"MYCOTOXIN- PRODUCING ABILITY AND CHEMOTYPE DIVERSITY OF *Aspergillus flavus* FROM SOILS OF GROUNDNUT GROWING REGIONS OF WESTERN MAHARASHTRA"

A thesis submitted to the

MAHATMA PHULE KRISHI VIDYAPEETH,

RAHURI-413 722, DIST. AHMEDNAGAR

MAHARASHTRA STATE (INDIA)

in partial fulfilment of the requirements

for the degree

of

DOCTOR OF PHILOSOPHY (AGRICULTURE)

in

PLANT PATHOLOGY

by

GUD MANOJ ARUN
Reg. No. 12/59

DEPARTMENT OF PLANT PATHOLOGY AND AGRICULTURAL MICROBIOLOGY
POST GRADUATE INSTITUTE
MAHATMA PHULE KRISHI VIDYAPEETH,
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Approved

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(Chairman & Research Guide)

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CANDIDATE'S DECLARATION

I hereby declare that this thesis or a part thereof has not been submitted by me or any other person to any other University or Institute for a Degree or Diploma

Place : M.P.K.V., Rahuri

Date : / /2015 (Gud M. A.)

Dr. C. D. Deokar
Professor,
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Rahuri, Dist. Ahmednagar.

C E R T I F I C A T E

This is to certify that the thesis entitled "**MYCOTOXIN-PRODUCING ABILITY AND CHEMOTYPE DIVERSITY OF *Aspergillus flavus* FROM SOILS OF GROUNDNUT GROWING REGIONS OF WESTERN MAHARASHTRA**" submitted to the Faculty of Agriculture, Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist. Ahmednagar, Maharashtra State, India, in partial fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY (AGRICULTURE) in PLANT PATHOLOGY**, embodies the results of piece of bonafide research work carried out by **Mr. GUD MANOJ ARUN**, under my guidance and supervision and that no part of the thesis has been submitted to any other University for degree or diploma in other form.

The assistance and help received during the course of this investigation and sources of reference have been duly acknowledged.

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Place: M.P.K.V., Rahuri (B. R.Ulmek)
Date : / /2015

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LIST OF ABBREVIATIONS

AFB	:	Aflatoxin B_1, B_2
AFB_1	:	Aflatoxin B_1
AFB_2	:	Aflatoxin B_2
AFG	:	Aflatoxin G_1, G_2
ADM	:	Aspergilus Differential Medium
AFPA	:	*Aspergillus flavus* and *parasiticus* agar
AOAC	:	Association of Official Analytical Chemists
BOD	:	Biological oxygen demand
CAM	:	coconut agar medium
CV	:	Coefficient of Variation
CD	:	Critical Difference
cm	:	Centimeter(s)
cm^2	:	square centimetre
c.f.u.	:	Colony forming unit
$CHCl_3$	:	Chloroform
CPA	:	Cyclopiazonic Acid
CRD	:	Completely Randomized Design
CZ	:	Czapeks medium
et al.	:	and others
etc	:	Et cetera (and so forth)
FAO	:	Food and Agriculture Organization
fig.	:	Figure(s)
g	:	Gram
HPLC	:	High Performance Liquid Chromatography
hr	:	Hours
i.e.	:	That is
ICC	:	Indian Childhood Cirrhosis
in vitro	:	In Laboratory
kg	:	Kilogram(s)
l	:	Litre(s)

mg	:	Milligram
min	:	Minute
ml	:	Milliliter
mm	:	Millimeter
N	:	Normality
$NaNo_3$	:	Sodium Nitrate
ng	:	nanogram
nm	:	nanometer
°C	:	degrees Celsius
OD	:	Optical Density
PDA	:	Potato Dextrose Agar
PFT	:	poison food technique
psi	:	per square inch
pH	:	Potential of hydrogen ion concentration
pp.	:	Page (s)
ppb	:	Parts per Billion
ppm	:	Parts per Million
%	:	Per cent
SE±	:	Standard Error
SMKY	:	Sucrose Magnesium sulphate Potassium nitrate Yeast extract
spp.	:	Species
Temp	:	Temperature
TLC	:	Thin Layer Chromatography
ug	:	Microgram
ul	:	Microlitre
um	:	Micrometer
UV	:	Ultra Violet
v/v	:	Volume per Volume
YES	:	Yeast Extract Sucrose
viz.,	:	Videlicet (namely)
vol	:	Volume

ABSTRACT

"MYCOTOXIN- PRODUCING ABILITY AND CHEMOTYPE DIVERSITY OF *Aspergillus flavus* FROM SOILS OF GROUNDNUT GROWING REGIONS OF WESTERN MAHARASHTRA"

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Research Guide	:	Dr. C. D. Deokar
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Abstract

The word mycotoxin is derived from the Greek language (mekes-mushroom and toxikon-toxic or poison) is non-volatile, low molecular weight compound produced as byproduct during the primary metabolic process of the fungi that grow on seeds and feed in the field, or in storage. Among mycotoxins, aflatoxin is one of the most important as these are compounds with a hepatotoxic, carcinogenic, and teratogenic potential that can affect either humans or animals health even if present in microgram quantities in the diet. Primary target organ for aflatoxin is the liver and it causes liver damage, resulting in liver cancer. Indian Childhood Cirrhosis (ICC) is a serious disorder of the liver in children. Aflatoxin B_1 is classified as a group I carcinogen and comsumption of food contaminated with it by dairy cows results in the excretion in milk and found in animal urine, milk or tissues. Now a days aflatoxins are considered a major public health problem worldwide, especially in developing countries.

Groundnut (*Arachis hypogea* L.) is an important oilseed as well as legume crop and their products are called "high aflatoxin risk materials". Ability of *Aspergillus flavus* isolates to produce aflatoxins varies greatly depending on geographic conditions and strains isolated. Harmful effects posed by aflatoxins are deformation of developing fetus, reduction in RBC, WBC and hemoglobin content in blood, delayed blood clotting and suppression of immune system in case of chronic poisoning while acute exposure to high levels of aflatoxin leads to aflatoxicosis, which can result in rapid death from liver failure.

Africa, India and South-East Asia are regarded as the "high-aflatoxin-risk areas" as aflatoxin production is more of a concern in humid tropical regions. Soil itself act as the main reservoir for its survival. *A. flavus* is a major pathogen associated with aflatoxin production and was isolated from 76 per cent soil samples out of 37 soil samples collected from different districts of groundnut fields *viz.* Nashik, Dhule, Jalgaon, Ahmednagar, Solapur, Pune, Satara, Sanagli and Kolhapur in western Maharashtra during summer season of 2013 indicates fungus is distributed through out all districts.

Data obtained during present investigation observed that soil pH influences the *A. flavus* population density. Maximum population (0.67 X 10^3) was noticed in acidic condition whereas minimum population (2.67 X 10^3) in basic soil condition indicates that soil pH negatively influences population density of it. Also, less density of it was observed in field sample in which onion, turmeric crop was grown as previous crop where as more population was observed in field sample in which maize, groundnut, cotton etc was taken as previous crop. This suggest that groundnut crop should be rotated with onion, turmeric etc crops to minimize population density of the pathogen. However soil type *viz.,* light, medium, heavy black etc does not found any correlation with population density of it.

Variation in pathogenic nature of different isolates was observed on pods and kernels during pathogenicity test. Colonization severity ranged from 21.43 to 64.29 per cent on pods while 17.86 to 53.57 per cent on kernels in cultivar SB-11 on 5^{th} day of incubation during *in-vitro* study. Also variation in macroscopic and microscopic character *viz.,* group of isolates, sclerotial colour, diameter, type, shape, conidial colour, size, wall etc was observed. CZ medium containing 3 per cent concentration of $NaNO_3$ was found to be most effective medium for sclerotia production study on which 85.72 per cent isolates produced sclerotia. Growth of all isolates on different medium showed a significant variation. Aspergillus differential medium (ADM) was non-sporulating medium, on which only white mycelial growth on upper side whereas pink pigmentation on back side of petriplate was observed. However, on Sucrose Magnesium sulphate Potassium nitrate (SMKY), Czapeks medium (CZ) and Yeast Extract Sucrose (YES) medium, poor to profuse sporulation was observed.

All the three methods *viz.,* ammonia vapour test, UV fluorescence and orange-yellow pigmentation were found simple, economical, less time consuming and effective for rapid screening of isolates for aflatoxin detection. Coconut agar medium (CAM) was found to be best medium for aflatoxin detection by UV fluorescence and orange-yellow pigmentation method. Hence, use of these methods combined act as an important tool for detecting aflatoxin contamination when limited resources are available. Aflatoxin G_1, G_2 was not observed during qualitative detection under UV florescence.

Dry mycelial mat weight of fungus ranged from 1.70 (AF-16) to 3.42 (AF-25) g per 50 ml of SMKY broth on 9th day of incubation and aflatoxin B_1 synthesized by all isolates in broth quantified by chromatography technique ranged from 600 (AF-07, AF-12) to 1380 (AF-14) ng/ml. Aflatoxin B_2 synthesis was not detected in any isolate during present investigation. However dry mycelial weight of *A. flavus* was not showed any correlation with aflatoxin production. Isolates of *A. flavus* were analyzed for their diversity on the basis of similarity coefficient analysis. Dendrogram of it resulted in two major clusters, cluster A and cluster B at similarity coefficient in between 0.18 to 0.91. All the isolates studied were classified into different chemotypes based on mycotoxin production patterns, phenotype characters and diversity among them.

Different released cultivars of groundnut were evaluated for studying resistance to *A. flavus* and observed that none of the cultivar was resistant to *A. flavus* pod and kernel colonization. However, minimum colonization severity of 43.33 per cent on Phule Unnati and maximum of 90.00 per cent on JL-24 in case of pods while minimum colonization severity of 40.00 per cent on Phule Unap and maximum of 86.67 per cent on JL-24 in case of kernels was observed on 7th day of incubation during *in-vitro* screening. Hence, in aflatoxin management programme, use of cultivars showing resistance or less colonization severity may be a good option.

In antifungal effect of different plant extract studied by poisoned food technique, water extract of pomegranate fruit peel showed minimum growth having poor sporulation and maximum growth inhibition over control. Antifungal effect of methanolic extract of pomegranate fruit peel was studied in laboratory at different concentrations, among them the treatment of 3000 ppm concentration showed 64.07 percentage growth inhibition over control having 32.33 mm colony diameter without sporulation on 8^{th} day of incubation. Also, aflatoxin synthesis was not detected by qualitative methods during this study. On pods and kernels of groundnut, methanolic extract of pomegranate fruit peel was found effective. It showed minimum pod colonization severity of 26.67 per cent with 72.88 per cent growth inhibition over control whereas minimum kernel colonization severity of 26.67 per cent with 70.37 per cent growth inhibition over control on 8^{th} day of incubation at 3000 ppm concentration. Also, no aflatoxin B_1 synthesis was detected in this treatment by chromatography technique.

Kernels treated with methanolic extract of pomegranate fruit peel and then inoculated with *A. flavus* showed increase in germination percent (2.54 to 9.57 %) and vigour index (9.63 to 25.50 %) respectively over untreated methanolic extract of pomegranate fruit peel but inoculated with isolates of *A. flavus* at laboratory condition. Hence, this have an utility during sowing of seed as seed treatment. Thus, pomegranate fruit peel which is considered as waste material, is abundant, available free of cost and simple to apply. These renewable bio-resources can successfully replace chemical antifungal agents. Thus, results support the idea that extract of pomegranate fruit peel which is considered as a waste material are economical, simple candidate for management of *A. flavus* colonization, thereby aflatoxin synthesis in groundnut kernels with enhancement in seed germination and vigour index.

1. INTRODUCTION

Mycotoxins are non-volatile, low molecular weight compounds produced as byproducts during the primary metabolic process of the fungi that grow on seeds and feed in the field, or in storage. The word mycotoxin is derived from the Greek language where mekes translates to mushroom and toxikon for toxic (Waring, 2002) or poison (Rustom, 1997). At present about 300 mycotoxins are known and they are produced by several species of fungi. Mycotoxigenic fungal contamination not only causes deterioration of foods, but also causes food born intoxicants in human and animals as they produces toxic secondary metabolites (Murthy *etal.,* 2009). The major mycotoxin producing fungi are the species of *Aspergillus*, *Fusarium*, *Penicillium* etc. Mycotoxin contamination in certain agricultural commodities *viz.*, cereals, oilseeds, treenuts, milk, eggs, meat, medicinal plants, fermented products and beverages has been a serious hindrance for health hazard and the export houses (Agrios, 2006).

Aflatoxin is one of the most important mycotoxin as these are compounds with a hepatotoxic, carcinogenic, and teratogenic potential that can affect either humans or animals health even if present in microgram quantities in the diet (Moss, 2002) and it causes the greatest amount of management costs because it is associated with stresses such as drought, temperature, humidity, nutrients, insect damage etc. Aflatoxins are considered a major public health problem worldwide, especially in developing countries where facilities for long term storage of food and food products are often inadequate and high temperature and high humidity encourage the growth of molds. Among several quality parameters, aflatoxin contamination is one of the major non-tariff trade barriers especially in the international trade. Africa, India and South-East Asia are regarded as the "high-aflatoxin-risk areas" (Mirocha and Christensen, 1974) as aflatoxin production is more of a concern in humid tropical regions when grain is not stored within the correct parameters. According to FAO (2002) developing countries account for approximately 95 percent of world peanut production, but are unable to sale large quantities of peanut in the international market because of aflatoxin contamination. In recent years, the export of Indian groundnut has been drastically reduced because of stringent tolerance limits imposed by the importing countries.

Groundnut (*Arachis hypogea* L.) is an important oilseed as well as legume which is also known as peanut, earthnut, monkey-nut and goobers. It plays an essential role in terms of income for the rural people having marginal and sub-marginal land. It is a major source of nutrients, particularly for children, due to its high protein, fat, and carbohydrate content, as well as its high concentration of the micronutrients *viz.,* calcium, potassium, phosphorus, magnesium and vitamin E. It is grown primarily for high quality edible oil (36 to 54% on dry matter basis) and easily digestible protein (12 to 36 %) in its seeds. The area under groundnut production in India was 5 million hectares and production of 5.4 million tons with 1086 kg per hectare productivity (Anonymous, 2013). Eighty percent of the total groundnut area in India is confined to five states *viz.,* Gujarat, Andhra Pradesh, Tamil Nadu, Karnataka and Maharashtra, which account for 84 percent of the total production. The oil may be extracted and used for cooking, and the residual cake is used for production of food or more commonly, in animal feeds (McDonald *et al.,* 1998). Furthermore, as legumes, groundnut provide an important source of soil nutrients through nitrogen fixation and helps in improving soil fertility.

Groundnut is frequently contaminated with aflatoxins. In India, many commercial groundnut cake and seed samples tested contained aflatoxin, levels even reaching as high as 8000 µg kg^{-1}. Aflatoxins were also detected in unrefined groundnut oil (Farid, 2006). The aflatoxin problem was first recognized in 1960 with the report of an outbreak of disease among approximately one lakh Turkey poultry in England. Since the etiology of this disease was unknown, it was called "Turkey X disease" (Blount, 1961). Sargeant *et al.,* (1961) demonstrated that an isolate of the common mold *A. flavus* Link ex Fries as the responsible agent. *A. flavus* belongs to the Genus *Aspergillus*, Subdivision Deutoromycotina (Alexopoulos *et al.,* 1996). The toxin produced by the strains of the *A. flavus* caused the disease and hence these toxins were named aflatoxins. Toxigenic strains of *A. flavus* typically produce only two aflatoxins, B_1 and B_2 however 18 different types of aflatoxins are produced in nature (Reddy and Waliyar, 2005).

Aflatoxin B_1 is the most toxic and is therefore listed as a group I carcinogen by the International Agency for Research on Cancer. American Food and Pharmacology Administration stipulated the content of AFB should not exceed 20 ppm in edible food and 30 ppm in animal feed. The permissible content of AFB is also regulated in china 20 ug/kg for corn, peanut core, peanut oil (Ruiqian *et al.,* 2004). Harmful effects posed by aflatoxins are teratogenicity i.e. deformation of developing fetus (Dipaolu *et al.,* 1967), reduction in RBC, WBC and hemoglobin content in blood (Panda *et al.,* 1975), delayed blood clotting (Clark *et al.,* 1986) and suppression of immune system in case of chronic poisoning.

Moreover, aflatoxin was shown to act synergistically with Hepatitis virus B and C infections and increases the risk of liver cancer by 30–60 times (Groopman, 1993; Henry *et al.,* 2002).

Acute exposure to high levels of aflatoxin leads to aflatoxicosis, which can result in rapid death from liver failure. Cow, calves, buffalo, fattening pig, sheep, goat, poultry and other domestic animals fed with infected grains are reported to results in severe disorders like cancer, cirrhosis, abortion and paralysis (Agrios, 2006). Also, chronic exposure to aflatoxin affects humans and livestock in many ways (Annonymous, 2010). Infection by *A. flavus* become the second leading cause of human aspergillosis (Krishnan *et al.,* 2009). In August 1981, the Ministry of Agriculture in the United Kingdom banned the feeding of groundnut products to diary cows because of the possible hazards of aflatoxins to the health of milk drinkers (Swindale, 1989). There were some reports of aflatoxicosis in farm animals from other European countries (Allcroft and Carnaghan, 1962). Several outbreaks of aflatoxicosis in poultry have been reported from India (Kishan, 1980; Char *et al.,* 1982). Shastry *et al.,* (1965) reported aflatoxicosis in Murrah breed of buffaloes in Andhra Pradesh. In 1962, a heavy mortality occurred among ducklings due to groundnut meal contaminated with a aflatoxin in Tamil Nadu State (Bhat *etal.,* 1978).

Indian childhood cirrhosis disease was observed in India due to ingestion of aflatoxin (Amla *et al.*, 1971 and Bhat *et al.,* 1989). In 2004, during the worst known outbreak of aflatoxicosis in Kenya, 317 cases were reported and 125 people died. Aflatoxins producing pathogen, *A. flavus* pathogen is saprophytic in nature and grows on a wide variety of substrates including decaying plant and animal debris (Payne, 1998). Soil itself serve as the major reservoir for this pathogen and is dominant under groundnut production system, isolated from soil of groundnut field (Vaamonde *et al.,* 1995; Barros *et al.*, 2003). The diverse populations of *A. flavus* strains producing mycotoxins are present in peanut field soils as potential threats for agriculture and public health. Knowledge of chemotype diversity of *A. flavus* species in each region would be very useful in local agricultural management practices.

Groundnut pods when come in direct contact with spores of *A. flavus* in soil are frequently invaded before harvest (Hill *et al.,* 1985 ; Horn *et al.,* 1994). The mode and extent of invasion of this fungus depends on soil population density of *A. flavus*, soil moisture and soil temperature during the pod development to maturity period. However, only a few workers have reported aflatoxin production by *A. flavus* isolates, their population load obtained from soils and there is no information on the possible association between soil type, their density and the aflatoxin-producing potential of these isolates. The entry of *A. flavus* to the developing groundnut pods is directly from the soil and distribution of *Aspergillus* in soil is influenced significantly by agro-ecological locations, soil temperature, soil p^H, soil type, cropping system etc. These fungi can invade and produce toxins in groundnut kernels before harvest, during drying and in storage. Aflatoxin production varied from strain to stain, usually begins during idiophase, upon completion of nutrients necessary for primary metabolism and depends on substrate, temperature of the media, pH, relative humidity, and storage or incubation time (Trail *et al.,* 1995 ; Belli *et al.,* 2004).

A. flavus isolates produces sclerotial bodies and on the basis of its size, isolates can be grouped into two phenotypes. One group as S strains with numerous small sclerotia (average diameter < 400 µm) and high levels of aflatoxins, while other group as L strains produce fewer, larger sclerotia and generally, less aflatoxin (Cotty, 1989). The fungi produce aflatoxins under favorable temperature, humidity and moisture content in the substrate (Sinha, 1998). Post-harvest contamination occurs when storage conditions favour germination of spores. Mycotoxin contamination of feed grains is problematic in years with a dry growing season and wet harvest season, and such conditions are common in tropical, subtropical and semi-arid tropical parts of the world. *A. flavus* is capable of growing over the temperature range from 6^{o}C to 54^{o}C with optimal growth occurs within a broad range of 25^{o}C to 42^{o}C (Fandialan and Ilag, 1972). Its better growth is ranged from 3.9 to 5.5 p^{H}. Since, not all isolates of *Aspergillus* are toxigenic, there is a need to characterize the isolates for their toxigenicity.

Aflatoxin formation in groundnut pod or karnel, before or after crop harvest cannot be prevented but it can be reduced by appropriate management practices. The spraying of fungicides due to its chemical and hazardous nature is less reliable under storage condition of groundnut pod. One of the possible means of reducing aflatoxin contamination of groundnut is the use of' cultivars resistant to seed invasion by aflatoxin producing fungi, but limited efforts have been made to identify resistant cultivars. Therefore, there is a need to identify cultivars with resistance to *A. flavus* seed colonization.

Aflatoxin was recognized as field as well as storage problem. The extracts and powders of some local plants have shown the ability to suppress the growth of toxigenic fungi. Many plant extracts and oils have been reported as safe and effective inhibitors of toxigenic fungi. Hence, in the absence of acceptable levels of host plant resistance, use of botanicals could be a promising alternative for the management of aflatoxin contamination of pod or karnel in groundnut. *A. flavus* being a soil born in nature, its invasion play a major role in decreasing the germination, vigour index of a seed in groundnut. This lead to screen a botanicals for antifungal activity against *A. flavus* in a laboratory with the ultimate aim of developing plant based formulations for its management and improving germination of kernels. Hence, by considering the importance of mycotoxin especially aflatoxin on health hazard of human and animal, study was carried out with following objectives.

1. Isolation and identification of *Aspergillus flavus* from soil samples collected from soils of groundnut growing regions of western Maharashtra,
2. Morphological and cultural study of *Aspergillus flavus* isolates,
3. Detection of toxin production by qualitative method,
4. Quantification of toxin production by quantitative method,
5. To study chemotype diversity of *Aspergillus flavus* isolates,
6. To evaluate the released groundnut cultivars for resistance against *Aspergillus flavus* colonization and
7. Efficacy of botanicals against *Aspergillus flavus* inhibition, aflatoxin synthesis and growth parameters in groundnut.

2. REVIEW OF LITERATURE

Critical reviewing and reporting of the previous studies are important for formulating of concept and framework the future line of work with defined objectives. An exhaustive review of relevant literature was done and has been presented below.

2.1. Mycotoxin

The term mycotoxin was coined in 1962 in the aftermath of an unusual veterinary crisis near London, England, during which approximately 100,000 turkey poults died (Blount, 1961 and Forgacs, 1962). Crops often become contaminated by mycotoxins, toxic fungal metabolites. Groundnut is especially vulnerable to infection by mycotoxins especially aflatoxin producing fungi in tropical and subtropical countries (Cotty *et al.,* 1994). Dietary, respiratory, dermal exposures to toxic fungal metabolites produce the diseases collectively called mycotoxicosis. The diseases caused by aflatoxin consumption are loosely called aflatoxicosis. Aflatoxin contamination in groundnut can occur in the field during pre-harvest, harvest and during storage (Nahdi, 1997).

2.2 Isolation and identification of *A. flavus* from soil samples collected from groundnut growing soils of western Maharashtra

2.2.1 Collection of soil samples, isolation and identification of *A. flavus*

Griffin and Gareen (1974) collected soil samples from peanut field at depth of 0-10 cm in the vicinity of Holland, Virginia. Soils were then assayed by dilution plate technique and plated on M3S1B medium. The population of *A. flavus* in the fruiting zone of peanut fields ranged from 0 to 48.7 propagules/g soil which was low. It might be due to low soil temperature in Virginia.

Salkin and Gordon, (1975) developed new ADM which distinguishes *A. flavus* group from other *Aspergillus* spp. All 8 strains of *A. flavus* were tested and found to produce the yellow-orange pigment on the reverse of ADM slants and no color change was observed with any of the other species of *Aspergillus* after 6 days of incubation.

Takahashi, (1993) studied distribution and characteristics of aflatoxin producing *A. flavus* in the soil in Kanagawa Prefecture, central Japan in 160 field soil samples by using ADM on which these isolates develop characteristic reverse orange color after 42 hr incubation at 30^{0}C. Thirty two fungal strains isolated from different food and feed commodities using ADM showed characteristic orange yellow reverse coloration confirming that these isolates were *A. flavus* strains (Pitt *et al.,*1983; Somashekar *et al.,* 2004).

Ehrlich *et al.,* (2007) isolated aflatoxin-producing *Aspergillus* species from soil samples collected during late December and January 2000 from ten different regions within Thailand and found that *A. flavus* species was present in all of the soil samples. Vinod Kumar *et al.,* (2008) collected soil samples from groundnut fields during summer season in Gujrat state. A total of 262 isolates of *Aspergillus* spp. mostly *A. flavus* (71 %) were isolated by dilution plating and maintained as single spore culture on agar slants.

Soil samples were collected by Singh (2010) from groundnut fields of different locations *viz.* Amreli, Anand, Bhavnagar, Bhuj, Jamnagar, Junagadh, Porbandar, Rajkot, S.K. Nagar and Surendranagar districts of Gujarat state during summer- 2005 and isolated 187 isolates of *A. flavus* from it. Sepahvand *et al.,* (2011) examined *A. flavus* isolated from air (indoors and outdoors), levels (surfaces), and soils of five hospitals in Southwest Iran and reported 63 isolates as a *A. flavus* on the basis of colony morphology, microscopic criteria and mycotoxin profiles.

Thirty soil samples were collected from five peanut fields of Astaneh Ashrafieh (Iran) during June- 2009, isolated 53 strains of *A. flavus* and all isolates produced yellow-green colonies on CZ medium and showed yellow-orange reverse coloration on AFPA. (Amani *etal.,* 2012).

2.2.2 Influence of soil pH and soil type on *A. flavus* population density

Determination of soil microbial community structures in peanut soils may be an important step in aflatoxin management (Lisker *et al.*, 1993 ; Garber and Cotty 1997). The mode and extent of invasion by *A. flavus* to groundnut pods depends on soil population density of *A. flavus*, soil moisture and soil temperature during the pod development to maturity period (Hill *et al.,* 1985; Horn *et al.,* 1994). The relative abundance of *A. flavus* is subject to change with changes in soil P^H, soil type, soil texture and cropping sequences etc (Jaime-Garcia and Cotty, 2006).

In Georgia, Bell and Crowford (1967) have indicated the population of *A.flavus* in soil collected from each of two fields was greater than 10^4 propagules/g soil. Similar results have also been reported from field soil in Israel and $10^2$to 10^3 for Nigerian field soil (MC Donald, 1969). However, Joffe (1969) observed *A. flavus* more commonly in soil of medium and heavy types while Bell and Crowford (1967) reported similar populations in a clay loam soil and a sandy loam soil.

Different soils may have significant different levels of seed infection by *A. flavus*. Its infection and aflatoxin contamination are lower in groundnut seeds of all genotypes harvested from vertisols (silty clay loam) than in those from alfisols (light sandy and red sandy loam). Vertisols also have significantly lower populations of *A. flavus* than alfisols as they have high water-holding capacity, this may be partially responsible for the lower levels of infection and aflatoxin contamination in groundnuts grown on such heavy soils (Mehan *et al.,* 1991). Ahmad and Singh (1994) found that soil types differed in their capability for harbouring *A. flavus* soils. Also, Smith *et al.*, (1995) reported these fungi are widespread in light and sandy soils.

Thompson (1990) studied the influence of pH on the toxigenicity of *A. flavus* and found that optimum pH for its growth was at pH 6 in un-amended potato dextrose broth medium. Significantly less mycelial growth was observed at pH 4 and 8, respectively. Variability in the density of *A. flavus* in soil may result from regional differences in the frequency of drought and in soil temperature, pH, and the influence of these factors on the susceptibility of groundnut seeds to fungal invasion (Horn and Dorner, 1998).

Khandar *et al.*, (2004) in his survey of some districts of Saurashtra region of Guajrat reported a wide variation in distribution of *Aspergillus* spp. in soil. Also, Barros *et al.*, (2005) studied the distribution of *A. flavus* population in the soil of Argentina's peanut-growing regions and found wide variation in distribution of different phenotypes.

Groundnut pods are formed in soil and *A. flavus* is a ubiquitous saprophytic soil fungus. Under normal condition does not invade a healthy growing pod. Therefore, the entry of the fungi in the pods is facilitated by certain factors during the pre-harvest, harvest and post-harvest stages (Vinod *et al.*, 2005).

Horn (2006) reported an average of two or fewer propagules of each *Aspergillus* spp. in soil is required at the wound site for colonization of 20% of peanut seeds and the invasion by other *Aspergillus* spp. occurs when densities of Flavi species are low and studied the aflatoxin producing fungi, from soils of three peanut-growing regions of Cordoba Province (Argentina) and, observed no significant differences in population level between planting and harvest time in two regions .

Vinod *etal.,* (2008) conducted studies at National Research Centre on Groundnut at Junagadh to determine soil population of *A. flavus* in major groundnut growing districts of Gujarat from different locations and observed low population of its in summer crop (< 4.0 x 10^3 c.f.u./g soil) than in rainy season crop (10 to 44 x 10^3 c.f.u./g soil).

2.2.3 Influence of previous crop on *A. flavus* population density

Decaying plant residues have large influences on *A. flavus* propagule counts in soils, and temporal variation in its density partially reflects the distribution and quantity of such residues as well as *A. flavus* colonization of crop components prior to incorporation into the soil (Stephenson and Russel, 1974; Boyd and Cotty, 2001). Horn *etal.*,(1995) reported high level of *A. flavus* population among common soil inhabitants of soils with peanut, corn, and cotton cultivation due to continuous availability of host crop that favours multiplication of these fungi.

Sclerotia and conidia produced by *A. flavus* growing in the soil serve as primary inoculum for young plants (Scheidegger and Payne, 2003). Jaime-Garcia and Cotty (2004) reported that corn-cotton rotations harbor more aflatoxin producing *A. flavus* due to colonization of the fungus on corn cobs that reside on the soil surface.

Mazzola (2004) reported that rotation sequences affect the relative abundance of *A. flavus* group of fungi in peanut soils. Crop rotation with peanut production is one viable option to bring changes in soil microbial communities in order to manage aflatoxin-producing fungi as the population of *A. flavus* in the soil is greatly influenced by crop residues (Jaime-Garcia and Cotty, 2004; Jaime-Garcia, 2006; Abbas *et al.*, 2008).

Vinod *et al.*, (2008) recorded low soil population ($< 4 \times 10^3$ c.f.u./g soil) in Bhuj district sample and the possible reasons for this low soil population may be attributed to soil type and intercropping with pearl millet and sorghum. In Bhavnagar district lowest population ($< 4 \times 10^3$ c.f.u./g soil) was observed as the farmers from this district commonly followed groundnut-onion groundnut rotation. The root exudates of onion may have the adverse effect on the population of *A. flavus* in soil as it was observed by earlier workers (Sharma *et al.*, 1979; Bilgrami *et al.*,1992).

Crop rotation study conducted using bahiagrass in rotation with peanuts resulted in significant reduction in population levels of *A. flavus* from 0.7 to 0.2 $X10^3$ c.f.u./g soil whereras, in peanut-cotton sequence and continous peanut sequence population increase from 1.0 to 1.2 $X10^3$ c.f.u./g. Both AFB_1 and total aflatoxin contents decreased in bahiagrass rotated fields of peanuts when compared to fields where peanuts were grown as monoculture(Sudini, 2009).

Garcia and Cotty (2010) observed that soils previously cropped to corn had significantly greater quantities of *A. flavus* (1628 c.f.u./g) than soils previously cropped to either cotton (1628 c.f.u./g) or sorghum (237 c.f.u./g). The c.f.u./g of soil of *A. flavus* was ranged between $1X10^3$ to $7X10^3$ before sowing of *rabi* crops *viz.*, onion, sorghum, maize, groundnut, wheat etc. After harvest of rabi crops, lowest c.f.u./g of *A. flavus* was observed in fallow plot. Crop rotation with onion crop reduces the population of it from $6X10^3$ to $2.67X10^3$ where on groundnut-groundnut system increases its population (Anonymous, 2013a).

In summer crop of groundnut in rotation with different crops and fallow, the population of *A. flavus* was smaller in soil after harvest compared to that of before sowing. The decrease in soil population of it during summer season could be attributed to rise in soil temperature and rotation of groundnut with non-host crops (Anonymous, 2013b).

2.2.4 Pathogenic nature of *A. flavus* strains on pods and kernels

A. flavus being a saprophyte can grow on debris, dormant tissues and or damaged weakened crops in which infection propagules may be conidia, sclerotia or mycelia. It can colonize all stages of corn growth including infection of the developing embryo (Ashworth *etal.,*1969 ; Marsh and Payne 1984). Klich *etal.,* (1986) reported that 78 per cent of the naturally contaminated bolls with *A. flavus* in the seed, also had the fungus in the peduncle and stem, whereas only 31 per cent of the naturally contaminated bolls with no *A.flavus* in the seed had the fungus in the stem and peduncle.

Vijay Kumar *et al.,* (2001) reported natural seed infection of *A. flavus* in groundnut seed sample in Tumkur district of Karnataka was in the range of 10 to 22 per cent. Upadhyaya *et al.* (2001) studied seed colonization by *A. flavus* under artificial inoculation conditions in the laboratory and found 13.6 per cent in J-11 and 46.6 per cent in JL-24 cultivar.

Mohan *et al.,* (2003) confirmed seed colonization on 13 groundnut genotypes against *A. flavus* and none of the genotypes of cultivated groundnut was found to be free from *A. flavus* colonization but certain degree of resistance to seed colonization in the genotypes was observed. Scheidegger and Payne (2003) reported that *A. flavus* become pathogenic to any stored crop like corn, cottonseed, peanuts, tree nuts and resulting food products.

Rahmianna *et al.* (2004) reported pathogenic nature of *A. flavus* by doing seed colonization study and observed infection ranged between 3.3 and 14.7 per cent. Very low seed colonization by *A. flavus* compared to resistant check J-11 in groundnut genotypes was observed in *in-vitro* conditions (Harish Babu *et al.* 2005; Yugandhar, 2005).

Bora (2008) isolated *A. flavus* fungus from infected groundnut kernels and inoculated on healthy seeds of groundnut *in-vitro* for proving the pathogenicity. It was observed that isolated fungus was pathogenic in varying degree and expressed typical symptoms of pale yellow green which later rot and rapidly reduced to a shriveled, dried brown and covered by yellow and green spores causing storage rot disease.

Pod and kernel infection levels of *A. flavus* in different genotypes were studied in lab condition at Directorate of Gorundnut Research, Junagadh (Gujrat). At pod level, infection ranged from 0 to 7 per cent in resistant check J-11 and 26 to 97 per cent in susceptible check GG-20. At kernel level, infection ranged from 25 to 28 per cent in resistant check J-11 and 25 to 47 per cent in susceptible check GG-20 (Anonymous, 2013).

2.3 Morphological and cultural study of *A. flavus* isolates

2.3.1 Microscopic study of *A. flavus*

Raper and Fennel (1965) described the morphology of *A. flavus* (Link ex. Fries). The conidia were globose at maturity, echinulated, somewhat variable in size and measured 4-5 um in diameter. Pitt and Klich, (1988) observed that conidial wall texture is the most effective criterion for distinguishing *A. flavus* and *A. parasiticus* and mycotoxin production correlated with morphological speciation where as, Mahajan (1994) studied the morphological characters of *A. flavus* (green strain) and revealed that colonies were green, conidiophores hyline, 0.3 – 1 um long and rough walled, conidia globose to subglobose and 3 um in diameter.

Colonies of *A. flavus* grow rapidly, colour of colony is initially yellow and turns in to yellow green or olive green. The old colony appears dark green. The shape of colony is smooth and some have radial wrinkles (Govrama and Bullerman, 1995). The hyphae of *A. flavus* are well developed, profusely branched, septate, and hyaline; their cells are, as a rule, multinucleate (Alexopoulos *et al.,* 1996).

Conidia are smooth to slightly roughened, 3.5 um in diameter, globose to subglobose. Certain species produces brown sclerotia, when grown on CZ medium, colonies being yellow-green

(Pitt and Hocking, 1985; Ruiqian *etal.,* 2004; Rodrigues *etal.,* 2007; Bora 2008). Rodrigues *etal.,* (2009) reported a polyphasic approach consisting of morphological characterization of 31 isolates of *Aspergillus* Section Flavi originating from Portuguese almonds. On the basis of morphological characters mainly colony color on Czapek-Dox agar and conidia morphology, they found 13 isolates (42%) had yellow-green colonies and smooth to finely rough globose conidia, and were classified as *A. flavus*.

Reddy *et al.,* (2010) observed the conidia of different isolates were light sparse grey green to pale blue green or parrot green, mycelium fluffy creamy white to dull white colour and exudates were present on surface, reverse uncoloured to yellowish or orange wrinkled mycelial growth, few sclerotia were present in wheat brown colour.

2.3.2 Grouping of different *A. flavus* isolates

Rinyu *et al.,* (1995) studied 61 strains of *Aspergilllus fumigatus* and found that strains were highly variable in colony morphologies, growth rates and levels of pigment production. Horn *et al.* (1996) also reported variation in the colony color of *A. flavus, A. parasiticus* and *A. tamarii* and summarized that there is high remarkable variation among isolates.

Raina (2005) has also reported a considerable amount of variations in colony colour of 150 *Aspergillus* isolates. On the basis of colony colour these isolates were classsifed into different classes. The isolates differed for their colony color considerably and could be grouped into three classes *viz.,* Parrot green, White fluffy with yellow sporulation and Olive green. Out of 187 isolates 155 belonged to Parrot green and grouped as Group A isolates; 20 belonged to White fluffy with yellow sporulation and grouped as

Group B and 12 belonged to Olive green and grouped as Group G (Singh, D. 2010)

2.3.3 Phenotypic study based on sclerotial morphology of *A. flavus*

Fungal mycelium found to be the predominant structure in the soil, but sclerotia can be formed, thus contributing to the long-term survival of the fungus (Scheidegger and Payne, 2003). The sclerotia are produced by many fungi to overcome the adverse climatic conditions. Many species of genus *Aspergillu*s also produce sclerotia (Hesseltine *et al.,* 1970). Several studies have shown that aflatoxin production and sclerotial formation is interrelated.

Influences of sodium nitrate and ammonium sulfate with respect to pH on aflatoxin synthesis and sclerotial production was observed. Various studies refer to a positive correlation between high aflatoxin production and presence of small sclerotia. (Geiser *et al.,* 2000 ; Chang *et al.,* 2001; Cotty, 1997; Novas and Cabral, 2002; Pildain *et al.,* 2004). El Abyad, (1988) studied the influence of salinity stress on sclerotia production of 11 rhizosphere fungi of sugar beet.

Cotty (1989) observed that L-strain isolates were more aggressive than S-strain isolates at deteriorating cotton bolls locules and reported reduction in sclerotial production by 50% accompanied with maximum aflatoxin synthesis at pH 4.0 or below in *A. flavus*. Bilgrami *et al., (*1991) detected aflatoxin in the sclerotia of toxigenic and non-toxigenic strains of *A. flavus,* found that strains of tropical origin were more toxigenic than strains from temperate regions. Out of 200 *A. flavus* strains isolated from groundnuts, 30 percent of isolates produced sclerotia, among which five percent produced sclerotia profusely in potato dextrose broth while 70 % to be non-sclerotial types (Lisker *et al.,*1993).

Abbas *et al.*, (2005) isolated *A. flavus* from different crops, soils and grown on CZ slants and incubated for 2 weeks at 30 ^{0}C in darkness. The isolates differed significantly in production of sclerotia. Notably, the S-strains produced higher levels of mycotoxins than the L-strains and non-sclerotial strains. (Barros *etal.,* 2005; Rodrigues, *etal.,* 2009). Singh (2010) studied 187 isolates of *A. flavus* isolated from groundnut field soil and found 127 isolates able to produce sclerotia, while 60 did not produce it. The sclerotial size across the isolates varied from 587 to 1349 µm.

Junxia and Minzhang (2009) analyzed characteristics of *A. flavus* strains from soil in Liaoning Province, by using CZ medium. About three-fifth of the isolates were L strains and only about two-fifth of the isolates were S strains. Sepahvand, *etal.,* (2011) isolated 60 *A. flavus* isolates from air, surface and soils of five hospitals in Southwest Iran and sclerotia production were determined by culturing the isolates on CZ, Czapek yeast extract and malt extract agar medium. Plates were incubated for 7 days at 25°C and at 42°C on CYA and observed that around 40% of the isolates produced sclerotia on CZ media. Amani *etal.,* (2012) observed macroscopically the presence of sclerotia as an identifiable criterion for *A. flavus* isolates obtained from groundnut field and reported 56.6 per cent strains of *A. flavus* were capable to produce sclerotia all from L-type on CZ medium at 30 ^{o}C.

2.3.4 Cultural study of *A. flavus* isolates

A higher colony diameter reflects better ability of the fungus to utilize the available nutrition. It reflects on the ability of the fungus to utilize the available nutrients and conversion of the same into effective biomass.

Colonies of *A. flavus* on PDA medium are olive to lime green with a cream reverse, rapid growth and texture was wooly to cottony to somewhat granular (Raper and Fennel, 1965; Patil, 1963). Nartey (1966) isolated the *A. flavus* on Czapeck Agar and revealed that colonies on the media were green-yellow to yellow green. Good sporulation was observed on Richards medium with mycelial growth of 66.40 mm and green pigmentation.

Bora (2008) evaluated cultural characters of *A. flavus* on different media and found varying degrees of mycelial growth ranging from 51.2 to 89.0 mm in colony diameter in seven days. An excellent mycelial growth (89.0 mm) was observed on PDA followed by CZ (74.20 mm) and Richards medium (66.40 mm) where as Reddy *etal.,* (2010) used four different media for cultural study of *A. flavus* and observed that, maximum growth was observed on CYA medium (73.0±4.0 mm) and minimum on CZ medium (68.0±2.0 mm).

Colonies of *A. flavus* grow rapidly and the diameter will reach 6-7 cm in 10 to 14 days. The colour of colony is initially yellow and turns into yellow green or olive green. The old colonies appear as dark green. The shape is smooth and some have radial wrinkles (Ruiqian, 2004). Singh (2010) reported that the isolates of *A. flavus* isolated from soils of groundnut field grown on PDA medium varied significantly for their colony diameter. Out of 187 isolates, 11 produced maximum colony diameter after 4 days of inoculation. Variability in growth among isolates of fungi due to various edaphic and nutritional factors were reported (Olutiola, 1976; Desai *et al.* 2003; Raina, 2005).

2.4 Aflatoxin Detection by qualitative method

There are several methods used for qualitative detection of aflatoxins. Inexpensive cultural methods *viz.,* ammonia vapor test, observance under UV light, yellow orange pigmentation etc used for detecting contamination by A. *flavus* may be a suitable alternative when limited resources preclude the use of analytical methods.

2.4.1 Ammonia Vapour Test

Ammonia vapor test is useful technique for preliminary screening of large number of samples for aflatoxin detection. Saito and Machida, (1999) studied a rapid identification method of aflatoxin producing strains of *A. flavus* by ammonia vapor. They observed that the colony reverse of aflatoxin - producing strains of *A. flavus* turned pink when their cultures were exposed to ammonia vapor. The color change was visible for colonies grown on media suitable for AF production such as potato dextrose, coconut and yeast extract sucrose agars after 2 days incubation at 25^{o}C. The color change occurred immediately after the colony was contacted with ammonia vapour.

The yellow pigment and ammonium hydroxide vapor tests are based on the production of yellow anthraquinone biosynthetic intermediates in the aflatoxin pathway. These compounds act as pH indicator dyes, which are more visible when they have turned red at alkaline pH (Abbas *et al.,* 2004a). Also, color change after ammonium hydroxide vapor exposure was observed in *Aspergillus* isolates (Abbas *et al.*, 2004b),

Shier *et al.,* (2005 a) reported the color change in copra after ammonia test in cultures of aflatoxigenic isolates of *A. flavus* grown on PDA. The pigments that turn red in response to ammonia exposure are not aflatoxins, but rather anthraquinone biosynthetic intermediates (e.g., averufin, nidurufin, versicolorin C, averantin, norsolorinic acid, versicolorin A and versicolorin A hemiacetal) and side products (e.g., methylated anthraquinone pigments) associated with the aflatoxin biosynthetic pathway (Shier *et al.,* 2005b).

Sangitkumar, *et al.,* (2007) isolated twenty-five isolates of *A.flavus* from maize grains and tested by ammonia vapour test and observed four isolates were highly toxigenic, 8 were moderately toxigenic and 10 mildly toxigenic which showed dark pink and light pink colour, respectively after treatment. The results of this test were in confirmation up to 92% with the result of toxin detection by thin layer chromatography. Out of 187 isolates of *A. flavus*, 83 were non-toxigenic, 80 were moderately toxigenic and only 24 isolates (12.8 %) were highly toxigenic (Singh, 2010).

Shier *et al.,* (2012) reported ammonia exposure resulted in the development of red pigmentation in the copra inoculated with the aflatoxigenic strain of *A. flavus*, but not in the copra inoculated with the non-aflatoxigenic strain. Also, observed the color change of *A. flavus* sporulation from green to light brown on exposure to ammonia in both the aflatoxigenic and non-aflatoxigenic strains.

2.4.2 Observance under UV light

Aflatoxin producing strains of *A. flavus* produced a blue fluorescence under ultraviolet (UV) light when grown on a modified Czapek's medium was reported by Hara *et al.,* (1973). Also, Hara *et al.,* (1974) examined plates inoculated on modified CZ medium with different strains of *A. flavus* under UV (366 nm) illumination from the 7^{th} through the 10^{th} day of incubation for the presence or absence of blue fluorescence in the agar surrounding the colonies. The B-type aflatoxins are characterized by a cyclopentane E-ring and these compounds have a blue fluorescence under long-wavelength ultraviolet light while the G-type aflatoxins have a xanthone ring in place of the cyclopentane with a green fluorescence.

On coconut agar medium, aflatoxin positive isolates showed a characteristic blue or blue-green fluorescence in agar surrounding the colonies when observed under ultra-violet light. Also, the fluorescence produced by strong aflatoxin producers began to appear within 32 hours after transfer to the medium and the intensity of fluorescence increased with prolonged incubation. An incubation time of 3 days was sufficient for detection of even very weak aflatoxin production (Lin and Dianese, 1976). This technique may be used as a presumptive screening method, but not as an analytical method since fluorescence may occur without aflatoxin being present (Duncan and Hagler, 1986).

Davis, *et al.,* (1987) screened nine isolates of *A. flavus* and *A. parasiticus* for aflatoxin production on a coconut extract agar medium by incubating at 26 to 28°C. Aflatoxin-producing colonies were detected under (365 nm) UV light by blue fluorescence on the reverse side after 2 to 5 days of growth. Also reported that when cultures were incubated longer than 7 days, they became difficult to evaluate because mycelial growth reached the margin of the plate and obscured the blue fluorescence. Also, Pallavi *et al.,* (1997) studied the aflatoxins detection of *A. flavus* strains based on fluorescence at 365 nm under UV light when grown in coconut milk agar medium.

Tests based on the fluorescence of aflatoxins, in which *A. flavus* colonies are examined under ultraviolet light for blue or green fluorescence, the sensitivity of fluorescence can be increased by incorporating β-cyclodextrin into the medium. Here, β-cyclodextrin binds aflatoxin B_1 in the core of the molecule, which is a more hydrophobic environment that results in more intense fluorescence by the aflatoxin (Fente *et al.,* 2001; Ordaz *et al.,* 2003).

Abbas *et al.,* (2004b) studied the comparison of cultural and analytical methods for detecting aflatoxin production by *Aspergillus* species by different cultural methods which were evaluated on potato dextrose agar: fluorescence (FL) on beta-cyclodextrin containing media (CD), yellow pigment (YP) formation in mycelium and medium.

All toxigenic *A. flavus* isolates produce B aflatoxins, The lack of G aflatoxin production by *A. flavus* results from deletions in the norB cypA intergenic region in the aflatoxin gene cluster. The cypA gene is necessary for the synthesis of the cytochrome P450 monooxygenase, CypA, which is required for G aflatoxin formation (Ehrlich *et al.*, 2004; Chang *etal.*, 2010)

Rodrigues, *et al.,* (2009) reported the analysis of aflatoxin production of *A. flavus* isolates by fluorescence in CAM showed a good correlation with the HPLC results and observed that all strains producing a strong signal for AFBs on the HPLC chromatogram showed a marked blue fluorescence pattern on CAM after 3 days of incubation, whereas those producing a weak signal by HPLC showed a weak violet fluorescence on CAM, only detectable after 5 days of incubation.

2.4.3 Production of pigments

Production of yellow to orange pigments by aflatoxigenic A. *flavus* was first identified by Wiseman *et al.* (1967). Also, production of abundant amounts of yellow pigment by *A. flavus* was reported by Arseculeratne *et al.* (1969), who observed that pigment production was noticeable by the second day in colonies on coconut agar medium. The yellow pigmentation was observed earlier than the blue fluorescence and was not observed in colonies that did not produce aflatoxins.

Ultra violet fluorescence producing colonies of *A. flavus* on coconut agar medium always produced a conspicuous orange-yellow pigmentation. The pigmentation was seen before the appearance of fluorescence and the degree of pigmentation seemed to be proportional to the intensity of UV fluorescence (Lin and Dianese 1976 ; Gupta and Gopal, 2002). Therefore, the aflatoxin producing ability of an isolate could be estimated presumptively by the intensity of pigmentation in coconut-agar medium without the use of a UV lamp.

Coconut-agar medium offers easy detection of aflatoxin production as the presence of fluorescence is rapid, specific due to white colour of medium and association of aflatoxin production with an orange-yellow pigmentation permits the quick identification of aflatoxin positive isolates.

2.5 Morphological diversity of different *A. flavus* isolates

Pitt and Klich, (1988) reported that conidial wall texture is the most effective criterion for distinguishing *A. flavus* and mycotoxin production correlated with morphological speciation. Population of *A.flavus* is extremely diverse and comprise large numbers of vegetative compatibility groups, even with in a restricted geographic area (Horn and Greene, 1995). Variability in the density of *A. flavus* in soil may result from regional differences in the frequency of drought and in soil temperature (Horn and Dorner, 1998).

Klich *et al.*, (2003) studied morphological variations of some *A. ochraceoroseus* strains and compared with other sections *Circumdati, Flavi, Nidulantes*, and *Versicolores*.

Singh, (2010) was done diversity analysis of all 187 *Aspergillus flavus* isolates on the basis of morphological characters (morphological group of isolates, colony diameter, number of sclerotia and size of sclerotia) and aflatoxigenicity. On the basis of diversity analysis they grouped isolates into fifteen distinct clusters.

2.6 Chemotype Classification of *A. flavus* isolates

Different geographical areas demonstrate a great variability in the mycotoxin producing potential of *A. flavus*. The diverse populations of *A. flavus* strains producing mycotoxins are present in peanut field soils as potential threats for agriculture and public health. Knowledge of population diversity of *A. flavus* species in each region would be very useful in local agricultural management practices.

Huang *et al.*, (1994) examined 153 isolates of *A. flavus* group, including 130 *A. flavus*, 15 *A. parasiticus* and 8 *A. tamarii* for production of both aflatoxin and CPA and found that 12 isolates of *A. parasiticus* produced aflatoxin but none of them produced CPA. All *A. tamarii* produced CPA where as 22.3 percent of *A. flavus* (29 of 130 isolates) that produced CPA also yielded little or no aflatoxin. Most *A. flavus* isolates (44.6 %) produced both aflatoxin and CPA. Approximately 9.2 percent of the *A. flavus* were low CPA producers but yielded higher amounts of aflatoxin. Twelve of 130 *A. flavus* isolates (9.2 %) produced neither CPA nor aflatoxin.

Vaamonde *et al.,* (2003) studied various strains of Aspergillus section Flavi isolated from peanuts, wheat and soybean for aflatoxin production and found that aflatoxigenicity of *A. flavus* strains was higher in peanuts (69 %) than in wheat (13 %) or soybeans (5 %).

Pildain *et al.,* (2004) shows characteristics of *A. flavus* strains isolated from peanuts seeds from Formosa (Argentina). Seventy eight percent of the isolates were sclerotia producers under our culture conditions and on peanuts seeds. Three of these sclerotia producers were classified as S strains. Eighty-nine percent of the L strain isolates produced AFB and CPA, and no isolates that produced only aflatoxin were observed. Two of the three S strain isolates produced AFB, AFG and CPA where as, *A. flavus* strains isolated from corn field soils, the group of non-producers of both aflatoxin and cyclopiazonic acid was the most abundant chemotype was reported by Razzaghi *etal,* (2006).

Rodrigues, *et al.,* (2009) studied the incidence of chemotypes of *A. flavus* based on mycotoxigenic profile and found that *A. flavus* isolates were assigned to 3 of the 5 chemotypes proposed by Vaamonde *et al.*, (2003): the vast majority (77%) were atoxigenic, whereas 2 isolates (15%)were CPA and AFB producers and one isolate (8%) produced the 3 groups of mycotoxins.

Sepahvand, *et al.,* (2011) isolated 60 *A. flavus* isolates and classified into four chemotypes (I to IV) based on their ability to produce AFB_1, CPA, and sclerotia. Chemotype IV (non-toxigenic isolates) was the most prominent group comprising 55% of the isolates. Twenty-two isolates (34.9%) only produced CPA (chemotype III) and four isolates (6.3%) produced both AFB_1 and CPA (chemotype I). A low percentage (3.2%) of the isolates classified as chemotype II produced only AFB_1. None of the toxigenic isolates were reported as producer of AFB_2 or AFG series.

Amani, *et al.,* (2012) studied distribution and toxigenicity of 53 strains of *A. flavus* isolated from peanut fields located in Guilan province, Northern Iran. Chemotype classification of them based on the ability for producing mycotoxins and sclerotia showed that 43.4 per cent were producers of CPA, AFB_1 and sclerotia (group I), 13.2 per cent of CPA and AFB_1 (group II), 9.4 per cent of AFB_1 and sclerotia (group III), 13.2 per cent of AFB_1 (group IV), 5.7 per cent of CPA and sclerotia (group V) and 15.1 per cent were non-toxigenic with no sclerotia (group VI). No strain was found as producer of only CPA or sclerotia.

2.7 Aflatoxin quantification by chromatography technique

The qualitative methods for aflatoxin detection were useful for screening of large number of samples but for accurate quantitation of it several methods *viz.,* Chromatography Assay, Enzyme-linked Immunosorbent Assay, Tube fluorescence etc have been developed.

Taber and Schroeder, (1967) assayed seventy-eight samples of farmer stock peanuts for aflatoxin by TLC method and observed that aflatoxin production by individual isolates ranged from 0 to 349 ppb under the test conditions employed. Maggon *etal.,* (1969) screened nine strains of *A.flavus* isolated from Delhi soils for their ability to produce aflatoxins on different media by TLC technique. Among them, seven strains produced AFB_1 and AFB_2 but no aflatoxin G. Also, Hara *etal.,* (1974) reported the quantification of various aflatoxins by TLC technique.

Lin and Dianese, (1976) verified the aflatoxin producing ability of a twenty-seven isolates of the *A. flavus* by UV fluorescence and thin layer chromatography technique and found that both tests are agreed well. Aflatoxin spots were identified under a UV light and relative concentration of aflatoxin was estimated by comparing the intensity and size of fluorescent aflatoxin spot and found that seven of 27 isolates produced aflatoxin. To confirm the presence of aflatoxins, the suspect spot is reacted with trifluoroacetic acid or glacial acetic acid, and developing the reaction products in a new solvent system and comparing with known standards. This method is used by several laboratories (Duncan and Hagler, 1986).

Davis *et al.,* (1987) analyzed isolates of *A. flavus* grown in 2% yeast extract-20% sucrose broth and on coconut agar medium plate for aflatoxin B_1 production by thin-layer chromatography and found 0 to 27 ug/ml and 0 to 2.20 ug/g aflatoxin B_1, respectively.

Mehan *et al.,* (1995) reported aflatoxin production potential of 48 *A. flavus* isolates obtained from groundnut fields of different soils types in India by TLC technique and observed that all isolates tested produced only aflatoxin B_1 ranging from 1.0 to 290 ug/g seed, confirming their earlier observations that most *A. flavus* isolates from groundnut fields in India produced only AB_1.

Dutta and Das, (2001) examined feed samples for detection of AFB_1 and reported that AFB_1 content of the feeds, as estimated by TLC and ELISA technique were very high (average 0.412 ±0.154 ppm) in comparison to the permissible Indian regulation level (0.03 ppm). Arrus *et al.,* (2005) isolated *A. flavus* from Brazil nuts and reported mycelial growth range between 1.64 to 2.90 g per 100 ml YES broth on 10^{th} day while aflatoxin synthesis between 10.02 $\pm$

2.43 to 778.09± 25.98 ng per ml. Also observed biomass in terms of mycelial growth did not appear to correlate with aflatoxin production.

Hatti *et al.,* (2009) grown different isolates of *A. flavus* isolated from groundnut pods on liquid SMKY medium and analyzed by TLC method for detection of aflatoxin and found that about 80% of isolates were found to produce aflatoxin. AFB_1 producing potential of eighty-five strains of *A. flavus,* isolated from 43 locations in rice growing states in India was investigated by Reddy *etal.,* (2009) and reported that among these, 43 strains were identified as AFB_1 producers ranging from 0.2 – 40 µg/g agar medium.

Amani *et al.,* (2012) done the chromatographic analysis of *A. flavus* isolates obtained from groundnut field by tip culture method and found that 84.9 percent isolates were toxigenic, while 15.1 per cent isolates were non-toxigenic. Amounts of AFB_1 produced by the isolates were reported in the range of 53.3 to 7446.3 ug/gm fungal dry weights, respectively.

Ouattara-Sourabie *et al.,* (2012) screened seven strains of *Aspergillus* spp. for their ability to produce aflatoxins in coconut broth media by chromatography technique and observed that, the two strains of NRRL5862 (73.67 ng/ml) and CDCB5333 (165.73 ng/ml) produced AFB_1. Also, UV observance results were in concordance with those obtained by chromatography technique.

Chakranarayan and Pati (2013) isolated aflatoxigenic *Aspergillus* species from rhizosphere of groundnut fields from the different sites of Akola and Amravati district (MS) India and observed that richard broth were able to produce the aflatoxin at maximum level of 966.66 – 266.66ug/l and SMKY broth 933.33 –

233.33 ug/l) which further indicated by high fluorescence on TLC plates.

2.8 *In-vitro* screening of groundnut cultivars against *A. flavus*

Besides adopting certain cultural, harvest, and storage practices, one of the possible means of reducing atlatoxin contamination of groundnut is the use of cultivars resistant to seed invasion by aflatoxin producing fungi (Mixon and Rogers, 1973).

The fungi *A. flavus* have to penetrate the pod wall and the seed coat to reach the cotyledons from which they derive their sustenance. Resistance to pod infection is attributed to pod-shell structure, while resistance to seed invasion and colonization is mostly physical, and has been correlated with thickness, density of palisade cell layers, absence of fissures and cavities, and presence of wax layers was reported by Upadhyay *et al.,*(2002).

Blankenship *et al.,* (1985) reported that all groundnut genotypes tested to *A. flavus* infection under laboratory conditions are found to be resistant. Mehan *et al.,* (1988) evaluated several groundnut genotypes in four rainy seasons and found that IVSCAF-resistant genotypes had significantly lower seed infection with *A. flavus* than the IVSCAF-susceptible genotypes

Various genotypes screened *in- vitro* for resistance to *A. flavus* using seed inoculation method, and reported considerable variation in resistance reaction seed colonization severity. Maximum colonization severity was recorded on ICGV 95492 (3.85) on 1-4 scale followed by the susceptible control JL24 (3.64). (Nayak *et al.,* 1992 ; Nur 2004).

Thakur *et al.,* (2000) evaluated 35 wild *Arachis* germplasm for *in-vitro* seed colonization with a highly aggressive and toxigenic strain of *A. flavus* and for aflatoxin production. Large variation existed for seed colonization severity (1.0– 4.0 grade) and aflatoxin production. Also, reported that the, most of the cultivated varieties of Karnataka exhibited colonization comparable to the most susceptible variety, TMV-2. The mutant 28-2 had a colonization severity of 3.0 for *A. flavus* compared to 3.7 for JL-24 on a 1 to 4 scale (Varma *et al.,* 2001; Gowda *et al.* 2002).

Narasimhulu (2007) screened 18 selected genotypes under *in vitro* condition to know their reaction to artificial seed inoculation by *A. Flavus* and observed seed colonization severity ranged from 1-4 scale. Eight advance breeding lines were evaluated for *in-vitro* colonization of pod and kernel of peanut by *A. flavus*. At both pod and kernel levels, two genotypes PBS-22065 and PBS-22067 did not show any infection in comparison with 3 per cent infection seen in resistant check J-11 and 20 per cent in susceptible check GG-20 (Anonymous, 2012).

2.9 Efficacy of botanicals against *A. flavus* seed colonization and afaltoxin synthesis

2.9.1 Antifungal activity of plant extract against *A. flavus* by poison food technique

Eventhough effective and efficient control of seed born fungi of seeds can be achieved by the use of synthetic chemical fungicides, the same cannot be applied to grains for reasons of pesticide toxicity (Barnard *et al.*, 1997; Dukic *et al.*, 2004). Also, WHO banned many agriculturally important pesticides due to wide range of toxicity against non-target organisms including humans, which are known to cause pollution problem. Plant extracts of many higher plants have been reported to exhibit antibacterial, antifungal and insecticidal properties (Satish *et al.*, 1999; Mohana and Raveesha, 2006). These plant metabolites appear to be one of the better alternatives to the synthetic pesticides.

Extracts of *Argemone mexicana* and *Cypress rotundus* inhibited aflatoxin production by inhibiting the growth of *A. flavus* (Masood and Ranjan, 1991; Sharma and Verma,1991). Also, found that leaf extracts of *Clerodendron* spp. exhibited inhibitory action on rotting fungi, *A. flavus*.

Masood *et al.,* (1994) reported the influence of colouring and pungent agents of red chilli (*Capsicum annum*) on growth by *A. flavus* and reported capsanthin (the coloring agent of red chilli) treatment, growth of the *A.flavus* were completely checked up to the fourth day of incubation.

Mahmoud (1999) studied the inhibition of growth and aflatoxin biosynthesis of *A. flavus* by extracts of some Egyptian plants. The effect of different concentrations (2,4,6,8 and10 mg/ml) of an a aqueous extracts of *Lupinious albus, Ammi visnaga* and *Xanthium pungens* was tested. All plant extracts inhibited mycelial growth and aflatoxin production. The application of *Lupinus albus* extract @ 10 mg/ml reduced both fungal growth and aflatoxin production by 45.3 per cent and 60.0 per cent respectively, while *Xanthium pungens* was recorded as 26.7 per cent and 32.7 per cent respectively.

Nur, (2004) tested different neem commercial formulations for their potential to reduce the growth of A. *flavus* in *vitro,* using the standard PFT. All the neem formulated products tested were significantly controlled the growth of *A. flavus*. Irkin and Korukluoglu (2007) studied the antifungal activity of Allium vegetables *viz.,* garlic (*Allium sativum* L), onion (*Allium cepa* L) and leek (*Allium porrum L.*) against *Aspergillus niger*. More inhibition of the fungus was obtained by garlic followed by onion and leek.

Bora (2008) reported that extract of *Allium sativum* L was found to be effective in poison food technique against *A. flavus* isolated from groundnut seed with the inhibition of 79.22 per cent followed by *Allium cepa* (78.71 %), *Azadirachta indica* (74.07 %). Sudhakar *etal.,*(2009) reported Methyleugenol 0.5 per cent inhibited *A. flavus* colonization completely on PDA and PDA having peanut substrate.

In vitro antifungal activity assay of methanol extract of 12 plants belonging to different families were tested against patho-genic fungal species *A. flavus* by poisoned food technique and observed that, methanol extract of *Acacia nilotica, Caesalpinia coriaria*, *Decalepis hamiltonii*, *Emblica officinalis, Lawsonia inermis* and *Mimosops elengi* showed significant antifungal activity at 3500 µg/ml concentration (Mohana *etal.,* 2011).

Raji and Raveendran (2013) were examined *Abrus precatorius* L., *Aegle marmelos* L., *Aporosa lindleyana* B., *Areca catechu* L. and *Brassica juncea* L. these plants used in traditional Indian medicine against *A. niger* by poison food technique on PDA medium and observed that these extracts exhibited varying degrees of inhibition activity against the fungi.

2.9.2 Inhibition of *A. flavus* colonization and aflatoxin synthesis in groundnut pods and kernels by botanicals

It has been reported by earlier workers that at least 25 per cent of the grains produced worldwide each year get contaminated with mycotoxin. Among mycotoxins, aflatoxins are produced by *A. flavus* fungi. Among different types of aflatoxins, AFB_1 is the most toxic form of toxin. Currently, about 100 nations have regulations for aflatoxin limits in peanut and its product, which vary from 2ug/kg to 200 mg/kg. Therefore, plant produced compounds are becoming of interest as a source of safer and more effective substances compare to available chemical pesticides or fungicides to preserve peanut pods and kernels from aflatoxin producing fungi.

Sanchez, *et al.,* (2005) studied the effect of aqueous extract of *Agave asperrima* and *Agave striata* on aflatoxin production in storage of maize kernels placed in Petridishes, incubated for 14 days at 28 ^{0}C and observed the production of aflatoxin in corn under storage conditions. Also, Vinod and Basu (2006) reported the frequent incidence of these toxins, generally found in developing countries, where adequate harvesting and post harvesting techniques for the prevention of fungal growth are rarely implemented or available.

Eugenol extracted from clove showed complete inhibition of mycelial growth of *A. flavus* and AFB_1 production in rice grains at a concentration 2.4 mg/g (Reddy *et al.,* 2007 ; Faria *et al.,* (2006) Bluma *et al.,*(2008) reported the most antifungal effect of clove, mountain thyme and poleo essential oils against *Aspergillus* section Flavi and suggested these essential oils could be used alone or in conjunction with other substances to control the presence of aflatoxigenic fungi in stored maize.

In-vitro effect of methyleugenol in inhibiting *A. flavus* colonization and aflatoxin production on peanut pod and kernels has been studied by Sudhakar *etal.,* (2009) and reported that spray of methyleugenol at 0.5 per cent on peanut pod and kernels checked the colonization of *A. flavus* and aflatoxin synthesis. Also, suggested that this chemical can be used as both prophylactic or post infection spray on peanut pods before storage.

The potential of certain plant extracts for the reduction of AFB_1 in stored rice was investigated by Reddy *etal.*, (2009). Among the plant extracts tested, *Syzigium aromaticum* (5 g/kg) showed complete inhibition of *A.flavus* growth and AFB_1 production. *Curcuma longa*, *Allium sativum* and *Ocimum sanctum* also effectively inhibited the *A. flavus* growth (65–78%) and AFB_1 production (72.2–85.7%) at 5 g/kg concentration.

Thanaboripat *et al.,* (2004) studied the activity of citronella oil on aflatoxin production and growth of *A. flavus* by growing on PDA and maize grain for 28 days. They reported that the fungal growth on PDA was inhibited by citronella oil at 0.2 per cent (v/v) where as the growth, aflatoxin production and spore production in maize grain were inhibited at 1.0 per cent or at more concentration.

Antifungal activity of lemon and pomegranate peels was studied by Naseer *etal.,* (2014) against *A. flavus* by mixing powdered peels @ 5, 10, 20% w/w in inoculated rice and inhibitory effect on fungal growth, aflatoxins synthesis was investigated and observed that addition of pomegranate peels inhibited aflatoxins production to 100% during four month-storage of rice at $25^{o}C$ and 18 per cent moisture. Also, showed that fruit-wastes are potent preventer of aflatoxin production in rice, useful for a safer and longer storage of rice.

2.10 Effect of botanicals on growth parameters of kernels inoculated with *A. flavus*

Khadem (1966) reported some macroscopic observations which shows that aflatoxins synthesized by *A. flavus* affect the plants by inhibition of seed germination, elongation of the hypocotyls or roots of developing seeds. The production of aflatoxins in food grains interferes with protein synthesis by inhibiting the incorporation of amino acids into protein, resulting in non-germination of embryo. It also binds to DNA and thus prevents RNA synthesis (Truelove *etal.,* 1970).

Charles, (1979) reported that the species of *A. flavus* are externally seed borne in nature, kernels infected with this pathogen leads to the surface contamination and it will decrease in germinability and other seed quality characters. Externally seed borne pathogen may compete with the embryo for oxygen supply and thus inhibits the germination (Harper and Lynch, 1981).

Janardhan *et al.,* (2011) observed that the germinibility of seeds had vigorously reduced when soaked on culture filtrate of *A. flavus* strain. *A. flavus* infection showed significant differences for germination, shoot length, root length, dry matter production and vigour index in groundnut seeds. Among different concentrations, 5.0% inoculated seeds recorded 60% germination and vigour index 2427 and 1452 was reported by Begum *etal.,*(2013).

3. MATERIAL AND METHODS

The present investigation was carried out at Department of Plant Pathology and Agril. Microbiology in association with Department of Biochemistry, Mahatma Phule Agricultural University, Rahuri (Maharashtra).

3.1 Material

3.1.1 Equipments and Glasswares

The laboratory equipments used were autoclave, incubator, laminar air flow cabinet, refrigerator, research microscope, electronic top pan balance, BOD incubator, P^H meter, Silica gel G60 plates (20 X 20 cm; Merk). UV Spectrophotometer, UV Scanner, Semi-rigid plastic boxes, Ultra violet chamber, chromatography tank, germination paper etc. All glasswares used were of Borosil brand *viz.,* petri plates, conical flasks, measuring cylinder, sprit jar, volumetric flask, microscopic glass slide, test tubes, micro pipettes etc were used for laboratory work.

3.1.2 Sterilization

All glasswares were sterilized in an autoclave at 15 lb psi (1.038 kg/cm^2) steam pressure for 30 minutes. All the culture media and reagents were sterilized at 15 lb psi (1.038 kg/cm^2) steam pressure in autoclave for 20 minutes. The antibiotics added to the medium were filter-sterilized and then added to luke warm sterile medium just before pouring.

3.1.3 Chemicals

Ethyl alcohol 70% (v/v) was used for surface sterilization, Tween - 80 for uniform distribution of spores in the suspension, 25 % Ammonia solution, Toluene : Isoamyl alcohol : Methanol (90:32:2) for preparation of solvent system, chloroform for extraction of aflatoxin from culture filtrate, Aflatoxin standards B_1 and B_2 were procured from Himedia Chemicals Pvt. Ltd. in crystal form for quantification of aflatoxin from samples etc.

3.1.4 Groundnut seeds

Groundnut pods and kernels of different cultivars *viz.,* Phule Unap, Phule Unnati, Phule Vyas, Phule Warna, SB-11, TAG-24, TPG-41, Phule-6021, Jl-501 and JL-24 were obtained from Agricultural Research Statin, K. Digraj, Oilseed Research Station, Jalgaon and AICRP project on Groundnut, Mahatma Phule Agriculture University, Rahuri, Dist. Ahmednagar, MS, India.

3.1.5 Plants used for preparation of extracts

Leaves of Neem (*Azadiracta indica* A. Juss), leaves of Pomegranate (*Punnica granatum*), flowers of Ghaneri (*Lantana camara*), bulb of onion (*Allium cepa* L.), cloves of garlic (*Allium sativum* L.) and fruit of Pomegranate (*Punnica granatum*). The extracts were prepared and used for screening of *A. flavus* by poison food technique.

3.2 Methods

3.2.1 Sample Collection

Thirty six soil samples were collected from different locations of nine districts *viz.,* Nashik, Dhule, Jalgaon, Ahmednagar, Solapur, Pune, Satara, Sanagli and Kolhapur of Western Maharashtra during summer 2013. The crop was grown under irrigated condition during summer season. Soil samples were collected from groundnut fields before flowering. At each sampling, soil samples from the upper 10 cm soil layer were collected from five randomly selected spots from between the plants and were pooled for each plot. All information in respect of village, tahsil, district, soil type, previous crop etc was collected.

3.2.2 Fungus Isolation

The fungus was isolated using AFPA medium by dilution plating (Horn and Dorner, 1999). One gram soil sample was powdered finely in a pestle and mortar and transferred to a test tube. To the test tube, 10 ml of sterile distilled water was added and shaken thoroughly on a vortex blender and allowed to stand. From the supernatant, 1 ml aliquot was drawn and transferred to 9 ml of sterile distilled water serially up to 10^3 ml^{-1}. From the final serial dilution, 100 µl of the soil extract was poured in petri-plates containing AFPA and incubated for five days at 28 ±2 ^oC. Population load of *A. flavus* was counted.

The *A. flavus* isolates were confirmed by growing on Aspergillus Differential Medium (ADM). For this purpose conidia from test culture were transferred to a plate containing ADM and incubated at 28^oC for 3 days. Fungi of this group do not sporulate on ADM; thus, secondary colonies do not develop from the conidia disseminated during handling. The pH of soil samples were calculated with the help of pH meter (Jackson, 1973). Then influence of soil pH, soil type and previous crop grown, on population of *A. flavus* were studied with PDA medium. Before pouring of PDA either to the petri-plates and test tubes, streptomycin was added to the media at the rate of 75 mg l^{-1}. Typical colonies of *A. flavus* developed on the petri-plates from each of the samples were transferred aseptically to PDA slants. The cultures were purified using single spore isolation technique. The cultures were identified at Disease Diagnosis Centre, Department of Plant Pathology and Agricultural Microbiology, MPKV, Rahuri (Maharashtra).

3.2.3 Pathogenic nature of *A. flavus* strains on groundnut pod and karnel (*in-vitro*)

Pathogenicity test of *A. flavus* isolated from soil samples collected from groundnut field was conducted *in vitro* on healthy pods and karnels of groundnut popular cultivar SB-11. Healthy pods were first washed under tap water, surface sterilized with 70 per cent aqueous solution of alcohol for 2 minutes and subsequently washed in two changes of distilled sterilized water to remove any traces of mercuric chloride. Slight injury to the pods were made with the help of sand paper. The seven days old culture of the isolate was used for preparation of spore suspension in sterile water. To this, about 1-2 drops of Tween-80 were added for uniform dispersal of conidia in the suspension. Serial dilution and pour plate method was used for enumeration of spores or conidia per ml of spore suspension. Accordingly, the original spore suspension was diluted using distilled sterile water to get final concentration of 1×10^6 spores/ml and was used for inoculation of pods. Pods were kept in petri plate containing moist blotting paper so as to maintain congenial condition for growth of isolate. These pods were securely placed in a moist chamber at room temperature ($27 \pm 1^{\circ}C$) having relative humidity more than 95 per cent for a period of 5 days in dark and the symptoms development of yellow mold was critically examined regularly. The pods without inoculation of isolate was kept as a control. Symptom development were recorded five days after inoculation and per cent colonization severity were calculated. Procedure for groundnut karnel was followed as above. Reisolation of inoculated fungal pathogen was carried out on PDA from artificially infected pod showing typical yellow mold symptoms of disease.

3.2.4 Morphological and cultural study of *A. flavus* isolates

3.2.4.1 Morphological study

In order to carry out morphological study, the major and remarkable macroscopic features in species identification *viz.,* colony diameter, colour of conidia, mycelial colour, colony reverse, colony texture, shape etc were observed. Lacto-phenol cotton blue staining procedure was used for microscopic study. Slides were prepared from two week old cultures, because they were covered with too many spores by placing few spores of *A. flavus* isolate in the center of a petri-plate containing PDA and incubated at 27±2^{o}C. These plates were used as initial inoculum. The identification of *A. flavus* was completed by taking into account a combination of all the observed criteria, including macroscopic and microscopic characters.

3.2.4.2 Grouping of fungal isolates

Based on colony colour all isolates on PDA medium were grouped into three different groups *viz.,* Group A, Group B and Group G. Group A isolates were identified with parrot green colony color in front and light greenish yellow in the reverse. Group B isolates were identified with white fluffy and yellow sporulation colony colour in front and light lemon yellow in the reverse. Finally group G isolates were identified with olive green colony colour in front and light greenish yellow in the reverse.

3.2.4.3 Phenotype study based on sclerotia producing ability

A 5 mm mycelial disc of the fungus was cut from the periphery of a 7 days old culture and was aseptically inoculated in the centre of the petri plate containing CZ medium with different concentration of $NaNO_3$. Each set was replicated three times. All the petri plates were incubated at room temperature (27$\pm$1°C) in darkness for 14 days. Sclerotia were obtained by scraping the surface of the plate over a Whatman filter paper during irrigation with water containing Tween 20 (100 µl/l), followed by rinsing with running tap water. Sclerotia were further cleaned in a beaker with repeated rinses and decanting, and later were air-dried. The observation on sclerotial diameter were recorded and classified as S or L strain (Cotty, 1989 ; Novas and Cabral, 2002). Also observations *viz.,* sclerotial colour and shape etc were recorded.

3.2.4.4 Cultural studies on different media

The isolated fungus was grown on four different media *viz.,* Aspergilus Differential Medium (ADM), Sucrose Magnesium sulphate Potassium nitrate Yeast extract (SMKY) medium, CZ (Czapeks) medium, Yeast Extract Sucrose (YES) medium in order to study the growth characters and ability of the pathogen to sporulate. All the media were prepared freshly by following standard methods in the laboratory with almost all possible cares. Before pouring of media to the petri-plates, streptomycin was added to the media at the rate of 75 mg l^{-1}. The sterilized petri plates of size 90 mm diameter were poured with 20 ml quantity of media in triplicate. The plates were inoculated with uniform mycelial disc of the isolate with the help of cork borer and placed at the centre of the petri plate. Seven days old cultures were used for inoculation. The inoculated petri plates were then incubated at room temperature 27±2°C. For each isolate, three replications were maintained. Observations on mean colony diameter, sporulation etc were recorded four days after inoculation.

3.2.5 Aflatoxin Detection by qualitative method

Several simple methods of aflatoxin detection have been used in this study *viz.,* ammonia vapour test, observance under UV light (365 nm), orange-yellow pigmentation.

3.2.5.1 Ammonia vapour test

This test was conducted according to the protocol of Singh (2010). Each isolate was inoculated at the center of solidified PDA medium in 9 cm glass petri-plates and incubated at 28±2^{o}C. To observe the color change of colony reverse after incubation, plates were placed upside down and a drop (0.2 ml) of 25% ammonia solution was put into the lid of the petri-plate on 3rd day and 5th day of incubation. Observation regarding colour change, mycelial diameter etc were recorded. Immediately after the ammonia solution was put into petri-plate, the colony reverse of aflatoxin producing isolate of *A. flavus* turned to pink. No change of color was observed with plates of non-aflatoxin producing isolate of *A. flavus*. The color change was restricted to the colony reverse. The surrounding agar did not show any color change. Then few drops of glacial acetic acid were added into lid of' ammonia vapour treated plates, which immediately converted the colour of cultures back from pink to normal as before the ammonia vapour treatment.

3.2.5.2 Observance under UV light

Coconut, reported to be an excellent substrate for aflatoxin production, hence coconut agar medium (CAM) was used for this study (Hara *et al.* 1974). Local brand of coconut were purchased, 100 g of shredded coconut was homogenized for 5 min in 100 ml of distilled water. The homogenate was filtered through muslin cloth and the pH of the filtrate was adjusted to pH 6.5 with 2 N NaOH, 20g of agar was added and the mixture was heated upto boiling. The pH was again checked and adjusted to 6.5 and the mixture was then autoclaved. Inoculations were done by conidial transfer to center of plates. The CAM plates were then incubated at 28 ˚C in the dark for 2-7 days. Plates were daily examined visually for colony morphology and then, reverse side of colonies under (365 nm wave length) UV light for blue or green fluorescence. Observance of blue fluorescence indicates presence of B type aflatoxin where as green fluorescence indicates presence of G type aflatoxin.

3.2.5.3 Observance for orange yellow pigmentation

A. flavus inoculated coconut agar medium plates were incubated at 28 ˚C in the dark for 2-5 days and these plates were daily examined visually for orange yellow pigmentation on reverse side of plates (Lin and Dianese, 1976).

3.2.6 Aflatoxin quantification by Chromatography technique

The aflatoxin was quantified by chromatography technique as per method described by Kabnoorkar (2005).

3.2.6.1 Preparation of extract

Isolates of *A. flavus* obtained were screened for their Aflatoxin-producing potentials in SMKY liquid media (Diener and Davis, 1966). Fifty ml of SMKY liquid media was taken in 250 ml flasks and culture of *A. flavus* was inoculated in it aseptically. Triplicates were maintained for each isolates of *A. flavus.* Following incubation for 8 days in dark at 26 to 30^{o}C temperature, on 9th day the flask contents were filtered through Whatman No. 1 filter paper. Mycelial mat dried at 70^{o}C for 24 hours in a convection microwave air oven and weight (Davis *etal.,* 1966). Then 5ml of culture filtrate was extracted twice with 10 ml of chloroform in a separating funnel. The pooled chloroform extract was passed through a bed of anhydrous sodium sulphate, which was evaporated, to dryness on water bath (60°C). The residue was dissolved in 1 ml of chloroform and kept in a small vial.

3.2.6.2 Spotting on TLC plate

Prepared extract was used for spotting on silica gel plates (Merck, Silica Gel 60, 25 mm, 20x20). The final sample extract was dissolved in 5 ml chloroform in a small vials. Fifty µl of chloroform extract was spotted on TLC plate along with the standard aflatoxin at 4 cm from bottom edge. Spotting was done with micro pipette and TLC guide was used for making all the spots in straight line, paralleled to bottom edge.

3.2.6.3 Development of Chromatogram

Spotted chromatography plates were developed in TLC tank containing running solvent of Toluene : Isoamyl alcohol : Methanol in ratio of 90:32:2 as suggest by Reddy *et al.,* (1970) 100 ml of solvent system was poured in TLC tank well before the development of chromatoplates for homogenous saturation. The developed plates were air dried and then observed under ultraviolet light (365 nm.).

3.2.6.4 Quantitative estimation of aflatoxin

The amount of aflatoxins was quantified by spectrophotometer (Nabney and Nesbitt, 1965). The spots of aflatoxins on chromatograms were scrapped and dissolved in 5 ml cold methanol and centrifuged. The ultraviolet absorption spectrum of the methanolic solution was recorded in UV spectrophotometer and amount of aflatoxin present in the sample was calculated.

3.2.7 Chemotype classification of *A. flavus* isolates

A. flavus strains were classified into different chemotype groups based on the ability for producing mycotoxins and sclerotia (Amani *etal.,* 2012). Chemotype I include the strains capable to produce AFB1 and sclerotia, Chemotype II include the strains capable to produce AFB1 only, Chemotype III include the strains capable to produce sclerotia only, Chemotype IV include the strains not capable to produce AFB_1, AFB_2 and sclerotia, Chemotype V include the strains capable to produce AFB_1, AFB_2 and sclerotia, Chemotype VI includes only AFB_1 and AFB_2, and Chemotype VII includes only AFB_2 producer only.

3.2.8 Morphological diversity of *A. flavus* isolates

Clustering of all isolates were done using Euclidean distance parameters (average linkage method) and principal component analysis (PCA) was carried out.

3.2.9 *In-vitro* screening of groundnut cultivars against *A. flavus*

Various groundnut cultivars *viz.,* Phule Unap, Phule Unnati, Phule Vyas, Phule Warna, SB-11, TAG-24, TPG-41, Phule-6021, JL-501 and JL-24 released for cultivation were screened against *A. flavus.* Surface sterilized healthy pods of each cultivar were used for study. The seven days old culture of the virulent isolate was used for preparation of spore suspension in sterile water. To this, about one to two drops of Tween-80 were added for uniform dispersal of conidia in the suspension. Spore suspension of 1×10^6 spore/ml dilution was used for inoculation of pods. Pods were kept in petri plate containing moist blotting paper so as to maintain congenial condition for growth of isolate. These pods were securely placed in a moist chamber at room temperature (27$\pm$1^{o}C) having relative humidity more than 95 per cent for a period of one week in dark and the symptoms development was critically examined regularly. The pods without inoculation of isolate was kept as a control. Symptom development were recorded five and seven days after inoculation and per cent colonization severity were calculated. Individual seeds were scored for surface colonization by *A. flavus* and for colonization severity rating scale given by Thakur *et al.*, (2000) was used.

3.2.10 Efficacy of botanicals against *A. flavus*

3.2.10.1 Antifungal activity of plant extract against *A. flavus*

Antifungal activity of plant extract against *A. flavus* was carried out by poisoned food technique (Bora, 2008). Plant parts used for preparation of extracts were mentioned in 3.1.5. Hundred grams of the plant material was weighed accurately and washed thoroughly under tap water. The plant material was then made into a paste using mortar and pestle by adding 100 ml of sterilized distilled water. The homogenate was then filtered through a double layered muslin cloth and filter paper (Whatman No. 1). The filterate thus obtained was used in this experiment. (Bambode and Shukala, 1973; Mohana *et al.,* 2007). Plant extracts were used as hundred per cent concentration.

All the six plant extracts were tested against the *A. flavus* on the sterilized potato dextrose agar (PDA) in 1:2 propertions (Tripathi and Dixit, 1977) wherein, the poisoned medium was poured in petriplates of 90 mm diameter. A 5 mm mycelial disc of the fungus was cut from the periphery of a 7 days old culture and was aseptically inoculated in the centre of the petri plate. Each set was replicated three times. The control set was runned using distilled water instead of plant extract. All the petri plates were incubated at room temperature (27$\pm$1^{o}C). The observation on colony diameter, sporulation of the test pathogen, aflatoxin detection were recorded for each treatment were recorded daily. The inhibition of mycelial growth of inoculated pathogen was worked out by using formula suggested by Vincent (1947).

$$I = \frac{C-T}{C} \times 100$$

Where,

I= Per cent inhibition of fungal pathogen

C= Growth of fungus colony diameter (mm) in control

T= Growth of fungus colony diameter (mm) in treatment

3.2.10.2 Antifungal activity of methanolic extract of pomegranate fruit peel against *A. flavus* (PFT)

The peel of fruits were manually removed, shade dried and powdered in grinder to 40 mesh. Dried powder of peels (15g) was extracted with 100 ml (80%) of methanol at room temperature for 1 hours. The extract was filtered through Whatman No. 42 filter paper to remove fine particles. The residue was re-extracted again with methanol. After extraction, the solvent was evaporated on a rotary evaporator under vacuum at 30^{0}C up to 20 ml and the concentrated extract was stored in a freezer. The antimicrobial activity of extract was tested against *A. flavus* by poison food technique as mentioned above at different concentrations. The control set was runned using methanol instead of pomegranate peal extract. Observation on colony diameter, sporulation, aflatoxin detection were recorded daily. The inhibition of mycelial growth of inoculated pathogen was worked out by using formula suggested by Vincent (1947).

3.2.10.3 Inhibition of *A. flavus* colonization in groundnut pods and kernels by methanolic extract of pomegranate fruit peel

Inhibitory effect of pomegranate peel extract was studied on pod and kernel of groundnut cultivar SB-11 as suggested by Sudhkar *etal.,* (2009). Pods and kernels were first washed under tap water, surface sterilized with 70 per cent aqueous solution of alcohol for 2 minutes and subsequently washed in two changes of distilled sterilized water to remove any traces of mercuric chloride. Five pods and five kernels each were placed separately on filter paper in sterile petri plate. Pods and kernels were inoculated with *A. flavus*, by gently putting conidial spore suspension (1×10^6 spore/ml) on pods and kernels so that inoculam get lodged on its surface. About 1-2 drops of Tween-80 was added in conidial spore suspension before inoculation for uniform dispersal of conidia. Petri plates were kept in incubation chamber at room temperature (27$\pm$1oC). Then after 24 hours, pomegranate peel extract was added gently at different concentration and incubated at room temperature (27$\pm$1^{o}C) having relative humidity more than 95 per cent in dark. To maintain high humidity, sterile distilled water (1-2 ml) was added everyday during the first five days. Number of pods and kernels contaminated by isolate was counted daily. Observations on pod, kernel per cent colonization severity, per cent growth inhibition and sporulation of the test pathogen were recorded for each treatment on 8th day of incubation. Also, aflatoxin B_1 synthesis in kernels was quantified by TLC technique.

3.2.11 Effect of methanolic extract of pomegranate fruit peel on growth parameters of groundnut kernels

The seeds of groundnut cultivar JL-24 was used for this study. The germination test was conducted with 50 seeds. Three replications were maintained. Surface sterilized seeds were dipped for 5 minutes in methanolic extract of pomegranate fruit peel having 3000 ppm_concentration, dried in shade for 15 minutes and then inoculated with conidial suspension of *A. flavus* at 1×10^6 spore/ml concentration and placed in germination papers. The test conditions of 27±1°C and 95 per cent relative humidity were maintained in a germination room. At the end of ten days, the number of normal seedlings was counted and the mean was expressed as percentage. Seedling length was measured and expressed in cm (ISTA, 2010). Vigour index (Germination percentage × Seedling length in cm) was computed by adopting the method suggested by Abdul-Baki and Anderson (1973) and expressed as whole number.

3.2.12 Statistical analysis

Statistical analysis of the data obtained from various experiments were done with the help of appropriate statistical designs. 'ANOVA TABLE' were prepared using the values of D.F.(Degree of freedom), S.S.(Summation square), M.S.S. (Mean of summation square), Variance ratio and Probability value etc. Tabular value of 'F' was recorded. Level of significance were determined at 1% and 5% per cent.

4. EXPERIMENTAL RESULTS

The present investigation were carried out on mycotoxin producing ability and chemotype diversity of *A. flavus* in soils of groundnut fields of western Maharashtra in respect of collection of soil samples, isolation and identification of *A. flavus*, its pathogenic nature on pods and kernels, morphological study, qualitative and quantitative detection of various aflatoxins, chemotype diversity, screening of released cultivar against pathogen, aflatoxin management with botanicals and its effect on growth parameters. The results of these aspects are presented here as under.

4.1 Isolation of *A. flavus* and its identification

4.1.1 Collection of soil samples, isolation and identification of *A. flavus*

Data in respect of soil samples collected from groundnut fields of western Maharashtra are presented in Table 1. A total of 37 soil samples from different location in nine districts *viz.,* Nashik, Dhule, Jalgaon, Ahmednagar, Solapur, Pune, Satara, Sanagli and Kolhapur of western Maharashtra were collected (Figure I and II). The soil samples were collected in the month of February- 2013 to April-2013. Crop stage was between seedling to pre-flowering at the time of soil sample collection. Out of total soil samples collected, *A. flavus* strains were isolated from 76 per cent soil samples by serial dilution method on AFPA medium. *A. flavus* isolates were confirmed by macroscopic, microscopic characters and by growing on Aspergillus Differential Medium which develop non-sporulating white mycelial growth and characteristic reverse orange yellow color after 42 hr incubation at 30^{o}C.

Table1- Details of soil samples collected from groundnut field of western Maharashtra

Sr. No.	Village	Tahasil	District	*A. flavus*
1	Vaygaon	Satana	Nashik	+
2	Andarsul	Yeola	Nashik	-
3	Kundewadi	Niphad	Nashik	+
4	Malegaon	Malegaon	Nashik	+
5	Phagne	Dhule	Dhule	-
6	Nandvan	Sakri	Dhule	+
7	Koyna	Patan	Satara	+
8	Padegaon	Phaltan	Satara	-
9	Surul	Wai	Satara	+
10	Medha	Medha	Satara	+
11	Nira	Purandar	Pune	+
12	Nimbgaon	Indapur	Pune	-
13	Lonavala	Lonavala	Pune	+
14	Ranjangaon	Ranjangaon	Pune	+
15	Kolhapur	Kolhapur	Kolhapur	-
16	Tapodi	Karveer	Kolhapur	+
17	Gadhinglaj	Gadhinglaj	Kolhapur	+
18	Jeur	Karmala	Solapur	+
19	Pandharpur	Pandharpur	Solapur	+
20	Angar	Mohal	Solapur	+
21	Baliramnagar	Barshi	Solapur	+
22	Kandar	Karmala	Solapur	+
23	Rahuri	Rahuri	Ahmednagar	+
24	Moha	Jamkhed	Ahmednagar	+
25	Rashin	Karjat	Ahmednagar	-
26	Vilad	Nagar	Ahmednagar	+
27	Shirdi	Kopergaon	Ahmednagar	+

\+ Presence of *A. flavus*

\- Absence of *A. flavus*

4.1.2 Influence of soil pH and soil type on *A. flavus* population density

Soil samples collected were analyzed for its pH with the help of pH meter and data was presented in Table 2. Data showed that *A. flavus* population observed in soil samples having pH between 5.0 to 7.8 indicating acidic to basic nature of soil. Maximum population load 2.67 x 10^3 of *A. flavus* was observed in soil sample having pH 5.0 and 5.1 however minimum of 0.33 x 10^3 in pH 7.5 and 7.8. Correlation study showed that, *A. flavus* population decreases when the soil pH increases indicating that maximum population was observed in acidic whereas minimum population in basic soil condition suggest that soil pH negatively influences its population density. This means the light acidic condition of soil is necessary in groundnut crop to reduce the population load.

Table 2 - *A. flavus* population influenced by soil pH

A. flavus Population at 10^3	Soil pH	*A. flavus* Population at 10^3	Soil pH
0.67	7.1	2.00	5.7
2.00	5.4	0.33	7.5
1.00	6.9	1.67	6.9
0.67	7.2	2.67	5.1
0.33	7.8	2.33	5.6
0.67	7.2	1.67	6.6
0.67	7.4	1.33	6.9
2.33	5.6	1.33	6.8
1.67	6.5	1.67	6.4
2.67	5.0	1.67	6.5
2.00	5.2	0.67	7.0
0.33	7.8	1.33	6.9
1.67	6.3	0.67	7.0
1.00	6.9	r value at 0.1 %	0.3809
1.00	6.8	r value at 0.5 %	0.4869

Also data in respect of soil type presented in Table 3 showed no significant correlation with *A. flavus* population. There was a variation in *A. flavus* population in different soil types *viz.,* light medium, medium red, medium black, black etc. Population in black soil ranged between 0.67 to 2.67 at 10^3 dilution. This indicates that *A. flavus* population present in soil depends upon biochemical properties of soil and not on soil type.

Table 3 - *A. flavus* population influenced by soil type

***A. flavus* Population at 10^3**	**Soil type**	***A. flavus* Population at 10^3**	**Soil type**
0.67	Medium Black	1.00	Black
2.00	Black	2.00	Medium Black
1.00	Black	0.33	Black
0.67	Black	1.67	Black
0.33	Black	2.67	Light medium
0.67	Black	2.33	Medium black
0.67	Medium Black	1.67	Black
2.33	Medium Black	1.33	Black
1.67	Medium Black	1.33	Black
2.67	Medium Red	1.67	Medium black
2.00	Medium Red	1.67	Medium black
0.33	Black	0.67	Black
1.67	Medium Black	1.33	Black
1.00	Black	0.67	Medium black

4.1.3 Influence of previous crop of different locations on *A. flavus* population density

Information regarding previous crop grown in the field were obtained during collecting soil samples and presented in Table 4. Groundnut growers were grown crop *viz.,* sunflower, wheat, vegetable, onion, turmeric, soybean, sugarcane, rice, sorghum, groundnut, chilli, mung, cotton, maize etc during previous *kharif* or *rabi* season. It was observed that *A. flavus* population ranged from 0.33 x 10^3 to 2.67 x 10^3 was significantly influenced by previous crop grown in the field. Completely less population of *A. flavus* was observed in field sample in which onion (0.33, 0.67), turmeric (0.67, 1.33), chilli (0.33) etc were grown as previous crop where as more population was observed in field sample in which maize (1.67, 2.33), groundnut (2.00, 2.67), cotton (1.67) etc were taken as previous crop. This may be due to antifungal properties of onion, turmeric crop grown during previous season. This suggest that groundnut crop should be rotated with crops *viz.,* onion, turmeric, chilli etc to minimize *A. flavus* population density.

Table 4 - *A. flavus* population influenced by previous crop in groundnut field

***A. flavus* Population at 10^3**	**Previous crop**	***A. flavus* Population at 10^3**	**Previous crop**
0.67	Sunflower	1.00	Sugarcane
2.00	Wheat	2.00	Mung
1.00	Vegetable	0.33	Onion
0.67	Sunflower	1.67	Cotton
0.33	Onion	2.67	Groundnut
0.67	Turmeric	2.33	Maize
0.67	Soyabean	1.67	Wheat
2.33	Sugarcane	1.33	Turmeric
1.67	Rice	1.33	Not known
2.67	Sorghum	1.67	Maize
2.00	Groundnut	1.67	Cotton
0.33	Chilli	0.67	Onion
1.67	Sorghum	1.33	Fallow
1.00	Soyabean	0.67	Wheat

4.1.4 Pathogenic nature of *A. flavus* isolates on groundnutpods and kernels

Data in respect of pathogenic nature of twenty eight *A. flavus* isolates on groundnut pods of cultivar SB-11 (Plate I) were presented in Table 5. Colonization severity on pods ranges from 21.43 per cent to 64.29 per cent. Maximum colonization severity was noticed by isolate AF-16 (64.29 %) having grade 4 and minimum by isolate AF-26 (21.43 %) having grade 2.

Table 5 - Pathogenic nature of different *A. flavus* isolates on pods of groundnut.

Isolate No.	Colonization severity (%)	Grade	Isolate No.	Colonization severity (%)	Grade
AF-01	53.57	4	AF-15	53.57	4
AF-02	50.00	3	AF-16	64.29	4
AF-03	42.86	3	AF-17	46.43	3
AF-04	28.57	3	AF-18	42.86	3
AF-05	46.43	3	AF-19	25.00	2
AF-06	46.43	3	AF-20	46.43	3
AF-07	42.86	3	AF-21	42.86	3
AF-08	46.43	3	AF-22	28.57	3
AF-09	50.00	3	AF-23	39.29	3
AF-10	46.43	3	AF-24	35.71	3
AF-11	42.86	3	AF-25	60.71	4
AF-12	50.00	3	AF-26	21.43	2
AF-13	46.43	3	AF-27	25.00	2
AF-14	50.00	3	AF-28	57.14	4

Data in respect of pathogenic nature of twenty eight *A. flavus* isolates on groundnut kernels of cultivar SB-11 (Plate II) were presented in Table 6. Colonization severity trend was observed on kernels ranging from 17.86 per cent to 53.57 per cent. Maximum colonization severity was noticed by isolate AF-16, AF-25 (53.57 %)

having grade 4 and minimum by isolate AF-19, AF-26, AF-27 (17.86 %) having grade 2.

Table 6 - Pathogenic nature of different *A. flavus* isolates on kernels of groundnut

Isolate No.	Colonization severity (%)	Grade	Isolate No.	Colonization severity (%)	Grade
AF-01	46.43	3	AF-15	46.43	3
AF-02	42.86	3	AF-16	53.57	4
AF-03	35.71	3	AF-17	39.29	3
AF-04	21.43	2	AF-18	35.71	3
AF-05	39.29	3	AF-19	17.86	2
AF-06	39.29	3	AF-20	39.29	3
AF-07	35.71	3	AF-21	35.71	3
AF-08	39.29	3	AF-22	21.43	2
AF-09	42.86	3	AF-23	32.14	3
AF-10	39.29	3	AF-24	28.57	2
AF-11	35.71	3	AF-25	53.57	4
AF-12	42.86	3	AF-26	17.86	2
AF-13	39.29	3	AF-27	17.86	2
AF-14	42.86	3	AF-28	50.00	3

4.2 Morphological and cultural study of *A. flavus* isolates

4.2.1 Microscopic study of *A. flavus*

Morphological characters of twenty eight isolates of *A. flavus* were studied and presented in Table 7. Conidia colour was observed from parrot green to olive green. The 67.85 per cent isolates shows parrot green colour whereas 32.15 per cent isolates shows olive green colour. Conidial wall, observed from finelly rough to smooth wall which was an important character in *A. flavus* identification.

53.57 per cent isolates shows finely rough whereas 46.43 per cent isolates shows smooth conidial wall during study. Conidial shape was observed from globose (64.29% isolates) to subglobose (35.71% isolates). Size of conidia ranges from 3.00 um (AF-07) to 4.25 um (AF-11 and AF-26) (Table 8).

Table 7 - Morphological characterization of different isolates of *flavus* grown on PDA

Sr. No.	Characters	Number of isolates	Details of isolates
1	Conidia colour		
	a. Parrot green	19 (67.85%)	AF-01, 02, 03, 05, 06, 07, 08, 09, 10, 12, 13, 18, 19, 20, 21, 22, 23, 25, and 28.
	b. Olive green	09 (32.15%)	AF-04, 11, 14, 15, 16, 17, 24, 26 and 27.
2	Conidia wall		
	a. Smooth	13 (46.43%)	AF-01, 03, 05, 06, 09, 10, 13, 17, 18, 19, 21, 22 and 25
	b. Finely rough	15 (53.57 %)	AF-02, 04, 07, 08, 11, 12, 14, 15, 16, 20, 23, 24, 26, 27 and 28.
3	Conidia shape		
	a. Globose	18 (64.29%)	AF-01, 02, 05, 06, 07, 09, 11, 13, 14, 15, 16, 20, 21, 23, 25, 26, 27 and 28.
	b. Subglobose	10 (35.71%)	AF-03, 04, 08, 10, 12, 17, 18, 19, 22 and 24

4.2.2 Grouping of isolated *A. flavus* strains

On the basis of colony color of all twenty eight isolates on PDA medium were grouped into two different groups and presented in Table 9. Group A consist of 67. 85 per cent isolates (AF-01, AF-

02, AF- 03, AF-05, AF-06, AF-07, AF-08, AF-09, AF-10, AF-12, AF-13, AF-18, AF-19, AF-20, AF-21, AF-22, AF-23, AF-25 and AF-28) showing parrot green colony colour in front and light greenish yellow in the reverse whereas group G consist of 32.15 per cent isolates (AF-04, AF-11, AF-14, AF-15, AF-16, AF-17, AF-24, AF-26 and AF-27) olive green colour in front and light greenish yellow in the reverse.

Table 8 - Conidial size of different *A. flavus* isolates

Isolate No.	Size of conidia (um)	Isolate No.	Size of conidia (um)
AF-01	3.15	AF-15	3.90
AF-02	4.20	AF-16	3.78
AF-03	3.76	AF-17	3.40
AF-04	4.23	AF-18	3.10
AF-05	3.91	AF-19	3.45
AF-06	3.15	AF-20	3.60
AF-07	3.00	AF-21	3.90
AF-08	3.75	AF-22	3.67
AF-09	3.60	AF-23	3.27
AF-10	4.15	AF-24	4.10
AF-11	4.25	AF-25	3.90
AF-12	3.70	AF-26	4.25
AF-13	3.87	AF-27	3.15
AF-14	3.27	AF-28	3.70

Table 9- Grouping of different *A. flavus* strains isolated from groundnut field of western Maharashtra

Group of isolates	Number of isolates	Details of isolates
A group	19 (67.85%)	AF-01, AF-02, AF-03, AF-05, AF-06, AF-07, AF-08, AF-09, AF-10, AF-12, AF-13, AF-18, AF-19, AF-20, AF-21, AF-22, AF-23, AF-25 and AF-28.

G group	09 (32.14%)	AF-04, AF-11, AF-14, AF-15, AF-16, AF-17, AF-24, AF-26 and AF-27.

4.2.3 Phenotype study based on sclerotia producing ability

The phenotypic study of *A. flavus* isolates based on sclerotial morphology was studied on CZ medium having 0 per cent, 2 per cent and 3 per cent concentration of $NaNO_3$ and results were presented in Table 10.

Out of twenty eight isolates, none of the isolate produced sclerotia on media having 0 per cent concentration of $NaNO_3$, only twenty five per cent isolates (AF-5, AF-6, AF-7, AF-8, AF-12, AF-24 and AF-25) produced sclerotia on media having 2 per cent concentration of $NaNO_3$ while maximum of 85.72 per cent isolates produced sclerotia on CZ media containing 3 per cent concentration of $NaNO_3$. Three colour of sclerotia *viz.,* Light brown (37.50% isolates), Dark brown (33.33% isolates) and Black (29.17% isolate) with globose (62.50% isolates) to subglobose (37.50% isolates) type of sclerotial shape were observed during present investigation. On the basis of sclerotial diameter 54.17 per cent isolates comes in L group and 45.83 per cent isolates comes in S group isolates.

Data in respect of sclerotial diameter was presented in Table 11. Sclerotia diameter ranged from 295.87um (AF-10) to 536.88um (AF-23). It was observed from this study that CZ media containing 3 per cent concentration of $NaNO_3$ found to be most effective medium for sclerotia production (Figure III).

Table 10 - Production of sclerotia by *A. flavus* isolates on CZ media

Sr. No.	Characters	No. of isolates	Details of isolates
1	Sclerotia production		
	0% $NaNo_3$	00 (00.00%)	Nil
	2% $NaNo_3$	07 (25.00%)	AF-05, 06, 07, 08, 12, 24 and 25
	3% $NaNo_3$	24 (85.72%)	AF-01, 02, 03, 05, 06. 07, 08, 09, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 23, 24, 25 and 28.
2	Sclerotia colour		
	Light brown	09 (37.50%)	AF-02, 06, 10, 12, 15, 18, 21, 25 and 28
	Dark brown	08 (33.33%)	AF-01, 05, 08, 11, 13, 14, 19 and 24
	Black	07 (29.17%)	AF-03, 07, 09, 16, 17, 20 and 23.
3	Sclerotia shape		
	Globose	15 (62.50%)	AF-01, 05, 06, 09, 10, 12, 14, 16, 17, 19, 21, 23, 24, 25 and 28.
	Subglobose	09 (37.50%)	AF-02, 03, 07, 08, 11, 13, 15, 18 and 20.
4	Sclerotia group		
	L-type	13 (54.17%)	AF-01, 02, 03, 05, 06, 07, 08, 12, 16, 19, 23, 25 and 28
	S-type	11 (45.83%)	AF-09, 10, 11, 13, 14, 15, 17, 18, 20, 21 and 24

Table 11- Sclerotial diameter of different *A. flavus* isolates

Isolate No.	Sclerotial diameter (um)	Isolate No.	Sclerotial diameter (um)
AF-01	521.08 ± 7.96	AF-15	345.53 ± 8.91
AF-02	515.52 ± 5.85	AF-16	474.02 ± 12.83
AF-03	477.32 ± 3.55	AF-17	297.96 ± 3.65
AF-04	-	AF-18	346.57 ± 4.49
AF-05	496.77± 5.42	AF-19	484.27 ± 7.80
AF-06	526.29 ± 7.30	AF-20	314.45 ± 6.70
AF-07	466.73 ± 7.81	AF-21	352.48 ± 9.40
AF-08	486.52 ± 13.78	AF-22	-
AF-09	340.15 ± 6.01	AF-23	536.88 ± 6.94
AF-10	295.87 ± 9.04	AF-24	304.9 ± 5.22
AF-11	330.25 ± 4.97	AF-25	499.55 ± 5.80
AF-12	533.41 ± 4.45	AF-26	-
AF-13	323.31 ± 5.52	AF-27	-
AF-14	369.49 ± 5.76	AF-28	494.51 ± 1.20

4.2.4 Cultural study of *A. flavus*

Maximum mycelial growth of isolates on ADM media ranged from 3.10cm (AF-20) to 6.63cm (AF-16) whereas minimum on YES medium ranged from 2.97cm (AF-26) to 5.30cm (AF-28). Also, on SMKY and CZ medium mycelial growth ranged from 3.87cm to 6.37 cm and 3.67 cm to 5.77 cm, respectively (Table 12). As ADM was non-sporulating medium, only white mycelial growth was observed on upper side whereas pink pigmentation on back side of petriplate and no sporulation was observed on it. However, on SMKY, CZ and YES medium poor to profuse sporulation was observed.

Table 12 – Mycelial growth of *A. flavus* grown on various media (4 days after inoculation in cm)

Isolate No	ADM	SMKY	CZ	YES
AF-01	5.40 ± 0.95	4.87 ± 0.58	4.87 ± 0.90	3.63 ± 0.75
AF-02	5.03 ± 1.10	4.97 ± 0.90	4.60 ± 0.70	4.00 ± 0.66
AF-03	5.13 ± 0.85	4.83 ± 0.85	4.83 ± 0.86	4.10 ± 0.56
AF-04	4.30 ± 1.01	5.10 ± 1.01	3.67 ± 0.78	3.13 ± 0.76
AF-05	4.10 ± 0.90	5.70 ± 0.72	4.87 ± 0.67	3.83 ± 0.81
AF-06	4.23 ± 1.11	6.40 ± 0.82	5.07 ± 1.05	4.03 ± 0.25
AF-07	4.17 ± 1.05	5.67 ± 0.46	5.13 ± 1.00	3.80 ± 0.70
AF-08	4.00 ± 0.85	6.37 ± 0.75	5.27 ± 0.91	3.90 ± 0.89
AF-09	4.43 ± 0.95	4.93 ± 1.15	4.47 ± 0.78	3.83 ± 0.72
AF-10	4.60 ± 1.05	5.73 ± 1.01	5.7 ± 0.35	3.97 ± 1.11
AF-11	6.17 ± 0.72	5.60 ± 0.46	5.30 ± 0.70	4.43 ± 1.07
AF-12	3.20 ± 0.92	5.03 ± 0.70	3.47 ± 0.55	3.50 ± 0.60
AF-13	5.40 ± 0.79	5.10 ± 0.95	3.30 ± 0.17	3.30 ± 0.56
AF-14	4.53 ± 1.30	5.17 ± 0.60	4.13 ± 1.00	3.27 ± 0.76
AF-15	4.07 ± 1.17	3.97 ± 0.21	4.27 ± 1.22	3.47 ± 0.50
AF-16	6.63 ± 0.87	4.37 ± 0.74	5.47 ± 1.38	4.37 ± 0.87
AF-17	6.23 ± 1.00	4.93 ± 0.46	4.72 ± 0.64	3.60 ± 0.46
AF-18	5.23 ± 1.01	4.47 ± 1.48	4.42 ± 0.98	3.37 ± 0.65
AF-19	5.00 ± 0.56	4.63 ± 0.45	4.20 ± 1.25	3.50 ± 0.56
AF-20	3.10 ± 0.70	6.03 ± 1.01	4.23 ± 1.07	4.67 ± 1.27
AF-21	4.60 ± 0.78	3.87 ± 1.12	4.22 ± 1.13	3.60 ± 0.26
AF-22	5.10 ± 1.15	4.53 ± 1.24	3.63 ± 0.81	3.03 ± 0.32
AF-23	3.40 ± 1.41	4.80 ± 1.71	4.67 ± 0.91	4.13 ± 0.61
AF-24	4.30 ± 0.95	6.03 ± 1.01	4.57 ± 0.61	3.73 ± 0.32
AF-25	4.53 ± 0.86	5.93 ± 1.66	5.77 ± 0.68	4.37 ± 0.67
AF-26	4.90 ± 1.10	4.23 ± 1.53	4.57 ± 0.98	2.97 ± 0.12
AF-27	4.43 ± 0.76	3.97 ± 0.70	3.53 ± 1.27	3.23 ± 0.67
AF-28	4.23 ± 1.15	3.93 ± 1.33	5.50 ± 0.87	5.30 ± 0.79

SE	0.57	0.57	0.52	0.40
CD at 5%	1.61	1.62	1.48	1.15

4.3 Aflatoxin Detection by qualitative method

4.3.1 Ammonia Vapour Test

In this study, different isolates of *A. flavus* were tested for their aflatoxin producing ability. These results were presented in Table 13.

Table 13- Aflatoxin detection of different *A. flavus* isolates by Ammonia vapour test on PDA medium

Group	Number of Isolates	Details of isolates*
A. 3rd day of incubation- (18 isolates + test) 64. 29 %		
Light red	17	AF-01(3.1), AF-03(3.6), AF-5(3.4), AF-06(3.3), AF-08(3.2), AF-10 (3.1), AF- 11(3.2), AF-13(2.2), AF-14(2.1), AF15-(2.4), AF-16(3.6), AF-20(2.7),AF-21(2.4), AF-23(2.6), AF-24(2.5), AF-26(1.9) and AF-28(3.3)
Medium red	01	AF-18(2.4)
Dark red	00	Nil
No colour change	10	AF-02(3.3), AF-04(1.6), AF-07 (3.3), AF-09(3.3), AF12-(2.5), AF17-(2.5), AF-19(1.9), AF-22 (1.8), AF-25(2.8) and AF-27(1.8)
B. 5th day of incubation- (26 isolates + test) 92.86%		
Light red	17 (65.38%)	AF-01(6.0), AF-02(6.2), AF-03 (6.1),AF-04(4.1), AF-05(5.9), AF-06(5.8), AF-08(5.7), AF-09(5.8), AF-10(5.6), AF-13(4.7), AF-15 (4.9), AF-16(6.1),AF-17(5.0), AF-19 (4.4), AF25-(5.7), AF-26(4.4) and AF-27(4.3)
Medium red	05 (19.25%)	AF-18(4.9), AF-20(5.2), AF-21 (4.9),AF-22(4.3) and AF-23-(5.1)
Dark red	04 (15.38%)	AF-11(5.7), AF-14(4.6), AF-24(5.0) and AF-28(6.2)
No colour change	02	AF-07(5.8) and AF-12(5.0)

*Figures in brackets indicates colony diameter in cm.

It was observed that among the isolates screened 64. 29 per cent isolates showed toxic nature on 3rd day while on 5th day of incubation 92.86 per cent isolates showed toxic nature. Among these isolates 17 isolates (65.38 %) showed light red colour, 5 isolates (19.23 %) showed medium red colour and 4 isolates (15.38 %) *viz.,* AF-11, AF-14, AF-24, AF-28 showed dark red colour indicating light, medium and high toxic nature, respectively while two isolates *viz.,* AF-07, AF-12 showed no colour change indicating non-toxic nature on 5th day of incubation.

It was noticed that the colour change of mycelium developed rapidly and color of sporulation changed from green to light brown on exposure to ammonia in both the aflatoxigenic and non-aflatoxigenic strains. All the isolates turned into its original colour as these were before ammonia treatment when treatment of glacial acetic acid was given to ammonia treated petri plates.

4.3.2 Observance under UV light

Observations recorded under UV light were presented in Table 14. It was observed that 42.86 per cent isolates showed blue fluorescence ring surrounding the colony while 57.14 per cent isolates did not produced it on the reverse side of the colony on 3rd day of incubation while on 5th day of incubation all the isolates observed blue fluorescence ring on the reverse side of the colony under UV light of 365 nm wavelength. Among them 7 isolates (25 %) observed light fluorescence, 16 isolates (57.14 %) observed medium fluorescence and 5 isolates (17.86 %) *viz.,* AF-11, AF-14, AF-22, AF-23, AF-28 observed dark fluorescence. It indicates that all the isolates observed aflatoxin either B_1 or B_2 or both. None of the isolate observed green fluorescence on the reverse side of the colony under UV light indicates the absence of aflatoxin G_1 or G_2 or both. On 7th day incubation cultures were became difficult to evaluate because mycelial growth reached the margin of the plate and obscured the blue fluorescence.

Table 14 - Aflatoxin detection of different *A. flavus* isolates by UV florescence on CAM medium

Group	**Aflatoxin B_1/ B_2 (Blue florescence)***	**Aflatoxin G_1/G_2 (Green florescence)**
A. 3rd day of incubation (12 isolates +)		
Light florescence	**12 isolates** *viz.,* AF-02(2.2), AF-06(2.6), AF-07(2.6), AF-09(2.8), AF-11(3.1), AF-14(2.0), AF-16(1.3), AF-18(1.4), AF-19(2.1), AF-22(2.3), AF-24(2.8) and AF-28(2.6)	00
Medium florescence	00	00
Dark florescence	00	00
B. 5th day of incubation (28 isolates +)		
Light florescence	**07 isolates** *viz.,* AF-05(5.3), AF-08 (5.2), AF-10(5.4), AF-12(4.9), AF-13(3.4), AF-20(5.0) and AF-25(6.1)	00
Medium florescence	**16 isolates** *viz.,* AF- 01(4.6), AF-02(4.7), AF-03(4.5), AF-04(3.2), AF-06(5.2), AF-07(5.1), AF-09(5.3), AF-15(3.7), AF-16(3.8), AF-17(4.2), AF-18(3.9), AF-19(4.6), AF-21(3.9), AF-24(5.3), AF-26(3.2) and AF-27(3.7)	00
Dark florescence	**05 isolates** *viz.,* AF-11(5.6), AF-14(4.5), AF-22(4.8), AF-23(4.7) and AF-28(5.1)	00
C. 7th day of incubation		
	Difficult to detect florescence	

*Figures in brackets indicates colony diameter in cm.

4.3.3 Orange yellow pigmentation

A. flavus isolates inoculated on coconut agar medium plates were incubated at 28 ˚C temperature in the dark, observed visually on 3rd and 5th day of incubation and results were presented in Table 15. Among all isolates observed, only 32.14 per cent isolates observed orange yellow pigmentation on reverse side of plates while 67.86 per cent isolates did not observed it on 3rd day. However, on 5th day it was observed that 22 isolates (78.57 %) observed orange yellow pigmentation on reverse side of plates while 6 isolates (21.43 %) *viz.,* AF-05, AF-08, AF-10, AF-12, AF-20 and AF-25 did not observed it indicating non-toxic nature of isolates. It was also observed that degree of pigmentation seemed to be proportional to the intensity of fluorescence.

Table 15 - Aflatoxin detection of different *A. flavus* isolates by Orange- Yellow Pigmentation on CAM medium

Group	Number of isolates
3rd day of incubation	
Positive test	09 (32.14%) isolates *viz.,* AF-02, AF-07, AF-11, AF-16, AF-18, AF-19, AF-22, AF-24 and AF-28
Negative test	19 (67.86%) isolates *viz.,* AF-01, AF-03, AF-04, AF-05, AF-06, AF-08, AF-09, AF-10, AF-12, AF-13, AF-14, AF-15, AF-17, AF-20, AF-21, AF-23, AF-25, AF-26 and AF-27
5th day of incubation	
Positive test	22 (78.57%) isolates *viz.,* AF-01, AF-02, AF-03, AF-04, AF-06, AF-07, AF-09, AF-11, AF-13, AF-14, AF-15, AF-16, AF-17, AF-18, AF-19, AF-21, AF-22, AF-23, AF-24, AF-26, AF-27 and AF-28
Negative test	06 (21.43%) isolates *viz.,* AF-05, AF-08, AF-10, AF-12, AF-20 and AF-25.

Mycotoxin producing ability of all the isolates tested by above methods were compared with each other for confirmation of accuracy of method and results were presented in Table 16. Among the isolates observed, only two isolates *viz.,* AF-07 and AF-12 showed non-toxic nature by ammonia vapour test. However, all the 100 per cent isolates were showed toxic nature by UV florescence method while six isolates *viz.,* AF-05, AF-08, AF-10, AF-12, AF-20 and AF-25 showed non-toxic nature by orange yellow pigmentation method on 5th day of incubation.

Table 16 - Comparison of aflatoxin detection of different *A. flavus* isolates by various methods (5th day)

Method of detection	Positive test	Negative test
Ammonia vapour test	26 isolates	2 isolates *viz.,* AF-07 and AF-12
UV florescence	28 isolates	Nil
Orange-Yellow Pigmentation	22 isolates	6 isolates *viz.,* AF-05, AF-08, AF-10, AF-12, AF-20 and AF-25

4.4 Morphological diversity of different *A. flavus* isolates

Twenty eight isolates of *A. flavus* were analyzed for their morphological diversity on the basis of similarity coefficient analysis and presented in (Figure-IV). Dendrogram of it resulted in two major clusters, cluster A and cluster B. Similarity coefficient in between samples ranged from 0.18 to 0.91. Cluster A consist of 18 isolates however cluster B consist 10 isolates.

Isolates with in cluster A shows two sub-clusters, A_1 and A_2. Subcluster A_1 consist of 11 isolates *viz.,* AF-01, AF-06, AF-05, AF-25, AF-09, AF-10, AF-03, AF-19, AF-13, AF-21 and AF-18. Within this cluster, AF-01 and AF-06, AF-05 and AF-25 shows maximum similarity coefficient of 0.91 followed by AF-13 and AF-21 (0.73), AF-03 and AF-19 (0.64). Isolates within this cluster show similarity in conidial colour, conidial wall, conidial size, isolate group, yellow orange pigmentation and dissimilarity in conidial shape, sclerotial shape, sclerotial size, sclerotial colour, growth pattern, aflatoxin synthesis ability. Subcluster A_2 with in cluster A consist of 7 isolates *viz.,* AF-02, AF-28, AF-07, AF-23, AF-20, AF-08 and AF-12. Within this subcluster AF-02 and AF-08 shows maximum similarity coefficient of 0.73 followed by AF-07 and AF-23 (0.64), AF-08 and AF-12 (0.64). Isolates within this subcluster show similarity in conidial colour, conidial wall, isaolate group, sclerotial colour and dissimilarity in conidial shape, conidial size, sclerotial size, sclerotial shape, growth pattern, yellow orange pigmentation, aflatoxin synthesis ability.

Isolates with in cluster B shows two sub-clusters, cluster B_1 and B_2. Subcluster B_1 consist of 04 isolates *viz.,* AF-04, AF-26, AF-27 and AF-22. Within this cluster, AF-04 and AF-26, shows maximum similarity coefficient of 0.91 having similarity in sclerotial shape, sclerotial size, sclerotial colour, growth pattern, yellow orange pigmentation while dissimilarity in conidial colour, conidial wall, conidial size, conidial shape, isolate group, aflatoxin synthesis ability. Subcluster B_2 consist of 6 isolates *viz.,* AF-11, AF-14, AF-24, AF-15, AF-16 and AF-17. Within this cluster AF-14 and AF-24 showing maximum similarity coefficient of 0.82 followed by AF-15 and AF-16 (0.64). Isolates within this cluster show similarity in conidial colour, isolate group, sclerotial size, yellow orange pigmentation while dissimilarity in conidial shape, conidial wall, conidial size, sclerotial shape, sclerotial colour, growth pattern, aflatoxin synthesis ability. Also 2-D scatter plot analysis depicts that isolates AF-12, AF-14, AF-17, AF-18 and AF-19 in distinguished by first component and isolates AF-05, AF-08, AF-09, AF-20, AF-25 and AF-28 in second component showed more similarity as compare to other isolates.

4.5 Aflatoxin quantification by chromatography technique

Results obtained in the present investigation in respect of dry mycelial mat weight was presented in Table 17. Dry mycelial mat weight ranged from 1.63 g (AF-21) to 3.42 g (AF-25) per 50 ml of SMKY broth on 9th day of incubation (Plate III).

Table 17 - Dry mycelial weight of different *A. flavus* isolates on 9th day of incubation in SMKY broth

Isolate No.	Mycelia g/50 ml broth	Isolate No.	Mycelia g/50 ml broth
AF-01	1.98	AF-16	1.70
AF-02	2.05	AF-17	2.23
AF-03	1.98	AF-18	1.94
AF-04	1.73	AF-19	2.02
AF-05	3.24	AF-20	2.83
AF-06	3.20	AF-21	1.63
AF-07	3.16	AF-22	1.80
AF-08	3.22	AF-23	2.42
AF-09	2.67	AF-24	2.88
AF-10	2.73	AF-25	3.42
AF-11	3.03	AF-26	1.71
AF-12	2.13	AF-27	1.72
AF-13	2.02	AF-28	2.85
AF-14	2.41	SE$\pm$	0.02
AF-15	1.73	CD at 5%	0.07

Data in respect of aflatoxin B_1 synthesized by different isolates was presented in Table 18 (Plate IV). Significantly aflatoxin B_1 synthesized by isolate AF-14 (1380 ng/ml) followed by AF- 28 (1346.67 ng/ml) while minimum by isolates AF-07, AF-12 (600 ng/ml) on 9th day of incubation. Also aflatoxin B_2 was not detected in any of the isolate. No significant differences in dry mycelial mat weight and aflatoxin B_1 synthesis were observed during present investigation.

Aflatoxin in individual flask was extracted with methanol and the extract was rated for colour intensity. The intensity of yellow colour of the methanolic extract was correlated with concentration of aflatoxin B_1 analyzed by chromatography technique and data presented in Table 19. It showed a positive correlation means higher the aflatoxin more dense was the yellow colour of the extract.

Table 18 - Aflatoxin B_1 producing potential of different *A. flavus* isolates in SMKY broth by chromatographic technique

Isolate No.	Aflatoxin B1 ng/ml	Isolate No.	Aflatoxin B1 ng/ml
AF-01	760.00	AF-16	786.67
AF-02	773.33	AF-17	1166.67
AF-03	766.67	AF-18	1053.33
AF-04	753.33	AF-19	780.00
AF-05	693.33	AF-20	1060.00
AF-06	760.00	AF-21	1066.67
AF-07	600.00	AF-22	1026.67
AF-08	720.00	AF-23	993.33
AF-09	873.33	AF-24	1320.00
AF-10	786.67	AF-25	846.67
AF-11	1253.33	AF-26	986.67
AF-12	600.00	AF-27	993.33
AF-13	1120.00	AF-28	1346.67
AF-14	1380.00	SE$\pm$	31.77
AF-15	753.33	CD at 5%	90.01

4.6 Chemotype Classification of *A. flavus* Strains

During the present investigation, all the 28 isolates studied were classified into seven chemotypes based on AFB_1, AFB_2 and sclerotia production patterns and presented in Table 20. It was observed that 85.71 per cent isolates produced both AFB_1, sclerotia and grouped into chemotype-I while 100 per cent isolates produced only AFB_1, grouped into chemotype-II and found that the most represented chemotype. No isolate were found able to produce both AFB_1 and AFB_2. None of the isolates were found in chemotype-III, IV, V, VI and VII.

Table 19 - Colour of $CHCl_3$ extract of different *A. flavus* isolates

Isolate No.	Colour of $CHCl_3$ extract	Isolate No.	Colour of $CHCl_3$ extract
AF-01	+	AF-15	+
AF-02	+	AF-16	+
AF-03	+	AF-17	++
AF-04	+	AF-18	++
AF-05	+	AF-19	+
AF-06	+	AF-20	++
AF-07	+	AF-21	++
AF-08	+	AF-22	++
AF-09	+	AF-23	+++
AF-10	+	AF-24	+++
AF-11	+++	AF-25	++
AF-12	+	AF-26	++
AF-13	++	AF-27	++
AF-14	+++	AF-28	+++

+ Very pale yellow, ++ Pale yellow, +++ Yellow

Table 20 - Chemotype diversity of *A. flavus* isolates based on aflatoxins and sclerotia producing ability

Chemotype	AFB_1	AFB_2	Sclerotia production	No. of isolates
I	+	-	+	24
II	+	-	-	28
III	-	-	+	0
IV	-	-	-	0
V	+	+	+	0
VI	+	+	-	0
VII	-	+	-	0

4.7 *In-vitro* screening of groundnut cultivars against *A. flavus*

Ten groundnut cultivars released for cultivation were studied in the laboratory for *in vitro* pod and seed colanization by *A. flavus*. Results obtained were depicted in Table 21 and Table 22 respectively.

Table 21 - Per cent colonization severity caused by *A. flavus* on pods of different peanut cultivars

Sr. No.	Cultivar	Pods			
		5th Day	Grade	7th Day	Grade
1	TPG-41	48.33 (44.04)	3	61.67 (51.76)	4
2	Phule Unnati	26.67 (31.07)	3	43.33 (41.13)	3
3	TAG-24	61.67 (51.81)	4	66.67 (54.83)	4
4	Phule Unap	28.33 (32.14)	3	45.00 (42.12)	3
5	JL-24	71.67 (57.86)	4	90.00 (71.57)	4
6	Phule Warna	48.33 (44.04)	3	50.00 (44.99)	3
7	Phule Vyas	31.67 (34.23)	3	45.00 (42.12)	3
8	JL-501	58.33 (49.80)	4	68.33 (55.77)	4
9	SB-11	63.33 (52.74)	4	88.33 (70.50)	4
10	Phule 6021	60.00 (50.79)	4	66.67 (54.78)	4
	SE	1.34		2.11	
	CD at 0.05%	3.95		6.24	
	CV	5.18		6.91	

Figures in the parenthesis are arc sin transformed values

Pod colonization severity ranged from 26.67 to 63.33 per cent on 5th day of incubation while 43.33 to 90.00 per cent on 7th day of incubation (Plate V). Minimum colonization severity (26.67%) was recorded on Phule Unnati which was at par with Phule Unap (28.33%) and Phule Vyas (31.67%) while maximum colonization severity was recorded on JL-24(71.67%) on 5th day of incubation. Similar trend was also observed on 7th day of incubation, showing minimum colonization severity on Phule Unnati (43.33%) which was at par with Phule Unap (45.00%), Phule Vyas (45.00%) and Phule Warna (50.00%) while maximum was recorded on JL-24 (90.00%). Individual seeds were scored for surface colonization by *A. flavus* and observed that in pod colonization study all the cultivars falls in 3 or 4 grade on 5th and 7th day of incubation.

Table 22 - Per cent colonization severity caused by *A. flavus* on kernels of different peanut cultivars.

Sr. No.	Cultivar	Kernel			
		5th Day	Grade	7th Day	Grade
1	TPG-41	43.33 (41.16)	3	53.33 (46.91)	4
2	Phule Unnati	25.00 (30.00)	2	41.67 (40.20)	3
3	TAG-24	38.33 (38.16)	3	55.00 (47.89)	4
4	Phule Unap	25.00 (30.00)	2	40.00 (39.23)	3
5	JL-24	55.00 (47.88)	4	86.67 (68.66)	4
6	Phule Warna	40.00 (39.23)	3	48.33 (44.04)	3
7	Phule Vyas	28.33 (32.14)	3	43.33 (41.16)	3
8	JL-501	53.33 (46.91)	4	63.33 (52.74)	4
9	SB-11	46.67 (43.08)	3	83.33 (66.26)	4
10	Phule 6021	43.33 (41.16)	3	56.67 (48.84)	4
	SE	1.60		1.73	
	CD at 0.05%	4.72		5.12	
	CV	7.11		6.06	

Figures in the parenthesis are arc sin transformed values

Kernel colonization severity ranged from 25.00 to 55.00 per cent on 5th day of incubation while 40.00 to 83.33 per cent on 7th day of incubation (Plate VI). Minimum colonization severity was recorded on Phule Unnati (25.00%) Phule Unap (25.00%) and at par with Phule Vyas (28.33%) while maximum colonization severity was recorded on JL-24 (55.00%) on 5th day. Similar trend was also observed on 7th day of incubation, showing minimum colonization severity on Phule Unap (40.00%) which was at par with Phule Unnati (41.67%), Phule Vyas (43.33%) and Phule Warna (48.33%) while maximum colonization severity was observed on JL-24 (86.67%). Individual seeds were scored for surface colonization by *A. flavus* and observed that in kernel colonization severity study only two cultivars *viz.*, Phule Unnati and Phule Unap falls in 2 rating scale while remaining cultivars falls in 3 or 4 grade on 5th day of incubation and all cultivars falls in 3 and 4 grade on 7th day of incubation.

4.8 Efficacy of botanicals against *A. flavus* seed colonization and aflatoxin synthesis

4.8.1 Antifungal activity of plant extract against *A. flavus* by poison food technique

The data presented in the Table 23 revealed that all the treatments were significantly superior over control in respect of colony growth diameter and growth inhibition percentage of *A. flavus* on 5th day of incubation. The extract of pomegranate (*P.granatum*) fruit peel was statistically found to be effective with minimum colony diameter of 32.00 mm having poor sporulation with 52.71 per cent growth inhibition over control and no aflatoxin was detected. The extract of pomegranate leaves shows 39.00 mm colony diameter with 42.36 per cent growth inhibition was at par with *A. indica* leaves showing 41.33 mm colony diameter with 38.92 per cent growth inhibition and bulb of *A. cepa* showing 42.00 mm colony diameter with 37.93 per cent growth inhibition

Table 23- Antifungal activity of different plant extracts against *A. flavus* (on 5th day) by poisoned food technique

Tr. No.	Treatment details	*Mean colony diameter (mm)	Growth inhibition (%)	Degree of sporulation	Aflatoxin detection
T1	*A. indica* (Leaves)	41.33 (40.01)	38.92	Moderate	+
T2	*A. cepa* L (Bulb)	42.00 (40.39)	37.93	Poor	-
T3	*L.camara* (Flowers)	52.67 (46.53)	22.17	Moderate	+
T4	*P. granatum* (Leaves)	39.00 (38.64)	42.36	Moderate	-
T5	*A.sativum* L (Cloves)	42.67 (40.78)	36.95	Moderate	-
T6	*P.granatum* (Fruit peel)	32.00 (34.44)	52.71	Poor	-
T7	Control	67.67 (55.35)	-	Abundent	++
	SE±	0.65			
	CD (0.05)	1.97			
	CV	2.66			

Figures in the parenthesis are arc sin transformed values

Data on 8th day of incubation in respect of above study presented in Table 24 observed similar trend showing 33.00 mm colony diameter having poor sporulation with 63.33 percentage growth inhibition over control and no aflatoxin was detected in treatment of pomegranate fruit peel extract. However in control treatment colony diameter was 67.67 mm and 90.00 mm with abundant sporulation on 5th day and 8th day of incubation, respectively and aflatoxin was detected.

Table 24 -Antifungal activity of different plant extracts against *A.flavus* (on 8th day) by PFT

Tr. No.	Treatment details	*Mean colony diameter (mm)	Growth inhibition (%)	Degree of sporulation	Aflatoxin detection
T1	*A. indica* (Leaves)	42.00 (40.39)	53.33	Moderate	+
T2	*A. cepa* L (Bulb)	43.00 (40.98)	52.22	Poor	+
T3	*L.camara* (Flowers)	53.00 (46.72)	41.11	Moderate	+
T4	*P. granatum* (Leaves)	40.67 (39.62)	54.81	Moderate	-
T5	*A.sativum* L (Cloves)	43.67 (41.36)	51.48	Moderate	+
T6	*P.granatum* (Fruit peel)	33.00 (35.04)	63.33	Poor	-
T7	Control	90.00 (71.57)	-	Abundant	++
	SE±	0.77			
	CD (0.05)	2.33			
	CV	2.95			

Figures in the parenthesis are arc sin transformed values

4.8.2 Antifungal activity of methanolic extract of pomegranate fruit peel against *A. flavus* by poison food technique

The data presented in the Table 25 (Plate VII) revealed that all the treatments were significantly superior over control in respect of colony growth diameter and growth inhibition of *A. flavus* on 5th day of incubation. The treatment T7 having concentration 3000 ppm was statistically found to be effective with minimum colony diameter of 30.67 mm without sporulation with 54.68 per cent growth inhibition over control and no aflatoxin was detected. This treatment was followed with treatment T6, T5 and T4 which were at par showing colony diameter of 33.67mm, 37.33 mm and 38.00 mm, respectively with 50.25, 44.83 and 43.84 per cent growth inhibition, respectively over control.

Table 25 - Antifungal activity of methanolic extract of pomegranate fruit peel against *A. flavus* (on 5th day) by PFT

Tr. No.	Treatment details	*Mean colony diameter (mm)	Growth inhibition (%)	Degree of sporulation	Aflatoxin detection
T1	100 ppm	61.00 (51.36)	9.85	Abundant	+
T2	500 ppm	49.33 (44.62)	27.09	Abundant	+
T3	1000 ppm	39.67 (39.02)	41.38	Moderate	-
T4	1500 ppm	38.00 (38.05)	43.84	Moderate	-
T5	2000 ppm	37.33 (37.65)	44.83	Poor	-
T6	2500 ppm	33.67 (35.46)	50.25	Poor	-
T7	3000 ppm	30.67 (33.62)	54.68	No sporulation	-
T8	Control	67.67 (55.36)	-	Abundant	++
	SE±	0.94			
	CD (0.05)	2.82			
	CV	3.89			

Figures in the parenthesis are arc sin transformed values

Data on 8th day of incubation in respect of above study presented in Table 26 observed similar trend showing 32.33 mm colony diameter without sporulation with 64.07 percentage growth inhibition over control and no aflatoxin was detected in treatment

T7 having concentration 3000 ppm followed by treatment T6 showing 35.33 mm colony diameter with 60.74 percentage growth inhibition over control. However in control treatment colony diameter was 67.67 mm and 90.00 mm with abundant sporulation on 5th day and 8th day of incubation, respectively and aflatoxin was detected.

Table 26 - Antifungal activity of methanolic extract of pomegranate fruit peel against *A. flavus* (on 8th day) by PFT

Tr. No.	Treatment details	*Mean colony diameter (mm)	Growth inhibition (%)	Degree of sporulation	Aflatoxin detection
T1	100 ppm	62.67 (52.34)	30.37	Abundant	++
T2	500 ppm	51.00 (45.57)	43.33	Abundant	++
T3	1000 ppm	41.33 (40.01)	54.07	Moderate	+
T4	1500 ppm	39.67 (39.04)	55.93	Moderate	+
T5	2000 ppm	39.00 (38.64)	56.67	Poor	-
T6	2500 ppm	35.33 (36.47)	60.74	Poor	-
T7	3000 ppm	32.33 (34.65)	64.07	No sporulation	-
T8	Control	90.00 (71.57)	-	Abundant	+++
	SE±	0.45			
	CD (0.05)	1.34			
	CV	1.73			

Figures in the parenthesis are arc sin transformed values

4.8.3 Antifungal activity of methanolic extract of pomegranate fruit peel on pods and kernels against *A. flavus*

The data presented in the Table 27 revealed that all the treatments were significantly superior over control in respect of colonization severity and growth inhibition percentage of *A. flavus* on 8th day of incubation. The treatment T7 having concentration 3000 ppm was statistically found to be effective with minimum pod colonization severity 26.67 per cent with 72.88 per cent growth inhibition over control (Figure V). This treatment was at par with treatment T6 showing colonization severity of 36.67 with 62.71 per cent growth inhibition over control. All the treatments showed varied degree of sporluation except treatment T7, in which no sporulation was observed (Plate VIII).

Table 27 - Antifungal activity of methanolic extract of pomegranate fruit peel against *A. flavus* on pods on 8th day

Tr. No.	Treatment details	*Colonization severity (%)	Growth inhibition (%)	Degree of sporulation
T1	100 ppm	81.67 (64.69)	16.95	Abundant
T2	500 ppm	66.67 (54.75)	32.20	Abundant
T3	1000 ppm	65.00 (53.76)	33.90	Moderate
T4	1500 ppm	65.00 (53.76)	33.90	Moderate
T5	2000 ppm	60.00 (50.79)	38.98	Poor
T6	2500 ppm	36.67 (37.26)	62.71	Poor
T7	3000 ppm	26.67 (31.07)	72.88	No sporuation
T8	Control	98.33 (85.69)	-	Abundant
	SE±	2.01		
	CD (0.05)	6.01		

Figures in the parenthesis are arc sin transformed values

Similarly, data presented in the Table 28 in respect of kernel colonization severity and growth inhibition percentage of *A. flavus* revealed that all the treatments were significantly superior over control on 8th day of incubation. The treatment T7 having concentration 3000 ppm was statistically found to be effective with minimum kernel colonization severity 26.67 per cent with 70.37 per cent growth inhibition over control (Figure V). This treatment was at par with treatment T6 (2500 ppm) showing colonization severity of 31.67 with 64.81 per cent growth inhibition over control. All the treatments showed varied degree of sporulation except treatment T7, in which no sporulation was observed (Plate VIII). Also, no aflatoxin B_1 synthesis was detected in this treatment by chromatography technique (Plate IX).

Table 28- Antifungal activity of methanolic extract of pomegranate fruit peel against *A. flavus* on kernels on 8th day

Tr. No.	Treatment details	*Colonization severity (%)	Growth inhibition (%)	Degree of sporulation	Aflatoxin B1 (mg/kg)
T1	100 ppm	75.00 (60.07)	16.67	Abundant	2.960
T2	500 ppm	63.33 (52.74)	29.63	Abundant	2.180
T3	1000 ppm	60.00 (50.79)	33.33	Moderate	1.720
T4	1500 ppm	60.00 (50.79)	33.33	Moderate	0.960
T5	2000 ppm	55.00 (47.88)	38.89	Poor	Not detected
T6	2500 ppm	31.67 (34.23)	64.81	Poor	Not detected
T7	3000 ppm	26.67 (31.07)	70.37	No sporulation	Not detected
T8	Control	90.00 (71.95)	-	Abundant	
	SE±	1.71			
	CD (0.05)	5.14			

Figures in the parenthesis are arc sin transformed values

4.9 Effect of methanolic extract of pomegranate fruit peel on growth parameters of groundnut kernels

4.9.1 Germination per centage

Significant differences in germination per centage were observed among the 28 isolates in case of kernels inoculated with *A. flavus* isolates and untreated with methanolic extract of pomegranate fruit peel (Table 29). Significant differences in germination per centage were observed among isolates. Isolate AF-26 showed maximum germination of 90.67 % followed by AF-19 (90.00%), AF-22(89.33%), AF-04(88.00%), AF-27 (86.67%) which were at par with each other while minimum germination was showed in AF-12 (74.67%).

Table 29 – Effect of different *A. flavus* isolates on germination of groundnut kernels

Isolate No.	**Germination %**	**Isolate No.**	**Germination %**
AF-01	76.67	AF-17	80.67
AF-02	76.67	AF-18	82.00
AF-03	81.33	AF-19	90.00
AF-04	88.00	AF-20	78.67
AF-05	80.67	AF-21	82.67
AF-06	80.67	AF-22	89.33
AF-07	82.67	AF-23	86.00
AF-08	80.67	AF-24	84.00
AF-09	77.33	AF-25	82.00
AF-10	80.67	AF-26	90.67
AF-11	82.67	AF-27	86.67
AF-12	74.67	AF-28	78.67
AF-13	80.67	Control	92.67
AF-14	76.67	SE±	1.60
AF-15	78.67	CD at 5%	4.53
AF-16	78.67		

Significant differences were observed in case of kernels treated with methanolic extract of pomegranate fruit peel and then inoculated with *A. flavus* isolates (Table 30). AF-19 and AF-26 recorded maximum germination of 94.00% and were at par with AF-22 (92.67%), AF-04 (92.67%), AF-27 (92.00%) and AF-23

(90.00%). However, AF-12 recorded minimum germination of 81.33%.

Table 30 - Effect of methanolic extract of pomegranate fruit peel on germination in groundnut kernels inoculated with *A. flavus* isolates

Isolate No.	Germination %	Isolate No.	Germination %
AF-01	84.00	AF-17	86.00
AF-02	83.33	AF-18	88.00
AF-03	88.00	AF-19	94.00
AF-04	92.67	AF-20	86.00
AF-05	85.33	AF-21	88.00
AF-06	87.33	AF-22	92.67
AF-07	87.33	AF-23	90.00
AF-08	88.00	AF-24	88.67
AF-09	82.67	AF-25	88.00
AF-10	86.00	AF-26	94.00
AF-11	86.67	AF-27	92.00
AF-12	81.33	AF-28	80.67
AF-13	87.33	Control	94.00
AF-14	82.00	SE±	1.79
AF-15	84.00	CD at 5%	5.08
AF-16	82.67		

Kernels treated with methanolic extract of pomegranate fruit peel and inoculated with *A. flavus* showed increase in germination per centage from 2.54 to 9.57 per cent over untreated methanolic extract of pomegranate fruit peel and inoculated with *A. flavus* in different isolates at laboratory condition (Table 31). Also, 1.44 per cent increase in germination was observed in case of control where kernels treated with methanolic extract of pomegranate fruit peel but not inoculated with *A. flavus* (Figure VI).

4.9.2 Vigour index

Effect of methanolic extract of pomegranate fruit peel on vigour index of kernels was studied. Significant differences in vigour index in case of kernels inoculated with *A. flavus* isolates and untreated with methanolic extract of pomegranate fruit peel were observed (Table 32). Isolate AF-19 showed maximum vigour index of 1160.67 which was at par with AF-26(1109.73) while minimum by isolate AF-16(469.73).

Table 31 - Effect of methanolic extract of pomegranate fruit peel on increase in germination (%) of kernels inoculated with *A. flavus* over untreated but inoculated with *A. flavus*

Isolate No.	Increase in germination (%)	Isolate No.	Increase in germination (%)
AF-01	9.57	AF-16	5.08
AF-02	8.70	AF-17	6.61
AF-03	8.20	AF-18	7.32
AF-04	5.30	AF-19	4.44
AF-05	5.79	AF-20	9.32
AF-06	8.26	AF-21	6.45
AF-07	5.65	AF-22	3.73
AF-08	9.09	AF-23	4.65
AF-09	6.90	AF-24	5.56
AF-10	6.61	AF-25	7.32
AF-11	4.84	AF-26	3.68
AF-12	8.93	AF-27	6.15
AF-13	8.26	AF-28	2.54
AF-14	6.96	Control	1.44
AF-15	6.78		

Also, significant differences were observed in case of kernels treated with methanolic extract of pomegranate fruit peel and then inoculated with *A. flavus* isolates (Table 33). AF-19 showed maximum vigour index of 1272.40 and at par with AF-26(1222.07) while AF-16 showed minimum vigour index of 562.13.

Table 32 – Effect of different *A. flavus* isolates on vigour index of groundnut kernels

Isolate No.	Vigour index	Isolate No.	Vigour index
AF-01	522	AF-17	654
AF-02	507	AF-18	771
AF-03	749	AF-19	1161
AF-04	933	AF-20	614
AF-05	630	AF-21	711
AF-06	646	AF-22	1045
AF-07	736	AF-23	912
AF-08	638	AF-24	815
AF-09	503	AF-25	541
AF-10	654	AF-26	1110
AF-11	736	AF-27	953
AF-12	508	AF-28	559
AF-13	630	Control	1211
AF-14	514	SE±	23.63
AF-15	564	CD at 5%	66.99
AF-16	470		

Table 33 - Effect of methanolic extract of pomegranate fruit peel on vigour index in groundnut kernels inoculated with *A. flavus* isolates

Isolate No.	Vigour index	Isolate No.	Vigour index
AF-01	655	AF-17	765
AF-02	633	AF-18	897
AF-03	871	AF-19	1272
AF-04	1056	AF-20	748
AF-05	742	AF-21	832
AF-06	778	AF-22	1168
AF-07	844	AF-23	1026
AF-08	783	AF-24	931
AF-09	620	AF-25	655
AF-10	757	AF-26	1222
AF-11	840	AF-27	1094
AF-12	613	AF-28	637
AF-13	760	Control	1325
AF-14	601	SE±	18.22
AF-15	683	CD at 5%	51.64

AF-16	562		

Kernels treated with methanolic extract of pomegranate fruit peel and inoculated with *A. flavus* showed increase in vigour index from 9.63 to 25.50 per cent over untreated methanolic extract of pomegranate fruit peel and inoculated with *A. flavus* in different isolates at laboratory condition (Table 34). Increase in vigour index by 9.47 per cent was also observed in case of control where kernels treated with methanolic extract of pomegranate fruit peel but not inoculated with *A. flavus* (Figure VII).

Table 34 - Effect of methanolic extract of pomegranate fruit peel on increase in vigour index of kernels inoculated with *A. flavus* over untreated but inoculated with *A. flavus*

Isolate No.	Increase in vigour index (%)	Isolate No.	Increase in vigour index (%)
AF-01	25.50	AF-16	19.67
AF-02	25.03	AF-17	16.94
AF-03	16.36	AF-18	16.39
AF-04	13.20	AF-19	9.63
AF-05	17.89	AF-20	21.82
AF-06	20.40	AF-21	17.03
AF-07	14.66	AF-22	11.72
AF-08	22.83	AF-23	12.51
AF-09	23.41	AF-24	14.27
AF-10	15.73	AF-25	20.97
AF-11	14.22	AF-26	10.12
AF-12	20.55	AF-27	14.77
AF-13	20.73	AF-28	13.99
AF-14	16.95	Control	9.47
AF-15	21.06		

5. DISCUSSION

Mycotoxin especially aflatoxin produced by *A. flauvs* fungus is a major constraint in groundnut production. Results obtained in the present investigation were discussed here under as follows.

5.1 Isolation of *A. flavus* and its identification

5.1.1 Collection of soil samples, isolation and Identification of *A. flavus*

A total of 37 soil sample were collected from groundnut fields of different location in nine districts of western Maharashtra and *A. flavus* isolated from 76 per cent soil samples. Similar work of soil sample collection from groundnut field soil of different geographic regions was done by several researchers and isolated the A. *flavus*. Present findings were in agreement with Vinod Kumar *et al,* (2008) who isolated *A. flavus* from 71 per cent soil samples of groundnut fields in Gujrat and maintained as single spore culture on agar slants. Also, Singh, (2010) collected soil samples from groundnut fields of Gujarat during summer season of 2005 and isolated 187 isolates of *A. flavus* from it.

These isolates were confirmed by macroscopic, microscopic characters and by growing on Aspergillus Differential Medium which develop non-sporulating, white mycelial growth and characteristic reverse orange yellow color after 42 hr incubation at 30°C. These results were in conformity to the work done by various research workers (Pitt *et al.,*1983 ; Somashekar *etal.,* 2004). Sepahvand *et al.,* (2011) identified 63 isolates as *A. flavus* by a combination of colony morphology, microscopic criteria. 53 strains were isolated by Amani *etal.,* [2012] from peanut fields of Iran and they produced yellow-green colonies on CZ, and CYA and showed yellow-orange reverse coloration on AFPA medium.

5.1.2 Influence of soil pH and soil type on *A. flavus* population density

Soil microbial community structures in peanut soils may be an important step in aflatoxin management since these communities greatly influence aflatoxin contamination (Lisker, *etal.*, 1993; Garber and Cotty 1997). Findings of the present investigation showed that *A. flavus* population observed in soil samples having wide range of soil pH (5.0 to 7.8). Maximum population load of *A. flavus* (2.67×10^3) in soil sample having pH 5.0 and 5.1 however minimum population load of 0.33×10^3 was observed in pH 7.5 and 7.8. Correlation study showed that, *A. flavus* population was negatively influenced by soil pH, means that maximum population was observed in acidic whereas minimum population in basic soil condition. This suggest that light acidic soil is most suitable for groundnut cultivation to reduce the population density of *A. flavus.* These findings were in confirmation with researchers (Hill *et al.,* 1985; Horn *et al.,* 1994) who studied mode and extent of invasion by *A. flavus* to groundnut pods which depends on soil population density during the pod development to maturity period which influenced by pH.

Variation in density of *A. flavus* population was observed in different soil types. However, soil type does not showed any relation with population load during investigation. This indicates that *A. flavus* population present in soil does not depends upon soil type but may be related to biochemical properties of soil. These findings are in support with Khandar *et al.*, (2004) who reported a wide variation in distribution of *Aspergillus* species in soil in some districts of Saurashtra region of Gujrat. Similarly, Barros *et al.*, (2005) studied the distribution of *A. flavus* population in the soil of Argentina's peanut growing regions and found wide variation in distribution of different phenotypes.

5.1.3 Influence of previous crop of different locations on *A. flavus* population density

Information regarding previous crop grown in the field were obtained during survey and observed that *A. flavus* population ranged from 0.33 x 10^3 to 2.67 x 10^3 was significantly influenced by previous crop grown in that field. Less population was observed in field soil sample in which onion, turmeric etc was grown as previous crop where as more population was observed in which maize, groundnut, cotton etc was taken as previous crop. Present findings were in confirmation with workers (Horn *etal.*,1995; Jaime-Garcia and Cotty, 2004; Mazzola, 2004) who reported the less population of *A. flavus* may be due to antifungal properties of onion, turmeric, garlic crop grown during previous season. Rotation sequences affect the relative abundance of *A. flavus* group of fungi in peanut soils were reported [Jaime-Garcia and Cotty, 2004; Mazzola, 2004). Vinod *etal.*, (2008) reported low soil population of *A. flavus* in Bhavnagar district (Gujrat), as the farmers from this district commonly followed groundnut-onion groundnut rotation. This may be due to root exudates of onion which have the adverse effect on the population of *A. flavus* in soil crop rotation with onion crop reduces the population of it from 6X10^3 to 2.67X10^3 where as in groundnut-groundnut system increases its population (Anonymous, 2013a). Thus rotation of groundnut crop with onion, turmeric, garlic etc reduces *A. flavus* population which serve as the best option for aflatoxin management.

5.1.4 Pathogenic nature of *A. flavus* isolates on groundnut pods and kernels

A. flavus isolates have different ability of pathogensis. Hence, twenty eight *A. flavus* strains isolated during present investigation were studied for seed colonization study on groundnut pods and kernels in laboratory. All the isolates were found to be pathogenic in nature. Colonization severity on pods (21.43 to 64.29 %) and on kernels (17.86 to 53.57 %) was observed. Present findings were in confirmation with Upadhyaya *et al.* (2001) who studied seed colonization by *A. flavus* under artificial inoculation conditions in the laboratory and found 13.6 per cent in J-11 and 46.6 per cent seed colonization in JL-24 cultivar. Also, Mohan *et al.* (2003) confirmed seed colonization on different groundnut genotypes against *A. flavus* and observed certain degree of variation. Present findings were also in confirmation with Rahmianna *et al.* (2004) who reported pathogenic nature of *A. flavus* and observed infection ranged between 3.3 to 14.7 per cent seed colonization. Also, Bora (2008) isolated *A. flavus* fungus from infected groundnut kernels and inoculated on healthy seeds of groundnut *in-vitro* for proving the pathogenicity and expressed typical symptoms of pale yellow green which later rot and rapidly reduced to a shriveled, dried brown to covered by yellow and green spores causing storage rot disease. At pod level, infection ranged from 0 to 7 per cent in resistant check J-11 and 26 to 97 per cent in susceptible check GG-20. At kernel level, infection ranged from 25 to 28 per cent in resistant check J-11 and 25 to 47 per cent in susceptible check GG-20 (Anonymous, 2013). This indicates the wide variation in pathogenic potential of different isolates isolated from different locations in western Maharashtra.

5.2 Morphological and cultural study of *A. flavus* isolates

5.2.1 Microscopic study of *A. flavus*

Microscopic study is an essential tools for identification of isolate. Microscopic characters of twenty eight isolates of *A. flavus* studied during present investigation and found variation in their microscopic characters *viz.,* conidia colour, conidia wall, conidia shape, conidial size etc. 67.85 per cent isolates showed parrot green whereas 32.14 per cent isolates shows olive green colour conidia. Conidial wall, observed from finely rough to smooth wall. 53.57 per cent isolates showed finely rough whereas 46.43 per cent isolates showed smooth conidial wall during study. Conidial shape was observed from globose (64.29 % isolates) to subglobose (35.71 % isolates) and its size ranges from 3.00 um to 4.25 um in diameter. These results were in confirmation with Mahajan (1994) who studied the morphological characters of *A. flavus* (green strain) and revealed that colonies were green, conidiophores hylines, 0.3 – 1 um long and rough walled, conidia globose to subglobose and 3 um in diameter. Various workers reported that the hyphae of *A. flavus* are well developed, profusely branched, septate, and hyaline; their cells are, as a rule, multinucleate, yellow-green colonies and smooth to finely rough globose conidia (Govrama and Bullerman, 1995; Alexopoulos *et al.,* 1996; Rodrigues *etal.,* 2009 and Reddy *etal.,* 2010).

5.2.2 Grouping of different *A. flavus* isolates

Twenty eight isolates of *A. flavus* were grouped into two different groups, A (67. 85 % isolates) and G (32.14 % isolates) on the basis of colony morphology on PDA medium. These results were in confirmation with Singh, (2010) who grouped 187 isolates of *A. flavus* isolated from groundnut field soils from Gujrat state into three classes *viz.,* parrot green, white fluffy with yellow sporulation and olive green. They observed 155 isolates into group A showing parrot green colour colony. High variability in colony morphologies, growth rates, colony color and levels of pigment production in *Aspergillus* spp were reported (Rinyu *et al.,*1995).

5.2.3 Phenotype study based on sclerotia producing ability

Several workers reported that aflatoxin production and sclerotia size is interrelated. Out of twenty eight isolates, CZ medium containing 3 per cent concentration of $NaNO_3$ was found to be most effective medium for sclerotia production on which 85.72 per cent isolates produced sclerotia. Findings of the present investigation were in confirmation with Cotty, (1989) who reported 3 per cent concentration of $NaNO_3$ was most effective for sclerotial production. Also, three colour of sclerotia *viz.,* Light brown, Dark brown and Black with globose to subglobose type of sclerotial shape were observed.

Sclerotial diameter range observed from 295.87um (AF-10) to 536.88um (AF-23) which grouped into L-phenotype (54.17 per cent isolates) and S-phenotype on the basis of their size during study. Similar findings were reported by Abbas, *etal.*, (2005) who proved that the L phenotype of the *A. flavus* was isolated more frequently than the S phenotype and represented 59 per cent of the total isolates (369 strains). Junxia and Minzhang (2009) analyzed *A. flavus* strains by using CZ medium and reported that isolates were of S and L type strains. About three-fifth of the isolates were L strains (60%) and only about two-fifth of the isolates were S strains

(40%). These findings were in additional confirmation with Amani *etal.,* (2012) who studied phenotype characters of *A. flavus* isolated from groundnut field and reported 56.6 per cent strains were capable to produce sclerotia all from L-type on CZ medium at 30^{o}C.

5.2.4 Cultural study of different isolates of *A. flavus*

More colony diameter indicates better ability of the fungus to utilize the available nutrition. With this view twenty eight isolates were grown on four different media viz., ADM, SMKY, CZ, YES for evaluating the suitability of medium. Growth of all the isolates among different medium showed a variation on 4th day of incubation. These findings were in confirmation with Bora (2008) who evaluated cultural characters of *A. flavus* on different media and found varying degrees of mycelial growth ranging from 51.2 to 89.0 mm in colony diameter in seven days. Colonies of *A. flavus* grow rapidly and the diameter will reach 6-7 cm in 10 to 14 days. The colour of colony is initially white, yellow and turns into yellow green or olive green. The shape is smooth and some have radial wrinkles (Patil 1963; Ruiqian *et al,* 2004 ; Singh 2010) Also, reported that the isolates of *A. flavus* isolated from soils of groundnut field grown on PDA medium varied significantly for their colony diameter.

5.3 Aflatoxin Detection by qualitative method

Several inexpensive cultural methods have been used for qualitative detection of aflatoxins *viz.,* Ammonia vapor test, observance under UV light, yellow orange pigmentation etc. However during the present investigation, comparative detection by these methods gives confirmation about aflatoxin synthesis by different isolates. Hence, these methods act as an important tool for detecting aflatoxin contamination when limited resources are available.

5.3.1 Ammonia Vapour Test

During the present investigation, among the isolates showing toxic nature, variation in toxicity observed on the basis of differences in red colour intensity of mycelium. Also, colour of sporulation changed from green to light brown on exposure to ammonia in both the toxic and non-toxic strains. Present findings were in confirmation with Saito and Machida, (1999) and Sangit kumar, *etal.,* (2007) who observed that the colony reverse of aflatoxin-producing strains of *A. flavus* turned pink when their cultures were exposed to ammonia vapor after 2 days incubation. Also, Singh, (2010) reported similar results in assessing *A. flavus* isolates.

This test is based on the production of yellow anthraquinone biosynthetic intermediates in the aflatoxin pathway which act as pH indicator dyes, which are more visible when they have turned red at alkaline pH (Abbas *et al.,* 2004a]. The pigments that turn red in response to ammonia exposure are not aflatoxins, but rather anthraquinone biosynthetic intermediates (e.g., averufin, nidurufin, versicolorin C, averantin, norsolorinic acid, versicolorin A and versicolorin A hemiacetal) and side products (e.g., methylated anthraquinone pigments) associated with the aflatoxin biosynthetic pathway (Shier *et al.,* 2005b). Abbas, *et al.* (2004b) reported the color response to ammonia vapour was in 100 per cent agreement with ELISA in the identification of the nontoxigenic isolates. Hence this technique would be ideal for pre screening of large numbers of *A. flavus* isolates in short time.

5.3.2 Observance under UV light

Observance of *A. flavus* grown on suitable medium under ultraviolet (UV) light of 365 nm wavelength for observance of a blue or green fluorescence, is one of the test for identifying aflatoxigenic or non- aflatoxigenic strain. During present investigation, 42.86 per cent isolates observed blue fluorescence ring surrounding the colony under UV light on 3^{rd} day of incubation while on 5^{th} day all the isolates observed blue fluorescence ring on the reverse side of the colony under UV light. But none of the isolate observed green fluorescence on the reverse side of the colony under UV light indicates the absence of G type aflatoxin. But on 7^{th} day incubation cultures were became difficult to evaluate as mycelial growth reached the margin of the plate and obscured the blue fluorescence. Principal involved in this test is that B-type aflatoxins are characterized by a cyclopentane E-ring and these compounds have a blue fluorescence under long-wavelength ultraviolet light while the G-type aflatoxins have a xanthone ring in place of the cyclopentane with a green fluorescence. These findings were in confirmation with Hara *et al.,* (1974); Lin and Dianese, (1976) who observed the fluorescence produced by strong aflatoxin producers began to appear within 32 hours after transfer to the medium. Rodrigues, *etal.,* (2009) reported the analysis of aflatoxin production of *A. flavus* isolates by fluorescence in CAM showed a good correlation with the HPLC results and observed that all strains producing a strong signal for AFBs on the HPLC chromatogram showed a marked blue fluorescence pattern on CAM after 3 days of incubation, whereas those producing a weak signal by HPLC showed a weak violet fluorescence on CAM. Hence, this method has been used as a presumptive screening method.

5.3.3 Orange yellow pigmentation

This technique was first identified by Wiseman *et al.* (1967). Also, production of abundant amounts of yellow pigment by *A. flavus* was reported by Arseculeratne *et al.* (1969), who observed the pigment production was noticeable from the second day in colonies on coconut agar medium. Results obtained during present investigation showed that 32.14 per cent isolates observed orange yellow pigmentation on 3rd day while 78.57 isolates observed orange yellow pigmentation on reverse side of plates on 5th day of incubation. It was also observed that degree of pigmentation seemed to be proportional to the intensity of fluorescence. These findings were in confirmation with Lin and Dianese (1976); Gupta and Gopal, (2002) who reported the UV fluorescence producing colonies of *A. flavus* on coconut agar medium always produced a orange-yellow pigmentation. The pigmentation was seen before the appearance of fluorescence and the degree of pigmentation seemed to be proportional to the intensity of UV fluorescence. Therefore, the aflatoxin producing ability of an isolate could be estimated presumptively by the intensity of pigmentation in coconut-agar medium without the use of a UV lamp is one simple and chief technique for quick identification of aflatoxin positive isolates.

Mycotoxin producing ability of all the isolates tested by above methods were compared with each other for confirmation of suitability of method. Among the isolates observed, only two isolates *viz.,* AF-07 and AF-12 showed non-toxic nature by ammonia vapour test. However, all the 100 per cent isolates were showed toxic nature by UV florescence method while six isolates *viz.,* AF-05, AF-08, AF-10, AF-12, AF-20 and AF-25 showed non-toxic nature by orange yellow pigmentation method on 5th day of incubation. Hence, if all the above methods are used combinely then it gives more accuracy in identification of aflatoxigenic and non-aflatoxigenic strains of *A. flavus*. Also for doing the test of UV observance and orange yellow pigmentation coconut-agar medium found to be best as this medium offers easy detection of aflatoxin production as the presence of fluorescence is rapid, specific and association of aflatoxin production with an orange-yellow pigmentation due to white colour of medium which permits the quick identification of aflatoxin positive isolates.

5.4 Morphological diversity of different *A. flavus* isolates

Morphological variability of 28 isolates of *A. flavus* were analyzed and found significant variation among them. Dendrogram showed two major clusters, cluster A and cluster B at similarity coefficient in between 0.18 to 0.91. The number of isolates included in each cluster varied. Cluster A consist of 18 isolates however cluster B consist 10 isolates and showed similarity, dissimilarity in morphological characters *viz.,* conidial colour, conidial wall, conidial size, conidial shape, sclerotial shape, sclerotial size, sclerotial colour, growth pattern, isolate group, yellow orange pigmentation and aflatoxin synthesis ability. Thus, it may be said that morphological characters of *A. flavus* may be good enough for diversity analysis. Similar findings were reported by Pitt and Klich, (1988) who showed that conidial wall texture is the most effective criterion for distinguishing *A. flavus.* Variability in the *A.flavus* in soil reported by Horn and Dorner (1998). These findings were in confirmation with Singh (2010) who done diversity analysis of all 187 *A. flavus* isolates on the basis of morphological characters and grouped isolates into fifteen distinct clusters.

5.5 Aflatoxin quantification by chromatography technique

Chromatography is one of the efficient technique for accurate quantification of aflatoxins. Maximum aflatoxin B_1 synthesized by isolate AF- 14 (1380 ng/ml) followed by AF- 28 (1346.67 ng/ml) while minimum by isolate AF-07, AF-12 (600 ng/ml) on 9^{th} day of incubation was observed. However aflatoxin B_2 synthesis was not detected in any isolate during present investigation. Findings were in confirmation with earlier workers Taber and Schroeder, (1967); Maggon *etal.,* (1969) and Hatti *etal.,* (2009) who studied aflatoxin synthesis ability of *A. flavus* isolates and reported its range from 0 to 349 ppb under the test conditions employed. Also, Mehan *etal.,* (1995) reported similar results of aflatoxin production potential of 48 *A. flavus* isolates obtained from groundnut fields of different soils types in India by TLC technique and observed that all isolates tested produced only AFB_1 ranging from 1.0 to 290 ug/g seed (Amani *et al.,* 2012; Chakranarayan and Patil, 2013).

Methanolic extract of aflatoxin B_1 in individual flask was rated for colour intensity and found positive correlation showing higher the aflatoxin more dense was the yellow colour of the extract. Thus, the apparent correlation of the aflatoxin production with methanolic extract colour may be used in some instances for quickly estimating the amount of aflatoxin produced by a fungus culture, However, dry mycelial mat weight ranged from 1.63 (AF-21) to 3.42 (AF-25) g per 50 ml of SMKY broth on 9^{th} day of incubation and did not appeared to correlate with aflatoxin synthesis. These findings were in support with Arrus *etal.,* (2005) who reported the biomass in terms of mycelial growth did not appear to correlate with aflatoxin production.

5.6 Chemotype Classification of *A. flavus* Strains

Knowledge of population diversity of *A. flavus* species in each locality would be very useful in local agricultural management practices. During present investigation, all isolates were classified into seven chemotypes based on AFB_1, AFB_2 and sclerotia production patterns. 85.71 per cent isolates produced both AFB_1, sclerotia, grouped into chemotype-I while all isolates produced only AFB_1, grouped into chemotype-II and found that the most represented chemotype. None of the isolates were found in chemotype-III, IV, V, VI and VII. These results were in accordance with Pildain *etal.,* (2004) who showed characteristics of *A. flavus* strains isolated from peanuts seeds from Formosa (Argentina). Rodrigues, *etal.,* (2009) studied the incidence of chemotypes of *A. flavus* based on mycotoxigenic profile and found that *A. flavus* isolates were assigned to 3 of the 5 chemotypes. Also, Sepahvand, *etal.,* (2011) isolated 60 *A. flavus* isolates from air, surface and soils of five hospitals in Southwest Iran and classified into four chemotypes (I to IV). Also, Amani *etal.,* (2012) studied distribution and toxigenicity of *A. flavus* and chemotype classification of *A. flavus* isolates based on the ability for producing mycotoxins and sclerotia.

In the present study, no relationship between production of sclerotia/size in *A. flavus* isolates with aflatoxin production was observed. However production of sclerotia by *A. flavus* isolates in relation to mycotoxin production has been a matter of controversy. Overall, the data from present study clearly showed that the diverse populations of *A. flavus* strains producing mycotoxins are present in peanut field soils as potential threats for agriculture and public health. Strains have a considerable variation in the aflatoxin producing ability and their chemotype group.

5.7 *In-vitro* screening of groundnut cultivars against *A. flavus*

Use of cultivars resistant to seed invasion by aflatoxin producing fungi is one of the possible means of reducing aflatoxin contamination of groundnut as it is effective and low-cost part of an integrated aflatoxin management programme. During present investigation none of the cultivar was found to be resistant to *A. flavus* seed colonization. However pod and kernel colonization severity observed wide range of variation from 43.33 to 90.00 per cent and 40.00 to 83.33 per cent, respectively on 7th day of incubation. Cultivars Phule Unnati (43.33%) showed less colonization severity on pod while Phule Unap (40.00 %) on kernel on 7th day of incubation. However maximum colonization severity was recorded on JL-24 cultivar up to 90 per cent.

These results are in confirmation with Varma *et al.,* (2001); Gowda *et al.* (2002); Narasimhulu (2007) and Annonymous, (2012) who conducted several laboratory test for colonization study and observed resistant as well as susceptible groundnut genotypes. Resistance to pod infection is attributed to pod-shell structure, while resistance to seed invasion and colonization is mostly physical, and has been correlated with thickness, density of palisade cell layers, absence of fissures and cavities, and presence of wax layers (Upadhyay *et al.,* 2002). Hence, in aflatoxin management programme, use of cultivars showing resistance or less colonization severity may be a good option.

5.8 Efficacy of botanicals against *A. flavus* seed colonization and aflatoxin synthesis

5.8.1 Antifungal activity of plant extract against *A. flavus* (PFT)

Chemical fungicides is an effective and efficient way for the management of *A. flavus.* But same cannot be applied to grains for reasons of pesticide toxicity, also WHO banned many agriculturally important pesticides due to wide range of toxicity against non-target organisms including humans. This causes soil, air pollution and there is problem of label clam in most of the commodities. Moreover, the increasing resistance of microbes against antimicrobial drugs has posed another problem in the use of synthetic preservatives. Plant extracts of many higher plants have been reported antibacterial, antifungal and insecticidal properties. Hence, with this intension during present investigation plant extracts of various botanicals were used and observed significant differences among them.

Among the different plant extracts tested, the extract of pomegranate (*P.granatum*) fruit peel was statistically found to be effective as compare to other plant extracts with minimum colony diameter of 33.00 mm having poor sporulation with 63.33 percentage growth inhibition over control and no aflatoxin was detected. However in control treatment colony diameter was observed 90.00 mm with abundant sporulation on 8th day of incubation and aflatoxin was detected qualitatively. Plant extracts of *A. indica* leaves, Bulb of *A. cepa* were also found effective.

5.8.2 Antifungal activity of methanolic extract of pomegranate fruit peel against *A. flavus* (PFT)

Antifungal activity of methanolic extract of pomegranate fruit peel against *A. flavus* was observed effective and concentration having 3000 ppm, minimum (32.33mm) colony diameter without sporulation with maximum (64.07%) growth inhibition was observed over control and no aflatoxin was detected. It was followed by concentration of 2500 ppm. These findings were in confirmation with Mahmoud (1999) and Nur, (2004) who studied laboratory experiments by using various plant extracts and found significant differences among them. Also, Bora (2008) reported that extract of *Allium sativum* L was found to be effective against *A. flavus* with the inhibition of 79.22 per cent. Also, Sudhakar *etal.,*(2009) reported Methyleugenol 0.5 per cent inhibited *A. flavus* colonization completely on PDA which contain peanut substrate.

5.8.3 Antifungal activity of methanolic extract of pomegranate fruit peel on pods and kernels against *A. flavus*

Methanolic extract of pomegranate fruit peel at 3000 ppm concentration was proved to be effective interms of inhibition of fungal growth on pods and kernels. Also, inhibition in aflatoxin synthesis was observed in kernels under laboratory condition. These findings were in confirmation with several workers who demonstrated the antifungal activity of plant derived chemicals. Sudhakar *etal.,* (2009) who reported the spray of methyleugenol at 0.5 per cent on peanut pod and kernels checked the colonization of *A. flavus* and aflatoxin synthesis. Also the potential of certain plant extracts for the reduction of AFB_1 in maize by Sanchez, *et al.,*(2005) and in stored rice was investigated by Reddy *etal.*,(2009).

Thus the present study demonstrated the usage of extract of pomegranate fruit peel, a natural waste material is an ideal alternative to protect groundnut pods and kernels from post-harvest infection of *A. flavus* and aflatoxin synthesis therein. Also, it is safer to use as antifungal agents compared to available chemical fungicides which limit groundnut for human consumption.

5.9 Effect of methanolic extract of pomegranate fruit peel on growth parameters of kernels inoculated with *A. flavus*

5.9.1 Germination Per centage

Germination per centage were significantly influenced in case of kernels inoculated with different *A. flavus* isolates. Also, significant differences of methanolic extract of pomegranate fruit peel were observed in kernels treated with this extract and then inoculated with *A. flavus* isolates. Germination per centage ranged from 81.33 to 94.00 per cent. Kernels treated with methanolic extract of pomegranate fruit peel and inoculated with *A. flavus* showed increase in germination per centage from 2.54 to 9.57 per cent over untreated methanolic extract of pomegranate fruit peel and inoculated with *A. flavus* in different isolates at laboratory condition. Also, 1.44 per cent increase in germination was observed in case of control where kernels treated with methanolic extract of pomegranate fruit peel but not inoculated with *A. flavus*.

5.9.2 Vigour index

Vigour index in case of kernels untreated with methanolic extract of pomegranate fruit peel but inoculated with *A. flavus* isolates influenced significantly. Also, significant differences of methanolic extract of pomegranate fruit peel were observed in case of kernels treated with it and then inoculated with *A. flavus* isolates. Increase in vigour index over untreated methanolic extract of pomegranate fruit peel and inoculated with *A. flavus* in different isolates were observed. In case of control where kernels treated with methanolic extract of pomegranate fruit peel but not inoculated with *A. flavus*. 9.47 per cent increase in vigour index was observed.

These results were in confirmation with earlier workers (Khadem *et al,* 1966; Truelove *etal.,* 1970) findings who reported that *A. flavus* affect the plants by inhibition of seed germination, elongation of the hypocotyls or roots of developing seeds. Increase in germination, vigour index in groundnut kernels may be attributed due to presence of metabolites such as phenolics, alkaloids, saponins, glycosides, steroids, terpenoids and flavonoids etc. Findings of Janardhan *etal.,* (2011) also confirmed that *A. flavus* infection showed significant differences for germination, shoot length, root length, dry matter production and vigour index in groundnut seeds. Among different concentrations, 5.0% inoculated seeds recorded 60% germination and vigour index 2427 and 1452 was reported by Begum *etal.,*(2013).

This suggests that pomegranate fruit peel which is consider as a waste material, abundant, available free of cost and simple to apply could be an important source of new antimicrobial compounds which treatment to seed enhances seed germination, vigour index etc hence have an utility during sowing of seed as seed treatment.

6. SUMMARY AND CONCLUSIONS

The word mycotoxin is derived from the Greek language (mekes-mushroom and toxikon-toxic or poison) is non-volatile, low molecular weight compound produced as byproduct during the primary metabolic process of the fungi that grow on seeds and feed in the field, or in storage. The major mycotoxin producing fungi are species of *Aspergillus*. Among mycotoxins, aflatoxin is one of the most important as these are compounds with a hepatotoxic, carcinogenic, and teratogenic potential that can affect either humans or animals health even if present in microgram quantities in the diet. Aflatoxins are considered a major public health problem worldwide, especially in developing countries where facilities for long term storage of food and food products are often inadequate and high temperature and high humidity encourage the growth of molds. Primary target organ for aflatoxin is the liver and it causes liver damage, resulting in liver cancer. Indian Childhood Cirrhosis (ICC) is a serious disorder of the liver in children. Aflatoxin B_1 is classified as a group I carcinogen and comsumption of food contaminated with it by dairy cows results in the excretion of AFM_1 in milk and found in animal urine, milk or tissues. Now a days aflatoxins are considered a major public health problem worldwide, especially in developing countries.

Groundnut (*Arachis hypogea* L.) is an important oilseed as well as legume crop which is also known as peanut, earthnut, monkey-nut, goobers etc and their products are called “high aflatoxin risk materials”. Ability of *A. flavus* isolates to produce aflatoxins varies greatly depending on geographic conditions and strains isolated. The aflatoxin problem was first recognized in 1960 with the report of an outbreak of disease among approximately one lakh Turkey poultry in England. Harmful effects posed by aflatoxins are teratogenicity i.e. deformation of developing fetus, reduction in RBC, WBC and hemoglobin content in blood, delayed blood clotting and suppression of immune system in case of chronic poisoning. Acute exposure to high levels of aflatoxin leads to aflatoxicosis, which can result in rapid death from liver failure. Africa, India and South-East Asia are regarded as the “high-aflatoxin-risk areas” as aflatoxin production is more of a concern in humid tropical regions. Soil itself act as the main reservoir for its survival. *A. flavus* is a major pathogen associated with aflatoxin production and was isolated from 76 per cent soil samples out of 37 soil samples collected from different districts of groundnut fields *viz.* Nashik, Dhule, Jalgaon, Ahmednagar, Solapur, Pune, Satara, Sanagli and Kolhapur in western Maharashtra during summer season of 2013 indicates fungus is distributed through out all districts. These isolates were confirmed by macroscopic and microscopic characters and by growing on Aspergillus Differential Medium.

Data obtained during present investigation observed that soil pH influences the *A. flavus* population density. Maximum population was noticed in acidic condition whereas minimum population in basic soil condition that indicates soil pH negatively influences population density of it. Also, less density of it was observed in field sample in which onion, turmeric crop was grown as previous crop where as more population was observed in field sample in which maize, groundnut, cotton etc was taken as previous crop. This suggest that groundnut crop should be rotated with onion, turmeric etc crops to minimize population density of pathogen. However soil type *viz.,* light, medium, heavy black etc does not found any correlation with population density of it.

Variation in pathogenic nature of different isolates was observed on pods and kernels during pathogenicity test. Colonization severity ranged from 21.43 to 64.29 per cent on pods while 17.86 to 53.57 per cent on kernels in cultivar SB-11 on 5^{th} day of incubation during *in-vitro* study. Also variation in macroscopic and microscopic character *viz.,* group of isolates, scloerotial colour, diameter, type, shape, conidial colour, size, wall etc was observed. CZ medium containing 3 per cent concentration of $NaNO_3$ was found to be most effective medium for sclerotia production study on which 85.72 per cent isolates produced sclerotia. 54.17 per cent isolates produced L- type whereas 45.83 per cent isolates produced S-type sclerotia. Relation between sclerotial production/size and aflatoxin synthesis was not observed during present investigation. However, earlier workers reported S-type isolates produces more aflatoxin. Growth of all isolates on different medium showed a significant variation. Aspergillus differential medium (ADM) was non-sporulating medium, on which only white mycelial growth on upper side whereas pink pigmentation on back side of petriplate was observed. However, on Sucrose Magnesium sulphate Potassium nitrate (SMKY), Czapeks medium (CZ) and Yeast Extract Sucrose (YES) medium, poor to profuse sporulation was observed.

Mycotoxin producing ability of all the isolates were tested by different methods *viz.,* ammonia vapour test, UV fluorescence and orange-yellow pigmentation and compared with each other for confirmation of suitability of method. All the three methods were found simple, economical, less time consuming and effective for rapid screening of isolates for aflatoxin detection. Coconut agar medium (CAM) was found to be best medium for aflatoxin detection by UV fluorescence and orange-yellow pigmentation method. Total

26, 28 and 22 isolates showed positive test to ammonia vapour test, UV fluorescence and orange-yellow pigmentation, respectively. Hence, use of these methods combinely act as an important tool for detecting aflatoxin contamination when limited resources are available. Aflatoxin G_1, G_2 was not observed during qualitative detection under UV florescence.

Dry mycelial mat weight of fungus ranged from 1.70 (AF-16) to 3.42 (AF-25) g per 50 ml of SMKY broth on 9^{th} day of incubation and aflatoxin B_1 synthesized by all isolates in broth quantified by chromatography technique ranged from 600 (AF-07, AF-12) to 1380 (AF-14) ng/ml. Aflatoxin B_2 synthesis was not detected in any isolate during present investigation. Also, intensity of yellow colour of the methanolic extract was correlated with concentration of aflatoxin B_1 analyzed by chromatography technique and found positive correlation. However dry mycelial weight of *A. flavus* was not showed any correlation with aflatoxin production. Isolates of *A. flavus* were analyzed for their diversity on the basis of similarity coefficient analysis. Dendrogram of it resulted in two major clusters, cluster A and cluster B at similarity coefficient in between 0.18 to 0.91. All the isolates studied were classified into different chemotypes based on mycotoxin production patterns, phenotype characters and found diversity among them.

Different released cultivars of groundnut were evaluated for studying resistance to *A. flavus* and observed that none of the cultivar was resistant to *A. flavus* pod and kernel colonization. However, minimum colonization severity of 43.33 per cent on Phule Unnati and maximum of 90.00 per cent on JL-24 in case of pods while minimum colonization severity of 40.00 per cent on Phule Unap and maximum of 86.67 per cent on JL-24 in case of kernels was observed on 7th day of incubation during *in-vitro* screening. Hence, in aflatoxin management programme, use of cultivars showing resistance or less colonization severity may be a good option.

In antifungal effect of different plant extract studied by poisoned food technique, water extract of pomegranate fruit peel showed minimum of 33.00 mm colony diameter having poor sporulation and 63.33 percentage growth inhibition over control on 8th day of incubation. Antifungal effect of methanolic extract of pomegranate fruit peel was studied by poisoned food technique at different concentrations, among them the treatment of 3000 ppm concentration showed 64.07 percentage growth inhibition over control having 32.33 mm colony diameter without sporulation on 8th day of incubation. Also, aflatoxin synthesis was not detected by qualitative methods during this study. On pods and kernels of groundnut, methanolic extract of pomegranate fruit peel was found effective at laboratory condition. It showed minimum pod colonization severity of 26.67 per cent with 72.88 per cent growth inhibition over control whereas minimum kernel colonization severity of 26.67 per cent with 70.37 per cent growth inhibition over control on 8th day of incubation at 3000 ppm concentration. Also, no aflatoxin B_1 synthesis was detected in this treatment by chromatography technique.

Effect of methanolic extract of pomegranate fruit peel on different growth parameters were studied in germination paper. Kernels treated with methanolic extract of pomegranate fruit peel and then inoculated with *A. flavus* showed increase in germination percent (2.54 to 9.57 %) and vigour index (9.63 to 25.50 %) respectively over untreated methanolic extract of pomegranate fruit peel but inoculated with *A. flavus* in different isolates at laboratory condition. Hence, this have an utility during sowing of seed as seed treatment to enhance seed germination and vigour index.

Thus, pomegranate fruit peel which is considered as waste material, are abundant, available free of cost and simple to apply. These renewable bio-resources can successfully replace chemical antifungal agents. Thus, results support the idea that extract of pomegranate fruit peel which is considered as a waste material are economical, simple candidate for management of *A. flavus* colonization, thereby aflatoxin synthesis in groundnut kernels with enhancement in seed germination and vigour index.

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8. APPENDIX

Media composition:

1. *Aspergillus flavus* and *parasiticus* agar medium (AFPA): Peptone-10.0 g, Yeast extract-20.0 g, Ferric Ammonium Citrate-0.5 g, Agar powder-15.0 g, Dichloron-2.0 mg, Chloramphenicol- 0.2 g and distilled water-1000 ml

2. Potato Dextrose Agar medium (PDA): Pealed potato-200 g, Dextrose-17.0 g, Agar powder-17.0 g and distilled water-1000 ml.

3. Aspergilus Differential Medium (ADM): Tryptone-15 g, Yeast Extract- 10 g, Ferric citrate- 0.5 g, Agar powder- 15 g and distilled water- 1000 ml.

4. Coconut agar medium (CAM): Coconut milk-600 ml, Agar powder-16 g and distilled water-200 ml.

5. Sucrose Magnesium sulphate Potassium nitrate Yeast extract (SMKY liquid) media: Sucrose-200 g, Magnesium sulphate- 0.5 g, Potassium nitrate-3 g, Yeast extract-7 g and distilled water-1000 ml.

6. CZ (Czapeks) medium: Sucrose-30 g, $NaNO_3$-3 g, KH_2PO_4- 0.5 g, K_2HPO_4-0.5 g, $MgSO_4$:$7H_2O$-0.5 g, KCL-0.5 g, Micronutrients-1ml, Agar- 20 g and distille water-1000 ml.

7. Yeast Extract Sucrose: Yeast extract-20 g, Saccharose-150 g, Magnesium sulphate-0.5 g, Agar powder-20 g and distilled water-1000 ml.

9. VITA

Manoj Arun Gud

A candidate for the degree

of

DOCTOR OF PHILOSOPHY (AGRICULTURE)

in

PLANT PATHOLOGY

Title of the thesis	:	"Mycotoxin- Producing Ability and Chemotype Diversity of *Aspergillus flavus* from soils of Groundnut Growing Regions of Western Maharashtra"
Major field	:	Plant Pathology
Minor field	:	Agriculture Botany, Entomology and Agriculture Biochemistry
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Control

Inoculated

Plate I - Pathogenic nature of *Aspergillus flavus* on pods of groundnut

Control

Inoculated

Plate II - Pathogenic nature of *Aspergillus flavus* on kernels of groundnut

Plate III- Growth of different *Aspergillus flavus* isolates on SMKY medium

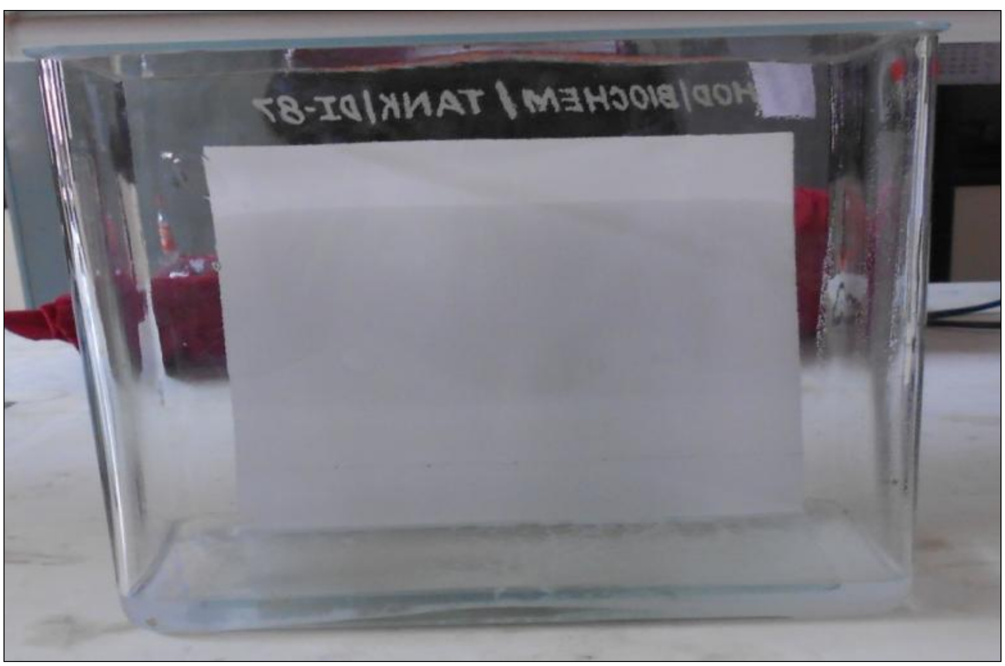

Plate IV- Development of Chromatogram for aflatoxin quantification

Plate V- Screening of pods of groundnut cultivars against *Aspergillus flavus*

Plate VI- Screening of kernels of groundnut cultivars against *Aspergillus flavus*

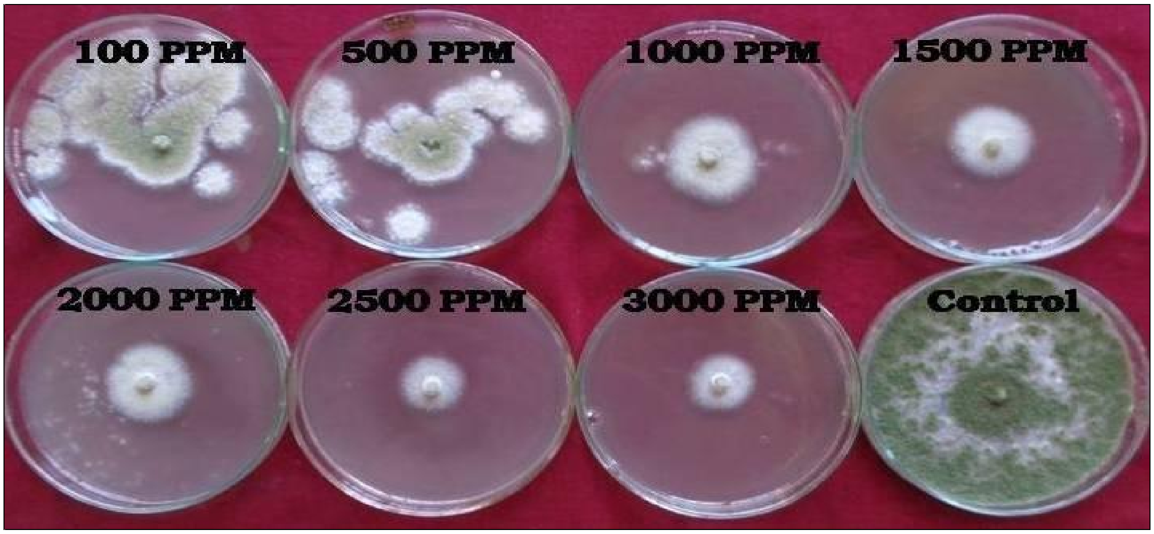

Plate VII- Antifungal activity of methanolic fruit peel extract of pomegranate against *Aspergillus flavus* (PFT)

Control **Treated**

Control **Treated**

Plate VIII - Antifungal effect of methanolic fruit peel extract of pomegranate against *Aspergillus flavus* on kernels and pods

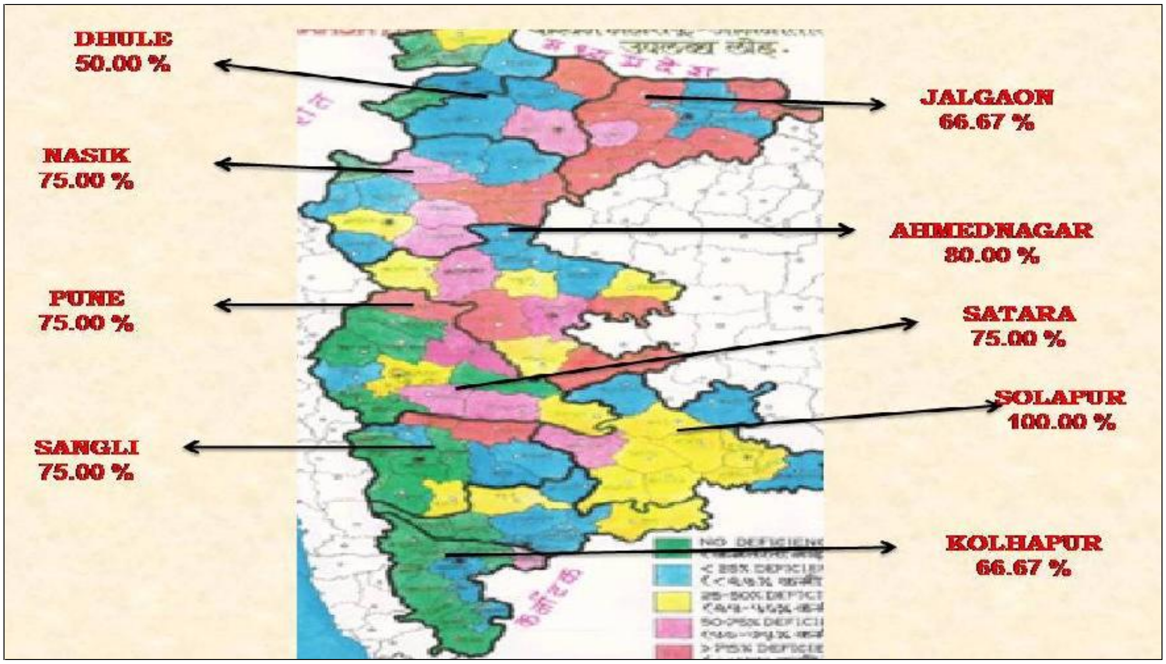

Fig. I - Percent soil samples in which *Aspergillus flavus* obtained in groundnut fields of western Maharashtra

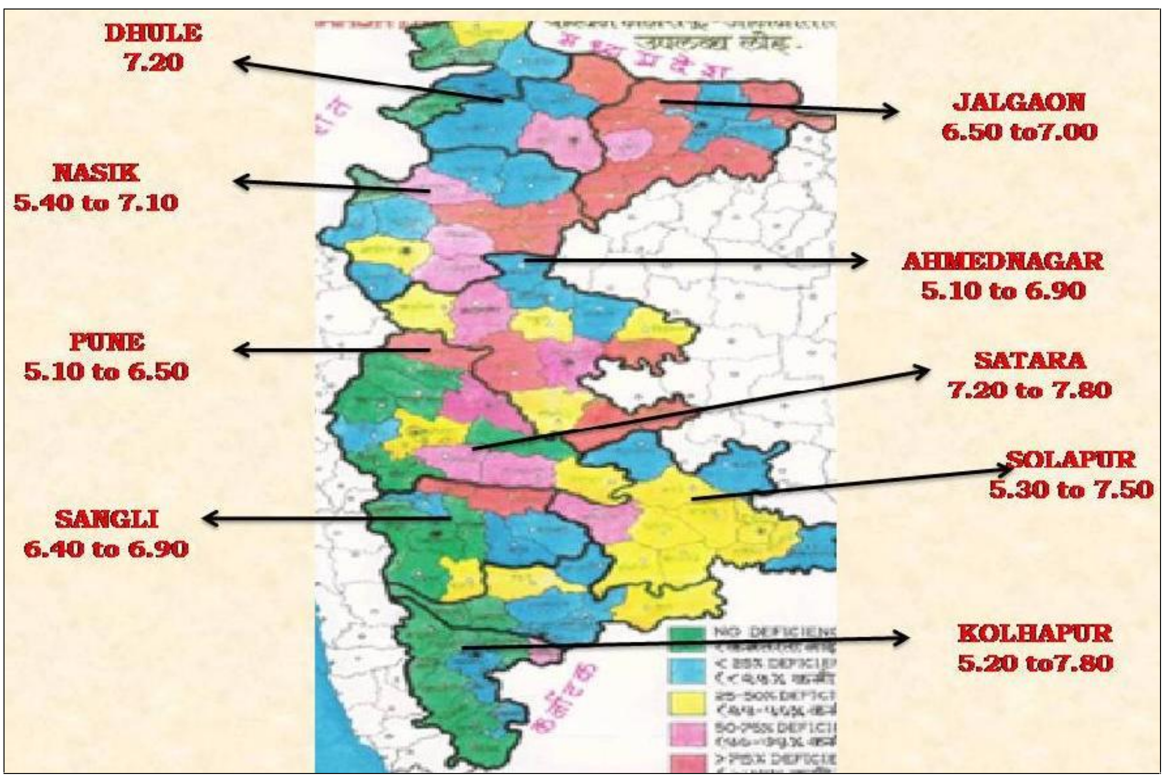

Fig. II - *Aspergillus flavus* (average CFU per g of soil at 10^3) isolated from soil samples of groundnut fields of western Maharashtra

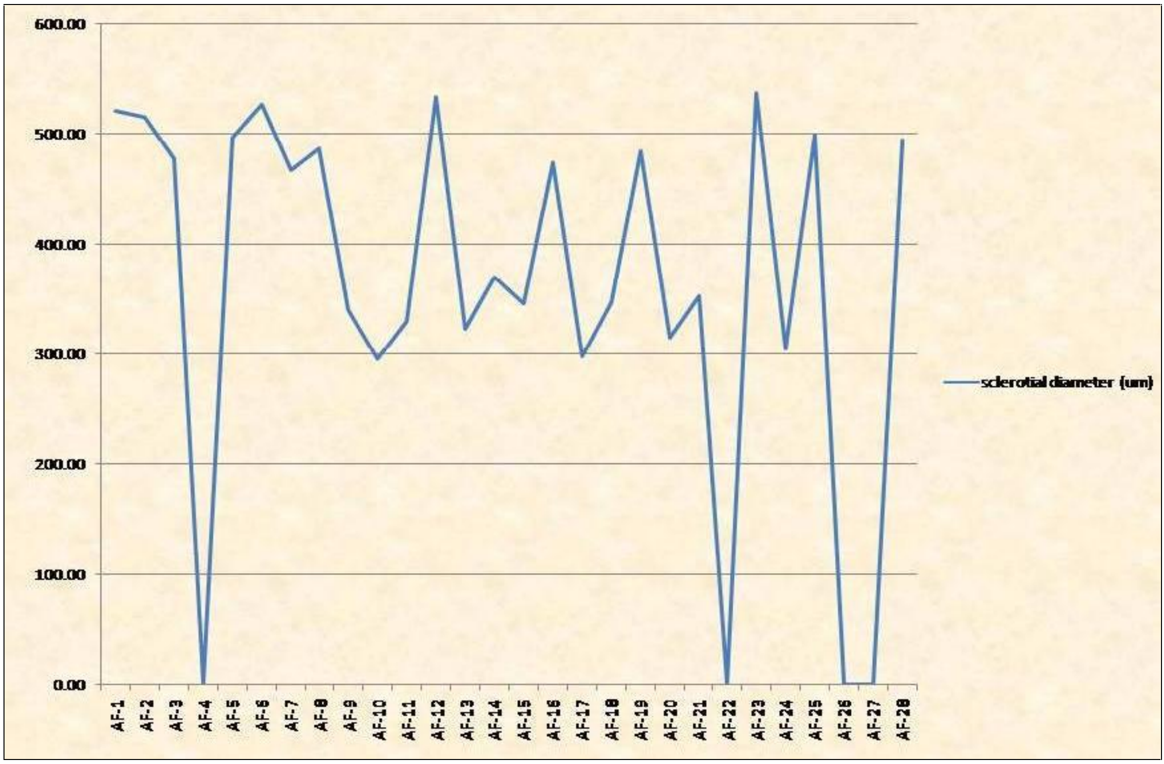

Fig. III - Sclerotial diameter of different *Aspergillus flavus* isolates in um

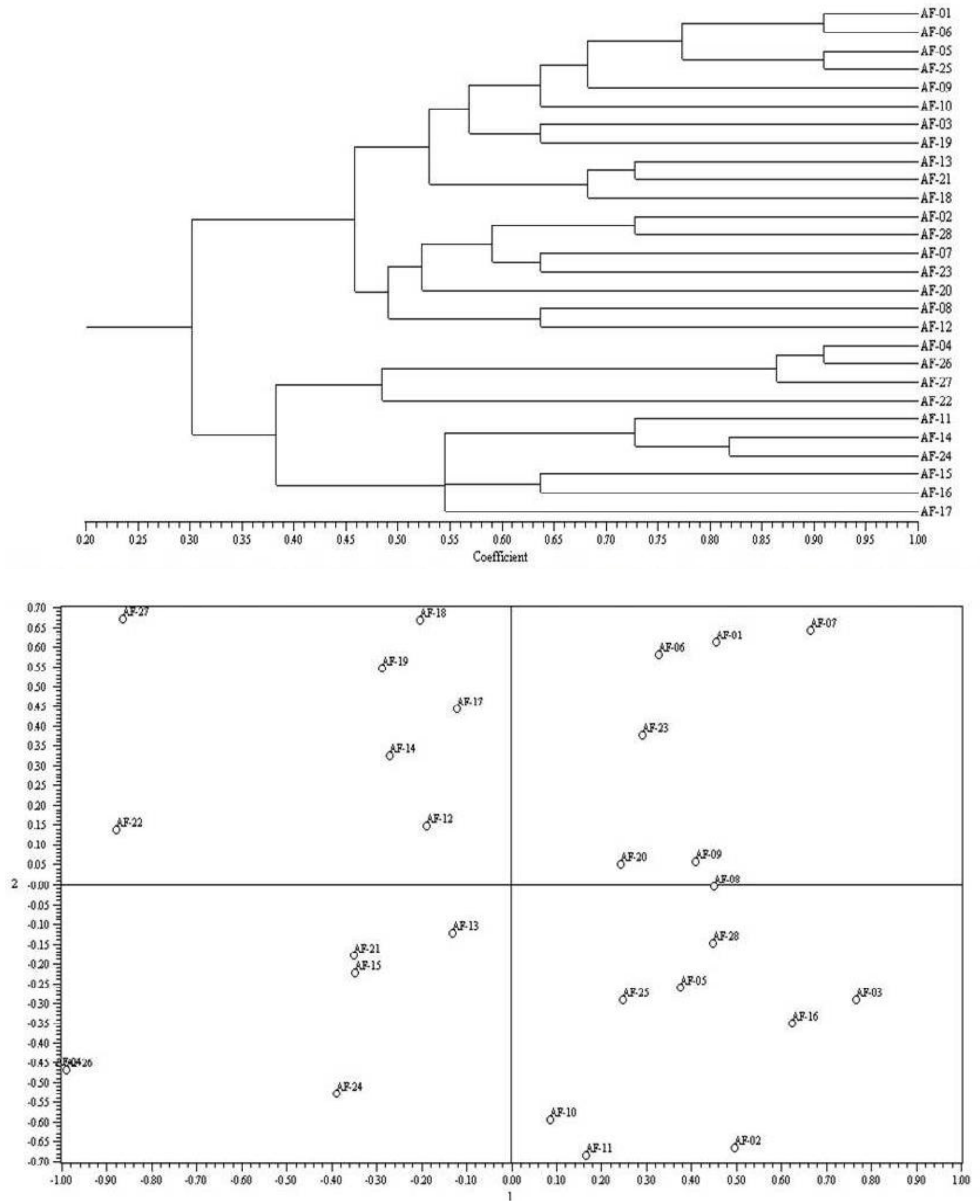

Fig. IV - Morphological diversity of different *Aspergillus flavus* isolates based on similarity coefficient and 2-D scatter plot analysis

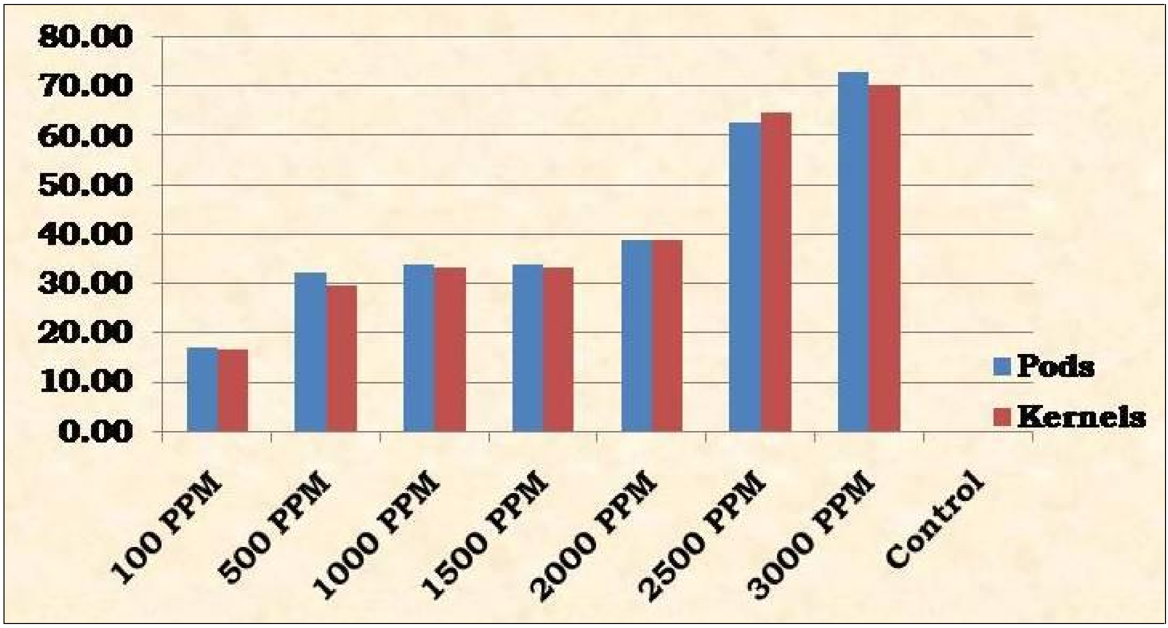

Fig. V -Effect of methanolic extract of pomegranate fruit peel on growth inhibition (%) of *Aspergillus flavus* (on 8th day) on pods and kernels of groundnut

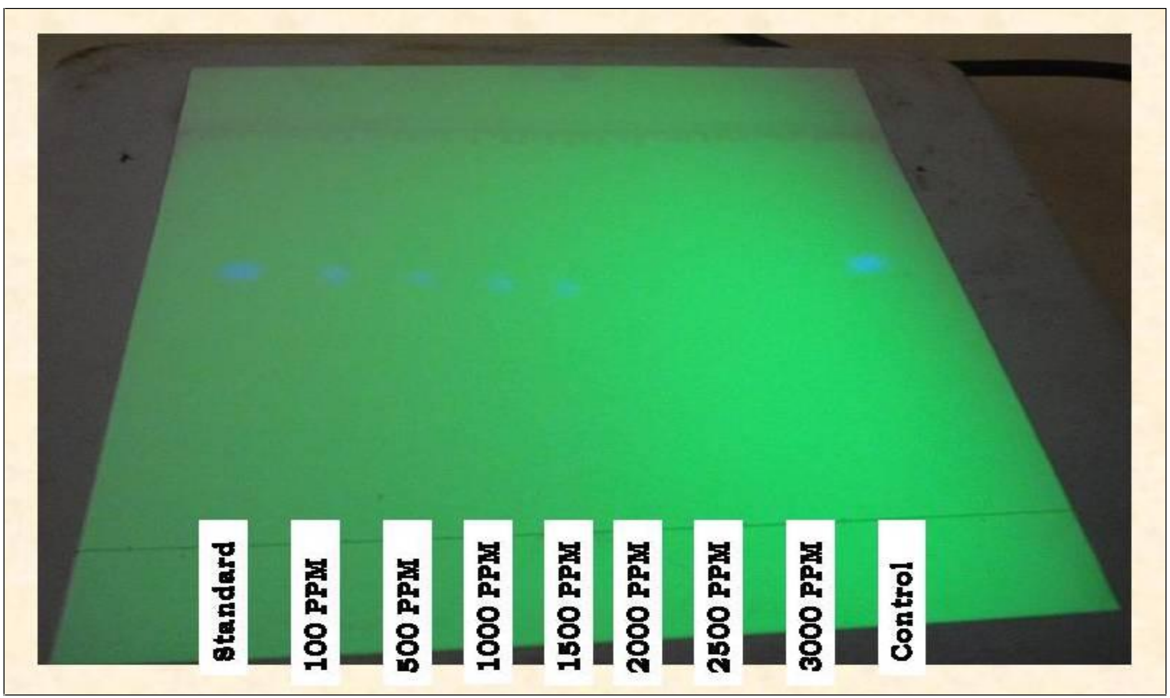

Plate IX- Inhibition of aflatoxin B_1 synthesis due to methanolic extract of pomegranate fruit peel in groundnut kernel (8th day)

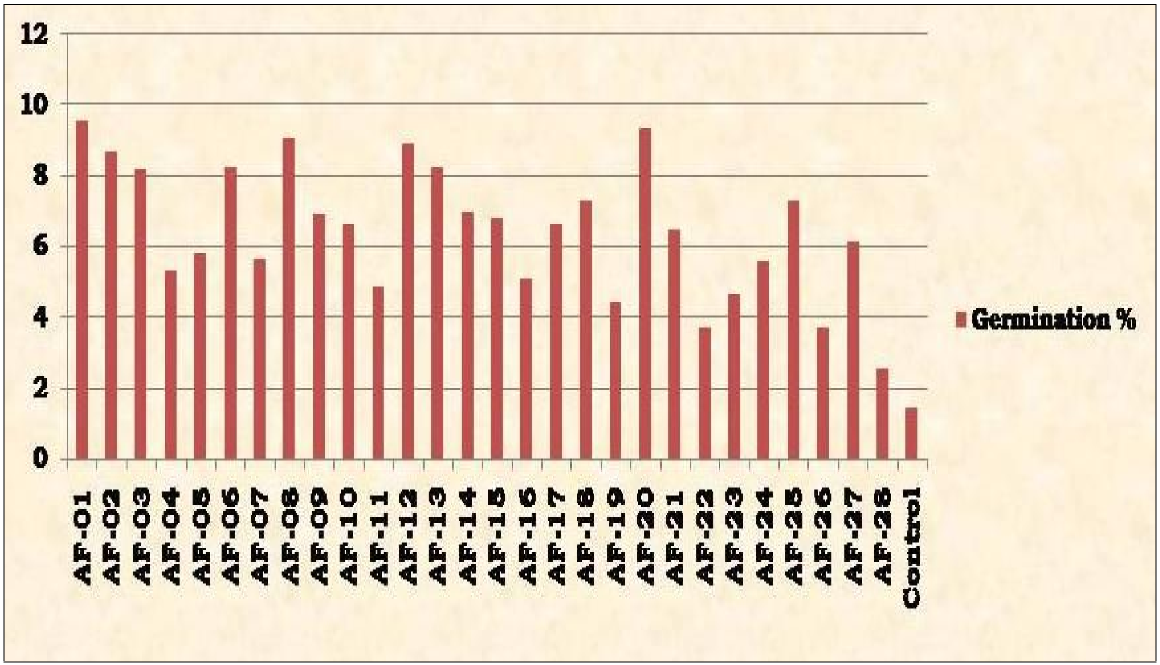

Fig. VI - Effect of methanolic extract of pomegranate fruit peel on increase in germination percentage of kernels inoculated with *Aspergillus flavus*

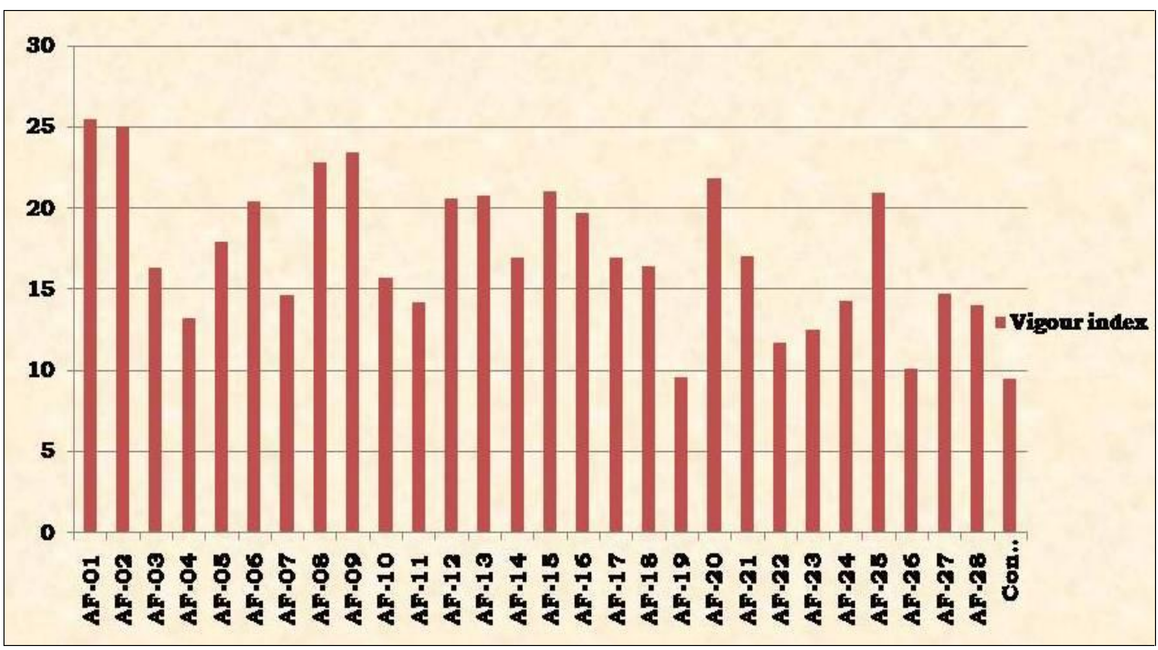

Fig. VII - Effect of methanolic extract of pomegranate fruit peel on increase in vigour index of kernels inoculated with *Aspergillus flavus*