

**Investigation on the Mycoflora of Nutmeg in Storage and the
Associated Mycotoxin**

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KERALA, INDIA**

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**Investigation on the Mycoflora of Nutmeg in Storage and the
Associated Mycotoxin**

by

ANJALI KRISHNA K. P.

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THESIS

**Submitted in partial fulfilment of the
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**DEPARTMENT OF PLANT PATHOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM- 695 522
KERALA, INDIA**

2014

DECLARATION

I, hereby declare that this thesis entitled “**Investigation on the mycoflora of nutmeg in storage and the associated mycotoxin**” is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

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LIST OF ABBREVIATIONS

| | | |
|---------------|---|----------------------------------|
| μ l | - | Micro litre |
| μ m | - | Micrometre |
| CD (0.05) | - | Critical difference at 5 % level |
| cfu | - | Colony forming units |
| cm | - | Centimetre |
| <i>et al.</i> | - | Co-workers/ Co-authors |
| Fig. | - | Figure |
| g | - | Gram |
| g^{-1} | - | Per gram |
| <i>i.e.</i> | - | that is |
| KAU | - | Kerala Agricultural University |
| kg | - | Kilogram |
| Max. | - | Maximum |
| Min. | - | Minimum |
| NS | - | Non significant |
| PDA | - | Potato dextrose agar |
| ppb | - | Parts per billion |
| RH | - | Relative Humidity |
| CRM | - | Certified Reference Material |
| UV | - | Ultra Violet |
| Rf | - | Retention factor |
| sp. | - | Species |
| spp. | - | Species |
| OD | - | Optical Density |
| <i>viz.</i> | - | Namely |

LIST OF SYMBOLS

| | | |
|--------------|---|----------------|
| % | - | per cent |
| $^{\circ}$ C | - | Degree celsius |

Introduction

1. INTRODUCTION

In India average production of spices is 4.01 million tonnes and about 10-12 per cent of this is exported annually. Of the important spices, nutmeg is cultivated in an area of 15,500 ha with the production of 8000 tonnes. About 3275 tonnes of nutmeg and mace was exported to other countries. The share of export in total production is about 72 per cent in nutmeg. The estimated post harvest loss in agricultural commodities in India ranged between 20-50 per cent (Lagvankar, 2012). The losses are primarily due to microbial contamination. It has been reported that 5–10 per cent of agricultural products in the world are contaminated by moulds to the extent that these products cannot be consumed by humans and animals (Aziz *et.al.*, 1998).

Nutmeg is exposed to a wide range of microbial contamination during pre- and post- harvest operations and in storage. Microbial spoilage is a major problem in the tropics and subtropics, where climatic conditions and storage practices were conducive to fungal growth and toxin production (Christensen and Kaufmann, 1965).

The Food and Agricultural Organization (FAO) of the United Nations has estimated that 25 per cent of the world food crops are contaminated by mycotoxins each year (Dahman-levinson *et al.*, 2006). Spices are largely produced in countries with tropical climates that have high range of temperature, humidity, and rainfall (Bilgrami, 1985). Furthermore, improper storage, extended drying times, and elevated moisture contents can cause development of mycotoxins in spices.

‘Mycotoxin’ is derived from the Greek word “mykes” meaning fungus and the Latin word “toxicum” meaning poison. The term mycotoxin was coined in 1962, during an investigation on the cause of turkey ‘X’ disease during which approximately 100,000 turkey poultries died due to the consumption of peanut meal contaminated with secondary metabolites from *Aspergillus flavus* (Blount, 1961). Mycotoxins are low molecular weight, toxic compounds produced by certain strains of a variety of filamentous fungi (Forgas and Carll, 1962). Most

mycotoxins are chemically stable and survive food processing. The diseases caused by the ingestion of food and feed contaminated by mycotoxins are called mycotoxicoses. Because of their pharmacological activity, some mycotoxins or mycotoxin derivatives are used as antibiotics, growth promotants and in other kinds of drugs. Some others have been implicated as chemical warfare agents. Mycotoxins are secondary metabolites of fungi. The major fungal genera producing mycotoxins include *Aspergillus*, *Fusarium* and *Penicillium*, that can grow on foods such as cereals, nuts, dried fruits, spices and legumes under certain environmental conditions.

The most common mycotoxins are aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, T-2 toxin and zearalenone. The fungi, *A. flavus*, *A. parasiticus*, *A. clavatus*, *A. niger* and *A. nominus* produce aflatoxins. *A. flavus* is the most common producer of Aflatoxins (Bradburn *et al.*, 1993). Aflatoxins are among the most carcinogenic substances known and the four major aflatoxins are Aflatoxin B₁, B₂, G₁ and G₂. Ochratoxin A (OA) is produced by the fungi *A. ochraceus*, *A. parasiticus* and *Penicillium verrucosum* (Kuiper-Goodman, 1991).

Aflatoxin contamination in spices exported from India is an issue that caused serious repercussions for the exporting community and farmers. Now-a-days, every container of spices exported from India is being inspected by concerned authorities in Europe. This has affected Indian exports of Spices.

Nutmeg is widely used by manufacturers in food application and medicine as whole spices, oils and oleoresins, and is facing severe quality issues, and rejection at ports due to aflatoxin contamination. The Spices Board of India, along with World Spice Organisation has initiated awareness campaigns at nutmeg growing areas in India to battle the aflatoxin menace. An audit was carried out by European Commission Food and Veterinary Office in India to assess the systems in place to control aflatoxin contamination in spices intended for export to the European Union (EU). In 2010 the Rapid Alert System for Food and Feed (RASFF) was put in place to provide food and feed control authorities in Europe with an effective tool to exchange information about measures taken responding to serious risks detected in relation to food or feed. The spices which

were identified by RASFF with aflatoxin contamination included *Capsicum* spp (whole or ground chillies, chilli powder, cayenne and paprika), nutmeg, ginger and turmeric. Several countries have introduced legislation concerning mycotoxins, specifying maximum limits for mycotoxins such as aflatoxins, ochratoxin A (OTA), patulin, fumonisins, zearalenone and deoxynivalenol for different foodstuffs.

In the recent past the antimicrobial properties of some plant products and essential oils are being exploited for protecting food and feed materials from moulds and their mycotoxins (Murcia *et al.*, 1996). However, authentic information on mycotoxins in spices and also systematic studies on the magnitude of the problem of 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. Based on the foregoing considerations the aim of the present project was to reveal the mycoflora associated with spoilage of nutmeg and mace in storage, to provide useful information on the production and detection of mycotoxins and to evolve suitable methods to minimise fungal contamination in storage. The work envisaged in the present study are

- Survey and collection of nutmeg and mace samples from collection centres
- Quantitative and qualitative estimation of fungal population in samples.
- Detection of mycotoxin in the samples.
- Characterization and identification of fungal flora.
- Ability of fungi to elaborate mycotoxin under *in vitro* and *in vivo* conditions
- Methods to minimize fungal contamination in nutmeg and mace.

Review of literature

2. REVIEW OF LITERATURE

A large number of fungi are known to cause spoilage of stored products. Very little information was available on the mycoflora of spices, especially nutmeg and mace in storage. A perusal of literature revealed that some of the fungi were found to cause spoilage of spices in storage.

2.1. MYCOFLORA ASSOCIATED WITH SPICES

In India, fungi were recorded on samples of spices, which included *Alternaria solani* Ellis & Martin, *A. tenuis* Wiltsh, *Aspergillus niger* Van Tieghem, *Fusarium solani* Martius and *Helminthosporium* sp. on chilli (Pavgi and Singh, 1964); *Fusarium oxysporum* Schldl and *F. sambucinum* Fuckelon ginger (Sharma and Joshi, 1977); *Fusarium* sp. and *Sclerotium rolfsii* on pepper and *Aspergillus flavus* Link, *A. fumigatus* Wilhelm, *A. niger*, *A. ochraceus*, *Fusarium moniliforme* Sheldon and *Penicillium oxalicum* Corrie on cardamom (Prasad, 1980). Three genera of fungi viz., *Aspergillus*, *Penicillium* and *Fusarium* (*Gibberella*) were found most frequently in spices (Betina, 1984; Frisvad and Thrane, 1987). Christensen (1988) had detected relatively high incidence of toxigenic moulds, including *A. flavus*, *A. parasiticus*, *A. fumigatus*, *A. ochraceus*, *Penicillium citrinum* and *P. islandicum* in some spices.

During the investigation on the mycoflora of spices, Misra (1981) found that *A. flavus*, *A. fumigatus*, *A. niger*, *A. ochraceus*, *A. sydowii* Thom & Church, *Chaetomium indicum* Corda, *C. globosum* Kunze, *C. biapiculatum* Lodha, *P. oxalicum* and *Rhizopus arrhizus* A. Fisch were associated with all the samples of black pepper, ginger and cardamom. Geeta and Kulkarni (1987) recorded species of *Aspergillus* from black pepper and turmeric with total cfu of 0.6×10^4 to $16 \times 10^5 \text{ g}^{-1}$ and 0.5×10^3 to $11.1 \times 10^5 \text{ g}^{-1}$ respectively in the samples collected from retail shops in Bombay. Garrido and Pozo (1992) screened spices, namely, clove, cumin and nutmeg for the presence of mycoflora, and found that these were contaminated mainly with species of *Aspergillus* and *Penicillium*. According to Bartine and Tantaoui-Elaraki (1997), fungal growth was weak on curcumin, black pepper and white pepper. Giridhar and Reddy (1997) surveyed for the occurrence

of mycoflora of spices in Andhra Pradesh and found that samples of chilli, ginger and turmeric were infested at 28.60, 23.80 and 23.60 per cent with toxigenic strains of *Aspergillus* species. Of the different fungi, *Aspergillus* spp. were the dominant ones and *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 during the survey were *Alternaria*, *Cladosporium*, *Chaetomium*, *Curvularia*, *Fusarium*, *Memnoniella*, *Macrophomina*, *Phoma*, *Penicillium*, *Rhizopus*, *Trichothecium* (Giridhar and Reddy, 1997).

Holley and Patel (2005) recorded mold count in the assorted pepper and the spore count was $2.0-3.4 \times 10^6 \text{ g}^{-1}$. Among the isolates of fungi collected from pepper, 49 per cent of *A. flavus* were found to be toxigenic. During the survey Shadanaika (2005) reported that important fungi belonging to species of *Aspergillus*, *Fusarium* and *Penicillium* were associated with chilli, ginger and turmeric. Chilli samples were infected with toxigenic strains belonging to *A. flavus*, *A. parasiticus* and *Fusarium sporotrichoides*, while *A. flavus* group only was associated with ginger and turmeric samples. *A. ochraceus* was observed on chilli and ginger. According to Bokhari (2007), the most common genera of fungi present in black pepper seeds were *Aspergillus*, *Penicillium* and *Fusarium*.

Ginger samples were found to be contaminated with *A. flavus* (Flannigan and Hui 1976; Madhyashta and Bhat 1985). Zakka *et al.* (2010) isolated *A. flavus*, *A. niger*, *Fusarium* sp. and *Rhizopus* sp. from samples of dried ginger and found that *A. flavus* occurred in highest frequency, followed by *F. oxysporum*, *A. niger* and *Rhizopus* sp.

During the study on the mycoflora associated with spoilage of cinnamon, Aziz *et al.*, (1998) reported that *A. flavus*, *A. niger*, *A. ochraceus*, *A. parasiticus* Speare and *A. versicolor* Tirab were the most common fungi. In cinnamon out of the 20 fungal species isolated, *A. flavus*, *A. niger*, *Penicillium* sp, *Rhizopus* sp. and *Syncephalastrum* sp. were the most dominant. (Abdulkhadir *et al.*, 2003).

2.2. EFFECT OF ENVIRONMENTAL FACTORS ON THE MYCOFLORA OF SPICES

Heintzeler (1939) conducted a comprehensive survey to understand the relationship of atmospheric humidity with growth of moulds. She found that the optimum relative humidity (RH) for the growth of *A. niger* and *A. glaucus* in steam sterilized atmosphere was 98 and 90 per cent respectively. The optimum temperature for *A. glaucus* was 30°C, while 20°C was favourable to *Penicillium glaucum*, *Rhizopus nigricans*, *Phycomyces niteus* and *Oidium lactis*. Bonner (1948) conducted *in vitro* studies on temperature and humidity requirements of *A. niger*. The results showed that optimum temperature requirement for the growth of *A. niger* was related to relative humidity (RH). At 95 per cent RH, the temperature requirements were around 40°C, at 100 per cent RH, the optimum temperature was 30°C.

Deiner and Davis (1969) reported that in dried ginger, for the invasion of mycoflora, optimum RH was between 89-99 per cent at 30°C for eight weeks incubation whereas under the field conditions it occurred rapidly at moisture content of 12-20 per cent. The optimal temperature range for aflatoxin production on ginger was 25-36°C in controlled environment (Detroy *et al.*, 1971). Moisture content or relative humidity (RH) surrounding the substrates is the most important factor for the growth and aflatoxin production by *A. flavus*. Substrate moisture determines the growth and production of toxins by the fungi, no matter how favourable is the temperature (Deiner, 1976). According to Northolt *et al.*, (1977) optimum temperature range for fungal growth and aflatoxin production in spices was 25 – 30°C. Moulds are widely distributed as environmental contaminants under favourable conditions of temperature and humidity; moulds grow on many commodities including spices (Bartine and Tantaoui-Elaraki, 1997).

2.3. PRODUCTION OF MYCOTOXIN

Mycotoxins are secondary metabolites produced by fungi particularly belonging to species of *Aspergillus*, *Penicillium* and *Fusarium* (Forgas and Carll, 1962). Aflatoxin contamination was more during transit and storage of

agricultural commodities (Bhat, 1988). Comparatively lesser information was available on aflatoxin problem in spices which constitute an important fraction of human diet. These substrates were also known to favour the growth of toxigenic fungi and help in aflatoxin production (Pal and Kundu 1972; Hansen *et al.*, 1994). Improper storage, extended drying times and elevated moisture contents resulting development of mycotoxins in spices (Jalili and Jinap, 2012).

2.3.1. Aflatoxin

Aflatoxins consist of a group of approximately 20 related fungal metabolites, although only aflatoxins B₁, B₂, G₁ and G₂ are normally found in foods. They are produced by at least three species of *Aspergillus* namely, *A. flavus*, *A. parasiticus* and *A. niger*, and can occur in a wide range of important raw food commodities including spices (Bennett, 1988; Devi *et al.*, 2001). According to Seenappa and Kempton (1980) of the different dried spices, nutmeg and red pepper appeared to be more prone to aflatoxin production. Aflatoxin are the most common mycotoxins present in spices (Ozbey and Kabak, 2012).

Hansen and Jung (1973) detected upto 15ppb of Aflatoxin B₁ content in samples of nutmeg. Scott and Kennedy (1973) reported the results of their survey and analysis of ground black, white and capsicum peppers for aflatoxins. It was found that crushed and whole pepper corns were good substrates for the production of aflatoxins by *A. flavus*. Scott and Kennedy (1975) recorded low to high incidence of aflatoxins in ginger (upto 25 ppb B₁) and nutmeg (upto 37.5 ppb B₁).

Seenappa and Kempton (1980) conducted a study on aflatoxin contamination in spices and observed aflatoxin levels upto 120 ppb in 18 of 125 samples of black pepper and ginger collected from drying yards of Kerala and warehouses of Karnataka. In India, a study conducted by Madhyastha (1985) on aflatoxin contamination in spices, reported an aflatoxin level of 1.5 - 57.5, 10 - 60 and 12.5 - 25 $\mu\text{g kg}^{-1}$ in black pepper, red chilli and ginger samples respectively. Aflatoxin contamination of *Piper nigrum* at 1.20 $\mu\text{g g}^{-1}$ was detected in Bihar by Roy *et al.* (1988). In India, common spices such as coriander, cardamom, cumin and nutmeg were found to be contaminated with aflatoxin B₁ above the tolerance

level fixed by World Health Organisation (WHO) (Roy and Chaurasia 1990). A total of 22 pepper (*Capsicum annuum*) samples from local markets and farms of six districts in Punjab, NWFP (North West Frontier Province) and Sindh, Pakistan were screened for aflatoxins. The aflatoxin B₁ and B₂ contamination in pepper ranged from 32.20 - 48.10 $\mu\text{g kg}^{-1}$ (Jaffar *et al.*, 1994). Shadanaika (2005) conducted a survey in Mysore on three spices *viz.*, chilli, ginger and turmeric for the presence of aflatoxin B₁ and found that the whole form of product show lower level of contamination than their powder form. Whole chilli samples showed a contamination level of 23.0 per cent, while 40.0 per cent of the powdered chilli was contaminated with aflatoxin B₁. However, only 10 per cent were showing contamination level above permissible limit of 10 ppb. The ginger and turmeric powdered samples showed a contamination level of 2.80 and 3.60 per cent respectively. Only one sample of turmeric out of 55 tested was found to be contaminated with 20 ppb of aflatoxin B₁. Eighteen of the 70 ground red pepper samples contained aflatoxin B₁ and concentrations in seven of these ranged from 6.1 to 15.7 $\mu\text{g kg}^{-1}$.

In Thailand, high aflatoxin levels were reported in dried chilli peppers and the quantity was 966 $\mu\text{g kg}^{-1}$ (Shank *et al.*, 1972). In another study conducted by Gerald *et al.* (1992) found that in Thailand 18 per cent of spices and herbs were contaminated with aflatoxin B₁ with a range of 40-160 ppb level. Aflatoxin B₁ to the range of 1-5 μg was detected in chilli, cumin, curry powder, saffron and white pepper where as aflatoxins were not detected in ginger, cardamom, clove and mustard. In another study, Holley and Patel (2005), recorded the presence of aflatoxin B₁ in the commercial samples of spices from Thailand with 84 – 175 ppb levels.

Among the spices, red chillies were reported to be showing highest aflatoxin levels and frequency of occurrence (Scott and Kennedy 1973; Flannigan and Hui 1976). In 1997, European Commission conducted a coordinated programme for the control of mycotoxin in food stuff and found that among the 3098 spice samples including nutmeg, pepper, chilli and paprika analysed, 183 samples (5.9 per cent) contained more than 10 $\mu\text{g kg}^{-1}$ aflatoxins. Tabata *et al.*

(1998) during their study on aflatoxin contamination in imported spices (white and red pepper, paprika and nutmeg) reported that 106 (19.4 per cent) of 546 samples contained aflatoxin B₁ at levels of 0.2–27.7 µg kg⁻¹.

In Germany, where a total of 185 samples of spices were analysed for mycotoxins, aflatoxins were detected in four samples of red chillies, ranging from 8.4–24 µg kg⁻¹. When a total of 15 samples of chilli powder were analysed for aflatoxins, seven samples were positive, containing aflatoxins B₁ upto 15.3 µg kg⁻¹ (El- Dessouki, 1992). Highest contamination of aflatoxins of upto 120 µg kg⁻¹ was found in red chillies by Llewellyn *et al.* (1992). Samples of sun dried, matured red pepper were analysed for aflatoxin content and it was reported that aflatoxin B₁ values varied from non-detectable to 2.2 µg kg⁻¹ (Adegoke *et al.*, 1996). In Hungary, 91 spice samples, which included 70 ground red pepper, six black pepper, five white pepper, five pice mix and five chilli samples, were analysed for aflatoxins (Fazekas *et al.*, 2005).

Aflatoxin contamination was investigated in ground red pepper samples collected from government owned food stores, retail shops and open markets of Addis Ababa, Ethiopia. Out of 60 samples each of ground red pepper, eight (13 per cent) were positive for aflatoxins and aflatoxin B₁ was detected at a range of 100 - 150 µg kg⁻¹ (Fufa and Urga, 1996).

Samples of black pepper, cayenne pepper, chilli powder, ground ginger and paprika from retail outlets in UK were analysed for aflatoxin contamination. More than 50 per cent of the samples were contaminated with more than 1 ppb aflatoxin. Among these, chilli powders were most often contaminated with aflatoxins and some samples contained more than 20 ppb total aflatoxins (Gamer *et al.*, 1993). In UK, Mac Donald and Castle (1996) conducted a survey of aflatoxins in 157 retail samples of spices which included curry powders, pepper, cayenne pepper, chilli, ginger, and cinnamon and found that nearly 95 per cent of samples contained below 10 µg kg⁻¹ total aflatoxins and only nine samples had higher levels. The highest concentration in a retail sample of chilli powder was 48 µg kg⁻¹.

With respect to incidence of aflatoxin contamination in black pepper, contradictory reports have been documented. The earlier studies by Scott and Kennedy (1973); Beljaars *et al.*, (1974); Awe and Schranz (1981) reported lack of aflatoxin contamination in black pepper. However fungal invasion by *A. flavus* and production of aflatoxins has been documented for black pepper (Christensen *et al.*, 1967 and Moreno-Martinez and Christensen, 1973). Scott and Kennedy (1975) reported low to high incidence of aflatoxins, upto 25 ppb aflatoxin B₁ in ginger samples. In a survey carried out to detect aflatoxins in spices, out of the 100 samples analysed, four black pepper samples were found to contain about 35 $\mu\text{g kg}^{-1}$ of aflatoxin B₁ (Aziz and Youssef, 1991).

A total of 120 different samples belonging to 24 kinds of spices collected from different places in Assiut, Egypt were examined for natural occurrence of mycotoxins. The analysis of these spices extracts revealed aflatoxins (8-35 $\mu\text{g kg}^{-1}$) in 16 samples of black pepper (El Kady *et al.*, 1995). Samples of different spices *viz.*, black pepper, white pepper and red chilli were collected from markets in Cairo and Giza for aflatoxin analysis by Selim *et al.* (1996). Bokhari (2007) reported that the most common genera of fungi present in black pepper seeds were *Aspergillus*, *Penicillium* and *Fusarium* and aflatoxin was detected in the range of 12 - 14 $\mu\text{g kg}^{-1}$.

Lebrihi (1986) tested the ability of fungi isolated from spices for production of aflatoxin under laboratory conditions. The result showed that out of 568 isolates of *A. flavus* screened, 280 were found to be toxigenic and produced different fractions of aflatoxins in varying concentrations (0.5 – 15 ppm). Sixty five isolates of *A. flavus* produced aflatoxin B₁ in the range of 5 – 15 ppm, whereas 215 isolates elaborate aflatoxin B₁ only up to 5ppm. All toxigenic isolates of *A. flavus* produced aflatoxin B₁ in liquid medium. However six isolates were capable of producing all the four aflatoxins *viz.*, B₁, B₂, G₁ and G₂, whereas 104 toxigenic isolates produced aflatoxin B₁ and B₂ in the medium. Even G₁ was produced in addition to B₁ and B₂ by 30 isolates. About 50% isolates produced only aflatoxin B₁. Shadanaika (2005) screened strains of *A. flavus* and *A. parasiticus* isolated from various spice samples for their ability to produce

aflatoxins on YES medium. *A. flavus* was abundant in all the spice samples. *A. flavus* isolates from chilli produced aflatoxin B₁ and B₂ at the range of 0.0 - 2400 and 0.0-1200 ppb level respectively. Similarly, isolates from ginger and turmeric produced aflatoxin B₁ and B₂ at lower range of 0.0 - 120 and 0.0 - 80 ppb respectively. All the isolates of *A. parasiticus* from chilli produced aflatoxin B₁, B₂, G₁ and G₂ at the range of 130 - 400, 60 - 180, 200 - 380 and 100 - 260 ppb levels respectively.

Shadanaika (2005) investigated the suitability of various kinds of spice, viz., chilli, ginger and turmeric as substrate for fungal growth and elaboration of mycotoxin and were artificially inoculated with toxigenic strain of *A. flavus*. The chilli, ginger and turmeric substrates supported production of aflatoxin B₁ at 260, 140, 40 ppb and aflatoxin B₂ at 40, 30 and 10 ppb levels respectively, while inoculated with *A. parasiticus*, chilli showed to be better substrate for aflatoxins. Chilli was showing contamination level of B₁, B₂, G₁ and G₂ at 200, 40, 120 and 30 ppb level respectively. Ginger supported B₁, B₂, G₁ and G₂ at 100, 30, 80 and 10 ppb level respectively. Turmeric supported B₁, B₂ and G₁ at 60, 10 and 20 level respectively. Amongst the three spices, chilli and ginger were the better substrates for aflatoxin elaboration than turmeric.

Separation, detection and quantification of aflatoxins in foods have been achieved mostly by chromatographic techniques such as Thin layer chromatography (TLC), High Pressure Liquid Chromatography (HPLC) and Mini column chromatography (MCC) (Romer 1984). In TLC determination of aflatoxins in dry ginger roots and ginger oleoresin, aflatoxins were found in the majority of the samples examined. Most of the reported results were below the Food and Drug Administration (FDA) guidelines of 20 ppb total aflatoxins (Trucksess *et al.*, 1989). Detection of aflatoxins in spices was done mostly by chromatographic techniques, namely, Thin Layer Chromatography (TLC) (Shank *et al.*, 1972; Beljaars *et al.*, 1975), Enzyme Linked Immuno Sorbent Assay (ELISA), High Performance Liquid Chromatography (HPLC) (Awe and Schranz 1981), Liquid Chromatography (LC) (Taguchi *et al.*, 1995). Biological or

immuno-assay method were also employed for detecting aflatoxin (Selim *et al.*, 1996).

2.3.2. Ochratoxin

Ochratoxin A (OTA) was discovered in 1965 as a secondary metabolite of *A. ochraceus*, later several other fungi, *viz.*, *Penicillium* and *Eurotium herbariorum* were established as OTA producers (Betina, 1984 and Chelkowski *et al.*, 1987). Krogh *et al.* (1974) reported that OTA was produced by *A. ochraceus* and *P. viridicatum*. Awe and Schranz (1981) used HPLC for the first time to determine ochratoxins in spices. Ochratoxin A is the most toxic member of the ochratoxins detected in spices (Kuiper and Scott, 1989; EFSA, 2006). Cultures of 205 isolates of fungi were screened for OTA and showed that 74.2 per cent of *Aspergillus carbonarius* and 14.3 per cent of *A. ochraceus* isolates produced OTA at levels ranging from 1.2-3530 $\mu\text{g ml}^{-1}$ and from 46.4 to 111.5 $\mu\text{g ml}^{-1}$ respectively (Medina, 2005).

In India, study was conducted by Devi *et al.* (2000) to determine 100 chilli samples for OTA. Out of which 26 samples were found to contain over 10 mg kg^{-1} OTA, in 12 samples, the OTA concentration varied from 10-30 mg kg^{-1} , in ten samples from 30-50 mg kg^{-1} , in three samples from 50-100 mg kg^{-1} , and in one sample it was 120 mg kg^{-1} . In another study they detected OTA in two of the 25 samples of ginger at 23 and 80 mg kg^{-1} (Devi *et al.*, 2001).

In Hungary, Fazekas *et al.* (2005) analysed 91 spice samples (70 ground red pepper, six black pepper, five white pepper, five spice mix and five chilli samples) for OTA. They found that 32 of the 70 ground red pepper samples contained OTA, eight of them in a concentration range of 10.6 - 66.2 mg kg^{-1} . One chilli sample was contaminated with OTA at 2.1 mg kg^{-1} . Shadanaika (2005) investigated the suitability of various spices *viz.*, chilli, ginger and turmeric for the production of ochratoxin. When *A. ochraceus* was grown on these substrates, 160, 20 and 10 ppb of ochratoxin A respectively were produced. Also found that among these spices tested, chilli was found to be the better substrate for ochratoxin A than ginger and turmeric.

2.4. CONTROL OF FUNGI CAUSING DETERIORATION OF NUTMEG AND MACE

Natural plant essential oils are the new source of antimicrobial agents to improve shelf life and safety of perishable foods. Bullerman *et al.* (1977) reported that plant products, especially essential oils, are recognized as one of the most promising groups of natural compounds for the development of safer antifungal agents for spices. They found that oil inhibit the growth of several mycotoxigenic moulds such as *A. flavus*, *A. niger*, *A. versicolor*, *A. ochraceus* and also reduced mycotoxin production in black and white pepper.

Mabrouk and El-Shayed (1980) found that clove oil completely inhibited the mycelial growth of *A. flavus* and aflatoxin formation. Gerald *et al.* (1992) reported that thymol from thyme leaves found to inhibit mycelial growth as well as aflatoxin production. Patkar *et al.* (1993) reported that cinnamon and clove oils inhibited *A. flavus* growth and subsequent aflatoxin B1 production. Nielsen and Rios (2000) reported that mustard and clove oil reduced growth of *A. flavus* by 100 and 40 per cent respectively. The oils of anise and cinnamon inhibited growth of *A. parasiticus* and toxin production completely (Rao *et al.*, 2000). 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). Soliman and Badeaa (2002) investigated the *in vitro* anti fungal effect of cinnamon oil and found that 1000 ppm induced total inhibition of growth of *A. flavus* and *A. parasiticus*. Bankole and Joda (2004) observed efficacy of lemongrass (*Cymbopogon citratus*) powder and essential oil on *A. flavus* growth and aflatoxin contamination. Omidbeygi *et al.* (2007) evaluated the antifungal activity thyme and clove oils in culture medium. Results clearly showed that each essential oil had notable antifungal activity. Thyme oil has the highest antifungal activity, followed by clove. Complete inhibition of growth of *A. flavus* was observed at 350 ppm thyme oil, while 500 ppm of clove oil had inhibition of 87.5 per cent. Reddy *et al.* (2009) reported that clove oil effectively inhibited the mycelial growth of *A. flavus* and aflatoxin production.

In vitro antifungal activities of some spice essential oils in culture media were demonstrated by Shadanaika (2005). All the 12 spice oils tested were able to inhibit growth of mycotoxigenic fungi namely, *A. flavus*, *A. ochraceus*, *A. parasiticus* and *Fusarium* spp. at different concentration when incorporated in the culture medium. Among the spice oils cinnamon oil was found to be effective in inhibiting the growth of *A. flavus* and *A. parasiticus* at 0.002 per cent concentration. Nutmeg and clove oil completely inhibited the growth of *A. flavus* and *A. parasiticus* at 0.006 per cent.

Various essential oils namely allspice oil, cinnamon oil, clove oil, turmeric leaf oil and plant materials like curry leaf, cinnamon leaf, garlic and neem leaf were found to have inhibitory effect on the growth of *Aspergillus* and aflatoxin production in black pepper, turmeric, ginger, nutmeg and mace (IISR, 2008). Turmeric leaf oil and cinnamon bark oil were tested for its inhibitory effect on aflatoxin production by *A. flavus* at concentration ranging from 0.01-1.5 per cent and 0.01-0.5 per cent respectively. Complete inhibition was seen at 1.5 per cent, with a drastic reduction in aflatoxin concentration from 163 ppb at 0.75 per cent to 4.3 ppb at 1.0 per cent of allspice oil (IISR, 2012).

Bilgrami *et al.* (1979; 1980) screened more than hundred wild and medicinal plant extracts against aflatoxin inhibition, and achieved aflatoxin inhibition in liquid culture above 70 per cent through the use of 20 plant extracts. Extracts of *Ricinus communis*, *Euphorbia hirta*, *Thuja orientalis* and *Adiantum* sp. were capable of preventing aflatoxin elaboration significantly on some solid substrates (Bilgrami *et al.*, 1979 and Singh, 1981). Aflatoxin elaboration has also been checked by onion and potato extracts (Sharma *et al.*, 1978; 1981). Some naturally occurring phenolic compounds like ferulic acid and O-vanilin also prevented aflatoxin production up to 85 per cent on some important cereals and oil-seeds (Bilgrami *et al.*, 1981; 1982). The extracts of clove and ginger (Mabrouk and El-Shayeb, 1980), turmeric (Madhyasta and Bhat, 1985), garlic and onion (Yin and Cheng, 1998) inhibited the growth of *A. flavus*.

According to Bilgrami *et al.* (1992) maximum inhibition in the mycelia growth occurred with garlic extract (62 per cent), whereas the inhibition of aflatoxin production was highest (60 per cent) with onion extract in liquid medium. Soni *et al.*, (1992) proved that extracts of turmeric, and garlic inhibited aflatoxin production considerably (more than 90 per cent) at concentrations of 5 - 10 mg ml⁻¹. Fan (1999) reported that mycelia growth of *A. flavus* and *A. parasiticus* and aflatoxin production were completely inhibited by onion extract at a concentration of 10 mg ml⁻¹. Latif *et al.* (2006) tested the efficacy of plant extracts prepared from garlic, neem leaf, ginger and onion bulb were studied on reduction of *A. flavus* on mustard. They found that garlic extract was more effective followed by neem.

Some traditionally useful plants have been shown to exhibit fungitoxic properties. Awuah (1996) reported that the plants extracts of *Ocimum gratissimum*, *Cymbopogon citrates*, *Xylopia aethiopica*, *Monodera myristica*, *Sizgium aromaticum*, *Cinnamomum verum* and *Piper nigrum* were effective in inhibiting formation of non sorbic acid, a precursor in aflatoxin synthesis pathway; also leaf powder of ocimum has been successfully used in inhibiting mould development on stored soybean for nine months. Paster *et al.* (1995) reported that aqueous plant extracts of cinnamon, piper mint, basil, organum, clove and thyme caused total inhibition of fungal development on maize kernels and optimal dosage varies from three to eight per cent.

Capsanthin from *Capsicum annum* (Red chilli) was also reported to check growth and toxin production at all tested concentrations. Phenolics like tannic acid, caffeic acid and phloroglucinol at 0.01 M concentration prevented aflatoxin production by more than 55per cent (Pafumi, 1986).

Storage containers can influence the moisture content of the substrate and ultimately mould development. Marar and Padmanabhan (1960) noted that copra stored in alkathene lined gunny bags remained in good condition for six months. Philip (1978) and Niza (1981) reported that polythene lined gunny bag was superior to ordinary gunny bag for the storage of copra as there was less

contamination by fungi in those stored in polythene lined gunny bags. In another study conducted by Naseema (1989) on the effect of storage containers in reducing the deterioration of oil cakes by fungi, it was noticed that polythene lined gunny bags were superior to other containers tested. The oil cakes stored in polythene lined gunny bag had the least population of fungi. Those stored in ordinary gunny bag which is most commonly used for the storage and transport had very high population of fungi.

Materials and methods

3. MATERIALS AND METHODS

Laboratory studies were carried out at the College of Agriculture, Vellayani, Triruvananthapuram to study the mycoflora associated with the spoilage of nutmeg and mace in storage, to provide useful information on the production of mycotoxin and to evolve suitable methods to minimize fungal contamination in storage.

3.1. COLLECTION OF NUTMEG AND MACE SAMPLES

Samples of nutmeg and mace were collected from spice collection centres of Kozhikode and Thrissur districts during two seasons of the Year: February-March, 2013 and June-July, 2013. From each district samples were collected from five locations (Table 1).

Table 1. Locations surveyed and observatories from where the weather data collected

| District | Collection centres | Observatory |
|------------------|---------------------------|---|
| Kozhikode | Kozhikode | IISR, Kozhikode and Water management (Agricultural) division, Centre for Water Resources Development and Management (CWRDM) Kozhikode |
| | Vellimadukunnu | ” |
| | Kunnamangalam | ” |
| | Vengeri | ” |
| | Chevayoor | ” |
| Thrissur | Thrissur | Dept of Meteoriology, College of Horticulture, Vellanikkara |
| | Thottapadi | ” |
| | Chalakkudi | ” |
| | Madakathara | ” |
| | Mannuthy | ” |

Approximately 50 g of nutmeg and mace samples were brought to laboratory for further studies. Quantitative and qualitative estimation of the fungal flora associated with the nutmeg and mace samples were carried out as soon as they were brought to the laboratory. The fungi isolated were maintained on potato dextrose agar (PDA) slants (Appendix 1) for characterization and identification.

3.2. QUANTITATIVE AND QUALITATIVE ESTIMATION OF FUNGI

3.2.1. Quantitative Estimation of Fungi in Nutmeg

The fungal population in different samples of nutmeg collected during the survey was estimated as soon as it was brought to the laboratory by dilution plate method (Booth, 1971) using peptone dextrose agar with Rose-bengal and streptomycin (Appendix 1). Five grams of powdered nutmeg sample was transferred into 250 ml conical flask containing 100 ml sterile, distilled water and thoroughly shaken for 30 min. in a mechanical shaker. Serial dilutions were made by transferring one ml of aliquot to a flask containing 99 ml of sterile distilled water. The flask containing suspension was shaken for five min. The final dilution used for the estimation of fungal population was one in ten thousand (10^{-4}). One ml of this diluted suspension was pipetted out into sterile Petridish and about 20 ml of melted medium cooled to 45 to 50°C was added and allowed to solidify. Two replications were maintained for each sample. The plates were then incubated at room temperature ($28\pm 2^\circ\text{C}$). Fungal count was taken at three, seven and twelve days after plating. The number of colony forming units (cfu) was expressed in millions per gram of dry sample.

3.2.2. Quantitative Estimation of Fungi in Mace

The fungal population in different samples of mace collected during the survey was estimated as soon as it was brought to the laboratory as per the procedure followed in item 3.2.1. Fungal count was taken at three, seven and twelve days after plating. The number of colony forming units (cfu) was expressed in millions per gram of dry sample.

3.2.3. Qualitative Estimation of Fungi in Nutmeg and Mace

The fungal colonies obtained from samples of nutmeg and mace from item 3.2.1 and 3.2.2 were transferred to Potato Dextrose Agar (PDA) slants. The cultures were purified by hyphal tip method (Rangaswami and Mahadeva, 2004) and maintained on PDA slants.

3.3. CHARACTERIZATION AND IDENTIFICATION OF FUNGAL FLORA

The cultural characters of fungi obtained in item 3.2.3, such as type, texture and colour of mycelium were studied by growing on Czapek (Dox) agar plates (Appendix 1). The morphological characters such as type of mycelium, shape of spores and spore bearing structures were studied by preparing wet mount and by slide culture technique described by Riddel (1974). The measurements of spores and spore bearing structures were taken by using ocular micrometer.

3.3.1. Preparation of Wet Mount

The fungal growth with spores and spore bearing structures maintained in Petri plates were teased out on a clean slide with a drop of lactophenol cotton blue stain, with the help of two clean needles and a cover slip was kept over it. The slides were sealed with DPX mountant and observed under the microscope to study the morphological characters such as nature of mycelium, shape and size of spores and spore bearing structures.

3.3.2. Preparation of Slide Culture

Sterile plain agar medium was poured on Petri dishes to a thickness of two mm. After solidification, six mm square pieces were cut using a sterile needle. One square disc was placed at the centre of a sterile slide and each of the four sides of the agar block was inoculated with mycelial bits of the fungi. A cover slip was placed on the top of the inoculated agar disc and the slides were placed inside the Petri dishes, consisting of wet filter paper in the bottom in which two glass rods were kept as support for the slides. The dish with the slide was then incubated at room temperature for two to three days. After this, the cover slip was lifted off gently and mounted on another slide using lactophenol stain. The agar block was removed from the culture slide and another mount was prepared on it

without disturbing the fungal growth on it. The slides were examined under low and medium power objectives of a compound microscope and micro morphological characters were observed and photo micrographs were taken. Measurements of spores and spore bearing structures were recorded using ocular micrometer. The fungi were provisionally identified.

The provisionally identified fungal cultures were sent to National Centre for Fungal Taxonomy (NCFT), Inderpuri, New Delhi for confirmation and deposition.

3.3.3. Frequency of Occurrence of Fungi

Frequency of occurrence of each of fungi identified as per item 3.3 from the two districts during two seasons such as February- March and June-July were calculated.

$$\text{Frequency of occurrence of fungi} = \frac{\text{No. of times a fungus occurred in a year}}{\text{Total locations surveyed}} \times 100$$

3.3.4. Effect of Weather Parameter and Sample Moisture on the Population of Fungi.

Weather parameters such as maximum and minimum temperature, relative humidity (RH) and total rain fall of both districts, namely, Thrissur and Kozhikode were collected from observatories (Table 1).

Moisture content of the samples of nutmeg and mace was estimated as soon as it was brought to the laboratory using Essae moisture analyser, which dries the sample using a halogen lamp and gives the moisture content in percentage based on the principle of thermo gravimetric analysis.

Correlation studies were carried out between weather parameters, moisture content of the sample and population of fungi.

3.4. DETECTION OF MYCOTOXIN IN THE NUTMEG AND MACE SAMPLES

The natural incidence of total aflatoxin and the different fractions of aflatoxin such as B₁, B₂, G₁ and G₂ in the samples of nutmeg and mace collected during the survey were assayed.

3.4.1. Detection of Total Aflatoxin

Total aflatoxin content in the nutmeg and mace samples collected during the survey was estimated by ELISA using the Agra Quant Aflatoxin test kit (Romer labs, Inc. USA)

3.4.1.1. *Sample Preparation \ Extraction*

1. Weighed 20g of ground sample into a clean jar and tightly sealed.
2. Added 100ml of methanol : water (70:30) extraction solution into the sealed jar.
3. the jars were vigorously shaken/ blended for three minutes.
4. Allowed the sample to settle and filtered the top layer of extract through a Whatman no. 1 filter and filtrate was collected.

3.4.1.2. *Immunoassay*

Assay was performed as per the manufacturer's protocol.

1. Placed 12 numbers (six standards and six samples) of blue/ green bordered dilution strips in a microwell strip holder. One dilution well (blue/ green bordered) was kept for each standard (*ie.*, 0,1.0, 2.0, 4.0, 10.0 and 20.0 ppb) and sample.
2. An equal number (12 numbers) of antibody coated microwell strips was placed in a microwell strip holder.
3. 250µl per well of conjugate from the green- capped bottle was dispensed into a reagent boat. Using an eight channel pipette, 200µl of conjugate was dispensed into each blue/green bordered dilution well.
4. Using a single channel pipette, 100 µl of each standard and sample was dispensed into the appropriate dilution well containing 200 µl of conjugate. Fresh tips were used for each standard and sample.

5. The microwell strips were emptied into a waste container. Each microwell was washed with distilled water. This step was repeated for three times.
6. Several layers of absorbent paper towels were laid on a flat surface and microwell strips were tapped on the towel to expel as much water.
7. 120µl per well of substrate was dispensed into a reagent boat. Using an eight channel micropipette 100µl of substrate was dispensed into each microwell and incubated at room temperature for five minutes.
8. 100 µl of stop solution from red capped bottle was taken and dispensed into each microwell strip.
9. The colour change from blue to yellow was observed and the strips were read with a micro plate reader (Bio-Rad model 680) at 630 nm.
10. The OD value was recorded for another analysis.

3.4.1.3. Quantification

The total aflatoxin content of the samples (ppb) was computed from a standard curve, prepared by plotting the OD values of different concentrations of standard toxin.

3.4.2. Detection of Different Fractions of Aflatoxin

The different fractions of aflatoxins *viz.*, B₁, B₂, G₁ and G₂ present in the samples of nutmeg and mace collected during the survey were assayed qualitatively and quantitatively.

3.4.2.1. Qualitative Detection of Aflatoxins

The qualitative detection of different fractions of the toxins in the samples of nutmeg and mace was carried out by TLC.

3.4.2.1.1. Extraction of Aflatoxin

Samples of nutmeg and mace were extracted for the detection of natural contamination of different fractions of aflatoxin, *viz.*, B₁, B₂, G₁ and G₂ by following the methods of Roberts and Patterson (1975).

Twenty five gram of each sample of nutmeg and mace were crushed separately and extracted with 150 ml methanol: water (60:40 v/v) for five min. at 2000 rpm in a blender. The filtrate was then extracted thrice with 40ml *n*-hexane.

The methanolic fraction was extracted with 10 ml of chloroform. The chloroform layer was then passed through anhydrous Na₂SO₄ bed to remove moisture. The combined chloroform extract was then evaporated to dryness and redissolved in 1 ml chloroform and used for Thin layer chromatography (TLC).

With the help of micropipette, 10 µl of sample were spotted on TLC plates (coated with silica gel). Two µl of Certified reference material (CRM) of aflatoxins B₁, B₂, G₁ and G₂ obtained from Sigma chemical company, U.S.A. were also spotted on the plate.

The plates were placed in the solvent chamber containing chloroform and methanol (98:2) and allowed to run till the solvent reached ¾ height of the plates. The plates were then air dried and observed under UV chamber (320-360nm). The characteristic fluorescent spots at the same R_f value as that of the standard toxins under UV excitation were observed.

3.4.2.1.2. Quantification

The characteristic fluorescent spots at the same R_f value as that of the standard toxins under UV excitation were marked by means of a sharp needle. The silica gel covering each spot was scraped carefully with a blade and collected individually in clean, dry centrifuge tubes and five ml of methanol was added to each. The tubes were centrifuged at 3000 rpm for five minutes. The methanol layer was decanted and used for spectrophotometry (NIN, 1983). The aflatoxin content was determined by recording the absorbance in a spectrophotometer (Spectronic 2000) at 360 nm (for B₁ and G₁) and 362 nm (for B₂ and G₂). The amount of aflatoxin present in 10 µl of the chloroform extract was computed from a standard curve. The aflatoxin content (µg kg⁻¹ or ppb) of the original sample was calculated by using the following formula (Nabney and Nesbitt, 1965):

$$\text{Aflatoxin content of sample} = \frac{D \times M \times 10^6}{E \times I \times 1000}$$

D = Optical density

M = Molecular weight of aflatoxin

E = Molecular extinction coefficient

I = Path length (1cm cell was used)

Molecular weight (M) and molecular extinction coefficient (E) of different fractions of aflatoxins at respective wave lengths are indicated below:

| Aflatoxins | Wave length (nm) | Molecular weight (M) | Molecular extinction coefficient (E) |
|------------|------------------|----------------------|--------------------------------------|
| B1 | 360 | 312 | 22,000 |
| B2 | 362 | 314 | 23,400 |
| G1 | 360 | 328 | 18,700 |
| G2 | 362 | 330 | 21,000 |

3.5. QUALITATIVE AND QUANTITATIVE ESTIMATION OF THE MYCOTOXIN PRODUCED BY THE FUNGI OBTAINED.

The mycotoxin producing ability of the species of *Aspergillus viz., A. flavus, A. niger* and *A. ochraceus* obtained during the survey was studied.

3.5.1. Detection of Aflatoxin

Production of aflatoxin by nine isolates of *A. flavus* and 12 isolates of *A. niger* was studied under *in vitro* condition in culture and under *in vivo* in nutmeg and mace separately.

3.5.1.1. Qualitative Estimation of Aflatoxin

Five isolates of *A. flavus* and seven isolates of *A. niger* obtained from nutmeg and four isolates of *A. flavus* and five isolates of *A. niger* of mace (Table 2. and Table 3.) collected from different regions during different periods of the year were tested for their ability to elaborate different fractions of aflatoxins, namely, B₁, B₂, G₁ and G₂ in culture and substrate.

3.5.1.2 In Culture Medium

3.5.1.2.1 Sample Preparation

Aflatoxin produced in culture medium was estimated by the method of Diener and Davis (1966). The isolates of *A. flavus* and *A. niger* were allowed to grow in sterile Petri dishes containing PDA for seven days. 250ml conical flask each containing 100ml of sterile SMKY liquid medium (Appendix 1) was inoculated with 8 mm fungal disc from 7 days old culture of each fungi and

Table 2. *Aspergillus* isolates of nutmeg tested for aflatoxin elaboration

| <i>Aspergillus flavus</i> | | <i>Aspergillus niger</i> |
|---------------------------|-------------|--------------------------|
| Sl. no | Isolates * | Isolates * |
| 1 | CLT – I-1 | CLT – II -1 |
| 2 | CLT – II -2 | CLT – I-3 |
| 3 | THR – II-3 | THR – I-4 |
| 4 | THR – I-4 | CLT – II -5 |
| 5 | CLT – II-7 | THR – II-6 |
| 6 | | THR – II-7 |
| 7 | | CLT – II -8 |

*CLT- Kozhikode
THR- Thrissur

I- February-March
II- June-July

Table 3. *Aspergillus* isolates of mace tested for aflatoxin elaboration

| <i>Aspergillus flavus</i> | | <i>Aspergillus niger</i> |
|---------------------------|------------|--------------------------|
| Sl. no | Isolates * | Isolates * |
| 1 | THR –I-3 | CLT – I-2 |
| 2 | THR – I-4 | CLT – I-3 |
| 3 | CLT – II-5 | CLT – II-5 |
| 4 | THR – I-6 | THR – II-7 |
| 5 | | CLT –II-8 |

*CLT- Kozhikode
THR- Thrissur

I- February-March
II- June-July

incubated without agitation for 7 days at room temperature ($28\pm 2^\circ\text{C}$), chloroform was sprayed over the culture and the culture filtrate was taken in a 250ml conical flask. Aflatoxins in the culture filtrate were extracted by vigorously refluxing with chloroform (2:1 v/v) for one hour. The chloroform layer was separated into a 100ml beaker using a separating funnel and then evaporated in a hot water bath.

3.5.1.2.2. Detection of Total Aflatoxin

Total aflatoxin produced by the isolates of *A. flavus* and *A. niger* in culture was estimated by ELISA. The samples prepared were resuspended in 1ml methanol and ELISA was carried out as per the procedure in item 3.4.1.2.

3.5.1.2.3. Detection of Different Fractions of Aflatoxin

The different fraction of aflatoxin viz., B₁, B₂, G₁ and G₂ elaborated by *A. flavus* and *A. niger* in SMKY liquid medium were detected qualitatively and quantitatively. For that the residue was dissolved in 1ml of chloroform and assayed for aflatoxins B₁, B₂, G₁ and G₂ by means of TLC.

3.5.1.3. Aflatoxin production on nutmeg and mace

Isolates of *A. flavus* and *A. niger* (Table 2 and 3) were grown in sterile Petri dishes containing PDA. After seven days nutmeg samples were artificially inoculated with five isolates of *A. flavus* and seven isolates of *A. niger* (Table 2), mace samples with two isolates of *A. flavus* and five isolates of *A. niger* (Table 3) by dusting the spores over the samples (25 g each) taken in a Petri dish. Inoculated samples were placed in desiccators maintained at 95 per cent relative humidity using sulphuric acid and water (10:100) (CMI,1983) and incubated for two weeks at room temperature ($28\pm 2^\circ\text{C}$) (Plate 1. A and C). Aflatoxins were estimated following the procedure of Pons *et al.* (1971).

3.5.1.3.1. Aflatoxin on Nutmeg and Mace

3.5.1.3.1.1. Sample Preparation

Fungal growth over the incubated nutmeg and mace sample were scrapped off using a sharp razor and the samples were ground finely, twenty five grams of sample was transferred to a 250 ml conical flask containing 125 ml of 70 per cent acetone and kept in a mechanical shaker for 30 min. The material was filtered through Whatman No. I filter paper. 75 ml of filtrate was taken in a 250 ml beaker



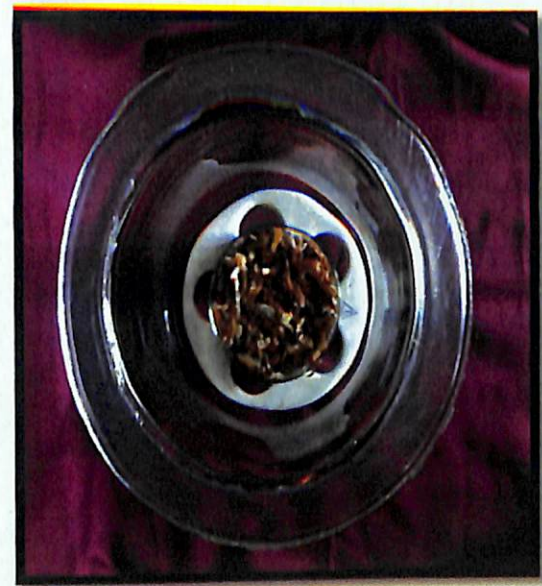
A. Nutmeg sample



B. Nutmeg sample (overview)



C. Mace sample



D. Mace sample (overview)

Plate 1. Inoculated sample for incubation in the desiccator

and level was marked. Ten ml of two per cent lead acetate was added to this to remove protein and carbohydrates which are likely to interfere with aflatoxin estimation. Thirty ml of distilled water was then added and the beaker was then placed in a hot water bath till the contents evaporated up to the marked level. After cooling to room temperature the contents were filtered through Whatman No. I filter paper into a separating funnel and extracted in three changes of 25 ml chloroform. Two columns were separated in the separating funnel and the bottom layer of chloroform containing aflatoxins was allowed to run off through a bed of anhydrous sodium sulphate in a conical flask, to remove traces of water.

3.5.1.3.1.2. Detection of Total Aflatoxin

Total aflatoxin produced by the isolates of *A. flavus* and *A. niger* in substrate (nutmeg and mace) was estimated by ELISA. The samples prepared were resuspended in 1 ml methanol and ELISA was carried out as per the procedure in 3.4.1.2.

3.5.1.3.1.3. Detection of Different Fractions of Aflatoxin

Different fractions of aflatoxin *viz.*, B₁, B₂, G₁ and G₂ elaborated by *A. flavus* and *A. niger* in substrate, *viz.*, nutmeg and mace were detected qualitatively and quantitatively. For that the residue was dissolved in 1ml of chloroform and assayed for aflatoxins B₁, B₂, G₁ and G₂ by means of TLC as per procedure in 3.4.2.

3.5.1.4. Quantitative Estimation of Aflatoxin

Total aflatoxin produced by different isolates of *A. flavus* and *A. niger* (Table 2 and 3) in culture and substrate detected by ELISA was quantified from a standard curve as per item 3.4.1.3. The quantity of different fractions of aflatoxin, *viz.*, B₁, B₂, G₁ and G₂ produced by above isolates in culture and in substrate was estimated by spectronically reading the spots marked over the TLC plates as per the procedure in 3.4.2.1.2.

3.5.2. Detection of Ochratoxin

The following five isolates (Table 4.) of *A. ochraceus* obtained from nutmeg samples collected during the survey were studied for their ability to produce ochratoxin in culture media and in substrate by High Performance Liquid Chromatography (HPLC).

Table 4. *A. ochraceus* isolates tested for ochratoxin elaboration

| Sl. No. | Isolate |
|---------|---------|
| 1 | AO- 1 |
| 2 | AO -2 |
| 3 | AO -3 |
| 4 | AO -4 |
| 5 | AO -5 |

AO- *Aspergillus ochraceus*

3.5.2.1. In Culture Medium

The pure culture of each *Aspergillus ochraceous* isolates were maintained on Potato Dextrose Agar (PDA) plate. For ochratoxin production assay, the isolates were grown in 50 ml of Yeast Extract-Sucrose broth (YES: two per cent Yeast Extract, 15 per cent Sucrose) (Appendix 1) in 125 ml conical flasks. Broth was inoculated with eight mm disc from seven days old culture of each isolate and incubated without agitation for ten days at 30°C in the dark (Bayman *et al.*, 2002). Chloroform was sprayed over the culture and the culture filtrates were collected and filtered through a 0.2 µm syringe filter and used for ochratoxin A (OTA) detection by High Performance Liquid Chromatography (HPLC) after passing through Immunoaffinity Column (OchraTest™, VICAM, USA).

3.5.2.2. Ochratoxin in Substrate (Nutmeg)

3.5.2.2.1. Extraction of Ochratoxin

Ochratoxin A extraction from collected samples was done by the method of Moller and Nyberg (2003). Isolates of *A. ochraceus* were grown in sterile Petri dishes containing PDA. After seven days nutmeg samples were artificially inoculated with five isolates of *A. ochraceus* by scrapping off the culture and the spore was dusted over the samples taken in a Petri dish. Inoculated samples were placed in desiccators maintained at 95 per cent relative humidity using sulphuric acid and water and incubated for two weeks at room temperature ($28\pm 2^{\circ}\text{C}$).

Five gram portion of the above nutmeg samples were taken and fungal growth were removed, ochratoxin extracted with methanol: sodium bicarbonate at 1 per cent (70:30, v/v) and blended at high speed for 1 min. The extract was filtered to remove particulate matter and 10 ml of the same was diluted with 40 ml of phosphate buffer saline (PBS) containing 0.01 per cent Tween 20. The diluted extract was filtered through a microfiber filter. A ten ml portion was taken and added to an immunoaffinity column (IAC) at a flow rate of one drop per second. Then IAC was washed with 5 ml of washing solution (2.5 per cent NaCl, 0.5 per cent NaHCO_3) and then with water at 1 - 2 drop per second flow rate. Column was dried by passing air through it.

3.5.2.2.2. Preparation of Sample Solution for Loading in HPLC.

Ochratoxin A was eluted from the column into vial by passing 2 ml of methanol at one drop per second flow rate. The elute was evaporated to dryness in a eppendorf concentrator plus at 45°C . The elute was immediately redissolved in 250 μl HPLC mobile phase (water: acetonitrile: glacial acetic acid, 99:99:2) and stored at 4°C for HPLC analysis.

3.5.2.2.3. HPLC Analysis

The samples were analysed using HPLC system consisting of an isocratic unit with a quaternary pump at a flow rate of 0.2 - 10 ml min^{-1} with manual injector with Rheodyne 7725i7- port sample injection valve. 20 μl of the sample was loaded into the external 50 μl sample loop through the injection port. A 15 cm

long reverse-phase column containing C18 modified silica packing material of 3 - 5 μm particle size was present inside the system for the separation of the compounds. The HPLC system was attached to a fluorescence detector where the luminescence takes place based on the excitation wave length of 333 nm and emission wave length of 460 nm.

3.6. METHODS TO MINIMISE FUNGAL CONTAMINATION IN NUTMEG AND MACE.

The following 15 treatments with two replications in Completely randomized design (CRD) were carried out to test their ability to minimize fungal contamination in nutmeg and mace (Plate 2 and 3).

Table 5. Treatments tested to minimize fungal contamination in nutmeg and mace

| Sl. No. | Treatments | Concentration |
|-----------------------------------|--------------------|---------------|
| i. Essential oils | | |
| T1 | Allspice oil | 0.05% |
| T2 | Cinnamon oil | 0.05% |
| T3 | Clove oil | 0.05% |
| T4 | Lemon grass oil | 0.05% |
| ii. Leaf powder/Plant extracts | | |
| T5 | Curry leaf | 10% |
| T6 | Garlic extract | 10% |
| T7 | Neem leaf | 10% |
| T8 | Onion extract | 10% |
| T9 | Ocimum leaf | 10% |
| iii. Containers/ Packing material | | |
| T10 | Cloth bag | - |
| T11 | Hessian cloth bag | - |
| T12 | Plastic bag | - |
| T13 | Polylined jute bag | - |
| T14 | Polypropylene bag | - |
| T15 | Gunny bag | - |

Essential oils, leaf powders and plant extracts (T1 - T9) were applied on nutmeg and mace and 20 g each of nutmeg and mace were separately packed in



T1- Allspice oil



T2- Cinnamon oil



T3- Clove oil



T4- Lemon grass oil



T5- Curry leaf



T6- Garlic extract



T7- Neemleaf



T8- Onion extract



T9- Ocimum leaf



T10- Cloth bag



T11- Hessian cloth bag



T12- Plastic bag



T13- Polylined jute bag



T14- Polypropylene bag



T15- Gunny bag

Plate 2. Treatments to minimize deterioration of nutmeg in storage



T1- Allspice oil



T2-Cinnamon oil



T3- Clove oil



T4-Lemon grass oil



T5-Curry leaf



T6-Garlic extract



T7-Neemleaf



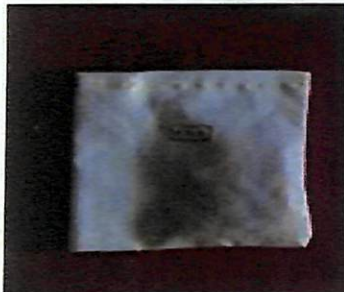
T8-Onion extract



T9-Ocimum leaf



T10-Cloth bag



T11-Hessian cloth bag



T12-Plastic bag



T13-Polylined jute bag



T14-Polypropylene bag



T15-Gunny bag

Plate 3. Treatments to minimize deterioration of mace in storage

polypropylene bags (15×10 cm) having a pocket with an opening. Essential oils *viz.*, allspice oil, cinnamon oil, clove oil and lemon grass oil (T1 - T4) at the required concentration (0.05 per cent) were prepared by impregnating 10µl of the same on absorbent cotton balls and placed in perforated sachet. The treatments of leaf powder *viz.*, curry leaf, neem leaf and ocimum leaf (T5, T7 and T9) were dried and powdered. The required concentration of ten per cent was prepared by filling two gram of the powder into perforated sachet. The treatments of plant extract, namely, onion and garlic (T6 and T8) were made by crushing ten gram of the material in a mortar and pestle. The crude extract was collected by passing through a muslin cloth. The required concentration of ten per cent was made by impregnating two ml of the extract on absorbent cotton balls and placed in perforated sachets. Each of the sachets was placed in the pocket, so that the fumes were diffused through the opening and got contact with the material inside the polypropylene bag.

For the treatments T11 - T15, 20 g of each of nutmeg and mace samples were stored in each of the containers (15×10 cm) at room temperature (28±2°C). Two replications were maintained for each treatment. Observations on the growth of fungi and their population (cfu) were taken at two week interval for a period of three months.

Moisture content of the sample before and after the treatments was recorded as per item 3.3.4.

Cost of treatments for storing 1 kg of nutmeg and mace were worked out.

3.7. STATISTICAL ANALYSIS

Data relating to different experiments were analysed by applying appropriate statistical methods (Panse and Sukhatme, 1967)

Results

4. RESULTS

4.1. COLLECTION OF NUTMEG AND MACE SAMPLES

Samples of nutmeg and mace were collected from collection centres of two districts, namely, Thrissur and Kozhikode during two seasons of the year: February- March and June-July, 2013. Ten sample each of nutmeg and mace were collected from each district (Thrissur and Kozhikode) during each season (February- March and June-July), 80 samples were thus collected and brought to the laboratory for further studies to be carried out.

4.2. QUANTITATIVE AND QUALITATIVE ESTIMATION OF FUNGI

4.2.1. Quantitative Estimation of Fungi in Nutmeg

Quantitative estimation of fungal propagules present in the samples of nutmeg collected from different districts, namely, Kozhikode and Thrissur during two seasons of the year *viz.*, February – March and June – July 2013 was carried out (Table 6). The results of the study revealed that there was variation in the population of fungi in the samples of nutmeg from different regions during different seasons of the year. The variation in the population between different regions was not statistically significant. However the variation in the population of fungi from the samples collected from Thrissur district during different periods of the year, February – March and June – July was found to be significant, whereas it was not significant in Kozhikode. The population of fungi was highest during June – July in both the regions, namely, Thrissur and Kozhikode being 9.20×10^4 and 5.64×10^4 respectively. The population was lowest in February - March and the population being 1.52×10^4 and 4.04×10^4 in Thrissur and Kozhikode district respectively (Plate 4).

4.2.2. Quantitative Estimation of Fungi in Mace

There was variation in the population of fungi in samples of mace collected from Kozhikode and Thrissur during the two seasons of the year namely February – March and June – July (Table 7). The variation in the population of fungi between region and season were not statistically significant. The highest

Table 6. Quantitative estimation of fungi in nutmeg

| Region | Population of fungal propagules (10^4 g^{-1}) | | | |
|--------------|---|----------------|--------------------|----------------|
| | Thrissur District | | Kozhikode District | |
| Season | February-March | June-July | February-March | June-July |
| Mean | 1.52 (1.59) | 9.20 (3.19) | 4.04 (2.25) | 5.64 (2.58) |
| t-value | 3.34* | | NS | |
| Overall mean | 4.60 (2.37) | | 4.94 (2.44) | |
| t- value | NS | | | |

*Figures in the parenthesis indicates $\sqrt{x+1}$ transformation

Table 7. Quantitative estimation of fungi in mace

| Region | Population of fungal propagules (10^4 g^{-1}) | | | |
|--------------|---|----------------|--------------------|----------------|
| | Thrissur District | | Kozhikode District | |
| Season | February-March | June-July | February-March | June-July |
| Mean | 2.61 (1.90) | 3.08 (2.02) | 3.75 (1.98) | 4.40 (2.02) |
| t-value | NS | | NS | |
| Overall mean | 2.79 (1.94) | | 4.55 (2.35) | |
| t- value | NS | | | |

*Figures in the parenthesis indicates $\sqrt{x+1}$ transformation



Plate 4. Fungal colonies from nutmeg sample

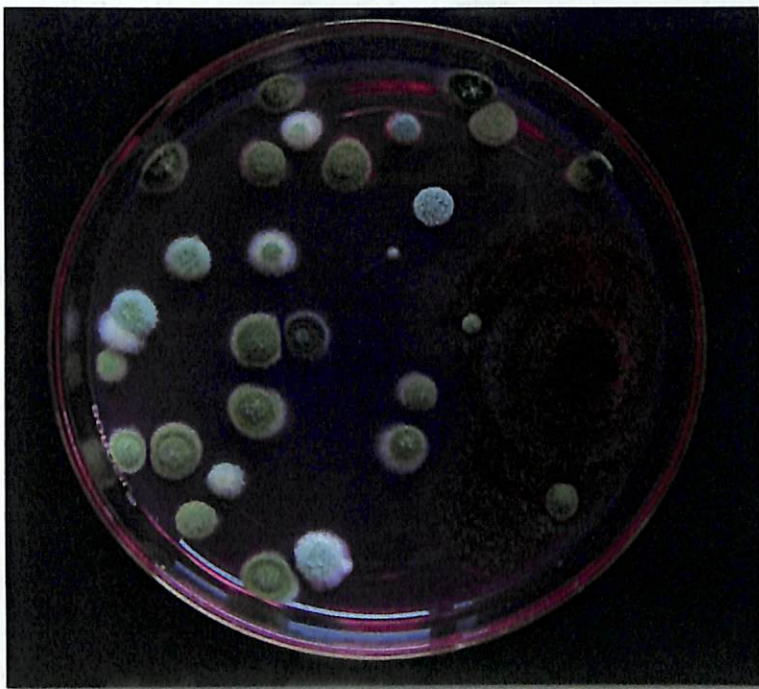


Plate 5. Fungal colonies from mace sample

population was recorded during June – July in all the samples of mace collected from the two regions, namely, Thrissur and Kozhikode being 3.08×10^4 and 4.40×10^4 respectively. The lowest population of 2.61×10^4 and 3.75×10^4 respectively was recorded during February- March from Thrissur and Kozhikode (Plate 5).

4.2.3. Qualitative Estimation of Fungi in Nutmeg and Mace

The qualitative estimation of fungi from samples of nutmeg and mace collected from the two regions during two seasons of the year were carried out. The cultural and morphological characters of the fungi were studied. The cultural characters such as type, texture and colour of mycelium were studied by growing on Czapek's (Dox) agar plates. The cultures were subjected to wet mounting and slide culturing for studying the morphological characters of the fungi such as nature of mycelium, shape and colour of spore and spore bearing structures. The size of spores and spore bearing structures were measured by using the ocular micrometer. Photo micrographs of the fungi were taken and provisionally identified. The identification of the fungi was confirmed by submitting the cultures to National Centre for Fungal Taxonomy (NCFT) (Table 8).

4.3. CHARACTERIZATION AND IDENTIFICATION OF FUNGAL FLORA

The cultural and morphological characters of the 13 identified fungi were as follows.

***Acremonium restrictum* W. Gams**

Colonies on Czapek's (Dox) agar medium were white in colour, compact, moist and slow growing (Plate 6.i). Phialides were formed at the tip of hyaline hyphae. Conidia were one celled, hyaline and cylindrical $2.0 \times 3.0 \mu\text{m}$ diameter and aggregated in slimy heads at the apex of each phialides (Plate 7.i).

***Acremonium strictum* W. Gams**

Colonies on Czapek's (Dox) agar medium were creamy white, slow growing, initially compact and moist (Plate 6.ii). The hyphae were fine, hyaline and produced phialides at the tip. The conidia were one-celled and pigmented,

globose to cylindrical ($3.0 \times 1.25 \mu\text{m}$) and aggregated on the apex of each phialide (Plate 7.ii).

***Aspergillus flavus* Link**

Colonies on Czapek's (Dox) agar medium were yellowish green or lime green in colour (Plate 6.iii). Conidiophores erect and formed on specialized thick walled foot cells. Conidiophore terminated in to a globose vesicle ($10\text{-}13\mu\text{m}$) bearing numerous phialides. Conidia were one celled, globose ($3\text{-}4 \times 4\text{-}5\mu\text{m}$) and produced from the tip of the phialides (Plate 7.iii).

***Aspergillus niger* van Tieghem**

Colonies on Czapek's (Dox) agar medium were rapidly growing with abundant aerial mycelium, brown to black in colour and reverse side without colour (Plate 6.iv). Conidiophore ($200\text{-}400 \mu\text{m}$) smooth, septate ends in globose vesicle ($20\text{-}45 \mu\text{m}$). Conidial heads were brown to black. Phialides were born directly on the vesicle over which globose conidia ($2.5\text{-}4.0\mu\text{m}$) were arranged in chains (Plate 7.iv).

***Aspergillus ochraceus* Wilhelm**

Colonies on Czapek's (Dox) agar medium were golden yellow to ochre coloured, consisting of conidiophores and conidial heads with little aerial mycelium (Plate 6.v). Conidiophores were long bearing globose vesicle ($35 - 50 \mu\text{m}$) with phialides arranged in two series. Conidia globose ($3.5\text{-}5.0\mu\text{m}$), smooth, yellow to orange and arranged in long chains (Plate 7.v).

***Aspergillus oryzae* E. Cohn**

Yellowish brown rapidly growing colonies were produced on Czapek's (Dox) agar medium (Plate 6.vi). Conidiophores were long with large globose conidial heads adhered with chains of conidia, which are pale yellow in colour. Vesicles were sub-globose to globose ($30 - 50\mu\text{m}$) over which phialides were arranged. Conidia were globose, smooth and $3.0 - 4.5\mu\text{m}$ in size (Plate 7.vi).

***Aspergillus sclerotiorum* G. A. Huber**

White to creamy colonies was developed on Czapek's (Dox) agar medium. Yellowish to ochre coloured sclerotia were scattered over the mycelium (Plate 6.vii). Conidiophores yellow $200\text{-}400 \times 700 \mu\text{m}$; conidial heads were sulphur

yellow in colour. Vesicles were sub-globose (6.0 - 32.0 μm), phialides born on metulae. Conidia smooth walled, spherical and 2.0 - 3.5 μm (Plate 7.vii).

***Emericella nidulans* Thom**

Yellow colonies were formed on Czapek's (Dox) agar medium with reverse side purplish red (Plate 6.viii). Conidiophores were cinnamon brown, 75 - 100 \times 2.5 - 3 μm , vesicle hemispherical 8-10 μm diameter and phialides were formed on metulae. Conidia were globose (3 - 3.5 μm) and rough walled. Cleistothecia was formed (Plate 7.viii).

***Eurotium amestelodami* L. Mangin**

Colonies on Czapek's (Dox) agar medium were more or less wrinkled, zonated bright yellow in colour, conidial heads olive brown (Plate 6.ix). Conidiophores colourless to pale yellowish green 275 - 350 \times 10 - 12 μm . Vesicles sub-globose 18 - 25 μm . Over which phialides were produced. Conidia formed were sub-globose to ellipsoidal and 3.5 - 5.2 μm in length (Plate 7.ix).

***Fusarium moniliforme* Sheldon**

Aerial mycelium was white initially, turning rosy peach colour when grown on Czapek's (Dox) agar medium. Powdery growth was also seen towards the centre of the colony (Plate 6.x). Conidiophores were simple and hyaline. Chlamydospores were present. Macroconidia were somewhat straight, tapering towards ends, hyaline, 3 - 5 septate and 3.6-12.2 \times 1.8-3.1 μm in size (Plate 7.x).

***Hansfordia pulvinata* Berk and M. A. Curtis**

Colonies on Czapek's (Dox) agar medium were pale yellow in colour (Plate 6.xi). Conidiophores were hyaline to brown, main stem of conidiophores were unbranched whereas upper part of conidiophores were branched. Conidia were globose, 5.5 - 8.5 μm in diameter, rough walled and hyaline (Plate 7.xi).

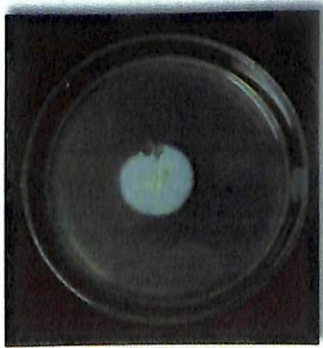
***Penicillium chrysogenum* Thom**

Colonies on Czapek's (Dox) agar medium were grey green becoming brownish in old and reverse side yellow (Plate 6.xii). Conidiophores were long (275 μm). Phialides were formed in two layer 8.0 \times 2.5 μm . Conidia were elliptical to globose and pale green and 3.0 - 4.0 μm (Plate 7.xii).

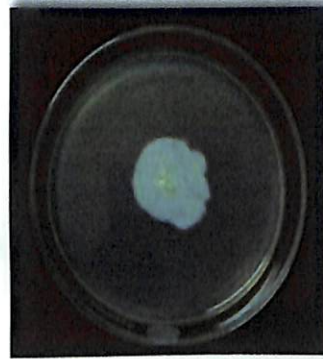
Table 8. Frequency of occurrence of fungi in samples of nutmeg and mace

| Sl. No | Fungi | Identification code | Percent occurrence | | | |
|--------|---|---------------------|--------------------|----------|-----------|----------|
| | | | Nutmeg | | Mace | |
| | | | Kozhikode | Thrissur | Kozhikode | Thrissur |
| 1 | <i>Acremonium restrictum</i> W. Gams | NCFT 5761.13 | 30 | 20 | 25 | 15 |
| 2 | <i>Acremonium strictum</i> W. Gams | NCFT 5888.14 | 30 | 15 | 15 | 15 |
| 3 | <i>Aspergillus flavus</i> Link | NCFT 5891.14 | 85 | 90 | 90 | 80 |
| 4 | <i>Aspergillus niger</i> van Tieghem | NCFT 5773.13 | 95 | 95 | 100 | 95 |
| 5 | <i>Aspergillus ochraceus</i> Wilhelm | NCFT 5775.13 | 10 | 15 | - | - |
| 6 | <i>Aspergillus oryzae</i> E Cohn | NCFT 5776.13 | - | - | - | 5 |
| 7 | <i>Aspergillus sclerotiorum</i> G.A. Huber | NCFT 5890.14 | 20 | 10 | 5 | 5 |
| 8 | <i>Emericella nidulans</i> Thom | NCFT 5892.14 | 20 | 5 | 20 | 10 |
| 9 | <i>Eurotium amestelodami</i> L. Mangin | NCFT 5769.1340 | 35 | 10 | 35 | 30 |
| 10 | <i>Fusarium moniliforme</i> Sheldon | NCFT 5768.13 | 45 | 40 | 55 | 45 |
| 11 | <i>Hansfordia pulvinata</i> Berk. & M.A. Curtis | NCFT 5889.14 | - | - | 5 | - |
| 12 | <i>Penicillium chrysogenum</i> Thom | NCFT 5770.13 | 50 | 45 | 45 | 40 |
| 13 | <i>Syncephalastrum racemosum</i> J Schrot | NCFT 5772.13 | 25 | 30 | 35 | 35 |

*Specific identification was provided by National Centre for Fungal Taxonomy (NCFT), New Delhi



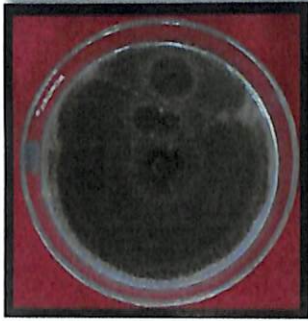
6. i. *A. restrictum*



6. ii. *A. strictum*



6. iii. *A. flavus*



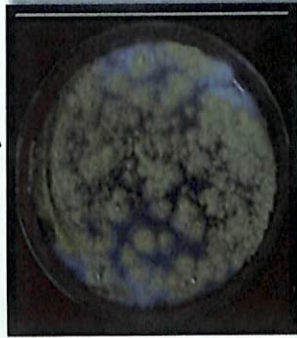
6. iv. *A. niger*



6. v. *A. ochraceus*



6. vi. *A. oryzae*



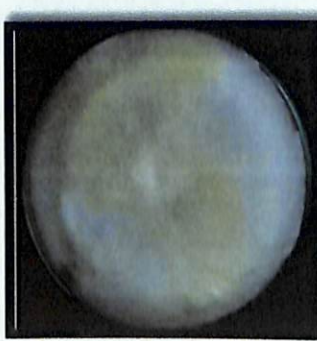
6. vii. *A. sclerotiorum*



6. viii. *E. nidulans*



6. ix. *E. amestelodami*



6. x. *F. moniliforme*



6. xi. *H. pulvinata*



6. xii. *P. chrysogenum*



6. xiii. *S. racemosum*

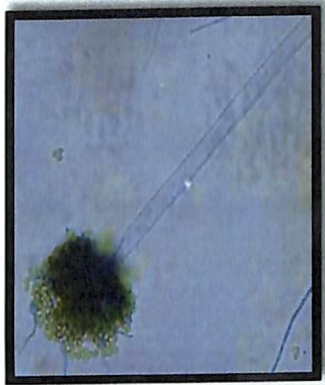
Plate 6. Growth of fungi on culture



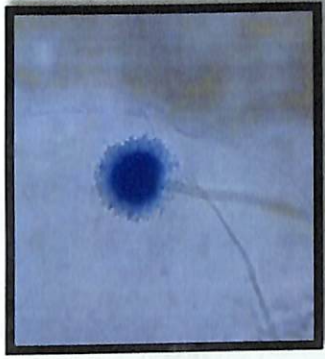
7. i. *A. restrictum*



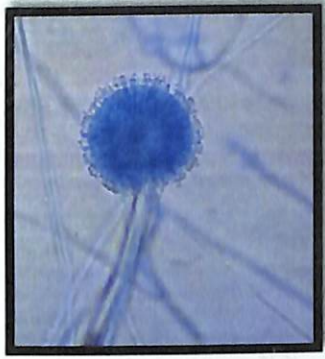
7. ii. *A. strictum*



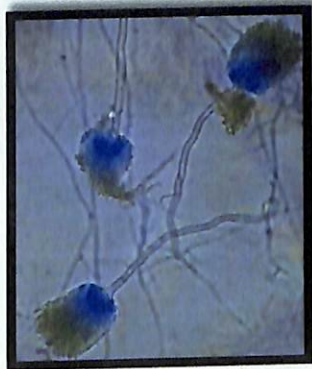
7. iii. *A. flavus*



7. iv. *A. niger*



7. v. *A. ochraceus*



7. vi. *A. oryzae*



7. vii. *A. sclerotiorum*



7. viii. *E. nidulans*



7. ix. *E. amestelodami*



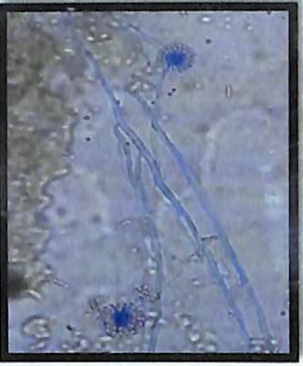
7. x. *F. moniliforme*



7. xi. *H. pulvinata*



7. xii. *P. chrysogenum*



7. xiii. *S. racemosus*

Plate 7. Microphotographs of different fungus

***Syncephalastrum racemosum* J Schrot**

Colonies on Czapek's (Dox) agar medium were rapidly growing with tuft of white to grey mycelium (Plate 6.xiii). Sporangiohores were long, fruiting head globose or oval, brown to grey with a terminal vesicle (80 μm diameter). Around this vesicle were the merosporangia (4-6 x 9-60 μm), which were filled with chains of sporangiospores. Each merosporangium contained a single row of 3-18 merosporangiospores. Merosporangiospores were one celled (3.0-10 μm diameter) and spherical to cylindrical in shape (Plate 7.xiii).

Altogether 13 fungi were obtained, of which 11 were isolated from samples of nutmeg (Table 8). All the 11 fungi were recorded from both the regions namely Kozhikode and Thrissur districts. This included: *Acremonium restrictum*, *A. strictum*, *Aspergillus flavus*, *A. niger*, *A. ochraceus*, *A. sclerotiorum*, *Emericella nidulans*, *Eurotium amestelodami*, *Fusarium moniliforme*, *Penicillium chrysogenum* and *Syncephalastrum racemosum*.

Among the twelve fungi isolated from samples of mace, ten were recorded from both the regions namely Kozhikode and Thrissur districts, viz., *A. restrictum*, *A. strictum*, *A. flavus*, *A. niger*, *A. sclerotiorum*, *E. nidulans*, *E. amestelodami*, *F. moniliforme*, *P. chrysogenum* and *S. racemosum*. Along with these *A. oryzae* and *H. pulvinata* was recorded from Thrissur and Kozhikode respectively.

4.3.1. Frequency of Occurrence of Fungi

The frequency of occurrence of each of the above fungi obtained during the survey was worked out (Table 8).

A. restrictum was obtained from 30 and 20 per cent samples of nutmeg and 25 and 15 per cent samples of mace collected from Kozhikode and Thrissur respectively. *A. strictum* was present in the frequency of 30 and 15 per cent in nutmeg samples from Kozhikode and Thrissur respectively. In mace *A. strictum* was present in 15 per cent each in the samples of Kozhikode and Thrissur districts.

A. flavus was present in 85 and 90 per cent of nutmeg samples and 90 and 80 per cent samples of mace respectively from Kozhikode and Thrissur districts. *A. niger* was obtained in nutmeg at a per cent frequency of 95 from both

Kozhikode and Thrissur and 100 and 95 per cent samples of mace respectively from Kozhikode and Thrissur. *A. ochraceus* was present in 10 and 15 per cent samples of nutmeg from Kozhikode and Thrissur districts. *A. oryzae* was isolated from mace samples collected from Thrissur only and occurred at a per cent frequency of five. *A. sclerotiorum* was collected from nutmeg samples of Kozhikode and Thrissur, and was present in 20 and 10 per cent of samples respectively and it was also present in five per cent each of mace samples collected from Kozhikode and Thrissur districts.

E. nidulans was isolated from 20 and five per cent samples of nutmeg and 20 and ten per cent of mace samples respectively from Kozhikode and Thrissur districts. *E. amestelodami* was obtained from 35 and 10 per cent samples of nutmeg and 35 and 30 per cent of mace samples respectively from Kozhikode and Thrissur. *F. moniliforme* was isolated from nutmeg and mace samples each at a frequency of 45, 40 and 55, 45 per cent of samples obtained from Kozhikode and Thrissur districts respectively.

H. pulvinata was isolated from mace samples collected from Kozhikode only and occurred in the per cent frequency of five. *P. chrysogenum* was obtained from 50 and 45 per cent nutmeg samples and 45 and 40 per cent mace samples from Kozhikode and Thrissur districts respectively. *S. racemosum* was obtained from Kozhikode and Thrissur district and present in 25 and 30 per cent of nutmeg and 35 per cent each of mace samples respectively.

4.3.2. Effect of Weather Parameters and Sample Moisture on the Population of Fungi

The effect of weather parameters, moisture content of the samples of nutmeg and mace collected from different regions *viz.*, Thrissur and Kozhikode and in two season *viz.*, February-March and June-July and on the occurrence of each of the 13 fungi isolated were studied.

4.3.2.1. Nutmeg

In the samples of nutmeg, population of fungi was higher at low daily temperature and high relative humidity in both the regions studied (Table 9). The species of fungi namely *A. restrictum*, *A. flavus*, *A. niger*, *A. ochraceus*, *A.*

sclerotiorum, *E. nidulans*, *E. amestelodami*, *F. moniliforme*, *P. chrysogenum* and *S. racemosum* were isolated from nutmeg samples of Kozhikode during February-March. Along with these *A. strictum* was present in the samples isolated during June-July.

A. restrictum, *A. strictum*, *A. flavus*, *A. niger*, *A. sclerotiorum*, *E. nidulans*, *E. amestelodami*, *F. moniliforme*, *P. chrysogenum* and *S. racemosum* are the fungi obtained from Thrissur during February-March. Of these ten fungi, except *A. strictum* was recorded during June-July.

Positive and significant correlation was noticed between fungal population and its moisture content. Positive correlation was obtained between minimum temperature, RH and fungal population was also positive, whereas maximum temperature showed negative correlation with fungal population (Table 10).

4.3.2.2. Mace

In mace samples, maximum number of fungi was obtained at high relative humidity and total rain fall (Table 11). The fungi isolated from the samples of mace during February- March from Kozhikode were *A. restrictum*, *A. strictum*, *A. flavus*, *A. niger*, *A. sclerotiorum*, *E. nidulans*, *E. amestelodami*, *F. moniliforme*, *P. chrysogenum* and *S. racemosum*. Along with these ten fungi, *H. pulvinata* was also present in June-July. The following were fungi isolated from Thrissur, during February- March, *A. restrictum*, *A. strictum*, *A. flavus*, *A. niger*, *A. oryzae*, *A. sclerotiorum*, *E. nidulans*, *F. moniliforme*, *P. chrysogenum* and *S. racemosum*.

A. restrictum, *A. strictum*, *A. flavus*, *A. niger*, *A. sclerotiorum*, *E. amestelodami*, *F. moniliforme*, *P. chrysogenum* and *S. racemosum* were recorded during June- July.

In the case of mace samples positive and significant correlation was noticed between fungal population and its moisture content. Positive correlation was obtained between minimum temperature, RH, rainfall and fungal population. Maximum temperature showed negative correlation with fungal population (Table 12).

Table 9. Seasonal occurrence of fungi on nutmeg in different regions

| Districts | Season | Fungal population (cfu) | Sample Moisture (%) | Temperature(°C) | | RH (%) | Total rainfall (mm) | Fungi isolated |
|-----------|-----------|-------------------------|---------------------|-----------------|------|-----------|---------------------|---|
| | | | | Max | Min | | | |
| Kozhikode | Feb-March | 4.04 | 8.21 | 34.5 | 23.3 | 78.6 2 | 24.3 | <i>Acremonium restrictum</i> , <i>Aspergillus flavus</i> , <i>A. niger</i> , <i>A. ochraceus</i> , <i>A. sclerotiorum</i> , <i>Emericella nidulans</i> , <i>Eurotium amestelodami</i> , <i>Fusarium moniliforme</i> , <i>Penicillium chrysogenum</i> and <i>Syncephalastrum racemosum</i> |
| | June-July | 5.64 | 9.193 | 29.6 | 22.2 | 94.0 5 | 38.6 | <i>A. restrictum</i> , <i>A. strictum</i> , <i>A. flavus</i> , <i>A. niger</i> , <i>A. ochraceus</i> , <i>A. sclerotiorum</i> , <i>E. nidulans</i> , <i>E. amestelodami</i> , <i>F. moniliforme</i> , <i>P. chrysogenum</i> and <i>S. racemosum</i> |
| Thrissur | Feb-March | 1.52 | 6.983 | 35.1 | 23.2 | 61 | 0.1 | <i>A. restrictum</i> , <i>A. strictum</i> , <i>A. flavus</i> , <i>A. niger</i> , <i>A. sclerotiorum</i> , <i>E. nidulans</i> , <i>E. amestelodami</i> , <i>F. moniliforme</i> , and <i>P. chrysogenum</i> <i>S. racemosum</i> |
| | June-July | 9.20 | 9.027 | 30.0 | 23.8 | 85 | 15.2 | <i>A. restrictum</i> , <i>A. flavus</i> , <i>A. niger</i> , <i>A. sclerotiorum</i> , <i>E. nidulans</i> , <i>E. amestelodami</i> , <i>F. moniliforme</i> , <i>P. chrysogenum</i> and <i>S. racemosum</i> |

Table 10. Relation between weather parameters, sample moisture and fungal population of nutmeg

| | Fungal population (cfu) | Sample moisture (%) | Max temp (°C) | Min. Temp (°C) | Relative humidity (%) | Rain fall (mm) |
|-------------------------|-------------------------|---------------------|---------------|----------------|-----------------------|----------------|
| Fungal population (cfu) | 1 | | | | | |
| Sample moisture (%) | 0.732* | 1 | | | | |
| Max temp (°C) | -0.079 | -0.208 | 1 | | | |
| Min. Temp (°C) | 0.118 | 0.059 | 0.342 | 1 | | |
| R H (%) | 0.084 | 0.201 | -0.931 | -0.528 | 1 | |
| Rain fall (mm) | -0.082 | -0.035 | -0.679 | -0.143 | 0.717 | 1 |

t-value at 0.05 level- 0.312

Table 11. Seasonal occurrence of fungi on mace in different regions

| Districts | Season | Fungal population (cfu) | Sample Moisture (%) | Temperature(°C) | | RH (%) | Total rainfall (mm) | Fungi isolated |
|-----------|-----------|-------------------------|---------------------|-----------------|------|--------|---------------------|--|
| | | | | Max | Min | | | |
| Kozhikode | Feb-March | 3.75 | 8.21 | 34.5 | 23.3 | 78.62 | 24.3 | <i>Acremonium restrictum</i> , <i>A. strictum</i> , <i>Aspergillus flavus</i> , <i>A. niger</i> , <i>A. sclerotiorum</i> , <i>Emericella nidulans</i> , <i>Eurotium amestelodami</i> , <i>Fusarium moniliforme</i> , <i>Penicillium chrysogenum</i> and <i>Syncephalastrum racemosum</i> |
| | June-July | 4.4 | 9.193 | 29.6 | 22.2 | 94.05 | 38.6 | <i>A. restrictum</i> , <i>A. strictum</i> , <i>A. flavus</i> , <i>A. niger</i> , <i>A. sclerotiorum</i> , <i>E. nidulans</i> , <i>E. amestelodami</i> , <i>F. moniliforme</i> , <i>Hansfordia pulvinata</i> , <i>P. chrysogenum</i> and <i>S. racemosum</i> |
| Thrissur | Feb-March | 2.61 | 6.983 | 35.1 | 23.2 | 61 | 0.1 | <i>A. restrictum</i> , <i>A. strictum</i> , <i>A. flavus</i> , <i>A. niger</i> , <i>A. oryzae</i> , <i>A. sclerotiorum</i> , <i>E. nidulans</i> , <i>F. moniliforme</i> , <i>P. chrysogenum</i> and <i>S. racemosum</i> |
| | June-July | 3.08 | 9.027 | 30.0 | 23.8 | 85 | 15.2 | <i>A. restrictum</i> , <i>A. strictum</i> , <i>A. flavus</i> , <i>A. niger</i> , <i>A. sclerotiorum</i> , <i>E. amestelodami</i> , <i>F. moniliforme</i> , <i>P. chrysogenum</i> and <i>S. racemosum</i> |

Table 12. Relation between weather parameters, sample moisture and fungal population of mace

| | Fungal population (cfu) | Sample moisture (%) | Max temp (°C) | Min. Temp (°C) | Relative humidity (%) | Rain fall (mm) |
|----------------------------|----------------------------|------------------------|---------------|----------------|-----------------------------|-------------------|
| Fungal population (cfu) | 1 | | | | | |
| Sample moisture (%) | 0.762* | 1 | | | | |
| Max temp (°C) | -0.118 | -0.198 | 1 | | | |
| Min. Temp (°C) | 0.057 | -0.054 | 0.342 | 1 | | |
| R H (%) | 0.155 | 0.216 | -0.931 | -0.528 | 1 | |
| Rain fall (mm) | 0.245 | 0.125 | -0.679 | -0.143 | 0.7167 | 1 |

t-value at (0.05) level- 0.312

4.4. DETECTION OF MYCOTOXIN IN THE SAMPLES OF NUTMEG AND MACE

The total aflatoxin and the different fractions of aflatoxin *viz.*, B₁, B₂, G₁ and G₂ present in the samples of nutmeg and mace collected from Thrissur and Kozhikode districts during February-March and June-July were estimated.

4.4.1. Detection of Total Aflatoxin

Total aflatoxin content in the nutmeg and mace samples collected during the survey were estimated by ELISA using the Agra Quant Aflatoxin test kit (Romer labs, Inc. USA) (Plate 8). Among the 80 samples tested only six samples, three each of nutmeg and mace showed the presence of aflatoxin (Table 13). The three nutmeg samples that showed the presence of aflatoxin were from Kozhikode, one of June-July with a total aflatoxin content of 808 ppb and the other two were of February – March with 824 and 925 ppb respectively.

The three of mace samples that showed the presence of aflatoxin included two samples obtained from Kozhikode during June-July with 805 and 921 ppb of total aflatoxin respectively. The other sample was collected from Thrissur during June –July with a total aflatoxin content of 964 ppb.

The maximum quantity of total aflatoxin, 964 ppb was detected in sample THR-MII-9, the mace sample collected during June-July from Thrissur. The minimum quantity of 805 ppb was detected in the sample, CLT-MII-4, the sample of mace collected from Kozhikode during June-July.

4.4.2. Detection of Fractions of Aflatoxin

The different fractions of aflatoxin, namely, B₁, B₂, G₁ and G₂ was estimated qualitatively and quantitatively, in the six samples of nutmeg and mace obtained during the survey (Table 14). The six samples, where the total aflatoxin content was detected by ELISA were subjected to TLC for estimating the different fractions of aflatoxin, qualitatively and quantitatively (Plate 9.A-E).

The results of the study revealed that, of the six samples tested aflatoxin B₁ was elaborated in three samples namely CLT-NII-9, CLT-MII-8 and THR-MII-9. Aflatoxin B₂ was detected in two samples namely CLT-MII-4 and CLT-NI-5, and only one sample, CLT-NI-4 showed aflatoxin G₁.

Table 13. Total aflatoxin content in samples of nutmeg and mace

| Sample | Sl. No. | Samples * | Quantity of total aflatoxin (ppb) |
|--------|---------|-----------|-----------------------------------|
| Nutmeg | 1 | CLT-NII-9 | 808 |
| | 2 | CLT-NI-4 | 824 |
| | 3 | CLT-NI-5 | 925 |
| Mace | 1 | CLT-MII-4 | 805 |
| | 2 | CLT-MII-8 | 921 |
| | 3 | THR-MII-9 | 964 |

*CLT- Kozhikode

N- nutmeg

I- February-March

THR- Thrissur

M- Mace

II- June-July

Table 14. Fractions of aflatoxins in the samples of nutmeg and mace

| Sl. No. | Aflatoxin produced | Samples* | Quantity of aflatoxin (ppb) |
|---------|--------------------|-----------|-----------------------------|
| 1 | B ₁ | CLT-NII-9 | 241 |
| 2 | B ₂ | CLT-MII-4 | 617 |
| 3 | B ₁ | CLT-MII-8 | 896 |
| 4 | G ₁ | CLT-NI-4 | 192 |
| 5 | B ₂ | CLT-NI-5 | 214 |
| 6 | B ₁ | THR-MII-9 | 921 |

*CLT- Kozhikode

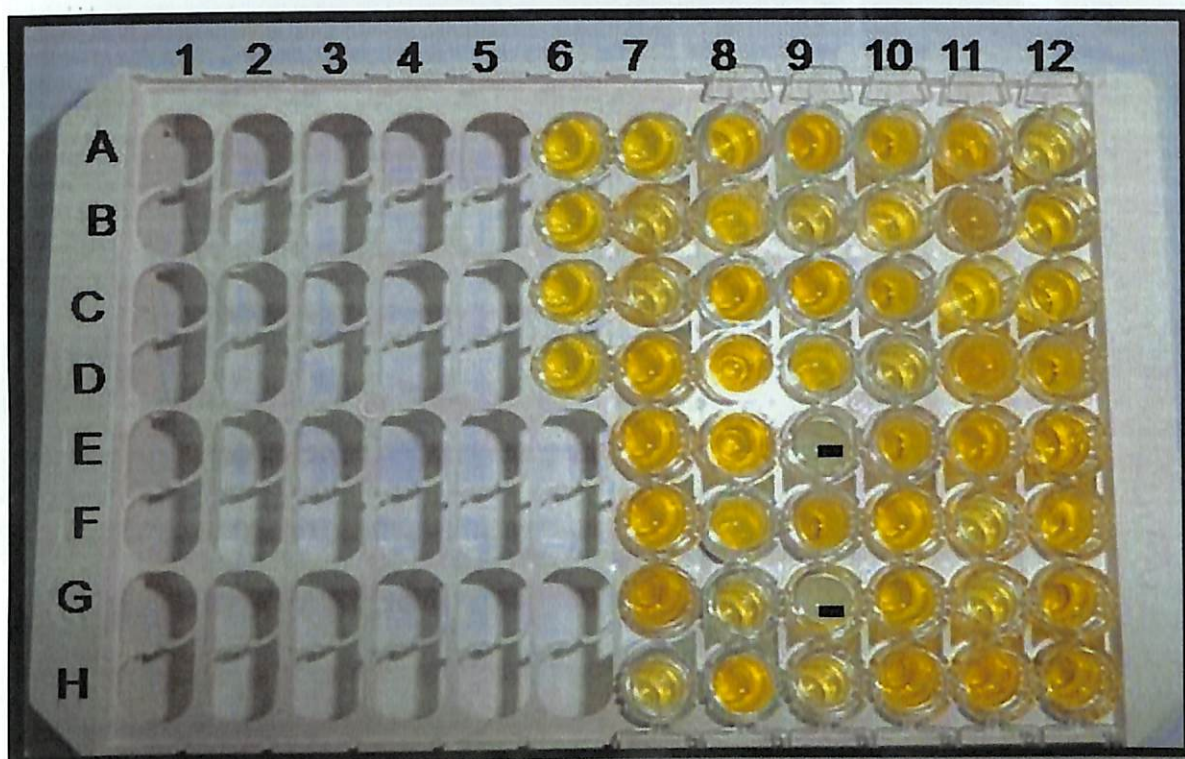
N- nutmeg

I- February-March

THR- Thrissur

M- Mace

II- June-July



| Aflatoxin standard | |
|--------------------|----------|
| A12 - | 0.00 ppb |
| B12 - | 1.0 ppb |
| C12 - | 2.0 ppb |
| D12 - | 4.0 ppb |
| E12 - | 10.0 ppb |
| F12 - | 20.0 ppb |

| Nutmeg and mace sample collected during survey | |
|--|-----------|
| G12 - | CLT-NII-9 |
| H12 - | CLT-NI-4 |
| H11 - | CLT-NI-5 |
| G11 - | CLT-MII-4 |
| F11 - | CLT-MII-8 |
| E11 - | THR-MII-9 |

Plate 8. ELISA plate showing aflatoxin in samples of nutmeg and mace

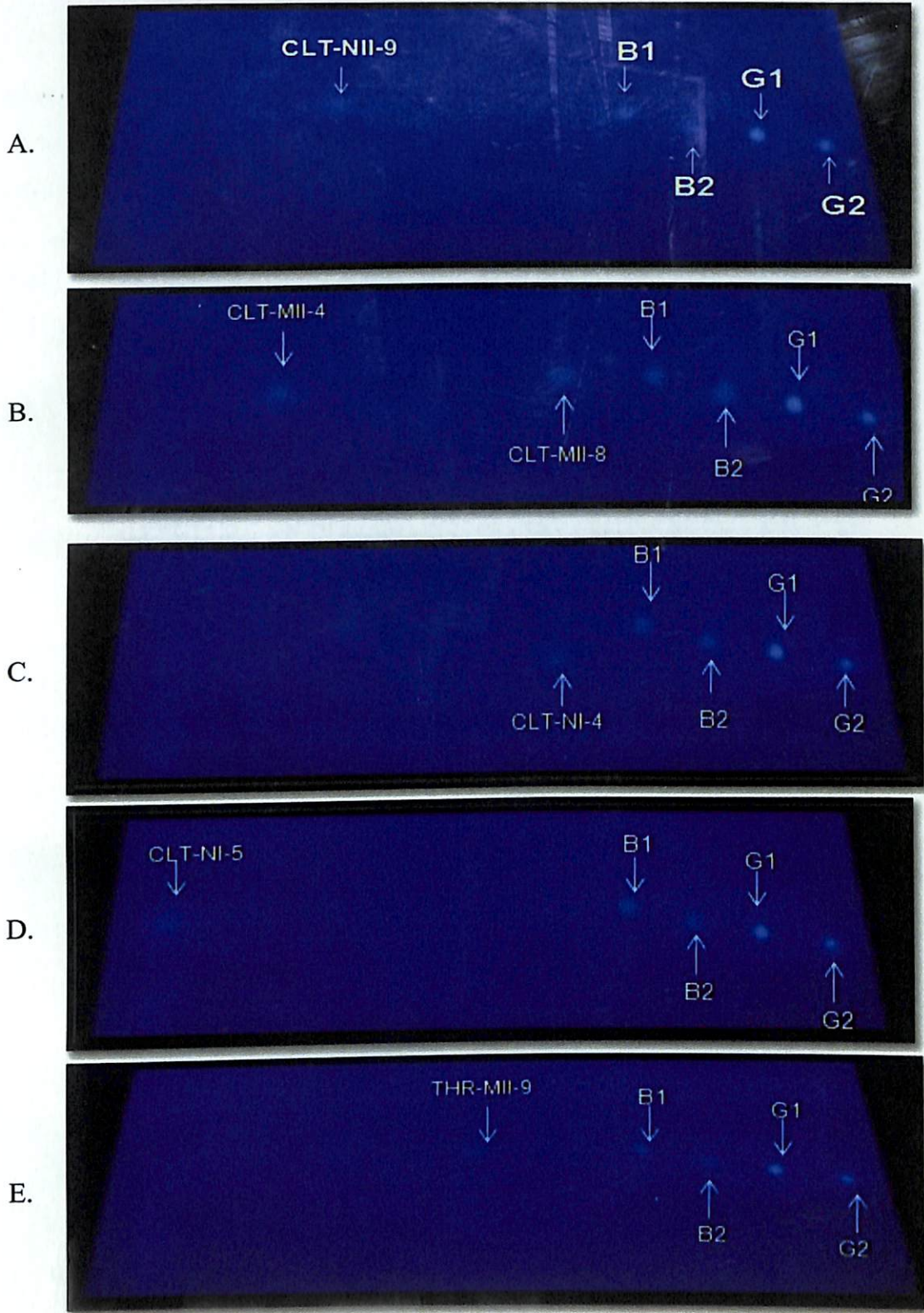


Plate 9. TLC plate showing aflatoxin in samples of nutmeg and mace

The quantity of aflatoxin B₁ produced by the three samples namely CLT-NII-9, CLT-MII-8 and THR-MII-9 being 241, 896 and 921 ppb respectively. The quantity of aflatoxin B₂ detected in samples CLT-MII-4 and CLT-NI-5 being 617 and 214 ppb respectively. The quantity of G₁ produced in the sample CLT-NI-4 being 192 ppb.

In the present study the maximum quantity of aflatoxin, being 921 ppb was B₁, detected in mace sample collected from Thrissur during June-July. The minimum quantity of aflatoxin, 192 ppb being G₁ was estimated from the nutmeg sample collected from Kozhikode during February-March.

4.5. QUALITATIVE AND QUANTITATIVE ESTIMATION OF THE MYCOTOXIN PRODUCED BY THE FUNGI OBTAINED

The mycotoxin production ability of the species of *Aspergillus*, namely, *A. flavus*, *A. niger* and *A. ochraceus* obtained during the survey, under *in vitro* (culture medium) and *in vivo* (substrate) conditions was estimated.

4.5.1. Detection of Aflatoxin

The fungi that occurred in the highest frequency, viz., *A. flavus* and *A. niger* were tested for their ability to produce aflatoxin in the culture and in the substrate from they were isolated.

4.5.1.1 Aflatoxin in Culture

The ability of nine isolates of *A. flavus* and 12 isolates of *A. niger* obtained from nutmeg and mace were tested for their ability to elaborate aflatoxin by inoculating on SMKY medium (Plate 10 and 11).

4.5.1.1.1. Detection of Total Aflatoxin

Total aflatoxin elaborated by 12 isolates of *Aspergillus* spp. of nutmeg, which included five isolates of *A. flavus* and seven isolates of *A. niger* in culture were detected by ELISA (Plate 12), was presented in Table 15. The results of the study indicated that, all the five isolates of *A. flavus*, obtained from nutmeg were found to be toxigenic. Total aflatoxin produced by these isolates of *A. flavus* in culture medium ranged from 865- 8483 ppb. Maximum quantity of aflatoxin was produced by isolate THR-II-3 and least by isolate THR-I-4. The other isolates



A. *A. flavus*

Plate 10. Growth of isolates of *Aspergillus* spp. from nutmeg in SMKY medium



B. *A. niger*

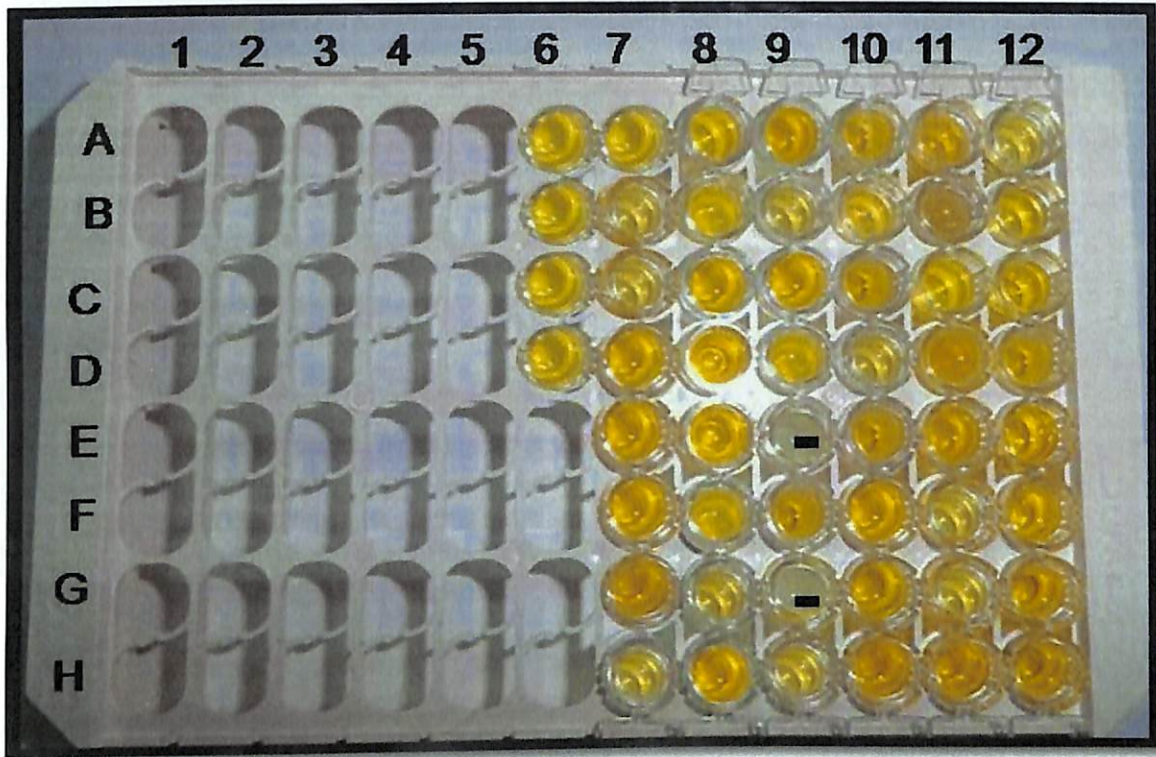


A. *A. flavus*

Plate 11. Growth of isolates of *Aspergillus* spp. from mace in SMKY medium



B. *A. niger*



| Aflatoxin standard | |
|--------------------|----------|
| A12 - | 0.00 ppb |
| B12 - | 1.0 ppb |
| C12 - | 2.0 ppb |
| D12 - | 4.0 ppb |
| E12 - | 10.0 ppb |
| F12 - | 20.0 ppb |
| | |

| Aspergillus isolates from nutmeg and mace in culture | | | | | |
|--|----------|-------|----------|------|----------|
| D11 - | CLT-I-1 | D10 - | THR-I-4 | F9 - | THR-I-4 |
| C11 - | CLT-II-2 | E10 - | CLT-II-5 | E9 - | THR-I-6 |
| B11 - | THR-II-3 | F10 - | THR-II-6 | D9 - | CLT-I-2 |
| A11 - | THR-I-4 | G10 - | THR-II-7 | C9 - | CLT-I-3 |
| A10 - | CLT-II-7 | H10 - | CLT-II-8 | B9 - | CLT-II-5 |
| B10 - | CLT-II-1 | H9 - | THR-II-3 | A9 - | CLT-II-7 |
| C10 - | CLT-I-3 | G9 - | CLT-II-5 | A8 - | CLT-II-8 |

Plate 12. ELISA plate showing results for aflatoxins by isolates of *Aspergillus* from nutmeg and mace in culture medium

CLT-II-7, CLT-I-1 and CLT-II-2 produced 1106, 5889 and 5913 ppb of aflatoxin respectively.

All the seven isolates of *A. niger*, obtained from nutmeg were found to be toxigenic. Total aflatoxin produced by these isolates ranged from 1904-12964ppb. Isolate THR-II-7 produced the maximum quantity of aflatoxin and least by isolate CLT-II-5. The isolates, namely, CLT-I-3, THR-II-6, CLT-II-1, THR-I-4 and CLT-II-8 produced 3882, 7243, 8413, 9136 and 9845ppb of total aflatoxin respectively.

Of the nine isolates of *Aspergillus* spp. obtained from mace (Table 16), viz., two isolates of *A. flavus* and five of *A. niger* were found to be toxigenic in culture media. The quantity of total aflatoxin produced by the two isolates of *A. flavus* namely THR-II-3 and THR-I-4 being 8483 and 865 ppb respectively.

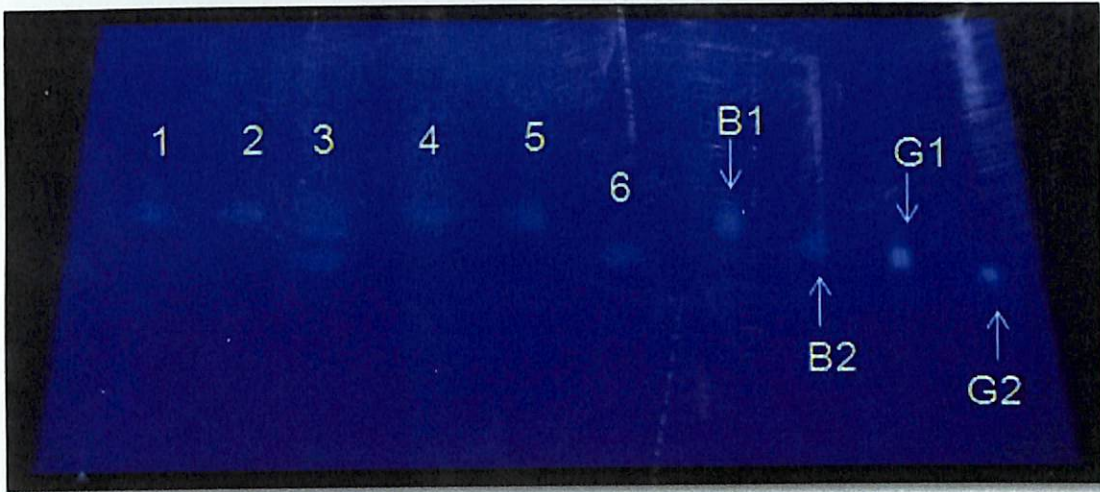
The quantity of aflatoxin elaborated by the *A. niger* isolates of mace ranged 1364-9845ppb and the maximum and minimum being produced by CLT-II-8 and THR-II-7. The isolates namely CLT-II-5, CLT-I-2 and CLT-I-3 produced 1904, 3376 and 3885 ppb of total aflatoxin respectively.

4.5.1.1.2 Detection of Different Fractions of Aflatoxin

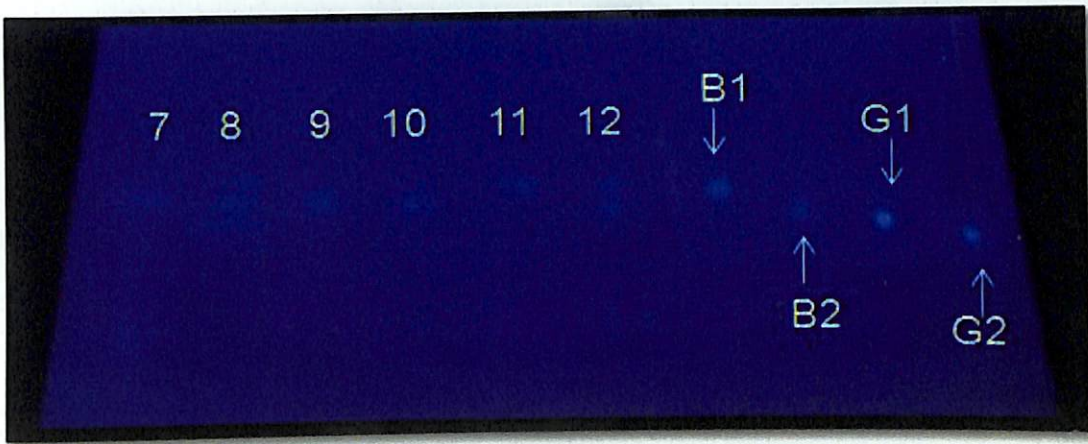
The toxigenic isolates of *A. flavus* and *A. niger* obtained from nutmeg and mace were subjected to TLC for their ability to elaborate different fractions of aflatoxin viz., B₁, B₂, G₁ and G₂ in culture medium (Plate 13 and 14).

Of the five toxigenic isolates of *A. flavus* obtained from nutmeg one (20 per cent) produced aflatoxin B₁, B₂ and G₁, four isolates (80 per cent) produced B₁ alone in culture medium. Of the seven toxigenic isolates of *A. niger*, one isolate each (14.28 per cent) produced B₁, B₂ and G₁, B₁ and B₂ and B₁ alone, respectively and four isolates (57.14 per cent) elaborated B₂ alone (Table 17).

The quantity of aflatoxin B₁ produced by the isolates of *A. flavus* from nutmeg, in culture medium ranged from 70-595 ppb (Table 18) with the maximum and minimum by isolate THR-I-4 and CLT-II-7 respectively. The quantity of aflatoxin B₂ being 201 ppb and G₁ being 122 ppb were produced by isolates THR-II-3 of nutmeg.

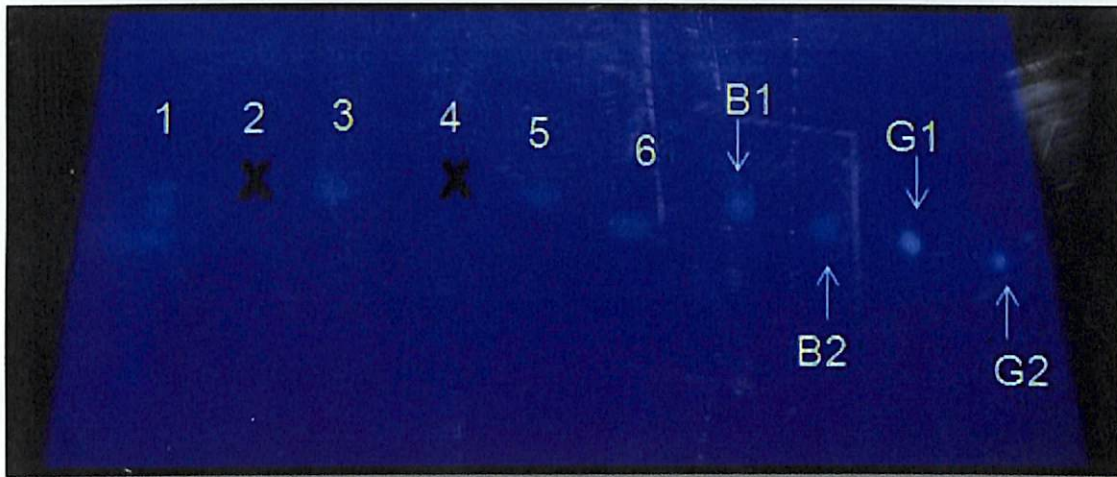


| | | | |
|-------------|-------------|-------------|-----|
| 1- CLT-I-1 | -B1 | 4- THR-I-4 | -B1 |
| 2- CLT-II-2 | -B1 | 5- CLT-II-7 | -B2 |
| 3- THR-II-3 | -B1, B2, G1 | 6- CLT-II-9 | -B2 |



| | | | |
|-------------|--------------|--------------|---------|
| 7- CLT-I-3 | -B2 | 10- THR-II-6 | -B2 |
| 8- THR-I-4 | - B1, B2, G1 | 11- THR-II-7 | -B1 |
| 9- CLT-II-5 | -B2 | 12- CLT-II-8 | -B1, B2 |

Plate 13. TLC plates showing aflatoxin elaboration in culture by *Aspergillus* isolates of nutmeg



| | | | |
|-------------|-------------|------------|-----|
| 1- THR-II-3 | -B1, B2, G1 | 4- THR-I-6 | - |
| 2- CLT-II-7 | - | 5- CLT-I-2 | -B1 |
| 3- THR-I-4 | -B1 | 6- CLT-I-3 | -B2 |



| | |
|-------------|---------|
| 7- CLT-II-5 | -B2 |
| 8- THR-II-7 | -B1 |
| 9- CLT-II-8 | -B1, B2 |

Plate 14. TLC plates showing aflatoxin elaboration in culture by *Aspergillus* isolates of mace

Table 15. Total aflatoxin produced by *Aspergillus* spp. from nutmeg in culture medium

| Sl. No. | Isolates | | Percentage of toxigenic isolates | Quantity of aflatoxin (ppb) |
|---------|---------------------------|--------------------|----------------------------------|-----------------------------|
| | <i>Aspergillus flavus</i> | Toxigenic isolates | | |
| 1 | CLT-I-1 | CLT-I-1 | 100 | 5889 |
| 2 | CLT-II-2 | CLT-II-2 | | 5913 |
| 3 | THR-II-3 | THR-II-3 | | 8483 |
| 4 | THR-I-4 | THR-I-4 | | 865 |
| 5 | CLT-II-7 | CLT-II-7 | | 1106 |
| | <i>A. niger</i> | | | |
| 1 | CLT-II-1 | CLT-II-1 | 100 | 8413 |
| 2 | CLT-I-3 | CLT-I-3 | | 3882 |
| 3 | THR-I-4 | THR-I-4 | | 9136 |
| 4 | CLT-II-5 | CLT-II-5 | | 1904 |
| 5 | THR-II-6 | THR-II-6 | | 7243 |
| 6 | THR-II-7 | THR-II-7 | | 12964 |
| 7 | CLT-II-8 | CLT-II-8 | | 9845 |

CLT- Kozhikode

I- February-March

THR- Thrissur

II- June-July

Table 16. Total aflatoxin produced by *Aspergillus* spp. from mace in culture medium

| Sl. No. | Isolates | | Percentage of toxigenic isolates | Quantity of aflatoxin (ppb) |
|---------|---------------------------|--------------------|----------------------------------|-----------------------------|
| | <i>Aspergillus flavus</i> | Toxigenic isolates | | |
| 1 | THR-II-3 | TRH-II-3 | 50 | 8483 |
| 2 | CLT-II-5 | THR-I-4 | | 865 |
| 3 | THR-I-4 | | | |
| 4 | THR-I-6 | | | |
| | <i>A. niger</i> | | | |
| 1 | CLT-I-2 | CLT-I-2 | 100 | 3376 |
| 2 | CLT-I-3 | CLT-I-3 | | 3885 |
| 3 | CLT-II-5 | CLT-II-5 | | 1904 |
| 4 | THR-II-7 | THR-II-7 | | 1364 |
| 5 | CLT-II-8 | CLT-II-8 | | 9845 |

CLT- Kozhikode

I- February-March

THR- Thrissur

II- June-July

Table 17. Fractions of aflatoxin produced by *Aspergillus* spp. from nutmeg

| Fungus | Number of isolates | Number of toxigenic isolates | % of toxigenic isolates | Aflatoxin produced |
|---------------------------|--------------------|------------------------------|-------------------------|--|
| <i>Aspergillus flavus</i> | 5 | 5 | 100 | |
| | | 1 | 20 | B ₁ , B ₂ and G ₁ |
| | | 4 | 80 | B ₁ |
| <i>A. niger</i> | 7 | 7 | 100 | |
| | | 1 | 14.28 | B ₁ , B ₂ and G ₁ |
| | | 1 | 14.28 | B ₁ and B ₂ |
| | | 1 | 14.28 | B ₁ |
| | | 4 | 57.14 | B ₂ |

Table 18. Fractions aflatoxin produced by *Aspergillus* spp. from nutmeg in culture medium

| Sl. No. | Isolates | Quantity of aflatoxin (ppb) | | | |
|---------------------------|----------|-----------------------------|----------------|----------------|----------------|
| | | B ₁ | B ₂ | G ₁ | G ₂ |
| <i>Aspergillus flavus</i> | | | | | |
| 1 | CLT-I-1 | 482 | – | – | – |
| 2 | CLT-II-2 | 198 | – | – | – |
| 3 | THR-II-3 | 198 | 201 | 122 | – |
| 4 | THR-I-4 | 595 | – | – | – |
| 5 | CLT-II-7 | 70 | – | – | – |
| <i>A. niger</i> | | | | | |
| 1 | CLT-II-1 | – | 348 | – | – |
| 2 | CLT-I-3 | – | 80 | – | – |
| 3 | THR-I-4 | 198 | 40 | 52 | – |
| 4 | CLT-II-5 | – | 40 | – | – |
| 5 | THR-II-6 | – | 53 | – | – |
| 6 | THR-II-7 | 212 | – | – | – |
| 7 | CLT-II-8 | 326 | 389 | – | – |

CLT- Kozhikode
THR- Thrissur

I- February-March
II- June-July

Table 19. Fractions of aflatoxin produced by *Aspergillus* spp. from mace

| Fungus | Number of isolates | Number of toxigenic isolates | % of toxigenic isolates | Aflatoxin produced |
|---------------------------|--------------------|------------------------------|-------------------------|--|
| <i>Aspergillus flavus</i> | 4 | 2 | 50 | |
| | | 1 | 50 | B ₁ , B ₂ and G ₁ |
| | | 1 | 50 | B ₁ |
| <i>A. niger</i> | 5 | 5 | 100 | |
| | | 1 | 20 | B ₁ , B ₂ and G ₁ |
| | | 2 | 40 | B ₁ |
| | | 2 | 40 | B ₂ |

Table 20. Fractions of aflatoxin produced by *Aspergillus* spp. of mace in culture medium

| Sl. No. | Isolates | Quantity of aflatoxin (ppb) | | | |
|---------|---------------------------|-----------------------------|----------------|----------------|----------------|
| | | B ₁ | B ₂ | G ₁ | G ₂ |
| | <i>Aspergillus flavus</i> | | | | |
| 1 | THR-II-3 | 198 | 201 | 122 | – |
| 2 | CLT-II-5 | – | – | – | – |
| 3 | THR-I-4 | 595 | – | – | – |
| 4 | THR-I-6 | – | – | – | – |
| | <i>A. niger</i> | | | | |
| 1 | CLT-I-2 | 226 | – | – | – |
| 2 | CLT-I-3 | – | 80 | – | – |
| 3 | CLT-II-5 | – | 40 | – | – |
| 4 | THR-II-7 | 497 | – | – | – |
| 5 | CLT-II-8 | 326 | 389 | – | – |

CLT- Kozhikode
THR- Thrissur

I- February-March
II- June-July

The quantity of aflatoxin B₁ elaborated by isolates of *A. niger*, ranged from 198-326 ppb, with the maximum and the minimum being produced by isolate CLT-II-8 and isolate THR-I-4 respectively. The quantity of aflatoxin B₂ produced ranged from 40 – 389 ppb with the maximum and the minimum being recorded in isolate CLT-II-8 and isolate THR-I-4 and CLT-II-5 each respectively. Aflatoxin G₁ (52 ppb) was produced by the isolate THR-I-4 from nutmeg.

None of the *A. flavus* and *A. niger* isolates of nutmeg elaborated aflatoxin G₂ in culture medium.

Of the two toxigenic isolates of *A. flavus* obtained from mace, one each (50 per cent) produced B₁, B₂ and G₁ and B₁ alone. Of the five toxigenic isolates of *A. niger* one (20 per cent) elaborated B₁, B₂ and G₁ and two isolates each (40 per cent) produced B₁ and B₂ respectively (Table 19).

The quantity of aflatoxin B₁ elaborated by two isolates of *A. flavus* from mace *viz.*, THR-II-3 and THR-I-4 being 198 and 595 ppb respectively. The aflatoxins B₂ and G₁ of 201 and 122 ppb being elaborated by isolate THR-II-3 (Table 20).

The five toxigenic isolates of *A. niger*, produced B₁ in the range of 226-497 ppb, with the maximum and minimum being produced by isolate THR-II-7 and CLT-I-2 respectively. The aflatoxin B₂ was produced in the range of 40-389 ppb, with the maximum and minimum being elaborated by isolates CLT-II-8 and CLT-II-5 respectively.

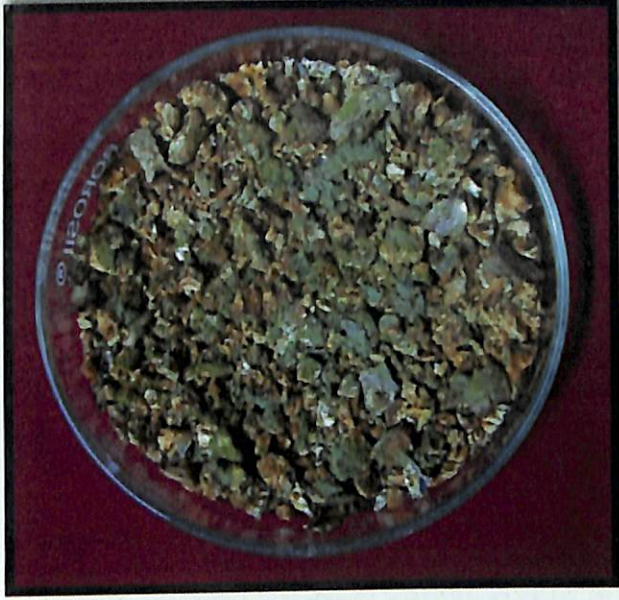
None of the *A. flavus* and *A. niger* isolates obtained from mace elaborated aflatoxin G₂ in culture medium.

4.5.1.2. Aflatoxin in Substrate

The ability of nine isolates of *A. flavus* and 12 isolates of *A. niger* obtained from nutmeg and mace were tested for their ability to elaborate aflatoxin in the substrate from where they were isolated (Plate 15 and 16).



Nutmeg sample before inoculation



Growth of *A. flavus* isolates in nutmeg



Growth of *A. niger* isolates in nutmeg

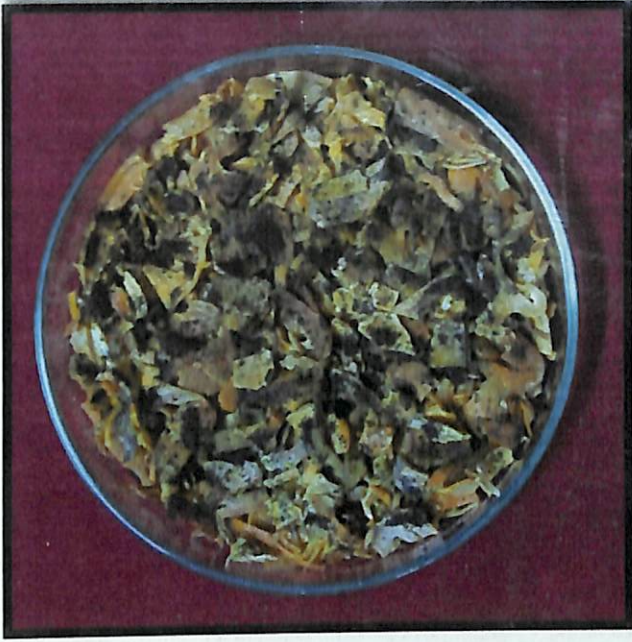
Plate 15. *In vivo* evaluation of *Aspergillus* spp. isolates for aflatoxin elaboration in nutmeg



Mace sample before inoculation



Growth of *A. flavus* isolates in mace



Growth of *A. niger* isolates in mace

Plate 16. *In vivo* evaluation of *Aspergillus* spp. isolates for aflatoxin elaboration in mace

4.5.1.2.1. *Aflatoxin in Nutmeg*

4.5.1.2.1.1. *Total Aflatoxin*

Of the 12 isolates of *Aspergillus* spp., five of *A. flavus* and seven of *A. niger* were tested for total aflatoxin production in nutmeg by ELISA (Plate 17; Table 21).

All the five isolates of *A. flavus* were found to be toxigenic on nutmeg. Total aflatoxin elaborated by these isolates in nutmeg ranged from 871- 3261ppb with the maximum and minimum being recorded in isolates CLT-I-1 and THR-I-4 respectively. The other isolates, namely, CLT-II-7, THR-II-3 and CLT-II-2 recorded 962, 979 and 2901 ppb of aflatoxin in nutmeg respectively

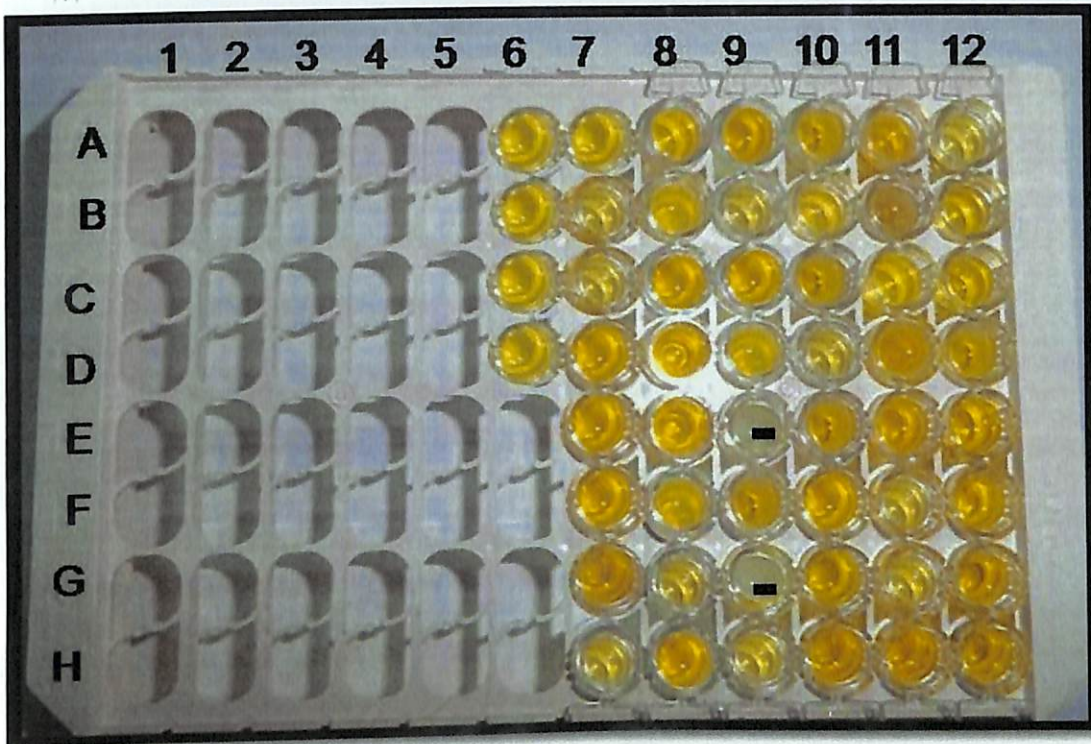
All the seven isolates of *A. niger* were found to be toxigenic on nutmeg . Total aflatoxin produced by these isolates in nutmeg ranged from 487 - 8410 ppb with the maximum and minimum being recorded by CLT-II-8 and CLT-II-5 respectively. The other isolates, namely, THR-II-6, THR-I-4, CLT-II-1, CLT-I-3, and THR-II-7 elaborated 609, 652, 657, 697 and 5502 ppb of total aflatoxin in nutmeg respectively.

4.5.1.2.1. 2. *Fractions of Aflatoxin*

All the five toxigenic isolates of *A. flavus* produced aflatoxin B₁ alone (100 per cent) when inoculated on nutmeg (Table 22). Of the seven toxigenic isolates of *A. niger* one (14.28 per cent) elaborated aflatoxin B₁ and B₂, two (28.57 per cent) produced aflatoxin B₁ alone and four isolates (57.14 per cent) elaborated B₂ alone (Plate 18).

The quantity of aflatoxin B₁ elaborated by the *A. flavus* on nutmeg ranged from 127-879 ppb with the maximum and minimum being recorded by isolates CLT-I-1 and CLT-II-7 respectively. None isolates of *A. flavus* elaborated aflatoxins B₂, G₁ and G₂ in nutmeg (Table 23).

Aflatoxin B₁ produced by isolates of *A. niger* on nutmeg ranged from 99-368 ppb, with the maximum and the minimum was recorded in CLT-II-8 and THR-I-4 respectively. The quantity of aflatoxin B₂ produced in nutmeg ranged from 107-456 ppb, and the maximum and minimum was recorded by CLT-I- 3 and THR-II-6 respectively. The other isolates, namely, CLT-II-8, CLT-II-1 and



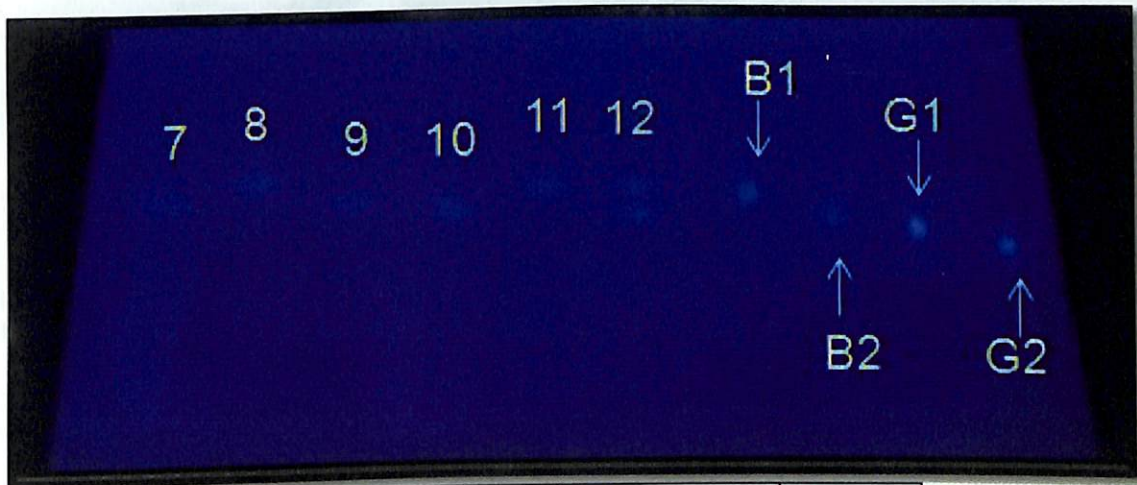
| Aflatoxin standard | |
|--------------------|----------|
| A12 - | 0.00 ppb |
| B12 - | 1.0 ppb |
| C12 - | 2.0 ppb |
| D12 - | 4.0 ppb |
| E12 - | 10.0 ppb |
| F12 - | 20.0 ppb |

| <i>Aspergillus</i> isolates in nutmeg | | | |
|---------------------------------------|----------|------|----------|
| B8 - | CLT-I-1 | H8 - | CLT-I-3 |
| C8 - | CLT-II-2 | H7 - | THR-I-4 |
| D8 - | THR-II-3 | G7 - | CLT-II-5 |
| E8 - | THR-I-4 | F7 - | THR-II-6 |
| F8 - | CLT-II-7 | E7 - | THR-II-7 |
| G8 - | CLT-II-1 | D7 - | CLT-II-8 |

Plate 17. ELISA plate showing results for aflatoxin by isolates of *Aspergillus* in nutmeg



| | | | |
|-------------|-----|-------------|-----|
| 1- CLT-I-1 | -B1 | 4- THR-I-4 | -B1 |
| 2- CLT-II-2 | -B1 | 5- CLT-II-7 | -B1 |
| 3- THR-II-3 | -B1 | 6- CLT-II-9 | -B2 |



| | | | |
|-------------|-----|--------------|---------|
| 7- CLT-I-3 | -B2 | 10- THR-II-6 | -B2 |
| 8- THR-I-4 | -B1 | 11- THR-II-7 | -B1 |
| 9- CLT-II-5 | -B2 | 12- CLT-II-8 | -B1, B2 |

Plate 18. TLC plates showing aflatoxin elaboration in nutmeg by isolates of *Aspergillus* spp.

CLT-II-5 produced 362, 241 and 174 ppb of aflatoxin B₂. None of the isolates of *A. niger* elaborated aflatoxin G₁ and G₂ in nutmeg.

4.5.1.2.2. Aflatoxin in Mace

4.5.1.2.2.1. Total Aflatoxin

The seven toxigenic isolates of *Aspergillus* spp. from mace, which were found to be toxigenic in culture, were tested for their ability to produce aflatoxin under *in vivo* condition (substrate). Both the isolates of *A. flavus*, namely, THR-II-3 and THR-I-4 were found to produce aflatoxin in mace (substrate), being 921 and 1035 ppb respectively (Plate 19; Table 24).

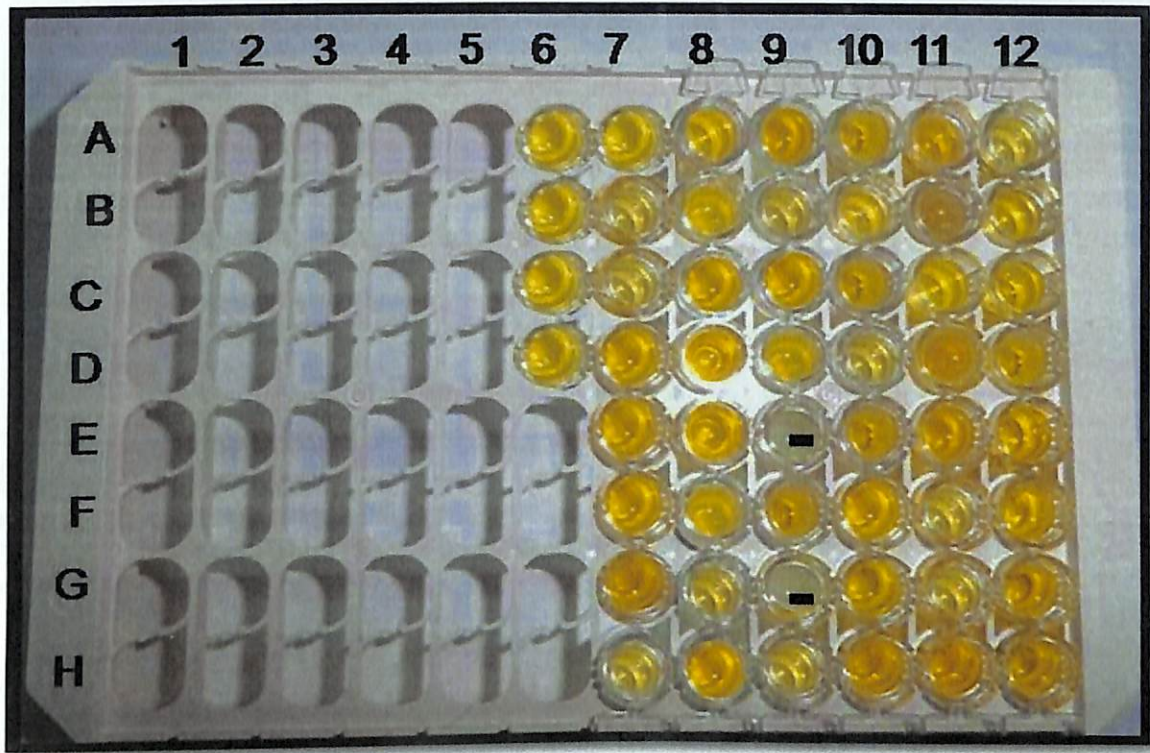
All the five toxigenic isolates of *A. niger* were found to produce aflatoxin in mace also. The quantity of aflatoxin produced by these isolates in mace ranged from 947-1113 ppb and the maximum and minimum being produced by isolate CLT-II-5 and CLT-I-2 respectively. The other isolates, namely, THR-II-7, CLT-I-3 and CLT-II-8 recorded 992, 979 and 1021 ppb of total aflatoxin in mace respectively.

4.5.1.2.2.2. Fractions of Aflatoxin

The aflatoxin elaborated by isolates of *A. flavus* (two), *A. niger* (five) were subjected to TLC to determine the different fractions, namely, B₁, B₂, G₁ and G₂ (Plate 20). Both the two isolates of *A. flavus* produced aflatoxin B₁ alone in mace (100 per cent). Of the five isolates of *A. niger*, three produced (60 per cent) B₁ alone and two produced (40 per cent) B₂ alone in mace respectively (Table 25).

The quantity of aflatoxin B₁ elaborated by the two isolates of *A. flavus viz.*, THR-II-3 and THR-I-4 in mace being 916 and 326 ppb respectively (Table 26). None of the isolates of *A. flavus* produced B₂, G₁ and G₂ in mace.

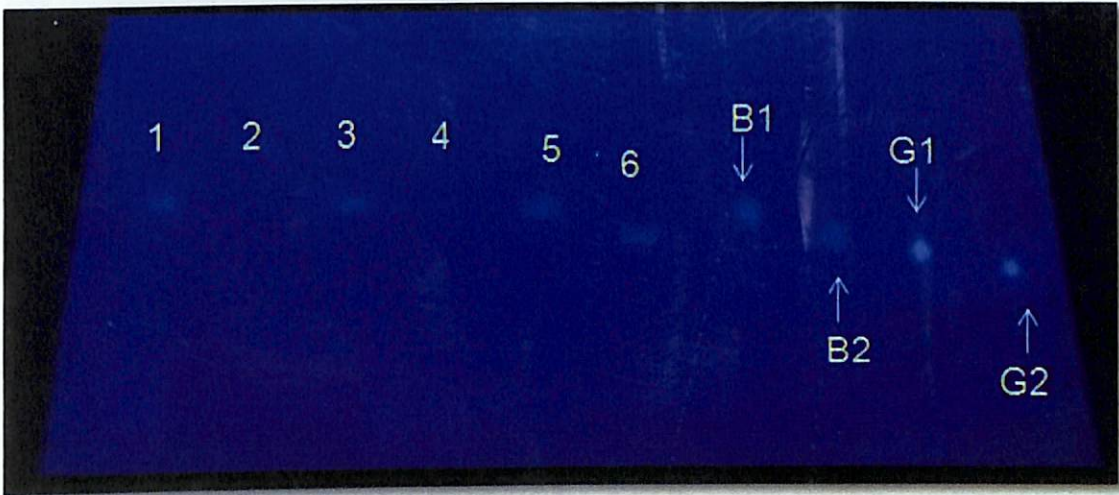
The quantity of aflatoxin B₁ elaborated by the three toxigenic isolates of *A. niger*, namely, CLT-I-2, THR-II-7 and CLT-II-8 in mace being, 539, 589 and 978 ppb respectively. The quantity of aflatoxin B₂ elaborated by isolate CLT-I-3 and CLT-II-5 in mace being 550 and 161 ppb respectively. None of the isolates of *A. niger* produced aflatoxin G₁ and G₂ in mace.



| Aflatoxin standard | |
|--------------------|----------|
| A12 - | 0.00 ppb |
| B12 - | 1.0 ppb |
| C12 - | 2.0 ppb |
| D12 - | 4.0 ppb |
| E12 - | 10.0 ppb |
| F12 - | 20.0 ppb |
| | |

| <i>Aspergillus</i> isolates in mace | |
|-------------------------------------|----------|
| C7 - | THR-II-3 |
| B7 - | THR-I-4 |
| A7 - | CLT-I-2 |
| A6 - | CLT-I-3 |
| B6 - | CLT-II-5 |
| C6 - | CLT-II-7 |
| D6 - | CLT-II-8 |

Plate 19. ELISA plate showing results of aflatoxin production by *Aspergillus* spp. in mace



| | | | |
|-------------|-----|------------|-----|
| 1- THR-II-3 | -B1 | 4- THR-I-6 | - |
| 2- CLT-II-7 | - | 5- CLT-I-2 | -B1 |
| 3- THR-I-4 | -B1 | 6- CLT-I-3 | -B2 |



| | |
|-------------|-----|
| 7- CLT-II-5 | -B2 |
| 8- THR-II-7 | -B1 |
| 9- CLT-II-8 | -B1 |

Plate 20. TLC plates showing aflatoxin elaboration in mace by isolates of *Aspergillus* spp.

Table 21. Total aflatoxin produced by *Aspergillus* spp. in nutmeg

| Sl. No. | Isolates | | Percentage of toxigenic isolates | Total aflatoxin (ppb) |
|---------|---------------------------|--------------------|----------------------------------|-----------------------|
| | <i>Aspergillus flavus</i> | Toxigenic isolates | | |
| 1 | CLT-I-1 | CLT-I-1 | 100 | 3261 |
| 2 | CLT-II-2 | CLT-II-2 | | 2901 |
| 3 | THR-II-3 | THR-II-3 | | 979 |
| 4 | THR-I-4 | THR-I-4 | | 871 |
| 5 | CLT-II-7 | CLT-II-7 | | 962 |
| | <i>A.niger</i> | | | |
| 1 | CLT-II-1 | CLT-II-1 | 100 | 657 |
| 2 | CLT-I-3 | CLT-I-3 | | 697 |
| 3 | THR-I-4 | THR-I-4 | | 652 |
| 4 | CLT-II-5 | CLT-II-5 | | 487 |
| 5 | THR-II-6 | THR-II-6 | | 609 |
| 6 | THR-II-7 | THR-II-7 | | 5502 |
| 7 | CLT-II-8 | CLT-II-8 | | 8410 |

CLT- Kozhikode
THR- Thrissur

I- February-March
II- June-July

Table 22. Production of aflatoxins by species of *Aspergillus* in nutmeg

| Fungus | Number of isolates | Number of toxigenic isolates | % of toxigenic isolates | Aflatoxin produced |
|---------------------------|--------------------|------------------------------|-------------------------|-----------------------------------|
| <i>Aspergillus flavus</i> | 5 | 5 | 100 | B ₁ |
| <i>A. niger</i> | 7 | 7 | 100 | |
| | | 1 | 14.28 | B ₁ and B ₂ |
| | | 2 | 28.57 | B ₁ |
| | | 4 | 57.14 | B ₂ |

Table 23. Fractions of aflatoxin produced by *Aspergillus* spp. in nutmeg

| Sl. No. | Isolates | Quantity of aflatoxin (ppb) | | | |
|---------|---------------------------|-----------------------------|----------------|----------------|----------------|
| | | B ₁ | B ₂ | G ₁ | G ₂ |
| | <i>Aspergillus flavus</i> | | | | |
| 1 | CLT-I-1 | 879 | – | – | – |
| 2 | CLT-II-2 | 283 | – | – | – |
| 3 | THR-II-3 | 198 | – | – | – |
| 4 | THR-I-4 | 156 | – | – | – |
| 5 | CLT-II-7 | 127 | – | – | – |
| | <i>A. niger</i> | | | | |
| 1 | CLT-II-1 | – | 241 | – | – |
| 2 | CLT-I-3 | – | 456 | – | – |
| 3 | THR-I-4 | 99 | – | – | – |
| 4 | CLT-II-5 | – | 174 | – | – |
| 5 | THR-II-6 | – | 107 | – | – |
| 6 | THR-II-7 | 312 | – | – | – |
| 7 | CLT-II-8 | 368 | 362 | – | – |

CLT- Kozhikode
THR- Thrissur

I- February-March
II- June-July

Table 24. Total aflatoxin produced by *Aspergillus* spp. in mace

| Sl. No. | Isolates | | Percentage of toxigenic isolates | Total aflatoxin produced (ppb) |
|-----------------|---------------------------|--------------------|----------------------------------|--------------------------------|
| | <i>Aspergillus flavus</i> | Toxigenic isolates | | |
| 1 | THR-II-3 | THR-II-3 | 100 | 921 |
| 2 | THR-I-4 | THR-I-4 | | 1035 |
| <i>A. niger</i> | | | | |
| 1 | CLT-I-2 | CLT-I-2 | 100 | 947 |
| 2 | CLT-I-3 | CLT-I-3 | | 979 |
| 3 | CLT-II-5 | CLT-II-5 | | 1113 |
| 4 | THR-II-7 | THR-II-7 | | 992 |
| 5 | CLT-II-8 | CLT-II-8 | | 1021 |

CLT- Kozhikode
THR- Thrissur

I- February-March
II- June-July

Table 25. Production of aflatoxins by *Aspergillus* spp. in mace

| Fungus | Number of isolates | Number of toxigenic isolates | % of toxigenic isolates | Aflatoxin produced |
|---------------------------|--------------------|------------------------------|-------------------------|--------------------|
| <i>Aspergillus flavus</i> | 2 | 2 | 100 | B ₁ |
| <i>A. niger</i> | 5 | 5 | 100 | |
| | | 3 | 60 | B ₁ |
| | | 2 | 40 | B ₂ |

Table 26. Fractions of aflatoxin produced by *Aspergillus* spp. in mace

| Sl. No. | Isolates | Quantity of aflatoxin (ppb) | | | |
|---------|---------------------------|-----------------------------|----------------|----------------|----------------|
| | | B ₁ | B ₂ | G ₁ | G ₂ |
| | <i>Aspergillus flavus</i> | | | | |
| 1 | THR-II-3 | 916 | – | – | – |
| 2 | THR-I-4 | 326 | – | – | – |
| | <i>A. niger</i> | | | | |
| 1 | CLT-I-2 | 539 | – | – | – |
| 2 | CLT-I-3 | – | 550 | – | – |
| 3 | CLT-II-5 | – | 161 | – | – |
| 4 | THR-II-7 | 589 | – | – | – |
| 5 | CLT-II-8 | 978 | – | – | – |

CLT- Kozhikode
THR- Thrissur

I- February-March
II- June-July

4.5.2. Detection of Ochratoxin

The isolates of *A. ochraceus* obtained from samples of nutmeg collected during the survey were tested for their ability to produce ochratoxin under *in vitro* (culture) and under *in vivo* (substrate) conditions by High Performance Liquid Chromatography (HPLC).

4.5.2.1. Ochratoxin in Culture Medium

All the five isolates of *A. ochraceus* isolated from the samples of nutmeg were found to produce Ochratoxin A in culture medium, in the range of 970.44-1835.95 ppb and the maximum and minimum being recorded by isolate AO-5 and AO-1. The other isolates, namely, AO-2, AO-3 and AO-4 recorded 1732.97, 1487.19 and 1074.51 ppb Ochratoxin A respectively (Fig. 1.A-E; Table 27).

4.5.2.2. Ochratoxin in Substrate

The five toxigenic isolates of *A. ochraceus* were found to produce ochratoxin A when inoculated on nutmeg (substrate). The quantity of Ochratoxin A produced by these isolates in nutmeg ranged from 6.02 to 212.33 ppb. The maximum quantity detected in nutmeg was by the isolate AO-4 and minimum by isolate AO -2. The other isolates, namely, AO -1, AO -3 and AO -5 produced 8.12, 10.30 and 37.59 ppb of ochratoxin A in nutmeg respectively (Fig. 1. F-J; Table 28).

4.6. METHODS TO MINIMIZE FUNGAL CONTAMINATION IN NUTMEG AND MACE

An experiment was conducted with 15 treatments including essential oils (four numbers), leaf powder/plant extracts (five numbers) and packing materials (six numbers) to minimize the fungal deterioration in nutmeg and mace during storage. The fungal population (cfu g⁻¹) of the samples of nutmeg and mace exposed to different treatments was recorded at two week intervals upto 12 weeks. The moisture content of the samples of nutmeg and mace in each treatment was also recorded before and after storage.

Table 27. Ochratoxin produced in culture medium

| Sl. No. | Isolates | Quantity of ochratoxin A (ppb) |
|---------|----------|--------------------------------|
| 1 | AO -1 | 970.44 |
| 2 | AO -2 | 1732.97 |
| 3 | AO -3 | 1487.19 |
| 4 | AO -4 | 1074.51 |
| 5 | AO -5 | 1835.95 |

AO- *Aspergillus ochraceus*

Table 28. Ochratoxin produced in substrate (nutmeg)

| Sl. No. | Isolates | Quantity of ochratoxin (ppb) |
|---------|----------|------------------------------|
| 1 | AO -1 | 8.12 |
| 2 | AO -2 | 6.02 |
| 3 | AO -3 | 10.30 |
| 4 | AO -4 | 212.33 |
| 5 | AO -5 | 37.59 |

AO- *Aspergillus ochraceus*

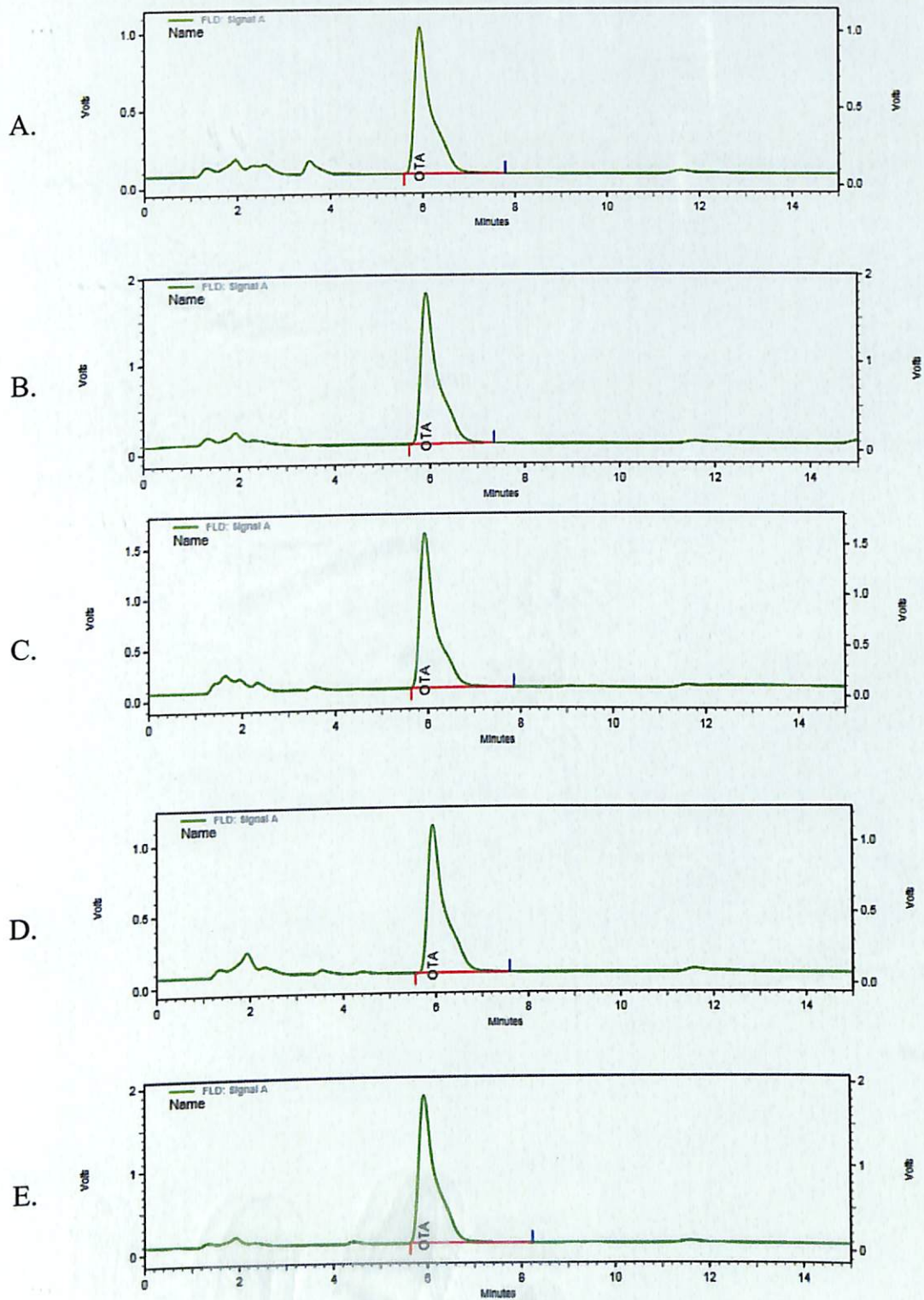


Fig. 1. HPLC Chromatograph for ochratoxin A

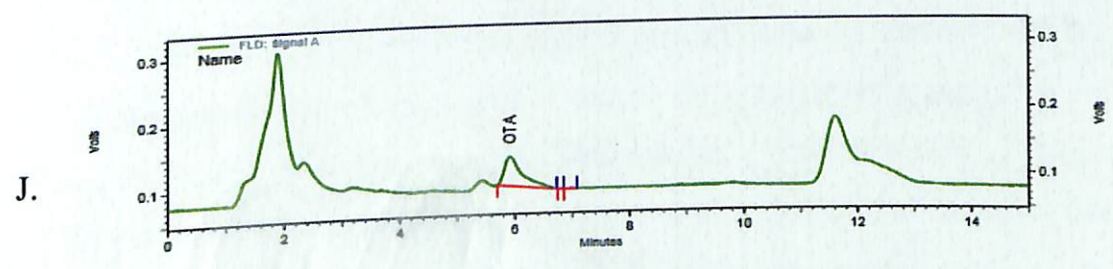
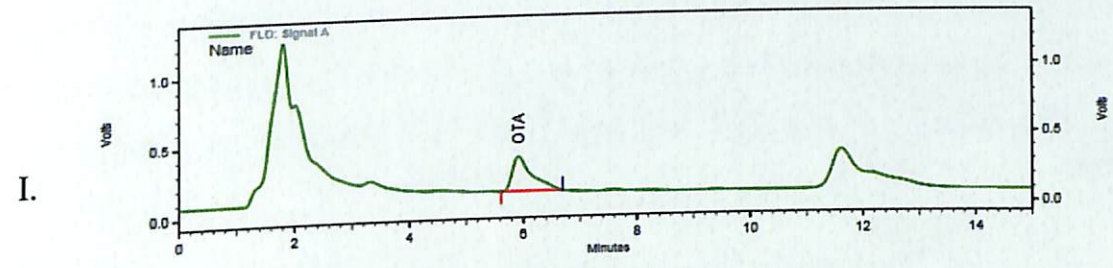
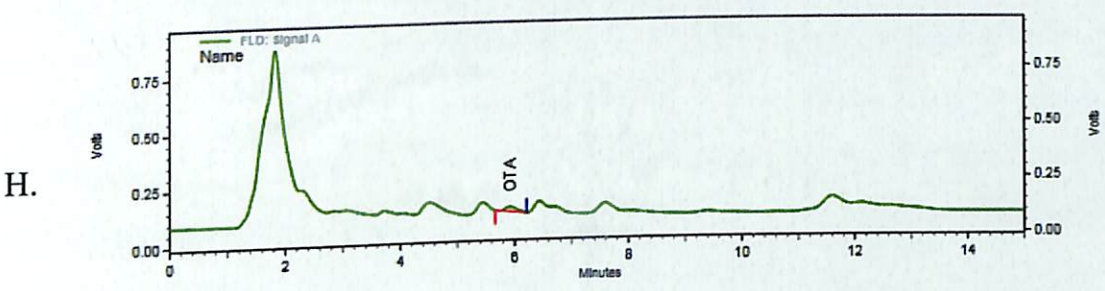
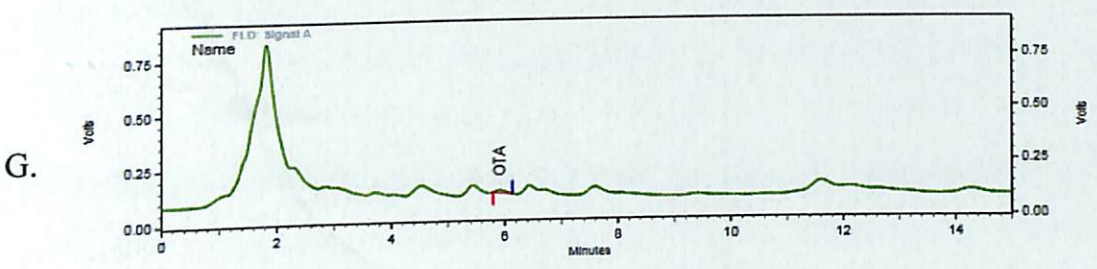
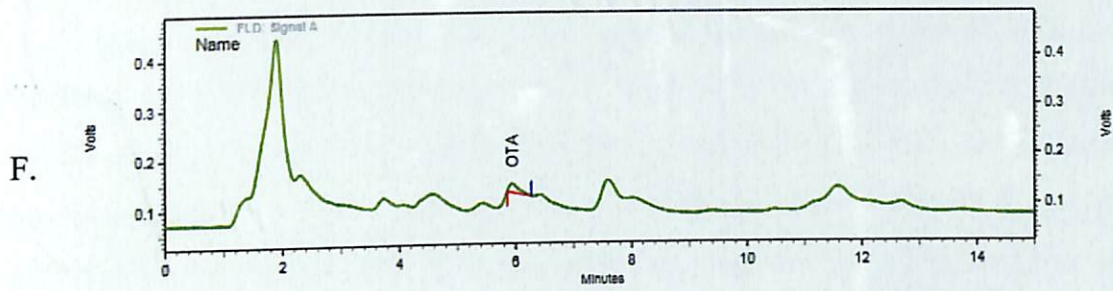


Fig. 1. HPLC Chromatograph for ochratoxin A (continued)

4.6.1. Nutmeg

The fungal population (cfu g⁻¹) of nutmeg samples exposed to different treatments were recorded at two week intervals upto 12 weeks (Table 29). There was no visible growth of fungi on nutmeg samples exposed to different treatments, except in treatments onion extract (T8) and garlic extract (T6) one week after storage. In general, population of fungi was lowest in the samples of nutmeg samples (54.32×10⁴ g⁻¹) stored in polylined jute bags. Comparatively higher population was noticed in nutmeg samples treated with garlic extract (285.91×10⁴ g⁻¹) and onion extract (260.37×10⁴ g⁻¹).

Statistical analysis of the data on the fungal population of nutmeg in different treatments, revealed that polylined jute bag was superior and was on par with plastic bag (56.04×10⁴ g⁻¹), cinnamon oil (81.78×10⁴ g⁻¹), polypropylene bag (84.84×10⁴ g⁻¹), ocimum leaf (88.34×10⁴ g⁻¹), allspice oil (89.19×10⁴ g⁻¹), clove oil (105.51×10⁴ g⁻¹), lemon grass oil (114.15×10⁴ g⁻¹) and neem leaf (129.48×10⁴ g⁻¹). However of the above treatments which were on par, plastic bag was found to be the cheapest in storing nutmeg.

The moisture content of nutmeg samples before treatment was 11.5 per cent and the moisture content of treatments varied from 7.06 – 14.52 per cent after three months of storage (Table 30). The minimum and maximum moisture content were recorded in treatment T-13 (polylined jute bag) and T-8 (onion extract) respectively.

4.6.2. Mace

The fungal population (cfu g⁻¹) mace samples exposed to different treatments were also recorded at two week intervals up to 12 weeks (Table 31). Samples treated with onion extract (T8) and garlic extract (T6) exhibited visible fungal growth one week after storage, whereas there was no visible growth of fungi on mace samples exposed to other treatments. In the case of mace samples fungal population was generally low (62.38×10⁴ g⁻¹) when stored in polylined jute bags. Comparatively higher population was noticed in mace samples treated with garlic extract (266.70×10⁴ g⁻¹) and onion extract (390.50×10⁴ g⁻¹).

Statistical analysis of the data on the fungal population of mace samples in different treatments, revealed that polylined jute bag exhibited minimum fungal population and was on par with plastic bag ($77.72 \times 10^4 \text{ g}^{-1}$), cinnamon oil ($88.83 \times 10^4 \text{ g}^{-1}$), polypropylene bag ($96.24 \times 10^4 \text{ g}^{-1}$), clove oil ($102.86 \times 10^4 \text{ g}^{-1}$), allspice oil ($105.99 \times 10^4 \text{ g}^{-1}$), ocimum leaf ($108.72 \times 10^4 \text{ g}^{-1}$) and lemon grass oil ($118.31 \times 10^4 \text{ g}^{-1}$). Of the above treatments which were on par, plastic bag was found to be the cheapest in storing mace.

The moisture content of mace samples before treatment was 9.5 per cent and the moisture content of treatments varied from 2.01-18.29 per cent after three months of storage (Table 32). The minimum and maximum moisture content were recorded in treatment T-13 (polylined jute bag) and T-8 (onion extract) respectively.

Table 29. Effect of different treatments on the population of fungi in nutmeg

| Treatments | Mean fungal population (10^4 g ⁻¹) after (weeks) | | | | | | | Mean | Treatment cost per kg storage |
|------------------------|---|--------------------------------|-------------------|-------------------|-------------------|--------------------------------|------------------------------|----------------------------------|-------------------------------|
| | 0 | 2 | 4 | 6 | 8 | 10 | 12 | | |
| T1-Allspice oil | 11.5 | 77.44 (8.8) | 87.05 (9.33) | 119.03 (10.91) | 104.86 (10.24) | 74.13 (8.61) | 76.56 (8.75) | 89.19 (9.44) ^a | 4.9 |
| T2-Cinnamon oil | 11.5 | 65.45 (8.09) | 85.93 (9.27) | 74.13 (8.61) | 90.82 (9.53) | 95.45 (9.77) | 80.28 (8.96) | 81.78 (9.04) ^a | 2.45 |
| T3-Clove oil | 11.5 | 157.00 (12.53) | 134.56 (11.6) | 121.44 (11.02) | 122.77 (11.08) | 89.68 (9.47) | 34.93 (5.91) | 105.51 (10.27) | 3.1 |
| T4-Lemon grass oil | 11.5 | 143.76 (11.99) | 114.92 (10.72) | 151.54 (12.31) | 108.16 (10.4) | 109.20 (10.45) | 67.24 (8.2) | 114.15 (10.68) ^{ab} | 2.67 |
| T5-Curry leaf | 11.5 | 174.24 (13.2) | 283.25 (16.83) | 229.83 (15.16) | 230.43 (15.18) | 196.56 (14.02) | 191.27 (13.83) | 216.35 (14.70) ^{bcd} | 77.0 |
| T6-Garlic extract | 11.5 | 279.2 (16.71) | 314.00 (17.72) | 358.72 (18.94) | 286.29 (16.92) | 301.72 (17.37) | 189.61 (13.77) | 285.91 (16.90) ^d | 14.5 |
| T7-Neem leaf | 11.5 | 144.00 (12) | 134.56 (11.6) | 149.82 (12.24) | 113.42 (10.65) | 162.82 (12.76) | 81.18 (9.01) | 129.48 (11.37) ^{ab} | 22 |
| T8-Onion extract | 11.5 | 224.70 (14.99) | 235.01 (15.33) | 300.68 (17.34) | 275.89 (16.61) | 293.78 (17.14) | 239.95 (15.36) | 260.37 (16.13) ^d | 3.9 |
| T9-Ocimum leaf | 11.5 | 138.53 (11.77) | 179.83 (13.41) | 117.94 (10.86) | 57.91 (7.61) | 48.30 (6.95) | 33.29 (5.77) | 88.34 (9.39) ^a | 92 |
| T10-Cloth bag | 11.5 | 168.48 (12.98) | 296.53 (17.22) | 219.34 (14.81) | 213.45 (14.61) | 167.44 (12.94) | 138.53 (11.77) | 197.68 (14.06) ^{bcd} | 15 |
| T11-Hessian cloth bag | 11.5 | 115.13 (10.73) | 170.82 (13.07) | 221.71 (14.89) | 184.14 (13.57) | 107.74 (10.38) | 78.32 (8.85) | 143.59 (11.98) ^{bc} | 20 |
| T12-Plastic bag | 11.5 | 188.51 (13.73) | 90.82 (9.53) | 53.29 (7.3) | 35.28 (5.94) | 22.37 (4.73) | 12.89 (3.59) | 56.04 (7.48) ^a | 1.3 |
| T13-Polylined jute bag | 11.5 | 81.00 (9) | 69.22 (8.32) | 58.68 (7.66) | 52.27 (7.23) | 44.76 (6.69) | 27.98 (5.29) | 54.32 (7.37) ^a | 25 |
| T14-Polypropylene bag | 11.5 | 115.99 (10.77) | 106.71 (10.33) | 112.78 (10.62) | 65.45 (8.09) | 61.00 (7.81) | 57.76 (7.6) | 84.84 (9.21) ^a | 1.5 |
| T15-Gunny bag | 11.5 | 103.02 (10.15) | 173.98 (13.19) | 309.06 (17.58) | 258.24 (16.07) | 206.21 (14.36) | 189.06 (13.75) | 201.30 (14.18) ^{bcd} | 5 |
| Mean | 11.5 | 140.04 (11.83) ^e | 156.29 de | 161.06 cd | 132.71 bc | 118.83 (10.90) ^b | 87.74 (9.36) ^a | | |

*Figures in the parenthesis indicates \sqrt{x} transformation

CD (0.05) for treatments = (4.58)

CD (0.05) for periods of storage= 0.740

CD (0.05) for interaction=0.685

Table 30. Moisture content and fungal population of nutmeg in different treatments

| Treatments | Mean fungal population (10^4 g^{-1}) | Moisture content (%) |
|------------|--|----------------------|
| T1 | 10.3 | 8.72 |
| T2 | 9.43 | 8.47 |
| T3 | 10.14 | 9.22 |
| T4 | 10.88 | 9.98 |
| T5 | 15.67 | 12.52 |
| T6 | 16.33 | 12.82 |
| T7 | 11.93 | 10.68 |
| T8 | 19.76 | 14.52 |
| T9 | 10.43 | 9.45 |
| T10 | 12.61 | 12.05 |
| T11 | 12.56 | 11.17 |
| T12 | 8.82 | 7.64 |
| T13 | 7.9 | 7.06 |
| T14 | 9.81 | 8.04 |
| T15 | 11.78 | 10.27 |

Table 31. Effect of different treatments on the population of fungi in mace

| Treatments | Mean fungal population (10^4 g ⁻¹) after (weeks) | | | | | | | Mean | Treatment cost per kg storage |
|------------------------|---|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|----------------------------------|-------------------------------|
| | 0 | 2 | 4 | 6 | 8 | 10 | 12 | | |
| T1-Allspice oil | 9.5 | 123.43 (11.11) | 126.79 (11.26) | 112.78 (10.62) | 131.56 (11.47) | 67.57 (8.22) | 82.08 (9.06) | 105.99 (10.30) ^{ab} | 4.9 |
| T2-Cinnamon oil | 9.5 | 156.75 (12.52) | 157.25 (12.54) | 78.50 (8.86) | 104.65 (10.23) | 57.76 (7.62) | 22.75 (4.77) | 88.83 (9.43) ^{ab} | 2.45 |
| T3-Clove oil | 9.5 | 111.09 (10.54) | 134.56 (11.60) | 117.29 (10.83) | 89.87 (9.48) | 95.06 (9.75) | 78.15 (8.84) | 102.86 (10.14) ^{ab} | 3.1 |
| T4-Lemon grass oil | 9.5 | 67.57 (8.22) | 37.95 (6.16) | 79.03 (8.89) | 173.98 (13.19) | 169.52 (13.02) | 248.06 (15.75) | 118.31 (10.88) ^{ab} | 2.67 |
| T5-Curry leaf | 9.5 | 327.61 (18.1) | 320.41 (17.9) | 188.51 (13.73) | 314.71 (17.74) | 242.11 (15.56) | 120.34 (10.97) | 245.61 (15.67) ^{cd} | 77.0 |
| T6-Garlic extract | 9.5 | 153.76 (12.4) | 137.83 (11.74) | 167.96 (12.96) | 265.36 (16.29) | 437.23 (20.91) | 559.80 (23.66) | 266.70 (16.33) ^{de} | 14.5 |
| T7-Neem leaf | 9.5 | 180.10 (13.42) | 185.50 (13.62) | 199.94 (14.14) | 106.09 (10.3) | 119.03 (10.91) | 99.80 (9.99) | 142.37 (11.93) ^{bc} | 22 |
| T8-Onion extract | 9.5 | 175.03 (13.23) | 163.84 (12.8) | 322.20 (17.95) | 504.00 (22.45) | 603.68 (24.57) | 757.90 (27.53) | 390.50 (19.76) ^e | 3.9 |
| T9-Ocimum leaf | 9.5 | 158.26 (12.58) | 171.35 (13.09) | 161.54 (12.71) | 109.20 (10.45) | 62.09 (7.88) | 33.87 (5.82) | 108.72 (10.43) ^{ab} | 92 |
| T10-Cloth bag | 9.5 | 153.51 (12.39) | 193.49 (13.91) | 223.50 (14.95) | 194.88 (13.96) | 131.56 (11.47) | 80.10 (8.95) | 159.01 (12.61) ^{bcd} | 15 |
| T11-Hessian cloth bag | 9.5 | 138.06 (11.75) | 118.16 (10.87) | 111.09 (10.54) | 169.52 (13.02) | 247.43 (15.73) | 180.63 (13.44) | 157.83 (12.56) ^{bcd} | 20 |
| T12-Plastic bag | 9.5 | 124.99 (11.18) | 112.15 (10.59) | 135.96 (11.66) | 69.56 (8.34) | 45.16 (6.72) | 19.18 (4.38) | 77.72 (8.82) ^{ab} | 1.3 |
| T13-Polylined jute bag | 9.5 | 106.09 (10.3) | 53.44 (7.31) | 166.15 (12.89) | 37.95 (6.16) | 45.83 (6.77) | 15.44 (3.93) | 62.38 (7.90) ^a | 25 |
| T14-Polypropylene bag | 9.5 | 115.78 (10.76) | 112.15 (10.59) | 131.56 (11.47) | 129.96 (11.4) | 56.40 (7.51) | 50.55 (7.11) | 96.24 (9.81) ^{ab} | 1.5 |
| T15-Gunny bag | 9.5 | 129.28 (11.37) | 106.92 (10.34) | 133.17 (11.54) | 94.67 (9.73) | 165.89 (12.88) | 219.34 (14.81) | 138.84 (11.78) ^b | 5 |
| Mean | 9.5 | 143.76 ab | 135.02 ab | 150.06 b | 150.80 b | 141.85 ab | 126.56 a | | |

*Figures in the parenthesis indicates \sqrt{x} transformation

CD (0.05) for treatments = 3.879

CD (0.05) for periods of storage = 0.796

CD (0.05) for interaction = 0.77

Table 32. Moisture content and fungal population of mace in different treatments

| Treatments | Mean fungal population (10^4 g^{-1}) | Moisture content (%) |
|------------|--|----------------------|
| T1 | 9.44 | 6.69 |
| T2 | 9.39 | 5.28 |
| T3 | 10.27 | 8.95 |
| T4 | 10.68 | 12.32 |
| T5 | 14.7 | 13.14 |
| T6 | 16.9 | 13.41 |
| T7 | 11.37 | 12.34 |
| T8 | 16.13 | 18.29 |
| T9 | 9.41 | 9.82 |
| T10 | 14.06 | 12.37 |
| T11 | 11.98 | 12.13 |
| T12 | 7.48 | 2.01 |
| T13 | 7.37 | 2.54 |
| T14 | 9.21 | 6.26 |
| T15 | 14.18 | 12.91 |

Discussion

5. DISCUSSION

Spices are one of the major foreign exchange earners to India, which includes black pepper, white pepper, red chillies, nutmeg, mace, cinnamon, clove etc. Among which nutmeg and mace constitutes a major share. Aflatoxin contamination of spices affects the international trade of these commodities. It is also a fact that very little information was available from India on mold infestation of spices by toxigenic fungi and the elaborations of mycotoxins. So the present investigation on the mycoflora of nutmeg during storage and the associated mycotoxin was taken up.

The results of the present study indicated that nutmeg and mace were subjected to deterioration and spoilage by a number of fungi and elaborated aflatoxin during storage. Fungi such as *Acremonium restrictum*, *A. strictum*, *Aspergillus flavus*, *A. niger*, *A. sclerotiorum*, *Emericella nidulans*, *Eurotium amestelodami*, *Fusarium moniliforme*, *Penicillium chrysogenum* and *Syncephalastrum racemosum* were found commonly associated with nutmeg and mace sample.

In addition *Aspergillus ochraceus* was present in nutmeg, *A. oryzae* and *Hansfordia pulvinata* were isolated from mace.

The morphological and cultural characters of the fungi were studied by growing them in Czapek Dox plates and by preparing wet mount and slide culture. The fungi were provisionally identified and confirmed by sending cultures to National Centre for Fungal Taxonomy (NCFT).

Our result was confirmed by previous work with the same fungi on different spices. *A. flavus* was reported in nutmeg (Hansen and Jung, 1973), black pepper (Misra, 1981; Bokhari, 2007), ginger (Flannigan and Hui 1976, Madhyashta and Bhat, 1985; Zakka *et al.*, 2010) and cinnamon (Aziz *et al.*, 1998; Abdulkhadir *et al.*, 2003). Occurrence of *A. niger* was reported in chilli on chilli (Pavgi and Singh, 1964), black pepper (Misra, 1981), dried ginger (Zakka *et al.*, 2010) and cinnamon (Aziz *et al.*, 1998; Abdulkhadir *et al.*, 2003). Giridhar and Reddy (1997) surveyed chilli for the occurrence of fungal flora, *Aspergillus* spp.

were found dominant and *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*, *Cladosporium*, *Chaetomium*, *Curvularia*, *Fusarium*, *Memnoniella*, *Macrophomina*, *Phoma*, *Penicillium*, *Rhizopus* and *Trichothecium*. Shadanaika (2005) reported that important fungi belonging to species of *Aspergillus*, *Fusarium* and *Penicillium* were found associated with chilli, ginger and turmeric. Chilli samples recorded toxigenic strains belonging to *A. flavus*, *A. parasiticus* and *Fusarium sporotrichoides*, while ginger and turmeric samples were found associated with *A. flavus* only. The species *A. ochraceus* was also observed on chilli and ginger.

Among the fungi isolated from nutmeg during the present investigation, *A. restrictum*, *A. strictum*, *A. ochraceus*, *A. sclerotiorum*, *E. nidulans* and *E. amestelodami* were isolated from nutmeg, *A. restrictum*, *A. strictum*, *A. oryzae*, *A. sclerotiorum*, *E. nidulans*, *E. amestelodami* and *H. pulvinata* from mace have not been reported earlier.

The studies on occurrence of different fungi in the samples of nutmeg and mace collected during two seasons from different regions, it was showed that *A. flavus* and *A. niger* were present in almost all the samples of nutmeg and mace and occurred in highest frequency in both the districts surveyed. *A. flavus* occurred at a frequency of 85 and 90 per cent from nutmeg samples (Fig 2) of Kozhikode and Thrissur respectively, whereas as 90 and 80 per cent from mace samples (Fig 3). *A. niger* occurred at a frequency of 95 per cent from nutmeg samples of both Kozhikode and Thrissur respectively, whereas as 100 and 95 per cent from mace samples. Bilgrami (1985) during their survey observed that *A. flavus* was the most dominant fungus with frequency of 61 to 82 per cent. Other mycotoxigenic fungi viz., *A. niger*, *A. ochraceus*, *P. citrinum* and *F. moniliforme* also exhibited a fairly high frequency of 20 to 67 per cent. In the present investigation fungal flora associated with dried nutmeg and mace were *Acremonium restrictum*, *A. strictum*, *Aspergillus flavus*, *A. niger*, *A. ochraceous*, *A. sclerotiorum*, *E. nidulans*, *E. amestelodami*, *F. moniliforme*, *P. chrysogenum*

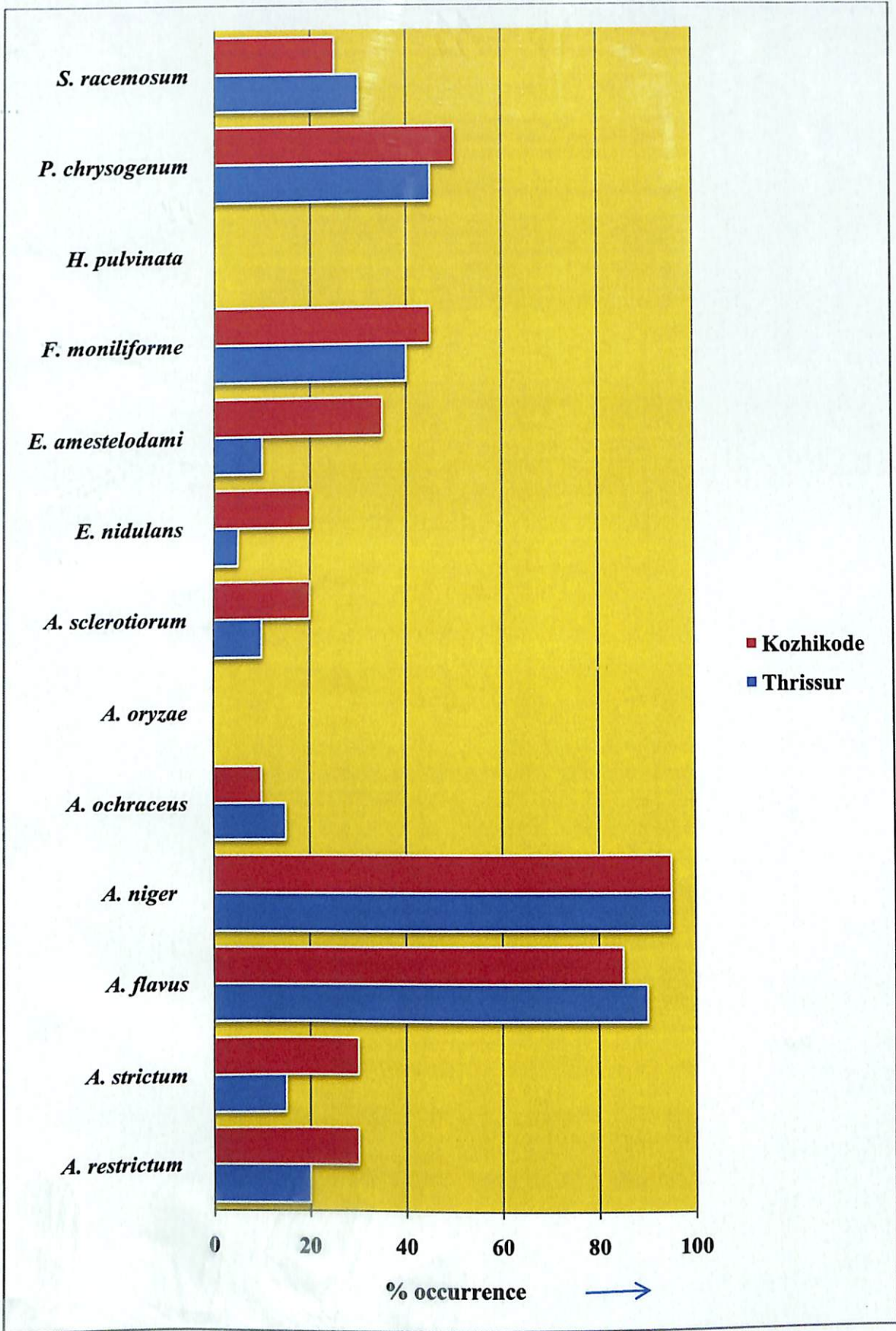


Fig. 2. Frequency of fungi from samples of nutmeg

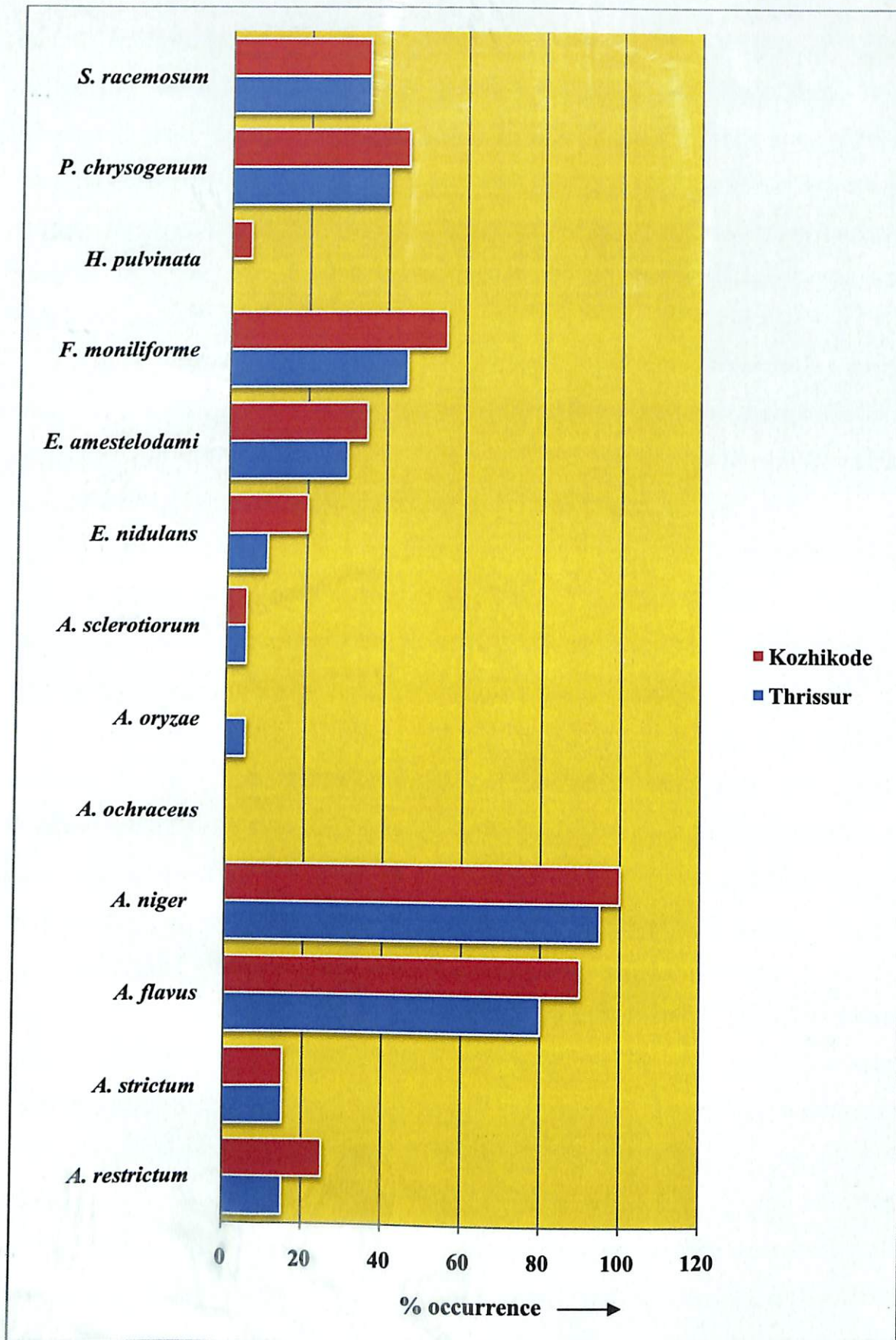


Fig. 3. Frequency of fungi from samples of mace

and *S. racemosum*. Those fungi classified in the deuteromycotina or Fungi imperfecti, belongs mainly to the genera *Aspergillus* and *Penicillium*, well adapted to grow on solid substrates with low water content (Lesage *et al.*, 1993). This property was 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 and Kaufmann, 1965).

During the survey, relatively lower fungal population was recorded in the samples of nutmeg and mace collected from different regions, during the two seasons. This may be due to the fact that these spices contain antimicrobial fractions, which would have inhibited the growth of fungi.

Higher population of fungi in nutmeg and mace from both regions was obtained during June-July than February- March. The high relative humidity and rainfall conditions prevailing in these regions during the monsoon periods can be attributed as plausible explanation for the above observation.

Nutmeg and mace samples collected during June-July had high moisture content, during which RH and associated mycoflora was high and natural incidence of aflatoxins was also recorded during that period. Moisture control of the substrate is the best and most economical means to prevent mould growth and mycotoxin production. To prevent microbiological growth, especially that of toxigenic mould, a safe moisture level should be achieved. Bottomley *et al.* (1950) reported that in the case of stored yellow corn, as the RH of atmosphere was raised from 75 to 100 per cent, the total moisture content increased logarithmically, consequently increasing the internal mould infection and decreasing the viability of seeds. The composition of mycoflora varied with moisture, temperature and oxygen concentration in the atmosphere. Moisture content was closely bound to temperature. Under certain circumstances, temperature differences can cause the re-distribution of moisture leading to local mould growth. As the RH increases the moisture content of the sample also increased. At maximum moisture content the RH was also highest and there was corresponding increase in fungal flora (Bilgrami, 1985).

The study also proved a positive and significant correlation between fungal population and moisture content of the samples, also showed positive correlation with RH and total rain fall. Shadanaika (2005) noticed a positive correlation between increase in the moisture content and increase in mycoflora as well as mycotoxin production in the samples of chilli, ginger and turmeric exposed to higher RH levels. According to Deiner (1976) moisture content or relative humidity surrounding the substrates was the most important factor for the growth and aflatoxin production by *A. flavus*. Also substrate moisture determines the growth and production of toxins by the fungi, no matter how favourable was the temperature. The optimal relative humidity (RH) range was between 89-99 per cent at 30°C for eight weeks incubation whereas under the field conditions the fungal invasion occurred rapidly at kernel moisture content of 12-20 per cent (Deiner and Davis, 1969). The optimal temperature range for aflatoxin production on seed substrates was 25-36°C in controlled environment (Detroy *et al.*, 1971). Bonner (1948) conducted *in vitro* studies on the temperature and humidity requirements of *A. niger*. The results showed that optimum temperature requirement for growth of *A. niger* was related to RH. At 95 per cent RH, the temperature requirements was around 40°C, at 100 per cent RH, the optimum temperature was near 30°C.

The natural incidence of mycotoxin in the samples of nutmeg and mace collected during the survey were assessed. Out of the 80 samples, 40 each of nutmeg and mace were analysed, only six samples, three of nutmeg and three of mace samples showed the presence of aflatoxin. The reason could be, fungal growth was weaker in spices as they contained essential oils with anti microbial effects (Ahamed *et al.*, 1994; Bartin and Tantaoui-Elaraki., 1997). Of the six samples which showed the presence of total aflatoxin four (75 per cent) were obtained during June- July, when the RH and moisture content were high. These reasons may be contributed to the mold growth and subsequent aflatoxin production. Even though aflatoxin was detected in 3.5 per cent of the samples each in nutmeg and mace, the quantity of aflatoxin detected was far above the recommended limit 10 ppb of the European union (EFSA, 2006). Also found that

B₁ which was found to be the most potent toxin among the aflatoxins (Langseth *et al.*, 1995) was detected in the highest quantity (921 ppb) in the six samples. The sample, THR-MII-9 which recorded maximum quantity of 964 ppb of total aflatoxin and 921 ppb of aflatoxin B₁ respectively was obtained from Thrissur district during June-July which coincided with high moisture content of the samples. The high relative humidity and warm temperature prevailed in the locality which enhances the mould growth and toxin production (Fig. 4). Cho *et al.* (2008) reported that spices are largely produced in countries with tropical climates that have high range of temperature, humidity, and rainfall. Furthermore, improper storage, extended drying times, and elevated moisture contents caused development of mycotoxins in spices.

The problem of aflatoxin was reported from spices, namely, nutmeg (Hansen and Jung, 1973; Scott and Kennedy, 1975; Llewellyn *et al.*, 1992; Tabata, 1998), ginger (Llewellyn *et al.*, 1992) black pepper and white pepper (Selim *et al.*, 1996; Tabata *et al.*, 1998) red chilli (Scott and Kennedy 1973; Flannigan and Hui, 1976).

In a study conducted by Scott and Kennedy (1975) found the presence of Aflatoxin B₁ content was upto 15 ppb in samples recorded low to high incidence of aflatoxins in ginger (upto 25 ppb B₁) and nutmeg (upto 37.5 ppb B₁). In another study of aflatoxins in various spices like ginger, pepper, nutmeg and red chillies, highest contamination of aflatoxins up to 120 µg kg⁻¹ was found in red chillies (Llewellyn *et al.*, 1992).

During 1997, European Commission conducted a coordinated programme for the control of mycotoxin in foodstuff and found that among the 3098 spice samples including nutmeg, pepper, chilli and paprika analysed, 183 samples (5.9 per cent) contained more than 10 µg kg⁻¹ aflatoxins.

The fungi, namely, *A. flavus* and *A. niger* which occurred in highest frequency in the present investigation were tested for their ability to elaborate aflatoxins in culture and in the substrate from where it was isolated.

All the nutmeg isolates and two of four mace isolates of *A. flavus* were found to be toxigenic, produced aflatoxin and one or more of its fractions,

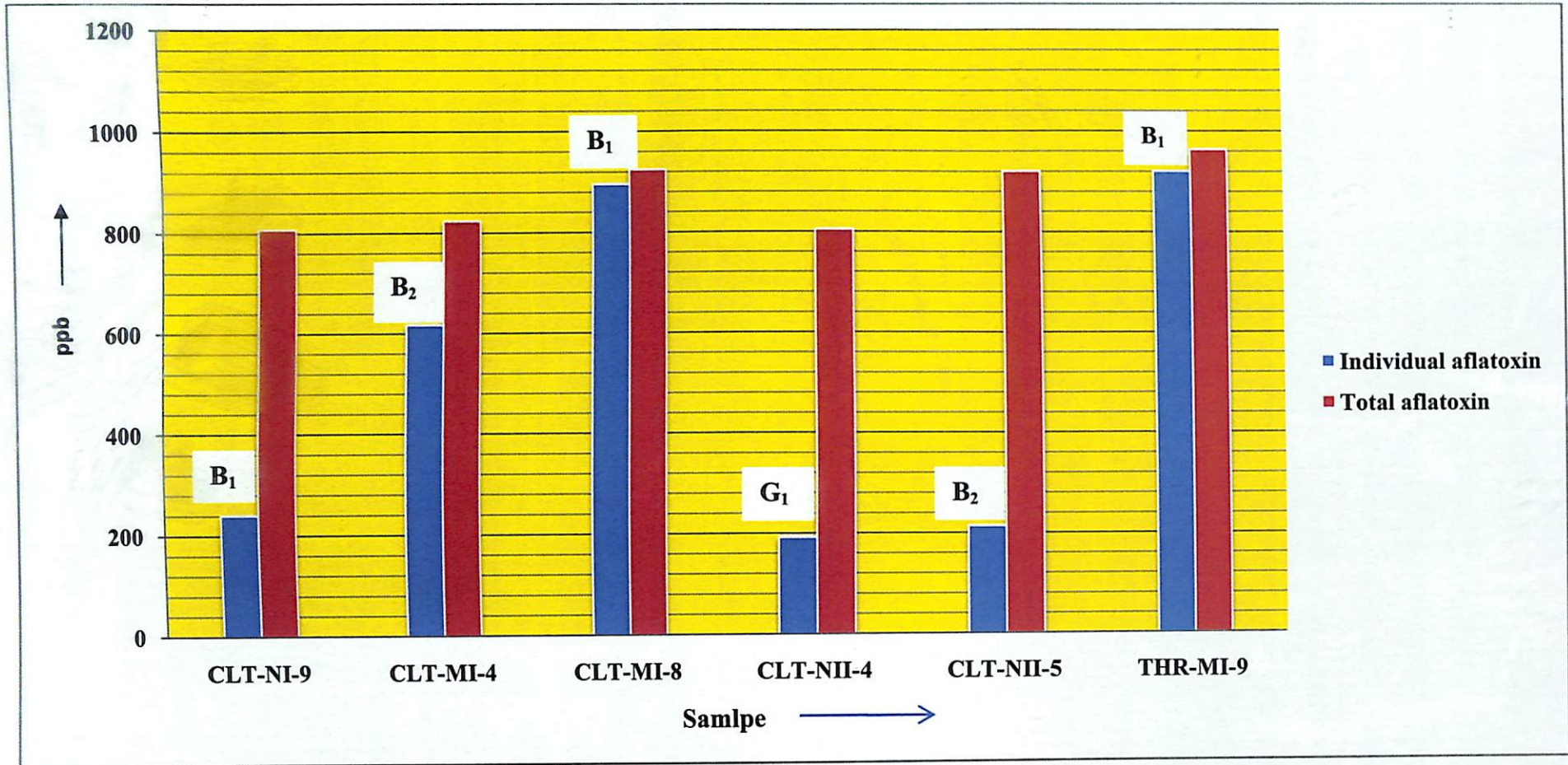


Fig. 4. Aflatoxin produced in samples of nutmeg and mace

namely, B₁, B₂ and G₁ in SMKY liquid medium. The production of aflatoxin by the isolates of *A. flavus* has been reported by Sinha *et al.* (1988) that, all the strains of *A. flavus* did not have the capacity to produce aflatoxin. All the nutmeg (seven) and mace (five) isolates of *A. niger* were found to be toxigenic in the medium and produced one or more of the aflatoxins, namely, B₁, B₂ and G₁. Production of these aflatoxins by *A. niger* has been reported by Kulik and Holaday (1967) and Bilgrami (1985). Naseema (1989) during her study reported that eight out of the 19 isolates of *A. niger* produced aflatoxin B₁ in medium.

The same isolates of *A. flavus* obtained from nutmeg and mace were found to produce maximum quantity of aflatoxin B₁ (THR-I-4), B₂ (THR-II-3) and G₁ (THR-II-3) in culture (Fig. 5).

In the case of *A. niger*, isolate CLT-II-8 of nutmeg and THR-II-7 of mace, CLT-II-8 of nutmeg and mace, THR-I-4 of nutmeg produced maximum quantity of aflatoxins B₁, B₂ and G₁ respectively in culture medium (Fig. 6). None of the isolates of *A. flavus* and *A. niger* produced aflatoxin G₂ in culture medium. Lebrihi (1986) screened the fungi obtained from spices for their ability to produce mycotoxin under laboratory conditions. The result shows that out of 568 isolates of *A. flavus* screened, 280 were found to be toxigenic and produced different fractions of aflatoxins in varying concentrations (0.5-15 ppm). Sixty five isolates of *A. flavus* produced aflatoxin B₁ in the range of 5-15 ppm, whereas 215 isolates elaborate aflatoxin B₁ only up to 5 ppm. All toxigenic isolates of *A. flavus* produced aflatoxin B₁ in liquid medium. However six isolates were capable of producing all the four aflatoxins *viz.*, B₁, B₂, G₁ and G₂, whereas 104 toxigenic isolates produced aflatoxin B₁ and B₂ in the medium. Even G₁ was produced in addition to B₁ and B₂ by 30 isolates. About 50 per cent isolates produced only aflatoxin B₁. Shadanaika (2005) screened strains of *A. flavus* and *A. parasiticus* isolated from various spice samples for their ability to produce aflatoxins on Yeast extract sucrose medium. *A. flavus* was abundant in all the spice samples. *A. flavus* Isolates from chilli produce aflatoxin B₁ and B₂ at the range of 0.0 - 2400 and 0.0 - 1200 ppb level respectively. Similarly, isolates from ginger and turmeric produced aflatoxin B₁ and B₂ at lower range of 0.0 - 120 and 0.0 - 80 ppb

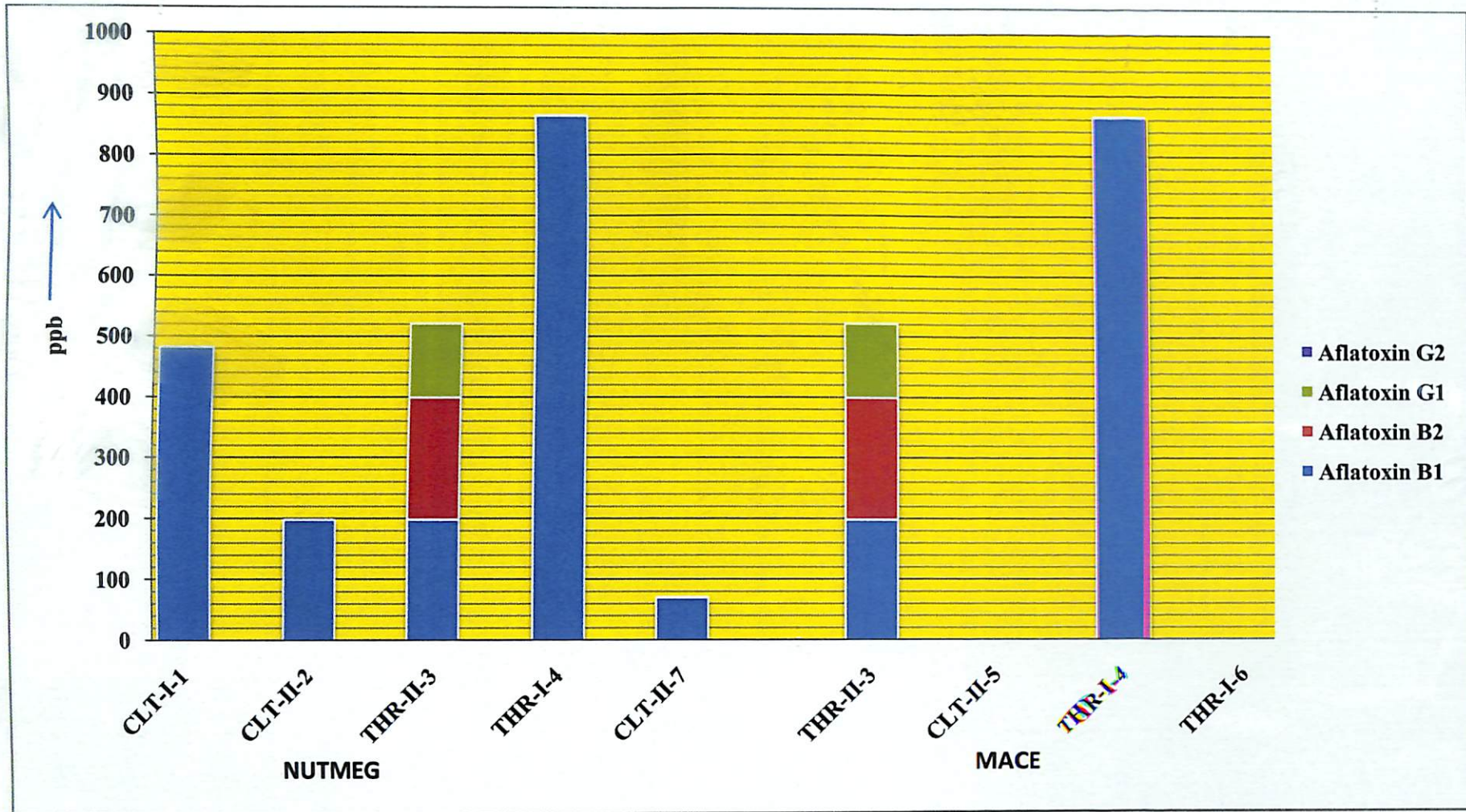


Fig. 5. Aflatoxins produced in culture by *Aspergillus flavus* from nutmeg and mace

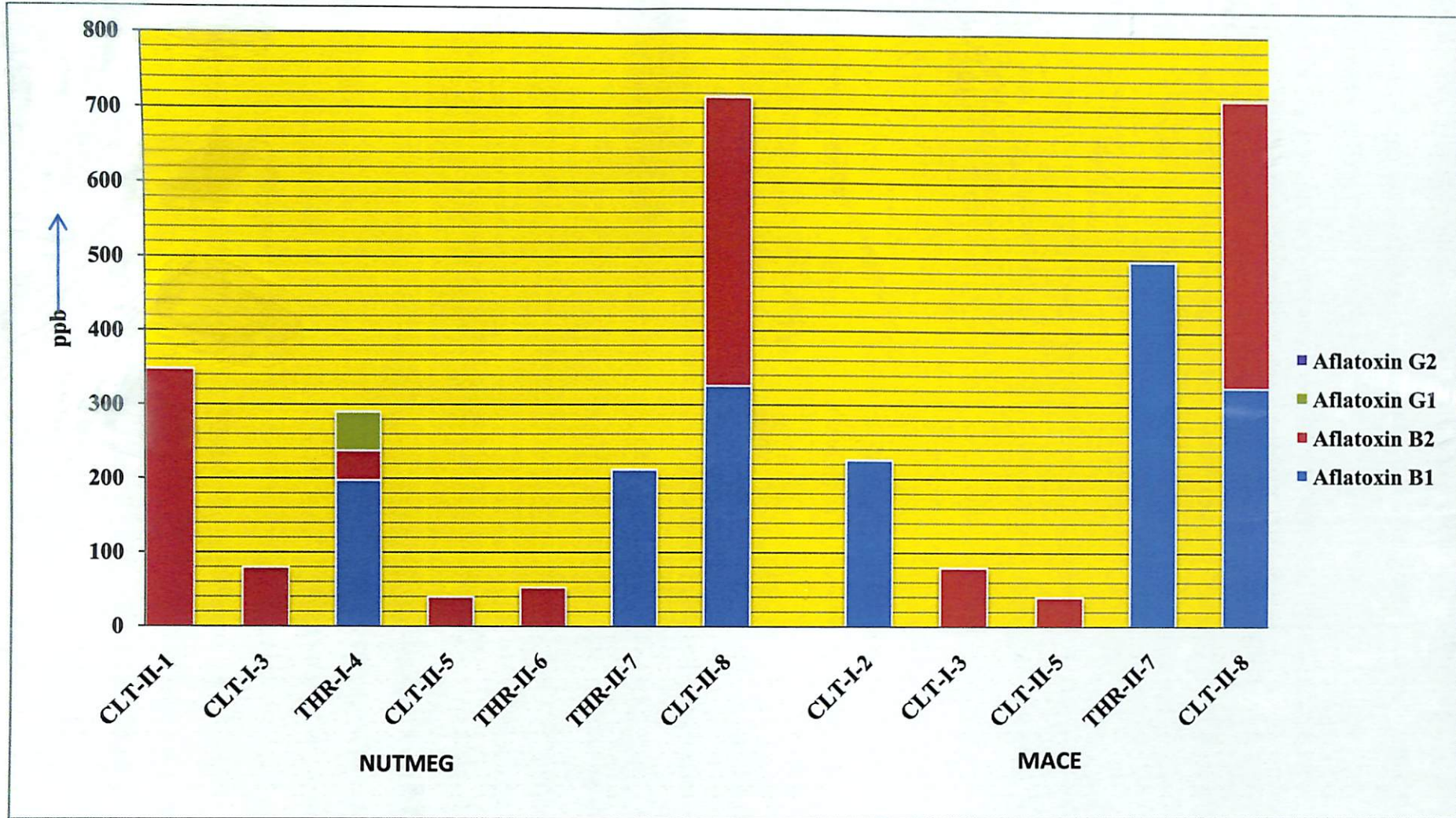


Fig. 6. Aflatoxins produced in culture by *Aspergillus niger* from nutmeg and mace

respectively. All the isolates of *A. parasiticus* from chilli produced aflatoxin B₁, B₂, G₁ and G₂ at the range of 130 - 400, 60 - 180, 200 - 380 and 100 - 260 ppb levels respectively.

In the present investigation all the isolates of *A. flavus* and *A. niger*, found to be toxigenic in culture elaborated aflatoxin when inoculated on the respective host material. In the case of *A. flavus* isolates, maximum quantity of total aflatoxin was produced by isolate THR-II-3 of nutmeg and THR-I-4 of mace and *A. niger* isolate CLT-II-8 of nutmeg and CLT-II-5 of mace respectively.

Among the three aflatoxins produced by isolates of *A. flavus*, CLT-I-1 of nutmeg and THR-II-3 of mace produced maximum quantity of aflatoxin B₁ in the substrate (Fig 7). None of the *A. flavus* isolates produced aflatoxin B₂, G₁ and G₂ in the host material from which they were isolated (nutmeg and mace). In the case of *A. niger* isolate CLT-II-8 and CLT-I-3 of nutmeg and mace produced aflatoxin B₁ and B₂ respectively (Fig 8). None of the *A. niger* isolate produced aflatoxin G₁ and G₂ in the substrate.

The present investigation further revealed that the isolate of *A. flavus* which produced maximum quantity of aflatoxin in culture medium could also produce similarly in their host material, there by indicating their inherent ability for aflatoxin production. It was also noticed that the highest amount of aflatoxin B₁ and B₂ was elaborated by the same *A. flavus* isolate of nutmeg and mace. Similarly in the case of *A. niger* the same isolate of nutmeg and mace produced maximum quantity of aflatoxin in culture.

Shadanaika (2005) investigated the suitability of various kinds of spice, viz., chilli, ginger and turmeric as substrate for fungal growth and elaboration of mycotoxin and were artificially inoculated with toxigenic strain of *A. flavus*. The chilli, ginger and turmeric substrates supported production of aflatoxin B₁ at 260, 140, 40 ppb and aflatoxin B₂ at 40, 30 and 10 ppb levels respectively. While inoculated with *A. parasiticus*, chilli showed to be better substrate for aflatoxins. Chilli was showing contamination level of B₁, B₂, G₁ and G₂ at 200, 40, 120 and 30 ppb level respectively. Ginger supported B₁, B₂, G₁ and G₂ at 100, 30, 80 and 10 ppb level respectively. Turmeric supported B₁, B₂ and G₁ at 60, 10 and 20 ppb

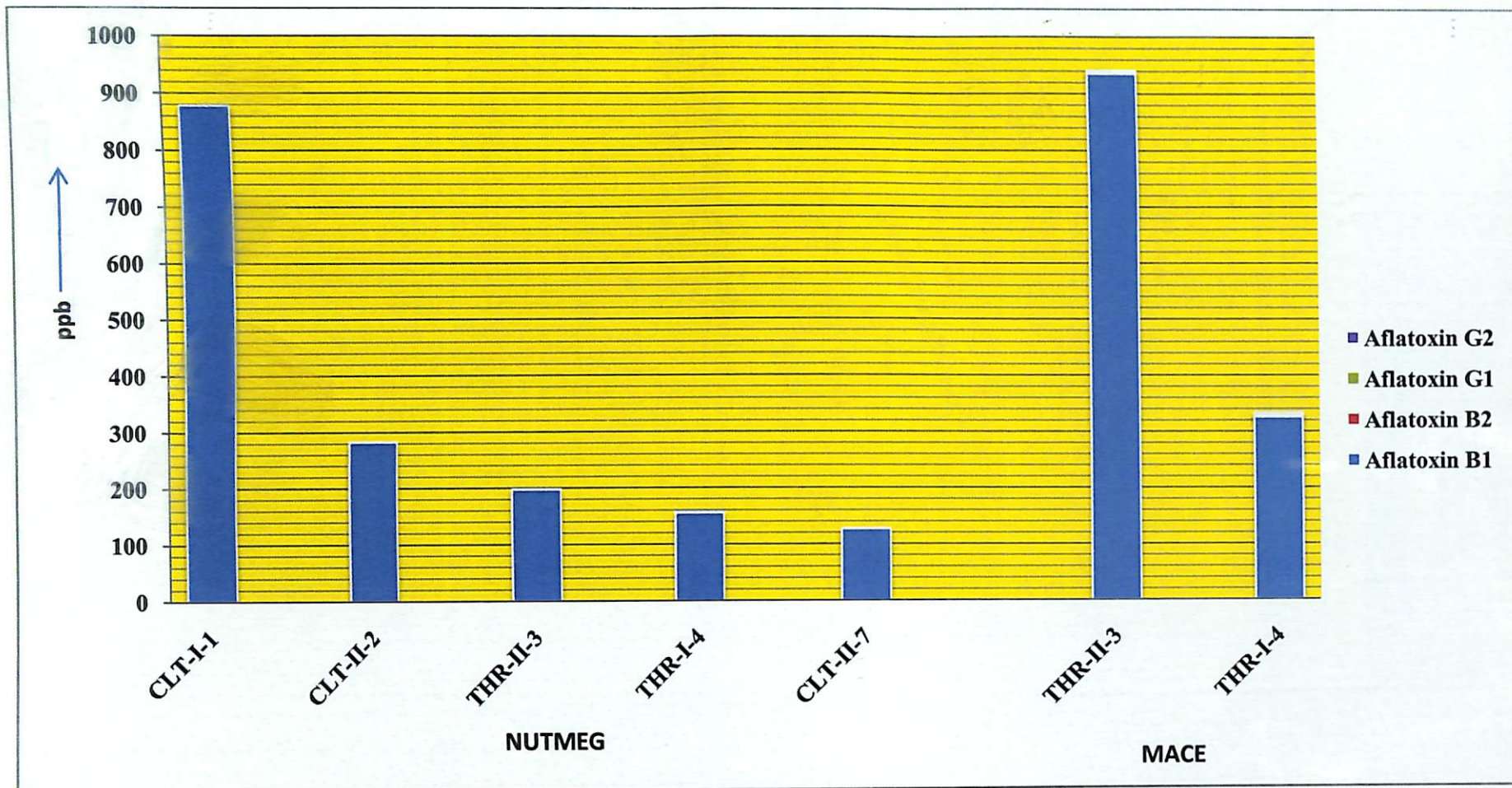


Fig. 7 Aflatoxin produced by *Aspergillus flavus* in nutmeg and mace

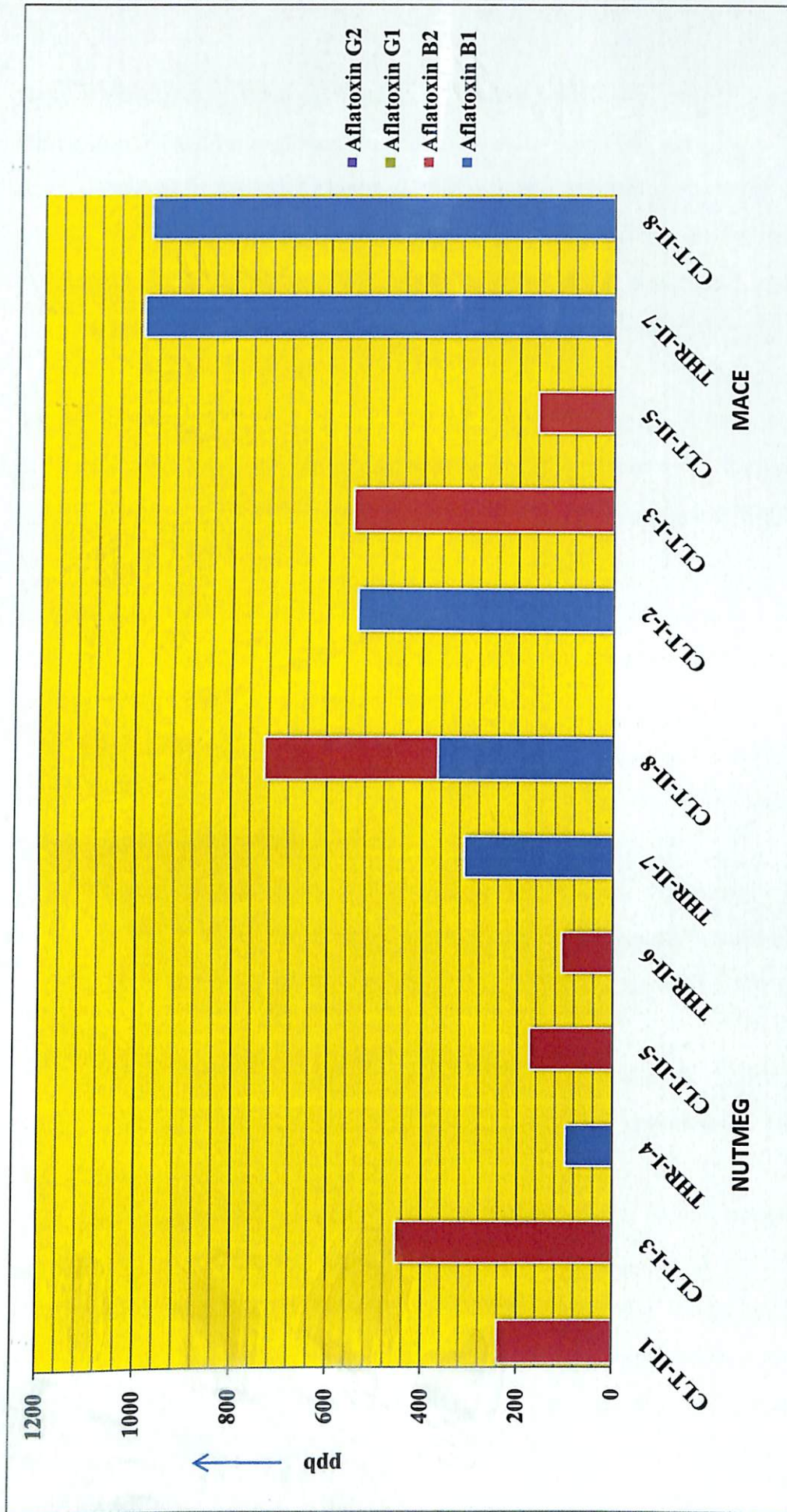


Fig. 8 Aflatoxin produced by *Aspergillus niger* in nutmeg and mace

level respectively. Amongst the three spices, chilli and ginger were the better substrates for aflatoxin elaboration than turmeric.

Aflatoxin and ochratoxin were the most common mycotoxin elaborated in spices (EFSA, 2006). In the experiment conducted to study the ability *A. ochraceus* isolate to elaborate ochratoxin in culture, it was found that all the five isolates tested were found to produce ochratoxin A. according to Shadanaika (2005) *A. ochraceus* elaborated 160, 20 and 10 ppb of ochratoxin A in chilli, ginger and turmeric respectively. Among the spices tested, chilli was found to be the better substrate for ochratoxin A than ginger and turmeric. Kuiper and Scott, (1989) reported that ochratoxin A is the most toxic member of the ochratoxins detected in spices. Medina (2005) screened cultures of 205 isolates of fungi for OTA and showed that 74.2 per cent of *Aspergillus carbonarius* and 14.3 per cent of *A. ochraceus* isolates produced OTA at levels ranging from 1.2 to 3,530 $\mu\text{g ml}^{-1}$ and from 46.4 to 111.5 $\mu\text{g ml}^{-1}$ respectively.

Spices appeared to be the poor substrate for ochratoxin A elaboration by *A. ochraceus* when compared with the aflatoxin produced by *A. flavus* and *A. niger* (Sahay and Prasad, 1990).

In the present study the *A. ochraceus* isolates which were toxigenic in culture (970.44 to 1835.95 ppb) were found to elaborate ochratoxin A when inoculated on the host material (6.01 to 212.33 ppb) (nutmeg) from which it was isolated (Fig. 9).

Earlier reports on elaboration of OTA in spices were of Singh (1983) in black pepper and cumin; Devi *et al.* (2001) in chilli; Fazekas *et al.* (2005) in red pepper, black pepper, white pepper, chilli and spice mix.

An experiment was conducted to minimise the fungal contamination in nutmeg and mace during storage using essential oils, plant extracts and leaf powders and packing materials. The search for antifungal agents especially spice oils which could safely be used as a substitute for fungicides was extensively tried. Therefore an attempt has been made to evaluate the various spice oils for their potential as antifungal agents for the safe storage of nutmeg and mace. In our study essential oils, namely, allspice oil, cinnamon oil, clove oil and lemon grass

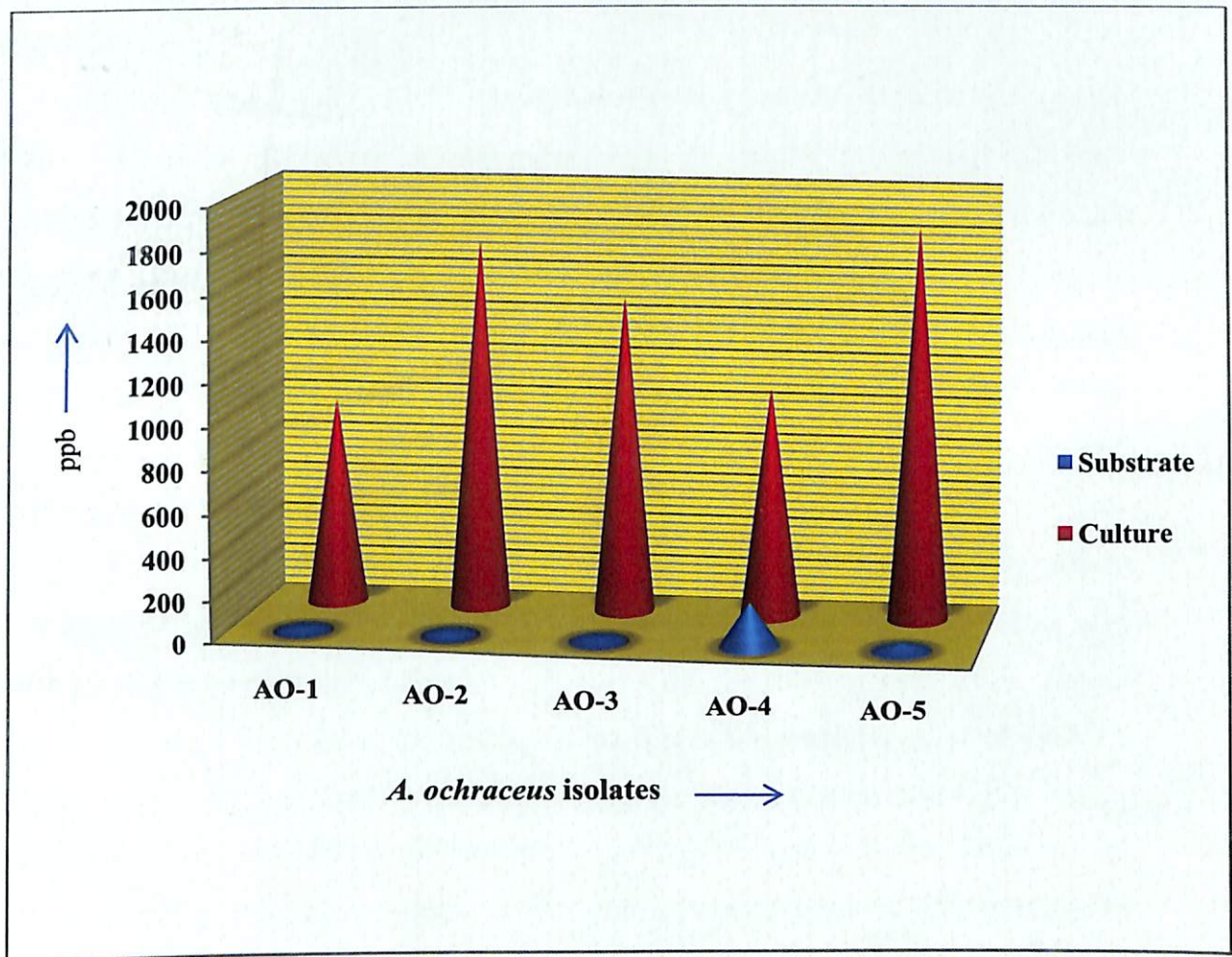


Fig. 9. Production of ochratoxin A in culture and substrate

oil could reduce the spoilage of fungi up to a period of three months (Fig. 10 and 11). *In vitro* activities of some spice essential oils have been demonstrated in culture media. However, this study reported the possibility in the control of mycotoxigenic fungi and mycotoxins using plant extracts and plant oils to fill the existing gaps and to develop effective anti-mycotoxigenic natural products for reduction of mycotoxigenic fungi and mycotoxins in nutmeg and mace.

Varma and Dubey (1999) reported that plant products, especially essential oils, are one of the most promising groups of natural compounds for the development of safer antifungal agents. For example cinnamon, clove and thyme inhibited the growth of several mycotoxigenic moulds such as *A. flavus*, *A. niger*, *A. versicolor*, *A. ochraceus* and also reduced mycotoxin production in black and white pepper (Bullerman *et al.*, 1977). Our study also confirmed the similar trend observed by Shadanaika (2005) on the efficacy of spice essential oils on the population of fungi in nutmeg and mace.

According to Mabrouk and El-Shayed (1980) clove oil completely inhibited the mycelial growth of *A. flavus* and aflatoxin formation. Nielsen and Rios (2000) reported that mustard and clove oil reduced growth of *A. flavus* with 100 and 40 per cent respectively. In another study Omidbeygi *et al.* (2007); Sindhu *et al.*, 2011 evaluated the antifungal activity of thyme and clove oils in culture medium and thyme oil had the highest antifungal activity, followed by clove. Complete inhibition of growth of *A. flavus* was observed at 350 ppm thyme oil, while 500 ppm of clove oil had inhibition of 87.5 per cent.

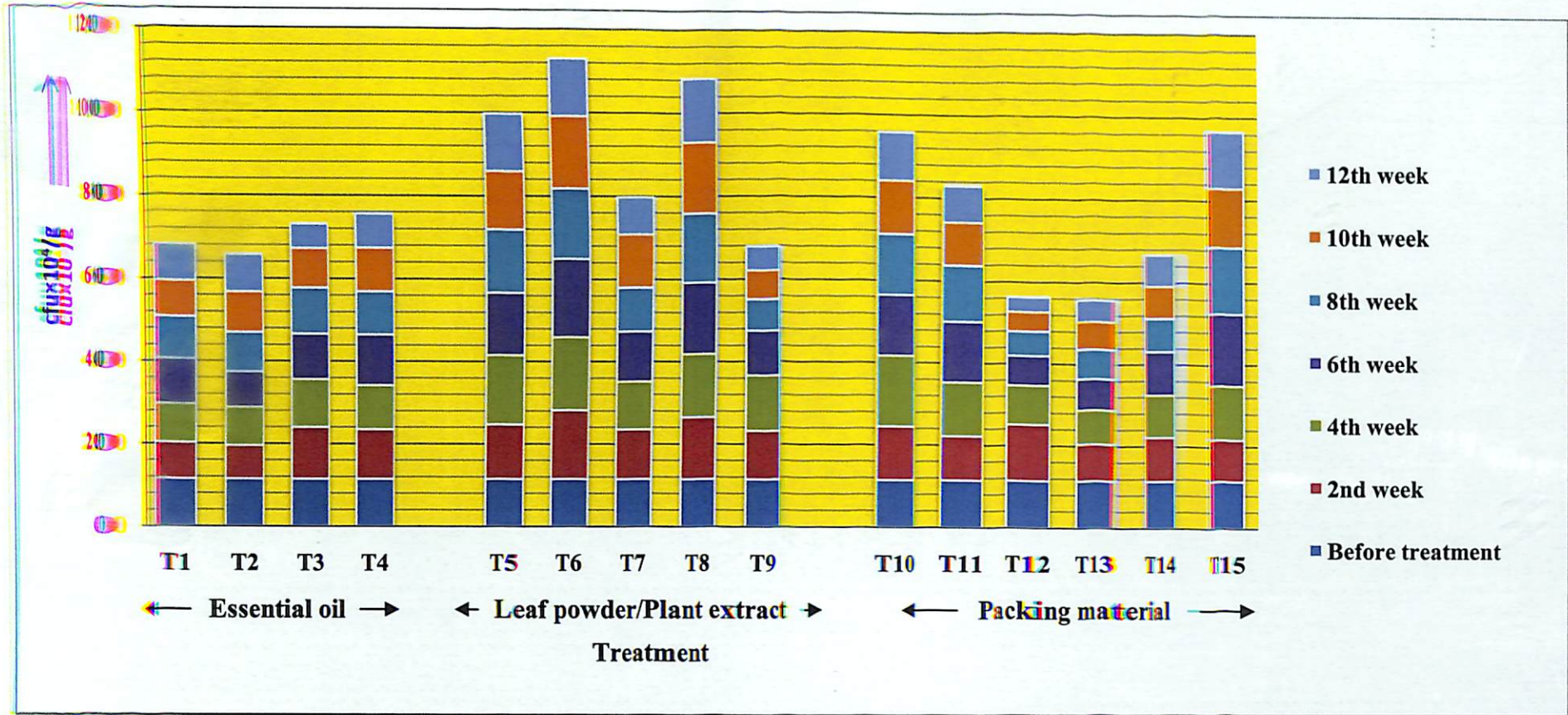
Several other reports were also available in the use of essential oils as antifungal agents: lemongrass oil (Bankole and Joda, 2004), clove oil (Hitokoko *et al.*, 1980; Azzouz and Bullerman 1982; Reddy *et al.*, 2007; Reddy *et al.*, 2009; Bullerman *et al.*, 1977), cinnamon oil (Bullerman *et al.*, 1977; Bullerman 1974), Mustard (Azzouz and Bullerman, 1982), Allspice (Hitokoko, 1980; Azzouz and Bullerman, 1982) and Thyme (Lewellyn *et al.*, 1981; Beuchat, 1987). During 2012, IISR conducted an investigation on the use of turmeric leaf and cinnamon

bark oil as antifungal agents, found that complete inhibition of fungal growth and drastic reduction in aflatoxin concentration.

Use of natural plant extracts to reduce deterioration of spices during storage provides an opportunity to avoid chemical preservatives. Over the years, efforts have been devoted to search for new antifungal materials from natural sources for food preservation (Galvano *et al.*, 2001; Onyeagba *et al.*, 2004). The present study revealed that treating nutmeg and mace samples with ocimum and neem leaf were effective, and in reducing the fungal growth throughout the period of observation of 12 weeks. The inhibitory effects of neem plant extracts on mycotoxin biosynthesis have also been examined (Bhatnagar *et al.*, 1990; Reddy *et al.*, 2009). Several edible botanical extracts have been reported to have antifungal activity (Reddy *et al.*, 2009; Pradeep *et al.*, 2003). By screening more than hundred wild and medicinal plant extracts, Bilgrami *et al.* (1979; 1980) had achieved aflatoxin inhibition in liquid culture above 70 per cent through the use of 20 plant extracts. Awuah (1996) observed that leaf powder of ocimum was found to inhibit mould development on stored soybean for nine months.

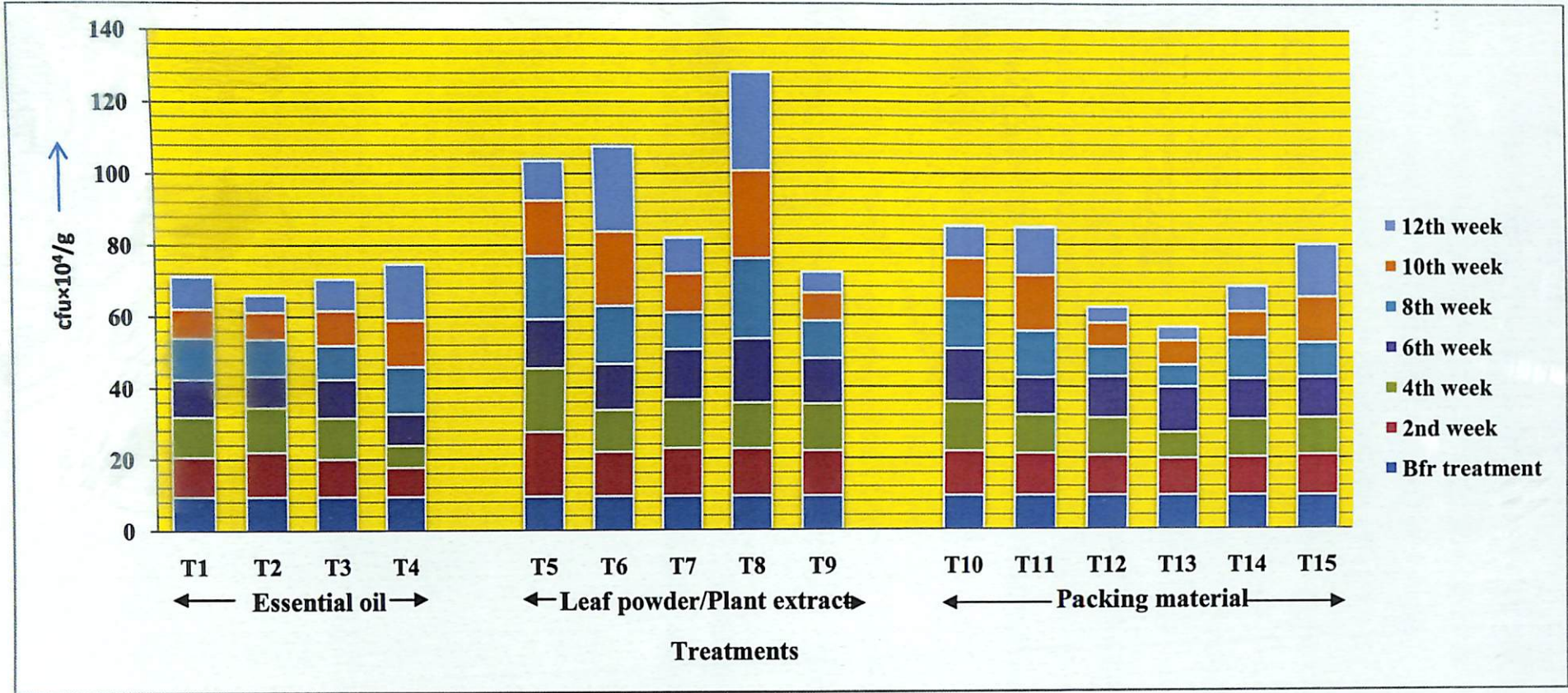
High fungal population was noticed in nutmeg and mace samples treated with onion and garlic extract (Fig. 10 and 11). Absorption of moisture from the plant products, namely, onion and garlic extract and development of humidity could be attributed to development of fungi in these treatments.

The study on the efficacy of storage containers revealed that polylined jute bag, plastic bag and polypropylene bag were equally effective in reducing fungal contamination in nutmeg and mace (Fig. 10 and 11). Marar and Padmanabhan (1960) noted that copra stored in alkathene lined gunny bags remained in good condition for six months. Philip (1978) and Niza (1981) reported that polythene lined gunny bag was superior to ordinary gunny bag for the storage of copra as there was less contamination by fungi in those stored in polythene lined gunny bags. Another study conducted by Naseema (1989) on the effect of storage containers in checking/ reducing the deterioration of oil cakes by fungi, it was noticed that polythene lined gunny bags were superior to other containers tested.



- | | | | | |
|-------------------------|-------------------|--------------------------|-------------------------|-----------------|
| T1 - Allspice oil | T2 - Cinnamon oil | T3 - Clove oil | T4 - Lemon grass oil | T5 - Curry leaf |
| T6 - Garlic extract | T7 - Neem leaf | T8 - Onion extract | T9 - Ocimum leaf | T10 - Cloth bag |
| T11 - Hessian cloth bag | T12 - Plastic bag | T13 - Polylined jute bag | T14 - Polypropylene bag | T15 - Gunny bag |

Fig. 10 Effect of treatments on the population of fungi in nutmeg



T1- Allspice oil T2-Cinnamon oil T3- Clove oil T4-Lemon grass oil T5-Curry leaf
 T6-Garlic extract T7-Neemleaf T8-Onion extract T9-Ocimum leaf T10-Cloth bag
 T11-Hessian cloth bag T12-Plastic bag T13-Polylined jute bag T14-Polypropylene bag T15-Gunny bag

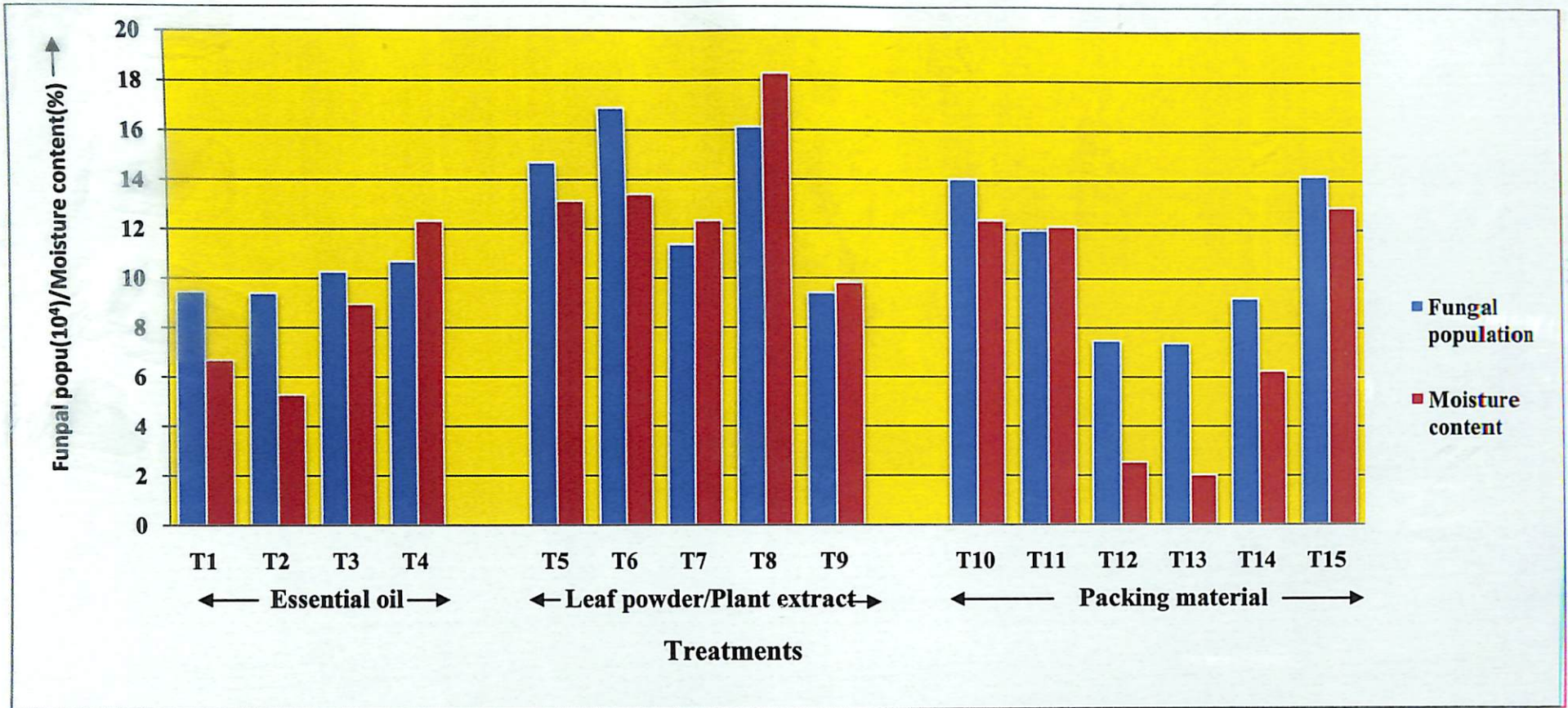
Fig. 11. Effect of treatments on the population of fungi in mace

The oil cakes stored in polythene lined gunny bag had the least population of fungi. Those stored in ordinary gunny bag which is most commonly used for the storage and transport had very high population of fungi.

With regard to the moisture content of nutmeg and mace samples subjected to different treatments, fungal population was found to increase with increase in moisture content of sample (Fig. 12 and 13). The nutmeg and mace samples stored in polylined jute bag, which had the lowest fungal population, were found to have the minimum moisture content after three months of storage. In this treatment moisture content was found to decrease from the initial moisture level. Absorption of moisture and development of humidity were minimum in samples stored in plastic bag (T-12) there by resulted in minimum fungal population. Whereas the moisture content was maximum in samples treated with onion extract (T-8), where in population of fungi was also highest.

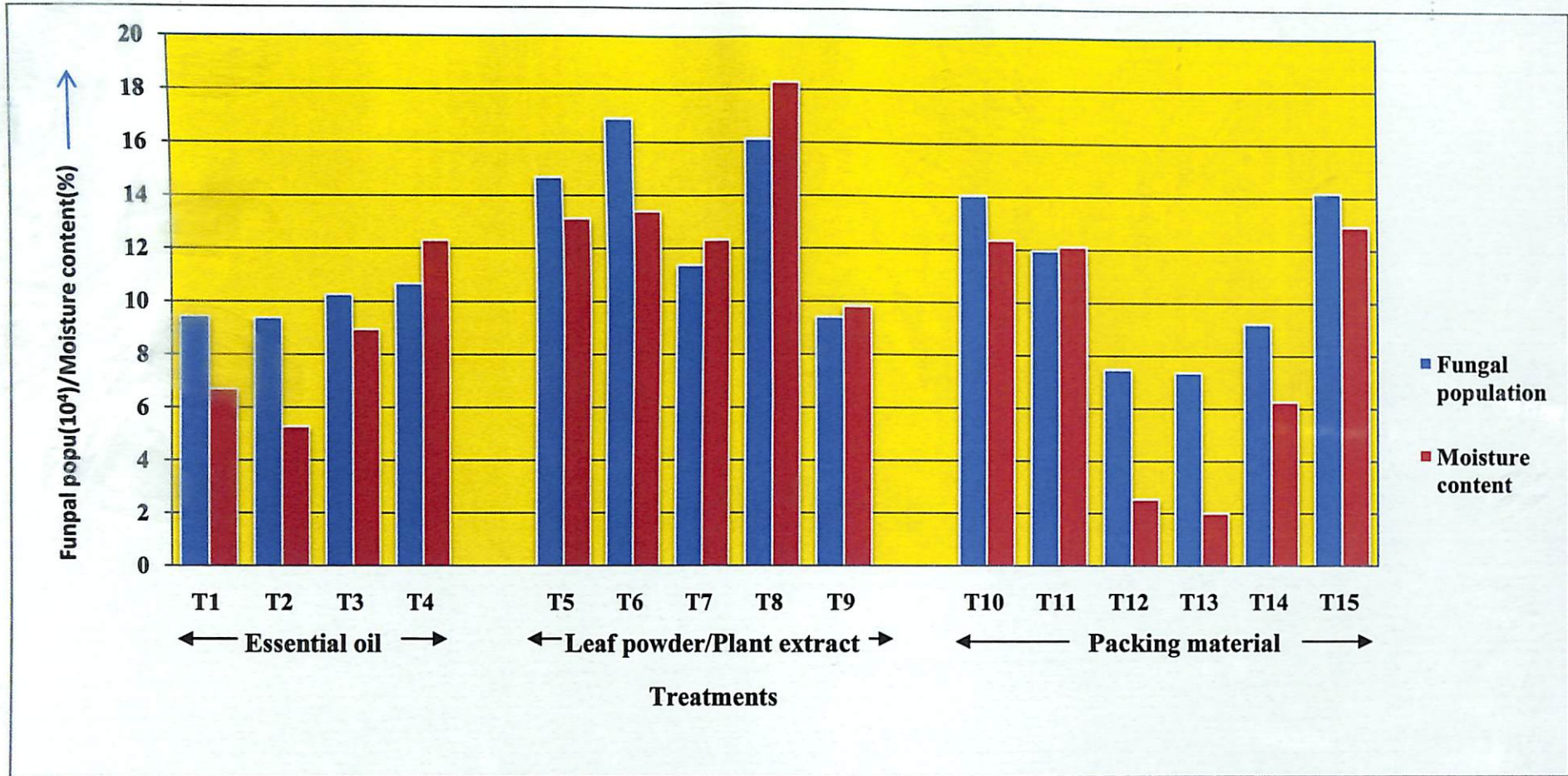
Among the effective treatments, plastic bag was found to be the cheapest, and the nutmeg and mace samples stored in this had comparatively lower moisture content and lowest population of fungi.

The foregoing considerations clearly indicate that fungal spoilage and deterioration of nutmeg and mace in storage and elaboration of mycotoxin can be reduced by storing in plastic bags.



- | | | | | |
|-----------------------|-----------------|------------------------|-----------------------|---------------|
| T1- Allspice oil | T2-Cinnamon oil | T3- Clove oil | T4-Lemon grass oil | T5-Curry leaf |
| T6-Garlic extract | T7-Neemleaf | T8-Onion extract | T9-Ocimum leaf | T10-Cloth bag |
| T11-Hessian cloth bag | T12-Plastic bag | T13-Polylined jute bag | T14-Polypropylene bag | T15-Gunny bag |

Fig. 12 Effect of moisture content on fungal population in nutmeg



- | | | | | |
|-----------------------|-----------------|------------------------|-----------------------|---------------|
| T1- Allspice oil | T2-Cinnamon oil | T3- Clove oil | T4-Lemon grass oil | T5-Curry leaf |
| T6-Garlic extract | T7-Neemleaf | T8-Onion extract | T9-Ocimum leaf | T10-Cloth bag |
| T11-Hessian cloth bag | T12-Plastic bag | T13-Polylined jute bag | T14-Polypropylene bag | T15-Gunny bag |

Fig. 13 Effect of moisture content on fungal population in mace

Summary

6. SUMMARY

An investigation was carried to study the mycoflora of nutmeg and mace in storage and associated mycotoxins. The study aimed to isolate the fungi causing deterioration of nutmeg and mace in storage, detection and elimination of mycotoxin and to develop methods to minimize fungal contamination. Survey covering two districts of Kerala was conducted and a total of 40 samples each of nutmeg and mace were procured during two seasons of the year namely February-March and June-July, 2013. Quantitative and qualitative estimation of the fungal flora associated with nutmeg and mace in storage was carried out. The variation in the population of fungi in the samples of nutmeg and mace from different regions during different seasons of the year was not statistically significant. However the population of fungi from the nutmeg and mace samples collected was higher during June – July than February – March.

During the survey altogether 13 fungi were obtained, of which 11 were isolated from samples of nutmeg. All the 11 fungi were recorded from both the regions namely Kozhikode and Thrissur districts. This included: *Acremonium restrictum*, *A. strictum*, *Aspergillus flavus*, *A. niger*, *A. ochraceus*, *A. sclerotiorum*, *Emericella nidulans*, *Eurotium amestelodami*, *Fusarium moniliforme*, *Penicillium chrysogenum* and *Syncephalastrum racemosum*. Among the twelve fungi isolated from samples of mace, ten were recorded from both the regions namely Kozhikode and Thrissur districts, viz., *A. restrictum*, *A. strictum*, *A. flavus*, *A. niger*, *A. sclerotiorum*, *E. nidulans*, *E. amestelodami*, *F. moniliforme*, *P. chrysogenum* and *S. racemosum*. Along with these *A. oryzae* and *H. pulvinata* was recorded from Thrissur and Kozhikode respectively. Among these fungi *A. flavus* and *A. niger* occurred in the highest frequency. *A. flavus* was present in 85 and 90 per cent of nutmeg samples and 90 and 80 per cent samples of mace respectively from Kozhikode and Thrissur districts. *A. niger* was obtained in nutmeg at a per cent frequency of 95 from both Kozhikode and Thrissur, and 100 and 95 per cent samples of mace respectively from Kozhikode and Thrissur districts.

Positive and significant correlation was obtained between fungal population and moisture content of both nutmeg and mace sample. Weather parameters *viz.*, minimum temperature and RH showed positive correlation, whereas maximum temperature showed negative correlation with fungal population in nutmeg samples. In the case of mace samples fungal population was positively correlated with between minimum temperature, RH, rainfall and negatively correlated with maximum temperature.

The samples when analyzed for aflatoxins, found that among the 80 samples tested only six samples, three each of nutmeg and mace showed the presence of aflatoxin. The three nutmeg samples that showed the presence of aflatoxin were from Kozhikode, with a total aflatoxin content of 808 - 925 ppb. The three mace samples that showed the presence of aflatoxin included two samples obtained from Kozhikode and one collected from Thrissur with a total aflatoxin content of 805 - 964 ppb. When tested for fractions of aflatoxin, B₁ was obtained from three isolates, namely, CLT-NII-9, CLT-MII-8 and THR-MII-9 at 241, 896 and 921 ppb respectively. Aflatoxin B₂ was recorded in isolates, *viz.*, CLT-MII-4 and CLT-NI-5 at 617 and 214 ppb respectively and G₁ in isolate CLT-NI-4 at 192 ppb.

The fungi isolated from nutmeg and mace samples were screened for their potential towards production of mycotoxins. All five *A. flavus* and seven *A. niger* isolates of nutmeg and two of four *A. flavus* and five *A. niger* isolates of mace was found to be toxigenic and produced aflatoxin *in vitro* and *in vivo*.

The quantity of total aflatoxin produced by *A. flavus* isolates in culture media ranged from 865 - 8485 ppb. *A. flavus* isolates produced aflatoxins *viz.*, B₁, B₂ and G₁ in culture medium. The same isolates of *A. flavus* obtained from nutmeg and mace were found to produce maximum quantity of aflatoxin B₁ of 595 ppb (THR-I-4); B₂, 201 ppb (THR-II-3) and G₁, 201 ppb (THR-II-3) in culture.

Total aflatoxin produced by *A. niger* isolates of nutmeg and mace in culture media ranged from 1904 - 12964 ppb and 1364 - 9845 ppb respectively. *A. niger* isolates of nutmeg *viz.*, CLT-II-8 produced maximum quantity of

aflatoxin B₁ (326 ppb) and B₂ (389 ppb) and THR-I-4 produced G1 (52 ppb) in culture media. The *A. niger* isolates of mace, namely, THR-II-7 and CLT-II-8 recorded 497 and 389 ppb of aflatoxin B₁ and B₂ respectively.

When tested for the ability to produce aflatoxin in host material, total aflatoxin produced by *A. flavus* isolates of nutmeg and mace ranged from 871-3261 ppb and 921- 1035 ppb respectively. The maximum quantity of B₁ produced by *A. flavus* in nutmeg was 879 ppb by isolate CLT-I-1 and in mace 916 ppb by isolate THR-II-3.

Total aflatoxin produced by *A. niger* isolates of nutmeg and mace ranged from 487 - 8410 ppb and 947 - 1113 ppb respectively. When tested for fractions of aflatoxin isolates viz., CLT-II-8 of both nutmeg and mace produced aflatoxin B₁ to the extent of 368 and 978 ppb and CLT-I-3 produced B₂ to the extent of 456 and 550 ppb in nutmeg and mace respectively.

Isolates of *A. ochraceus* obtained from nutmeg elaborated ochratoxin A in culture medium and substrate (nutmeg) ranged from 970.44 - 1835.95 ppb and 6.02 - 212.33 ppb respectively.

In the experiment conducted to minimize fungal contamination in nutmeg and mace, among the 15 treatments including essential oils (four numbers), leaf powder/plant extracts (five numbers) and packing materials (six numbers), plastic bag was found to be the cheapest, had comparatively lower moisture content and lowest population of fungi, whereas nutmeg treated with garlic extract (10 per cent) and mace with onion extract (10 per cent) and stored in polypropylene bag recorded higher fungal population throughout the period of observation (12 weeks).

The results of the study clearly indicated that nutmeg and mace collected during June – July had the highest population of fungi with positive and significant correlation on the sample moisture. *Aspergillus flavus* and *A. niger* occurred in the highest frequency and aflatoxin B₁ was the predominant mycotoxin. Also, storing in the plastic bag was the cheapest method to reduce fungal spoilage and likely to contain mycotoxin producing fungi such as *A. flavus*, *A. niger* and *A. ochraceus*.

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**Investigation on the Mycoflora of Nutmeg in Storage and the
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by

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ABSTRACT OF THE THESIS

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ABSTRACT

The study entitled “Investigation on the mycoflora of nutmeg in storage and the associated mycotoxin.” was carried out in the Department of Plant Pathology, College of Agriculture, Vellayani during the year 2012-2014 with the objective to isolate the fungi associated with nutmeg and mace in storage, detection and estimation of mycotoxin and methods to minimize fungal infection.

Survey was conducted in Kozhikode and Thrissur districts during February- March and June-July 2013 and collected 40 samples each of nutmeg and mace. Quantitative estimation of the fungal population revealed that samples of nutmeg and mace collected during June-July had the highest population. Positive and significant correlation was obtained between moisture content of the sample and fungal population. Qualitative estimation of the fungi indicated the presence of 13 different fungi, among which *Aspergillus flavus* and *A. niger*, were the predominant ones. The study on the detection of mycotoxin revealed that aflatoxin (805-964 ppb) was present in three samples of nutmeg and mace.

Nine isolates of *A. flavus* and 12 isolates of *A. niger* were tested for their ability to elaborate aflatoxin in culture and substrate, among which five isolates of *A. flavus* and seven of *A. niger* obtained from nutmeg was aflatoxigenic in culture media. Similarly two of the four isolates of *A. flavus* and all the five of *A. niger* from mace produced aflatoxin in culture.

Studies on the production of aflatoxin in the substrate indicated that five *A. flavus* and seven *A. niger* isolates elaborated aflatoxin in nutmeg, whereas, two *A. flavus* and five *A. niger* isolates produced aflatoxin in mace.

A. ochraceous isolates were tested for their ability to produce ochratoxin in culture and in substrate and found that all the five isolates produced ochratoxin A, 970.43 - 1835.95 ppb in culture and 6.01 - 212.33 ppb in nutmeg.

Studies on the methods to minimize fungal infection in nutmeg and mace revealed that among the treatments: essential oil, leaf powder/plant extracts and packing materials, plastic bag was found to be the cheapest, had comparatively lower moisture content and lowest fungal population; whereas nutmeg treated

with garlic extract (10 per cent) and mace with onion extract (10 per cent) and stored in polypropylene bag recorded higher fungal population throughout the period of observation (12 weeks).

The results of the study clearly indicated that nutmeg and mace collected during June – July had the highest population of fungi with positive and significant correlation on the sample moisture. *Aspergillus flavus* and *A. niger* occurred in the highest frequency and aflatoxin B1 was the predominant mycotoxin. Also, storing in the plastic bag was the cheapest method to reduce fungal spoilage and elaboration of mycotoxin.

Appendix

APPENDIX – 1

1. Potato Dextrose Agar (PDA)

| | | |
|-----------------|---|---------|
| Potato | - | 200 g |
| Dextrose | - | 20 g |
| Agar | - | 20 g |
| Distilled water | - | 1 litre |

2. SMKY Liquid Medium

| | | |
|--------------------|---|--------|
| Sucrose | - | 200g |
| Magnesium sulphate | - | 0.5g |
| Potassium nitrate | - | 3.0g |
| Yeast extract | - | 7.0g |
| Distilled water | - | 1000ml |

3. Czapek's (Dox) Agar

| | | |
|---------------------------------------|---|---------|
| Sucrose | - | 30 g |
| NaNO ₃ | - | 2 g |
| K ₂ HPO ₄ | - | 1 g |
| MgSO ₄ . 7H ₂ O | - | 0.5 g |
| KCl | - | 0.5 g |
| FeSO ₄ | - | 0.01 g |
| Agar | - | 15g |
| Distilled water | - | 1 litre |

4. Martin's Rose Bengal Agar

| | | |
|---------------------------------|---|---------|
| Dextrose | - | 10 g |
| Pepton | - | 5 g |
| KH ₂ PO ₄ | - | 1 g |
| MgSO ₄ | - | 0.5 g |
| Rose Bengal | - | 33 mg/l |
| Distilled water | - | 1 litre |
| Agar | - | 20 g |
| Streptomycin | - | 30 mg |

5. YES broth

| | | |
|-----------------|---|--------|
| Yeast extract | - | 20g |
| Sucrose | - | 150g |
| Distilled water | - | 1000ml |