with the common fungus, *Aspergillus flavus*. The active principles were extracted and isolated from *A.flavus* cultures by a group of scientists in England and the Netherlands (Van der Zijden. et al, 1962; Nesbitt et al, 1962), chemically identified by a research group in the U.S. (Asao et al, 1963), and hence named Aflatoxin, an acronym, has been formed from the following combination: the first letter "A" for the genus Aspergillus and the next set of three letters, "fla" for the species flavus, and the noun “toxin” meaning poison (Ellis et al., 1991). The aflatoxins are a group of secondary fungal metabolites which have been epidemiologically implicated as an environmental toxin and carcinogen (Massey et al., 1995). Aflatoxins are a group of closely related heterocyclic compounds formed predominantly by two species, *A.flavus* and *A.parasiticus* (Betina 1989). They are substituted coumarins containing a fused dihydrofurofuran moiety. The four primary aflatoxins, named on their characteristic fluorescence, B_1 and B_2, showing blue UV fluorescence and G_1 and G_2 showing green fluorescence, on thin-layer chromatographic plates. A potency series, namely AFB_1 > AFG_1 > AFB_2 > AFG_2 > has been established for aflatoxin - induced mutagenic activity and DNA damage. Typical occurrence ratios for aflatoxins B_1 and B_2 (mainly produced by *Aspergillus flavus*) average approximately 4:1. Typical occurrence ratios for aflatoxin B_1 and the sum of the aflatoxins B_2, G_1 and G_2 (the G toxins are mainly produced by *Aspergillus parasiticus*) average approximately 1:0.8, although variations do occur for both ratios (Van Egmond *et al.*, 1978). Aflatoxin B_1 is a human carcinogen (IARC, 1993a) and is one of the most potent hepatocarcinogens known to cause cancer in liver of human and in other animals (Heathecote and Hibbert 1978). Examples of human affected by aflatoxins include the first case of human systemic infection by *Aspergillus flavus*. This was the case of a 41-year-old man with acute myelogenous leukemia. He developed a complication of suspected pulmonary *aspergillus* infection during remission therapy. Antifungal chemotherapy was used and did bring on improvement, but the underlying disease was not diagnosed. Bone marrow transplantation also did not work. The patient died due to lesions in the lungs, myocardium, kidneys, brain, thyroid gland and skin due to suspected *Aspergillus* species. The fungus isolated from the right lung and skin lesions was *Aspergillus flavus*. Aflatoxins can also affect the immune system (Pier, 1991; Yamada et.al, 1998). Another example is the intake of aflatoxins by infants in Sierra Leone. The infants were exposed to aflatoxins through the

weaning of food or diet of the family or through breast milk (Jonsyn, 1999). Animals that have been affected by aflatoxicosis have been categorized as primary acute aflatoxicosis and primary chronic aflatoxicosis. Primary acute aflatoxicosis results from intake of moderate to high levels of aflatoxins and primary chronic aflatoxicosis is when low to moderate levels of aflatoxins are consumed. The chronic effects of low dietary levels of aflatoxin on livestock are also well documented (Coker, 1997) and include decreased productivity and increased susceptibility to disease. Epidemiological studies in Africa and Southeast Asia have shown correlations between the rate of human liver cancer and the level of aflatoxin contamination in the daily diet (Selim et al. 1996).

2.3.2 Ochratoxin A

Ochratoxin A (OTA) is a potent nephrotoxin and its inhalation can lead to renal failure (Dipaulo et al., 1994). In the early 1970s, observers in Denmark noted a high incidence of nephritis (kidney inflammation) in pigs at slaughter. In Yugoslavia, 6.5 percent of blood samples contained ochratoxin A at concentrations between 3 and 5 mg/g serum (Hurt et al, 1982). Krogh et al, (1974) presented preliminary evidence to associate the human disease with ingestion of ochratoxin A (OTA) produced by *Aspergillus och*raceus and *Penicillium viridicatum*. Nephropathy has also been reported in chickens. Ochratoxin is a dihydroisocoumarin derivative produced by seven species of *Penicillium* and six species of *Aspergillus* including *A.ochraceous*. Frequent complaints of headache, lassitude, fatigue and anorexia in pigs led to search for possible causes which eventually led to the presence of ochratoxin A, a mycotoxin originally reported from *Aspergillus ochraceus* in the feed. Analysis of pig feeds showed that 50% of samples contained ochratoxin A at levels up to 27 mg/kg. The mould responsible was reported to be *Penicillium viridicatum*, but has more recently been shown to be *P.verrucosum* (Pitt, 1987). This species occurs commonly in Danish barley (Frisvad and Viuf, 1985). Although cereal grains are considered to be the main human dietary source of OTA, it has been suggested (IARC, 1993e) that pork products may also be a significant source of this toxin. One of the highest reported levels is 100 ng/ml OTA in blood from Yugoslavia (Fuchs et al, 1991); whereas 6.6 ng/ml OTA in milk has been recorded in Italy (Micco et al, 1991). The risk to humans is difficult to assess, but as pig meat is an important part of the Danish diet and rural population usually raise their own pigs which were not inspected by regulatory authorities, a risk certainly exists. Death rates from kidney failure are high in some Danish rural areas and it was reasonable to implicate the cause to ochratoxin contamination. *Penicillium verrocusum* has not been reported to occur in Asia. Ochratoxin A, produced primarily by members of the *Aspergillus ochraceus* group and a number of species of

Penicillium, especially *P. viridicatum* have been found in some samples of food and feed grains. However, Meat and certain organs of pigs are safe for consumption, if the ochratoxin A level is below 25 and 10 ppb respectively (van Egmond, 1997). A provisional tolerable weekly intake of 100 ng/kg bw, of ochratoxin A, approximating to 14 ng/kg body weight per day, has been recommended by a WHO/FAO Joint Expert Committee on Food Additives, JECFA (JECFA, 1996a). Regulatory agencies at International level have fixed the limit for ochratoxin A at 5ppb for wheat, barley and rye.

2.3.3 Trichothecenes

Toxic trichothecenes are produced by species of *Fusarium sporotrichioides; F.tricinctum, F. graminearurm, F.roseum, F. equisetii, F.moniliforme* and *F. poae.* These fungi commonly attack grains and can grow at temperatures from slightly above freezing to about 15^{O}C. This group of toxins was associated with alimentary toxic aleukia (ATA), which killed thousands of human beings in the USSR in 1913 and also after World War II. Trichothecenes such as T-2 toxin, HT-2 toxin, Diacetoxiscerpinol (DAS), Deoxynivolenol (DON), Nivalenol are the common toxins produced by the genus *Fusarium*. During the war years of 1941-1945 larger outbreaks occurred frequently involving several districts in western Siberia and European Soviet Russia. Soon the toxicity of rye was related to the growth of toxigenic strains of *Fusarium* on the cereal seed which absorbed much moisture and provided a suitable medium for the growth of fungi. It has been suggested that the epoxytrichothecenes, T-2 toxin, played a role in Russian alimentary toxic aleukia (Bamburg, et al. 1969). The trichothecenes are a complex group of sesquiterpenoids containing the trichothecane nucleus, characterized by an olefinic bond at the 9, 10 position and an epoxy group at the 12, 13 positions. It is shown that T-2 poisoning in chickens resembles the mycotoxin poisoning associated with Russian over wintered grain. Apparently all domestic animals are susceptible to injury by dietary intake of T-2, HT-2, and DAS in the range of a few ppm. In poultry, T-2 toxin in feed contaminated with 1 to 3.5 ppm of T-2 and 0.7 ppm of HT-2 may produce lesions at the edges of the beaks, abnormal feathering in chicks, a drastic and sudden drop in egg production, eggs with thin shells, reduced weight gains, and mortality. T-2 and DAS in cattle feed results in unthriftiness, decreased feed consumption, slow growth, lowered milk production, and sterility. In swine, infertility with lesions in the uteri and ovaries result from consumption of feed contaminated with 1 to 2 ppm of T-2 toxin. When toxin free sound feed was provided to all domestic animals, the troubles quickly disappeared. T-2 toxin and DAS in amounts sufficient to cause toxicoses have been found in corn which was still in the field, in silage, and in

prepared feeds made from corn. These toxins have also been identified in weather-delayed harvest soybeans. Feed contaminated with these toxins could cause severe skin irritation**.**

2.3.4 Fumonisins

The fumonisins are a group of at least 15 closely related mycotoxins that frequently occur in maize, the most important being fumonisin B_1 (FB_1). The fumonisins are a group of recently characterized mycotoxins produced by *F. moniliforme*, a mold which occurs worldwide and is frequently found in maize (IARC, 1993d). Fumonisin B_1 has been reported in maize and its products from a variety of agro climatic regions including the USA, Canada, Uruguay, Brazil, South Africa, Austria, Italy and France. The toxins especially occur when maize is grown under warm, dry conditions. Fumonisins were only identified during the mid-1980, although their effects on horses had been recognized for at least 150 years. They are polar metabolites produced by several species of *Fusarium*, including *F.moniliforme, F.proliferatum, F nygamai, F.anthophilum, F.dlamini* and *F.napiforme*. Fumonisins often occur together with aflatoxins, deoxynivalenol and zearalenone. The range of effects that fumonisins cause in mammals appears to be species-related. The full significance of fumonisins in maize for humans and animal health still remains to be determined. When the fumonisins were first identified, it was considered that their occurrence was confined to maize. Subsequently, their presence is being noted in a range of products, which include rice, sorghum and navy beans, but so far in much lower concentrations than are common in maize. Significant accumulation of FB_1 in maize occurs when weather conditions favor *Fusarium* kernel rot. Surveillance has shown that fumonisins may be present in a number of finished foods, such as polenta, maize-based breakfast cereals and beer and snack products. The presence of the fumonisins in maize has been linked with the occurrence of human oesophageal cancer in the Transkei, southern Africa and China. The relationship between exposure to *F. moniliforme*, in home-grown maize, and the incidence of oesophageal cancer has been studied in the Transkei during the ten-year period 1976-86 (Rheeder et al, 1992). They have not been detected in milk, meat or eggs. Fumonisins commonly occur in concentrations of parts per million in maize; up to 300 mg/kg has been reported. This contrasts with aflatoxins, which are usually measured in the low parts per billion concentrations in foods. This is most evident when comparing fully developed and developing countries. For example, although fumonisins can occur in maize products in the USA, Canada and Western Europe, human consumption of those products is modest. In parts of Africa, South-Central America and Asia, some populations consume a high percentage of their calories as maize meal and this often coincides with the growing areas where contamination may be the highest. On a weight for weight basis, fumonisins are far less acutely toxic than the aflatoxins. The range of effects that fumonisins cause

in mammals appears to be species-related. The liver is also generally affected and, in severe cases, gross liver lesions may be seen with fibrosis of the centrilobular areas. In pigs, fumonisins induce pulmonary oedema and hydrothorax, with thoracic cavities filled with a yellow liquid. There may also be respiratory problems and foetal mortality.

2.3.5 Patulin

Patulin is a polyketide lactone, produced by certain fungal species of *Penicillium*, *Aspergillus* and *Byssochlamys* growing on fruit, including apples, pears, grapes and other fruit. In whole fruits, visual inspection will usually identify poor-quality items. The principal risk arises when unsound fruit is used for the production of juices and other processed products. It has also been reported in vegetables, cereal grains and silage. *Penicillium expansum* appears to be the mould usually responsible for patulin in apple juice. Patulin possesses wide-spectrum antibiotic properties and has been tested in humans to evaluate its ability to treat common colds. For patulin, the LD_{50} for the rat has been reported as 15 mg / kg body and 25 mg / kg after sub-cutaneous injection. Death was usually caused by pulmonary oedema. Lungs were oedematous, with the alveoli filled with protein-rich fluid and many leucocytes. The pulmonary vessels were congested but haemorrhages were few. It has also been shown to be immunotoxic, and neurotoxic. IARC (1986) concluded that no evaluation could be made of the carcinogenicity of patulin to humans and that there was inadequate evidence in experimental animals. By far the most important source of patulin for humans is apples and apple juice, particularly that produced by direct pressing of apples. Pears, grapes, bilberries and other fruit may also be affected. A limit of 50 mg/kg for patulin in apple juice and apple juice ingredients in other beverages was proposed for adoption at the Codex Alimentarius Commission (July 2001). The Committee was of the opinion that this aspect needed a closer examination with regard to the potential health risks for children and that it was premature to adopt the level of 50 mg/kg as a maximum level for patulin in apple juice

2.3.6 Sterigmatocystin

Sterigmatocystin is a toxic metabolite structurally closely related to the aflatoxins and consists of a xanthone nucleus attached to a bifuran structure. Sterigmatocystin is mainly produced by the fungi *Aspergillus nidulans* and *A.versicolor*. It has been reported in moldy grain, green coffee beans and cheese although information on its occurrence in foods is limited. It appears to occur much less frequently than the aflatoxins, although analytical methods for its determination have not been as sensitive until recently, and so it is possible that small concentrations in food commodities may not always have been detected. Although it is a potent liver carcinogen similar to aflatoxin B_1, current knowledge suggests that it is nowhere

near as widespread in its occurrence. A number of closely related compounds such o-methyl sterigmatocystin are known and some may also occur naturally. It is considered as a potent carcinogen, mutagen and teratogen. It is less acutely toxic to rodents and monkeys but appears to be slightly more toxic to zebra fish. Cattle exhibiting bloody diarrhea, loss of milk production and in some cases death were found to have ingested feed containing *Aspergillus versicolor* and high levels of sterigmatocystin of about 8 mg/kg. The acute toxicity, carcinogenicity and metabolism of sterigmatocystin has been compared with those for aflatoxin and several other hepatotoxic mycotoxins.

2.3.7 Ergot Alkaloids

Ergot were recognized in the latter part of the 16th century, and about 100 years later, cereal grains containing ergot were shown to be the cause of these epidemics in Europe. In 1951 there was an outbreak of "bread poisoning" in a small town in France. Apparently moldy rye was sold illegally in central France to a miller, who ground the rye and mixed it with flour which he then sold to a baker. Many people ate small amounts of bread which may have been made from this contaminated flour. *Claviceps purpura* is the common ergot on rye and wheat. The fungus lives over winter in the form of sclerotium, a dense mass of fungus cells.

2.3.8 Psoralens

Birmingham et al (1961) reported a phototoxic dermatitis which had been shown to be endemic among white harvesters of celery. It was believed that exposure to celery oil and sunlight was responsible for the dermatitis. Celery rot ("pink rot") is a fungus disease produced by *Sclerotinia sclerotiorum*. Two psoralens were isolated and identified from "pink rot" celery which were not detectable in healthy extracts.

2.3.9. Citrinin

Citrinin was first isolated as a pure compound from a culture *of Penicillium citrinum* in 1931. Later, yellowish colored rice imported from Thailand to Japan in 1951 was found to be contaminated with *P. citrinin* and subsequent investigations showed that isolates of the fungus produced citrinin. In mice, citrinin is embryocidal and fetotoxic while in rat's citrinin induces similar effects and high doses are teratogenic. There is only limited evidence for the carcinogenicity of citrinin to experimental animals. Number of other species of *Penicillium* including *P.verrucosum* have been reported to produce citrinin. Because this fungus is the major producer of ochratoxin A in cereals such as wheat and barley, it is not surprising that both mycotoxins often occur together although citrinin is reported much less frequently. Other fungi cited as producing citrinin include *Aspergillus terreus* and *A.niveus*. Early studies had suggested citrinin to have potential as a powerful antibacterial agent but its potent effect on mammalian kidneys quickly ruled out this

possibility.

2.3.10 Cyclopiazonic Acid

Cyclopiazonic acid is a toxic indole tetramic acid, first isolated from *Penicillium cyclopium* and subsequently from other *Penicillium* species (e.g. *P.commune* and *P. camembertii*), *Aspergillus flavus and A. versicolor*. It is thus an interesting mycotoxin because it is produced by a number of different fungi that infect different foodstuffs. CPA causes liver necrosis or gastrointestinal tissue necrosis (Cole, 1986). Because it can be formed by *A. flavus*, a species that is a major producer of aflatoxins, it has the potential to co-occur with these mycotoxins in a range of commodities. It has been detected at levels up to 10 mg / kg or higher in maize, millet, peanuts, pulses, cheese, ham, sausage, frankfurters, mixed feeds, hay, tomato, milk and other foods and feeds. However, there are only limited studies of this mycotoxin, possibly because the analytical methods are not straight forward. It only appears to be toxic in high concentrations and its significance for human health has not yet been fully appraised. It has been found to be a neurotoxin when injected intraperitoneally into rats and the LD_{50} in male rats was 2.3 mg/kg. Oral administration produced no convulsions and LD_{50} values found in rats for administration by this route were 36 mg/kg and 63 mg/kg for males and females respectively.

2.3.11 Citreoviridin

Citreoviridin is produced by *P.citreonigrum* (synonyms *P.citreoviride* and *P.toxicarium*), particularly in rice after harvest. Acute cardiac beriberi in humans was associated with the consumption of polished rice and was initially thought to be due to avitaminosis. Uraguchi (1971) suggested that acute cardiac beriberi may have resulted from eating "yellowed rice" and proved that such a foodstuff became pigmented and toxic to rats, and produced symptoms similar to those observed in humans affected with beriberi. Acute cardiac beriberi in Japan is now only of historical interest although *P.citreonigrum* is still reported in other parts of Asia. The fungus is said to be favored by the lower temperatures and shorter hours of daylight occurring in the more temperate rice growing areas. The toxin is also produced by *P.ochrosalmoneum.* Citreoviridin has been found in un-harvested corn in the USA.

2.3.12 Zearalenone

Zearalenone and zearalenol are produced almost exclusively by *Fusarium* species including *F.culmorum, F.graminearum* and *F.crookwellense*. These species are known to colonize wheat, barley, rice, maize, and other cereals and in some other food crops and produce their toxin. However, in 1986, these mycotoxins were detected in delayed harvest of soybeans. The most important effect of zearalenone is on the reproductive system. In New Zealand, zearalenone in pasture is a recognized cause of infertility in

sheep although its acute toxicity is low. Estrogenism in swine and dairy cows is usually more prevalent in the winter and early spring because, once the fungus is established in the grain, it generally requires a period of relatively low temperatures to produce biologically significant amounts of zearalenone. When some strains of *Fusarium graminearum* grow in corn they produce a mixture of toxins along with zearalenone. In cereals and animal feeds, closely related compounds or conjugated products are known to also occur. Some of these are considered to be more potent oestrogens than the parent compound. The effects of zearalenol are similar to zearalenone, but zearalenol is generally considered to produce estrogenic effects five to ten times greater than those of zearalenone. *Fusarium graminearum* requires a minimum of 22 to 25 percent moisture to grow in cereal grains (Jacobsen et al, 1993).

2.3.13 Moniliformin

Moniliformin is formed in cereals by a number of *Fusarium* species that include *F.avenaceum. F.subglutinans,* and *F.proliferatum* and occurs as the sodium or potassium salt of 1-hydroxycyclobut-1-ene-3, 4-dione. Data on the occurrence of moniliformin in food is scarce. Concentrations up to 12 mg/kg occurred in maize in the Transkei intended for human consumption. More recently, analysis of imported milled maize products, destined for incorporation into animal feed stuffs in the UK, showed 60% of samples contaminated with concentrations up to 4.6 mg/kg moniliformin. It has also been shown to occur in other cereals including wheat, rye and rice. Samples with similar maximum concentrations have been reported in maize from Gambia and South Africa while field samples of maize, oats, wheat, rye and triticale showing visible fungal damage in Poland contained levels ranging from 0.5 to nearly 400 mg/kg. In consumer products, moniliformin has been found in 12 out of 14 corn tortillas examined at levels of 0.022 to 0.1 mg/kg.

2.3.14 Tenuazonic acid, alternariol, altenuene, and altertoxin-1

Alternaria is a common genus with a number of species that can invade crops at the pre- and post-harvest stage and cause considerable losses due to rotting of fruits and vegetables. Under suitable conditions it may lead to production of a range of mycotoxins as well as other less-toxic metabolites. Some species are fairly specific to particular crops. *A.alternata* is probably the most important mycotoxin-producing species and occurs on cereals, sunflower seeds, oilseed rape, olives, various fruits etc. The principle *alternaria* mycotoxins that have been shown to occur naturally are tenuazonic acid, alternariol monomethyl ether, alternariol, altenuene, and altertoxin I. Iso-altenuene and altertoxin II have not been found in crops to date. These toxins are produced by *A. alternata* f. sp. *Lycopersici*, a rarely occurring pathotype of *A. alternata*, and are structurally related to fumonisins. There is only one report on their

natural occurrence in hay silage. *Alternaria* toxins have been implicated in animal and in human health disorders. During investigation into outbreaks of suspected mycotoxicoses, it was shown that cereal samples collected from affected farms in Germany were more frequently contaminated with *Alternaria* mycotoxins than samples from farms with healthy animals. Cases of death in rabbits and poultry have been reported as a result of toxic action of *Alternaria* spp. found in the fodder and feed.

2.3.15 Other mycotoxins

Griseofulvin is produced by *Penicillium griseofulvum*, which has been detected in moldy cereals, although its survival into food products does not appear to have been studied. It has strong fungistatic property and has been used for treating many fungal infections. Mycophenolic acid is produced by *Penicillium roqueforti,* which has been detected in cheese. It has antibiotic activity against bacteria. Monkeys fed 150 mg/kg daily for 2 weeks developed abdominal pain, and diarrhoea with bleeding and anaemia. Mutagenic activity has been reported. *A.oryzae* is used for the production of soy and other sauces. However, along with other food-borne moulds, it can produce b-nitropropionic acid. Its mode of action is apparently irreversible inhibition of succinate dehydrogenase that can cause a variety of symptoms, often neurological in nature. *A.oryzae* has been shown *to* produce this toxin in cooked sweet potato, potato and ripe banana. Ames type assays for mutagenicity showed positive responses on some strains of *Salmonella*. It has been implicated in food poisonings in China. Kojic acid is produced by koji moulds, including *A.oryzae,* which is a commonly produced metabolite that possesses antibacterial and anti fungal activity. *A.oryzae* strains tested in one study produced kojic acid. It has been reported to occur in dried fruit such as figs. Penicillic acid is produced by several species of *Penicillium* and *Aspergillus*. It has been reported in corn and dried beans and in commercial tobacco. Penicillic acid is a hepatocarcinogen in some animal species, and has also been reported to affect the heart. PR-toxin a metabolite formed by *P.roqueforti,* which is reported to cause symptoms like congestion and oedema of lung, kidney and brain and degeneration and haemorrhaging in the liver and kidney. It has been reported in cheese, moldy grains and silage. Penitrem A, is produced by *P.crustosum*, its occurrence in serious outbreaks of tremorgenic and other types of neurotoxicity in domestic animals has been recorded. Penitrem A is a potent neurotoxin and death or severe brain damage has been reported in field outbreaks involving sheep, cows, horses and dogs. Roquefortines are produces by several moulds including *P. roqueforti.* There is no evidence that roquefortines are formed in significant levels in cheese. They occur in infected feed grain, wilted grasses or whole-crop maize silages. Stachybotryotoxin, satratoxins G and H are suspected to be produced by *Stachybotrys atra*, on feeding its cultures, symptoms such as anorexia,

depression and death was recorded in horses and dogs. Viomellein, vioxanthin and xanthomegnin are produced in cereals by some *Penicillium* and *Aspergillus* species. They have been shown to co-occur with ochratoxin A and citrinin and because they can affect the kidneys, they may contribute to the overall nephrotoxic effect caused by ochratoxin A, particularly in pigs. Current analytical methods are insensitive and there is no evidence for or against their survival in human food products. Walleminols are the metabolites produced by a xerophilic fungi called *Wallemia sebi* on dry products including cereals, dried fish and hay. Cultures of this fungus can be very toxic probably due to the presence of walleminols and other compounds. The occurrence of these metabolites in materials used to produce human food is poorly studied and the possibility of significant amounts reaching the consumer has not been investigated. Information on mycotoxins mentioned above was taken from online internet source published by Jacobsen et al (1993) of University of Auburn, Alabama.

2.4 Natural Occurrence

2.4.1 Natural occurrence of mycotoxin in cereal, pulses and oil seeds

Natural occurrence of aflatoxins in various agricultural commodities such as cereals, millets, oil seeds, nuts and spices has been documented (FAO, 2003). Corn and other cereals (wheat, rice, sorghum and barley) are the globally or individually considered, foodstuffs and are most frequently investigated for the presence of aflatoxins B_1, B_2, G_1 and G_2. Peanuts and pistachios came next in importance with 31 papers, and spices appeared in 11. Other commodities that were subject of studies were edible oils, wine, meat, fruits, chicken, infant formulae and pet foods. The presence of Aflatoxin M_1 was investigated in milk and cheese. One article dealt with the natural occurrence of aflatoxins and *Aspergillus flavus* in water (Paterson, et al., 1997). Two articles were found about the presence of Aflatoxins in airborne dusts in workplaces (Ghosh, et al., 1997; Brera, et al., 2002). Apart of the conventional articles published in scientific journals, one of the most important pools of data was presented in the report of the Scientific Cooperation Task (SCOOP) on aflatoxins set up by the European commission to provide the scientific committee on food with information on European dietary exposure to aflatoxins. This task was undertaken during 1995 and assembled extensive data describing the occurrence and distribution of aflatoxins in foodstuffs in the EU. Aflatoxins have also been detected in cotton seeds, copra, nuts, cassava, eggs and cheese and spices. In Asia since cereals and groundnuts are the major items in the diet, relatively high incidence of aflatoxin in warmer areas aggravate this problem. In addition, harvesting seasons in Asian countries usually fall on the onset, of the wet season. During this period, sun drying may not be possible which is the usual method employed by most of the farmers. Thus, grain enters the storage system at high

moisture content. Aflatoxin contamination occurs when the crops could not be dried within 48 hours (FAO, 1979, Ilangantileke, 1987, Campos, 1987, Sanchis et al., 1988; Luxsanakoses, 1989). Mycotoxins like Aflatoxins B_1, Trichothecene, Fumonisin B_1 and Ergot alkaloids have been detected in cereals that have been harvested and left in the field during heavy rains or floods or improperly stored without adequate drying. The levels encountered under normal circumstances were otherwise relatively low. Aflatoxin B_1, the most toxic and abundantly found among the series of aflatoxins, continues to be a major problem in risk commodities like groundnut, maize and chillies. Earlier many isolated studies have been conducted on aflatoxin contamination in maize and groundnut in different parts of India. In order to assess the extent of aflatoxin contamination in these commodities on a national level, the Indian Council of Medical Research (ICMR) has conducted a multicentric study involving rural and urban regions in 11 states in India. The study revealed that 21 per cent of the 2062 samples of groundnut analyzed exceeded the Indian tolerance limit of 30 μg / kg (Vasanthi and Bhat, 1998). A similar analysis of maize samples showed that 26 per cent of 2074 samples analyzed exceeded the tolerance levels (Vasanthi and Bhat, 1998). Cereals as a group, and wheat and barley specifically, were the foodstuffs most investigated for the presence of ochratoxin A. Wine, coffee and beer also were investigated for ochratoxin A. Other commodities that were the subject of surveys or occurrence studies were spices, dried fruits, herbal drugs and milk. Two articles about the presence of OTA in airborne dusts in households and workplaces were also identified (Richard, et al, 1999; Brera, et al. 2002). In 1995, the European Commission (EC) established a collaborative scientific co-operation task (SCOOP Task 3.2.2), co-ordinated by Denmark, to provide data on dietary exposure to OTA in the EU, in parallel with a similar exercise carried out on aflatoxins. One of the conclusions of the report of the Task published in 1997 (EUR 17523) was that the main contributor to the OTA intake in the EU member States seemed to be cereals and cereal products. Other possible contributors were coffee, beer, pork, products containing pig blood/plasma, pulses and spices. Fumonisins produced by *Fusarium moniliforme* have come into prominence in recent years and gained much importance next only to aflatoxins. Fumonisins have been shown to occur naturally in maize-based animal feeds through out the world at levels ranging from a few nanograms to a few hundred micrograms. Aflatoxin B_1 was also found to co-occur with fumonisin but no correlation was observed between the two toxins indicating that both toxins exist independently in the sample. National Dairy Development Board (NDDB), Anand (Gujarat) under the technology mission for dairy has carried out a survey during 1993 to 1995 on aflatoxin B_1 in feeds and occurrence of aflatoxin M_1 in milk (NDDB, 1997**).** Aflatoxin M_1 has been widely found in a number of food products including infant formula, dried milk, cheese and yogurt (Galvano et al., 1996). Analysis of 334

feed samples comprising de-oiled groundnut cake and cattle feed with the presence of damaged grains, showed that the majority of samples contained up to 200 μg / kg of aflatoxin B_1. From a survey of 1000 samples of liquid milk collected from and analyzed in different parts of India, about 2.6 – 16.0 per cent of the samples had aflatoxin M_1 levels exceeding 0.5 ppb. In another detailed study conducted on 1135 milk samples by Meerarani et al (1997) in Chennai, revealed that 10 per cent of samples tested were found positive to aflatoxin M_1 ranging from 0.05 – 3.0 μg / L. An extensive 10 year survey of aflatoxin in 4818 samples of animal feeds comprising cereals, oilseed cake compound feed and other ingredients, showed the highest incidence of contamination (96 %) in groundnut cake and de-oiled groundnut cake (Vasanthi and Bhat, 1998). In general, mycotoxins seem to pose great problem in the tropics than in the temperate regions but no part of the world can be considered to be mycotoxin-free zone due to the movement of various foodstuffs from one part of the globe to the other. Cereals, especially maize, are the major source of carbohydrates in Asia and are important as export products in some Asian countries. As export crops, grain quality has been critically monitored to meet international standards. Aflatoxin contamination has affected maize and peanuts in Thailand and India and also in coconut in the Philippines, Sri Lanka and other Pacific countries. The highest incidence of aflatoxin contamination (56.7 %) of peanut butter with AFB_1 at 700ppb level was observed in Cyprus (Kakouri et al., 1999).

2.4.2 Natural occurrence of mycotoxin in spices

Some spices and common medicinal plant sample were found contaminated by fungi and their mycotoxins (Aziz et al, 1998). Spices comprise different plant parts such as aril, barks, bulbs, floral parts, fruits, kernal, leaves, rhizomes, roots, seeds etc, (Table – 3) and their common names vary (Spice India, 2005). Several studies, especially in the United States have been made on the microbiology of spices and herbs. In Australia, the commodities tested to be with high levels of microbial flora, which predominantly originated from under- developed, south-east Asian countries (Pafumi, 1986). Aflatoxin B_1 was recorded from 40 % of spice samples by Salem et al (1996) in Egypt. Fungal flora which mainly included species of *Aspergillus* were recorded by Geetha and Kulkarni (1987) from black pepper (ranging from 0.6×10^4 – 16×10^5 / g) and turmeric (ranging from 0.5×10^3 – 11.1×10^5 / g) samples collected from retail shops in Bombay. In their study, aflatoxin producing *Aspergillus flavus* and *A.parasiticus* were detected from 88 % of chilli samples. Fungal infestation and occurrence of mycotoxins in spices was observed by Gerald et al (1992). Natural contamination of agricultural produce with mycotoxins is fairly high in developing countries like India (Aktar and Khan, 1987). Spice samples from Andhra Pradesh were surveyed for occurrence of

mycoflora and found chilli, ginger and turmeric infested at 28.60, 23.8 and 23.6 % with toxigenic strains of *Aspergillus* species (Giridhar and Reddy 1997). According to Bartine and Tantaoui-Elaraki (1997), fungal growth was weak on curcumin, black pepper and white pepper. Dry chilli was found infested heavily by *Aspergillus flavus* and *A.fumigatus* (Giridhar and Reddy 1999). Amongst various fungi recorded in chilli (powder) samples collected from retail outlets of Bombay, *Aspergillus* group was predominant (Geetha and Kulkarni, 1987). A sampling from Thailand revealed that 18 % of spices and herbs were contaminated with aflatoxin B_1 with the range of 40 – 160 ppb level (Gerald et al., 1992). Aflatoxin contamination of *Piper nigrum* at 1.20 µg/g was detected in Bihar (Roy et al. 1988). Scott and Kennedy (1973) reported the results of their analysis and survey of ground black, white, and capsicum peppers for aflatoxins. In their study the authors reviewed the occurrence of toxin-producing moulds in spices such as pepper, Indian red pepper, and paprika. It was found that crushed and whole pepper corns were good substrates for the production of aflatoxins by *Aspergillus flavus*. In India, common spice such as coriander, cardamom, cumin, pippali and emblic were found contaminated with aflatoxin B_1 above the tolerance level by WHO (Roy and Chaurasia 1990). Aflatoxin B_1 ranging from 1 – 5 µg was detected in chilli, cumin, curry powder, saffron and white pepper and no aflatoxins were detected in ginger, cardamom, clove and mustard (Maria et al. 2001). Among some spices screened for

Table.3. Spices and their parts in daily diet

Clove	Flower bud	*Syzygium aromaticum*	Myrtaceae
Cumin	Seeds	*Cuminum cyminum*	Apiaceae
Ginger	Rhizome	*Zingiber officinale*	Zingiberaceae
Nutmeg / Mace	Seed & aril of fruit	*Myristica fragrans*	Myristicaceae
Pepper	Fruit Seed	*Piper nigram*	Piperaceae
Turmeric	Rhizome	*Curcuma longa*	Zingiberaceae

Source: Sethi and Meena, 1997; Spice Board, 2005.

presence of mycoflora, clove, ground cumin and nutmeg were contaminated mainly with species of *Aspergillus* and *Penicillium*, (Garrido and Pozo, 1992). Pepper yielded 49 % of toxigenic *A.flavus* isolates (Gerald et al., 1992). While mycotoxin contamination in such commodities assumes significance from the health point of view, its problem in cash crop like groundnut and plantation products including spices affect trade and economy. However, cereals and millets which form the staple food for many population groups in India are often the risk commodities. In addition to the other agricultural commodities, some work on contamination of spices with toxigenic fungi and toxins have been carried out, but systematic reports are not available. Most of the researchers have given attention towards identifying causal factors and formulating control measures. As per the data of National Nutrition Monitoring Bureau, the dietary intake of spices and condiments is more in South India. Natural contamination of aflatoxin in various samples of chillies, fennel, cumin, coriander, black pepper, ginger, cardamom and turmeric and presence of Ochratoxin, Citrinin and Zearalenone has also been reported in a few samples of spices. In a limited survey in Uttar Pradesh conducted by the Indian Grain Storage Institute, Hapur during 1984-85, it was revealed that out of 99 dry fruit samples only 19 samples were contaminated with aflatoxin B_1 in the range of trace to

50 ppb.

A relatively high incidence of toxigenic moulds, including *Aspergillus flavus*, *A.parasiticus*, *A.fumigatus*, *A.ochraceous*, *Penicillium.citrinum* and *P.islandicum* has been detected in some spices (Christensen**,** Anisa Ath-kar et al., 1988). Several investigators have found aflatoxins in a range of spices, viz, Black pepper, cumin and mustard seed (Scott and Kennedy, 1973, 1975; Flannign and Hui, 1976; Seenappa and Kempton, 1980a,b; Awe and Schranz, 1981; Emerole et al., 1982; Misra, 1987; Misra et al.,1989; Sahay and Prasad, 1990), although the levels of aflatoxins recorded were generally low, while certain spices and herbs, especially cinnamon, cloves and possibly oregano inhibit mycelial growth and subsequent toxin production, others particularly sesame seed, ginger and rosemary appear to be conducive to aflatoxin production (Llewellyn et al., 1981). Aflatoxins nave been isolated from spices like cayenne pepper, Indian chilli powder, dried pepper, black pepper, capsicum and nutmeg (Misra, 1987). Nutmeg and red pepper appear to be especially prone to aflatoxin production (Seenappa and Kempton, 1980 b). In a survey of 21 different imported spices by the U.S food and Drug administration (FDA), nutmeg and chilli were found to contain detectable levels of aflatoxins (Wood, 1989). A French survey implicated pepper as a source of toxigenic *Aspergillus flavus* which produced high levels of aflatoxin in sausages and pepper cheese (Jacquet and Teherani, 1974). Thus, available information on spices deals mostly with productional aspects, medicinal, nutritional properties and their importance in human health. Holley and Patel (2005) recorded mold count in the assorted pepper and the spore count was from 200 – 3400000 / g. Amongst isolates from pepper, 49 % of *A.flavus* were found to be toxigenic. They also recorded aflatoxin B_1 excess of 75 ppb in coriander. Sampling of commercial spices from Thailand recorded the presence of aflatoxin B_1 at the range between 40 – 160 ppb (Holley and Patel, 2005). However detailed information regarding the intake of spices is limited. Data available on per-capita consumption of spices in some countries indicate that though the production is almost confined to developing countries, the consumption is considerably more in developed countries like U.S.A and Sweden.

2.5 Environmental Factors

2.5.1 Water Activity

In 1952, W.J. Scott introduced the idea of water activity. Water activity (a_w) is the ratio of water vapor pressure of the substrate to the vapor pressure of pure water at the same temperature and pressure. At low a_w, water is bound by salts, sugars, proteins, and other solutes, therefore growth of moulds cannot occur since water is not present in an available form (Northolt and Verhulsdonk, 1976). Aflatoxins are

produced at water activity (a_w) values ranging from 0.95 to 0.99, with a minimum a_w value of 0.82 being reported for *A.flavus* (ICMSF, 1996). However, fungal growth can still occur at a_w values as low as 0.78 to 0.80. The minimum a_w for growth of *A.flavus* has been reported as 0.78, while the minimum a_w for aflatoxin production was found to be 0.84. *A.parasiticus* has a minimum a_w of 0.84 for growth and minimum a_w of 0.87 for aflatoxin production (Diener and Davis, 1970). The optimum a_w for aflatoxin production by both *A.flavus* and *A.parasiticus* is reported to be in the range of a_w 0.95 to 0.99(Diener and Davis, 1967). *A.ochraceus* require minimum a_w 0.77 and 0.87 for growth and ochratoxin A production respectively (Kozakiewicz and Smith, 1994).

2.5.2 Temperature

The optimum temperature range for fungal growth and aflatoxin production is 25^0C to 30^0C (Northolt et al., 1977). However, in nature, temperature varies due to seasonal variations or through spontaneous heating in stored food commodities. As a result of temperature variation, the yield of aflatoxins can vary considerably. At low temperature, approximately equal amounts of aflatoxins B and G are formed, while at higher temperatures aflatoxin B production predominates relative to aflatoxin G. It has been shown that while aflatoxins B_1 and G_1 were produced in equal amounts at 15^0C to 18^0C, they were produced at ratio of 12:1 at higher storage temperature of 32^0C (Diener and Davis,1969). The decrease in aflatoxin G_1 production has been attributed to the accelerated catabolism of B_1 at higher storage temperatures (Schroeder and Hein, 1967).

2.5.3 Atmospheric Gases

Fungi are aerobic organisms, but the relationship between oxygen and carbon dioxide requirements vary considerably among species and strains. Low concentrations of CO_2 have been shown to be beneficial to spore germination and are involved in fungal metabolism and in the synthesis of proteins, nucleic acids, and intermediates of the tricarboxylic acid cycle (TCA). Concentrations of CO_2 greater than 20 % inhibit mould spore germination and more than 10 % suppress toxin production (Tabak and Cooke, 1968). A decrease in oxygen (O_2) concentration to less than 20 % or an increase to 90 % or more have shown to inhibit aflatoxin formation (Landers et al., 1967; Shih and Marth., 1973). An increase in nitrogen concentration has also been shown to suppress aflatoxin formation (Epstein et al., 1970). The maximal production of aflatoxin depends not only on mould strain but also on the appropriate concentrations of headspace CO_2 and O_2. This is due to the fact that aflatoxin formation is neither associated with maximal concentrations of O_2 present nor with maximal mycelial growth (Shih and Marth., 1973). Inhibition of aflatoxin production by *A.flavus* and *A.parasiticus* has been reported to occur by the

reduction of available oxygen through modified atmosphere packaging of foods in barrier film or by using oxygen scavengers (Ellis et al., 1994).

2.5.4 Light

Light is essential to many mould species for the induction and completion of sporulation. It influences both the vegetative growth and aflatoxin production of toxigenic moulds in both liquid and solid media. With respect to species, the role of light may be either inhibitory or stimulatory due to photochemical effects on the medium (Charlile, 1970). *A.flavus* and *A.parasiticus* produced more conidiophores under light than dark (Bennett et al., 1978).

2.5.5 pH

The pH of the medium in which fungi grows has minimal effect on the primary metabolic patterns of fungi, but it appears to have a more pronounced effect on aflatoxin production. During fungal growth, pH may fluctuate to values of 4 to 5 as a result of fungal activities (Moreau and Moss, 1979). *A.flavus* and *A.parasiticus* are both able to grow over a wide range of pH values with optimum growth occurring in the pH range 5 to 8. *A.flavus* and *A.parasiticus* able to grow at pH between 1.7 to 9.34, with optimum growth occurring between pH 3.42 and 5.47(Lie and Marth, 1968). However, aflatoxin production did not occur at all pH levels. Therefore, while moulds in general can tolerate more acidic conditions, but they inhibit aflatoxin production (Northolt et al., 1977). Aflatoxin production was maximum when media was buffered between 5 – 6 pH (Basappa et al, 1976).

2.6 Biosynthesis

The mycotoxigenic fungi are the genes which code for the enzymes of the mycotoxin biosynthetic pathway (referred to as mycotoxin biosynthetic genes). However, to date only some of the genes of mycotoxin biosynthetic pathways have been cloned and sequenced. The best analyzed biosynthetic pathways at the genetic level are these from the aflatoxins (Trail et al., 1995a ; Yu et al., 1995), the trichothecenes (Mc. Cormick et al., 1996), Patulin (Beck et al.,1990) ; Wang et al., (1991), PR-toxin (Procter and Hohn, 1993) and for sterigmaticystin (Kelkar et al., 1996). Sterigmatocystin is a precursor of aflatoxin and the sterigmatocystin biosynthetic genes are rather homologous to the aflatoxin biosynthetic genes (Brown et al., 1996). Genes for biosynthetic enzymes of secondary metabolites are usually clustered (Hohn et al., 1993) and this is true for the aflatoxin (Trial et al., 1995a; Yu et al., 1995), sterigmatocystin (Brown et al., 1996), trichothecene (Hohn et al., 1993) and fumonisin biosynthetic genes (Desjardius, 1996). The polyketide synthase gene from *penicillium* patulin, which is the key enzyme in the biosynthetic pathway of the mycotoxin patulin, has been cloned (Beck et al., 1990). Polyketide synthesis is often

involved in fungal secondary metabolism and can be found in various species (Hopwood and Sherman, 1990). The polyketide synthesis is composed of different catalytic domains, each specific to a certain reaction. These domains, however, often share high sequence homology, even between species, which makes this gene unsuitable as a specific target gene for diagnostic PCR (Mayorga and Timberlake, 1992). Aflatoxins are secondary metabolites and their synthesis occurs through the polyketide pathway, the major pathway of secondary metabolism in moulds. Aflatoxins are formed by the polyketide process that involves the condensation of an acetyl unit with three or more malonyl units with the loss of CO_2 (Bennett and Lee, 1979). Polyketide intermediates are thought to be built up in a cyclic process similar to the fatty acid biosynthesis (Sedgwick and Moris, 1980), but omitting the reduction-elimination-reduction sequence, resulting in the loss of the acetate oxygen (Hsieh et al., 1976). The first step in the biosynthetic pathway of aflatoxins is the condensing of acetate units to form norsolorinic acid. Sixteen enzymes have been estimated to be involved in the bioconversion of norsolorinic acid to aflatoxins. Some of these enzymes have been purified and identified, but only a few have been purified to homogeneity (Yu, Chang, Lary 1995). Aflatoxin biosynthetic pathway is quite well understood and has recently been described in a number of reviews (Bhatnagar et al., 1994; Trail et al., 1995a; Bennett et al., 1997; Minto and Townsend, 1997). The generally accepted aflatoxin B_1 and B_2 biosynthetic pathway (Yu et al, 1995) in *A.parasiticus* and *A.flavus*, enzymes for some specific conversion steps, and cloned genes are schematically presented in the figure 1a. The regulatory gene, aflR, coding for the regulatory factor (AFLR protein), controls the expression of all the characterized structural genes (nor-1, ver-1 and omtA). The ver-1 gene product(s) has not been fully characterized; catalytic steps in the aflatoxin biosynthetic pathway of the uvm8 and aad gene products, fatty acid synthase and a dehydrogenase, respectively, are not defined; and the ord-1 and ord-2 gene products are under investigation. The approximate sizes, relative locations and directions of transcription of identified genes are indicated. The transcription of structural genes ord-1, ver-1 and omtA is regulated by AFLR (Yu et al, 1995).

2.7 Detection

2.7.1 Detection of mycotoxin

Methods of detecting mycotoxins are broad and complex. This complexity is due to the diversity of commodities prone for contamination. Several methods for the simultaneous determination of mycotoxins have been reported, offering a significant advantage over conventional techniques. Rundberget and Wilkins (2002) have determined 13 *Penicillium* toxins, ochratoxin A, citrinin, patulin, mycophenolic acid, cyclopiazonic acid, PR-toxin, rubratoxin B, verruculogen, chaetoglobosin B, penitrem A, griseofulvin,

roquefortin C, penicillic acid in standardized matrix samples, which included bread, rice, potatoes, vegetables and fruit. Several assay methods specific for commodities, such as peanuts, cottonseed, corn, green coffee and spices, have been developed based on the inherent nature of the commodity. The following are the generally adopted procedures:

2.7.1.1 Sampling

Sampling is the most important contributor to the variability of analyses for aflatoxin in agricultural commodities, particularly groundnuts, because of the nonhomogenous nature of aflatoxin distribution. The first consideration in any experimental or regulatory protocol should be the sampling method. Protocols have been published on sampling techniques (Dickens and Whitaker 1986) published an excellent review of sampling plans and collaboratively studied methods for aflatoxin analysis.

2.7.1.2 Extraction

Extraction involves removal of the aflatoxin from the sample for quantitation. Therefore, extraction procedures must be efficient, quantitative, and must not alter or have any effect on aflatoxin. Due to diverse nature of commodities that may be contaminated, no single method of extraction is adequate for all commodities. Commodities with high lipid and pigment content require a different treatment relative to those with a low content of these components. Most of the interfering substances are often soluble in the same solvents as aflatoxin; therefore, selective extraction or extensive purification methods are required to produce pure extracts. Aflatoxins are soluble in slightly polar solvents and insoluble in completely non-polar solvents. Practically all aflatoxins are extracted using mixtures of organic solvents such as acetone, chloroform, or methanol in combinations with small amounts of water (Bullerman, 1987). Aqueous solvents more easily penetrate hydrophilic tissues and enhance aflatoxin extraction. The presence of fats, lipids, and pigments in extracts reduces the efficiency of separation techniques. Therefore, addition of fat solvents, such as hexane, to the extraction solvent results in many fats and lipids being partitioned into the hexane portion of the solvent, which is subsequently discarded (Moss and Smith, 1985). Method of extraction of aflatoxins developed by Pon's was modified (Rati et al., 1987) by eliminating column clean up step and by introducing an additional TLC development in ether and hexane (1:1). Different methods of extraction by using different solvent systems as suggested by AOAC are shown in Table.4. The aflatoxins and many of the other important mycotoxins are fluorescent under ultraviolet light. Detection or "qualitative assay" is usually by TLC or mini-column and only requires the use of a qualitative standard. Various methods have been evaluated for the estimation of aflatoxin B_1 in chilli powder (Shantha, 1999).

2.7.1.3 Purification

The objective of the clean-up step is to remove co extracted substances in order to reduce the chemical complexity of the final extract used for identification and quantitation (Moss and Smith, 1985). Purification and clean-up involves liquid-liquid partitioning and precipitation of impurities, then removal using preparative thin-layer chromatography or column chromatography. Liquid-liquid partitioning can also occur during extraction when equilibrium extraction systems are used.

2.7.1.4 Separation

This step is achieved either by thin-layer chromatography or high-performance liquid chromatography.

2.7.1.5 Thin – layer Chromatography (TLC)

Thin-layer chromatography (TLC) involves coating a glass plate with silica gel and applying a concentrated sample of aflatoxin on a baseline and developing the TLC in appropriate solvent system. (Bullerman, 1987). It has been considered as the AOAC official method and the method of choice to identify and quantitate aflatoxins at levels as low as 1 ng/g. The TLC method is also used to

Table.4. Summary of extraction solvents used in Official AOAC methods for mycotoxin analysis.

Toxin	Commodity	Extraction solvent
Aflatoxins	Corn, cottonseed, green coffee beans, soyabeans, coconut, copra, copra meal, cocoa	Acetone: water (85:15) Chloroform : water (91: 9)
Ochratoxins	Barley	Chloroform + 0.1 M phosphoric acid
Patulin	Apple juice	Ethyl acetate, Acetone
Sterigmatocystin	Barley, wheat	Acetonitrile: Potassium chloride (4%) (9: 1)
Trichothecenes (Type A)	Cereals	Methonol : water (9: 1)

Trichothecenes (Type A)	Cereals	Methonol : water (9: 1)
Trichothecenes (Type B)		Acetonitrile : water (1:1), Chloroform : methanol (3:1)
Fumonisins		Methonol : water (3: 1), Acetonitrile : water (1:1)
Zearalenone		Ethyl acetate, methanol, acetonitrile and chloroform and mixtures thereof
Moniliformin		Acetonitrile : water (1:1)

Source: AOAC, 2002.

verify findings by newer, more rapid techniques. Detection is based on the fluorescent properties of aflatoxin. Quantitation can be achieved by several methods, with one of the most common methods being visual estimation. This involves comparison of aflatoxin standards of known R_f values with the color and intensity of fluorescence of sample over a range of concentrations of standards. The accuracy of the technique has been reported to be about 20 %. Recent methods involve the use of the fluorodensitometer, in which the TLC plates are examined under UV light and scanned with a photometer that allows the extract location of the position of fluorescent spots, as well a precise measurement of the intensity of their fluorescence, to be determined. When detection and / or quantification is by TLC, then derivatives can be formed "on-the-plate" either by spotting a reagent on the plate before development or by spraying on a reagent after development. An example of the former approach is the use of trifluoroacetic acid (TFA) to form the hemi-acetyl derivatives of aflatoxin B_1, G_1 and M_1 which are identified, if present, as slower running fluorescent spots(Przybylski 1975). A commonly used spray reagent for the aflatoxins is 50% sulphuric acid which reacts with the blue and green-blue fluorescent aflatoxins to give yellow fluorescent derivatives. The TFA procedure or direct acetylation (Cauderay 1979) can be carried out on a TLC plate before development.

2.7.1.6 High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC involves the separation of sample constituents, followed by their detection and quantification. Separation is achieved by a competitive distribution of the sample between a mobile liquid phase and a stationary liquid or solid phase that is supported in a column. The mobile phase moves under pressure and passes through a column that contains the extract and then flows to UV absorption / fluorescence detector. A change in electrical output is produced, which is recorded on a moving chart to give a chromatogram. The retention time for aflatoxin is constant under fixed conditions. A comparison of the retention times with those of the standards enables results to be compared on a quantitative basis as the area under each peak on the chromatogram is proportional to the concentration of the particular type of aflatoxin. Extremely low levels of aflatoxin can be detected by attaching sensitive detection and sophisticated data retrieval equipment to the HPLC. HPLC has been used in assaying aflatoxin in many foods, for example, cottonseeds (AOAC, 1984), peanut products, figs, corn (Pons and Franz, 1977), milk and milk products for aflatoxin M_1, and in blood of mammals (Thiel, 1986). Awe and Schrans (1981) used HPLC for the first time to determine aflatoxins in spices. Samples of chili, ginger and nutmeg were extracted according to previously published methods. Recoveries from aflatoxin free samples of chili and ginger spiked with 1-50 μg aflatoxin per kg ranged from 64 to 90 %. Trucksess et al. (1984) published a rapid TLC method using a disposable silica-gel column for cleanup and confirmation by gas chromatography-mass spectroscopy. Madhyastha and Bhat (1984) developed a minicolumn confirmation method for aflatoxins. These workers confirmed the identity of aflatoxins on the developed minicolumn by applying 20% H_2SO_4, 20% HCl, or trifluoroacetic acid (TFA) in 20% HNO_3. All acids changed the fluorescence from blue to yellow, with the TFA in 20% HNO_3 having the lowest detection limit. Method of detection by HPLC is widespread, because of their superior performance and reliability compared with thin layer chromatography (TLC). However, TLC remains the method of choice for rapid screening purposes and for situations where advanced HPLC equipment is not available. HPLC methods have been developed for almost all major mycotoxins in cereals and other agricultural commodities; where as the use of gas chromatography is restricted to trichothecenes.

2.7.1.7 Immunochemical Method

Thin layer chromatography and LC methods for determining toxins in food are laborious and time consuming. Often, these techniques require knowledge and experience of chromatographic techniques to solve separation and interference problems. Through advances in biotechnology, highly specific antibody-based tests are now commercially available that can identify and measure toxins in food in less than 10 minutes. These tests are based on the affinities of the monoclonal or polyclonal antibodies.

Three types of immunochemical methods are radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and immunoaffinity column assay (ICA). Additionally dipstick tests have been developed, which claim a detection limit of FB_1 as low as 0.04-0.06 µg/g in corn based foods (Schneider et al, 1995). Aflatoxin B_1 in groundnuts can be determined by using Immunoassays (solid-phase radio-immunoassay). (Langone and van Vunakis, 1976; Sun and Chu, 1977), monoclonal affinity column immunoassay (Groopman et al. 1984), or enzyme-linked Immunosorbent assay (ELISA) techniques (Chu and Ueno, 1977; El-Nakib et al. 1981; Lawellin et al. 1977; Pestka et al. 1980). The major advantages of the ELISA and affinity column methods include speed, ease of sample preparation, ease of use, and a potentially low cost per analysis ideally suited for screening. The disadvantages include different a antibody specificities for B1 and cross reactivity with other aflatoxins. Methods also need to be developed that will distinguish between B_1, B_2, G_1, and G_2 individually or collectively.

2.7.1.8 Biological method

Biological methods have been investigated as potential bioassay procedures for detection and quantitation of aflatoxins. Bio assay include cell and tissue cultures and the use of laboratory animals and microorganisms. Immuno assays here emerged as powerful analytical tools.

2.7.1.9 Detection of mycotoxigenic fungi

Various methods have been developed to detect the mycotoxins in food and agricultural commodities and none of the method is found to have only the merits. The newly emerging methods such as Polymerase Chain Reaction (PCR), though it has its own drawback, is now being employed all over the world to detect many microorganisms including pathogenic bacteria to mycotoxigenic fungi.

2.7.1.10 Polymerase Chain Reaction (PCR) method

Conventional methods for the detection of mycotoxigenic fungi are culture methods, which are time consuming and require exposure in fungal taxonomy. Taxonomic classification can be simplified by the use of selective media, but the time required is not reduced (Davies et al., 1987). These problems can be overcome by the use of the PCR which can reduce the detection time from several days to several hours. The diagnostic PCR approach for the detection of mycotoxigenic fungi is an indirect method. DNA or DNA fragments of the target organism can be amplified and detected by gel electrophoresis Nucleic acid probes and PCR. The Polymerase chain reaction (PCR) technique amplifies DNA by using a heat stable DNA polymerase in a repetitive cycle of heating and cooling (Saiki *et al*., 1988). After the cycle of heating and cooling is repeated 30 to 40 times the target DNA will have been amplified to a theoretical maximum of 10^9 copies. The resulting DNA is stained with ethidium bromide and visualized by agarose gel

electrophoresis with UV transillumination (312 nm). Negative control samples omitting DNA must be used in order to check for contamination of the PCR reaction by extraneous DNA. Many detection kits have been developed which are specific for food pathogens.

2.8 Control

Recognition of the problems of mycotoxins in various countries led to the development of appropriate programmes for their prevention and control. These programmes formed part of the strategies to minimize the problems of mycotoxin contamination and included not only the prevention of mycotoxin formation in agricultural commodities but also their removal through detoxification or decontamination, routine surveillance, regulatory measures to control the occurrence of mycotoxin in foods, both at national and international trade as well as an information, education and communication drive. Several approaches both preharvest and post harvest are currently being tried to reduce or to eliminate aflatoxin from the food chain. Prevention of mold contamination and /or mold growth can be achieved by methods viz, Improved farm management, sowing healthy seeds, proper irrigation, rotation of crops, harvesting after full maturity, drying the harvested crops within time, genetic engineering, rapid screening techniques, antifungal agents, control of environmental conditions (moisture, temperature and gaseous atmosphere) as per the review of Oldham (1991). But it is not possible to adopt these methods scrupulously because of lack of facilities, poor education of the farmer and adverse climatic conditions. Hence almost all the vulnerable commodities are contaminated with Aflatoxins, the level depending on the season. These toxins are highly resistant to food processing conditions. Hence the decontamination methods are inevitable. The decontamination methods are of three types; physical, chemical and biological.

2.8.1 Physical method

Physical method of decontamination involves physical removal of infected grains by hand picking or electronic color sorting, density segregation by floatation, removal by milling cereal grains, adsorption-cum-filteration technique of groundnut oil, removal by suitable solvents and inactivation by heat and irradiation are some of the methods found to be promising in reduction of aflatoxin levels in contaminated products (Basappa, 1983). By physical method, heating and cooking under pressure can destroy nearly 70% of aflatoxin in rice compared to under atmospheric pressure only 50% destroyed. Dry and oil roasting can reduce about 50-70% of aflatoxin B1. Only about 10% of total 1242 ppb of aflatoxin B. decreased in naturally contaminated peanut by heating at up to 100°C. Since aflatoxin resists to higher temperature up to 260°C, long-time cooking and overheating would destroy essential vitamins and amino acids in treated foods. Cooking at atmospheric pressure can destroy about 50 percent of the toxins. Dry roasting and oil

roasting of groundnut reduces aflatoxins to a significant degree. Cooking rice under 15 lbs. pressure for 5 minutes gave maximum destruction of aflatoxins (72 percent) as compared to ordinary cooking or cooking with excess water. The most practical approach was found to be that of segregation. This was successfully shown for aflatoxin removal from groundnuts and maize. Hand picking of visibly moldy nuts could remove a major percentage of aflatoxin from groundnut. For removal of aflatoxin and fumonisin contamination in maize, density segregation in water or NaCl was studied. It was found that 74 – 86 per cent of fumonisins could be removed by removal of buoyant grains in water or 30 per cent NaCl solution (Vasanthi and Bhat, 1998). A similar approach was also found to be effective in removing ergoty pearl millet by treatment of the millet with water and NaCl solutions. Segregation of contaminated portions of grains by milling procedures was earlier evaluated with rice. It was observed that while dehusking could remove 55 – 67 per cent of the toxin, subsequent polishing and parboiling reduced the toxin content to 72 – 93 per cent. Physical separation of infected grains is an efficient and feasible method of mycotoxin de-contamination (Table - 5). This is affected either by manual operation or with the help of an electronic sorter. It has been reported that gamma irradiation (5-10 M-rad) caused reduction of aflatoxin. The irradiation, however, could not completely destroy the toxin and its mutagenicity. Only about 30% of total 600 ppb at aflatoxin B_1, either pure toxin or in contaminated peanut, was destroyed by 1 and 5 M rad or gamma irradiation.

2.8.1.1 Irradiation method

Radiation are of two types: ionizing and non-ionizing. Gamma rays, X-ray, and ultraviolet rays are the ionizing radiation and radio waves, microwaves, visible light, and infrared waves are the non-ionizing radiation (Rustom, 1997). Irradiation is a process of preservation involving exposure of foods to high-energy rays to improve product safety and shelf life. Red meats, poultry, potatoes, onions, spices, seasonings, fresh fruits and vegetables may be irradiated to prevent growth of food poisoning bacteria, eliminate parasites, or delay ripening and spoilage. Also, irradiation could be used to replace chemical preservatives in foods. More than 40 years of research on food irradiation has shown that foods exposed to low levels of irradiation are safe and wholesome, and they retain high quality. Here the food is exposed to a controlled amount of gamma rays from a radioactive source such as $_{60}$cobalt. Irradiated foods do not alter the nature of food, are not radioactive since the rays do not remain in the food. Internationally, foods such as apples, strawberries, bananas, mangoes, onions, potatoes, spices, seasonings, meat, poultry, fish, and grains have been irradiated for many years. Since 1991, Japan has irradiated more than 20,000 pounds of potatoes each year to prevent sprouting. In the Netherlands, more than 18,000 pounds of foods such as strawberries, spices, poultry, and dehydrated vegetables are irradiated daily. Belgium irradiates more than

8,000 tons of food per year. Canada irradiates potatoes, onions, wheat flour, fish fillets, spices, and seasonings. More than 35 countries have approved irradiation of some 40 different food products. The toxicity of a contaminated peanut meal with AFB_1 was reduced by 75 and 100 % after irradiation at the dosage level of 1 and 10 KGy, respectively (Temcharoen and Thilly, 1982). In 1986, the United States Food and Drug Administration (FDA) approved irradiation of spices and seasonings up to 30 kGy to reduce microorganisms and insects. Irradiation has been most effective and safe method of treatment, yielding no toxic by-products and studied for application in developing countries (F.A.O, 1973).

Table.5. Reduction in mycotoxin content due to decontamination methods

Food	Mycotoxin	Method of decontamination	Level of mycotoxin (μg / kg)		
			Before treatment	After Treatment	% Reduction
Groundnut	Aflatoxin	Segregation by removal of visually moldy seeds	10 - 1600	ND	-
Rice	Aflatoxin	Milling : Dehusking & polishing	13.3 – 53.5	2.6 – 18.8	65 – 80
		Parboiling, Dehusking & polishing	13.3 – 53.5	2.1 – 4.5	72 - 93
Maize	Fumonisin	Density segregation :			
		With water	923.55	242.40	73.7
		With 30 % NaCl	973.6	164.41	82.9
Wheat	Deoxynivalenol	Washing with water	93.30	76.2	18.0
		Washing with 15 % NaCl	93.30	71.3	23.0

Source : Vasanthi and Bhatt, 1998.

Irradiation of spices and seasonings reduces the dependency on chemical fumigants. Food proteins, carbohydrates, and fats have been found to be relatively stable to irradiation up to 10 kGy. Minerals have also been reported to be stable to irradiation. However, vitamins A, C, E, and B1 tend to be susceptible to irradiation at doses of 1 kGy or above. However, these vitamins are also sensitive to heat processing. The reduction of these vitamins in foods is minimal and would not create a risk of deficiency in the diet. A joint committee of the FAO, WHO, and IAEA claim that losses of vitamins in foods treated with irradiation doses of 1 kGy or less are minimal and compatible with losses of vitamins in foods heat treated and stored for extended periods of time. Low-dose irradiation does not cause a significant decrease in the nutritional quality of foods. Food irradiation has been endorsed by FAO, WHO, USDA, the American Medical Association (AMA), and the Institute of Food Technologists (IFT) as a safe and practical method for preserving a variety of foods and reducing the risk of food borne disease. International imports and exports of fresh foods could be expanded, increasing the abundance of food worldwide. Food irradiation provides safer food, improves quality, and extends shelf life.

2.8.2 Chemical method

Chemical methods involves structural degradation and inactivation of aflatoxins by use of chemicals such as chlorinating agents, oxidizing agents and hydrolytic agents, acids and alkalis. Mycotoxins are removed from contaminated commodities. The method should be sure that the detoxification system is capable of converting the toxin to a nontoxic derivative (s) without deleterious change in the raw product. Toxicity and mutagenicity of the treated products should be assessed. These chemicals include the followings: acetic acid, ammonia gas ammonium salts, calcium hydroxide, formaldehyde, hydrogen peroxide, methylamine, ozone gas, phosphoric acid, phosphine gas, toxic sodium bicarbonate, sodium bisulfite, sodium bisulfite, sodium hypo chlorite. Ammoniation is an effective method for the elimination of aflatoxins in contaminated cottonseed meal (Mc Kinney et al., 1973). The

detoxification of aflatoxin are primary addition of the double bond of the furan ring and oxidation involving phenol formation and opening of the lactone ring. In the presence of acid, aflatoxins B. and G. will be converted into their hydroxy derivatives. Other mycotoxins which are like aflatoxin and have a lactone grouping in the molecule can be similarly destroyed by alkaline condition. Certain short-chain fatty acids have been shown to inhibit growth of fungi. Propionic acid has been shown to be effective fungi static agent against *A. flavus*. It is a volatile fatty acid that has been used to preserve high moisture corn without reducing its value as animal feed. The activity of propionic acid is enhanced by acid pH and certain feed ingredients. Salts of sorbic acid have also been shown to inhibit growth of *A.flavus* and *A.parasiticus*. The compound dichlorvos has been found to inhibit aflatoxin biosynthesis by *A. parasiticus* even though it has no effect on fungal growth. In recent times more and more antifungal agents of plants origin are being worked out by several investigators and some of those tried includes aliphatic aldehydes, ketones, alcohols, phenols, ethers and other hydrocarbons. Amongst them, two aliphatic aldehydes, cinnamaldehyde and omethyl cinnamaldehyde, three ethers, anethole, methyleugenol and methyl isoeugenol and three phenoloics, carvocrol, eugenol and thymol are the main components of spice oils having fungistatic activity in .some foods like pickles and chutneys etc. (Ayres et al.1980; Prakash, 1990; Mathews, 1992; Pruthi, 1993; Mehta et al. 1995). Thymol from thyme leaves shown to inhibit mycelial growth as well as aflatoxin production (Gerald et al. 1992). Shantha et al (1997) have tried detoxification method against aflatoxin B_1 using ammoniated groundnut cakes in albino rats. Spice like gercinia have antifungal property against aflatoxigenic fungi (Selvi et al. 2003). Natural plant essential oils are the new source of antimicrobials to improve shelf life and safety of perishable foods. Some of the spice oils which possesses antimicrobial properties are presented in Table - 6.

2.8.3 Biological method

Biologically many microorganisms, including bacteria, actinomycetes, yeasts, moulds and algae are used to degrade aflatoxins. Fungi like *Aspergillus niger*, *Absidia repens*, *Corynebacterium rubrum*, *Dactylium denroides*, *Mucor alternans*, *Mucor ambigus*, *Mucor griseo-cyanus*, *Rhizopus arrhizus*, *Rhizopus oryzae*, *Rhizopus stolonifer*, *Tetrahymena pyriformis* and *Trichoderma viride* are capable of converting AFB_1 to aflatoxicol. Strain of *A.flavus* and *A.parasiticus* have shown promise in reducing the level of the resident fungal population and has demonstrated a significant reduction (80 – 90 %) in aflatoxin contamination in green house and field studies (Cotty et al., 1994). Shantha (1999) found degradation of aflatoxin B_1 by species of Phoma, Mucor, and Trichoderma, when they were co-cultured with aflatoxigenic *A.flavus*.

Because this approach depends on survival and successful occupation of an ecological niche by the biocontrol strain, identification of the environmental factors that favor certain isolates of *A.flavus* and *A.parasiticus* over others must be understood. One interesting feature of this approach, which must be considered for its successful implementation, is that strains of *A.flavus* seem to replace other strains of *A.flavus* more effectively than *A.parasiticus* and vice versa (Horth et al., 1994). Therefore, it is likely that combinations of strains of both species will be required. Recent

Table.6. Antimicrobial components of Spices

Spice / Herb	Main component (%)	Identified compounds (%)
Eletaria cardamom	1,8-ceneole (30.7)	27 (97.0)
Amomum subulatum	1,8-cineole (72.7)	12 (99.9)
Cinnamomum verum	Cinnamaldehyde (79.8)	24 (95.5)
Cuminum cyminum	Cuminaldehyde (37.4)	20 (95.7)
Coriandrum sativum	Linalool (78.1)	24 (99.8)
Myristica fragrans (Mace)	terpinen-4-ol (20)	33 (99.9)

Source: Shelef, 1983; Sethi and Meena, 1997

studies have suggested that naturally occurring nontoxigenic isolates of A.flavus may have the genetic capability to synthesize AFB_1 (Rarick et al., 1994). With the use of a molecular genetics approach, genetically stable non-toxigenic biocontrol strains of Aspergillus that are known to compete well can be generated by specific deletion of key genes in the biosynthesis pathway once these genes have been identified. Using this gene disruption technology, at least one genetically engineered fungal biocontrol strain (nvm8 disruption strain – Dis3) has been made available for field testing (Mahanti et al., 1994). The most active organism discovered is *Flavobacterium aurantiacum* NRRL B-184, which in aqueous solution can irreversibly metabolize and degrade aflatoxins B_1, G_1 and M_1 (Ellis et al., 1991).

2.8.4 Regulation

Regulation of mycotoxins is another method of control measures for mycotoxins especially aflatoxins have been initiated by several countries to protect public health and to promote trade at national and International levels. Most of these limits have been established on the basis of analytical sensitivities of the detection method and economic compulsion, while a few countries have based their limits on toxicological data, risk analysis and expert group advice. These limits range from zero to 50 μg / kg. In India under the Prevention of Food Adulteration Act (PFA), a tolerance limit of 30 μg / kg has been established for aflatoxin in all food meant for human consumption on the basis of the 1967 Protein Advisory Group recommendation of the United Nations. In Europe, 39 countries, have been covered by specific mycotoxin regulations in 2003. Compared to other regions of the world, Europe has the most extensive and detailed regulations for mycotoxins in food. In the EU, harmonized regulations exist for aflatoxins in various foodstuffs, aflatoxin M_1 in milk, ochratoxin A in cereals and dried vine fruits, patulin in apple juice and apple products, and for aflatoxin B_1 in various feedstuffs. Guideline limits have been established for deoxynivalenol in cereals and cereal products. The major Latin American agricultural crops are maize, wheat, coffee, cotton, soybeans, barley, sunflower, groundnuts and tree nuts, cocoa and dairy products, which are highly susceptible to fungal contamination and mycotoxin production (Pineiro, 2004). Nineteen countries, accounting for 91 percent of the population of the region, were known to have specific mycotoxin regulations. The United States and Canada have had mycotoxin regulations in place for many years, and have implemented advanced techniques for sampling and analysis. In both countries, limits for aflatoxins are set for the sum of the aflatoxins B_1, B_2, G_1 and G_2. Canada has established tolerance limit for Fusarium-damaged kernels in wheat, other grains and ergot in various crops. In the United States detailed tolerance levels exist for the sum of the fumonisins B_1, B_2 and B_3 in a wide variety of maize products. This is the only country known to have limits for the sum of these three fumonisins. Aflatoxin B_1 is the most important of the aflatoxins, considered from both the viewpoints of toxicology and occurrence. It is most

MATERIALS AND METHODS

3.0. MATERIALS

3.01. Chemicals

Chemicals such as acetone, acetic acid, benzene, chloroform, celite powder, ethanol, ethyl acetate, formic acid, methanol, tartaric acid, tween-20, silica gel, silica gel – G (for TLC), hexane, sodium sulphate (anhydrous), trifluoroacetic acid (TFA), buffer saturated phenol, Isopropanol, Gib's reagent, KCl, NaCl, Na_2HPO_4, EDTA, SDS, Ethidium bromide, Tris-HCl, Boric acid All the chemicals used were of AR / HPLC grade procured from local dealers in chemicals.

3.01.1 Fine Chemicals

The PCR primers were procured from Sigma, Genosys Ltd, UK. Taq polymerase, dNTPs, Agarose, λ DNA marker (100bp ladder), loading dye was purchased from Bangalore Genei, Bangalore, India. The standard mycotoxins for this study were obtained from Sigma, USA. Other chemicals used in these studies were of molecular biology grade and purchased from standard chemical companies.

3.02. Equipments

Laminar Hood, TLC plate spreader (CAMAG), Hot air oven, autoclave, weighing balance, water bath, freezer, flash evaporator, UV cabinet, pH meter, hair drier, HPLC with fluorescent detector (Hewlett Packard) and thermal cycler for PCR (Primus), spectrophotometer, rotary shaker, camera (SLR), colony counter, chromatographic column and TLC equipment, balance (metler), binocular research microscope, microbalance, vacuum oven, electrophoresis unit, gel documentation system and gamma radiation unit.

3.03. Glass / Plastic Wares

Conical flasks, test tubes, petri plates, pipettes, plastic trays, reagent bottles, TLC plates, TLC tank, desiccators, microscopic glass slides, coverslips, haemocytometer, double distillation unit, micropipettes, measuring cylinder, funnels and separating funnels were from standard companies. Plastic wares like microtips, microsyringe, micropipettes, PCR tubes and Eppendorf tubes were from Tarsons Co.

3.04. Miscellaneous

Aluminum foils, bunsen burner, buchner funnel, buckets, cotton (absorbent & non-absorbent), cork borer, chromatographic paper, pH paper, disposable glouse, mortar and pestle, polyethylene bags, rubber bands, LPG connections, forceps, Inoculation loops, markers, muslin cloth,

needles, spatula, stickers, spirit lamp, surf washing powder, test tubes stands, and tissue paper were routinely purchased from local and national dealers.

3.05. Samples

Chilli, ginger and turmeric (whole and ground form) were purchased from local markets/retailers in Mysore, Mandya and Bangalore cities of Karnataka, India. Spice oils (food grade) viz, ajowan, celery, cinnamon, clove, cumin, chilli oleoresin, ginger, large cardamom, nutmeg, pepper, small cardamom and turmeric were procured from Plantation Products and Spices Flavoring Technology (PPSFT) dept. of CFTRI, Mysore, and also from local market in Mysore, India.

3.06. Media

Czapek Dox agar (CDA), Aspergillus Differential Medium (ADM) (Bothast and Fennell, 1974) and Plate Count Agar (PCA) medium were procured from Hi Media Co. Mumbai, India. Potato Dextrose Agar (PDA), Yeast Extract Sucrose (YES) and Glucose Yeast Extract Peptone (GYEP) were prepared and utilized as and when required.

3.06.1 Czapek dox broth (modified)

Ingredients :	**-**	**g/L**
$NaNO_3$	-	3.00
K_2HPO_4	-	1.00
KCL	-	0.50
Fe SO_4, $7H_20$	-	0.01
Mg SO_4, $7H_20$	-	0.50
Casaminoacids	-	3.50
Sucrose	-	30.0
Thiamine	-	0.004
Ethanol	-	20.0 ml
pH	-	4.5 ± 0.2

3.06.2 Potato Dextrose Agar

Ingredients	**-**	**g/L**
Potato (Infusion from)	-	200.00
Dextrose	-	20.00
Agar	-	20.00

Chloremphenicol	-	100 ppm
pH	-	5.5 ± 0.2

3.06.3 Aspergillus Differential Medium

Ingredients	**-**	**g/L**
Peptic digest of animal tissue	-	10.00
Yeast extract	-	20.00
Ferric ammonium citrate	-	0.50
Dichloran	-	0.002
Agar	-	15.00
pH	-	6.3 ± 0.2

3.06.4 Yeast Extract Sucrose (YES)

Ingredients	**-**	**g/L**
Yeast extract	-	0.20
Sucrose	-	0.40
pH	-	6.3 ± 0.2

3.06.5 Glucose Yeast Extract Peptone (GYEP)

Ingredients	**-**	**g/L**
Glucose	-	50.00
Peptone	-	1.00
Yeast extract	-	1.00
pH	-	6.3 ± 0.2

3.06.6 Plate Count Agar (PCA)

Ingredients	**-**	**g/L**
Dextrose	-	1.00
Tryptone	-	5.00
Yeast extract	-	2.50
Agar	-	15.00

	pH	-	7 ± 0.2

3.07. Buffers

3.07.1	**TRIS – SDS Buffers**	**-**	**100mL**
	Tris	-	50 mM
	EDTA	-	50 mM
	SDS	-	3 %
	pH	-	8.0

3.07.2	**TRIS – EDTA Buffers (stock 50 X)**	**-**	**100mL**
	Tris	-	24.2
	EDTA (0.5 M)	-	10 mL
	Glacial acetic acid	-	5.71 mL
	pH	-	8.0

3.08.	**Gel loading dye (6x)**	**-**	**g/100 mL**
	Bromophenol blue	-	0.25
	Xylene cyanol FF	-	0.25
	Glycerol	-	30.0

3.09. DNA staining solution

Ethidium bromide	-	0.5 %

3.10. Mycotoxin Standards

Aflatoxin standards B_1, B_2, G_1, G_2, Ochratoxin A, T-2 toxin and Diacetoxiscerpinol (DAS) were procured from Sigma chemicals Co. U.S.A. and stored in dark at 4^o C. Standard stock solutions were prepared in recommended solvents and stored at 4^o C. Suitable working solutions were prepared by diluting this stock, as and when required, with respective solvents as recommended.

3.11. Culture

Stock cultures of *Aspergillus flavus* (ATCC 46283), *A.parasiticus* (CFR 223), *A.ochraceus* (CFR 221) and *Fusarium sporotrichoides* (MTCC 1894) were revived by sub culturing on PDA slants

and incubating these slants at RT for 5 – 7 days. The stock cultures were maintained as slant cultures on Czapek Dox agar slants.

3.12. PCR Primers

Forward and reverse primers for aflatoxin regulatory gene (afl-R) was designed and ordered for synthesis from Genei research laboratory, and were used in PCR experiments.

3.20. METHODS

3.21. Collection of Samples

The samples were collected at retail outlets of market at different localities in and around Mysore and also from three districts viz, Mandy, Bangalore and Mysore. Spice samples viz, chilli, ginger and turmeric were purchased. Random sampling procedure was followed while collecting and minimum of 500 g of sample were drawn each time and sealed in polyethylene bags. The samples were stored in laboratory condition till further use. Required amount of sub-samples were utilized for investigations.

3.22. Estimation of fungal flora

Samples were powdered in sterile conditions and thoroughly mixed. The powdered sample in ten gram quantity was added to 90ml sterile water having 0.5 % sodium chloride in flask to make sample – saline water mixture. From this mixture, serial dilution of sample was prepared by drawing 1 ml of sample adding to 9 ml saline diluent and mixing with the help of vortex mixer. Subsequently, dilution at 10^{-2}, 10^{-4} and 10^{-6} level were prepared and appropriate dilutions were spread uniformly on previously poured plates of PDA and ADM by spread plate technique. The inoculated plates were incubated for 7 days at 28^{O} C ± 2^{O} C. The colonies appearing on these plates were periodically counted (3^{rd} – 7^{th} day) and also isolated for identification purposes. Each sample was analyzed in triplicate and the average of the replicates was recorded. The results were expressed as total colony forming units per gram of the sample (cfu / g).

3.22.1 Qualitative estimation of fungi

Fungal colonies growing on the PDA plates were aseptically picked up between 3^{rd} and 7^{th} day of growth and transferred to PDA slants for identification purposes. The percentage frequency of each fungal species was calculated. The *Aspegillus* species was identified by the following the procedure as per the manual of Genus *Aspergillus* (Raper & Fennel 1965).

3.23. Detection of Mycotoxins

Spices procured from retail shops were analyzed for mycotoxins especially aflatoxins using standard procedures of detection. The standard *Aspergillus* strains and the fungal isolates from various spice commodities were screened for their toxigenic potency. Aflatoxigenic *Aspergilli* was individually inoculated into sterilized modified Czapek Dox medium (Basappa *et al*, 1967). Aflatoxin in the broth medium was extracted and estimated by Pon's modified method (Rati *et al.* 1987).

3.23.1. Preparation of TLC plates

The Camag TLC automatic spreader was used to coat TLC plates with silica gel. 50g of Silica gel 'G' was mixed with 100ml of distilled water to form uniform slurry and poured into the spreader which was suitably adjusted to coat a film of 0.3 mm thickness. The TLC spreader was operated to spread uniform silica gel slurry on 20cm x 20cm glass plates and the plates were air dried at room temperature. The silica gel plates were activated in hot air oven at 110^{O}C for 1 hour. The activated plates were stored in a dust and moisture free chamber.

3.23.2. Preparation of standards

Aflatoxin B_1 (1mg) obtained from sigma chemicals was dissolved in benzene + acetonitrile (98: 2 v/v) and made up the volume to 100 ml in volumetric flask. It gave a concentration of 10 µg /ml. It was further diluted 10 times with benzene + acetonitrile solvent system. This would give a working standard solution with concentration of 1µg/ml. Similarly standards of G_1, B_2 & G_2 obtained from sigma chemicals were also prepared. Suitable aliquots of sample extract in chloroform were spotted on TLC with the help of micropipettes. The working standard aflatoxin solutions, B_1, G_1, B_2 & G_2 was spotted at various concentrations along with the sample on the plates. The spotted plates were developed first in ether: hexane (3:1) subsequently in chloroform + acetone (9:1) solvent systems in a chromatographic chamber. The developed plates were viewed under long wave U.V (360 nm) and B_1, G_1, B_2 & G_2 were visualized. Visual observations of comparison of sample spots and standard spots for type of fluorescence and their Rf was carried out.

3.23.3. Sample processing

Samples for the extraction of aflatoxins were ground to fine powder and weighed into 500 ml Erlenmeyer flask. Extraction solvent 85 % acetone was added in the ratio of 1: 5 (sample: solvent) and kept for extraction on a rotary shaker for 30 minutes (Rati et al., 1987). The contents of the flask were filtered through a Whatman no.1 filter paper and to 50 mL filtrate, 10 mL of 20 % lead acetate and 5 g of celite was added. The mixture was held for 5 minutes and was filtered over a bed of celite using

buchner funnel. The clear filtrate was transferred into a separating funnel and 100 mL distilled water was added. Toxin was extracted using chloroform (25 mL) by shaking the contents in separating funnel. The lower chloroform phase was passed through a bed of anhydrous sodium suphate into a dry flask. The extraction was repeated with another 25 mL chloroform. Chips of pumice was added into the flask and the extract was evaporated completely over water bath. The aflatoxin sample extract was resolved using thin layer chromatogram (TLC) (Rati et al, 1987).

3.23.4. Calculation:

The aflatoxin concentration in sample was calculated by using the following formula:

$$AFB_1\ (ppb) = \frac{\text{Dilution of the Sample (mL)} \times \text{Standard aflatoxin minimum fluorescence (ng)}}{\text{Observed minimum fluorescence of sample (µL)} \times \text{Weight of the sample (g)}} \times 100$$

3.23.5. Chemical confirmation test

Compounds exhibiting fluorescence and chromatographic properties similar to aflatoxin B_1 are frequently encountered in nature. Therefore confirmatory test becomes necessary to determine the authenticity of the aflatoxin. The test is based on the ability of the olefinic linkage of an enol ether bond of aflatoxin moety to react additively with a hydroxyl group under the catalytic influence of a strong acid.

Activated silica gel coated TLC plate was marked into two halves, and half of the plate was covered with a clean glass plate. Other half of the plate was spotted with two 10 µl equivalent of the sample extract containing approximately 0.5 – 5 µg aflatoxins. Approximately equal amount of aflatoxin B_1 standard was superimposed on one of the extract spots. Standard B_1 standard was spotted as a separate single spot. All the spots were reacted with Trifluoroacetic acid (1 µl) for 5 minutes. The acid was dried by blowing hot air at temperature 40^{O} C from lower surface of the plate for 15 minutes. The plate was uncovered and similar spots of sample, sample + aflatoxin B_1 superimposed and B_1 standard was spotted on this portion of the plate. The plate was developed in chloroform **:** acetone (85:15) solvent system in a unlined TLC tank. Beakers with water was placed in the developing chamber to provide moist atmosphere. Plates were examined under long wave UV light and observation were recorded. On reaction with strong acid, aflatoxin B_1 form highly fluorescent

hydroxylated compound having lower Rf value. If the samples show the strongly fluorescent spot with low Rf, then presence of aflatoxin B_1 in sample is confirmed. Aflatoxins G_1 and M_1 also form highly fluorescent compounds having typical characteristic fluorescence with low Rf value on reaction with TFA (Pyrzybylsky, 1975).

3.23.6. High-Performance Liquid Chromatography (HPLC)

The technique involves the separation of sample constituents, followed by their detection and quantification. Separation is achieved by a competitive distribution of the sample between a mobile liquid phase and a stationary liquid or solid phase that is supported in a column. The HPLC resolves the aflatoxins into individual toxin peaks having characteristics retention times. The sensitivity of the aflatoxins B_1 and G_1 is increased by derivatization of the aflatoxins into aflatoxin B_2a & G_2a. Toxin may be derivatized by the two methods viz, pre-column or post-column derivatization. The aflatoxins in sample is derivatized using strong acid (TFA) before injecting it into HPLC, it is said to be pre-column derivatization. The aflatoxin in sample after separation on the HPLC can be derivatized using Kobra cell before detection (post column derivatization).

3.23.7. Column Purification

Preparation of sample for HPLC involves column purification. Column chromatography grade silica gel (60 – 120, 0.063 – 0.2 mm) was activated by drying at 100^{O}C for 4 hours. After cooling, water (1 %) was added to silica gel, mixed thoroughly and stabilized at room condition for 18 hour. Silica gel column was prepared by adding silica gel slurry prepared in ether: hexane (3+1) to glass column. After settling of silica gel in the column, 1 g of anhydrous granular sodium sulphate was added to the column. The ether + hexane solvent was allowed to drain to top of sodium sulphate bed. Sample in chloroform (<1ml) was loaded on the column. Sample was washed with 25 mL benzene + acetic acid (9+1) and 30 mL of ether+ hexane (3+1). Aflatoxins were eluted with chloroform + acetone (90+10) and were collected in 250 mL beaker and the solvent was completely evaporated.

3.23.8. Pre Column Derivatization

The aflatoxin in sample is derivatized by Trifluroaceticacid (TFA) and injected (20 µL) to HPLC column (C – 18 reverse phase column). The fluorescent detector detects the toxin according to their retention times, which is recorded on the chart as peaks. Calculation of aflatoxin concentration is by comparing the standard toxin peak areas with that of sample (AOAC, 1995).

3.23.9. Post Column Derivatization

Toxin (20 µL) was injected to HPLC column (column C – 18 ODS supelco) where resolution of sample takes place in reverse manner (G_2, G_1, B_2, B_1). The HPLC column purified and resolved sample passes through the Kobra X cell (Bromine) where derivatization of aflatoxin takes place. The fluorescent UV detector detects the derivatized toxin and the resolution peaks are recorded on the HPLC chart according to retention time of each toxin (AOAC, 1995).

3.23.10. Calculation:

Concentration of toxin in sample is calculated by the following formula:

$$\text{Concentration of toxin in sample (µg)} = \frac{\text{Sample peak area}}{\text{Standard peak area}} \times \text{Standard concentration (µg)} \times \frac{1}{\text{Injected volume of sample (µL)}} \times D$$

3.24. Substrate specificity of spices

3.24.1 Elaboration of aflatoxins on spices

Spore suspension of toxigenic *Aspergillus flavus* was prepared at 10^6/ml and inoculated to sterilized spices such as chilli (whole, powdered and parts such as seeds, stalk and pericarp), ginger and turmeric (whole and powdered form) and incubated at temperature of 30 ± 3^{O}C for 7 days after raising the moisture content of substrate to 40 %. Substrate specificity of spices with toxigenic *A.parasiticus* was also studied under similar conditions and observation on their suitability for production of aflatoxins was recorded.

3.24.2 Elaboration of ochratoxin A on spices

Substrate specificity of spices for production of ochratoxin A was also studied with toxigenic *A.ochraceus* under similar conditions as in section 3.24.1.

3.24.3 Estimation of mycotoxin production on spices

After incubation the samples were subjected to extraction and the toxin was quantified according to standard procedures as in section 3.23.

3.25 Determination of the water adsorption isotherm of spices

3.25.1 Experimental protocol

The water adsorption isotherms were determined gravimetrically by exposing food samples to atmospheres of known equilibrium relative humidities ranging from 65 to 92 %. Ten grams of sub-

samples of chilli, ginger and turmeric each were placed in glass petri dishes and conditioned to a specific level of water activity in desiccators containing saturated aqueous solution of various salts. The salts used included sodium nitrite ($NaNO_2$, sodium chloride (NaCl), potassium chromate ($K_2 CrO_4$) and potassium nitrate (KNO_3) to obtain 65, 75, 86 and 92 % RH respectively, according to Stokes and Robinson (1949). The volume of the solution used was large enough so that moisture lost or gained by the spice being conditioned did not alter the composition of the controlling solution. The desiccators were kept in constant temperature incubators at 25 ± 0.5^{O} C. The samples from different RH levels were withdrawn during incubation process in various batches at different time intervals. The samples from 92 % RH were withdrawn in different batches at intervals of 7, 14, 21, 28 and 42 days. Samples from 86 % RH were withdrawn at 14, 28 and 42 days. Samples from 75 % and 65 % RH were withdrawn at 28 and 42 days respectively. All the samples were subjected for the estimation of moisture, mycoflora and mycotoxins. While estimating mycoflora, dilution plate technique using both PDA and ADM media was adopted (refer section 3.22). The fungal flora was recorded. Mycotoxins were estimated by adopting modified Pon's method (Rati et al. 1987) as described in section 3.23. The samples maintained under room conditions served as control. The specific gravities of the solutions were measured at the beginning and at the end of each conditioning experiment.

3.25.2 Determination of the initial moisture content

The moisture contents (m.c.) of the samples of each spice such as chilli, ginger and turmeric were determined by drying a weighed sample of spice in a vacuum oven set with temperature at 70^{O}C and pressure at 400 mbar for 6h. The samples were weighed until constant weight was achieved. The percentage moisture content was derived on a dry weight basis. For each sample three moisture determinations were included and moisture % was calculated.

3.25.3 Estimation of fungal load on samples

The fungal load was estimated as in section 3.22.

3.25.4 Fungal development on spices stored at various levels of water activity

The time taken for fungal development at various levels of a_w was determined by storing 25 g sub-samples in desiccators having relative humidities ranging from 65 to 92 % as described above. The samples were examined periodically under a binocular microscope (x40 magnification) for the appearance of fungi. The number of days before visible fungal growth was noted for each sample stored at various water activities. The experiment was repeated three times.

3.26 PCR Technique

3.26.1 Designing of Primers

Primers were designed specifically for *O- methyl transferase* (omt) gene which encodes the enzyme involved in the conversion of sterigmatocystin to *O*-methylsterigmatocystin of aflatoxin biosynthetic pathway (Yu et al, 1993) using Primer 3 software. The primers as mentioned above were designed based on published sequence strand for *A. flavus* and *A. parasiticus* from the NCBI databank and has been patented (Manonmani et al. 2004). With the assumption that aflatoxin-producing genes are unique for aflatoxigenic fungi, in our studies, the PCR reaction was targeted against *O - methyl transferase (omt)* gene.

3.26.2 Screening of food samples for aflatoxigenic fungi

Conventional dilution plating technique was employed to assess the total yeast and mold flora of the raw ingredients (Speck, 1984) as section 3.22. Toxigenic strains of *Aspergillus spp.* used in the present study were isolated from spices like ginger, turmeric, large cardamom, chillies and curry spice mix by specific selective isolation on Aspergillus Differential Medium (ADM). Diluted samples were spread plated on ADM plates and observed for specific orange coloration of medium after 48h (Pitt, 1983). These isolates were isolated onto PDA slants and maintained at 4^0C.

3.26.3 Enrichment of food samples

Fungal DNA was isolated from food samples by enrichment technique. The food samples were appropriately diluted in sterile saline and known amounts were inoculated into Potato Dextrose Broth tubes **(**PDB), which were incubated at 30°C for 24h.The template DNA was extracted from fungal mycelia (individual fungal isolates or their mixtures or from enrichment of food samples) as follows: fungal mycelia grown in PDB under stationery conditions was harvested by filtration. The mycelium was washed twice with phosphate buffered saline (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4) followed by centrifugation. The mycelium was transferred to a mortar and ground well. Freshly prepared, sterile Lysis buffer (50mM Tris, 150 mM EDTA, 1% (w/v) SDS, pH 8.0) was added to the pulverized mycelia and incubated at 65ºC for 1h. The suspension was centrifuged and supernatant was then extracted twice with phenol: chloroform: isoamylalcohol (25:24:1) and the aqueous layer was precipitated with two volumes of isopropanol. The precipitate was resuspended in 200µl of TE buffer (10mM TRIS-HCl, 1.0mM EDTA, pH 8.0) (Lee *et al.,* 1998).

3.26.4 Polymerase Chain Reaction

Polymerase Chain Reaction was used to amplify the o-methyl transferase regulatory gene fragments of aflatoxigenic fungal genomic DNA. Primers of aflatoxin biosynthetic pathway, the O-methyl transferase gene (*omt)* with sense and antisense strands were used in this study. PCR was performed in 25µl reaction mixture that contained 100ng of genomic DNA, dNTPs at 0.025 nM each, primers at 4nM each and reaction buffer (10mM TRIS – HCl, pH 8.0, 50mM KCl, 1.5mM $MgCl_2$, 0.1% Triton X-100 and 0.2 mg gelatin per mL). Each reaction mixture was heated to 94°C for 10 min before adding 0.3 units of Taq DNA polymerase. A total of 30 PCR cycles, each cycle start with denaturation 0.3 min at 94°C for , 0.45min at 50°C for annealing, 1.15 min at 72°C for extension and a 10 min final extension at 72°C was run on a programmable Thermal Cycler, model Perkin Elmer Cetus, Norwalk, Conn.

3.26.5 Gel electrophoresis

The PCR amplicons were visualized by gel electrophoresis. Agarose gel (1.2 %) was prepared in Tris-EDTA buffer (pH 8.0) and the gel was set with comb in place. The PCR samples (8 µL) was mixed with loading buffer (2 µl) and was loaded into each wells of the gel and DNA markers were also included. Electrophoresis was carried out at 90-110 volts for about 2h. Staining of the gel was done in ethidium bromide for 10-20 min. The gel was washed and viewed under UV illuminator and results were recorded by scanning the gel using Gel DOC system with HEROLAB computer program software.

3.27 Screening of spice oils for antifungal activity

3.27.1 Spice oils

Spice essential oils viz, ajowan, celery, cinnamon, clove, cumin, chilli oleoresin, ginger, large cardamom, nutmeg, pepper, small cardamom and turmeric were used in this study. Known concentration of spice oils at 0.001, 0.002, 0.004, 0.006, 0.008, 0.01 and 0.02 % was added to the culture flask containing pre-sterilized media. These oils were emulsified with 0.002 % of tween 20 before adding to the media. Appropriate controls were maintained.

3.27.2 Indicator organism

Aspergillus flavus (ATCC 46283)*, A.parasiticus* (CFR 223), *A.ochraceus* (CFR 221) and *Fusarium sporotrichoides* (MTCC 1894) were used in this study. Five day old culture was used for inoculation of the media. Ten mL of sterile water containing 0.001 ml tween 80 was added to five day old slant and transferred to a 100ml conical flask containing 15ml sterile water. The count of the

spores in the inoculum was determined using haemocytometer. Inoculum at 1 % level (100 cfu/ml) was added into each flask containing the spice oil (section 3.27.1). The inoculated flasks were incubated for 7 – 10 d at 28^0 C while flask with *Fusarium* inoculum was incubated for 25 days.

3.27.3 Toxin production by indicator organisms

The Yeast Extract (2%) Sucrose (4%) (YES) medium for the study on growth and toxin production by *A.flavus, A.parasiticus*, and *A.ochraceus* and Glucose Yeast Extract Peptone (GYEP) medium for the study of growth and mycotoxins production by *Fusarium sporotrichoides* were used. The pH of the media were adjusted to 6.5 before sterilization by autoclaving at 121^OC under 15 psi for 30 min. The inoculum suspension was diluted to obtain approximately 10^6 conidia / mL and inoculated into respective sterile culture media. The flasks thus inoculated individually with *A.flavus , A.parasiticus*, and *A.ochraceus* were incubated at 28 ± 2^OC for 7-10 days and flasks inoculated with *Fusarium sporotrichoidies* were incubated at 28 ± 2^OC for 25 days.

3.27.4 Estimation of fungal growth

The mycelial mat from the culture flasks were removed after inactivating spores by methyl alcohol (10ml/ 50ml media). Dry weight of mycelia was determined by collecting the mat on pre weighed what man no.1 filter paper and washing the mat with distilled water. The mat with filter paper was dried at 80^O C for 16 h to constant weight. The dry weight of the mat was determined.

3.27.5 Estimation of mycotoxins

Culture filtrate after separating the mycelial mat was extracted twice with chloroform (Scott et al, 1970) and the combined extract was dried through anhydrous sodium sulphate and the solvent evaporated to dryness on water bath. Aflatoxins and ochratoxin A were separated on TLC and quantified by dilution to extinction method (AOAC, 2000). The residue was dissolved in known quantities of chloroform applied on TLC along with the standard aflatoxins and ochratoxin A. The plate was developed in solvent system containing benzene – acetic acid – methanol (90:5:5) in a TLC chamber. Aflatoxins, B_1, B_2, G_1, G_2 and Ochratoxin A were visualized by viewing under UV light (365 nm) (Scott et al. 1971). Aflatoxins, B_1 and G_1 were confirmed by treating it with (TFA). T-2 toxin and Diacetoxiscerpinol (DAS) were detected by HPLC using fluorescence detector at excitation 292 nm and emission 425 nm. HPLC column (C-18 supelco) was used with acetonitrile: water: acetic acid (65: 35: 0.7 %) as mobile phase. The toxins were quantified by calculating resolution peaks areas obtained on the graph in comparison with standard toxin peaks.

3.28. Screening of spice oils for antibacterial activity

3.28.1 Indicator organism

Food pathogenic bacteria viz, *Bacillus cereus, Escherichia coli, Listeria onocytogenes, Staphylococcus aureus*, and *Yersinia enterocolitica* were included in this study. Freshly growing cells of the pathogens were used as inoculum.

3.28.2 Spice oils

Spice oils tested against mycotoxigenic fungi were used in this study to assess their antibacterial activity. Spice oils viz, ajowan, celery, cinnamon, clove, cumin, chilli (oleoresin), ginger, large cardamom, nutmeg, pepper, small cardamom and turmeric were tested.

3.28.3 Well assay

Pathogenic bacteria inoculum (0.1 mL/plate) were spread plated onto PCA media. Wells of 7 dia were bored on inoculated media. Spice oil at concentrations of 1, 2, 5, 10, 20 and 40 µL was introduced into wells and labeled. Each and every step was carried out under aseptic conditions and plates were incubated for 24 h at 37^{O} C. Replicates of each concentration were maintained and wells without spice oil served as the control. The inhibition of bacterial growth around the wells showing inhibitory zone was measured.

3.28.4 Disc assay

The plates inoculated with indicator organisms were taken. Spice oil at various concentration was added to filter discs. These discs were placed on the inoculated plates and incubated at 37^{O} C for 24 h. The inhibitory zones were measured around each disc.

3.29. Determination of effect of irradiation of spices on microbial flora

3.29.1 Preparation of samples

Samples each of chilli, ginger and turmeric were inoculated with known quantity of spores (10^6 spores / mL) from 7 day old cultures of fungi. The test cultures belonging to three genera. viz, species of Aspergillus, Fusarium and Penicillium were inoculated and the samples were sealed in polyethylene pouches under sterile condition. The samples which were not inoculated with test organism served as control.

3.29.2 Exposure to Gamma irradiation

The experimental samples inoculated with fungi and the control was exposed to gamma irradiation by ^{60}Cobalt at different levels of 2.5, 5.0, 7.5 and 10.0 KGy for 1h. A set of experimental and control samples without exposure to irradiation was also maintained.

3.29.3 Estimation of fungal flora

The fungal flora of all the samples (non irradiated, irradiated) were estimated by serial dilution technique. Specific dilutions were spread plated on PDA and ADM media and incubated. Observation on fungal flora was recorded on 5^{th}-7^{th} day. The percent survival of the fungi at various levels of treatment was calculated.

3.30 Bacteria

3.30.1 Preparation of samples

Samples of chilli, ginger and turmeric (30 gram each) were sealed in polyethylene pouches under sterile condition.

3.30.2 Exposure to Gamma irradiation

Sample sealed in pouches were exposed to gamma irradiation by ^{60}Cobalt at different radiation levels of 2.5, 5.0, 7.5 and 10.0 KGy for 1h. Samples without exposure to irradiation served as control.

3.30.3 Estimation of total count

Samples after exposure to radiation were analyzed for bacterial flora by serial dilution technique and respective dilutions were spread plated on PCA media. Plates were incubated at 37^{O} C for 24-48 h. The total count of bacteria was recorded and percent survival of bacteria in irradiated and non irradiated samples were calculated.

NATURAL OCCURRENCE OF MYCO TOXIGENIC FUNGI IN MAJOR SPICES

Contents

4.1 Introduction

4.1.1 Chilli

Chillies (*Capsicum annum*) native of Central America and the West Indies are grown in each and every tropical country of the world and has become the most important spice in a number of countries. It was introduced to India from the tropical South America in the 17th Century. It is grown in all parts of our country. Karnataka, Andhra Pradesh, Maharasthra and Tamilnadu shares major part, as for as, production of chilli in the country is concerned. Both green and ripe fruits of pungent type are used as spice mainly for preparation of sauces, curry powder and pickles. The chilli fruits are rich source of vitamin A, C and E. They are used in medicines as stimulant and carminative and to prevent fever.

Chilli crop is susceptible to many number of diseases. The fruit rot of chilli is caused by *Colletotrichum capsici*, leaf spot disease by *Cercospora capsici*, Periconia rot by *Perconia byssoides* (Kanaujia and Verma, 1979), damping off seedlings by *Rhizoctonia solani* (Surekha et al., 1986), Seed borne fungi of this crop has been reported (Anisha Athar et al., 1988; Prabhavathy, 1993; Bilgrami and Verma, 1993; Giridhar,1996). In the present studies mycoflora of chilli (dry fruit) collected from Bangalore, Mandya and Mysore cities of Karnataka, India, was investigated.

4.1.2 Ginger

Ginger (*Zinziber officinale*) an important rhizomatous member of the family Zingiberaceae has originated in tropical south-east Asia. China and India were the first country to grow it. The main ginger growing countries are India, China, Japan, Indonesia, Malaysia, Australia, Jamaica and other islands of the West Indies. Ginger is used as a condiment. It dilates the blood vessels, and thus cause a feeling of warmth. It increases the rate of perspiration and thus helps in lowering the body temperature. It is widely used in preparing soups, pudding, pickles, cookies and curries. It is widely used in preparing beverages and medicines. It is also used in the digestive disorders. The distilled ginger oil is used in perfumery. In china, the ginger is prepared by drying, cleaning, boiling, peeling, scraping and then boiling again in a sugar solution before they are packed. It is prepared by cleaning, pealing and drying in the sun in other countries.

Ginger is susceptible to yellow disease caused by *Fusarium. oxysporum* f. sp. Zinziberi (Sharma and Jain, 1979), Bacterial wilt by *Pseudomonas solanacearum* (Ojha et al., 1986) root knot nematode disease by *Meladogyne arenaria* (Kuruchave et el., 1991 and Koshy, 1991). But no

information on the fungi associated with ginger during storage is available. Hence an attempt to record the mycoflora associated with this spice was made.

4.1.3 Turmeric

Turmeric (*Curcuma longa*) is another important rhizomatous member of the family Zingiberaceae is believed to be originated in Southern Asia. India, China, Sri Lanka, Indonesia and West Indies are the main producers of this spice. In India, Tamilnadu, Andhra Pradesh, West Bengal and Orissa states are the main producers of this crop. The rhizome produces a yellow, orange dye, which is still used for dying purposes in India and China. It is used as flavoring agent. It is very sacred to the Hindus who use it as an offering on religious and ceremonial occasions. This crop is susceptible to number of diseases. Storage rot caused by *Macrophomina phaseolina* and *Cladosporium cladosporioides* are the main diseases of turmeric responsible for huge loss of produce during storage (Sharma and Roy, 1986).

Spices do get contaminated with fungi like other agricultural commodities. The extent of contamination and the nature of mycoflora may vary with geographical conditions, post harvest practices such as drying and storage methods and also the trade practices. During the past few decades, various fungi including mycotoxigenic fungi have been recorded in major as well as minor spices (Christensen et al, 1967; Flanningan and Hui, 1976; Seenappa, 1979). The mycotoxigenic fungi such as *Aspergillus flavus*, *A. ochraceus*, *A.versicolor*, *Penicillium* sps. and *Fusarium* sps. were commonly encountered in spices.

In the present study mycoflora of chilli (whole dry fruit and powder) ginger and turmeric was investigated and all the important mycotoxigenci fungi were recorded. Natural occurrence of mycotoxins on the various substrates and the mycotoxigenic potency of various fungal isolates were also investigated.

4.2 Natural occurrence of mycotoxins and mycotoxigenic fungi in spices

4.2.1 Survey and screening of samples for mycoflora

Samples of spices such as chilli, ginger and turmeric (whole and powder form) procured from various retail outlets representing three cities of Karnataka viz, Bangalore, Mandya and Mysore were analyzed for mycoflora by employing serial dilution technique and the fungal flora as cfu/g was recorded (as in section 3.22). The results of samples selected randomly are presented in the tables 7 to 12 in terms of $\log_{10}$ cfu/g. A total of 65 number of samples of chilli, 55 number each of ginger and turmeric were screened for the presence of total mycoflora and mycotoxins. The total fungal flora on

Table.7. Mycoflora in chilli (whole)*

Sample no	1		2	
	Other fungi	*Aspergillus flavus* / *A.parasiticus*	Other fungi	*Aspergillus flavus* / *A.parasiticus*
1	5.70	5.60	3.60	3.00
5	6.50	5.30	4.40	3.30
10	4.30	3.50	4.10	3.30
15	2.60	2.60	2.80	3.00
20	4.00	3.20	3.90	2.80
25	2.80	2.00	2.30	2.00
30	4.15	2.60	3.20	2.60
35	4.30	4.00	4.60	2.30
40	2.80	2.30	3.00	3.00
45	6.00	4.60	4.00	4.00

Note: * = $\log_{10}$ cfu/g, 1 = PDA & 2 = ADM.

Table.8. Mycoflora in chilli (powder) *

Sample no	1		2	
	Other fungi	*Aspergillus flavus* / *A.parasiticus*	Other fungi	*Aspergillus flavus* / *A.parasiticus*
1	4.10	3.10	3.00	3.00
5	5.30	5.00	4.10	3.20
10	3.30	3.40	3.10	3.00
15	1.80	1.60	2.60	2.30
20	3.20	3.10	2.60	1.20
25	2.00	2.10	2.20	1.10
30	2.10	2.30	3.10	1.80
35	2.20	3.20	3.30	1.60
40	2.60	2.10	2.00	1.10
45	2.50	2.30	2.60	1.80

Note: * = $\log_{10}$ cfu/g, 1 = PDA & 2 = ADM.

Table.9. Mycoflora in ginger (whole) *

Sample no	1		2	
	Other fungi	*Aspergillus flavus* / *A.parasiticus*	Other fungi	*Aspergillus flavus* / *A.parasiticus*
1	1.50	2.00	2.00	1.80
5	1.30	1.60	1.00	2.00
10	1.20	2.00	3.10	3.00
15	1.00	1.00	2.20	2.00
20	1.80	2.50	1.80	1.20
25	1.20	1.00	2.30	2.00
30	1.00	1.20	1.60	1.20
35	2.30	2.60	1.80	1.00
40	3.30	2.00	2.30	2.10
45	2.60	2.30	2.10	1.00

Note: * = $\log_{10}$ cfu/g, 1 = PDA & 2 = ADM.

Table.10. Mycoflora in ginger (powder) *

Sample no	1		2	
	Other fungi	*Aspergillus flavus* / *A.parasiticus*	Other fungi	*Aspergillus flavus* / *A.parasiticus*
1	2.70	2.50	3.00	2.00
5	2.30	2.00	2.60	2.60
10	2.00	<1.0	2.00	2.00
15	3.10	2.00	2.00	2.30
20	2.00	3.50	2.00	2.00
25	2.50	2.50	2.00	2.90
30	3.50	3.00	2.80	2.30
35	3.10	2.85	2.00	2.00
40	2.00	2.00	<1.0	<1.0
45	2.00	2.00	<1.0	<1.0

Note: * = $\log_{10}$ cfu/g, 1 = PDA & 2 = ADM.

Table.11. Mycoflora in turmeric (whole) *

Sample no	1		2	
	Other fungi	*Aspergillus flavus* / *A.parasiticus*	Other fungi	*Aspergillus flavus* / *A.parasiticus*
1	1.10	1.00	1.80	1.00
5	1.10	1.20	1.00	1.80
10	1.00	1.20	1.20	<1.0
15	1.20	1.00	1.00	1.00
20	1.60	1.00	1.00	1.00
25	1.00	1.00	1.00	1.00
30	1.00	1.00	1.20	1.00
35	2.60	<1.0	1.60	1.00
40	2.10	<1.0	1.00	<1.0
45	1.00	1.00	1.00	<1.0

Note: * = $\log_{10}$ cfu/g, 1 = PDA & 2 = ADM.

Table.12. Mycoflora in turmeric (powder) *

Sample no	1		2	
	Other fungi	*Aspergillus flavus* / *A.parasiticus*	Other fungi	*Aspergillus flavus* / *A.parasiticus*
1	2.80	2.30	2.30	2.00
5	2.30	2.0	2.30	2.0
10	2.00	<1.0	2.0	<1.0
15	3.50	<1.0	2.0	2.0
20	2.60	2.30	2.70	2.70
25	<1.0	< 0.0	3.50	2.30
30	2.90	2.50	2.00	2.00
35	2.00	<1.0	2.00	2.00
40	2.00	<1.0	2.60	2.60
45	2.00	<1.0	2.00	2.00

Note: * = $\log_{10}$ cfu/g, 1 = PDA & 2 = ADM.

PDA medium and the differential fungal flora particularly of *Aspergillus flavus* group on ADM medium is presented. The fungi isolated from these spices were screened for their potential towards production of mycotoxins (Tables 14 - 17). If any and the results on screening was given in the forgoing chapter. In PDA medium, the range of *Aspergillus* spp. and other fungi were at 2.0 to 5.60 and 2.60 to 6.50 $\log_{10}$ cfu/g respectively on chilli sample. In ADM medium it was 2.0 to 4.0 and 2.30 to 4.60 $\log_{10}$ cfu/g respectively (Table – 7). Similar trend was noticed on chilli powder which showed *Aspergillus* spp. and other fungi. In comparison to whole samples, powder yielded less fungal flora. In PDA media the flora of *Aspergillus* spp. and other fungi were 1.60 to 5.00 and 2.0 to 5.30 $\log_{10}$ cfu/g respectively (Table – 8). However, in comparision with PDA medium, the total fungal flora was lesser in ADM. Ginger samples were found to be the less supporter of Mycoflora when compared to chilli (table 9 to 10). Maximum of 2.60 and 3.00 $\log_{10}$ cfu/g of *Aspergillus* spp. was recorded from PDA and ADM media respectively in whole ginger, while in powder it was 3.50 and 2.90 $\log_{10}$ cfu/g. Other fungi at 3.30 and 3.10 $\log_{10}$ cfu/g was recorded from PDA and ADM medium respectively in whole ginger. In case of powder, maximum of 3.50 and 2.90 $\log_{10}$ cfu/g of *Aspergillus* spp. was recorded on PDA and ADM media respectively. The other fungal flora recorded were maximum at 3.50 and 2.80 $\log_{10}$ cfu/g on PDA and ADM media respectively. Turmeric was found to be the poor supporter of mycoflora when compared to chilli and ginger. The maximum of *Aspergillus* spp. recorded were at 1.20 and 1.80 $\log_{10}$ cfu/g from whole samples from PDA and ADM respectively. In the same media, the other fungi recorded were at 2.60 and 1.80 $\log_{10}$ cfu/g respectively. Compared to whole sample, powders were found to be the better supporter of mycoflora. Maximum of 2.50 and 2.60 $\log_{10}$ cfu/g were recorded from PDA and ADM medium respectively. The other fungi were recorded at 3.50 $\log_{10}$ cfu/g each on both the media.

Among the three spices surveyed for the presence of aflatoxin B_1, the chilli spice is found to have more contamination than ginger and turmeric (Table- 13). The whole form of product show lower level of contamination than their powder form. The whole chilli show a contamination level of 23.00 %, while 40.0 % of the powdered chilli is contaminated with aflatoxin B_1. However, it is interesting to note that only 10 % are showing contamination level above permissible limit of 30 ppb. Similarly, trend was noticed with respect to whole ginger and turmeric which do not show the presence of aflatoxin B_1. The procured products of ginger and turmeric powder show a contamination level of 21.80 and 3.60 % respectively. Only one sample of turmeric out of 55 tested was found to be contaminated with 20 ppb of aflatoxin B_1.

Table.13. Natural occurrence of Aflatoxin B_1 in Spices

Commodity	No. tested	No. having AFB_1	% B_1	B_1 range (ppb)	Contamination	
					<30 ppb	>30ppb
Chilli-A	65	15.0	23.00	20 - 160	10.0	5.0
Ginger-A	55	ND	-	-	ND	ND
Turmeric-A	55	ND	-	-	ND	ND
Chilli-B	65	26.0	40.00	60 - 140	16.0	10.0
Ginger-B	55	7.0	21.80	20 - 80	6.0	1.0
Turmeric-B	55	2.0	03.60	0 - 20	2.0	ND

Note: A= whole, B= powder, ND = not detected

The survey shows that the powdered form of spices show higher rate of contamination with aflatoxin B_1. None of the samples showed the presence of all the four Aflatoxins viz., aflatoxin B_1, B_2, G_1 and G_2. The necessity of hand picking and separating the contaminated chilli pods while powdering the chilli is clearly indicated. Physical removal of fungal contaminated spices at the stage of powdering step can definitely reduce the level of contamination in the final powdered product. An awareness on this issue to the producers, distributors and consumers is of prime importance.

4.3 Screening of isolates of Aspergillus species for aflatoxin production

The strains of *Aspergillus flavus* and *A.parasiticus* isolated from various spice samples were screened for their ability to produce aflatoxins on YES medium (as in section 3.26.20). *A.flavus* was abundant in all the spice samples. *A.parasiticus* was isolated only on chilli sample. Isolates from different spices produced varied levels of aflatoxins. *A. flavus* Isolates from chilli produce aflatoxin B_1 and B_2 at the range of 0-2400 and 0-1200 ppb level respectively. Similarly, isolates from ginger and turmeric produced aflatoxin B_1 and B_2 at lower range of 0 – 120 and 0 – 80 ppb respectively (Table-16). All the three isolates of *A.parasiticus* from chilli spice produced aflatoxin B_1, B_2, G_1 and G_2 at the range of 130-400, 60-180, 200-380 and 100-260 ppb levels respectively (Table-15). Samples of chilli harbor higher levels of aflatoxigenic strains (28.7 %) than ginger (13.7 %) and turmeric (9.2 %).

The data on the production of aflatoxins by each strain of *A.flavus* on YES medium is presented in the table 15. All the 19 isolates of *A.flavus* from chilli were found to produce aflatoxin B_1 and B_2. The range of aflatoxin B_1 and B_2 produced by them was from 60-2400 and 60- 600 ppb respectively. Out of 19 toxigenic isolates, only two of them produce aflatoxin B_1 above 1000 ppb. Isolate no. C-11 produced highest level, 2400 and 600 ppb level of aflatoxin B_1 and B_2 respectively and the isolate no. C-16 produced 1000 and 360 ppb of aflatoxin B_1 and B_2 respectively. Chilli isolates no. C-2, C-18 and ginger isolates no. G-1 produced same quantity of aflatoxin B_1 (300 ppb) and B_2 (60 ppb). Similarly, isolates C-6 and C-9, C-8 and C-13, C-15 and C-19 of *A.flavus* strain pairs produced same quantity of Aflatoxins (table-14). All the isolates of *A.flavus* from ginger (isolates of G.series table-14) produced aflatoxin B_1 and B_2. Only one isolate i.e. isolate no.G-5 produce aflatoxin B_1 above 1000 ppb and isolate no.G-1, G-2 and G-3 produced aflatoxin B_1 above 300 ppb. The turmeric is found to be a poor source for aflatoxigenic fungi compared to other spices like chilli and ginger. Only two isolates of *A.flavus* out of 55 samples tested were found to produce aflatoxin B_1 and B_2. Isolate no.T-1, produced B_1 and B_2 at 180 and 60 ppb and Isolate no.T-2, produced B_1 and B_2 at 120 and 30 ppb level respectively(Table-14).

Table.14. Screening of aspergilli from spices for the production of aflatoxins.

Isolates	No. of isolates	Toxigenic strains (%)	Aflatoxin range (ppb)			
			B_1	B_2	G_1	G_2
Aspergillus flavus (C)	65	28.7	0-2400	0-1200	ND	ND
A.parasiticus (C)	3	100.0	130-400	60-180	200- 380	100-260
A. flavus (G)	55	13.7	0-1200	0-140	ND	ND
A. flavus (T)	55	9.2	0-120	0-80	ND	ND

Note: C = Isolate from chilli, G = Isolate from ginger,
T = Isolate from turmeric, ND = Not detected.

Table.15. Aflatoxin production by isolates of *A.flavus* from chilli, ginger & turmeric.

A.flavus	Aflatoxin (ppb)	
	B_1	B_2
C-1	600	150
C-2, C-18 & G-1	300	60
C-3	60	20
C-4	360	120
C-5	680	240
C-6 & C-9	600	100
C-7	150	60
C-8 & C-13	600	200
C-10	360	60
C-11	2400	600
C-12	300	120
C-14	150	60
C-15 & C-19	300	80
C-16	1000	360
C-17	200	80
G-2	360	80
G-3	300	20
G-4	80	20
G-5	1200	140
T-1	180	60
T-2	120	30

The data on the production of aflatoxins by *A.parasiticus* is presented in the Table-16. It is clearly indicated that all three isolates of *A.parasiticus* from chilli produce aflatoxin B_1, B_2, G_1 and G_2. Isolate no. 1, produce aflatoxin B_1, B_2, G_1 and G_2 at 400, 60, 360 and 120 ppb level respectively. Where as isolate no.2, produce aflatoxin B_1, B_2, G_1 and G_2 at 360, 80, 280 and 80 ppb level and isolate no. 3, produce aflatoxin B_1, B_2, G_1 and G_2 at 260, 80, 200 and 40 ppb level respectively.

4.4 Screening of isolates of *Fusarium sporotrichoides* for the production of T-2 toxin and DAS

The strains of *F.sporotrichoides* isolated from chilli were tested for the production of T-2 toxin and DAS on GYEP medium. Out of 11 isolates, five were found to be toxigenic and three of them were able to produce both T-2 and DAS, while other two produced DAS only. The isolate no.1, 2 and 4 produced 2.90, 3.70 & 1.30 ppm level of T-2 and 2820, 2590 & 2050 ppm levels of DAS respectively. Isolate no.3 & 5 produced 2210 and 1250 ppm level of DAS respectively (Table – 17). None of the market samples of ginger and turmeric screened for mycoflora harbored species of *Fusarium*.

The picture (figure 1– 4) of *Aspergillus* spp. as mixed culture after 3 days of incubation. The figure – 5 showed the picture of isolates of *Aspergillus flavus, A.parasiticus* & *A.ochraceus* on PDA slants from chilli samples and Figure – 6 is the PDA slants containing isolates of *Fusarium sporotrichoides*. The figure 7 – 8 showed the T.L.C separation of aflatoxin B_1 and B_2 as viewed under UV at 365 nm.

4.5 Discussion

Studies on the Mycoflora of spices conducted at CFTRI, Mysore revealed that wide variety of fungi were found to occur at varied degree of infestation. Spices are part of agriculture and economy of producer countries such as India. Therefore it was important to know about the extent of natural contamination of spices and their potentiality for mycotoxins production. Several investigators have carried out seed health testing for past few decades, especially on various agricultural commodities including cereals, pulses, oil seeds and medicinal plants including feeds and fodders; (Bilgrami et al., 1980; Vijaya,1982; Reddy,1983; Basak and Mirdha, 1985; Kingsland, 1986; Mislivec et al., 1987; Shrivastava, 1989; Rodrigues and Thorne, 1990; Kanchan Lata, 1991; Niranjan Kumar and Sinha, 1992; Weidenborner and Kunz,1993; Frank Ross, 1994; Angelo Visconti and Brunno Loko, 1994; Nelson et al., 1994; Sinha,1994. It is also a fact that very little attention has been paid on spices and therefore very little information on spices, their mycoflora etc are available from India (Bilgrami, 1985; Anisa et al., 1988; Rajender singh et al., 1991; Jyotsna Sharma et al., 1992). Only few reports of

Table.16. Screening of *A.parasiticus* isolates of chilli for the production of aflatoxin.

A.parasiticus	Aflatoxin (ppb)			
	B_1	B_2	G_1	G_2
Cap-1	400	60	360	120
Cap-2	360	80	280	80
Cap-3	260	80	200	40

Note: C = Isolate from chilli, ap = *Aspergillus parasiticus.*

Table.17. Screening of *Fusarium sporotrichoides* isolates of chilli for the production of mycotoxins.

Fusarium sporotrichoides	Mycotoxins	
	T-2 (ppm)	DAS (ppb)
Cfs-1	2.90	2820
Cfs-2	3.70	2590
Cfs-3	0.0	2210
Cfs-4	1.30	2050
Cfs-5	0.0	1250

Note: C = Isolate from chilli, fs = *Fusarium sporotrichoides.*

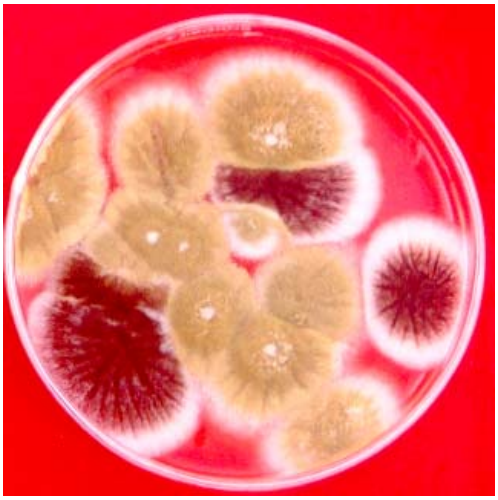


Fig.1. Mixed culture of *A.flavus* with *A.niger* on PDA medium.

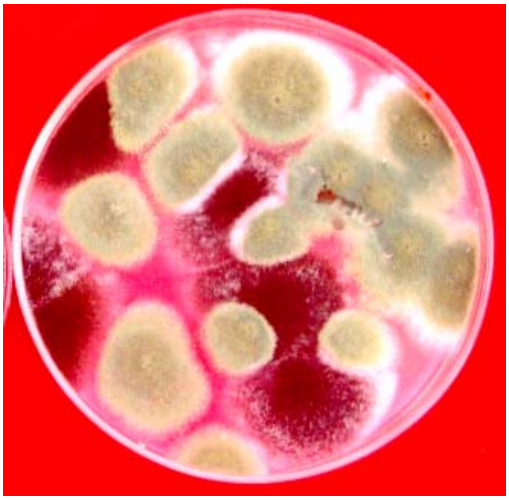


Fig.2. Mixed culture of *A.parasiticus* with *A.niger* on PDA medium

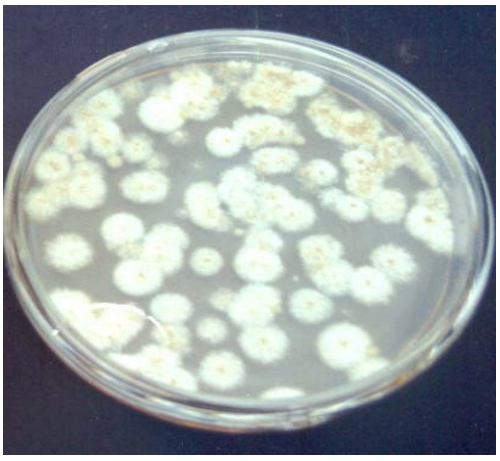


Fig.3. Pure culture of *A.flavus* on ADM medium (top view).

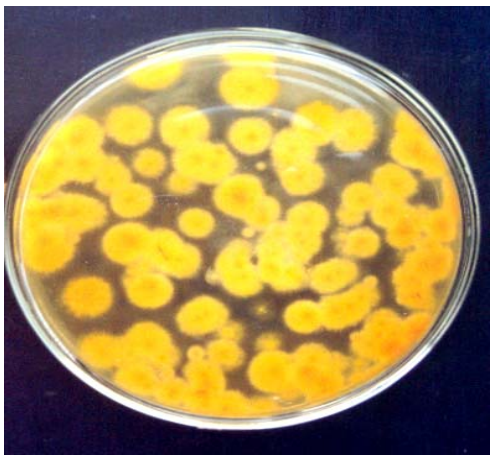


Fig.4. Pure culture of *A.flavus* on ADM medium (reverse view).

1 **2** **3**

Fig.5. Pure cultures of 1 (*Aspergillus flavus*), 2 (*A.parasiticus*) & 3 (*A.ochraceus*) isolates from chilli.

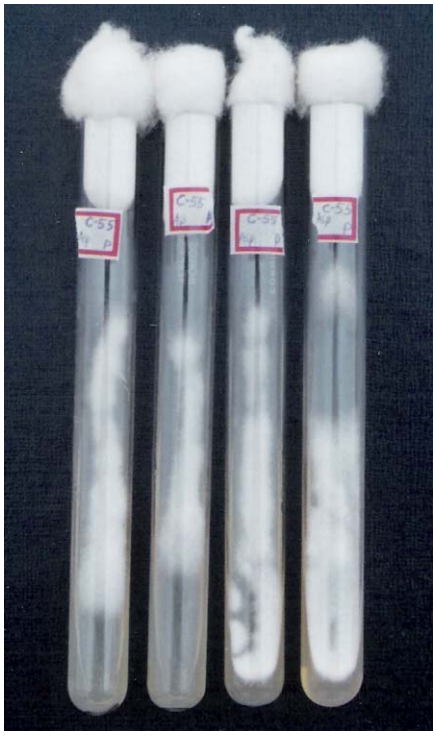


Fig.6. Pure culture of toxigenic *Fusarium sporotrichoides* isolate from chilli on PDA.

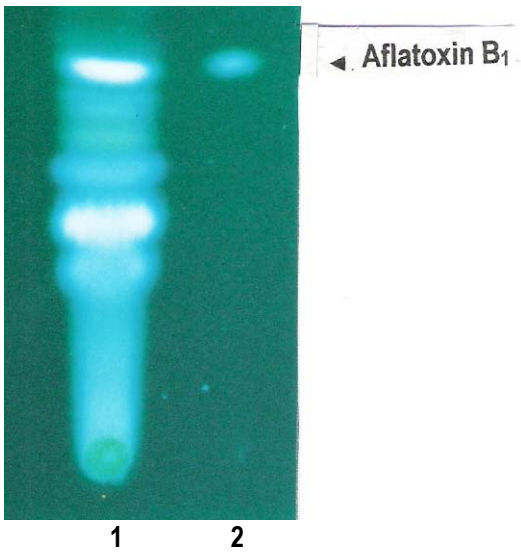


Fig.7. T.L.C separation of aflatoxin B_1 as viewed under UV at 365 nm. Lane 1= sample (chilli), lane 2= standard

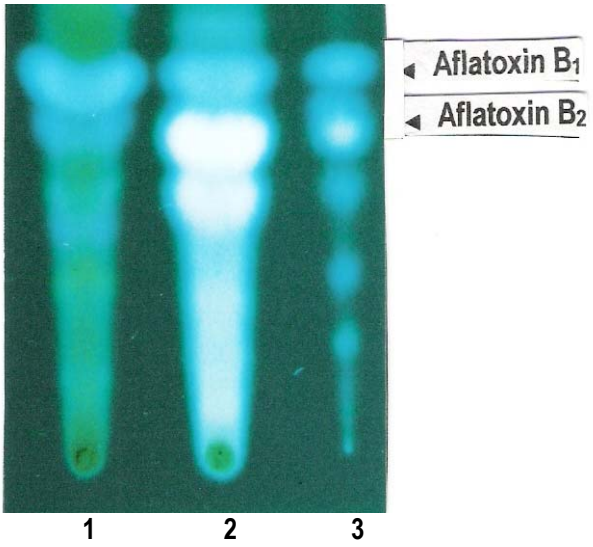


Fig.8. T.L.C separation of aflatoxin B_1 and B_2 as viewed under UV at 365 nm). Lane 1 = toxin from isolate (C-16), lane 2 = toxin from isolate (G-5) and lane 3 = standard.

mould infestation of spices by toxigenic fungi and elaboration of mycotoxins are available (Awe and Schranz, 1981; Llewellyn et al., 1981; Bata et al., 1983; Prasad et al., 1984; Bilgrami, 1985; Flannigan and Llewellyn, 1986; Rahman, 1987; Martnez et al., 1988; Anisha Athar et al., 1988 and Raja Kumai, 1992). Further such information is lacking.

Samples of chilli harbored fungal flora as high as 6.50 $\log_{10}$ cfu/g on PDA medium and fungal flora were represented as *Aspergillus flavus* group and other fungal flora (cover all fungi other than *Aspergillus flavus* group). In this study more importance was given to *Aspergillus flavus* group because these fungi could infest almost all agricultural commodities including majority of spices. It is shown in the tables that PDA medium being general isolation medium supported the growth of other fungi along with the genus *Aspergillus*. Where as the ADM medium being specific for the isolation of aflatoxigenic Aspergilli showed to be more selective and easy isolation of aflatoxigenic molds from those culture plates was possible. In this survey the occurrence of natural fungal flora was at higher level on chilli substrate than ginger and turmeric. Species of *Aspergillus* were found dominant in both the forms of chilli. *A.flavus, A.fumigatus. A.niger, A.glaucus, A.versicolor, A.terreus, A.parasiticus* and *A.candidus* were found to occur more frequently. Other fungi encountered frequently were belongs to species of *Alternaria, Cladosporuim, Chaetomium, Curvularia, Fusarium, Memnoniella, Macrophomina, Phoma, Penicillium, Rhizopus, Trichothecium* (Giridhar and Reddy, 1999). *Aspergillus flavus*, *A.niger* and *A.glaucus* were predominant among Aspergillus species. Reported interference of *A.niger* with toxin producing *Aspergillus flavus*, clearly demonstrate that there is competition amongst fungi in nature (Horn and Whicklow, 1983). Amongst spice samples tested, all the samples of the chilli, about 85 and 60 % samples from ginger and turmeric respectively showed presence of mould exceeding the limit specified by International Commission on Microbiological Specifications for foods (10^2 - 10^4). Although, important fungi belonging to species of *Aspergillus, Fusarium and Peneicillium* were found associated with chilli, ginger and turmeric, toxin producers were seen mainly from chilli source. Chilli samples recorded toxigenic strains belonging to *Aspergillus flavus*, *A.parasiticus* and *Fusarium sporotrichoides*, while ginger and turmeric samples were found associated with toxigenic strains belonging to *Aspergillus flavus* group only. Although *Aspergillus ochraceus* were encountered on chilli and ginger, toxin producers were absent. Amongst field fungi, *Fusarium sporotrichoides* capable of producing T-2 and Diacetoxiscerpinol were found associated with chilli samples at an incidence of 50 %.

Amongst samples analyzed for aflatoxins, chilli, 23 % (whole) and 40 % (powder) were found contaminated with B_1 in the range of 20 – 160 and 60 – 140 ppb levels respectively. Shank et al (1972) reported that 11 out of 106 samples of chilli were found contaminated with aflatoxin with mean concentration of 125 ppb, while Seenappa and Kempton (1980) reported contamination of chillies with aflatoxin in the range of 0.2 – 130 ppb. Singh (1983) observed that chilli samples were found contaminated with aflatoxin in the range of 120 – 1250 ppb. In the present survey, ginger and turmeric (whole) were found free from aflatoxins. Where as 22.8 % of powder form of ginger and 3.60 % of powder form of turmeric were found contaminated with B_1 in the range of 20 - 80 and 0 – 20 ppb level respectively.

Scott and Kennedy (1975) detected aflatoxin B_1, B_2, G_1 and G_2 in ginger with aflatoxin B_1 at 12.5 ppb as maximum level. Awe and Schrans (1981) detected B_1, B_2, G_1 and G_2 in ginger, while Singh (1983) reported that 4 out of 15 ginger samples were found contaminated with aflatoxin in the range of 66 – 720 ppb level. In the present survey, amongst turmeric samples tested, most of them were found free from aflatoxin contamination, Seenappa and Kempton (1980) observed aflatoxin contamination in the range of 10 – 45 ppb in 6 out of 25 samples analyzed. This clearly indicates that among spices, chilli and ginger appeared to be the spices at high risk and they require to be kept free from contamination with mycotoxigenic fungi. There may be several reasons for contamination of spices with mycotoxigenic fungi starting from growers level till reaching consumers, but in general it appears that spices contamination is due to inadequate drying practices, unhygienic handling, poor packaging followed by prolonged storage which might trigger fungal contamination and their elaboration of mycotoxins.

SUBSTRATE SPECIFICITY OF SPICES, CHILLI, GINGER AND TURMERIC

Contents

5.1 Introduction

Spices are grown mostly in tropical regions where climatic conditions are conducive for fungal growth and elaboration of various mycotoxins. Natural occurrence of *Aspergillus flavus* and aflatoxin on various spices have been documented by Udagava (1982). Aflatoxin has been produced in spices under artificial conditions (Flanningan and Hui, 1976; Seenappa and Kempton, 1980; Llewellyn et al, 1981a, b). Spices support growth and production of Aflatoxins by *Aspergillus flavus* and *A. parasiticus*. It has been documented that *Aspergillus ochraceus* also grow and produce toxin on spices like chilli and ginger (Singh, 1983). Yet on other hand spices possess preservative properties and are inhibitory against various microorganisms. Certain chemical constituents of spices such as eugenol, thymol, anethol were found to inhibit growth of fungus and production of mycotoxin. Therefore, to understand the potentiality of individual spices for their suitability as substrate for the growth and production of toxin by toxigenic fungi, the following study with chilli, ginger and turmeric was undertaken. Three toxigenic strains of Aspergilli such as *A. flavus* (ATCC 46283), *A. parasiticus* (CFR 223) and *A.ochraceus* (CFR 221) were tested for their potentiality to eloaborate mycotoxins under conducive experimental conditions with these spices as substrates and the results are presented below.

5.2 Substrate specificity of spices

5.2.1 Elaboration of aflatoxins by *A.flavus* on wholes spices

The suitability of various kinds of spice (whole form) as substrate for fungal growth and elaboration of mycotoxin is investigated (Table-18). The samples of chilli, ginger and turmeric (natural form) were artificially inoculated (as in section 3.24.1) with toxigenic strain of *A.flavus* (ATCC 46283). The cereal grain maize served as control. The chilli, ginger and turmeric substrates supported production of aflatoxin B_1 at 260, 140, 40 ppb and aflatoxin B_2 at 40, 30 and 10 ppb levels respectively. The control sample of maize (whole) supported highest levels of B_1 and B_2 at 800 and 200 ppb level respectively. Amongst the three spices, chilli and ginger were the better substrates for aflatoxin elaboration than turmeric.

5.2.3 Elaboration of aflatoxins by *A.flavus* on powdered spices

To assess the suitability of substrate in powder form spices were uniformly powdered to pass through ≥ 20 BSM size and inoculation with toxigenic mold and incubation was undertaken at ideal substrate moisture level and temperatures. The cereal grain maize (powder) served as control (as in section 3.24.1). The chilli, ginger and turmeric substrated were contaminated with aflatoxin B_1 at 180, 200, 60 ppb and aflatoxin B_2 at 30, 40 and 20 ppb levels respectively. The maize sample supported

Table.18. Elaboration of aflatoxins by *Aspergillus flavus* (ATCC 46283) on whole spices.

Substrate (whole)	Aflatoxins (ppb)		% yield	
	B_1	B_2	B_1	B_2
Chilli	260	40	32.5	20.0
Ginger	140	30	17.5	15.0
Turmeric	40	10	5.0	5.0
Control	800	200	-	-

Note: Substrate moisture – 40 %, Incubation period – 7 days, Incubation temperature – 30 ± 3^{O}C.

highest levels of B_1 and B_2 1200 and 320 ppb level respectively. Amongst spices, chilli and ginger were the better substrates for aflatoxin elaboration than turmeric (Table-19).

5.2.4 Elaboration of aflatoxins by *A.parasiticus* on whole spices

Whole form of spices such as chilli, ginger and turmeric were tested (as in section 3.24.1) with toxigenic *A.parasiticus* (CFR 223). On observation, chilli showed to be better substrate for aflatoxins (table 20). Chilli was showing contamination level of B_1, B_2, G_1 and G_2 at 200, 40, 120 and 30 ppb level respectively. Ginger supported B_1, B_2, G_1 and G_2 at 100, 30, 80 and 10 ppb level respectively. Turmeric supported B_1, B_2 and G_1 at 60, 10 and 20 level respectively. The maize substrate which was included as reference control supported more of B_1, B_2, G_1 and G_2 at 860, 300, 280 and 60 ppb respectively. Amongst spices, chilli and ginger were the better supporters aflatoxins than turmeric.

5.2.5 Elaboration of aflatoxins by *A.parasiticus* on powdered spices

Similarly, when toxigenic *A.parasiticus* (CFR 223) was inoculated to powder form of spices, chilli emerged as better substrate for aflatoxins production (table-21) (as in section 3.24.1). Chilli was showing contamination level of B_1, B_2, G_1 and G_2 at 180, 30, 100 and 20 ppb level respectively. Ginger supported B_1, B_2, G_1 and G_2 at 120, 30, 80 and 20 ppb level respectively. Turmeric supported B_1, B_2 and G_1 at 80, 20 and 40 level respectively. The maize substrate which was included as reference control supported more of B_1, B_2, G_1 and G_2. Amongst spices, chilli and ginger were the better supporters of aflatoxins than turmeric.

5.2.6 Elaboration of ochratoxin A by *A.ochraceous* on whole spices

As evidenced in the table 9, *A.ochraceus* (CFR 221) when grown on chilli, ginger and turmeric (whole) produced ochratoxin A at 160, 20 and 10 ppb level respectively. Where as, on maize (whole) substrate it produced highest amount of 260 ppb of ochratoxin A (as in section 3.24.2). Amongst spices tested, chilli was found to be the better substrate for ochratoxin A than ginger and turmeric (Table-22).

5.2.7 Elaboration of ochratoxin A by *A.ochraceous* on powdered spices

When powder form of spices used as substrate for *A.ochraceus* (CFR 221), chilli, ginger and turmeric supported production of ochratoxin A at 80 and 30 ppb level respectively. Turmeric (powder) did not support ochratoxin A production (Table-23). Where as, on maize (powder) substrate it produced highest amount of ochratoxin A. Amongst spices tested, chilli was found to be the better substrate for ochratoxin A than ginger.

Table.19. Elaboration of aflatoxins by *Aspergillus flavus* (ATCC 46283) on powdered spices.

Substrate (powder)	Aflatoxins (ppb)		% yield	
	B_1	B_2	B_1	B_2
Chilli	180	30	15.0	9.40
Ginger	200	40	16.70	12.5
Turmeric	60	20	5.0	6.25
Control	1200	320	-	-

Note: Substrate moisture – 40 %, Incubation period – 7 days, Incubation temperature – 30 ± 3^{O}C.

Table.20. Elaboration of aflatoxins by *A.parasiticus* (CFR 223) on whole spices.

Substrate (whole)	Aflatoxins ppb			
	B_1	B_2	G_1	G_2
Chilli	200 (23.25)	40 (13.33)	120 (42.85)	30 (50.0)
Ginger	100 (11.2)	30 (10.0)	80 (28.60)	10 (16.7)
Turmeric	60 (6.97)	10 (3.3)	20 (7.10)	-
Control	860	300	280	60

Note: Substrate moisture – 40 %, Incubation period – 7 days,
Incubation temperature – 30 ± 3^{O} C. %,
Figures in parenthesis % yield.

Table.21. Elaboration of aflatoxins by *A.parasiticus* (CFR 223) on powdered spices.

Substrate (powder)	Aflatoxins ppb			
	B_1	B_2	G_1	G_2
Chilli	180 (20.9)	30 (10.0)	100 (37.7)	20 (33.30)
Ginger	120 (13.95)	30 (10.0)	80 (28.6)	20 (33.30)
Turmeric	80 (9.3)	20 (6.6)	40 (14.30)	-
Control	860	300	280	60

Note: Substrate moisture – 40 %, Incubation period – 7 days,
Incubation temperature – 30 ± 3^{O} C. %,
Figures in parenthesis % yield.

Table.22. Elaboration of Ochratoxin A by *A.ochraceus* (CFR 221) on whole spices

Substrate (whole)	Ochratoxin A (ppb)	(%)
Chilli	160	61.53
Ginger	20	7.6
Turmeric	10	3.6
Control	260	-

Note: Substrate moisture – 40 %, Incubation period – 7 days, Incubation temperature –30 ± 3^{O}C.

Table.23. Elaboration of Ochratoxin A by *A.ochraceus* (CFR 221) on powdered spices

Substrate (powder)	Ochratoxin A (ppb)	(%)
Chilli	80	66.70
Ginger	30	25.00
Turmeric	0.0	-
Control	120	-

Note: Substrate moisture – 40 %, Incubation period – 7 days, Incubation temperature –30 ± 3^{O}C.

5.2.8 Elaboration of aflatoxins by *A.flavus* on various parts of chilli

To investigate the ability of the specific substrate that promote aflatoxin formation when ideal conditions are provided, pericarp, seeds and the stalks of whole chilli pods were separated and subjected to experimentation. Parts of chilli were inoculated (as in section 3.24.1) with toxigenic strain of A. *flavus* (ATCC 46283). Of the three parts tested, seed was found to support the formation of aflatoxin B_1 and B_2 more than pericarp and stalk (Table-24). When compared to control (whole pods), seed was found supporting production of aflatoxins at higher level, at 600 and 160 ppb respectively. The chilli pods were contaminated with 400 and 120 ppb of B_1 and B_2 respectively. The level of toxin in pericarp was 360 ppb of B_1 and 120 ppb of B_2, while stalk had 200 ppb of B_1 and 100 ppb of B_2. Seed supported more B_1 and B_2 at the level of 50.0 and 33.33 % respectively when compared to whole chilli pod.

5.2.9 Elaboration of aflatoxins by *A.parasiticus* on various parts of chilli

Similarly, when toxigenic strain of *A. parasiticus* (CFR 223) was allowed to elaborate its toxins on various parts of chilli such as pericarp, seeds and stalks, seeds remained as the better supporter of toxin production than pericarp, stalk and the whole pod (Table-25). Of the three different parts tested, seed supported majority of aflatoxins more than pericarp and stalk. Seed harbored B_1, B_2, G_1 and G_2 at 240, 60, 140 and 40 ppb level respectively. While pericarp had 80, 40, 40 and 20 ppb of B_1, B_2, G_1 and G_2 and stalk was contaminated with 80, 30, 60 and 20 ppb of B_1, B_2, G_1 and G_2. The seed supported highest level of contamination with B_1, B_2, and G_2 at the level of 100, 75 and 33.33 % respectively. The whole chilli pods were contaminated at 120, 60, 80 and 30 ppb level of B_1, B_2, G_1 and G_2 respectively, which was more than the toxin elaborated on pericarp and stalks. This clearly indicates that seeds are very good substrate for aflatoxin elaboration.

5.2.10 Elaboration of ochratoxin A by *A.ochraceous* on various parts of chilli

When toxigenic strain of *Aspergillus ochraceus* (CFR 221) was grown on different parts of chilli, variation in the trend was evident (table 13). Ochratoxin A contamination was highest on whole pod with decreasing trend in the order of pericarp (75 %), stalk (37.5 %) and seed (25 %). It is interesting to note that the seeds of chilli showed lowest level of contamination with ochratoxin A. Whereas the seeds supported highest level of contamination with aflatoxigenic fungi elaborating high amount of aflatoxins (Table- 26).

Table.24. Elaboration of aflatoxins by *A.flavus* (ATCC 46283) on various parts of chilli.

Part of chilli	Aflatoxins (ppb)		% yield	
	B_1	B_2	B_1	B_2
Pericarp	360	120	90	100
Seed	600	160	150	133
Stalk	200	100	50	83
Pods	400	120	-	-

Note: Substrate moisture - 40 %, Incubation period – 7 days, Incubation temperature – 30 ± 3^{O} C

Table.25. Elaboration of aflatoxins by *A.parasiticus* (CFR 223) on various parts of chilli.

Part of chilli	Aflatoxins (ppb)				Yield (%)			
	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
Pericarp	80	40	40	20	66.66	66.66	50.00	66.66
Seed	240	60	140	40	200	100	175	133.33
Stalk	80	30	60	20	66.66	50.0	75.0	66.66
Pods	120	60	80	30	-	-	-	-

Note: Substrate moisture - 40 %, Incubation period – 7 days,
Incubation temperature – 30 ± 3^{O} C

Table.26. Elaboration of ochratoxin A by *A.ochraceus* (CFR 221) on various parts of chilli.

Part of chilli	OTA (ppb)	% yield
Pericarp	60	75.0
Seed	20	25.0
Stalk	30	37.5
Pods	80	-

Note: Substrate moisture – 40 %, Incubation period – 7 days, Incubation temperature – 30 ± 3^{O} C.

5.3 Discussion

Whole chilli sample supported growth and aflatoxin production by *Aspergillus flavus* (ATCC 46283) better than sterilized whole ginger and turmeric under experimental conditions which is similar to that observed by Madhyastha and Bhat (1985). Chilli powder form did not support aflatoxin production as much as whole sample. While powder samples of ginger and turmeric supported better production of aflatoxin than whole form. Powder form of chilli and whole form of ginger and turmeric emerged as poor substrates as they did not support much aflatoxin production. It may be due to the reason that capsaicin an active principle known to inhibit toxin production may remain inside sample when whole form was used. When powder form was used, the capsaicin content get released outside which might induce production of aflatoxin. In case of ginger and turmeric substrate as whole samples, the toxin production which was less may be due to the reason that the rhizome was hard and did not support growth of fungi and thus production of aflatoxin. The results of our study coincides with observation made by Madhyasta and Bhat (1985).

Spices appeared to be the poor substrate for ochratoxin A elaboration by *Aspergillus ochraceus* (CFR 221), when compare to aflatoxin production by *A.flavus* and *A.parasiticus*. The mycotoxins other than aflatoxins are unlikely to occur on spices like chilli, ginger and turmeric. This study conducted on suitability of spices both whole and powder form, the production of mycotoxins by *Aspergillus* species revealed that production of aflatoxin B_1, B_2, G_1, G_2 and ochratoxin A was better on whole chilli sample than whole ginger or turmeric. Singh (1983) observed ochratoxin A contamination in spices such as black pepper, cardamom, cumin, coriander, chilli, fennel and ginger. Amongst powdered spices, ginger supported production of aflatoxin, while chilli powder was comparatively a poor substrate for aflatoxin production. Madhyasta et al (1985) observed that chilli powder was poor supporter of aflatoxin production. Flannigan and Hui (1976) observed that a total aflatoxin production of 4000 ppb was achieved on whole chilli. However, amount of toxin produced by particular fungus depends on various factors such as type of strain, nutrition, favorable environmental conditions etc. Turmeric appeared to be the poor supporter of aflatoxin production, may be due to the presence of curcumin which is found to have inhibitory properties against fungi. Tiwari et al (1983) observed that curcumin at higher concentrations inhibit aflatoxin production completely.

The present study clearly indicated that spices support growth of fungi such as *A.flavus*, *A.parasiticus* and *A.ochraceus*, but not the production of their respective toxins which totally depends on many factors, one such factor is antifungal properties of spices themselves. However, it is

established time and again that the spices are not good substrates for production of toxin unlike cereals and oil seeds (Hesseltine, 1977).

SORPTION BEHAVIOR OF SPICES WITH REFERENCE TO MYCOTOXIGENIC FUNGI

Contents

6.1 Introduction

Water is the single most important factor governing microbial spoilage in foods, and the concept of water activity (a_w) has been very valuable because measured values generally correlate well with the potential for growth and metabolic activity (Chirife and Buera, 1996). Chilli or red pepper, ginger and turmeric products are widely used as spices in many countries. After being harvested, these spices are usually dried and stored as whole or in powder form for several months before consumption. A major factor affecting the quality of stored agricultural products including spices is the change in moisture content. Moisture loss induces loss of food value or quality and moisture gain induces color change or change in other quality parameters. Therefore it was necessary to understand the moisture sorption characters of products of interest to our present study since the research work on moisture uptake and the critical environmental conditions for mycotoxins elaboration has been initiated as a concept on spices such as chilli, ginger and turmeric. In 1952, W.J. Scott introduced the idea of water activity. Water activity (a_w) is the ratio of water vapor pressure of the substrate to the vapor pressure of pure water at same temperature and pressure (Ellis et al., 1991). Those foods which are usually considered to be with a water activity of 0.60 – 0.85 are called intermediate moisture foods (Quast and Neto, 1976). Typically, this corresponds to a moisture content of 10-35 % on a moist basis. At water activities below 0.85, no pathogenic microorganisms develop, but quality deterioration due to non pathogenic microorganisms is a major problem. This deterioration is more serious in the upper part of water activity range. In tropical climates, intermediate- moisture foods usually absorb moisture. Small increase in the water activity may be sufficient to permit microbial growth. In tropical climates, however, ambient RH may remain above 90 % for several days or above 80 % for long periods. Under such conditions, moisture diffuses into the package, and the product gains moisture. Consequently, its water activity increases, and microbial spoilage may occur. In addition, undesirable physical changes may occur. The post-harvest losses in agricultural commodities, including spices, mainly due to fungal contamination and insect infestation have been viewed with serious concern particularly in developing countries like India. It is the prime requisite that all spices be harvested at correct stage of maturity without much physical damage after which they are processed properly for marketing. Moisture content is one of the most important factors in quality control of grain especially in controlling fungus infestation. The moisture content is an index of the probable keeping quality of the product and can be expressed on either the wet or dry basis. Fungal spores require moisture to germinate and if this is

excluded (i.e. if grain is stored at its safe moisture content or below) moulds infestation may be arrested. The maximum safe storage moisture content may be defined as the amount of absorbed water held within a commodity which is in equilibrium with an atmospheric RH 70 %. Moisture content is closely bound to temperature. Under certain circumstances, temperature differences can cause the re-distribution of moisture leading to local mould growth. Other factors that affect fungal development and the production of spores are availability of oxygen, light, acidity, and salt/sugar content.

Those molds classified in the deuteromycotina or Fungi imperfecti (Pitt, 1985), belongs mainly to the genera *Aspergillus* and *Penicillium*, well adopted to grow on solid substrates with low water content (Lesage et al, 1993). This property is a major factor in the predominance of *Aspergillus* species as 'storage fungi' on dry stored produce – including members of the *A. restrictus, A.glaucus, A.candidus, A.ochraceus* and *A.flavus* groups (Christensen & Kaufmann, 1974). Moisture control without question is the best and most economical means to control the environment to prevent mould growth and mycotoxin production. To prevent microbiological growth, especially that of toxigenic mould, a safe moisture level should be achieved.

6.2 Sorption behavior of spices

6.2.1 Effect of RH and moisture uptake on spices

Perusal of table 27 reveals the sorption pattern in chilli, ginger and turmeric at 65, 75, 86 and 92 Relative Humidity (RH) levels with different duration starting from 7 to 45 days at temperature of 25^0 C. The experimental design for the chapter on sorption behavior of spices has been detailed in the sections 3.25.1 of materials and method. Moisture stability starts after 45 days of exposure to 65 % RH amongst all spice samples. As the RH increases along with duration of exposure, the increase in the moisture of the sample is also noticed. The initial moisture content in chilli, ginger and turmeric was 9.30, 5.20 and 6.10 per cent respectively. After exposure at RH 65 % for 30 days, the moisture content of chilli, ginger and turmeric showed a steady rise to 12.10, 5.50 and 6.30 % respectively. After 45 days of exposure, the moisture content of chilli, ginger and turmeric further stabilized to 13.8, 5.60 and 6.62 % respectively. The sample kept at prevailing room temperature and humidity (RTH) for 45 days served as control, which showed a decrease in moisture content at 6.20, 3.20 and 3.80 % respectively. Similar trend was noticed amongst the samples exposed to 75 and 86 % RH. However, at highest level of RH (92 %), an initial rise in moisture was recorded with chilli (22.5 %) and ginger (13.6 %) on day 14. This showed a moisture gain of 142.0 and 161.53 % respectively. From there

onwards, there was stabilization of moisture between day 21 to 28. The turmeric showed maximum moisture of 17.18 % which corresponded with moisture gain of 181.63 %. It seemed to be stabilizing at

Table.27. Humidity – Moisture content data:

Relative Humidity (%)	Time period (days)	Moisture content % (% gain)		
		Chilli	Ginger	Turmeric
65	30	12.10 (30.10)	5.50 (5.76)	6.30 (3.27)
	45	13.80 (48.38)	5.60 (7.69)	6.62 (8.52)
75	30	13.80 (48.38)	5.66 (8.84)	6.68 (9.50)
	45	14.90 (60.21)	8.50 (63.46)	9.88 (62.0)
86	15	17.10 (83.87)	9.20 (76.92)	10.20 (67.20)
	30	17.80 (91.39)	10.10 (94.23)	12.20 (100.0)
	45	20.20 (117.20)	12.10 (132.70)	13.60 (123.0)
92	7	21.50 (131.18)	12.80 (146.15)	13.66 (123.93)
	14	22.50 (142.0)	13.60 (161.53)	15.30 (150.81)
	21	22.00 (136.55)	13.00 (150.0)	15.90 (160.65)
	28	18.00 (93.54)	12.26 (135.76)	17.18 (181.63)
RTH	0	9.30	5.20	6.10
	45	6.20	3.20	3.80

Note: RTH = Prevailing Room Temperature Humidity.

Table.28. Fungal flora in Chilli (powder) exposed to different RH.

Relative Humidity (%)	Time period (days)	Moisture (%)	Mycoflora (log $_{10}$ cfu / g) *Aspergillus flavus*/ *A.parasiticus*	Other fungi	Mycotoxins AFB_1	AFB_2
65	30	12.10	2.30	2.0	-	-
65	45	13.80	5.30	2.0	-	-
75	30	13.80	4.30	3.65	-	-
75	45	14.90	5.76	4.40	+	-
86	15	17.10	2.0	4.14	-	-
86	30	17.80	3.44	5.54	++	-
86	45	20.20	2.0	5.61	++	+
92	7	21.50	2.0	5.74	-	-
92	14	22.50	2.47	6.07	++	-
92	21	22.00	2.69	6.09	++	+
92	28	18.00	3.92	6.55	++	+
RTH	0	9.30	<1.0	2.0	-	-
	45	6.20	2.0	3.0	-	-

Note: RTH = Prevailing Room Temperature Humidity, + = < 50 ppb, ++ = < 100 ppb.

Table.29. Fungal flora in Ginger (powder) exposed to different RH.

Relative Humidity (%)	Time period (days)	Moisture (%)	Mycoflora (log $_{10}$ cfu / g) *Aspergillus flavus*/ *A.parasiticus*	Other fungi	Mycotoxins AFB_1	AFB_2
65	30	5.50	2.3	2.8	-	-
65	45	5.60	2.6	2.8	-	-
75	30	5.66	2.7	3.0	-	-
75	45	8.50	2.8	3.07	-	-
86	15	9.20	3.0	3.09	-	-
86	30	10.10	3.0	3.17	-	-
86	45	12.10	3.2	3.4	-	-
92	7	12.80	3.23	3.6	+	-
92	14	13.60	3.3	3.73	+	-
92	21	13.00	3.6	3.60	+	-
92	28	12.26	4.0	3.87	++	+
RTH	0	5.20	<1.0	2.06	-	-
	45	3.20	<1.0	2.0	-	-

Note: RTH = Prevailing Room Temperature Humidity, + = < 50 ppb, ++ = < 100 ppb.

15.3 to 15.9 % (14 to 21 days) but showed gain in moisture on day 28 (Table – 27). This behavior correlates with the increase in Mycoflora which is presented below.

6.3 Fungal flora on chilli

The pattern of fungal flora as well as mycotoxin in samples of chilli exposed to different RH with different time durations at 25^0 C is presented in table 28. Fungi were mainly represented as *Aspergillus flavus*, *A.parasiticus* and other fungi at $\log_{10}$ values and mycotoxins represented by Aflatoxins B_1 and B_2 at ppb level. Sample kept at prevailing room temperature and humidity (RTH) for 45 days served as control which had least of initial fungal flora and was free of mycotoxin. The positive correlation between increase in moisture and growth of mycoflora as well as mycotoxin production was noticed amongst samples exposed at higher RH levels. Increase in moisture content in chilli sample with increase in RH was noticed up to the extent of RH 92 %, when exposed for two week and beyond. Sample after exposed to RH 65 % for 30 and 45 days showed presence of fungal flora. Species of *Aspergillus* and other fungi at 2.3 and 2.0 $\log_{10}$ cfu/g respectively were seen after 30 days of exposure to RH 65 %. At the same RH, after 45 days of exposure, sample showed presence of *Aspergillus* spp. and other fungi at 5.3 and 2.0 $\log_{10}$ cfu/g respectively. *Aspergillus* spp. & other fungi were 4.3 & 3.65 $\log_{10}$ cfu/g and 5.76 & 4.40 $\log_{10}$ cfu/g amongst samples exposed for 30 & 45 days respectively at RH 75 %. Sample exposed for 45 days at RH 75 % have recorded aflatoxin B_1 at < 50 ppb level. Amongst samples exposed for 15, 30 and 45 days to RH 86 %, *Aspergillus* spp. was in the range of 2.0 – 3.0 $\log_{10}$ cfu/g which was comparatively low. However, other fungi were recorded in the higher range of 4.14 – 5.61 $\log_{10}$ cfu/g. Aflatoxin B_1 at > 50 ppb level was recorded from samples exposed for 30 & 45 days. *Aspergillus* spp. and other fungi at 2.0 - 3.92 $\log_{10}$ cfu/g and 5.74 - 6.55 $\log_{10}$ cfu/g respectively was recorded at 92 % RH. Aflatoxin B_1 at > 50 ppb level was found in samples exposed for 14, 21 & 28 days. Aflatoxin B_2 at < 50 ppb level was recorded after 21 & 28 days of exposure. Control sample showed an increase in *Aspergillus* spp. and other fungi from <1.0 to 2.0 $\log_{10}$ cfu/g and 2.0 to 3.0 $\log_{10}$ cfu/g respectively. However, there was no mycotoxin production in RTH samples and also amongst samples exposed at 65 and 75 %. Aflatoxin B_1 was found in samples exposed to 86 % RH (30 & 45 d period) and in 92 % RH (7 to 28 d period) samples. Aflatoxin B_2 was found in few samples (Table – 28).

6.4 Fungal flora on ginger

Table 29 reveals the presence of fungal flora as well as mycotoxin in samples of ginger exposed in similar way as in case of chilli. The positive correlation between increase in moisture and

Table.30. Fungal flora in Turmeric (powder) exposed to different RH.

Relative Humidity (%)	Time period (days)	Moisture (%)	Mycoflora (log $_{10}$ cfu / g) *Aspergillus flavus*/ *A.parasiticus*	Other fungi	Mycotoxins AFB_1	AFB_2
65	30	6.30	<1.0	1.13	-	-
65	45	6.62	<1.0	1.14	-	-
75	30	6.68	<1.0	1.16	-	-
75	45	9.88	<1.0	1.20	-	-
86	15	10.20	<1.0	1.20	-	-
86	30	12.20	1.25	1.21	-	-
86	45	13.60	1.34	1.38	-	-
92	7	13.66	1.36	1.17	-	-
92	14	15.30	1.41	1.27	+	-
92	21	15.90	2.25	2.0	+	-
92	28	17.18	2.69	3.17	+	+
RTH	0	6.10	<1.0	1.14	-	-
	45	3.80	<1.0	1.11	-	-

Note: RTH = Prevailing Room Temperature Humidity, + = < 50 ppb, ++ = < 100 ppb.

Fig.9.Chilli sample before exposure.

Fig.10. Chilli sample after exposure (RH 92 %).

Fig.11. Chilli (powder) before exposure.

Fig.12. Chilli (powder) after exposure (RH 92 %).

growth of mold as well as mycotoxin production was noticed at RH 92 %, when exposed for a week and beyond. At RH 75 %, after 30 days, 2.7 and 3.0 cfu/g and after 45 days, 2.8 and 3.07 cfu/g of *Aspergillus* spp. and other fungi respectively were recorded. After 15, 30 and 45 days at RH 86 %, *Aspergillus* spp. and other fungi were recorded at 3.0 - 3.2 $\log_{10}$ cfu/g and 3.09 - 3.4 $\log_{10}$ cfu/g range respectively. At 92 % RH also there was not much variation in *Aspergillus* spp. and other fungi which were in the range of 3.23 - 4.0 $\log_{10}$ cfu/g and 3.6 - 3.87 $\log_{10}$ cfu/g respectively. However, aflatoxin B_1 was recorded at > 50 ppb level only in sample exposed for 28 days at this RH. Control showed decline in fungal flora.

6.5 Fungal flora on Turmeric

Similarly with turmeric, positive correlation between increase in moisture and growth of mycoflora as well as mycotoxin production was noticed amongst samples exposed to RH of 92 %, when exposed for two weeks and beyond. At RH 65 and 75 %, no *Aspergillus* spp. was recorded. Other fungi was in the range of 1.13 – 1.20 $\log_{10}$ cfu/g. At RH 86 %, lower range of *Aspergillus* spp. (<1.0 – 1.34 $\log_{10}$ cfu) and other fungi (1.20 - 1.38 $\log_{10}$ cfu/g) were recorded. Similarly at 92 % RH also same trend continued. The samples from RH 92 % after 14, 21 and 28 days, recorded both aflatoxin B_1 and B_2 at < 50 ppb level after 28 days exposure at 92 % RH. Control recorded a marginal increase of other fungi from 1.14 – 1.17 $\log_{10}$ cfu/g (Table – 30).

The picture of chilli samples (whole and powder) taken (Fig. 9 to 12) before and after exposure to 92 % RH clearly showed the visible mold growth only on exposed samples (Fig. 10 & 12).

6.6 Discussion

Alternative approaches such as limiting water activity to control microbial growth in foods besides other techniques such as sterilization (heat or irradiation), pasteurization have come through in recent time. The most effective mycotoxin control measures is to dry the commodity such that the water activity (a_w) is too low to support mould growth and/ or prevent mycotoxin production. To prevent the growth of most moulds the a_w needs to be 0.70, which translates to a moisture content of approximately 14% for maize and 7.0% for groundnuts at 20°C (the corresponding moisture content decreases as the temperature increases). Each toxigenic mould has its own minimum water activity for growth and mycotoxin production and these translate into moisture contents for each commodity. These moisture contents are termed ‘safe’ and would be the critical limit for the control measure. If the

commodity is at an 'unsafe' moisture content for longer than 48 hours, then mould can grow and mycotoxins be produced. Hence limiting the time that the commodity spends in the 'unsafe' moisture content to less than 48 hours is an important aspect to be considered as part of control measure. This explains why timely sun-drying can sometimes be safer than delayed mechanical drying. Two days on a drying floor with occasional turning can often achieve the target 'safe' moisture content, whereas a back-log at the mechanical drier can result in the critical limit of 48 hours not being met. Once produced, it is not usually possible to remove mycotoxins, other than by physical separation (grading) techniques. To apply this type of control measure, representative samples of batches of commodity are collected and tested for selected mycotoxins. Only those batches containing less than the critical limit of mycotoxin, as specified in official regulations, are accepted. These are control measures that would also be suitable for application at a critical control point for aflatoxin, but only for the specified commodities. It is essential that GAP, GSP, and GMP pre-requisites are in place, and simply ensuring that this practice is followed to reduce the risk of the mycotoxin hazard.

The absolute limit for microbial growth seems to be at RH 60 % (Louise and Harry, 1991). Mould counts of untreated spices including turmeric in polyethylene pouches and at RH above 80 % increased up to 10^8 cfu/g during 1-3 months of storage at 30-35^O C (Ito et al., 1985). Seenappa and Kempton (1980a, b) observed that during storage of dried whole red peppers at 70 % RH, the moulds of the *Aspergillus glaucus* grew and colonized stalks, pods and seeds. At 85 % RH, *A.glaucus* group species were replaced in predominance by *A.niger, A.flavus* and *A.ochraceous* and at 95 % by the last two species or *A. flavus* alone. Thus a succession of fungi was recorded as the RH increased. At low a_w, water is bound by salts, sugars, protein, and other solutes, therefore growth of molds cannot occur since water is not present in available form (Vasanthi and Bhat, 1998). According to Ellis et al. (1991), aflatoxin production ceases or decreases at a_w values below 0.85. However fungal growth can still occur at a_w values as low as 0.78 to 0.80. The optimum a_w for production of aflatoxin by both *A.flavus* and *A.parasiticus* is reported to be in the range of 0.95 to 0.99. Insect infestation has been shown to contribute to the biodeterioration of spices (Seenappa et al., 1979). Kim et al (1994) observed browning and mold growth in red pepper at water activity of 0.93 after 25 days of exposure at 25^0 C. Equilibrium for aw <0.75 occurred within 21 days. Mold growth was observed after 20 days at a_w 0.93 after equilibrium moisture content were reached before mold growth occurred. According to Larry (1983), *A.flavus* requires 0.78 and 0.84 a_w for growth and toxin production respectively. Northolt et al (1976) found that *A.parasiticus* requires 0.82 and 0.87 a_w for growth and toxin production respectively.

A.ochraceus requires 0.85 a_w for production of ochratoxin as observed by Bacon et al (1973). Ballesteros (1993) observed that mold does not grow below a_w of 0.62. Chirife and Buera (1996) recorded critical a_w of 0.80 is necessary for mycotoxins production. According to Pitt and Miscamble (1995) *A.flavus, A.parasiticus* and *A.oryzae* requires minimum a_w of 0.81 for germination at 25^0 C. Aflatoxins are produced at a_w values ranging from 0.95 to 0.99, with a minimum a_w value of 0.82 being reported for *A.flavus* (ICMSF, 1996). Apart from aflatoxins a UV fluorescent Cyclopiazonic acid (CPA), which is also produced, is an indole-tetramic acid mycotoxin (Pitt, 1997). The minimum a_w values for growth of the *Penicillium* spp was 0.85 – 0.90 (Abellana and Ramos, 2001). According to Torres et al (2003), germination and growth of different fungi also depends on other factors, such as fungal interactions, temperature etc. His study showed that growth of *Fusarium verticillioides* was temperature dependant. *A.ocraceus* was inhibited when it was co-inoculated with other fungi, while *Alternaria alternata* was able to co-exist well with other fungi. Drying to safe moisture levels and storage of crops in moisture free condition was recognized as an important method for prevention of aflatoxin contamination (Vasanthi and Bhat, 1998). In our study also raise in moisture content with higher RH (92 %) was evident amongst all three spices tested. In case of chilli and ginger, maximum moisture gain was recorded on day 14. But from day 15 – 28, decrease in moisture was recorded after a steady increase from day 7 to 21. In case of turmeric, maximum moisture gain was recorded on day 28 after steady increase from day 7. This phenomenon was mainly due to more absorption of water by the competing fungal population over the substrates (chilli, ginger and turmeric). The study reveals interesting data as the chilli gained less moisture (142 %) than ginger (161 %) and turmeric (182 %). This varied sorption capacity of chilli may be related to its chemical composition pattern and retention capabilities. At RH 65 and 75 %, as the moisture increased up to 13 %, the *A.flavus* / *A.parasiticus* flora also showed an increase of 3 log cfu/g. However, at 86 and 92 % RH, the general fungal flora (other fungi) showed increasing trend. But the *A.flavus* / *A.parasiticus* flora did not show much increment. However, aflatoxin B_1 was found in most of the samples at these RH. This is because the fungi grew and in mycelial state elaborated mycotoxins. Moreover, though chilli showed comparatively less sorption characteristics than ginger and turmeric, the substrate showed to be better suited for growth of fungi and mycotoxin elaboration.

DETECTION OF AFLATOXIGENIC FUNGI BY MOLECULAR METHOD

Contents

7.1 Introduction

The rapid identification of spoilage microorganisms is of eminent importance to the food industry. Traditional identification methods, which are based on laborious morphological and physiological tests, are time-consuming, costly, require facilities and mycological expertise, often fail in rapidity, sensitivity and specificity. They lack discriminatory power and as a consequence, misidentification occurs frequently, resulting in the slow and incomplete/inaccurate release of data, for retrospective evaluation. Immunological methods and diagnostic media have limitations in identifying the aflatoxigenic fungi. Time is a major factor when analyzing highly perishable foods before releasing to the public (Hill, 1988). It provides the food industry with the opportunity to reduce economical losses by designing adequate intervention measures. Rapid and highly specific methods like PCR to detect aflatoxigenic fungal strains are needed to replace traditional methods. However, the presence of toxigenic molds in a product does not automatically mean the presence of mycotoxins, especially if growth has not occurred, but it gives an insight to the possible production of toxins by these fungi under favorable environmental conditions. PCR methods are generally rapid and easy to execute, only a small amount of DNA is required, which does not necessarily need to be highly purified (Jos *et al.* 1996). Farber, (1997) has reported the amplification of *nor*-1, *ver*-1, and *omt*-A genes of aflatoxigenic *A. flavus* in contaminated figs. Similarly, Shapira et al., (1996) and Geisen et al., (1996) described the amplification of *ver*-1, *omt*-1 and *apa*-2 of *A. flavus* in grains by PCR. There are the few reports on the detection of aflatoxigenic fungi using PCR technique. In this study, the PCR technique to selectively distinguish aflatoxigenic fungi in spice samples is described.

7.2 Detection of aflatoxigenic fungi ; by molecular method – PCR

7.2.1 Determination of the specificity of the primer pair

The primer pair designed for the O-methyltransferase gene (as in section 3.26.1) was tested for its specificity, as the detection of aflatoxigenic Aspergilli was most important. Various genera of fungi were screened by PCR using omt primer pair. The fungal isolates belonging to the genus *Rhizopus, Fusarium, Penicillium, Aspergillus ochraceus, A.niger* that did not produce aflatoxins did not show amplification product in PCR. Amplification was also not observed with non-aflatoxigenic *Aspergillus species*, thus showing the specificity of the *omt* primer pair for aflatoxigenic strains as shown in figure-13.

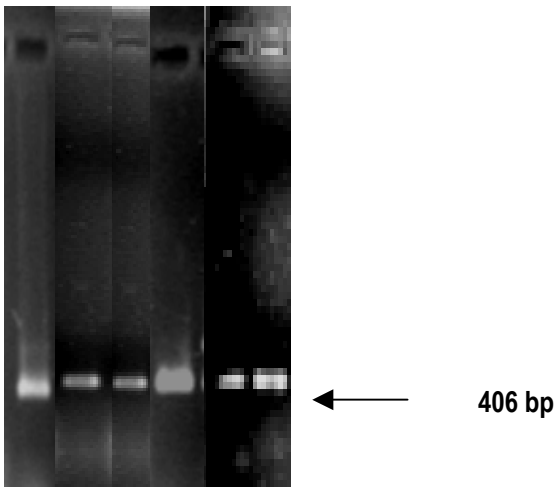


Fig.13. PCR product of *Omt* Gene for DNA of aflatoxigenic fungi

Lanes: 1. *Aspergillus flavus* NCIM 645
2. *A.parasiticus* CFR 223
3. *A.flavus* ATCC 46283
4. A.*flavus* MTCC 152
5. *A.flavus* NCIM No.538
6. *A.flavus* NCIM No.554

Table.31. PCR amplification of aflatoxigenic fungi

Sl. No.	Source	Organism	Aflatoxin production in modified czapek dox medium				Amplification with omt primer
			B_1	B_2	G_1	G_2	
1	Ginger	*A. flavus*	+	-	-	-	+
2	Turmeric	*A. flavus*	+	-	-	-	+
3	Turmeric	*A. flavus*	+	-	-	-	+
4	Ginger	*A. parasiticus*	+	+	+	+	+
5	Ginger	*A. parasiticus*	+	+	+	+	+
6	Large cardamom	*A. flavus*	+	-	-	-	+
7	Standard strain	A.parasiticus CFR 223	+	+	+	+	+
8	Large cardamom	*A. flavus*	+	-	-	-	+
9	Large cardamom	*A. flavus*	+	-	-	-	+
10	Large cardamom	*A. flavus*	+	-	-	-	+
11	Standard strain	*A. parasiticus*	+	+	+	+	+
12	Standard strain	*Aspergillus*	+	+	+	+	+
13	Standard strain	*A. flavus* ATCC 46283	+	+	-	-	+
14	Chilli	*A. flavus*	+	+	-	-	+
15	Chilli	*Aspergillus*	+	+	-	-	+
16	Standard strain	*A. flavus* NCIM 538	+	+	-	-	+
17	Chilli	*Aspergillus*	+	+	-	-	+

Table.32. Survey of market samples

Commodity	Mycoflora (cfu/g)		Dominant Genus	PCR amplification
	A. flavus group	Total fungi		
Chilli (C-1)	$10x10^4$	$42x10^4$	*A. niger* ($28x10^4$) *Rhizopus* ($1x10^4$)	+
Chilli (C-2)	$16x10^4$	$47x10^4$	*A.niger* ($21x10^4$) *A.niger* ($3X10^2$)	+
Chilli (C-4)	$1x10^2$	$10x10^2$	*Rhizopus* ($1X10^2$)	-
Chilli (C-5)	$3x10^4$	$23x10^4$	*A. niger* ($5x10^4$) *Rhizopus* ($1x10^4$)	+
Ginger (G-5)	$3x10^4$	$14x10^4$	*Cladosporium* ($1x10^4$) *Penicillium* ($4x10^4$)	+
Ginger (G-8)	$3.5x10^4$	$3.5x10^4$	Nil	+
Ginger (G-10)	$2.5x10^2$	$4.5x10^2$	Nil	-
Ginger (G-6)	$<10^2$	$13.5x10^2$	Nil	-
Turmeric (T-1)	$1.5x10^2$	$9.5x10^2$	Nil	-
Turmeric (T-2)	$1x10^2$	$2x10^2$	Nil	-
Turmeric (T-7)	$1.5x10^2$	$3.5x10^2$	Nil	-
Turmeric (T-3)	$5x10^4$	$12x10^4$	*A. niger* ($2x10^4$) *Rhizopus*($1x10^4$	+

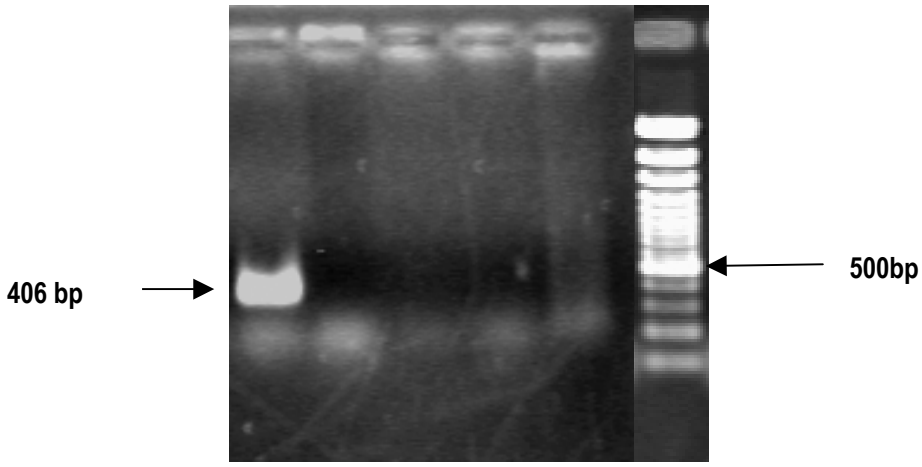


Fig.14. PCR product of *omt* gene for Genomic DNA from various fungi
Lanes: 1. *Aspergillus flavus* ATCC 46283
2. *A.oryzae*
3. *A.niger*
4. *Fusarium spp.,*
5. *Rhizopus spp.,*
6. DNA – Molecular weight marker

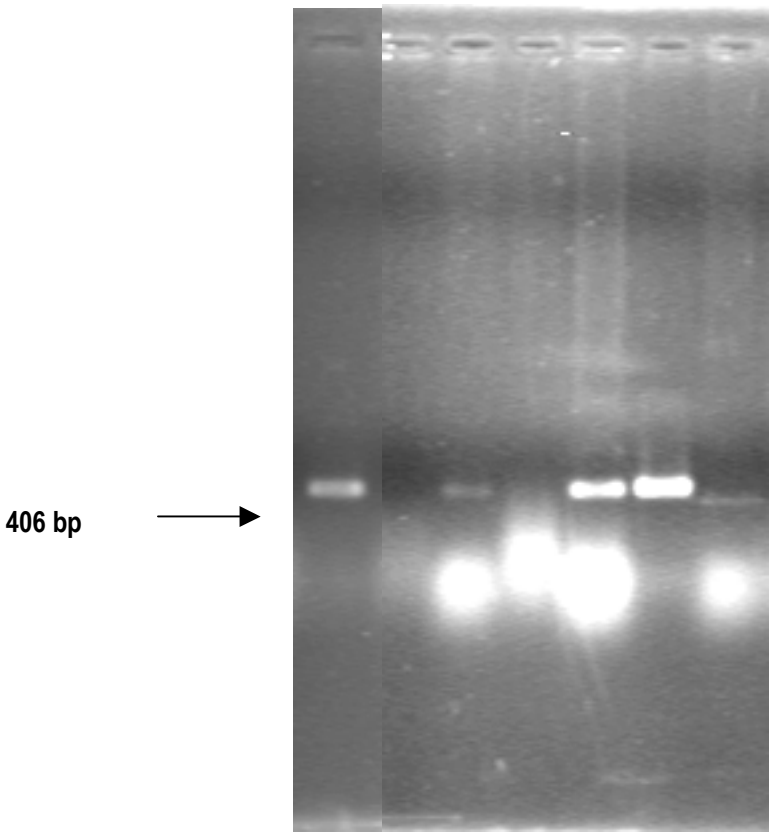


Fig.15. PCR Product of *Omt* gene for fungal DNA of market spice samples
Lanes: 1. Large Cardamom
2. Negative control
3. Turmeric
4. Chilli
5. Chilli
6. *A.flavus* ATCC 46283
7. Ginger

7.2.2 Screening of aflatoxigenic fungi by PCR

Aflatoxin producing strains of *A.flavus* and *A.parasiticus* isolated from food commodities were screened for the presence of *omt* gene by PCR (as in section 3.26.2). Out of 17 aflatoxigenic strains of *Aspergillus* screened, four isolates of *A. parasiticus* were found to produce all four types of aflatoxins (Table- 31). Seven strains were found to produce only aflatoxin B_1.

Five isolates namely *A. flavus* ATCC 46283, *A. flavus* MTCC 152, *A. flavus* NCIM 538, *A. flavus* NCIM 554 and *A. flavus*, a food isolate were found to produce both aflatoxin B_1 and B_2. Amplification with *omt* primer pair was observed with all aflatoxin positive isolates (Table-31). The PCR reaction of isolated strains was *in par* with standard strains and the size of the amplicon was 406bp in both standard and isolated strains. (Fig.13).

7.2.3 Detection of aflatoxigenic strains in market samples

DNA extracted from contaminated food samples such as large cardamom, chilli, ginger, turmeric and spice mix sample following enrichment on PDB for 24 hours showed amplification of *omt* gene (Fig.14). A few of the market samples did not give positive amplification with *omt* primer pair which was *in par* with the total plate count that indicated the absence of aflatoxigenic Aspergilli but showed the presence of some other molds (Table-32). The presence of these contaminating microflora did not give any interference in the form of either inhibition or non-specific amplification (Fig.15). This indicated that the *omt* gene primer pair was very specific and amplified only the aflatoxigenic Aspergilli.

7.3 Discussion

The counterparts for mycotoxigenic fungi are the genes which code for the enzymes of the mycotoxin biosynthetic pathway often referred to as mycotoxin biosynthetic genes. However, to date only some of the genes of mycotoxin biosynthetic pathways have been cloned and sequenced. The best analyzed biosynthetic pathways at the genetic level are those of the aflatoxins (Trail et al., 1995a ; Yu et al., 1995), the trichothecenes (Mc. Cormick et al., 1996), the patulin (Beck et al.,1990) ; Wang et al., (1991), PR-toxin (Procter and Hohn, 1993) and of sterigmatocystin (Kelkar et al., 1996). Sterigmatocystin is a precursor of aflatoxin and the sterigmatocystin biosynthetic genes are rather homologous to the aflatoxin biosynthetic genes (Brown et al., 1996). Genes for biosynthetic enzymes of secondary metabolites are usually clustered (Hohn et al., 1993) and this is true for the aflatoxin (Trial et al., 1995a; Yu et al., 1995), sterigmatocystin (Brown et al., 1996), trichothecene (Hohn et al.,

1993) and fumonisin biosynthetic genes (Desjardius, 1996). The polyketide synthase gene from *Penicillium* patulin, which is the key enzyme in the biosynthetic pathway of the mycotoxin, has been cloned (Beck et al., 1990). Polyketide synthases are often involved in fungal secondary metabolism and can be found in various species (Hopwood and Sherman, 1990). The polyketide synthases are composed of different catalytic domains, each specific to a certain reaction. These domains, however, often share high sequence homology, even between species, which makes this gene unsuitable as a specific target gene for diagnostic PCR (Mayorga and Timberlake, 1992). Interestingly, the genes are organized in such a way that the gene encoding the first enzyme in the pathway is located at one end of the cluster and the other genes follow in the same order as the enzymatic reaction in the biosynthetic pathway. Due to this arrangement, the gene for norsolorinic acid reductase (nor-1) is located at one end of the cluster; the gene for sterigmatocystin-O-methyltransferase is located at the other end of the cluster. By selecting these genes as target sequences in a multiplex,PCR, the whole aflatoxin biosynthetic gene cluster can be covered, and partial deletions of the cluster can be detected (Geisen,1996). Conventional morphological methods for the detection of aflatoxigenic fungi cannot distinguish between aflatoxin producing and non-producing fungi. If the non producing phenotype is due to a deletion of the biosynthetic gene cluster or a part thereof, or to nucleotide changes at the primer binding sites, the PCR approach is able to distinguish between both genetic alterations. The pattern of the aflatoxigenic *A.flavus* strains was identical to that of *A.parasiticus* strains, indicating the homology of the aflatoxin biosynthetic genes in both species, which is also described in the literature (Yu et al., 1995). Non-aflatoxigenic *A.flavus* gave variable results. One strain showed no signal at all, indicating a complete or nearly complete deletion of the aflatoxin biosynthetic gene cluster. Another strain exhibited a doublet biosynthetic pattern the band for the *Omt-A* gene was missing, suggesting a deletion of the gene cluster containing the genes for the last reactions in the biosynthetic pathway. A third strain possessed all three PCR DNA product bands, demonstrating another type of mutation, perhaps in one of the regulatory genes. Most of the non-aflatoxigenic strains which were analyzed showed changes in the triplet pattern, suggesting that their phenotype was due to deletions of the aflatoxin biosynthetic genes. Shapiro et al (1996) described a similar diagnostic PCR method for the detection of aflatoxigenic fungi. These target genes; the *Omt-1* gene, the Ver-1, and the apa-2 gene, from the aflatoxin biosynthetic gene cluster were used in three separate monomeric PCRs. The apa-2 (now named as aflR) gene is a regulatory gene, influencing the expression of the other aflatoxin biosynthetic genes (Chang et al., 1993). In contrast to the results

described by Geisen (1996), no strong positive PCR signals were found with DNA from aflatoxigenic *A.flavus* strains. After 30 cycles a weak signal for the *Omt-1* gene appeared, and after 40 cycles, a weak band for the Ver-1 gene became visible with DNA from *A.flavus* as the template, but a reaction with the aflR specific primer pair failed. The sequences for the aflR genes of *A.flavus* and *A.parasiticus* are highly homologous, but show distinct differences according to chang et al (1995). These sequence dissimilarities might be responsible for the failure to detect the aflR gene in *A.flavus* by PCR. Shapiro et al (1996) could not identify the Omt-1 gene in *A.nidulans*, a potential sterigmatocystin producing species, and they argued that no Omt-1 gene was present. The PCR approach described by Geisen (1996) however, demonstrates that at least in *A.versicolor*, an Omt-1 homologous sequence was present. Shapiro et al. (1996) used their PCR method for the detection of aflatoxigenic fungi in corn. They described an enrichment procedure to amplify the template DNA prior to PCR by suspending the corn sample in a rich medium; the sensitivity of the method increased with the incubation time but the detection time increased simultaneously. Only corn inoculated with aflatoxigenic *A.parasiticus* gave positive signals with the PCR and after 24 h incubation, 1x 10^{2} spores of *A.parasiticus* could be detected.

In this study specificity of *o-methyltransferase gene (omt)* has been used in the detection of aflatoxigenic *Aspergilli* in food systems. In our observation the purified DNA from *A. parasiticus* gave very good bands but weak signals were obtained from *A. flavus* DNA. No interference from spice samples were observed. However, the negative results in market samples could be correlated with the absence of aflatoxigenic strains in these spices. Rossen et al, (1992) observed that the PCR reactions with food samples often gave negative results due to interference from food components. The presences of fats and proteins in complex food samples have been described to cause interference in PCR reaction. Farber et al, (1997) described the inhibitory effects of carbohydrates in foods in PCR reaction. Difference in specificity in the detection of *Salmonella spp.,* was observed between pure cultures and from chicken meat spiked with the pathogen (Widjojoadmodjo et al., 1991). They observed 100 fold reduction in the sensitivity of the PCR reaction in spiked samples. In our studies the use of *omt* was able to detect aflatoxigenic *Aspergilli* in pure cultures and also in market samples of various spices. The interference from the food commodities was not observed in our studies. The presence of other microflora such as bacteria, yeasts and other molds did not give any inhibitory effects for non-specific amplifications, thus indicating the high specificity of *omt* primer pair. Only the toxigenic *Aspergilli* producing aflatoxin B_1, B_2, G_1 and G_2 gave positive amplification and

other non-toxigenic *A.flavus* and other fungal genera did not give any amplification with *omt* primer pair. The interference of PCR reaction from food commodities was overcome by using dilute template DNA for first PCR reaction followed by second reaction with nested PCR primers has been described (Kruetzinger et al., 1996). Modified DNA preparation protocol has been described for the detection of pathogenic *Listeria monocytogenes* in soft cheese (Lantz et al, 1994). In order to overcome problems of on-viable target microorganisms, a selective enrichment of microorganisms prior to amplification has been applied in order to increase the number of microorganisms of interest and to dilute the food components by Jarvis (1983) and Swaminathan *et al.* (1994).

Thus the PCR approach is a rapid assay method for the detection of aflatoxigenic fungi and the *omt* primer pair could be used for the specific detection of toxigenic Aspergilli in spice samples without inhibition from food and other microflora. However, the method needs to be made more sensitive to detect aflatoxigenic Aspergilli spores at $\leq 10^3$ cfu/g of food commodity which is in tune with the recommended regulatory standards for the yeast and mold count.

CONTROL OF MYCOTOXIGENIC FUNGI

Contents

8.1 Introduction

Recognition of the problems of mycotoxins in various countries led to the development of appropriate programmes for their prevention and control. These programmes formed part of the strategies to minimize the problems of mycotoxin contamination and included not only the prevention of mycotoxin formation in agricultural commodities but also their removal through detoxification or decontamination. Routine surveillance, regulatory measures to control the occurrence of mycotoxin in foods both at national and international trade as well as an information, education and communication drive formed part of these programmes. Several approaches both preharvest and post harvest are currently being tried to reduce or to eliminate mycotoxins from the food chain. Current preharvest approaches including irrigation, application of fungicides or insecticides and use of resistant or regionally adapted crop varieties fall short of effective control. Use of chemicals is often environmentally unacceptable being too costly. The search for antifungal agents especially spice oils which could safely be used as substitutes for fungicides is extensively tried and there is a renewed interest in harnessing the antimicrobial properties of spices. In vitro activities of some spice essential oils have been demonstrated in culture media. Therefore an attempt has been made to evaluate the various spice oils for their potential as antifungal agents.

8.2 Screening of spice oil for antifungal activity

8.2.1 Effect of spice oil on *A.parasiticus*

The strain of *A.parasiticus* (CFR 223) which produces all the four aflatoxins viz, B_1, B_2, G_1 and G_2, was used to evaluate various spice oils for their antifungal potential (as in section 3.27). The results on the effect of celery, chilli oleoresin, ginger, large cardamom, nutmeg, pepper and turmeric oil on mycelial growth and aflatoxin production by *A.parasiticus* (CFR 223) at different concentrations is presented in the table-33. The sample untreated with spice oil served as control and results of various treatments at different concentrations (0.002 – 0.02 %) are presented in comparison with control. The oil of celery was able to inhibit mycelial growth between 9.0 - 59.0 % at the range of oil tested. It had an inhibitory effect on production of aflatoxin B series (B_1 17.0 - 68.00; B_2 89 – 100.0 %) and aflatoxin G (G_1 & G_2) series was inhibited completely. Chilli oleoresin inhibited mycelial growth (10.0 - 23.0), production of aflatoxin B_1 (5.0 - 20.0), B_2 (22.0 - 60.0), G_1 (6.0 – 26.0) and G_2 (20.0 - 100.0) %. Ginger oil inhibited maximum of mycelial growth by 38.0 %, toxin production by B_1 (23.0), B_2 (100.0), G_1 (40.0) and G_2 (100.0) %. Large cardamom inhibited mycelial growth (9.0 - 92.0), B_1 (17.0 - 100.00), B_2 and G_1 (67.0 – 100.0) and G_2 (100.0) %. Nutmeg oil had the least inhibitory effect as it

Table.33. Effect of spice oil on *Aspergillus parasiticus* (CFR 223)

Name of spice oil		Spice oil concentration (%)					
		0.002	0.004	0.006	0.008	0.01	0.02
Celery	1	9.0	22.0	26.0	43.0	52.0	59.0
	2	17.0	33.0	50.0	50.0	67.0	68.0
	3	89.0	96.0	100	100	100	100
	4	100	100	100	100	100	100
	5	100	100	100	100	100	100
Chilli oleoresin	1	0.0	0.0	10.0	12.0	16.0	23.0
	2	0.0	0.0	5.0	8.0	10.0	20.0
	3	0.0	0.0	22.0	26.0	32.0	60.0
	4	0.0	0.0	6.0	10.0	23.0	26.0
	5	0.0	0.0	20.0	30.0	60.0	100
Ginger	1	0.0	0.0	2.0	14.0	20.0	38.0
	2	0.0	0.0	0.0	12.0	18.0	23.0
	3	0.0	0.0	0.0	80.0	90.0	100
	4	0.0	0.0	0.0	10.0	26.0	40.0
	5	2.0	0.0	0.0	86.0	100	100
Large cardamom	1	9.0	13.0	35.0	43.0	78.0	92.0
	2	17.0	17.0	17.0	33.0	80.0	100
	3	67.0	80.0	96.0	100	100	100
	4	67.0	67.0	80.0	100	100	100
	5	100	100	100	100	100	100
Nutmeg	1	0.0	0.0	0.20	0.40	1.0	1.0
	2	0.0	2.0	5.0	5.0	6.0	6.0
	3	0.0	20.0	20.0	23.0	33.0	50.0
	4	0.0	1.0	1.0	6.0	8.0	20.0
	5	0.0	1.0	6.0	12.0	18.0	20.0
Pepper	1	12.0	17.50	22.0	39.0	57.0	78.0
	2	6.0	18.0	36.0	80.0	100	100
	3	23.0	67.0	81.0	88.8	100	100
	4	16.0	10.0	18.0	80.0	100	100
	5	25.0	85.0	90.0	98.0	100	100
Turmeric	1	18.0	30.0	39.0	47.0	48.0	84.0
	2	45.0	62.0	67.0	83.0	91.0	100
	3	88.0	89.0	90.0	100	100	100
	4	100	100	100	100	100	100
	5	100	100	100	100	100	100

Note: 1 = % inhibition of growth of *A.parasiticus*,
2 = % inhibition of B_1 production,
3 = % inhibition of B_2 production,
4 = % inhibition of G_1 production,
5 = % inhibition of G_2 production

Table.34. Effect of spice oil on *Aspergillus flavus* (ATCC 46283)

Name of spice oil		Spice oil concentration (%)					
		0.002	**0.004**	**0.006**	**0.008**	**0.01**	**0.02**
Celery	1	30.0	30.0	32.00	40.00	50.0	70.00
	2	67.0	67.0	75.0	75.0	75.0	87.0
	3	87.0	100	100	100	100	100
Chilli oleoresin	1	0.0	8.00	8.0	10.0	16.0	22.0
	2	4.0	5.0	12.0	14.0	17.0	17.0
	3	0.0	0.0	17.0	30.0	33.0	33.0
Cinnamon	1	5.0	5.0	8.0	15.0	32.00	74.0
	2	17.0	17.0	17.0	58.0	100	100
	3	67.0	80.0	100	100	100	100
Clove	1	96.0	100	100	100	100	100
	2	100	100	100	100	100	100
	3	100	100	100	100	100	100
Cumin	1	100	100	100	100	100	100
	2	100	100	100	100	100	100
	3	100	100	100	100	100	100
Ginger	1	0.0	2.0	8.0	19.0	29.0	33.0
	2	17.0	22.0	33.0	63.0	83.0	90.0
	3	100	100	100	100	100	100
Large cardamom	1	23.0	23.0	24.0	26.0	34.0	61.0
	2	17.0	17.0	17.0	33.0	33.0	61.0
	3	65.0	86.0	90.0	100	100	100
Nutmeg	1	0.0	0.0	0.0	8.0	17.0	17.0
	2	0.0	0.0	0.0	12.0	33.0	37.0
	3	0.0	0.0	0.0	100	100	100
Pepper	1	39.0	42.0	48.0	50.0	55.0	80.0
	2	17.0	17.0	17.0	58.0	92.0	100
	3	33.0	68.0	87.0	93.0	97.0	100
Small cardamom	1	20.0	20.0	20.0	23.0	31.0	35.0
	2	19.0	25.0	25.0	25.0	25.0	67.0
	3	33.0	42.0	63.0	85.0	95.0	100
Turmeric	1	21.0	41.0	46.0	50.0	53.0	80.0
	2	17.0	58.0	58.0	67.0	83.0	100
	3	67.0	68.0	83.0	91.0	100	100

Note: 1 = % inhibition of growth of *A.flavus*,
2 = % inhibition of B_1 production,
3 = % inhibition of B_2 production.

Table.35. Effect of spice oil on *Aspergillus ochraceus* (CFR 221)

Name of spice oil		Spice oil concentration (%)					
		0.002	**0.004**	**0.006**	**0.008**	**0.01**	**0.02**
Celery	1	38.0	44.0	55.0	61.0	64.0	66.0
	2	40.0	40.0	60.0	60.0	100	100
Chilli oleoresin	1	0.0	9.0	13.0	15.0	18.0	25.0
	2	0.0	33.0	57.0	60.0	60.0	80.0
Cinnamon	1	100	100	100	100	100	100
	2	100	100	100	100	100	100
Clove	1	45.0	67.0	82.0	89.0	97.0	100
	2	60.0	83.0	100	100	100	100
Cumin	1	36.0	61.0	76.0	81.0	92.0	100
	2	26.0	100	100	100	100	100
Ginger	1	0.0	0.0	14.0	16.0	18.0	23.0
	2	0.0	0.0	33.0	62.0	70.0	80.0
Large cardamom	1	56.0	62.0	71.0	84.0	90.0	90.0
	2	10.0	16.0	32.0	41.0	60.0	81.0
Nutmeg	1	0.0	0.0	0.20	2.0	6.0	12.0
	2	0.0	0.0	2.0	10.0	18.0	27.0
Pepper	1	20.0	36.0	52.0	61.0	81.0	100
	2	8.0	22.0	60.0	88.0	96.0	100
Small cardamom	1	80.0	88.0	90.0	91.0	96.0	100
	2	40.0	50.0	60.0	64.0	80.0	100
Turmeric	1	30.0	45.0	50.0	52.0	54.0	100
	2	60.0	62.0	62.0	80.0	80.0	100

Note: 1 = % inhibition of growth of *A.ochraceus*,
2 = % inhibition of OTA production,

could inhibit growth only up to 1.0 % at the concentration of 0.02 %. Pepper oil inhibited mycelial growth (12.0 - 78.0) and could completely inhibit aflatoxins. Turmeric oil inhibited mycelial growth by 17.0 - 84.0, B_1 > 45.0 %, B_2 > 88 % and G_1 and G_2 were completely inhibited. Inhibitory effect of chilli oleoresin, ginger and nutmeg were comparatively less than that of celery oil (Table-33). At higher concentration of 0.02 %, the fungal inhibition was only 23.0, 38.0 and 1.0 in chilli oleoresin, ginger and nutmeg respectively.

8.2.2 Effect of spice oil on *A.flavus*

The effect of various oils on the growth and aflatoxin B_1 and B_2 production by *Aspergillus flavus* (ATCC 46283) is presented in the table-34. It reveals the results on the effect of celery, chilli oleoresin, ginger, large cardamom, nutmeg, pepper and turmeric oil mycelial growth and aflatoxin B_1 and B_2 production. Celery oil was found to inhibit maximum mycelial growth up to 70.0 %, production of B_1 (87.0) and completely inhibited B_2 at the concentration of 0.02 %. Chilli oleoresin inhibited maximum mycelial growth (22.0), B_1 (17.0) and B_2 (34.0) % at the concentration 0.02 %. The cinnamon oil inhibited maximum of mycelial growth (74.0 % and completely inhibited aflatoxin at the highest concentration of 0.02 %. Clove as well as cumin oil gave maximum inhibition of mycelial growth, B_1 and B_2 by 100.0 % at the concentration level of 0.004 %. The ginger oil inhibited maximum of mycelial growth up to 33.0 %, B_1 90.0 % by inhibiting B_2 completely at the concentration of 0.02 %. Large cardamom oil was able to inhibit maximum of mycelial growth (61.0), B_1 (61.0) and B_2 (100.0) %, at the concentration 0.02 %. Nutmeg oil inhibited maximum of mycelial growth (17.0), B_1 (37.0) and B_2 completely at the concentration 0.02 %. Pepper oil inhibited mycelial growth (80.0), B_1 and B_2 completely at the concentration 0.02 %. Small cardamom oil was able to inhibit maximum of mycelial growth (35.0), B_1 (67.0) and B_2 (100.0) % at the concentration 0.02 %. The oil of turmeric inhibited maximum of mycelial growth at 80.0 % and completely inhibited B_1 and B_2 at the concentration 0.02 %.

8.2.3 Effect of spice oil on *A.ochraceous*

Similar trend was noticed when spice oils were tested against *Aspergillus ochraceus* for their effect on mycelial growth and ochratoxin A production by *Aspergillus ochraceus* (CFR 221) and the results is presented in table-35. Oils of celery, Chilli oleoresin, ginger, large cardamom and nutmeg, inhibited maximum of mycelial growth by 66.0, 25.0, 23.0, 90.0 and 12.0 % respectively. On the other hand the inhibitory effect was more drastic on the production of ochratoxin A showing complete inhibition with celery oil, 80.0 % inhibition with ginger and large cardamom and 27 % with nutmeg at

Table.36. Effect of spice oil on *Fusarium sporotrichoides* (MTCC1894)

Name of spice oil		Spice oil concentration (%)					
		0.002	**0.004**	**0.006**	**0.008**	**0.01**	**0.02**
Celery	1	14.0	29.0	29.0	29.0	43.0	43.0
	2	14.0	23.0	27.0	87.0	100	100
	3	27.0	77.0	100	100	100	100
Chilli oleoresin	1	8.0	11.0	15.0	16.0	26.0	36.0
	2	62.0	82.0	88.0	98.0	100	100
	3	79.0	91.0	100	100	100	100
Cinnamon	1	20.0	40.0	48.0	50.0	66.0	72.0
	2	61.0	100	100	100	100	100
	3	100	100	100	100	100	100
Clove	1	33.0	44.0	44.0	100	100	100
	2	75.0	97.0	100	100	100	100
	3	17.0	50.0	100	100	100	100
Cumin	1	16.0	25.0	29.0	32.0	36.0	47.0
	2	95.0	98.0	98.0	100	100	100
	3	65.0	95.0	99.0	100	100	100
Ginger	1	5.5	14.0	30.0	37.0	46.0	66.0
	2	62.0	71.0	72.0	75.0	78.0	86.0
	3	91.0	92.0	100	100	100	100
Large cardamom	1	0.0	12.0	19.0	25.0	25.0	50.0
	2	61.0	64.0	69.0	72.0	98.0	100
	3	45.0	56.0	100	100	100	100
Nutmeg	1	0.3	0.3	1.0	2.0	3.0	9.0
	2	0.0	0.0	0.0	5.0	17.0	22.0
	3	0.0	0.0	0.0	2.0	4.0	32.0
Pepper	1	18.0	27.0	27.0	34.0	56.0	71.0
	2	96.0	99.0	99.0	100	100	100
	3	86.0	88.0	95.0	96.0	97.0	98.0
Small cardamom	1	11.0	13.0	22.00	26.0	33.0	50.0
	2	0.30	4.0	45.0	87.0	94.0	100
	3	20.0	52.0	71.0	100	100	100
Turmeric	1	10.0	20.0	34.0	45.0	57.0	64.0
	2	99.0	100	100	100	100	100
	3	84.0	100	100	100	100	100

Note: 1 = % inhibition of growth of *F.sporotrichoides*,
2 = % inhibition of T-2 production,
3 = % inhibition of DAS production.

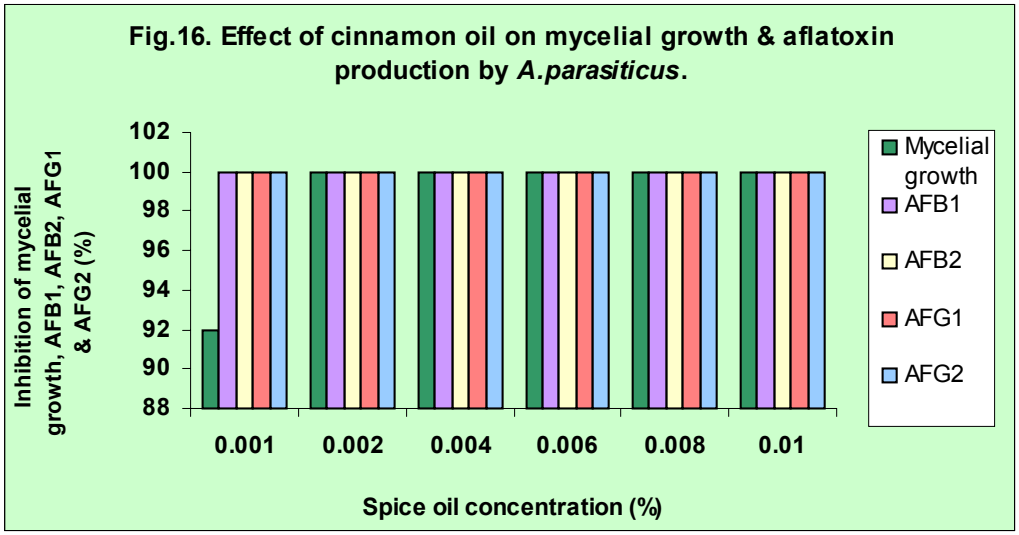


Fig.16. Effect of cinnamon oil on mycelial growth & aflatoxin production by A.parasiticus.
Inhibition of mycelial growth, AFB1, AFB2, AFG1 & AFG2 (%)
102
100
98
96
94
92
90
88
0.001
0.002
0.004
0.006
0.008
0.01
Spice oil concentration (%)
Mycelial growth
AFB1
AFB2
AFG1
AFG2

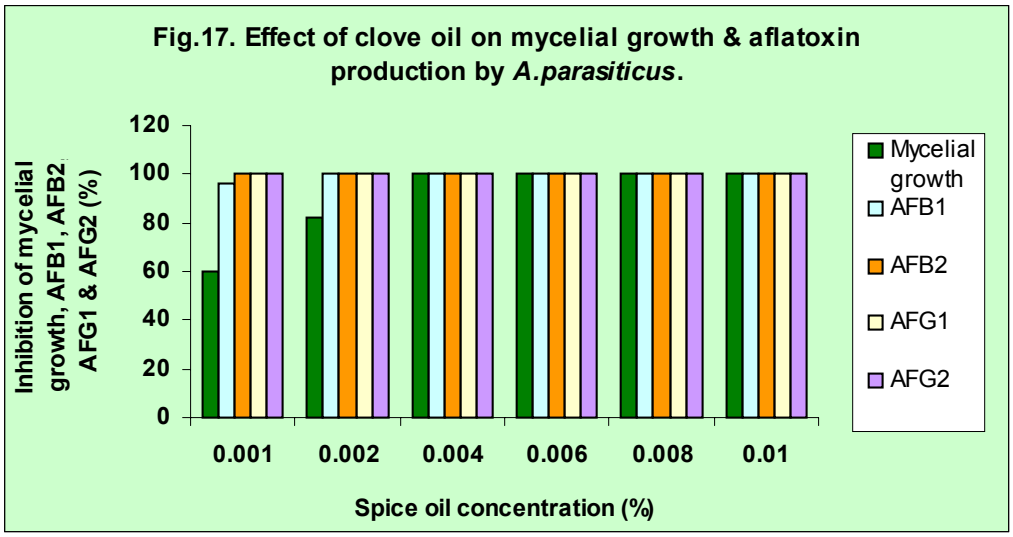


Fig.17. Effect of clove oil on mycelial growth & aflatoxin production by A.parasiticus.
Inhibition of mycelial growth, AFB1, AFB2 AFG1 & AFG2 (%)
120
100
80
60
40
20
0
0.001
0.002
0.004
0.006
0.008
0.01
Spice oil concentration (%)
Mycelial growth
AFB1
AFB2
AFG1
AFG2

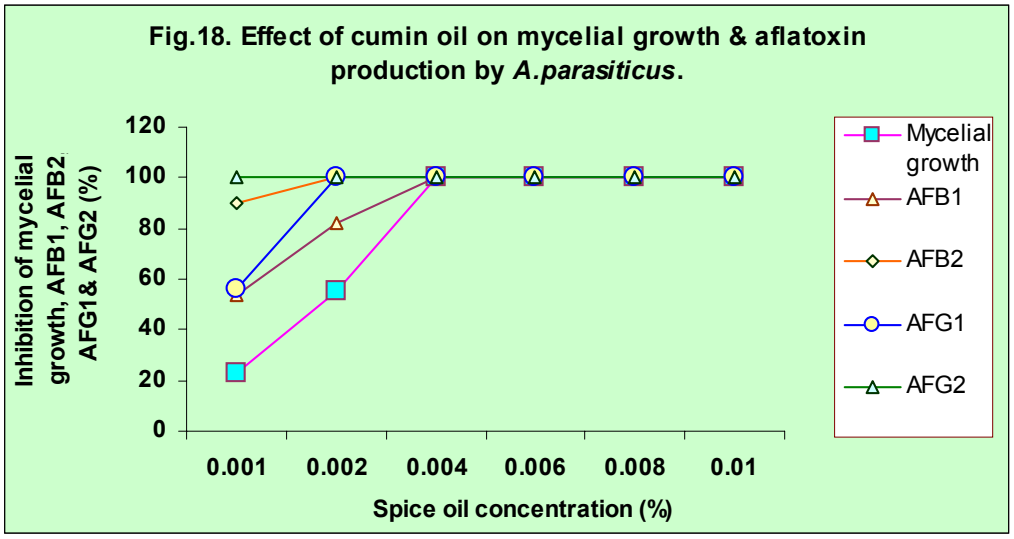


Fig.18. Effect of cumin oil on mycelial growth & aflatoxin production by A.parasiticus.
Inhibition of mycelial growth, AFB1, AFB2 AFG1& AFG2 (%)
120
100
80
60
40
20
0
0.001
0.002
0.004
0.006
0.008
0.01
Spice oil concentration (%)
Mycelial growth
AFB1
AFB2
AFG1
AFG2

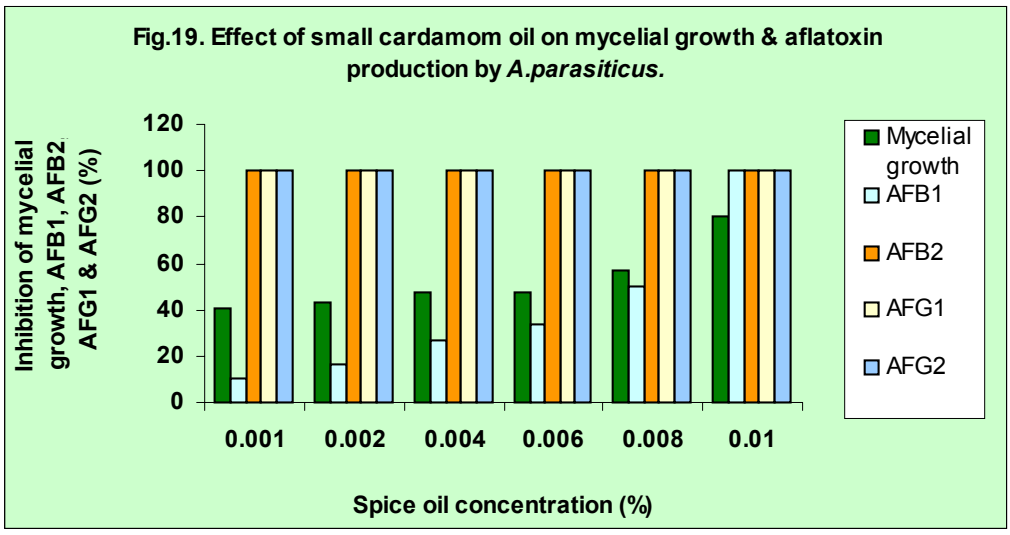


Fig.19. Effect of small cardamom oil on mycelial growth & aflatoxin production by A.parasiticus.
Inhibition of mycelial growth, AFB1, AFB2 AFG1 & AFG2 (%)
120
100
80
60
40
20
0
0.001
0.002
0.004
0.006
0.008
0.01
Spice oil concentration (%)
Mycelial growth
AFB1
AFB2
AFG1
AFG2

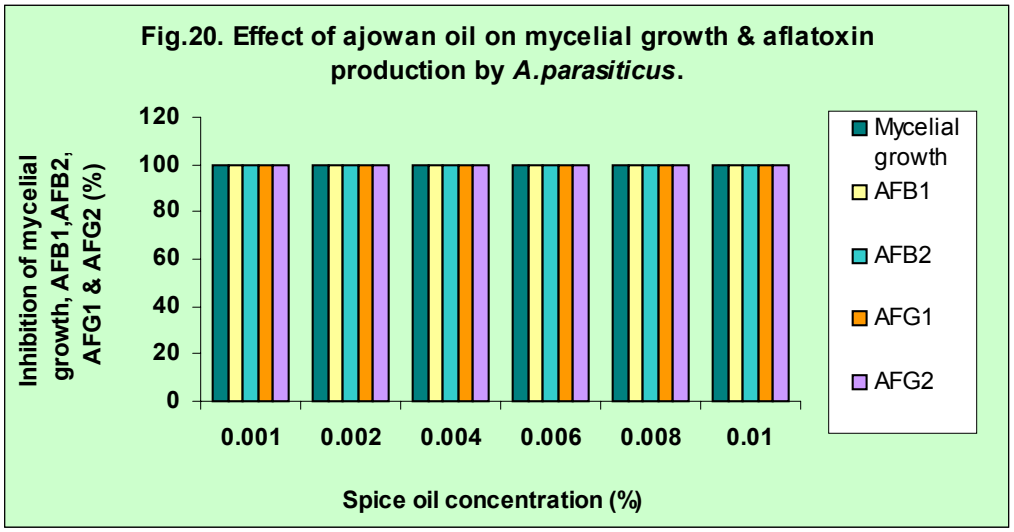


Fig.20. Effect of ajowan oil on mycelial growth & aflatoxin production by A.parasiticus.
Inhibition of mycelial growth, AFB1,AFB2, AFG1 & AFG2 (%)
120
100
80
60
40
20
0
0.001
0.002
0.004
0.006
0.008
0.01
Spice oil concentration (%)
Mycelial growth
AFB1
AFB2
AFG1
AFG2

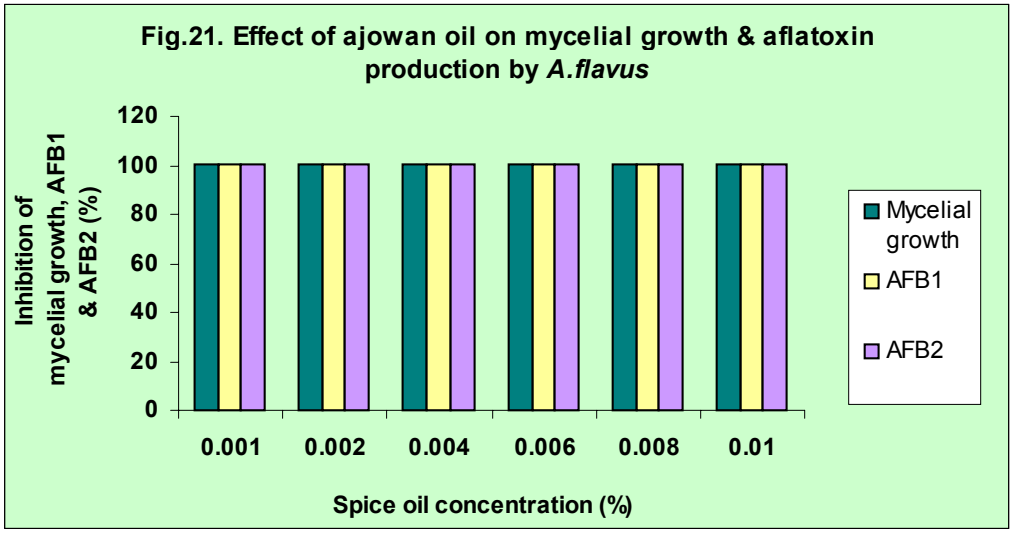


Fig.21. Effect of ajowan oil on mycelial growth & aflatoxin production by A.flavus
Inhibition of mycelial growth, AFB1 & AFB2 (%)
120
100
80
60
40
20
0
0.001
0.002
0.004
0.006
0.008
0.01
Spice oil concentration (%)
Mycelial growth
AFB1
AFB2

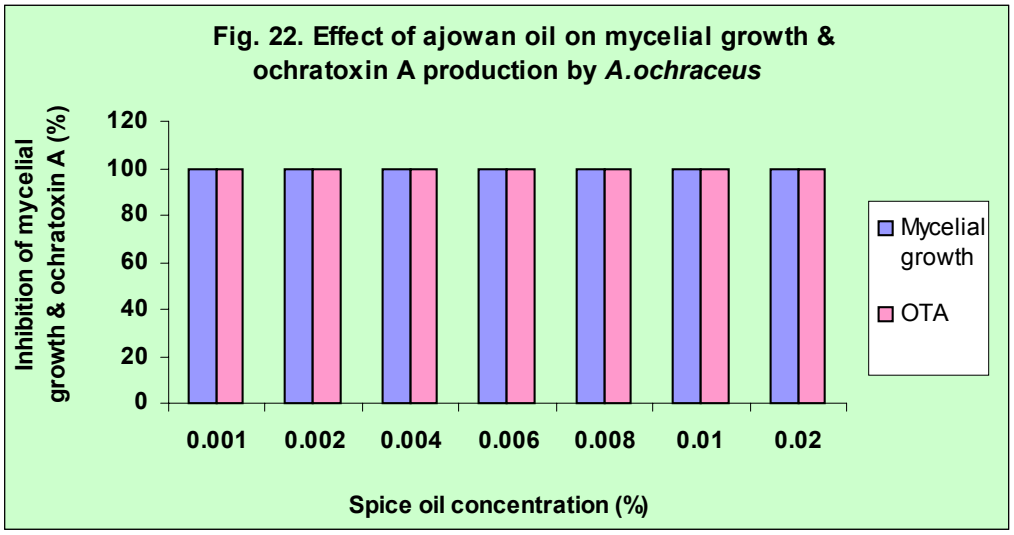


Fig. 22. Effect of ajowan oil on mycelial growth & ochratoxin A production by A.ochraceus
Inhibition of mycelial growth & ochratoxin A (%)
120
100
80
60
40
20
0
0.001
0.002
0.004
0.006
0.008
0.01
0.02
Spice oil concentration (%)
Mycelial growth
OTA

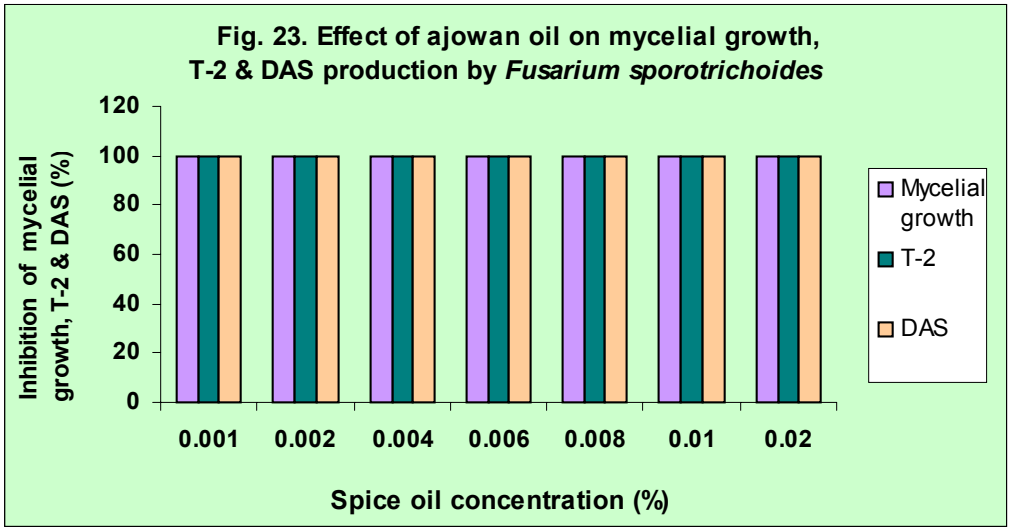


Fig. 23. Effect of ajowan oil on mycelial growth, T-2 & DAS production by Fusarium sporotrichoides
Inhibition of mycelial growth, T-2 & DAS (%)
120
100
80
60
40
20
0
0.001
0.002
0.004
0.006
0.008
0.01
0.02
Spice oil concentration (%)
Mycelial growth
T-2
DAS

1 2 3 4 5 6

Fig.24. Culture flask with YES medium showing inhibition of *Aspergillus flavus* due to ajowan oil at various concentration (no.1 to 5 and 6.control).

1 2 3 4 5 6

Fig.25. Culture flask with GYEP medium showing inhibition of *Fusarium sporotrichoides* due to ajowan oil at various concentrations (no.1 to 5 and 6. control).

Fig.26. Effect of Ajowan oil on mycelial growth & aflatoxin production by *A.parasiticus* at various time interval

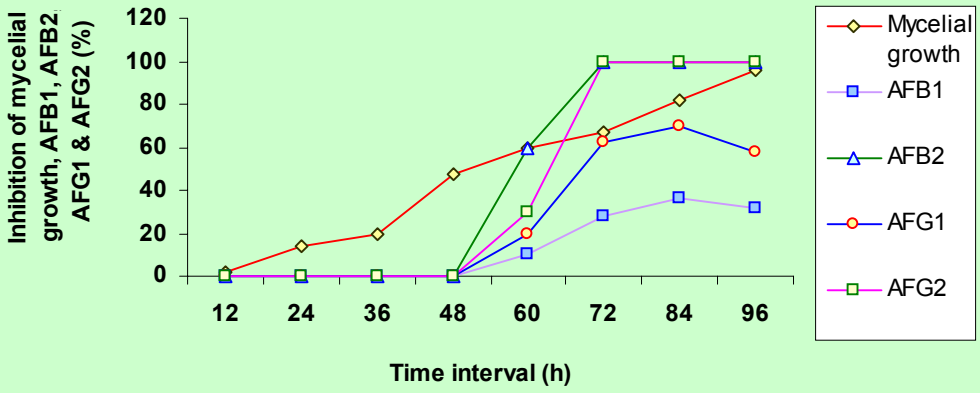


Fig.27. Effect of Ajowan oil on mycelial growth & aflatoxin production by *A.flavus* at various time interval

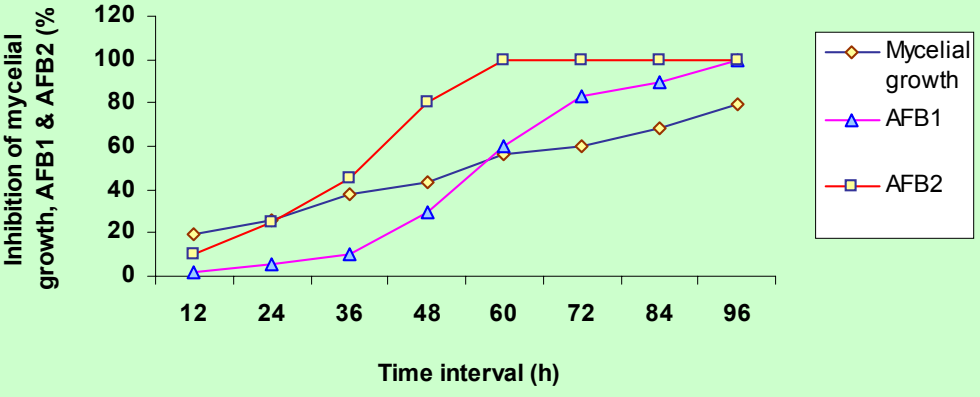
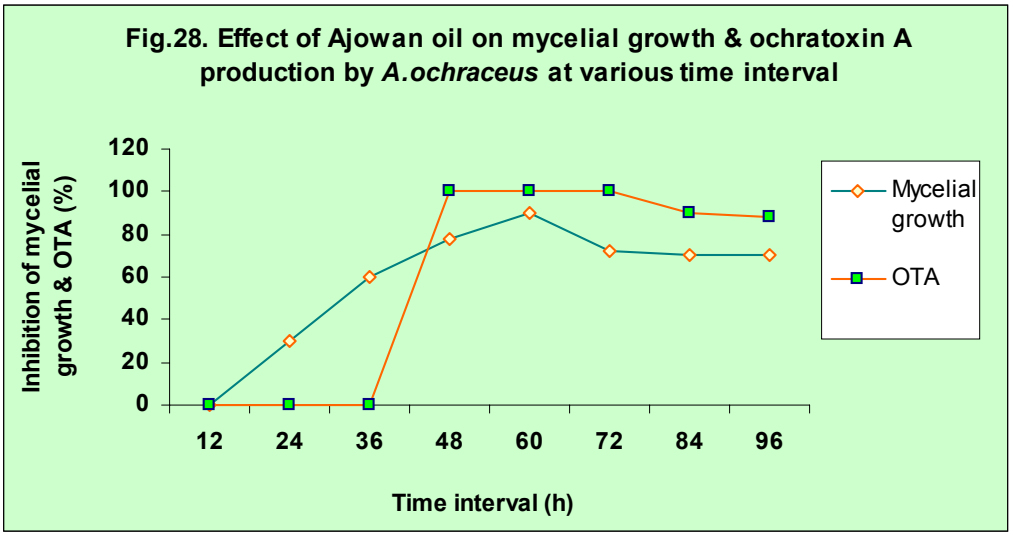


Fig.28. Effect of Ajowan oil on mycelial growth & ochratoxin A production by *A.ochraceus* at various time interval



the highest oil concentration of 0.02 %. The oils of cinnmon, clove, cumin, pepper, small cardamom and turmeric oil inhibited both mycelial growth and ochratoxin A completely at 0.02 %. At highest level of 0.02 % oil concentration, mycelial growth inhibition of 66.0, 25.0, 23.0, 90.0 and 12 % was achieved with the oils of celery, chilli oleoresin, ginger, large cardamom and nutmeg.

8.2.4 Effect of spice oil on *Fusarium sporotrichoides*

The strain of *Fusarium sporotrichoides* (MTCC1894) produces T-2 toxin and DAS was used to evaluate the effect of various spice oils against on mycelial growth, T-2 toxin and DAS production. The results of the study is presented in the table-36. Maximum mycelial growth inhibition of 43.0, 36.0, 72.0, 46.0, 66.0, 50.0, 9.0, 71.0, 50.0 and 64.0 % was recorded from the samples treated with oils like celery, chilli oleoresin, cinnamon, cumin, ginger, large cardamom, nutmeg, pepper, small cardamom and turmeric respectively at 0.02 % concentration. Oils of ginger, nutmeg and pepper inhibited T-2 toxin and DAS production by 86.0, 22.0, 91.0 and 100.0, 32.0, 98.0 % respectively. Where as other oils such as celery, chilli oleoresin, cinnamon, cumin, large cardamom, small cardamom and turmeric inhibited T-2 toxin and DAS production by 100.0 % at 0.02 % concentration. Clove oil completely inhibited mycelial growth, T-2 toxin and DAS production at 0.008 % itself.

The oils such as clove, cinnamon and cumin which gave complete inhibition of mycelial growth at 0.002 % concentration, were further evaluated at lower concentration of 0.001 % level for their efficacy. The figure 16 reveals the effect of cinnamon oil at various concentrations on mycelial growth and aflatoxin viz, B_1, B_2, G_1 and G_2, production by toxigenic strain of *Aspergillus parasiticus* (CFR 223). The oil inhibited mycelial growth by 92.0 % and complete inhibition of aflatoxins B_1, B_2, G_1 and G_2 at the concentration of 0.001 %. The sample untreated with spice oil served as control and results of treated samples are presented in comparison with control.

The figure 17 reveals the effect of clove oil at various concentrations on mycelial growth and aflatoxin viz, B_1, B_2, G_1 and G_2, production by toxigenic strain of *Aspergillus parasiticus* (CFR 223). The oil inhibited mycelial growth by 60.0 % and aflatoxin B_1 by 96 % with the complete inhibition of aflatoxin B_2, G_1 and G_2 at the concentration of 0.001 %.

The figure 18 reveals the effect of cumin oil at the concentrations mentioned above on mycelial growth and aflatoxin viz, B_1, B_2, G_1 and G_2, production by toxigenic strain of *Aspergillus parasiticus* (CFR 223). Minimum inhibition of mycelial growth by 23.0 % was observed at the 0.001 % concentration of oil. The aflatoxins B_1 and B_2 were inhibited by 53.0 and 90.0 % respectively at the

concentration of 0.001 %. However, the oil completely inhibited mycelial growth and production aflatoxins B_1, B_2, G_1 and G_2 at concentration oil 0.004 %.

The figure-19 reveals the effect of small cardamom oil at various concentrations on mycelial growth and aflatoxin viz, B_1, B_2, G_1 and G_2, production by toxigenic strain of *Aspergillus parasiticus* (CFR 223). The oil inhibited mycelial growth by 40.0 - 80.00 % at the concentrations of oil ranging between 0.001 - 0.02 %. Complete inhibition of aflatoxin B_1 was observed at concentration of 0.01 %. However, the other aflatoxins B_2, G_1 and G_2 were completely inhibited at the concentration of 0.001 %.

Ajowan oil at the lowest concentration of 0.001 % was evaluated for its efficacy towards inhibition of mycelial growth and toxin production by known mycotoxigenic fungi. The growth of fungi such as *Aspergillus flavus* (ATCC 46283), *A.parasiticus* (CFR 223), *A.ochraceus* (CFR 221) and *Fusarium sporotrichoides* (MTCC 1894) were found inhibited completely by ajowan oil at 0.001 % concentration (Fig. 20 to 23). The oils of cumin, clove and cinnamon were also found to be effective in inhibiting mycelial growth as well as toxin production next only to ajowan.

The growth inhibition of *Aspergillus flavus* due to ajowan oil at various concentration is presented in the figure - 24. The growth inhibition of *Fusarium sporotrichoides* due to ajowan oil at various concentration is shown in figure – 25.

The effect of ajowan oil at concentration of 0.001 % was tested on mycelial growth and production of aflatoxin B_1, B_2, G_1 and G_2 by toxigenic strain of *Aspergillus parasiticus* (CFR 223) at different time interval is presented in the figure.26. The ajowan oil showed a progressive inhibitory effect on the mycelial growth showing 2.0 to 96.0 % inhibition at 12 to 96 h. The production of aflatoxin B_1 was inhibited maximum by 36.0 % at 84 h and > 50 % inhibition of B_2, G_1 and G_2 production at 72 h time period (Fig. 26).

Similarly an attempt was made to assess the effect of ajowan oil at 0.001 % on mycelial growth and Aflatoxin B_1 and B_2 production by *Aspergillus flavus* (ATCC 46283) at different time interval 12, 24, 36, 48, 60, 72, 84 and 96 h. The figure 27 revealed the effect of ajowan oil at selected concentration of 0.001 %. The oil inhibited mycelial growth by 19.0 to 79.0 % at the interval of 12 – 96 h. Aflatoxins (B_1 and B_2) inhibition of > 50 % was recorded at 60 h time period.

The effect of ajowan oil at concentration mentioned above was tried with *Aspergillus ochraceus* (CFR 221 to assess the effect of oil on mycelial growth and ochratoxin A production by at various time interval between 12 – 96 h. The maximum mycelial growth inhibition of 90.0 % was

Table.37. Growth inhibition of mycotoxigenic fungi by spice oils

Spice oil (%)	Fungi *			
	A.flavus **(ATCC 46283)**	***A.parasiticus*** **(CFR 223)**	***A.ochraceus*** **(CFR 221)**	***F.sporotrichoides*** **(MTCC 1894)**
0.001	Ajowan	Ajowan	Ajowan	Ajowan
0.020	Cumin Ginger	Cinnamon Clove	Cinnamon Cumin	Cinnamon
0.040	-	Cumin	-	Turmeric
0.060	Nutmeg	-	Clove Nutmeg	Celery Clove Ginger Cardamom (small) Chilli oleoresin Nutmeg
0.080	-	-	-	Cardamom (large) Pepper
0.100	Clove	-	Celery	-

***** = 100 % inhibition of fungal growth.

Table.38 Effect of spice oils on growth of pathogenic bacteria (well assay).

Spice oil (µL/well)	Food borne pathogenic Bacteria (Z I)				
	I	II	III	IV	V
Ajowan (1)	17	17	17	17	17
Cardamom (large) (10)	_	17	17	_	17
Cardamom (small) (20)	12	_	_	_	_
Celery (20)	_	_	_	_	12
Chilli oleoresin (50)	_	_	_	_	_
Cinnamon (5)	12	_	10	17	17

Contd…

Clove (1)	_	_	9	17	17
Cumin (20)	_	_	_	_	9
Ginger (50)	_	_	_	_	_
Nutmeg (10)	_	_	9	_	8
Pepper (5)	_	_	8	_	8
Turmeric (2)	_	_	8	_	8
Control (no oil)	_	_	_	_	_

Note: I = Bacillus cereus, II = Staphylococcus aureus,
III = Listeria monocytogenes, IV = Escherichia coli,
V =Yersinia enterocolitica, - = Inhibition not noticed,
ZI = Zone of inhibition in mm, Incubation temperature - 37 ± 2^{O} C,
Incubation period – 24 h and Control: Sterile water.

recorded at 60 h. The complete inhibition of ochratoxin A was recorded between 48 to 72 h time period (Fig. 28).

All the 12 spice oils tested were able to inhibit growth of mycotoxigenic fungi comprising of *Aspergillus flavus* (ATCC 46283), *A.parasiticus* (CFR 223), *A.ochraceus* (CFR 221) and *Fusarium sporotrichoides* (MTCC 1894) completely at different concentrations when incorporated in culture medium. Amongst the spice oils, ajowan oil emerged as effective spice oil in inhibiting all the four toxigenic fungi belonging to two genera. Complete growth inhibition of *Aspergillus flavus*, *A.parasiticus* and *A.ochraceus* and *Fusarium sporotrichoides* was achieved by ajowan oil at the concentration of 0.001 %. Cinnamon oil was found effective in inhibiting *A.parasiticus, A.ochraceus* and *Fusarium sporotrichoides* at 0.002 %. The growth of *Aspergillus flavus* was also found inhibited completely by oils of cumin and ginger at 0.002 %. Nutmeg and clove oils completely inhibited the growth of *Aspergillus flavus* (ATCC 46283) and *A.parasiticus* (CFR 223) at 0.006 %. Celery, clove, ginger, small cardamom, chilli oleoresin and nutmeg oils completely inhibited *Fusarium sporotrichoides* (MTCC 1894) at 0.06 % concentrations. *A.flavus* and *A.ochraceus* were found inhibited completely by oils of clove as well as celery at 0.100 %. *A.ochraceus* was completely inhibited by cinnamon and cumin at 0.002 %. *Fusarium sporotrichoides* was found inhibited completely by oil of turmeric at 0.004 %. Large cardamom and pepper gave complete inhibition of *Fusarium sporotrichoides* at 0.008 %. Spice oils such as cinnamon, followed by cumin and clove emerged as effective inhibitors of fungal growth next to ajowan oil (Table-37).

8.3 Screening of spice oil on for antibacterial activity

The data on the inhibitory action of spice oil against food pathogenic bacteria such as *Bacillus cereus*, *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* and *Yersinia enterocolitica* by well assay is presented in table - 38. The experimental protocol for the study is presented in the section 3.28 of material and methods. Oil of ajowan was found inhibiting growth all five bacteria at the lowest concentration of 1µL/ well. In case of ajowan oil, the zone of inhibition achieved was 17 mm which is considered to be strong inhibition effect for all the bacteria tested irrespective of gram +ve or gram -ve. Large cardamom oil gave strong inhibition for bacteria such as, *Staphylococcus aureus, Listeria monocytogenes* and *Yersinia enterocolitica* at 10 µL/well. Oil of small cardamom gave inhibition of 12 mm a moderate inhibition on the growth of *Bacillus cereus* at 20 µL/well. Celery oil at 20 µL/well gave moderate inhibition of *Yersinia enterocolitica* by 12 mm. Oleoresin of chilli and ginger oil did not inhibit any of the bacteria even at highest concentration of 20 µL/well. Cinnamon oil at 5

Table.39 Effect of irradiation on fungi inoculated to chilli (powder).

Radiation (KGy/h)	Fungal flora of sample (cfu / g)				
	1	2	3	4	5
10	0	0	0	0	-
7.5	0	0	0	0	-
5.0	A - 60 O - 106	F - 20 O - 160	P - 180 O - 130	A - 20 P - 60 O – 20	-
2.5	A - 100 O - 210	F - 60 O - 160	P - 220 O - 160	A - 120 P - 100 O - 230	-
C	-	-	-	-	A - 130 P - 160 O - 260

Note: 1 = Aspergillus sp., 2 = Fusarium sp., 3 = Penicillium sp.,
4 = irradiated with out fungal inoculation,
5 = without irradiation & fungal inoculation,
C = Control & O = Other fungi.

Table.40.Effect of irradiation on fungi inoculated to ginger (powder).

Radiation (KGy/h)	Fungal flora of sample (cfu / g)				
	1	2	3	4	5
10	0	0	0	0	-
7.5	0	0	0	0	-
5.0	A - 20 O - 30	F - 0 O - 20	P - 60 O - 20	A - 5 P - 30 O - 20	-
2.5	A - 160 O - 110	F - 100 O - 100	P - 100 O - 100	A - 20 P - 60 O – 60	
C	-	-	-	-	A - 110 P - 60 O - 100

Note: 1 = Aspergillus sp., 2 = Fusarium sp., 3 = Penicillium sp.,
4 = irradiated with out fungal inoculation,
5 = without irradiation & fungal inoculation,
C = Control & O = Other fungi.

Table.41. Effect of irradiation on fungi inoculated to turmeric (powder).

Radiation (KGy/h)	Fungal flora of sample (cfu / g)				
	1	2	3	4	5
10	0	0	0	0	-
7.5	0	0	0	0	-
5.0	0	0	0	0	-
2.5	A - 86 O - 102	F - 100 O - 170	P - 100 O - 120	A - 10 P - 60 O - 120	-
C	-	-	-	-	A - 20 P - 60 O - 150

Note: 1 = Aspergillus sp., 2 = Fusarium sp., 3 = Penicillium sp.,
4 = irradiated with out fungal inoculation,
5 = without irradiation & fungal inoculation,
C = Control & O = Other fungi.

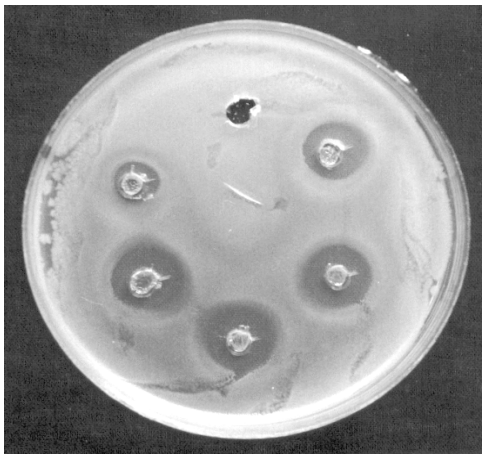


Fig.29. Inhibition of growth of *B.cereus* by ajowan oil.

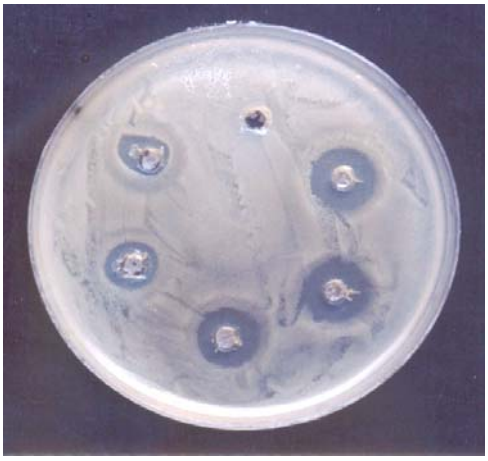


Fig.30. Inhibition of growth of *B.cereus* by clove oil.

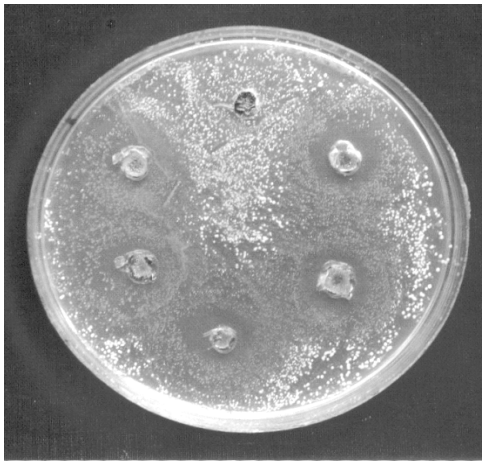


Fig.31. Inhibition of growth of *Y.enterocolitica* by ajowan oil.

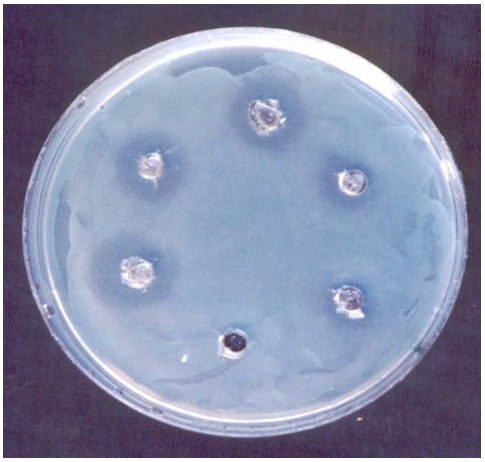


Fig.32. Inhibition of growth of *E.coli* by ajowan oil.

µL/well inhibited strongly *Escherichia coli* and *Yersinia enterocolitica* by 17 mm each by moderately inhibiting other bacteria such as *Bacillus cereus* and *Listeria monocytogenes* by 12 and 10 mm respectively. However *Escherichia coli* and *Yersinia enterocolitica* were strongly inhibited by clove oil at 1 µL/well. It showed low level of inhibition to *Listeria monocytogenes*. Cumin oil inhibited *Yersinia enterocolitica* by 9 mm at 20 µL/well. Nutmeg oil showed low level of inhibition to Listeria *monocytogenes* and *Yersinia enterocolitica*. Pepper oil at 5 µL/well showed low level of inhibition of *Listeria monocytogenes* and *Yersinia enterocolitica* by 8 mm each. Turmeric oil at 2 µL/well also indicated lowest level of inhibition to both *Listeria monocytogenes* and *Yersinia enterocolitica* by 8 mm each.

The picture taken after 24 h of incubation of assay plates clearly show the zone of inhibition of bacteria by spice oils as shown in figures - 29, 30, 31 & 32.

8.4 Effect of gamma radiation on mycoflora

The data on chilli samples exposed to Gamma radiation at the level of 10.0, 7.5, 5.0 and 2.5 KGy / hour is presented in table-39. The experimental protocol for irradiation experiments is presented in the section 3.29 of material and methods.Samples after exposure to radiation at 10 and 7.5 KGy for 1h did not show presence of fungi. The sample of chilli when exposed at 5.0 KGy showed survival of *Penicillium* spp (180.0) and other fungi (230.0) followed by species of *Aspergillus* (60.0) cfu/g. Samples after exposure to 2.5 KGy for 1h showed better survival fungi with *Penicillium* spp. (220.0) and *Aspergillus* spp. (100.0) cfu/g. Other fungi were also recorded as better survivors at 160- 210 cfu/g. However, the control sample was found having *Apergillus* spp. (130.0), *Penicillium* spp. (100.0) and other fungi (160.0) cfu/g.

The samples of ginger when exposed to Gamma radiation showed better survival of *Penicillium* at 5.0 KGy (Table – 40). At higher doses of 10.0 and 7.5 KGy, there was complete inhibition of fungal growth. At lower dose of 2.5 KGy, *Aspergillus* flora was at 160.0 cfu/g, while *Fusarium* and *Penicillium* spp. were 100.0 cfu/g each. There was no inhibition of other fungi at 2.5 KGy radiation as all of them showed the flora at equal or more than 100.0 cfu/g, which is comparable to untreated and unexposed control (C). The fungus *Fusarium* showed complete inhibition even at 5.0 KGy radiation

The data on the effect of irradiation on species of *Aspergillus, Fusarium* and *Penicillium* when inoculated to turmeric is presented in the table – 41. Complete inhibition of fungi was achieved even at 5.0 KGy level. At 2.5 KGy level of irradiation showed 50.0 % reduction in *Aspergillus* count. Only 20.0

Table.42. Effect of irradiation on Bacterial count in chilli (powder).

Radiation (KGy/h)	Total bacterial count (cfu x 10^4 / g)		
	In SR	In C	Inhibition (%)
10	-	880	100
7.5	-	880	100
5.0	34	880	96.0
2.5	140	880	84.0

Note: SR = Sample irradiated, C = Control.

Table.43. Effect of irradiation on Bacterial count in ginger (powder).

Radiation (KGy/h)	Total bacterial count (cfu x 10^4 / g)		
	In SR	In C	Inhibition (%)
10	0	640	100
7.5	0	640	100
5.0	28	640	96.00
2.5	30	640	95.00

Note: SR = Sample irradiated, C = Control.

Table.44. Effect of irradiation on total count in turmeric (powder).

Radiation (KGy/h)	Total bacterial count (cfu x 10^4 / g)		
	In SR	In C	Inhibition (%)
10	0	720	100
7.5	0	720	100
5.0	60.0	720	92.0
2.5	75	720	90.0

Note: SR = Sample irradiated, C = Control.

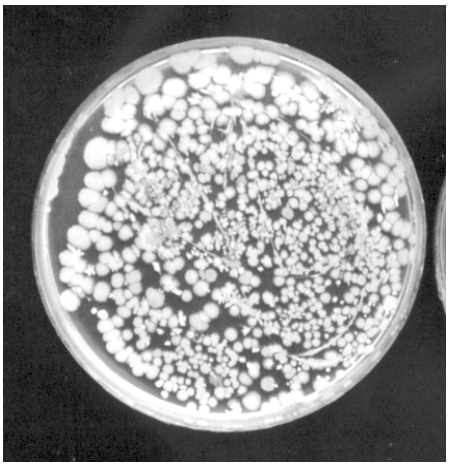


Fig.33. Microflora of un-irradiated chilli sample.

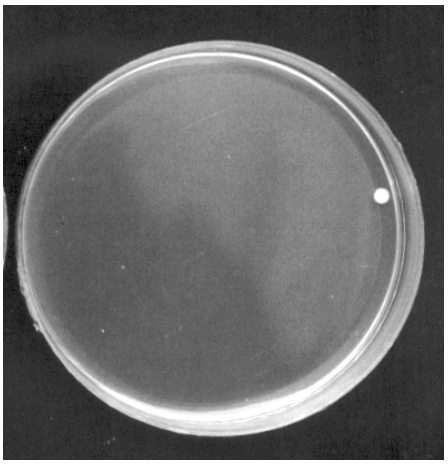


Fig.34. Microflora of irradiated chilli sample at 10 KGy.

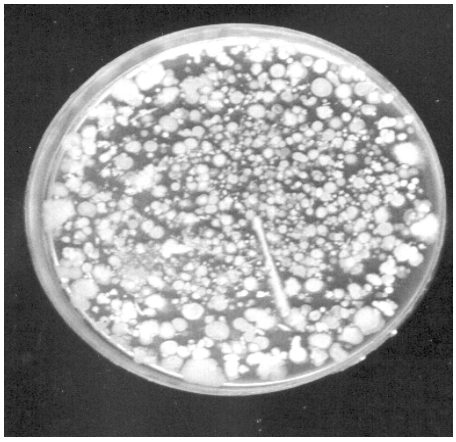


Fig.35. Microflora of un-irradiated chilli sample

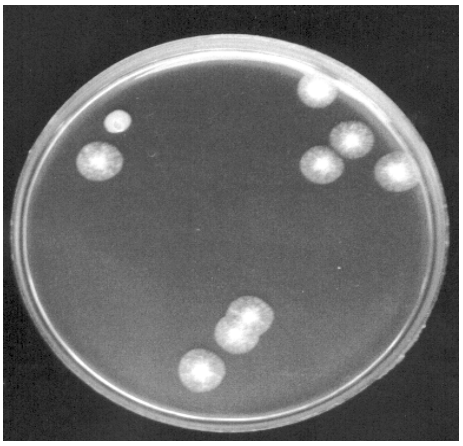


Fig.36. Microflora of chilli sample irradiated at 7.5 KGy.

% reduction of other fungal count and in sample which was not inoculated (no. 4). Turmeric was found to be a poor substrate for supporting the flora and low level of irradiation (2.5 KGy) was found sufficient to check fungi on turmeric.

Chilli samples exposed at radiation level of 10 KGy for 1h was quite effective than 7.5 KGy in inhibiting growth of microflora. The photograph of chilli samples taken before and after treatment clearly show the difference in microflora amongst treated samples in comparison with control (Fig. 33, 34, 35 & 36).

8.5 Effect of gamma radiation on bacteria in spices

The data on the effect of irradiation on bacterial count is presented in the tables 42, 43 and 44 respectively. At higher levels of irradiation (10.0 and 7.5 KGy) complete inhibition of bacterial count was achieved. Chilli, ginger showed more than 90.0 % inhibition at 5.0 and 2.5 KGy level while turmeric showed 84.0 % inhibition at 2.5 KGy irradiation.

8.6 Discussion

Studies on inhibitory effects against mycotoxigenic fungi using spice essential have been carried out by few workers earlier. As part of control measures, the avoidance of growth of mycotoxigenic fungi and mycotoxins elaboration in food commodities have been subjected through various means since the very first report of mycotoxicosis in animals. Strategies for the control of mycotoxins (Park, 1993) through fungicides (Dhanraj et al., 1992), irradiation (Diehl, 1992), detoxification of aflatoxin B_1 in groundnut using Sunlight (Shantha et al., 1986), and using plant extracts (Singh, 1983) have been worked out. Studies in recent past confirmed the growth inhibition of yeasts and molds by different spices viz, sage, oregano, cardamom, caraway, clove, cinnamon and others. In recent times more and more antifungal agents of plants origin are being worked out by several investigators and some of those tried includes aliphatic aldehydes, ketones, alcohols, phenols, ethers and other hydrocarbons. Amongst them, two aliphatic aldehydes, cinnamaldehyde and omethylcinnamaldehyde, the ethers - anethole, methyleugenol and methyl isoeugenol and the phenolics - carvocrol, eugenol and thymol are the main components of spice oils having fungistatic activity (Prakash 1990, Pruthi 1993, Mehta et al. 1995). Spices are reported to inhibit the growth of some mycotoxigenic fungi (Bullerman et al., 1977, Hitikto et al., 1980) ; Inhibition of fungal growth by black pepper have been reported (Scott and Kenney (1973); Hitokto et al. (1977) ; Mabrouk and El-Shayeb (1980). Essential oil of clove spices was found to inhibit mold growth and aflatoxin production completely (Liewellyn et al. 1992).The inhibition of growth and aflatoxin production by *Aspergillus*

flavus and *A.parasiticus* by spice oils and their active components has frequently been reported (Bullerman 1974; Hitokoto et al.1980; Farag et al.1989; Paster et al.1990) with varied inhibitory levels of spice oils. Sometimes toxin production may be inhibited without fungal growth being affected (Bullerman 1974). Considerable interest has been shown on the preservation of grains by the use of essential oils to effectively retard growth and mycotoxin production (Bullerman et al., 1977). Anise and cinnamon oils inhibited growth of *Aspergillus parasiticus* and toxin production completely (Veerabhadra Rao et al.1983). Clove and ginger (Mabrout and Elshayeb, 1980), turmeric (Madhyasta and Bhat, 1985), extract of garlic and onion inhibited the growth of *Aspergillus flavus* (Mei-chin yin 1998). Some varieties that restrict toxin production were developed (Bilgrami et al, 1992). Cinnamon and clove oils inhibited *Aspergillus flavus* growth and subsequent aflatoxin B_1 production (Patkar et al. 1993). Many investigators used essential oils such as cinnamon, peppermint, basil and thyme to protect maize kernels against infection, without affecting germination and mold growth (Montes-Belmont and Carvajall, 1998).Oils of clove oil (eugenol) was most inhibitory to the growth of *A.parasiticus* and *F.moniliforme* followed by cinnamon (cinnamic aldehyde), oregano (thymol) and mace (myristin) oils (Juglal et al.2002). Essential oils of thyme, cinnamon, anise and spearmint inhibited fungal growth and toxin production on wheat treated with oils (Soliman 2002). Effect of 13 chemically different essential oils on the mycelial growth and aflatoxin synthesis by *Aspergillus parasiticus* has been tried. Cinnamon, thyme, oregano, and cumin oils were able to stop mycelial growth at only 0.1% in the medium, while curcumin, ginger, lemon, and orange oils were unable to inhibit totally the growth even at 1% concentration. Coriander, black pepper, mugwort, bay, and rosemary oils caused the growth to stop at concentrations between 0.2 and 1%. The oils most active upon mycelial growth were also the most active against aflatoxinogenesis. However, aflatoxin synthesis was inhibited by all the oils tested (Tantaoui 1994) at higher concentrations though mycelial growth was not inhibited. Similarly with spice oils (Jaspal and Tripati, 1999) and extract of garcinia (Selvi et al., 2003). Growth of *A.parasiticus* was inhibited by 50 % and above by oils of celery, large cardamom, pepper and turmeric when concentration was 0.002 % (Table – 33). Chilli oleoresin, ginger and nutmeg oils were poor inhibitors of growth of *A.parasiticus*. Variable levels of inhibition of aflatoxin formation occurred with all spice oil tested. Complete inhibition of aflatoxins G_1, G_2 and more than 80 % inhibition of aflatoxin B_2 occurred when celery and turmeric oils were incorporated in the growth medium at lowest concentration of 0.002 % (Table - 37). At variable concentration, inhibition at > 50 % was clearly indicated with respect to the production of aflatoxin B_1 with turmeric (0.004 %), celery

(0.006 %), pepper (0.008 %) and large cardamom (0.0 %. The variable effect of spice oils on biosynthetic pathway of aflatoxin is clearly shown by this study.

The oils of clove and cumin had strong inhibitory effect on growth and toxin production by *A.flavus*. The oil of celery inhibited growth at 30 – 32 % at 0.004 – 0.006 % level. However, at the same level, inhibition effect on aflatoxin B_1 (67 – 75 %) and B_2 (99 – 100 %) was very drastic. Ginger oil had adverse effect on aflatoxin B_2 which was completely inhibited though growth of fungus was not much affected. The oils of large cardamom, turmeric and pepper also had similar trend wherein growth inhibition led to the inhibition of aflatoxin B_2 and B_1 (Table – 34). The oils of small cardamom, nutmeg and chilli oleoresin was less effective in controlling the fungus.

Fungal growth and ochratoxin A production by *A.ochraceus* was also adversely affected by oils of cumin and clove both of which showed inhibitory effect on OTA production at 0.006 % level. Oils of nutmeg ginger and chilli oleoresin proved to be less effective in controlling the fungi and toxin production (Table – 35).

Spice oils had less inhibitory effect on the growth of *Fusarium sporotrichoides*, but it had adverse effect on production of T-2, DAS production. Oils of cinnamon, turmeric, pepper, ginger, cumin and chilli oleoresin had inhibitory effect on both the T-2 toxin and DAS production at the lowest level of 0.002 % (Table – 36). It is also clearly indicated that oils of clove, large cardamom, pepper and turmeric had adverse effect on T-2 toxin production as compared to DAS.

It has been reported that some essential oils are capable of inhibiting foodborne bacteria and thus extending self life of processed food (Conner and Beuchat 1984; Kim et al. 1995; Smith-Palmer et al. 1998). Antimycotic effects were observed with cinnamon and clove which were found to have active principles such as cinnamic aldehyde and eugenol (Bullerman et al, 1977).

Ultra violet irradiation has little penetrating power and has limited effectiveness in reducing bacteria on spices (Walkowiak et al., 1971) despite continuous agitation to expose surfaces (Eschmann, 1965). Research and Development in the past 40 years on a large variety of dry food ingredients and herbs has proved that ionizing radiation is a viable process for destroying contaminating organisms (Farkas, 1988). For practical reasons, ionizing radiation applied for food irradiation is limited to either gamma rays from isotopic sources such as ^{60}Co and ^{137}Cs, machine produced X-rays. Gamma rays and X-rays have a high penetrating capacity as compared with accelerated electrons. Spices are best packaged before irradiation in order to avoid re-contamiation after radiation treatment. Depending on the number and type of micro organisms and the chemical

composition of the commodity; a radiation dose of up to 20 kGy may be required to achieve commercial 'sterility' (i.e. a total viable cell count of less than 10 cfu / g) in natural spices and herbs; however doses of 3 to 10 kGy can reduce viable cell counts to a satisfactory level (from 10^5-10^7 cfu / g to less than 10^3 –10^4 cfu / g) without affecting quality attributes (Zehnder and Ettel,1982; Sugimoto et al., 1986; Munasiri et al., 1987; Farkas, 1988; Singh et al., 1988; Narviaz et al., 1989; Ito and Islam,1994). The number of bacterial spores normally decreases by at least 10^2 fold as a result of irradiation with 5 kGy

Our study also confirmed the similar trends of spice essential oils against growth and toxin production by four mycotoxigenic fungi at even lowest concentrations especially the oil of ajowan was effective and emerged as best of all oils evaluated.

SUMMARY AND CONCLUSION

Summary

Spices generally gets contaminated at various stages of preharvest and processing. Therefore it was intend to screen the spice (chilli, ginger and turmeric) samples sold at market for presence of mycotoxigenic fungi. Survey covering three districts of Karnataka was conducted and a total of 55 – 60 samples each of chilli, ginger and turmeric were procured. These spices were analyzed for mycoflora and mycotoxins. Serial dilution technique was employed while screening samples for mycoflora and the observation on fungal flora was recorded in terms of log $_{10}$ cfu/g. The samples were analyzed for mycotoxins, mainly aflatoxins by employing modified Pon's method. The fungi isolated from these spices were screened for their potential towards production of mycotoxins. *A.flavus* followed by *A.niger* was encountered more frequently and *A.parasiticus* was noticed only in chilli samples. Amongst toxigenic field fungi, *Fusarium sporotrichoides* was found associated with chilli samples. The incidence of fungal flora was more in chilli (whole and powdered form) samples than ginger and turmeric samples. Samples of chilli showed contamination level of aflatoxin B_1 at 15 and 26 % on whole and powder form respectively. The level of aflatoxin B_1 was ranging from 20 – 160ppb. Ginger and turmeric samples (powder form) were found to be contaminated with aflatoxin B_1 at 7.0 and 2.0 % respectively. However, whole form of ginger and turmeric samples were found free from aflatoxin contamination. The study conducted on survey on spices showed that the incidence of aflatoxin contamination was higher in powdered market spice samples. In general these samples had total aflatoxin that exceeded the legal limit of 10 ppb. The potentiality of individual spices for their suitability as substrate for the elaboration of toxin by toxigenic fungi, the following study with spices such as chilli, ginger and turmeric was undertaken. Mycotoxigenic fungi such as *A. flavus* (ATCC 46283), *A. parasiticus* (CFR 223) and *A.ochraceus* (CFR 221) were tested for their potentiality to eloaborate mycotoxins on these commodities under conducive experimental conditions. Powdered samples of ginger and turmeric supported better production of aflatoxin than whole form. In case of ginger and turmeric, toxin production was less in whole samples, which may be due to the reason that the rhizome was hard and did not support growth of fungi for production of aflatoxin. However, chilli was found to be the better substrate for production of mycotoxins such as aflatoxin B_1, B_2, G_1, G_2 and ochratoxin A. Amongst parts of chilli tested, seeds were found to be the better substrate for the production of aflatoxins, In case of *A.ochraceus*, the pericarp supported the production of ochratoxin

A, rather than the seeds and stalk. Chilli substrate was a better supporter of mycotoxins than the ginger and turmeric. Study made on the sorption behavior of various spices by exposing the samples RH levels of 65 – 92 % for various time periods of 7 – 45 days proved beyond doubt that the moisture control is the most effective way of preventing the contamination of molds and their toxins. The growth and production of mycotoxins was noticed at higher moisture levels. This study proved a positive correlation between higher moisture level with that of growth of fungi and elaboration of toxins. The rapid identification of spoilage microorganisms is of eminent importance to the food industry. Traditional identification methods, which are based on laborious morphological and physiological tests, are time-consuming, costly, require facilities and mycological expertise, often fail in rapidity, sensitivity and specificity. They lack discriminatory power and as a consequence, misidentification occurs frequently, resulting in the slow and incomplete/inaccurate release of data, for retrospective evaluation. PCR methods are generally rapid and easy to execute, only a small amount of DNA is required, which does not necessarily need to be highly purified (Jos *et al.*1996). In this study, we describe the PCR technique to selectively distinguish aflatoxigenic fungi in spice samples. Studies conducted on enzymatic amplification of DNA sequence of fungi isolated from spices (chilli, ginger and turmeric) has revealed that the *Omt* primer was specific for the detection of aflatoxigenic fungi in spices. The technique could detect aflatoxigenic fungi even at 100 cfu/g, which is of immense value to food industry. The search for antifungal agents especially spice oils which could safely be used as substitutes for fungicides is extensively tried and there is a renewed interest in harnessing the antimicrobial properties of spices. Invitro activities of some spice essential oils have been demonstrated in culture media. All the 12 spice oils tested were able to inhibit growth of mycotoxigenic fungi comprising of *Aspergillus flavus* (ATCC 46283), *A.parasiticus* (CFR 223), *A.ochraceus* (CFR 221) and *Fusarium sporotrichoides* (MTCC 1894) at different concentrations when incorporated in culture medium. Amongst the spice oils, ajowan oil emerged as effective spice oil in inhibiting all the four toxigenic fungi belonging to two genera. Complete growth inhibition of *Aspergillus flavus*, *A.parasiticus* and *A.ochraceus* and *Fusarium sporotrichoides* was achieved by ajowan oil at a very low concentration of 0.001 %. Cinnamon oil was found effective in inhibiting *A.parasiticus, A.ochraceus* and *Fusarium sporotrichoides* at 0.002 %. The growth of *Aspergillus flavus* was also found inhibited completely by oils of cumin and ginger at 0.002 %. Nutmeg and clove oils completely inhibited the growth of *Aspergillus flavus* (ATCC 46283) and *A.parasiticus* (CFR 223) at 0.006 %. Celery, clove, ginger, small cardamom, chilli oleoresin and nutmeg oils completely inhibited *Fusarium*

sporotrichoides (MTCC 1894) at 0.06 % concentrations. *A.flavus* and *A.ochraceus* were found inhibited completely by oils of clove as well as celery at 0.100 %. *A.ochraceus* was completely inhibited by cinnamon and cumin at 0.002 %. *Fusarium sporotrichoides* was found inhibited completely by oil of turmeric at 0.004 %. Large cardamom and pepper gave complete inhibition of *Fusarium sporotrichoides* at 0.008 %. Spice oils such as cinnamon, followed by cumin and clove emerged as effective inhibitors of fungal growth next only to ajowan oil. Ajowan was found inhibiting growth of all the five bacteria tested at the lowest concentration of 1µL/ well. In case of ajowan oil, the zone of inhibition achieved was 17 mm which is considered to be strong inhibition effect for all the bacteria tested irrespective of gram +ve or gram –ve. Our study also confirmed the similar trends of spice essential oils against growth and toxin production by four mycotoxigenic fungi at even lowest concentrations. The study on irradiation of spices sourced by ^{60}cobalt at various dosage levels clearly suggests that samples after exposure to radiation at 10 and 7.5 KGy for 1h were free from fungi. The sample of chilli when exposed at 5.0 KGy for 1h showed survival of *Penicillium* spp and other fungi followed by species of *Aspergillus*. Samples after exposure to 2.5 KGy for 1h showed better survival of fungi such as *Penicillium* spp. (220.0) and *Aspergillus* spp. (100.0) cfu/g. Other fungi were also recorded as better survivors at the range of 160- 210 cfu/g. At higher doses of 10.0 and 7.5 KGy, there was complete inhibition of fungal growth. At lower dose of 2.5 KGy, *Aspergillus* flora was at 160.0 cfu/g, while *Fusarium* and *Penicillium* spp. were 100.0 cfu/g each. There was no inhibition of other fungi at 2.5 KGy radiation as all of them showed the flora which is comparable to untreated and unexposed sample control. The fungus *Fusarium* showed complete inhibition even at 5.0 KGy radiation. Complete inhibition of fungi was achieved even at 5.0 KGy level. At 2.5 KGy level of irradiation showed 50.0 % reduction in *Aspergillus* count only 20.0 % reduction of other fungal count and in sample which was not inoculated. Turmeric was found to be a poor substrate for supporting the flora and low level of irradiation (2.5 KGy) is sufficient to check growth of fungi on turmeric. Chilli samples exposed at radiation level of 10 KGy for 1h was quite effective than 7.5 KGy in inhibiting growth of microflora. At higher levels of irradiation (10.0 and 7.5 KGy) complete inhibition of bacterial count was achieved. Chilli, ginger showed more than 90.0 % inhibition at 5.0 and 2.5 KGy level while turmeric showed 84.0 % inhibition at 2.5 KGy irradiation.

Conclusion

- Chilli commodity was found to be the more prone to fungal contamination than ginger and turmeric.
- Amongst mycotoxigenic fungi, toxigenic strains of *Aspergillus flavus, A.parasiticus* and *Fusarium sporotrichoides* in chilli and only *A.flavus* in ginger and turmeric were encountered.
- About 8.0 % of whole and 15 % of powder form of chilli samples were found contaminated with the most potent mycotoxin i.e. AFB_1.
- Chilli, followed by ginger was found to be the better substrate for production of mycotoxins than turmeric.
- Amongst parts of chilli, seeds were found to be the better substrate for production of aflatoxins while the pericarp supported higher levels of OTA.
- The study on the sorption behavior of mycotoxigenic fungi on spices showed that a moisture level of 12 % was conducive for safety of these commodities
- The *Omt* primer was specific and gave positive amplification for aflatoxigenic fungal DNA in PCR. The PCR method could be applied to chilli, ginger and turmeric successfully.
- Ajowan oil (0.001 %), followed by cinnamon, clove and cumin oil were found to be effective Inhibitors of mycelial growth of mycotoxigenic fungi such as *Aspergillus flavus* (ATCC 46283), *A.parasiticus* (CFR 223), *A.ochraceus* (CFR 221) and *Fusarium sporotrichoides* (MTCC 1894).
- Studies conducted on irradiation of spices (chilli, ginger and turmeric) sourced by ^{60}Cobalt revealed that irradiation of spices at 7.5 KGy for 1h could eliminate both fungal and bacterial flora effectively.

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S Y N O P S I S

The occurrence of mycotoxigenic fungi and elaboration of their toxins in agricultural commodities including spices has been viewed with great concern world over, mainly because of their potential health hazards to humans and live stocks and also its impact on economy. Several mycotoxigenic fungi belonging to species of *Alternaria, Aspergillus, Cladosporium, Curvularia, Fusarium* and *Penicillium*, are known to contaminate food and agricultural commodities. Amongst mycotoxigenic fungi, *Aspergillus flavus* group continues to be the predominant fungi and occupies wide variety of human habitats. The mycotoxin contamination in some spices is suspected for two reasons; (1) many spices are cultivated and processed in warm tropical areas, where conditions favor growth of naturally occurring fungi; (2) most of the spice producing countries are developing countries where the drying and storage practices are not satisfactory. Out of 70 spices grown in different parts of the world, 52 of them are cultivated in India and have occupied a prominent place in the national economy with a tag of export potential. Chilli, ginger, turmeric, cardamom and pepper are considered as major spices and have considerable volume of international trade. India exported 246566.32 metric tones of spices worth of Rs 190508.50 during the year 2003-2004. Amongst them, chilli contributed 81500.00 metric tones worth of Rs 35511.25, ginger and turmeric contributed 5000.00 and 34500.00 tones worth of Rs 2340.50 and 12751.88 respectively (Spices Board, 2004). It has been estimated that roughly 25% of the world's food crops are affected by mycotoxin contamination annually (Magan and Aldred, 2003). Mycotoxins are important as evidenced by occasional outbreaks of human mycotoxicoses and the role of aflatoxins in liver cancer in West Africa and fumonisins in esophageal cancer in South Africa are well established (Shephard, 2004).

The most significant mycotoxigenic Aspergilli are *A. flavus, A. parasiticus* and *A.nomius* which produce aflatoxins, *A.ochraceus*, which produce ochratoxins, and *A.versicolor*, which produce sterigmatocystin. *Aspergillus flavus* is of ubiquitous occurrence in nature and produces aflatoxin B_1 and B_2, while *Aspergillus parasiticus* produces these same metabolites along with G_1 and G_2 (Mclean, Dutton 1995). Despite several efforts attempted throughout the world in the field of mycotoxins, some questions are still to be answered. The need for total surveillance, improve sampling and analytical methods, storage facilities and continuously evolving control measure against mycotoxigenic fungi is indispensable. In addition to this, priority towards management of mycotoxins through development of efficient detoxification and decontamination procedures for their regulations needs to be addressed. The presentation of investigations in this thesis deals with a systematic study on spices such as chilli, ginger and turmeric with the following objectives:

Objectives :

- Natural occurrence of mycotoxigenic fungi in spices.
- Evaluation of substrate specificity of spices for mycotoxin production.
- Determination of critical moisture level in spices through sorption studies.
- Detection of aflatoxigenic fungi in spices by molecular method.
- Studies on the control of mycotoxigenic fungi.

Chapter-I (iii)

Introduction

Safety of food has been a major focus of international and national action bodies over the years. Both microbiological and chemical hazards are of concern. Among chemical hazards, the contamination of food and feed by naturally occurring mycotoxins produced by certain fungi, have been recently characterized as significant sources of food-borne illnesses by the World Health Organization (WHO). In several parts of the world, mycotoxins currently represent a major food safety issue. Introduction to the background material showing the importance of spices, their role in the economy and the present need to look into the safety aspects are presented in this chapter.

Chapter-II

Review of Literature

This chapter provides an overview of current knowledge on occurrence of mycotoxigenic fungi on agricultural commodities including spices, conditions favoring their growth and elaboration of toxins, detection of mycotoxigenic fungi / mycotoxins, biosynthesis, regulations and various aspects of control. This chapter provides an overview of the current knowledge regarding the mycotoxigenic fungi.

Chapter -III

Materials and methods

The chapter covers the list of materials fine chemicals, spice samples, standards, common chemicals, materials and equipments along with the various methodologies adopted in carrying

out the research work. Furthermore, the chapter deals with chapter wise details of the methodologies adopted.

Chapter-IV

Natural Occurrence of mycotoxigenic fungi in spices

Spices generally gets contaminated at various stages of preharvest and processing. Therefore it was intend to screen the spice (chilli, ginger and turmeric) samples sold at market for presence of mycotoxigenic fungi. Survey covering three districts of Karnataka was conducted and a total of 55 – 60 samples each of chilli, ginger and turmeric were procured. These spices were analyzed for mycoflora and mycotoxins. Serial dilution technique was employed while screening samples for mycoflora and the observation on fungal flora was recorded in terms of $\log_{10}$ cfu/g. The samples were analyzed for mycotoxins, mainly aflatoxins by employing modified Pon's method. The results of samples selected randomly are presented in the table 7 to 12. The fungi isolated from these spices were screened for their potential towards production of mycotoxins. *A.flavus* followed by *A.niger* was encountered more frequently and *A.parasiticus* was noticed only in chilli samples. Amongst toxigenic field fungi, *Fusarium sporotrichoides* was found associated with chilli samples. The incidence of fungal flora was more in chilli (whole and powdered form) samples than ginger and turmeric samples. Samples of chilli showed contamination level of aflatoxin B_1 at 15 and 26 % on whole and powder form respectively. The level of aflatoxin B_1 was ranging from 20 – 160ppb. Ginger and turmeric

samples (powder form) were found to be contaminated with aflatoxin B_1 at 7.0 and 2.0 % respectively. However, whole form of ginger and turmeric samples were found free from aflatoxin contamination.

(v)

The survey indicated that the incidence of aflatoxin contamination was higher in powdered market spice samples. In general these samples had total aflatoxin the exceeded the legal limit of 10 ppb.

Chapter-V

Substrate specificity of spices - chilli, ginger and turmeric

To understand the potentiality of individual spices for their suitability as substrate for the elaboration of toxin by known fungi, the following study with spices such as chilli, ginger and turmeric was undertaken. Mycotoxigenic fungi such as *A. flavus* (ATCC 46283), *A. parasiticus* (CFR 223) and *A.ochraceus* (CFR 221) were tested for their potentiality to eloaborate mycotoxins on these commodities under conducive experimental conditions. Powdered samples of ginger and turmeric supported better production of aflatoxin than whole form. In case of ginger and turmeric, toxin production was less in whole samples, which may be due to the reason that the rhizome was hard and did not support growth of fungi for production of aflatoxin. However, chilli was found to be the better substrate for production of mycotoxins such as aflatoxin B_1, B_2, G_1, G_2 and ochratoxin A. Amongst parts of chilli tested, seeds were found to be the better substrate for the production of aflatoxins, In case of *A.ochraceus*, the pericarp supported the production of ochratoxin A, rather than the seeds and stalk. Chilli substrate was a better supporter of mycotoxins than the ginger and turmeric.

Chapter-VI

Sorption behavior of spices with reference to mycotoxigenic fungi

This chapter presents the data on the sorption behavior of various spices. The samples were exposed to RH levels of 65 – 92 % for various time periods of 7 – 45 days.

(vi)

The growth and production of mycotoxins at higher moisture levels proved beyond doubt that the moisture control is the most effective way of preventing the contamination of molds and their toxins. Samples of chilli, ginger and turmeric were placed inside desiccators having different built-in relative humidity (RH) levels of 65, 75, 86 & 92 % using appropriate saturated salt solutions and exposed for different time period starting from 7 to 45 days. The exposed samples were periodically withdrawn and analyzed for moisture content, mycoflora, mycotoxins (mainly aflatoxins) using standard procedures. The samples kept at prevailing room temperature and humidity served as control. Moisture control without question is the best and most economical means to control the environment to prevent mould growth and mycotoxin production. This study proved a positive correlation between higher moisture level with that of growth of fungi and elaboration of toxins. The safe moisture content for chilli, ginger and turmeric was found to be < 12 %.

Chapter-VII

Detection of aflatoxigenic fungi by molecular methods

The rapid identification of spoilage microorganisms is of eminent importance to the food industry. Traditional identification methods, which are based on laborious morphological and physiological tests, are time-consuming, costly, require facilities and mycological expertise, often fail in rapidity, sensitivity and specificity. They lack discriminatory power and as a consequence, misidentification occurs frequently, resulting in the slow and incomplete/inaccurate release of data, for

retrospective evaluation. PCR methods are generally rapid and easy to execute, only a small amount of DNA is required, which does not necessarily need to be highly purified (Jos *et al.*1996). In this study, we describe the PCR technique to selectively distinguish aflatoxigenic

fungi in spice samples. Studies conducted on enzymatic amplification of DNA sequence of fungi isolated from spices (chilli, ginger and turmeric) has revealed that the *Omt* primer was specific for the detection of aflatoxigenic fungi in spices. The technique could detect aflatoxigenic fungi even at 100 cfu/g, which is of immense value to food industry.

Chapter-VIII

Control of mycotoxigenic fungi

The search for antifungal agents especially spice oils which could safely be used as substitutes for fungicides is extensively tried and there is a renewed interest in harnessing the antimicrobial properties of spices. Invitro activities of some spice essential oils have been demonstrated in culture media. All the 12 spice oils tested were able to inhibit growth of mycotoxigenic fungi comprising of *Aspergillus flavus* (ATCC 46283), *A.parasiticus* (CFR 223), *A.ochraceus* (CFR 221) and *Fusarium sporotrichoides* (MTCC 1894) at different concentrations when incorporated in culture medium. Amongst the spice oils, ajowan oil emerged as effective spice oil in inhibiting all the four toxigenic fungi belonging to two genera. Complete growth inhibition of *Aspergillus flavus*, *A.parasiticus* and *A.ochraceus* and *Fusarium sporotrichoides* was achieved by ajowan oil at a very low concentration of 0.001 %. Cinnamon oil was found effective in inhibiting *A.parasiticus, A.ochraceus* and *Fusarium sporotrichoides* at 0.002 %. The growth of *Aspergillus flavus* was also found inhibited completely by oils of cumin and ginger at 0.002 %. Nutmeg and clove oils completely inhibited the

growth of *Aspergillus flavus* (ATCC 46283) and *A.parasiticus* (CFR 223) at 0.006 %. Celery, clove, ginger, small cardamom, chilli oleoresin and nutmeg oils completely inhibited *Fusarium sporotrichoides* (MTCC 1894) at 0.06 % concentrations.

(viii)

A.flavus and *A.ochraceus* were found inhibited completely by oils of clove as well as celery at 0.100 %. *A.ochraceus* was completely inhibited by cinnamon and cumin at 0.002 %. *Fusarium sporotrichoides* was found inhibited completely by oil of turmeric at 0.004 %. Large cardamom and pepper gave complete inhibition of *Fusarium sporotrichoides* at 0.008 %. Spice oils such as cinnamon, followed by cumin and clove emerged as effective inhibitors of fungal growth next only to ajowan oil. Ajowan was found inhibiting growth of all the five bacteria tested at the lowest concentration of 1µL/ well. In case of ajowan oil, the zone of inhibition achieved was 17 mm which is considered to be strong inhibition effect for all the bacteria tested irrespective of gram +ve or gram –ve. Our study also confirmed the similar trends of spice essential oils against growth and toxin production by four mycotoxigenic fungi at even lowest concentrations.

In addition to this another attempt under the control of mycotoxigenic fungi was made by exposing the samples of chilli, ginger and turmeric to irradiation sourced by ^{60}cobalt at various dosage levels with exposure time of 1 hour. Samples after exposure to radiation at 10 and 7.5 KGy for 1h did not show presence of fungi. The sample of chilli when exposed at 5.0 KGy for 1h showed survival of *Penicillium* spp and other fungi followed by species of *Aspergillus*. Samples after exposure to 2.5 KGy for 1h showed better survival of fungi such as *Penicillium* spp. (220.0) and *Aspergillus* spp. (100.0) cfu/g. Other fungi were also recorded as better survivors at the range of 160- 210 cfu/g. At higher doses of 10.0 and 7.5 KGy, there was complete inhibition of fungal growth. At lower dose of 2.5 KGy, *Aspergillus* flora was at 160.0 cfu/g, while *Fusarium* and *Penicillium* spp. were 100.0 cfu/g each. There was no inhibition of other fungi at 2.5 KGy radiation as all of them showed the flora which is

comparable to untreated and unexposed sample control. The fungus *Fusarium* showed complete inhibition even at 5.0 KGy radiation. Complete inhibition of fungi was achieved even at 5.0 KGy level. At 2.5 KGy level of

irradiation showed 50.0 % reduction in *Aspergillus* count only 20.0 % reduction of other fungal count and in sample which was not inoculated. Turmeric was found to be a poor substrate for supporting the flora and low level of irradiation (2.5 KGy) is sufficient to check growth of fungi on turmeric. Chilli samples exposed at radiation level of 10 KGy for 1h was quite effective than 7.5 KGy in inhibiting growth of microflora. At higher levels of irradiation (10.0 and 7.5 KGy) complete inhibition of bacterial count was achieved. Chilli, ginger showed more than 90.0 % inhibition at 5.0 and 2.5 KGy level while turmeric showed 84.0 % inhibition at 2.5 KGy irradiation.

RESEARCH DOCUMENTS

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