EFFECT OF *Aspergillus flavus* ON GROUNDNUT SEED QUALITY AND ITS MANAGEMENT

GUNTHA ADITHYA

B. Sc. (Ag.)

THESIS SUBMITTED TO THE
PROFESSOR JAYASHANKAR TELANGANA STATE AGRICULTURAL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE IN AGRICULTURE
(SEED SCIENCE AND TECHNOLOGY)

CHAIRPERSON: Dr. B. RAJESWARI

DEPARTMENT OF SEED SCIENCE AND TECHNOLOGY
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2016

DECLARATION

I**, Mr. G. ADITHYA** hereby declare that the thesis entitled **"EFFECT OF *Aspergillus flavus* ON GROUNDNUT SEED QUALITY AND ITS MANAGEMENT"** submitted to the Professor Jayashankar Telangana State Agricultural University for the degree of **Master of Science in Agriculture** is the result of original research work done by me. I also declare that any material contained in the thesis has not been published earlier in any manner.

Date: **(G. ADITHYA)**

Place: Hyderabad **I. D. No. RAM/14-51**

CERTIFICATE

Mr. G. ADITHYA has satisfactorily prosecuted the course of research and that the thesis entitled **"EFFECT OF *Aspergillus flavus* ON GROUNDNUT SEED QUALITY AND ITS MANAGEMENT"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that neither the thesis nor its part thereof has been previously submitted by him for a degree of any University.

Date:
Place: Hyderabad

(Dr. B. RAJESWARI)
Chairperson

CERTIFICATE

This is to certify that the thesis entitled **"EFFECT OF *Aspergillus flavus* ON GROUNDNUT SEED QUALITY AND ITS MANAGEMNT"** submitted in partial fulfilment of the requirements for the degree of **Master of Science** in Agriculture of the Professor Jayashankar Telangana State Agricultural University, Hyderabad is a record of the bonafide original research work carried out by **Mr. G. ADITHYA** under our guidance and supervision.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part and all assistance received during the course of the investigations have been duly acknowledged by the author of the thesis.

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*Date***:** **GUNTHA ADITHYA**

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ABBREVATIONS

A*f*	:	*Aspergillus flavus*
CFU	:	Colony forming units
cm	:	Centimeter
CRD	:	Completely Randomized Design
ELISA	:	Enzyme- linked immunosorbent assay
et al.	:	Co-workers
etc	:	And so on
Fig	:	Figure
g	:	Gram
i.e.,	:	That is
Kg	:	Kilogram
$KMNO_4$	:	Potassium permanganate
L	:	Litre
M	:	Molar
M	:	Million
mg	:	Milligrams
ml	:	Milliliter
mm	:	Millimeter
Mt	:	Million tones
N	:	Normal
NA	:	Nutrient Agar
ng	:	nano gram
nm	:	Nano metre
No.	:	Number
^0C	:	Degree Centigrade
PDA	:	Potato Dextrose Agar
Pg	:	Pictogram
pH	:	Hydrogen ion concentration
Sp	:	Species
viz.,	:	Namely
µg/Kg	:	Microgram per kilogram
%	:	Per cent
@	:	At the rate of
µg	:	Microgram(s)
µL	:	Microlitre
CD	:	Critical difference
DAS	:	Days after sowing
Fig	:	Figure
ha	:	hectares
TFC	:	Total fungal colonies
S.Em (±)	:	Standard error of mean
min	:	minutes
h	:	hours

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ABSTRACT

Groundnut (*Arachis hypogaea* L.) is an important oil seed crop in India. It contains oil to an extent of 48 - 51 %. The major problem associated with groundnut is aflatoxin contamination. It is mainly caused by *Aspergillus flavus* and *Aspergillus parasiticus.* Groundnut being an oil seed, it contains lesser amount of carbohydrates than cereals but more amount of oil and protein and they break down into simple sugars and amino acids which is essential for germinating seed as an energy source. Several management strategies were adopted to minimize the aflatoxin problem *viz.*, development of resistant varieties, use of biocontrol agents and cultural practices. Keeping this in view, the present findings pertaining to the present investigations were carried out on detection of seed mycoflora, mode of entry of *A. flavus* into groundnut seed, effect of *A. flavus* on seed and oil quality and evaluation of bioagents and fungicides in the management of *A. flavus* of groundnut under glasshouse conditions.

A total of seventy two groundnut (72) pod samples comprising farmer samples (36) and market samples (36) from major groundnut growing districts of Telangana state during 2015 - 2016. The seed samples were analysed for seed health by agar plate method as per ISTA (1996). Significant differences in occurrence of total number fungal colonies due to location and source of seed samples were observed. Irrespective of the districts, total per cent occurrence of seed mycoflora was found high in farmer samples (92.4 %) over market samples (45.3 %). Out of four districts, samples of Mahabubnagar district (47.1 % & 26.2 %) followed by Warangal district (43.5 % & 24.6 %) recorded more total number of fungal colonies in farmer and market samples. Irrespective of the samples, occurrence of six fungal flora *viz., A. flavus, A. niger, Fusarium* sp. *Alternaria* sp. *Macrophomina* sp. *Penicillium* sp. were observed. Among them, *A. flavus* (43.2 %), *A. niger* (26.7 %) were found predominant in both farmer and market samples.

External seed colonization due to *A. flavus* in groundnut resistant cv. J 11 and susceptible cv. JL 24 were observed at different days of incubation period indicated that resistant groundnut cv. J 11 inoculated with *A. flavus* colonized the seeds with severity score of 1, 2, 3, 4 and susceptible cv. JL 24 inoculated with *A. flavus* colonized the seeds with disease severity score of 2, 3, 4, 4 at 3, 5, 7 and 9 days after incubation period.

The mode of entry of pathogen into groundnut seed was studied by Scanning Electron Microscopy. Groundnut seeds of resistant and susceptible cultivars inoculated with *A. flavus*

toxigenic strain, the penetration and establishment of the fungi in case of J 11 was slow compared to JL 24. The present investigation reveals that *A. flavus* is seed borne in nature and contaminated seeds were important source of inoculum for seed infection and spread of the fungus from one seed to another during storage.

The per cent reduction in oil content was high in susceptible groundnut cv. JL 24 (18.3%) as compared to resistant groundnut cv. J 11 (9 %). While the reduction in oil content was less in the untreated seeds of groundnut cv. JL 24 and groundnut cv. J 11 (13.7% and 6%). Overall the per cent reduction in the protein content was found high in susceptible groundnut cv. JL 24 (16.3 %) as compared to resistant groundnut cv. J 11 (6.5 %). While the reduction in protein content was less in the untreated seeds of groundnut cvs. JL 24 and J 11 (14.2 % & 5.1 %).

The per cent reduction in the unsaturated fatty acids like linoleic and oleic acids were high in susceptible groundnut cv. JL 24 (17.5 % and 16.6 %) as compared to resistant groundnut cv. J 11 (15 % and 14 %). Whereas in the untreated seeds, the per cent reduction in linoleic and oleic acids were found low (11.3 % and 6 %) in cv. J 11 and 13.3 % and 8.2 % in cv. JL 24, respectively. The increase levels of saturated fatty acids viz., palmitic and stearic acids were high in susceptible cv. JL 24 (4.5 % & 4.5 %) as compared to resistant cv. J 11 (3.9 % & 2.93 %). Where as in untreated seeds, the increased levels in palmitic and stearic acids were found low (2.5 & 2 %) in cv. J 11 and 2.9 % and 1.94 % in groundnut cv. JL 24 respectively.

The aflatoxin content at 1 to 56 days after incubation increased in groundnut cv. J 11 (2.15 µg/kg - 2861.3 µg/kg) & 63.4 µg/kg - 4077.1 µg/kg in cv. JL 24, respectively. In the untreated seeds there was low level aflatoxin content of 2.15 µg/kg - 14.7 µg/kg in cv. J 11 and 2.15 µg/kg - 21.1 µg/kg in cv. JL 24 were recorded.

The efficacy of seed treatments against seed borne *A. flavus* were evaluated under glasshouse conditions. Groundnut seeds treated with *T. harzianum* was significantly superior in recording higher seed germination (96 %), plant height (4.75, 12.9 and 14.1 cm) and yield (4.60 g) followed by *T. viride* (91 %, 4.10, 11.5 and 13.5 cm, 4.20 g) which was on par with *P. fluorescens* (88.2 %, 3.40, 10.2 and 10.8 cm 4.10 g). The remaining seed treatments were also found effective in improving seed germination, plant height and yield in seeds treated with carbendazim (81 %, 2.77, 8.02 and 9.21 cm, 3.65 g), mancozeb (73.5 %, 2.72, 6.92 and 8.43 cm, 3.37 g) over untreated (65 %, 2.67, 6.62 and 7.37 cm, 29.2) and pathogen treated seeds (54.5 %, 2.57, 5.65, 6.41 cm, 2.55 g) at 15, 30, 45 DAS.

Aflatoxins were detected in pathogen treated seeds (1.38 µg/kg) and untreated seeds (0.69 µg/kg) which is below permissible level. While aflatoxin was not observed in the seed treated with *T. harzianum*, *T. viride* and *P. fluorescens.*

CHAPTER I

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is one of the important oil seed crop grown all over the world. India stands first in production and area under legumes in the world. The major crops include groundnut, gram, pigeon pea, green gram and black gram. India is one of the largest producers of oilseeds in the world and occupies an important position in the Indian agricultural economy. The seeds of groundnut contains oil to an extent of 48 - 51 %. The crop is a rich source of protein, dietary fiber, minerals and vitamins.

In India, groundnut is cultivated during *kharif* under rainfed conditions and in *rabi* and summer seasons under irrigated conditions. 90 - 95 % of the total crop area is grown in *kharif* season. It is cultivated in an area of 25.4 M ha worldwide with an annual production of 45.20 M t and productivity of 3824 kg ha^{-1} (FAO, 2013). In India, the crop is grown to an extent of 5.53 M ha with a production of 9.67 M t and productivity of 1750 kg ha^{-1} (INDIASTAT, 2013). In united Andhra Pradesh, the crop is grown to an extent of 1.37 M ha with a production of 1.01 M t and productivity of 890 kg ha^{-1}. In Telangana state, the crop is grown to an extent of 0.21 M ha with a production of 0.35 M t and productivity of 1690 kg ha^{-1} (Directorate of Economics and Statistics, 2015).

Availability of good quality seeds of high yielding varieties is the key to increase the productivity. Groundnut production all over the world is limited by various biotic and abiotic constraints that results in severe yield reduction. Seed borne pathogens affect the seed quality and lower the yield. Knowledge on the type of the pathogen associated with farmers seed and their effect on seed quality helps in adopting suitable strategies to manage them.

Deterioration in seed quality of groundnut is mainly due to *A. flavus* which makes the product unfit for marketing and consumption. In groundnut, seed and seedling decay and aflarot diseases were caused due to *A. flavus* pathogen. Aflatoxin contamination in groundnut kernels possesses a great threat to humans and live stock health as well as international trade. According to FAO estimates, 25 % of world food crops are affected by mycotoxins each year and also crop losses due to aflatoxin contamination. Considering the significance of the aflatoxins, several countries including FAO has fixed the tolerance limits for groundnut and its by-products. India and USA has fixed the tolerance limit of 30 and 20 µg/kg of seed which is meant for human consumption purpose. Aflatoxin contamination of agricultural crops such as groundnut and cereals causes annual

losses of more than $750 million in Africa. There are four major types of aflatoxins (namely Aflatoxin B_1, Aflatoxin B_2, Aflatoxin G_1 and Aflatoxin G_2) among 18 structurally related mycotoxins (Bennet, 2010). Aflatoxins designated by B_1 and B_2 shows strong blue fluorescence under UV light, whereas G_1 and G_2 forms shows greenish yellow fluorescence.

In addition to this, environmental conditions also play a major role in the attack of these molds and the crop is affected at various stages such as pre, post-harvest and storage conditions (Waliyar *et al*., 2008). The extent of aflatoxin contamination in groundnut oil mills/traders gives an indication of the prevalence of this qualitative problem. The pathogen produces aflatoxin as a secondary metabolite in the seeds of number of crops both before and after harvest. It is a potent carcinogen that is highly regulated in most of the countries.

Groundnut seeds are known to harbor several species of seed borne fungi *viz., Aspergillus flavus, Aspergillus niger, Macrophomina phaseolina, Rhizoctonia solani, Fusarium oxysporum, F. solani* were predominant in groundnut and seed coat was greatly infected by fungi followed by cotyledon and axis (Rasheed *et al.,* 2004). Species of *Aspergillus, Penicillium, Fusarium, Rhizopus* and *Alternaria* are commonly occurring post harvest moulds in storage conditions (Chavan, 2011). The major problems associated with groundnut is aflatoxin contamination. It is mainly caused by *A. flavus* and *A. parasiticus* (Shephard, 2003 and Strosnider *et al.,* 2006). The pathogen is saprophytic soil fungus that infects and contaminates during pre and post-harvest stages of the groundnut crop.

Groundnut being an oil seed, it contains lesser amount of carbohydrates than cereals but more amount of oil and protein and they break down into simple sugars and **amino acid**s which is essential for germinating seed as an energy source. Reduction in oil and protein content and increased levels of free fatty acids were noticed in the stored kernels than in the pods due to invasion of storage fungi (Ramamoorthy and Karivaratharaju, 1989). Presently, there are no tools that would measure the total oil content of groundnut seeds, economically and non-destructively. Groundnut pods have to be shelled and cleaned before oil content is measured which takes time and resources. Near-infrared reflectance spectroscopy (NIRS) is effectively utilised for analysis of chemical and physical properties without sample preparation and applied for the analysis of quality characteristics in food and agricultural commodities (Batten, 1998; Williams & Norris, 2001). The effect of *A. flavus* on seed quality *i.e*., aflatoxin levels in the groundnut seed samples were estimated by using an indirect competitive ELISA method (Enzyme-Linked Immuno Sorbent Assay). Ultrastructural studies in understanding the mode of entry of *A. flavus* pathogen in groundnut resistant and susceptible cultivars through scanning electron microscopy was useful to identify the fungal structures and facilities correct diagnosis and detailed examination of taxonomic characters of seed borne fungi.

For successful production of any crop the seed must be sound and free from seed mycoflora which interfere with seed germination and subsequent emergence of the crop. Seed treatment with bioagents and fungicides is an economical and viable approach to protect seed and seedlings from attack of the pathogens. Several management strategies were adopted to minimize the aflatoxin problem *viz*., development of resistant varieties, use of biocontrol agents and cultural practices. However, genetic resistance is not available in the cultivable groundnut germplasm. Hence, to control aflatoxin problem in groundnut biological control is considered as one of the viable options. Fluorescent *Pseudomonads* as bacterial biocontrol agents were effectively utilised in reducing pre-harvest aflatoxin contamination. During root colonization, these bacteria produce antifungal antibiotics and elicit induced systemic resistance in the host plant or interfere specifically with fungal pathogenicity factors. Several isolates of *Trichoderma* and *Pseudomonas* were characterized for their antagonism against *A. flavus* and for their biocontrol potential (Anjaiah and Thakur, 2000 and Desai *et al*., 2000).

Keeping this in view, the present study has been proposed with the following objectives.

1. To study the extent of seed mycoflora contamination in groundnut kernels at farmers and traders level.
2. To study the effect of *A. flavus* infection on seed and oil quality.
3. To study the effect of seed treatments under glasshouse conditions against *A. flavus* in groundnut.

CHAPTER II

REVIEW OF LITERATURE

The available literature on Effect of *Aspergillus flavus* on groundnut seed quality and its management has been reviewed in this chapter. As the available literature on these aspects is scanty, the literature pertaining to other crops has also been reviewed under the following headings.

2.1 Effect of aflatoxins on human beings

2.2 Economic importance

2.3 Etiology of *A. flavus*

2.4 Seed mycoflora and its detection

2.5 Ultra structural studies

2.6 Effect of *A. flavus* on oil quality in groundnut

2.7 Estimation of oil and fatty acids by Near Infrared Reflectance Spectroscopy (NIRS)

2.8 Estimation of aflatoxins by Enzyme Linked Immunosorbent Assay (ELISA)

2.9 Management of *A. flavus* in groundnut using bioagents and fungicides under glasshouse conditions

2.1 EFFECT OF AFLATOXINS ON HUMAN BEINGS

Aflatoxins are well recognized as a cause of liver cancer and other additional toxic effects. In farm and laboratory animals chronic exposure to aflatoxins reduces immunity and interferes with protein metabolism and multiple micronutrients that are critical to health. These effects have not been studied in humans but the available information indicates that at least some of the effects observed in animals were also observed in human beings.

Wild *et al*. (2002) reported the toxicity of aflatoxins by studying DNA adduct induction, mutagenicity and carcinogenicity which is paralleled by the development of biomarkers of aflatoxin exposure and biological effects which applied to human populations.

Agag *et al.* (2004) reported that aflatoxins affect the liver in which cytochrome P450 enzyme convert aflatoxins and capable of binding to both DNA and proteins. Inactivation of the p53 tumor suppressor gene leads to the development of liver cancer.

Crop storage conditions were frequently conducive for fungal growth and mycotoxin production which effect on human health. The study also reveals that Aflatoxin B_1 has been linked to liver cancer such as immune suppression and growth faltering (Shepard, 2008).

Liu *et al*. (2010) conducted a study of quantitative cancer risk assessment by collecting global data on food-borne aflatoxin levels, consumption of aflatoxin-contaminated foods and hepatitis B virus (HBV) prevalence. It indicates that cancer potency of aflatoxin for HBV-positive and HBV-negative individuals as well as uncertainty in all variables to estimate the global burden of aflatoxin - related hepatocellular carcinoma (HCC).

Zain (2011) studied the impact of different categories of mycotoxin like aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins and ergot alkaloids on human health. Further they reported that different factors which influence the presence of mycotoxins in food were related to storage and other extrinsic factors such as climate or intrinsic factors such as fungal strain specificity, strain variation and instability of toxigenic properties.

Eva *et al.* (2007) studied the impact of aflatoxins which causes different types of diseases and disorders in human beings and animals.

Ding *et al.* (2012) reported the effect of aflatoxins on human dietary risk and their effects on children by studying the 1040 groundnut seed samples collected from China after post harvest of the crop.

2.2 ECONOMIC IMPORTANCE

The economic importance of aflatoxins derive directly from crop and livestock losses as well as indirectly from the cost of regulatory programs designed to reduce risks to animal and human health. Aflatoxin losses to livestock and poultry producers from aflatoxin contaminated feeds include death and more effects on immune system suppression, reduced growth rates and losses in feed efficiency. Other adverse economic effects of aflatoxins were include lower yields for food and fiber crops.

The ability of aflatoxins to induce cancer and related diseases in humans indicates their unavoidable occurrence in food and feeds make the prevention and detoxification of mycotoxins is one of the most challenging toxicology issues of present time.

Health risks associated with aflatoxin consumption decreases labour productivity besides increasing health costs and overall income losses due to opportunity costs linked to lost days of work (Lubulwa and Davis, 1994).

According to Food and Agricultural Organization (FAO), 25 % of the world food crops are significantly contaminated with mycotoxins (Boutrif and Canet, 1998). It is estimated that

approximately $ 450 million annual loss to all food exporters if all nations harmonized to EU aflatoxin standards (Wu, 2004). Further, $ 670 million annual loss was incurred to African food exporters from attempting to meet EU aflatoxin standards (Otsuki *et al*., 2001).

Aflatoxins were considered to have significant negative impact on health, food and nutritional security and income at the household, community and national levels (Coulibaly *et al*., 2008).

Mathur *et al.* (2015) reported that biotechnological tools to reduce aflatoxin contamination through *A. flavus* by studying physical, biological and chemical methods.

2.3 ETIOLOGY OF *A. flavus*

Moreno *et al.* (1999) reported that *A. flavus* is propagated through conidial germination and by hyphal growth. Sclerotial formations on maize kernels naturally infected by *A. flavus* and buried sclerotia were able to withstand cold temperatures in the soil.

Afjal *et al.* (2013) studied *Aspergillus* species using macroscopic characteristics such as colony growth, conidial color, colony reverse and microscopic characteristics including conidiophore, vesicle, matulae, phialides and conidia for its identification.

Ihenacho *et al.* (2014) studied the morphological and molecular features of *A. flavus* and *A. parasiticus* with the help of electrophoretic technique by studying DNA patterns.

2.4 SEED MYCOFLORA AND ITS DETECTION

Kishore *et al*. (2002) recorded the presence of mycotoxins and toxigenic fungi in 182 groundnut samples collected from farmers fields in five districts of Andhra Pradesh i.e., Anantapur, Chittoor, Cuddapah, Kurnool and Mahabubnagar districts during rainy seasons of 1999 and 2000. The seed infection due to *A. flavus* range was 11.9 % to 18.3 % and 9.5 % to 14.1 % in 1999 and 2000, respectively were recorded in each district.

Rasheed *et al.* (2004) studied the seed mycoflora of 12 groundnut seed samples by blotter, agar plate and deep freeze method. Of the 14 genera, 28 species of fungi were isolated. All the groundnut seed samples were infected by *A. flavus* where as 17 % with *R. solani* and *Alternaria citri*, 33 % with *M. phaseolina* and 58 % with *Fusarium* sp. *Aspergillus* sp., as detected by blotter method.

Nakai *et al*. (2008) reported the occurrence of seed mycoflora in stored groundnut samples in Brazil. The results showed that predominance of *Fusarium* sp. (67.7 % in hulls and 25.8 % in kernels) and *Aspergillus* sp. (10.3 % in hulls and 21.8 % in kernels) and the presence of five other fungal genera. The toxigenic potential revealed that 93.8 % of the *A. flavus* strains isolated were producers of AFB1 and AFB2 toxins.

Ibiam *et al.* (2011) evaluated fresh, cooked and fried seeds of three varieties of groundnuts (*Arachis hypogaea* L.) Nwakara, Kaki and Campalla were screened to determine the post harvest seed borne fungi. Fungi were not observed in fresh seeds of Nwakara and kaki varieties, where as *A.niger, A. flavus, A. terreus, A. culmorum, A. fumigatus, A. nidulans, A. tamarii, F. moniliforme, Mucorrouxii, Penicillium spp, Cladosporium spp* and *Aureobasidium pullulans* were found associated with fried and cooked seeds of the three groundnut varieties Nwakara, Kaki and Campalla.

Rathod *et al.* (2012) assessed the seed mycoflora of different varieties of legumes by standard blotter paper, agar plate and seed wash methods. The results showed that agar plate method was found effective with less incubation time and recording high percentage of seed mycoflora.

Naqvi *et al.* (2013) studied seed quality *viz.*, seed germination percentage, per cent pathogen frequency and major seed-borne fungi of 30 groundnut seed samples. Fungi most frequently isolated in groundnut seed were *Alternaria, Aspergillus*, *Fusarium*, *Helminthosprium* and *Rhizopus.* The per cent pathogen frequency of seed-borne fungi was found high in groundnut (73.0 %).

Mohammed and Chala (2014) collected 270 groundnut samples from three districts of Eastern Ethiopia and the incidence of infected groundnut kernels ranged from 50 to 80 % at the district level. Heavy infestation of groundnut samples by various molds including *A. niger, A. flavus, A. ochraceus, A. parasiticus* and *Penicillium* species were recorded with the kernel infection varied kernel infection of 36.3 and 100 %.

Mohammed *et al*. (2015) studied 270 groundnut samples from farmers and storage fields and local markets of three districts of Eastern Ethiopia for mycological analysis. *A. flavus* and *A. niger* were isolated in higher frequencies from samples collected from farmers fields and stores than markets while *A. parasiticus* was consistently isolated at higher frequencies than market samples. At the district level, the incidence of infected groundnut kernels was ranged from 50 - 80 %.

Ramannuj *et al*. (2014) evaluated seed mycoflora of soybean, sorghum and groundnut in 18 villages of different zones of Madhyapradesh. Seed germination percentage, per cent pathogen

frequency and major seed-borne fungi were identified using blotter method. The per cent pathogen frequency of seed-borne fungi was high in groundnut 73.0 % and low in soybean 15.3 %.

Nagpurne *et al.* (2014) conducted survey from Udgir region to detect seed mycoflora of five different varieties of groundnut using blotter paper and agar plate methods. Seven genera of 12 species of fungi were isolated from this method. From the above study higher number of fungi *Aspergilli* was isolated by blotter paper method (80 %) as compared to agar plate method (70 %).

Gintling *et al*. (2015) studied the physical quality and infection levels by collecting 16 groundnut samples from farmers, collectors, retailers and food processors in Banjarnegara District, Central Java. On an average, the moisture content of groundnut kernels was 8.8 %, while the damaged kernels (46.7 %) and *A. flavus* infection (45.1 %) were considerably high. From the above study high damaged kernels and infection of *A. flavus* need to be decreased through proper handling and storage practices.

2.5 ULTRASTRUCTURAL STUDIES

Mohan *et al.* (2003) screened 13 confectionary groundnut genotypes against *A. flavus* seed colonization. The results revealed that cultivated groundnut genotypes showed stable resistance to *A. flavus* and certain degree of resistance to seed colonization.

Ultra-structural studies using scanning electron microscopy for characterization of *A. flavus* on sugarcane was found that conidia had two distinct ornamentations. The results confirmed that conidia of *A. flavus* have relatively thin walls which were finely to moderately roughened. Further, the conidial shape varied from spherical to elliptical (Rodrigues *et al*. 2007).

Achar *et al.* (2009) observed *A. flavus* infected groundnut kernels using light microscopy in combination with electron microscopy to describe the infection course established by the pathogen.

Alvis *et al.* (2012) studied the SEM methodology to observe the interaction of fungi on seed surface of cotton, common bean, soybean and maize. It has more advantage as compared to genome techniques by considering the small structures of the fungi were measured and quantified by the images stored in the computer and the results were analyzed in comparison with the conventional methods.

2.6 EFFECT OF *A. flavus* ON OIL QUALITY IN GROUNDNUT

Deshpande *et al*. (1979) studied the colonization and biochemical changes in groundnut seeds infected with *A. flavus*. The results showed that growth of *A. flavus* on various groundnut seed samples resulted an increased levels of free fatty acids and decreased levels of protein content.

Deteriorative changes in oilseeds due to *Aspergillus* sp. recorded loss in protein content due to *A. terreus.* It was found that fat content in groundnut and soybean was reduced due to *A. flavus* (Chavan *et al.* 2003).

Maharous (2007) studied the chemical properties of *A. flavus* infected seeds exposed to different levels of γ-irradiation during storage. The results revealed that there was no effect of irradiation at different dose levels on moisture, protein, total lipids and amino acid content of the seeds over a period of 60 days of seed storage.

Fagbohun *et al.* (2012) reported the nutritional and mycoflora changes in groundnut during twenty weeks of storage period. A total of seven fungal species *viz., A. flavus, A. niger, A. fumigatus*, *Rhizopus* sp., *Penicillium* sp., *Mucor* sp. and *Fusarium* sp. were recorded. Fungal colonization and contamination of stored groundnut were found to reduce the market value, edibility and depletion of nutrients.

Ameer *et al.* (2013) reported the relationship between seed borne pathogens and seed quality deterioration of stored groundnut. The study reveals that a progressive decrease in germination percentage, oil and protein content and increase in free fatty acid content in stored groundnut kernels than pods were observed.

Begum *et al.* (2013) studied the relationship of *A. flavus* infection on seed quality by artificial inoculation of *A. flavus* on seeds of groundnut cultivar VRI 2. Seeds with 0.25 % infection maintained germination up to 71 % at the end of the storage period. Hence, this stage could be the tolerable limit for the safe storage of groundnut seeds.

Bhushan *et al.* (2013) reported that high moisture content in the groundnut seeds resulted in more fungal infection with *Aspergillus* sp. Aflatoxin contamination was higher at 18 % moisture content as compared to other moisture regimes.

Adiver *et al.* (2015) conducted survey in 14 districts of Karnataka, India to assess the severity of *A. flavus.* The results revealed that high incidence of *A. flavus* in the samples collected

from market areas. In inoculated groundnut seeds, the infection led to the reduction in sugars, proteins, oil content and seed germination.

2.7 ESTIMATION OF OIL AND FATTY ACIDS BY NEAR INFRARED REFLECTANCE SPECTROSCOPY (NIRS)

Ki won *et al*. (2000) studied 46 perilia and 83 groundnut samples to estimate lipid and protein contents using NIRS equation development and validation and concluded that this method was useful for mass screening of lipid and protein contents.

Sundaram *et al.* (2010) reported that groundnut oil and fatty acid concentration of Virginia and Valencia types of in-shell groundnut using NIR reflectance spectroscopy. The average total oil concentrations of all samples were determined by a standard soxtec extraction method and fatty acids were converted to the corresponding methyl esters and measured using gas chromatography.

Patil *et al*. (2010) estimated fatty acid composition in 612 soybean seed samples using non destructive method using Near Infrared Transmittance Spectroscopy. Highest variability was observed for oleic and linoleic acid followed by palmitic and linolenic acid and least in stearic acid.

Sundaram *et al.* (2012) reported that moisture content of intact kernels of grain and nuts by Near infrared reflectance spectrometry and In-shell groundnuts of two different market types Virginia and Valencia were conditioned to different moisture levels between 6 % and 26 % and separated into calibration and validation groups.

Rehema *et al*. (2014) assessed groundnut fatty acids by using Hyper Spectral Imaging (HSI) method and it was an efficient and effective method for evaluating the quality and safety of oil.

Bansod *et al*. (2015) used non-destructive method to identify high oleic groundnut seeds to support the selection and cultivation of high oleic acid groundnut varieties through NIRS.

2.8 ESTIMATION OF AFLAOXINS BY ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Ramakrishna and Mehan (1993) studied direct and indirect competitive enzyme-linked immunosorbent assays to determine the aflatoxin B_1 in groundnut. For direct competitive assay, the monoclonal antibody was conjugated to horse radish peroxidase (HRP) and for indirect competitive ELISA a commercially available goat-antimouse Ig G-HRP conjugate was employed. The sensitivities of both the ELISAs were as low as 20 pg/well and useful for routine analysis of aflatoxin B1 in groundnut.

Aldao *et al.* (1995) quantified aflatoxin B1 by an indirect ELISA in groundnut samples and observed the cross reactivity of antibodies with aflatoxin B2, G1 and G2.

Sylos *et al.* (1996) estimated aflatoxin content in 10 samples of groundnut and nine samples of maize by ELISA and mini column chromatography and detected >20 μg/kg toxin in 50 per cent groundnut seeds and none in maize samples and also ELISA method took less time to complete than mini column chromatography.

Holbrook *et al.* (2000) tested 20 groundnut genotypes having drought tolerance and susceptibility. The results showed that susceptible groundnut genotypes had greater pre harvest aflatoxin contamination and drought tolerant genotypes had less pre harvest aflatoxin contamination.

Reddy *et al*. (2001) collected three grades of chilli pod samples from the principal market yards and cold storage facilities of the major chilli-growing areas of Andhra Pradesh (AP), India (grades 1 to 3) in a survey during 1998 and 1999. An indirect competitive ELISA was used for the estimation of aflatoxin B_1 (AFB_1) content. The results of indirect competitive ELISA revealed that maximum per cent of grade 3 chilli pods have shown AFB_1 levels higher than 30 μg kg^{-1} (non-permissible levels) and one sample from grade 3 has highest AFB_1 concentration (969 μg kg^{-1}).

Asis *et al.* (2002) reported that aflatoxin B_1 was highly contaminated groundnut samples using HPLC and ELISA.

Kolosoya *et al.* (2006) developed direct competitive ELISA based on monoclonal antibody and optimized for detection of aflatoxin and ELISA kit has been designed.

Lia Qi *et al.* (2006) detected aflatoxin B_1 by ELISA and thin layer chromatography.

Radoi *et al.* (2008) developed different clones of antibodies against aflatoxin and their efficacy was investigated by an indirect ELISA method.

Giray *et al.* (2009) used ELISA technique for the quantification of aflatoxin and ochratoxin from 47 maize samples.

Li *et al.* (2009) reported that development of class specific monoclonal antibody based ELISA for aflatoxin in groundnut.

Hong *et al.* (2010) studied the determination of aflatoxin B1 and B2 in groundnut and corn based products by collecting 20 groundnut samples and corn based products from retail shop and local market.

Ayejuyo *et al*. (2011) studied the assessment of aflatoxin levels in 99 samples of groundnut through ELISA in Nigeria and among these 50 were contaminated with aflatoxin (50.5 % incidence). The results showed that aflatoxin content of groundnut ranged from 6.25 ng/g to 7.80 ng/g.

Bakhiet and Musa (2011) analyzed sixty samples of stored groundnut kernels collected from four different locations in Sudan were examined for aflatoxin contamination. Among these, thirty five samples (58.3 %) were found positive with TLC technique and *A. flavus* was isolated from twenty six samples (43.3 %). The concentration of aflatoxin B_1 in these samples was ranged from low (17.5 μg kg^{-1} kernel) to very high (404 μg kg^{-1} kernel).

Aseefa *et al*. (2012) studied the natural occurrence of toxigenic fungal species and aflatoxins in freshly harvested groundnut kernels in Northern Ethiopia to detect the occurrence and severity of infection. A total of 168 groundnut kernel samples were collected from farmers and research center fields which recorded the aflatoxin concentrations with a range of 0.1 to 397.8 ppb.

Alemayechu *et al.* (2013) evaluated 120 samples from Ethiopia to assess the total aflatoxin concentration in groundnut samples using an ELISA test. Of these, 93 were found positive while the remaining 27 were found negative with aflatoxin level ranged from 15 mg kg^{-1} and 11,900 mg kg^{-1}, respectively.

Chala *et al.* (2013) studied 120 groundnut seed samples from farmers stores and markets in Eastern Ethiopia to assess the natural occurrence of aflatoxins in surveyed samples through ELISA method. Of these, 93 were found positive while the remaining 27 samples were found negative. The total aflatoxin levels in the positive samples varied between 15 mg kg^{-1} and 11,900 mg kg^{-1}.

Chen *et al*. (2013) reported that 1827 commercial groundnut products were analyzed for aflatoxin levels which revealed that 32.7 % of samples with aflatoxin levels ranging from 0.2 mg kg^{-1} to 513.4 mg kg^{-1}. Aflatoxin B1 recorded the highest frequency of detection followed by aflatoxin B2, aflatoxin G2 and aflatoxin G1.

Raarajan *et al.* (2013) assessed aflatoxin levels in groundnut seed samples collected from trader godowns which were stored for several months. The study revealed that aflatoxin B1 played a major role to study aflatoxin levels.

Khoraggani *et al*. (2013) evaluated the occurrence of aflatoxin in the groundnut samples collected from supermarkets of Ahvaz were analyzed for the determination of aflatoxin concentration in groundnut using TLC scanner. In total, 59.26 % of samples were contaminated with aflatoxin, 14.8 % of samples were contain above 20 ppb which was above the maximum level of total aflatoxin permitted in Iran.

Mohammed *et al.* (2015) studied different methods of mycological, biochemical and molecular methods for detection of aflatoxigenic *Aspergilli* from groundnut kernels using PCR and HPLC methods.

Rahmiana *et al*. (2015) studied the tolerant mechanism of different groundnut varieties collected from Indonesia. These genotypes consisted of two local varieties, three drought tolerant lines, one foliar disease tolerant line and four national-improved varieties. Aflatoxin content in groundnut kernels varied among these Indonesian genotypes. The highest level of aflatoxin content was in Tuban cultivar while the lowest was in GH 51 with 21 and 5 ppb, respectively.

Rahmiana *et al.* (2015) reported that factors leading to the post-harvest build up of aflatoxin in groundnut sold in traditional market and in supermarket in Indonesia to assess the seed moisture content, physical quality, *A. flavus* infection and aflatoxin B1 contamination. The results revealed that seed water content at wholesalers, collectors and retailers in traditional wet markets was almost lower than 10 %.

Waliyar *et al.* (2015) assessed aflatoxin contamination in three districts in Mali both in the field and storage. Ninety groundnut pod samples in each district were collected from fields (30 villages/district and 3 samples/village) during 2009 and 2010. Pre-harvest contamination was estimated at harvest whereas samples for post-harvest contamination were collected from granaries of the same farmers at a monthly interval for 3 months. The results indicated that Mali groundnuts are heavily contaminated with AFB1 at both pre and post-harvest stages and pose a serious threat to human and animal health.

Wu *et al*. (2016) studied the aflatoxin contamination of groundnut at harvest in China from 2010 to 2013 and its relationship with climatic conditions by collecting 2494 groundnut samples. The highest aflatoxin contamination level occurred under the climatic conditions where precipitation was rare and the mean temperature was close to 23^0C and the minimum temperature was approximately 20^0C and the maximum temperature was 29^0C.

2.9 MANAGEMENT OF *A. flavus* IN GROUNDNUT USING BIOAGENTS AND FUNGICIDES UNDER GLASSHOUSE CONDITIONS

Mixon and Rogers (1973) suggested that use of groundnut cultivars resistant to seed invasion and colonization by the aflatoxin producing fungi could be an effective means of preventing aflatoxin contamination. They developed laboratory inoculation method for screening groundnut genotypes resistance to *A. flavus / A. parasiticus* invasion and colonization.

Mixon *et al.* (1984) reported that chemical CGA 64250 and *T. harzianum* were found more effective in reducing seed colonization by *A. flavus* in groundnut in the gypsum treated than in the gypsum-untreated soils. There was no aflatoxin contamination of seeds in the gypsum-treated soil, but it was found in seeds from the non-treated controls.

Mehan *et al.* (1987) reported resistance groundnut to seed infection of eleven genotypes against *A. flavus* in field trials conducted in India. The results showed that positive correlations were found between seed infection and aflatoxin contamination.

Waliyar *et al.* (1994) tested 25 groundnut lines for resistance to *A. flavus* colonization and aflatoxin contamination. Average seed infection due to *A. flavus* varied with site and year from 5 % to 37 % in groundnut cultivars *viz.*, 55-437, J 11, and p1 337394 F were the least infected.

Romero *et al*. (2000) reported the efficacy of *P. fluorescens* isolates against *A. flavus* to determine the inhibition of fungal infection on maize ear grains at the milk stage. It was observed that mold infection was significantly reduced from 35.8 to 3.2 % on maize ears. Further, reduction in mold infection was proportionate with an increased bacterial antagonist concentration.

Anjaiah *et al.* (2001) tested the efficacy of biocontrol strains potentially antagonistic to *A. flavus* under *in vitro* conditions by dual culture plate method and concluded that 4 - 70 *Trichoderma* isolates were found antagonistic.

Vijay *et al*. (2002) reported that predominance of *A. fIavus* infection in plot with farmers practice (10 %) over improved package (2 %). It was due to inhibition of initial rhizosphere soil population build up of *Aspergillus* by seed treatment with systemic fungicide and application of biocontrol agent in the improved package.

Dharmaputra *et al*. (2003) studied the effect of non-toxigenic *A. flavus, A.niger* and *T. harzianum* inoculated into planting media against toxigenic *A. flavus* infection and its aflatoxin production in peanut kernels at harvest. Test fungi inoculated into planting media could inhibit

toxigenic *A. flavus* infection in groundnut kernels. Aflatoxin was detected in groundnut kernels originated from one plant whose planting medium was inoculated only with the toxigenic *A. flavus.*

Thakur *et al*. (2003) reported that six *Trichoderma* and three *Pseudomonas* strains were identified as highly antagonistic to *Af* 11-4 a highly toxigenic *A. flavus* strain in *A. flavus*-sick plots. The antagonists were applied as seed dressing and soil application at flowering stage. Among the biocontrol agents, two *T. viride* (Tv 17 and Tv 23), one *T. harzianum* (Th 23), and one *Pseudomonas* (Pf 2) isolates provided greater protection to seed infection by *Af* 11 - 4.

Anjaiah *et al*. (2006) reported that inoculation of selected antagonistic strains fluorescent *Pseudomonads, Bacillus* and *Trichoderma* sp. on groundnut have shown significant reduction of seed infection by *A. flavus*. Further a reduction of >50 % of the *A. flavus* populations in the geocarposphere of groundnut were observed.

Gachamo *et al.* (2008) studied the biocontrol management of aflatoxins using four *Trichoderma* isolates under laboratory conditions. Two isolates of *T. harzianum* i.e., Th1, Th2 and two isolates of *T. viride i.e.,* Tv1 and Tv2 suppressed the growth of groundnut moulds and significantly reduced the aflatoxins AFB1 and AFB2.

Reddy *et al.* (2009) reported that PGPR strains such as *P. fluorescens* and *B. subtilis* inhibited *A. flavus* growth up to 93 % and 68 %, respectively. The fungal bioagents, *T. virens* inhibited 80 % of the test pathogen in rice.

Palumbo *et al*. (2010) reported the efficacy of two bacterial strains, *Pseudomonas chlororaphis* strain (JP1015) and *P. fluorescens* (strain JP2175) were tested in inhibiting the growth of *A. flavus* under laboratory conditions. Further, three days after soil co-inoculation with *P. chlororaphis* strain JP1015 inhibited *A. flavus* growth up to 100 fold and up to 58 fold by *P. fluorescens* strain JP2175.

Rathod *et al*. (2010) studied the effect of fungicides on seed borne pathogen of groundnut. The results indicated that thiram, carbendazim and mancozeb were found more inhibitory as compared to other fungicides.

Verma *et al.* (2010) studied the interaction between antagonistic activity of *P. fluorescens* on different sps. of *Trichoderma.* The inhibition in growth of *Trichoderma* was 8.59 % when *Trichoderma* was used first and 59.4 % when *P. fluorescens* was used first in dual culture test. Highest growth inhibition (59.4 %) of *Trichoderma* (Th3) was recorded when *P. fluorescens* was

first inoculated in King's B medium 24 h prior to inoculation of *T. harzianum.* The population of *Trichoderma* was gradually increased over a period of 14 days incubation.

Bhagawan *et al.* (2011) studied eco friendly management using five biocontrol agents like *T. viride, T. harzianum, T. hamatum, B. subtilis* and *P. fluorescens* to reduce aflatoxin B1 in groundnut cultivar GG - 20. The combination of *T. viride, B. subtilis and P. fluorescens* were found effective in reducing *A. flavus* rhizospheric population, per cent incidence of afla root infection and colonization of kernels and aflatoxin B1 content.

Baig *et al.* (2012) evaluated the efficacy of biocontrol agents, i.e., *T. viride, T. harzianum, P. fluorescens* and *B. subtilis* against *A. flavus, A. niger, F. oxysporum* and *A.alternata* in oil seed crops and found were effectively.

Dey *et al.* (2004) assessed nine different isolates of plant growth-promoting rhizobacteria (PGPR) influence on plant growth, yield and nutrient uptake. Seed inoculation of these three isolates, *viz.,* PGPR1, PGPR2 and PGPR4 resulted significant increase in pod yield over control.

Pushpalatha *et al.* (2013) studied eco friendly management of seed borne fungi using *Trichococcus* species in groundnut samples collected from various places of Karnataka. The fungal pathogens were identified as *Penicillium* sp, *A. niger*, *A. flavus* and *Fusarium* sp. Among these, maximum disease incidence due to *A. niger* was observed.

Sudha *et al.* (2013) evaluated efficacy of fungicides, bioagents and plant extracts against *A. flavus* on chilli under both *in vitro* and field conditions. Among different bioagents tested *P. fluorescens* and *T. harzianum* inhibited *A. flavus* growth up to 74 % and 70.4 % where as mancozeb was effective with 91.1 % inhibition of *A. flavus*. Complete inhibition (100 %) of *A. flavus* was recorded by the plant extracts like neem seed kernel extract (NSKE), nimbicidine and pongamia.

Sanskriti *et al*. (2015) reported the antifungal activity of *P. fluorescens* against *A. flavus* in groundnut under laboratory and field trials. The results indicated that there was a significant reduction in seed infection due to *A. flavus* by inoculation of *P. fluorescens* on groundnut.

Vasundara *et al.* (2015) studied the effect of seed treating fungicides and insecticides and their combinations with *T. viride* on rhizosphere mycoflora and plant biometrics at 75 days after sowing in groundnut.

CHAPTER III

MATERIAL AND METHODS

The present investigation was carried out at Department of Seed Science and Technology, College of Agriculture, Rajendranagar, PJTSAU, Hyderabad, Telangana, in collaboration with International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, India. The details of the material and methods are presented here under the following headings.

3.1 Collection of groundnut pod samples

3.2 Isolation of seed mycoflora by Agar plate method

3.3 Scanning electron microscopic studies (SEM)

3.4 Effect of *A. flavus* infection on oil quality

3.5 Evaluation of seed treatments (bioagents and fungicides) against *A. flavus* in groundnut under glasshouse conditions

3.6 Estimation of seed quality (ELISA)

3.1 COLLECTION OF GROUNDNUT POD SAMPLES

A total of seventy two (72) pod samples were collected from the major groundnut growing districts of Telangana *viz*., Warangal (18) Karimnagar (18), Nizamabad (18) and Mahabubnagar (18) were collected. Out of 72 samples, 36 samples were collected from farmers and the remaining 36 samples were collected from market yards for assessment of seed mycoflora during 2015 - 2016. The collected pod samples were shade dried and shelled and used for further studies.

Table 3.1 Collection of groundnut pod samples from different districts of Telangana State

S. No	Name of the District Surveyed	Source of sample collection (Farmer sample)	Source of sample collection (Market sample)	Number of samples/district
1	Karmimnagar	Burampet	Sultanabad	Three
		Sultanpur	Choppadandi	Three
		Julapalli	Husnabad	Three
2	Warangal	Mulugu	Hanmakonda	Three
		Keshavapatnam	Warangal	Three

		Medaram	Hasanparthi	Three
3	Nizamabad	Balkonda	Banswada	Three
		Bichkunda	Nizamabad	Three
		Gandhari	Bhodan	Three
4	Mahabubnagar	Atmakur	Narayankhade	Three
		Khanapur	Jadcharla	Three
		Singampeta	Nagarkurnool	Three
Total number of samples collected				72

3.2 ISOLATION OF SEED MYCOFLORA BY AGAR PLATE METHOD

For isolation of seed mycoflora associated with groundnut samples, Agar plate method (ISTA, 1996) was employed.

3.2.1 Agar Plate Method (ISTA, 1996)

PDA medium was prepared by using the following components for isolation of the seed mycoflora in the laboratory.

Potato dextrose agar (PDA)	
Potato	200g
Dextrose	20g
Agar	20g
Water	1000 ml
PH	6.8

Peeled potato pieces were boiled in 500 ml of distilled water in a 1000 ml beaker till the pieces got softened and the extract were collected in a beaker by sieving through a double layered muslin cloth. Agar - agar (20g) was melted in another 500 ml of distilled water in 1000 ml beaker into which 20g dextrose was added. The final volume of the medium was made up to 1000 ml by adding sterile distilled water. The pH of the medium was adjusted to 6.8 with 0.1 N NaOH or 0.1 N HCl as the case may be with the pH meter. The medium was sterilized in an autoclave at 15 psi for 15 minutes. About 20 ml of the medium was distributed to each of the sterile Petri plate under

aseptic conditions. Groundnut seeds were transferred to the plates containing PDA medium. Ten seeds per plate were placed at equidistance in a circular fashion. Four hundred seeds from each sample were placed in the plates in four replications. The Petri plates were incubated at 25 ± 2^0C in an incubator for seven days and observed every day for the growth of fungi. Small quantity of streptomycin sulphate was added in each plate for the suppression of the bacterial pathogens. The characteristic features of the isolated seed borne fungi were tallied with the descriptions given for identification (Ellis, 1976). The total fungal colonies were calculated and per cent infection was assessed.

$$\text{Total fungal colonies (\%)} = \frac{\text{No. of seeds colonized in each plate by a Particular species}}{\text{Total number of seeds in each plate}} \times 100$$

3.3 EXTERNAL SEED COLONIZATION BY *A. flavus* IN GROUNDNUT cvs. J 11 AND JL 24

Seeds of groundnut cvs. J 11 and JL 24 were artificially inoculated with toxigenic strains of *A. flavus* (isolate of *Af* 11 - 4) @ 10^9 conidia/ml were placed on sterilized petri plates and incubated at 1, 3, 5, 7 and 9 days. Seeds of both the cvs. J 11 and JL 24 were assessed for surface seed colonization by pathogen as per the colonization severity rating scale (1 - 4) as given by Thakur *et al.* (2000).

3.4 SCANNING ELECTRON MICROSCOPIC STUDIES

Groundnut seeds of cv. JL 24 (susceptible) and cv. J 11 (resistant) were artificially inoculated with *A. flavus* @ 10^9 conidia/ml and placed on sterilized blotter papers and maintained at 25 ± 2^0 C in a BOD incubator. Further seed samples were prepared with an interval of 48 h i.e., 1, 3, 5, 7 and 9 days after incubation. Ultra-structural mycelial characters in the infected groundnut seeds were analyzed through Scanning Electron Microscopy (SEM) RUSKA laboratory, College of Veterinary Science, SPVNRTSUVAFS, Rajendranagar, Hyderabad, Telangana state.

Infected groundnut seeds were cut into sections measuring not more than 1-2 mm with a razor blade. Healthy groundnut seeds were aseptically washed and sectioned similarly to serve as

control treatments. Samples were fixed in 2.5 % glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for 24 h at 4^0C and post fixed in 2 % aqueous osmium tetroxide for 4 h. Dehydrated in series of graded alcohols and dried to critical point drying with CPD unit. The processed samples were mounted over the stubs with double - sided carbo conductivity tape and a thin layer of gold coat over the samples were done by using an automated sputter coater (Model – JEOL JFC-1600) for 3 min and observed under Scanning Electron Microscope (SEM-Model: JEOLJFC-1600) at required magnifications as per the standard procedures.

The basic steps involved in SEM sample preparation was surface cleaning, stabilizing the sample with a fixative, rinsing, dehydrating, drying, mounting the specimen on a metal holder and coating the sample with a layer of material that is electrically conductive.

3.4. 1 Cleaning the Surface of the Specimen

Cleaning of the sample was done to remove the media components and was permanently fixed to the specimen surface. The sample was rinsed three times for 10 min in 0.1 M phosphate buffer (pH 7.2) at room temperature.

3.4.2. Stabilizing the Specimen

Stabilization was done with fixatives. Samples were fixed by immersing the specimen in 2.5 % glutaraldehyde (GA) solution prepared in 0.1 M phosphate buffer (pH 7.2) and incubated at 4^0C for 24 h and post fixed in 2 % aqueous osmium tetroxide (OsO_4) for 4 h. The use of post fixative helps in improving the bulk conductivity of the specimen.

3.4.3 Rinsing the Specimen

After fixation samples were rinsed in 0.1 M phosphate buffer (pH 7.3) one time for 10 min and then three times for 20 min at 4^0C in order to remove the excess fixative.

3.4.4 Dehydration and Drying

Specimens were dehydrated in the following series of alcohols and dried in ethyl alcohol critical point drying (CPD) unit.

30 % Ethyl alcohol - 10 min

50 % Ethyl alcohol - 10 min

70 % Ethyl alcohol - 10 min

85 % Ethyl alcohol - 20 min

95 % Ethyl alcohol - 20 min

100 % Ethyl alcohol - 20 min

100 % Ethyl alcohol - 20 min

100 % Acetone - 20 min

100 % Acetone - 20 min

This process allows the water in the samples to be slowly exchanged through liquids with lower surface tensions.

3.4.5 Mounting the Specimen

The processed specimens were mounted on holder and inserted into the scanning electron microscope. Samples were mounted on metallic (aluminum) stubs using a double sided carbon conductivity tape.

3.4.6 Coating the Specimen

Specimens were coated with 20 nm to 30 nm thin layer gold coat over the samples by using an automated sputter coater (Model - JEOL JFC - 1600) for 3 minutes and scanned under Scanning Electron Microscope (SEM – Model: JOEL - JSM 5600) at required magnifications as per the standard procedure.

3.5 EFFECT OF *A. flavus* INFECTION ON OIL QUALITY

Toxin producing aflatoxigenic strain of *A. flavus* (*Af* 11–4) was grown on potato dextrose agar (PDA) and plates were kept in BOD incubator for 7 days at 25 ± 2^0C. Seeds of groundnut cv. JL 24 (susceptible) and cv. J11 (resistant) were surface sterilized using 0.01 % clorax solution for one min. Seeds were washed in three times with sterile water and placed in dry blotter paper to remove the excess moisture. Seeds of both the cultivars were artificially inoculated with *A. flavus* @ 10^9 conidia/ml and kept on sterilized blotter paper discs of 9 cm diameter and moistened with sterile distilled water. The excess water was drained off from the plates. Groundnut seeds (400 Nos) in four replications were placed equidistantly on sterile blotter paper and incubated at 25 ± 2^0C for a period of two months with an interval of 3, 7, 14, 21, 28, 35, 42, 49 and 56 days. After incubation at different intervals groundnut seeds of both the cultivars (infected and healthy seeds)

were assessed for oil quality by using near infrared reflectance spectroscopy (NIRS) with the following treatments.

Table 3.2. Effect of *A. flavus* infection on oil quality (Oil, Protein and fatty acids) in groundnut cvs. J 11 and JL 24

Treatments	Groundnut cv. J11 (R)	Groundnut cv. JL 24 (S)
T_1 (0 Day)	(J 11 + *Af* 11-4)	(JL 24 + *Af* 11-4)
T_2 (3^{rd} Day)	(J 11 + *Af* 11-4) Control (Untreated)	(JL 24 + *Af* 11-4) Control (Untreated)
T_3 (7^{th} Day)	(J 11 + *Af* 11-4) Control (Untreated)	(JL 24 + *Af* 11-4) Control (Untreated)
T_4 (14^{th} Day)	(J 11 + *Af* 11-4) Control (Untreated)	(JL 24 + *Af* 11-4) Control (Untreated)
T_5 (21^{st} Day)	(J 11 + AF 11-4) Control (Untreated)	(JL 24 + *Af* 11-4) Control (Untreated)
T_6 (28^{th} Day)	(J 11 + *Af* 11-4) Control (Untreated)	(JL 24 + *Af* 11-4) Control (Untreated)
T_7 (35^{th} Day)	(J 11 + *Af* 11-4) Control (Untreated)	(JL 24 + *Af* 11-4) Control (Untreated)
T_8 (42^{nd} Day)	(J 11 + *Af* 11-4) Control (Untreated)	(JL 24 + *Af* 11-4) Control (Untreated)
T_9 (49^{th} Day)	(J 11 + *Af* 11-4) Control (Untreated)	(JL 24 + *Af* 11-4) Control (Untreated)

T_{10} (56^{th} Day)	(J 11 + *Af* 11-4) Control (Untreated)	(JL 24 + *Af* 11-4) Control (Untreated)

The per cent oil, protein and fatty acids (saturated and unsaturated) contents were estimated by using NIRS (model XDS RCA, FOSS Analytical AB, Sweden, Denmark). Non-destructive method of estimation was used in NIRS. Approximately 70 - 100 g of each intact groundnut sample was kept in rectangular cup in the NIR machine and readings were taken at different days of incubation.

3.6 ESTIMATION OF SEED QUALITY BY USING ELISA (ENZYME LINKED IMMUNOSORBANT ASSAY)

3.6.1. Coating

ELISA plates were coated with 150 µl of AFB_1 – BSA conjugate (1 µl of AFB_1 – BSA in 10 ml of 0.2 % carbonate buffer).

⇩

Incubated for overnight in refrigerator or incubator at 37^0 C for one hour.

⇩

Washed the plate thrice with PBS – T 20 (phosphate buffer saline – tween for 3 min).

3.6. 2 Blocking

160 µl of 0.2 % BSA (Bovine serum albumin) was added and incubated at 37^0 C for one hour.

⇩

Wash the plate thrice with PBS – T 20.

⇩

Dilution of antiserum in a ratio of 1:2000 in a test tube and incubated at 37^0 C.

3.6.3 Competition

AFB_1 standards ranging from 0.1 to 50 ng/ml were prepared in groundnut extracts (diluted to 10 %) not containing any aflatoxin. Healthy groundnut kernels (20g) free of aflatoxin were powdered and extracted in 100 ml of 70 % methanol containing 0.5 % KCl. The extract was filtered and diluted to 1:10 in PBST – BSA. This was used as a diluent for aflatoxin standards.

Simultaneously pure toxin (AFB_1) was prepared by diluting with above prepared healthy groundnut (HGN) extract in a test tube.

⇩

100 µl of AFB_1 (50 ng/ml) was added to first two columns of first two rows.

⇩

100 µl of diluted HGN extract was added to remaining wells of first two rows.

⇩

The remaining wells were loaded with 90 µl of BSA + 10 µl of sample extract to be analyzed.

⇩

50 µl of antiserum was loaded to each well of ELISA plate and kept in shaker for 10 min.

⇩

Incubated the plate for one hour at 37^0 C to facilitate reaction between toxin and antibody and plate was washed thrice in PBS – T 20.

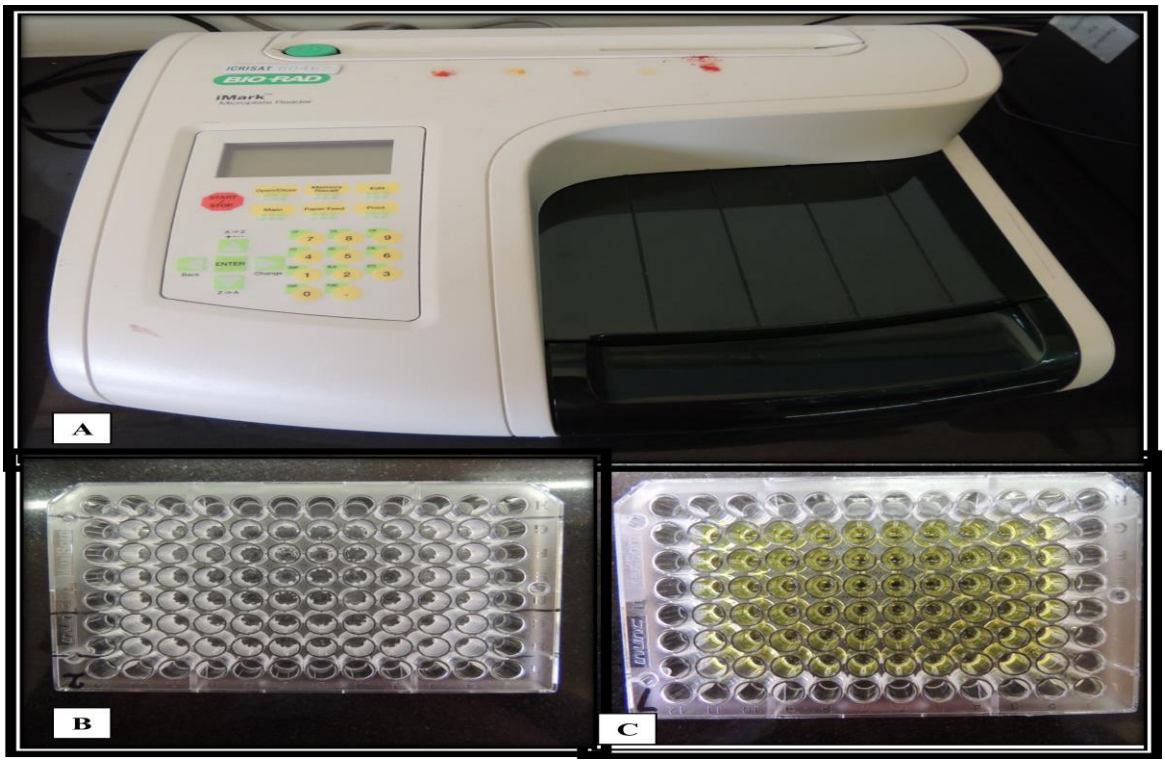

Plate 3.1. Enzyme linked immunosorbent assay for estimation of aflatoxin content in groundnut kernels. A) Microplate reader B) 96-well ELISA plate prior to experimentation C) ELISA plate after the reaction

3.6.4 Conjugation

150 µl of substrate buffer {PNPP (P – Nitro Phenyl Phosphate) in 10 % diethylene amine} was added to each well.

⇩

Simultaneously substrate was added to top left corner well as blank.

⇩

Incubated at normal temperature in dark for colour development at 15 minutes interval.

⇩

Absorbance was measured at 405 nm in ELISA reader.

ELISA Plate Reader (Bio-Rad)

Micropipettes: 1 - 40 µl, 40 - 200 µl and 200 - 1000 µl single channel pipettes, 40 – 200 µl multichannel pipettes (Finn pipette) were used.

ELISA plates: For high binding 'NUNC – Maxisorp^{TM}surface' plates were used.

Others

Polyclonal antibodies for total aflatoxins

Mortar and pestle, Muslin cloth, pH meter, incubator, Refrigerator

Aflatoxin B_1 standard (Sigma A6636)

Aflatoxin B_1 - BSA conjugate (Sigma A6655)

Bovine Serum Albumin (Sigma A6793)

Solutions

Carbonate buffer or coating buffer (pH 9.6)

Na_2CO_3 --1.59 g

$NaHCO_3$ --2.93 g

Distilled water --1000 ml

Phosphate buffer saline (PBS) (pH 7.4)

Na_2HPO_4 -- 02.38 g

KH_2PO_4 -- 00.40 g

KCl -- 00.40 g

NaCl -- 16.00 g

Distilled water --2000 ml

Phosphate buffer saline Tween (PBS-T)

PBS --1000 ml

Tween - 20 -- 0.5 ml

Antibody buffer

PBS-T --100 ml

Polyvinyl Pyrrolidone (PVP) 40,000 MW --2.0 g

Bovine serum albumin --0.2 g

3.6.5 Preparation of groundnut seed extracts

Groundnut seed (100 g) was grinded into powder using a blender. The seed powder was titrated in 70% methanol (v/v-70 ml absolute methanol in 30 ml distilled water) containing 0.5 % KCl (proportion used in 100 ml for 20 g seed) in a blender until the seed powder was thoroughly ground. The extract was transferred to a conical flask and shaken for 30 min at 300 rpm in the mechanical shaker. The extract was filtered through Whatman No. 1 filter paper. To estimate lower levels of AFB_1 (<10 µg Kg^{-1}), prior to ELISA, a simple liquid cleanup and concentration (5:1) procedure was adopted. Twenty ml of methanol extract, 10 ml of distilled water and 20 ml of chloroform were mixed in a separating funnel and used for cleanup. After vigorous shaking for one min, the lower chloroform layer was collected and evaporated to near desiccation in water bath at 60°C. To the residue, four ml of PBS - Tween containing 7 % methanol was added and used for analysis by ELISA.

AFB_1 - BSA conjugate was prepared in carbonate coating buffer at 100 ng/ml concentrations and 170 µl of the diluted AFB_1 - BSA is dispensed to each well of ELISA plate. The plate was incubated in a refrigerator overnight at 37° C for at least one and half-hour.

The plates were washed in three changes of PBS – Tween allowing 3 min gap between for each wash (To inhibit non-specific binding of antibodies and thus give false positive reaction). BSA (0.2 %) was prepared in PBS - Tween was added in 170 µl per each well of ELISA plate and

incubated at 37°C for 1h. The plates were washed in three changes of PBS - Tween allowing 3 min between each wash.

3.6.6 Preparation of Aflatoxin B_1 standards

Healthy groundnut seed extract was prepared as mentioned previously. Aflatoxin B_1 standards (using 1:10 healthy groundnut seed extract) were diluted at concentrations ranging from 100 ng to 10 picogram in 100 µl volume.

Using the values obtained for aflatoxin B_1 standards a curve was drawn with the help of a computer, taking aflatoxin concentrations on the X-axis and optical density values on the Y-axis. The amount of aflatoxin present in the sample was calculated using the formula given below:

$$\text{AFB1 } (\mu g/kg) = \frac{A \times D \times E}{G} \text{ or } \frac{A \times E}{C \times G}$$

A = AFB_1 concentration in diluted or concentrated sample extract (ng/ml)

D = Time dilution with buffer

C = Time concentration after clean up

E = Extraction solvent volume used (ml)

G = Sample weight (g)

3.7 EVALUATION OF SEED TREATMENTS (BIOAGENTS AND FUNGICIDES) AGAINST *A. flavus* IN GROUNDNUT UNDER GLASSHOUSE CONDITIONS.

3.7.1 Methodology

Seeds of groundnut susceptible cv. JL 24 were surface sterilized and artificially inoculated with *A. flavus* @ 10^9 conidia / ml. After 24h, the seeds were again treated separately with *T. harzianum* @ 10 g kg^{-1}, *T. viride* @ 10 g kg^{-1}, *P. fluorescens* @ 10 g kg^{-1}, mancozeb @ 2.5 g kg^{-1} and carbendazim @ 2 g kg^{-1} along with untreated and pathogen treated seeds. Seeds after imposition of treatments were sown in sterilized soil filled in earthen pots containing 5 kg soil @ five seeds per pot in replicated trial adopting CRD design under controlled glasshouse conditions. Observations were recorded on germination percentage, plant height and yield. Aflatoxin levels in the harvested produce were estimated through ELISA. The details of the experiment were provided in Table 3.5.1.

3.7.2 Treatments

Design	**CRD**
Replications	4
Treatments	5
T_1	*Trichoderma harzianum* @ 10 g/kg
T_2	*Trichoderma viride* @ 10 g/kg
T_3	*Pseudomonas flourescens* @ 10 g/kg
T_4	Mancozeb @ 2.5 g/kg
T_5	Carbendazim @ 2 g/kg
T_6	Inoculated control (Treated seeds)
T_7	Uninoculated control (untreated seeds)

Note: Seeds were prior inoculated with *A. flavus* @ 10^9 conidia / ml before imposition of seed treatments from T_1 to T_6 except T_7 treatment.

3.7.3 Observations

3.7.3.1 Germination (%)

The number of seeds germinated in each treatment was counted on seventh day after sowing. Four replications were maintained for each treatment.

3. 7.3.2 Plant height (cm)

The plant height was recorded in cm from the base of the plant at 15, 30 and 45 DAS.

Harvesting

The crop was harvested at maturity, threshed and sun-dried for 3 days.

3. 7.3.3 Yield (g)

Pod weight in grams was recorded in different treatments with replication wise.

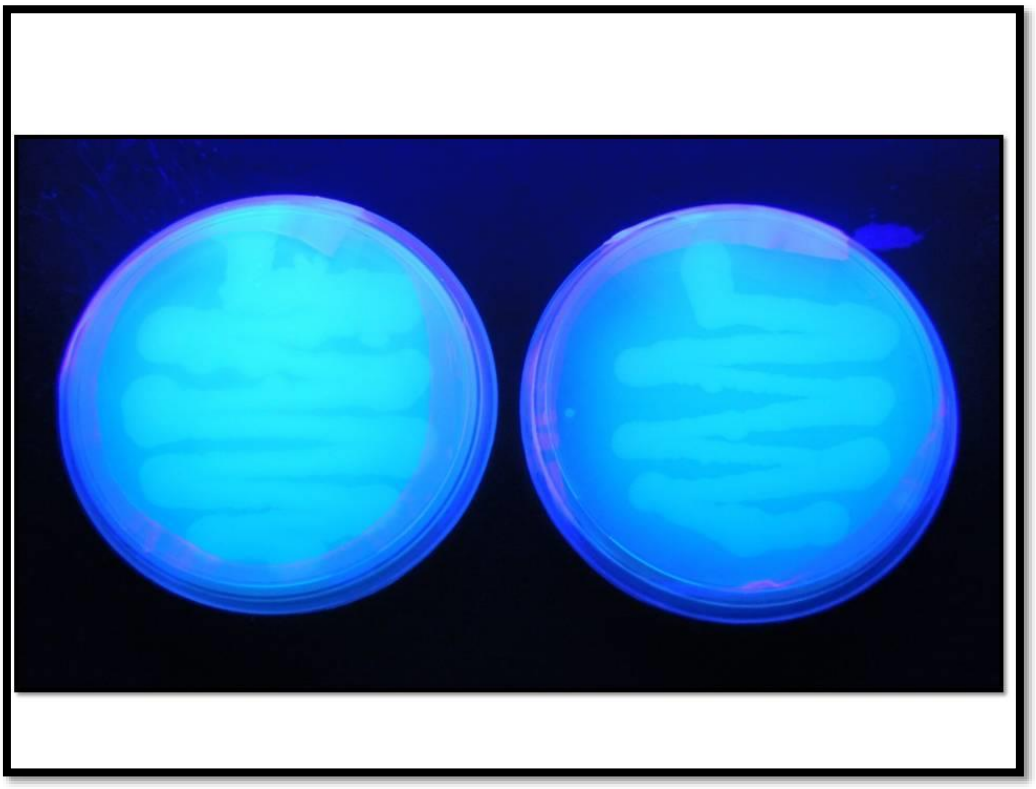

Plate 3.2. Pure culture of *Pseudomonas fluorescens* maintained on Kings B medium

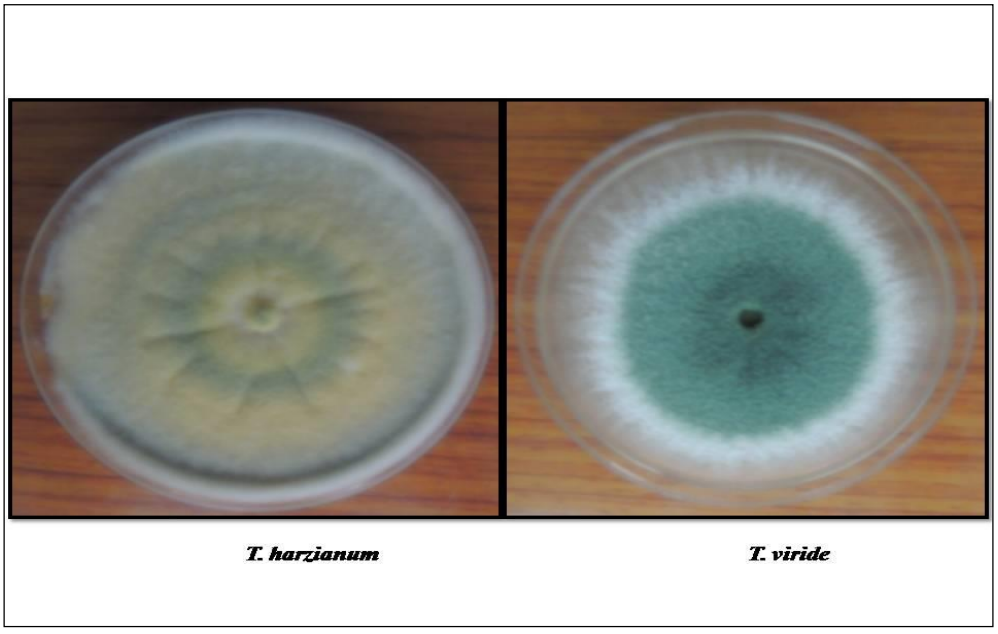

Plate 3.3. Pure culture of *T. harzianum* and *T. viride* maintained on PDA

3.7.3.4 Aflatoxin levels

Pods after harvest were manually shelled and seeds were assessed for aflatoxin by following the indirect competitive ELISA (Reddy *et al.* 2001).

3.8 STATISTICAL ANALYSIS

The data obtained in various laboratory/glasshouse experiments were statistically analyzed by using Completely Randomized Design (CRD) as suggested by Gomez and Gomez (1984). The data pertaining to percentage were angular transformed wherever necessary.

CHAPTER IV

RESULTS AND DISCUSSION

The results of the experiment conducted in the present investigation are presented here under the following headings.

4.1 DETECTION OF SEED MYCOFLORA ASSOCIATED WITH GROUNDNUT SEED SAMPLES COLLECTED FROM FARMERS

A total of 36 groundnut pod samples were collected from four major groundnut growing districts of Telangana state *viz*., Karimnagar (9), Warangal (9), Nizamabad (9) and Mahabubnagar (9). The seed samples were analyzed for seed health as per ISTA, 1996 by agar plate method. Seed mycoflora associated with groundnut seed samples were isolated and identified.

4.1.1 Agar plate method

Mycoflora associated with groundnut seed samples from farmers and market yards of Karimnagar, Warangal, Nizamabad and Mahabubnagar districts were detected following the agar plate method (Fig 4.1).

Significant differences in occurrence of seed mycoflora in different districts of Telangana state were observed. The results indicated that irrespective of the location and sources, a total of six fungal species belonging to five genera were detected from the seed samples tested. Six fungi *viz*., *Aspergillus flavus, A. niger, Fusarium* sp. *Alternaria* sp. *Macrophomina* sp. and *Penicillium* sp. were observed. Total per cent occurrence of seed mycoflora in different districts *viz*., Karimnagar (72.2 %), Warangal (82.1 %), Nizamabad (56.7 %) and Mahabubnagar (84.8 %), were observed respectively with respect to the mean total fungal colonies. Seed samples collected from Mahabubnagar district (84.8 %) and Warangal district (82.1 %) recorded more total number of fungal colonies followed by Karimnagar (72.2 %) and Nizamabad districts (56.7 %) which was lowest of all the districts (Table 4.1).

Out of six fungal species recorded, the occurrence of *A. flavus* was predominant in the seed samples analyzed from all the four districts (43.2 %). The occurrence of *A. flavus* was highest in the seed samples of Mahabubnagar district (47.1 %) followed by Warangal district (43.5 %). *A. flavus* was the most predominant fungus followed by *A. niger* (14.7 % to 34.4 %). The occurrence of pathogenic fungi *viz*., *Alternaria* sp. (0.67 % to 0.89 %), *Fusarium* sp. (0.78 % to 1.90 %), *Macrophomina* sp. (0.67 % to 1 %), *Penicillium* sp. (0.67 % to 1 %) were observed in these

Table 4.1. Detection of seed mycoflora associated with groundnut farmer samples collected from Karimnagar, Warangal, Nizamabad and Mahabubnagar of Telangana state following agar plate method

S. No.	DISTRICTS SURVEYED	*A. flavus* (%)	*A. niger* (%)	*Fusarium* sp. (%)	*Alternaria* sp. (%)	*Macrophomina* sp. (%)	*Penicillium* sp. (%)	TFC (%)
1	**Karimnagar**	43.1 (41.0)	25.5 (30.3)	1.00 (5.98)	0.89 (5.70)	1.00 (5.97)	0.67 (5.25)	72.2
2	**Warangal**	43.5 (41.2)	32.2 (34.5)	1.90 (7.61)	0.67 (5.26)	0.78 (5.45)	1.00 (5.97)	82.1
3	**Nizamabad**	39.1 (38.6)	14.7 (21.4)	0.78 (5.51)	0.67 (5.33)	0.67 (5.25)	0.78 (5.51)	56.7
4	**Mahabubnagar**	47.1 (43.4)	34.4 (35.8)	0.78 (5.51)	0.89 (5.62)	0.67 (5.97)	0.89 (5.70)	84.8
	Mean	43.2	26.7	1.10	0.78	0.78	0.84	
	SE(m) ±	2.11	1.69	1.08	0.91	1.05	0.96	
	CD at 5 %	6.18	4.95	3.15	2.65	3.06	2.80	

Figures in parenthesis are angular transformed values. Each value is mean of four replications. TFC: Total fungal colonies

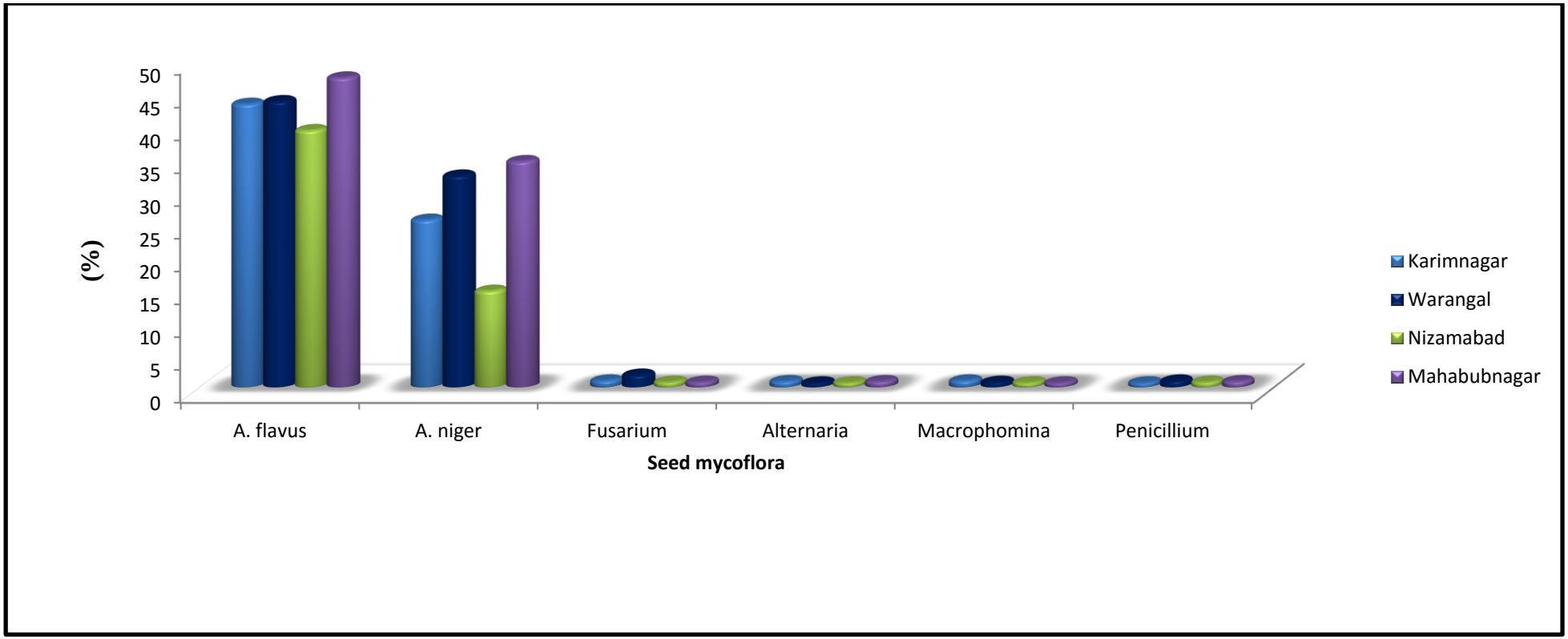

Fig 4.1. Total seed mycoflora detected in groundnut farmer samples collected from different districts of telangana state.

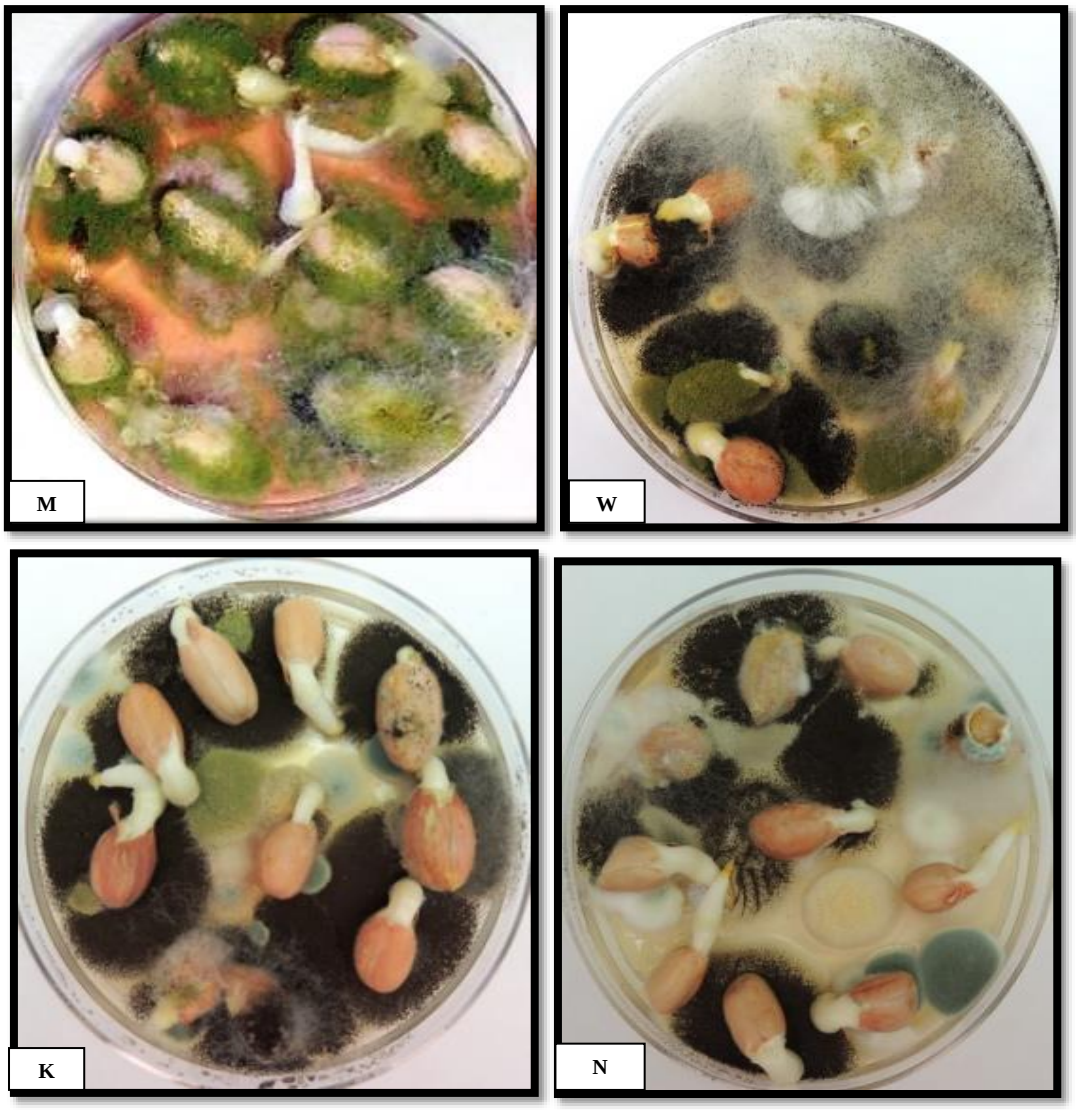

Plate 4.1. Seed mycoflora detected by agar plate method (Farmer samples)

M:Mahabubnagar, W:Warangal, K:Karimnagar, N:Nizamabad

samples. The differences in occurrence of seed mycoflora in groundnut seed samples collected from different districts may be attributed to the variations in the moisture content of the seed and storage environment (temperature, relative humidity and light) adopted by the farmers. Mycoflora associated with seed may varied from place to place due to change in conditions prevailing during seed development, harvesting and storage. Seed mycoflora was highest in the seed samples of Mahabubnagar district (84.8 %), while it was least in Nizamabad district (56.7 %). Storage fungi like *A. flavus, A. niger* and *Penicillium* sp. were found low in Nizamabad district with a range of 0.67 % to 39.1 %. The per cent occurrence of the individual fungi was ranged from 0.67 % to 47.1 %. Seed mycoflora viz., *A. flavus, A. niger, Fusarium* sp. *Alternaria* sp. *Macrophomina* sp. and *Penicillium* sp. were recorded in the groundnut seed samples indicated their seed borne nature in groundnut. There is need for reducing the mold growth and mycotoxin production by improving the storage conditions.

The present findings corroborates the report of Rathod *et al.* (2012) who reported that agar plate method was effective in detection of seed borne fungi in groundnut. Agar plate method favours the growth of fungi and gives highest per cent incidence due to potato dextrose agar contents. He further reported the highest occurrence of *A. flavus* and *A. niger*. Most of the fungal species detected in the present study were reported earlier in groundnut by Goldblatt (1969), Reddy and Rao (1980), Mukherjee *et al.* (1992), Lumpungu *et al*. (1989), Rasheed *et al.* (2004), Shazia *et al*. (2004), Aseefa *et al.* (2012), Naqui *et al.* (2013) and Nagapurne and Patwari (2014).

4.2 DETECTION OF SEED MYCOFLORA ASSOCIATED WITH GROUNDNUT SEED SAMPLES COLLECTED FROM MARKETYARDS

Significant differences in occurrence of seed mycoflora in different districts of Telangana state were observed. The results indicated that irrespective of the location and sources, six fungal species belonging to five genera were detected from the seed samples tested. *Aspergillus flavus, A. niger, Fusarium* sp. *Alternaria* sp. *Macrophomina* sp and *Penicillium* sp. Total per cent occurrence of seed mycoflora in different districts were ranged from Karimnagar (41.7 %), Warangal (50.4 %), Nizamabad (34.7 %) and Mahabubnagar (54.8 %), respectively with respect to the mean total fungal colonies. Seed samples collected from Mahabubnagar district (54.8 %) and Warangal district (50.4 %) recorded more total number of fungal colonies followed by Karimnagar district (41.7 %). Least total number of fungal colonies were observed in Nizamabad district (34.7 %) (Table 4.2, Plate4.2).

Table 4.2. Detection of seed mycoflora associated with groundnut market samples collected from Karimnagar, Warangal, Nizamabad and Mahabubnagar of Telangana state following agar plate method

S. No.	DISTRICTS SURVEYED	*A. flavus* (%)	*A. niger* (%)	*Fusarium* sp. (%)	*Alternaria* sp. (%)	*Macrophomina* sp. (%)	*Penicillium* sp. (%)	TFC (%)
1	**Karimnagar**	19.5 (22.7)	19.6 (26.2)	0.78 (5.53)	0.56 (5.06)	0.67 (5.25)	0.67 (5.26)	41.7
2	**Warangal**	24.6 (25.9)	21.9 (27.8)	0.78 (5.51)	0.78 (5.45)	1.00 (5.98)	1.33 (6.54)	50.4
3	**Nizamabad**	15.6 (21.6)	16.7 (23.9)	0.78 (5.45)	0.78 (5.51)	0.44 (5.25)	0.44 (4.79)	34.7
4	**Mahabubnagar**	26.2 (30.7)	25.0 (29.9)	1.44 (6.80)	0.78 (5.51)	0.67 (5.97)	0.67 (5.33)	54.8
	Mean	21.5	20.8	0.94	0.72	0.69	0.78	
	SE(m) ±	1.37	1.52	1.22	0.99	0.98	1.00	
	CD at 5 %	4.00	4.45	3.58	2.89	2.73	2.93	

Figures in parenthesis are angular transformed values. Each value is mean of four replications. TFC: Total fungal colonies

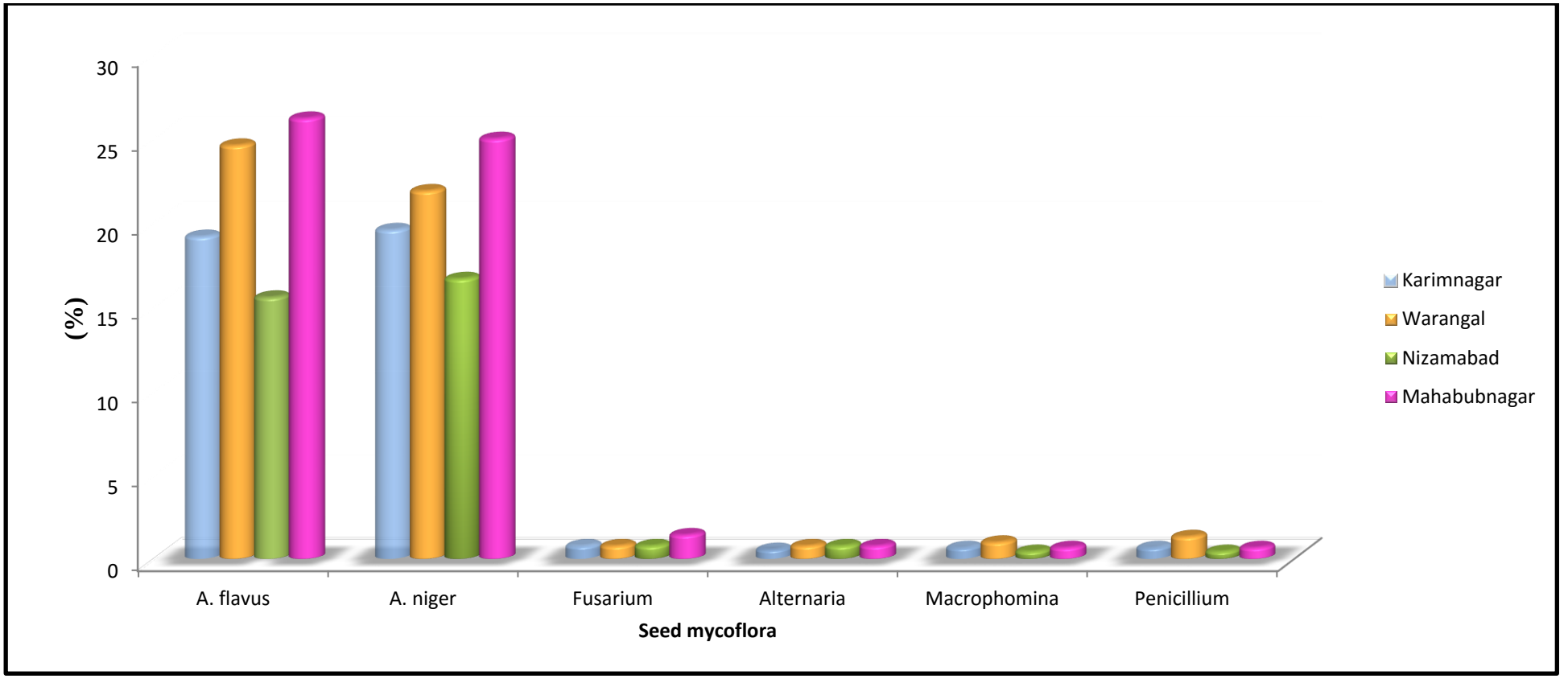

Fig 4.2. Total seed mycoflora detected in groundnut market samples collected from different districts of telangana state.

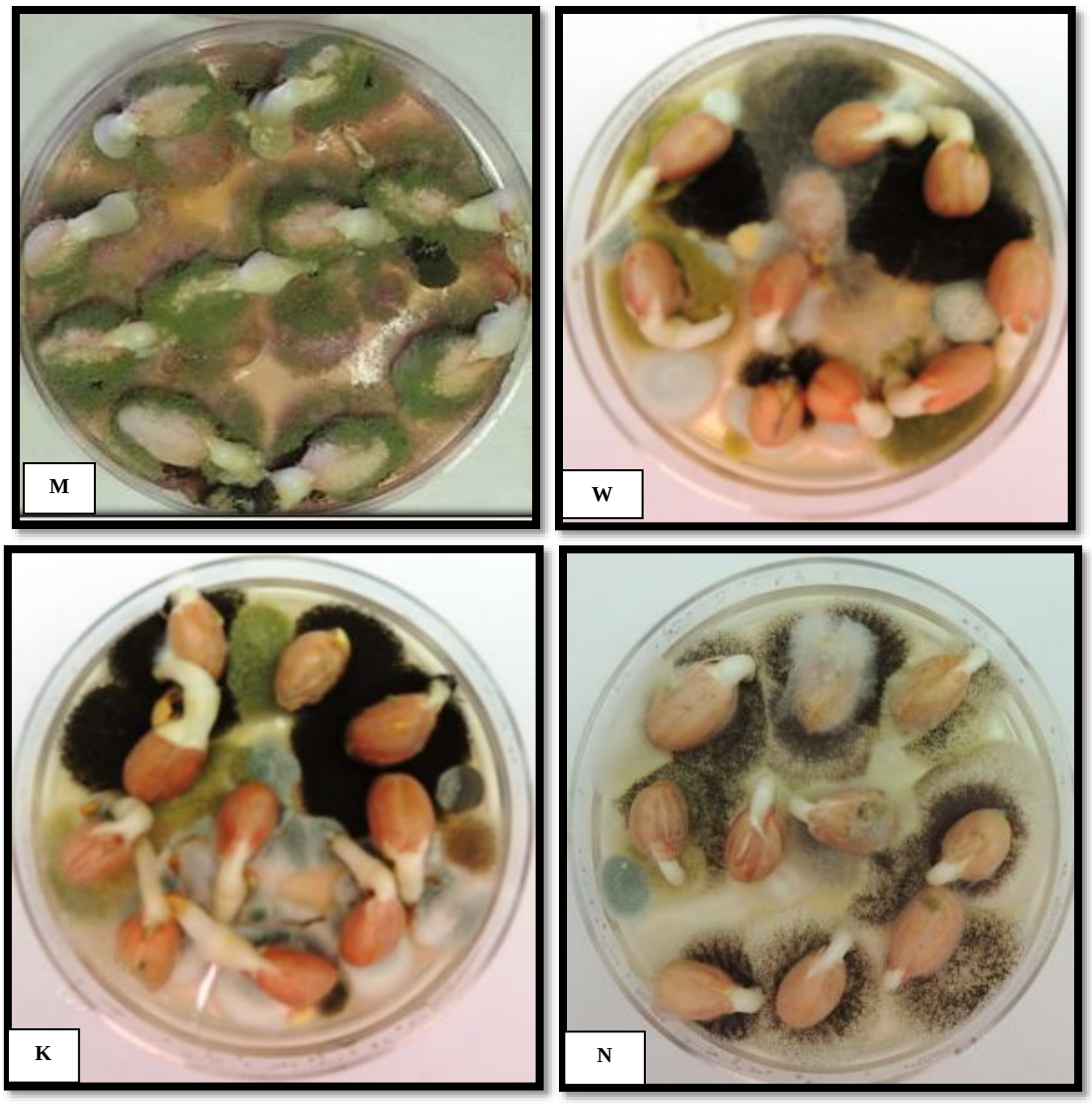

Plate 4.2. Seed mycoflora detected by agar plate method (Market samples)

M:Mahabubnagar, W:Warangal, K:Karimnagar, N:Nizamabad

Out of six fungal species recorded, the occurrence of *A. flavus* was found predominant in the seed samples analyzed from four districts (21.5 %). The occurrence of *A. flavus* was found highest in the seed samples of Mahabubnagar district (26.2 %) and Warangal district (24.6 %) and was the most predominant fungus followed by *A. niger* (16.7 % to 25 %). The occurrence of pathogenic fungi like *Alternaria* sp. (0.56 % to 0.78 %), *Fusarium* sp. (0.78 % to 1.44 %), *Macrophomina* sp. (0.44 % to 1 %), *Penicillium* sp. (0.44 % to 1.33 %) were observed in the seed samples analyzed from four districts, respectively. Seed mycoflora was found highest in the seed samples of Mahabubnagar district (54.8 %), while it was least in Nizamabad district (34.7 %). Storage fungi like *A. flavus, A. niger* and *Penicillium* sp. were low in Nizamabad district (0.44 % to 16.7%). The per cent incidence of the individual fungi was ranged from 0.44 % to 26.2 %. Seed mycoflora viz., *A. flavus, A. niger, Fusarium* sp. *Alternaria* sp. *Macrophomina* sp. and *Penicillium* sp. were recorded in the groundnut seed samples collected from market yards indicated their seed borne nature.

The present findings are in conformity with the earlier findings of Pitt and Hocking (1997), Guchi *et al*. (2014) and Kalyani *et al*. (2014) who reported that *A. flavus* and *A. niger* were more predominant in the field and stored foods than in the markets which might be attributed to source of seed samples, place of collection and location.

4.3 EXTERNAL SEED COLONIZATION BY *Aspergillus flavus* IN GROUNDNUT cvs. J 11 AND JL 24

Significant differences in seed colonization were observed in groundnut seeds of resistant cv. J 11 and susceptible cv. JL 24 which were artificially inoculated with *A. flavus* toxigenic strain (A*f* 11 - 4) @ 10^9 conidia/ml and incubated for 1, 3, 5, 7 and 9 days along with untreated seeds were externally examined for seed colonization. The percent surface area colonized due to *A. flavus* with a severity score of 1, 2, 3 and 4 were observed at 3, 5, 7 and 9 days of incubation in resistant cv. J 11. Whereas, in the susceptible cv. JL 24 recorded severity score of 2, 3, 4 and 4 at 3, 5, 7 and 9 days of incubation period indicating the differences in seed colonization in resistant and susceptible cultivars (Table 4.3, 4.4) (Plate 4.3,4.4).

Similar variation in seed colonization due to *A. flavus* pathogen in groundnut accessions was reported earlier by Deshpande and Pancholi (1979) and Thakur *et al*. (2000) and Nakai *et al.* (2008) recorded the susceptibility of groundnuts to colonization of *A. flavus* especially during storage.

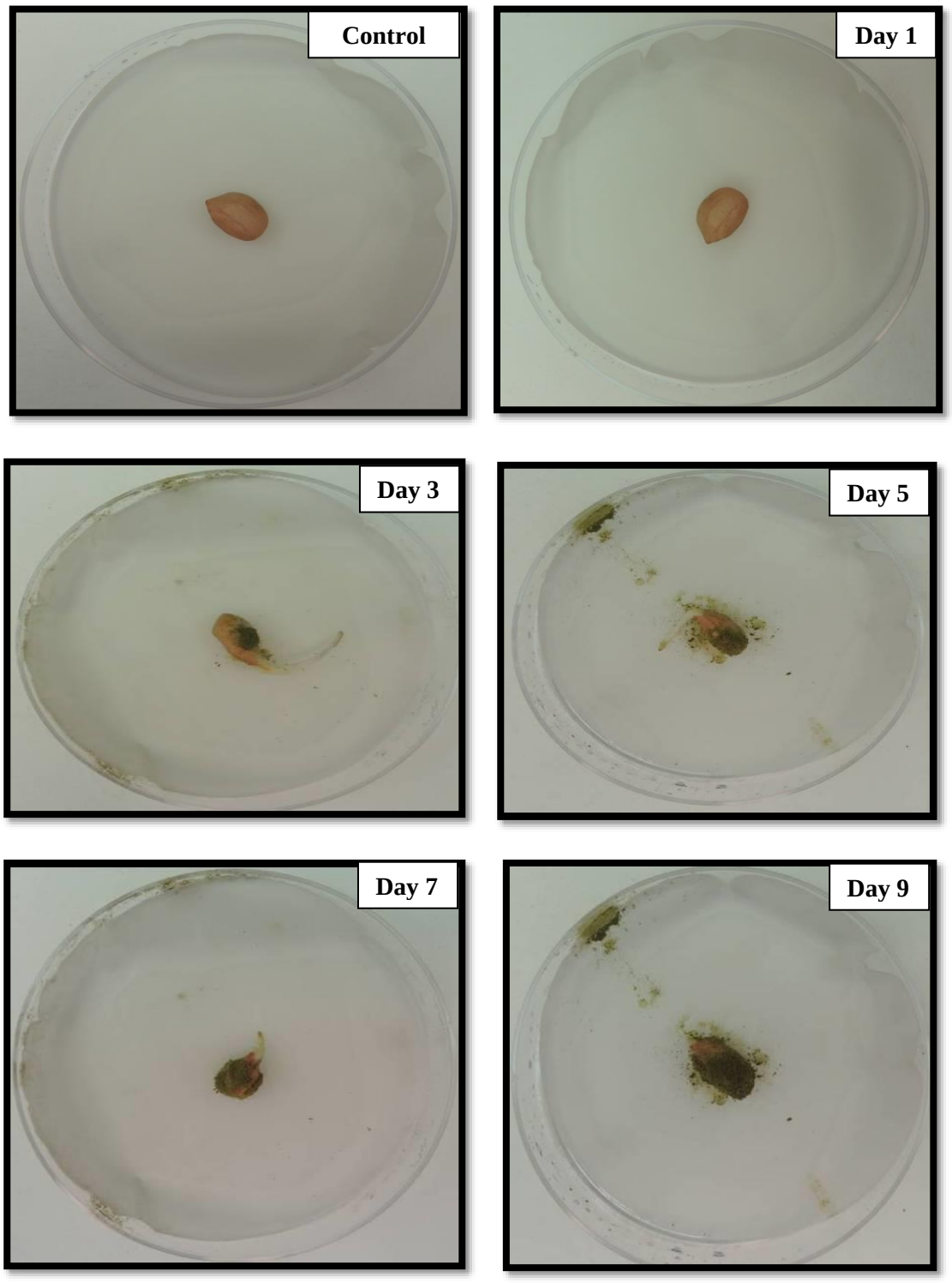

Plate 4.3. External seed colonization of *A. flavus* in groundnut cv. J 11 at different days of incubation period

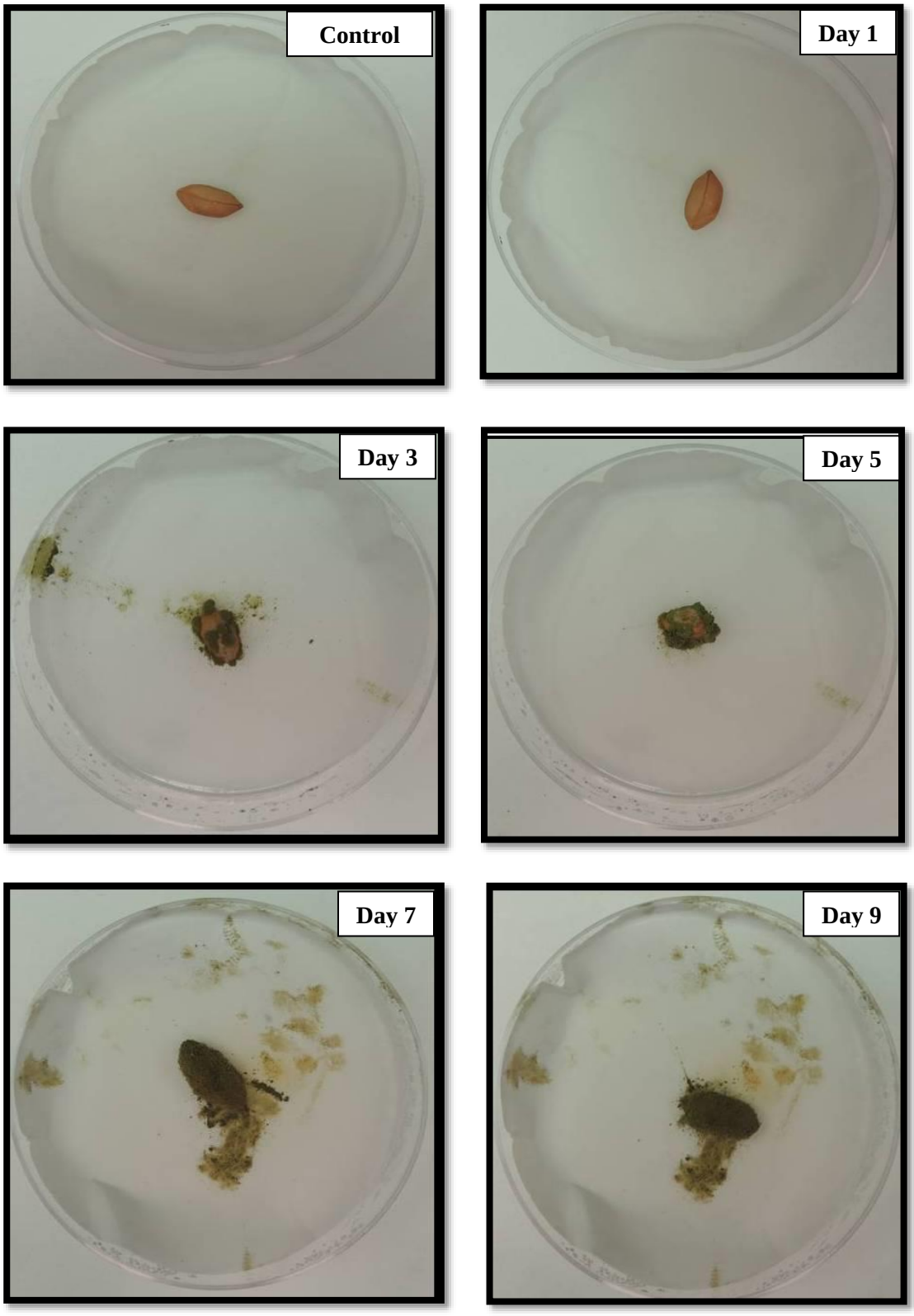

Plate 4.4. External seed colonization of *A. flavus* in groundnut cv. JL 24 at different days of incubation period

4.4 SCANNING ELECTRON MICROSCOPIC STUDIES

Groundnut seeds of resistant cv. J 11 and susceptible cv. JL 24 were artificially inoculated with *A. flavus* @ 10^9 conidia/ml and incubated at 1, 3, 5, 7 and 9 days along with untreated seeds. Nature of seed colonization by *A. flavus* and entry of the pathogen into the groundnut seeds was observed with Scanning Electron Microscopy (SEM). The results revealed that presence of pathogen mycelium in the damaged seed coat with fractured discontinuous epidermis with loose broken cell junctions between epidermal cells were observed. In addition to this rough, discontinuous, disorganised parenchyma, broken and missing cell walls and almost with total depletion of storage proteins were observed. Similarly, cells of healthy embryos of both cultures were well organized, unruptured cell walls with minimal intercellular space and abundant storage proteins. Hyphae penetrated into embryonic tissues and established intercellularly and intracellularly leading to an overall depletion of storage proteins. Intense hyphal branching with haustoria and abundant sporulation were observed in groundnut cv. JL 24 as compared to resistant cv. J 11 (Plate 4.5 & 4.6).

Seed coat responsiveness is a major key factor in establishing the pathogen when infection occurs. In the present study when the seeds of both the cultivars of groundnut were inoculated with *A. flavus* toxigenic strain, the penetration and establishment of the fungi in case of cv. J11 was slow as compared to cv. JL 24. The results are in conformity with Ae gendy *et al.,* (2001) who reported cell wall fortifications such as deposition of callose, cellulose, lignin and structural proteins directly below the point of attempted penetration to prevent the pathogen infection.

A thorough understanding of the host pathogen interaction between groundnut and *A. flavus* may provide information that might be used to develop novel detection and screening methods. The toxic properties of the aflatoxins produced by *A. flavus* are a major concern for growers and consumers of groundnut. Elimination of the threat of infection due to *A. flavus* is important before groundnut seeds goes into the storage. The present investigation reveals that *A. flavus* was seed borne in nature and contaminated seeds were important source of inoculum for seed infection and spread of the fungus from one seed to another during storage. SEM studies proved to be a reliable method to detect the intercellular and intracellular hyphae of *A. flavus* which is undetectable with the naked eye or by conventional light microscopy.

The present findings are in conformity with Achar *et al*. (2009) who reported that mycelium of *A. flavus* was seen established in the host tissues both intercellularly and intracellularly and continuous branching of young hyphae was seen in the groundnut seed. Rodriguez *et al*. (2007) who reported the structure of aflatoxigenic molds and their identification up to species level and

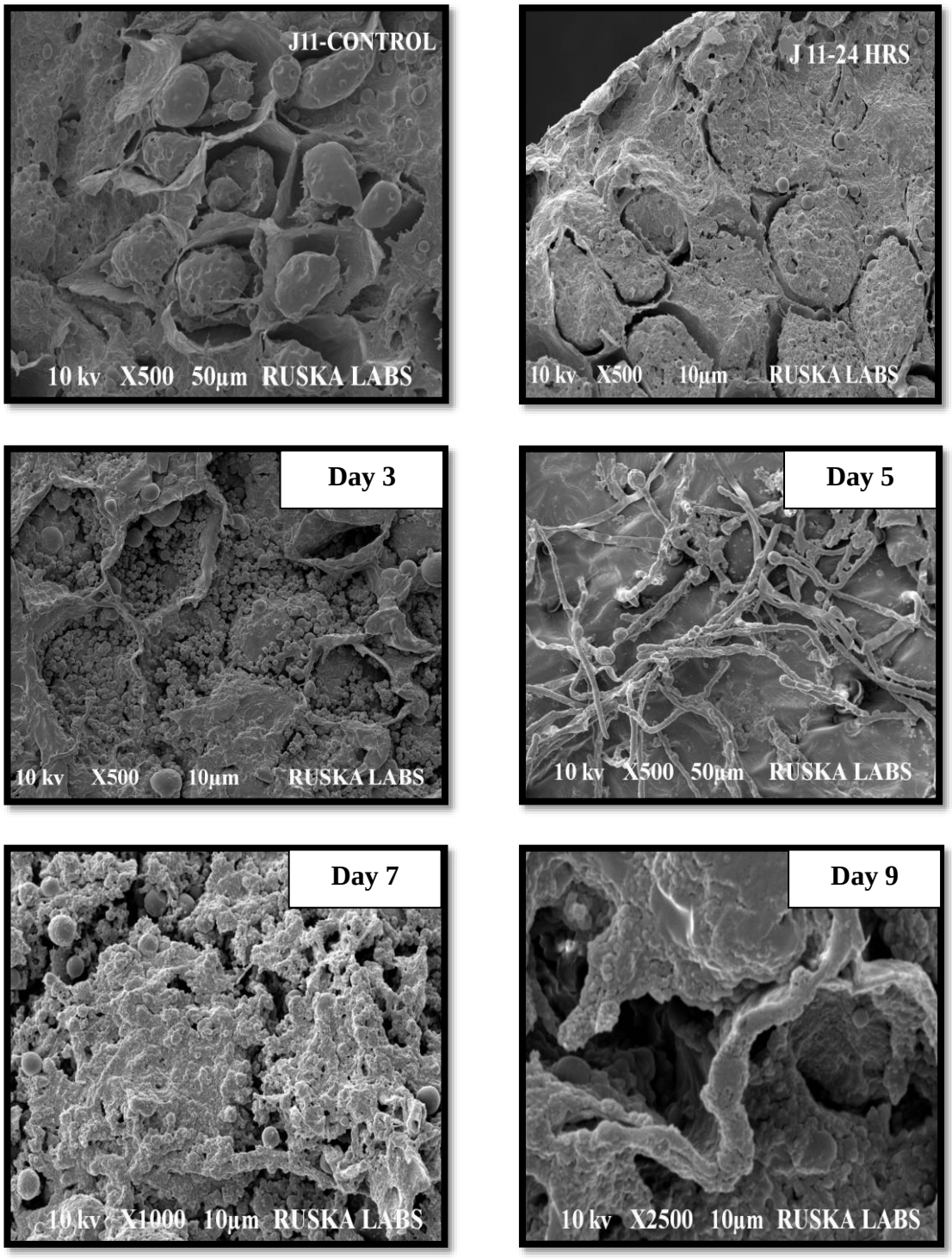

Plate 4.5. Scanning electron micrographs of *A. flavus* growth in treated seeds of groundnut resistant cv. J 11 at different days of incubation period

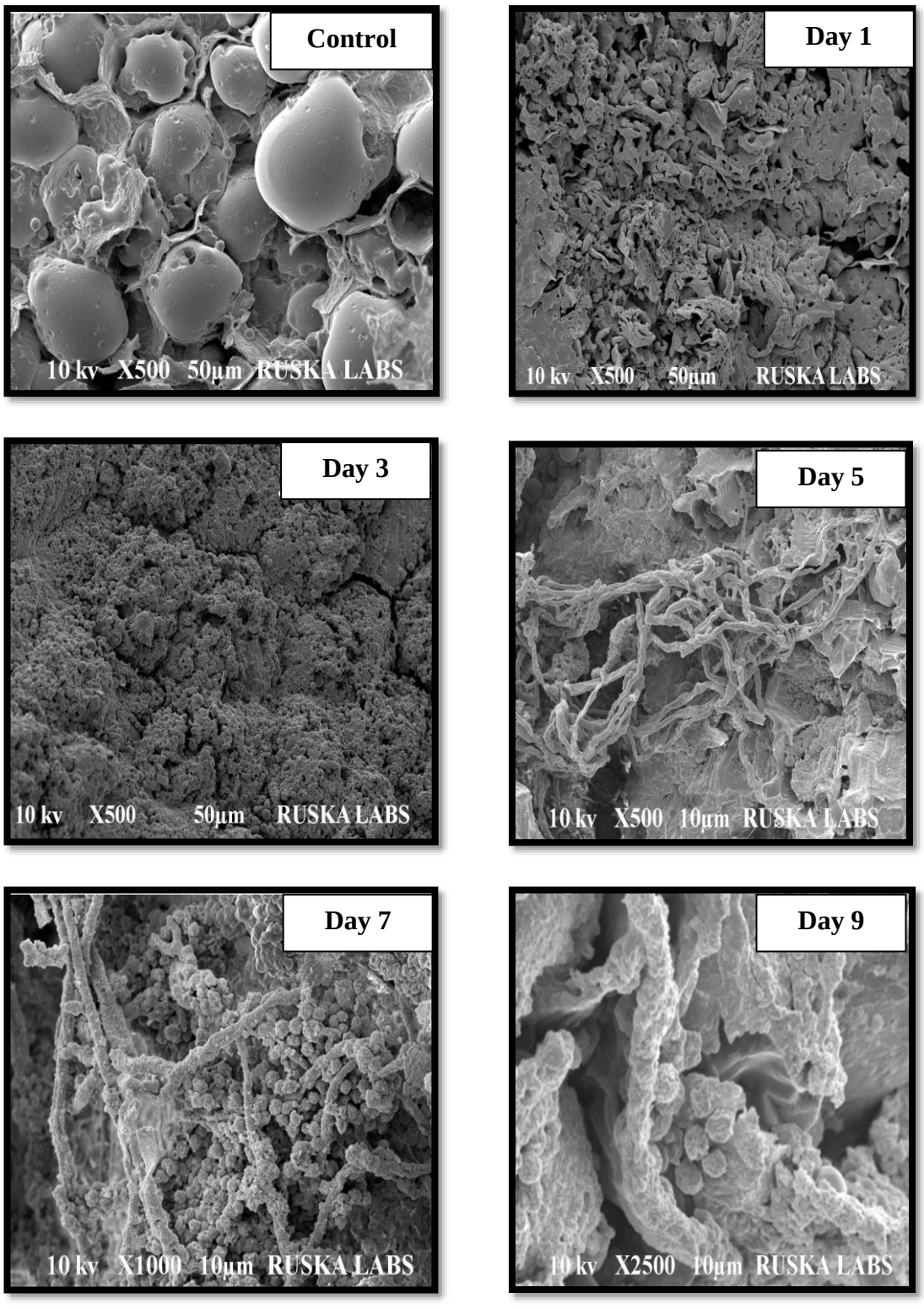

Plate 4.6. Scanning electron micrographs of *A. flavus* growth in treated seeds of groundnut susceptible cultivar JL 24 at different days of incubation period

characterization. Hermetz *et al.* (2014) observed that establishment of seed borne nature of *A. flavus* and its significance in seed and seedling infection using light microscopy, Scanning Electron Microscopy and Transmission Electron Microscopy.

Scanning Electronic Microscopic (SEM) methodology enabled to observe the interaction of fungi on surface of seeds and potential to increase the opportunities for teaching and learning in Seed Pathology depending on the level of detail of observed structures. The adoption of this technique in the future seed health analysis could be useful to identify fungal structures that enable ensuring the implementation of correct diagnoses as well as to facilitate conduction of more detailed taxonomic classification of seed-borne fungi.

4.5 EFFECT OF *A. flavus* INFECTION ON OIL CONTENT IN GROUNDNUT RESISTANT cv. J 11

The effect of *A. flavus* infection on oil content in groundnut seeds were recorded. Oil content was significantly differed in treated and untreated seed samples analyzed. The oil content was gradually reduced at 1 to 56 days after incubation to an extent of 50.3 % to 41.3 % in the seeds treated with *A. flavus*. In the untreated seeds (control) there was less reduction in oil content (50.6 % to 44.6 %) (Table 4.5).

4.5.1 Effect of *A. flavus* on oil content in groundnut susceptible cv. JL 24

The effect of *A. flavus* infection on oil content in groundnut seeds were assessed. Oil content was significantly differed in treated and untreated seed samples analyzed. The oil content was gradually reduced at 1 to 56 days after incubation. The reduction in oil content was high in treated seeds (50.6 % to 32.3 %) as compared with untreated seeds (50.6 % to 36.9 %) (Fig 4.3).

The per cent reduction in oil content was high in susceptible groundnut cv. JL 24 (18.3%) as compared to resistant groundnut cv. J 11 (9 %). While the reduction in oil content was low in the untreated seeds of groundnut cv. JL 24 and groundnut cv. J 11 (13.7 % and 6 %).

The reduction in oil content might be attributed to lipids present in the seeds were primarily neutral triglycerides and their hydrolysis to free fatty acids and glycerol were catalyzed by seed borne fungi which caused the oxidation of fatty acids and inactivation of enzymes. This might be one of the reasons for the reduction in oil content in the groundnut cultivars. The present results are in agreement with Deshpande and Pancholy (1979), Bhattacharya and Raha (2002) and Narayanswamy (2003) who reported that significant changes in oil content in the inoculated groundnut seed samples with the advancement in the storage period.

Table 4.5. Effect of *A. flavus* infection on oil content (%) in treated and untreated seeds of groundnut cvs. J 11 and JL 24

S. No	Interval (Days)	Oil content (%)			
		cv. J 11		cv. JL 24	
		Treated	Untreated	Treated	Untreated
1	1	50.3	50.6	50.6	50.6
2	3	47.2	48.4	47.9	47.9
3	7	47.1	47.5	46.8	47.8
4	14	47.0	47.5	46.8	47.5
5	21	45.8	47.4	46.4	47.3
6	28	45.8	47.4	46.3	47.0
7	35	45.5	47.2	43.5	42.5
8	42	45.5	46.1	38.3	39.3
9	49	44.8	45.8	34.9	38.0
10	56	41.3	44.6	32.3	36.9
	SE (m) ±	1.29	0.91	0.73	0.93
	CD at 5 %	3.80	2.70	2.17	2.76

Table 4.6. Effect of *A. flavus* infection on protein content (%) in treated and untreated seeds of groundnut cvs. J 11 and JL 24

S. No	Interval (Days)	Protein content (%)			
		cv. J 11		cv. JL 24	
		Treated	Untreated	Treated	Untreated
1	1	33.6	33.2	41.2	40.1
2	3	33.2	31.0	41.0	39.9
3	7	33.0	31.0	39.8	37.1
4	14	32.4	30.2	39.8	36.4
5	21	31.4	29.7	38.9	36.4
6	28	28.9	29.2	34.9	36.3
7	35	28.8	29.2	33.0	35.7
8	42	28.5	28.8	29.6	29.9
9	49	27.6	28.1	29.4	29.4
10	56	27.1	28.1	24.9	25.9
	SE (m) ±	1.19	0.68	1.62	1.57
	CD at 5 %	3.52	2.03	4.80	4.65

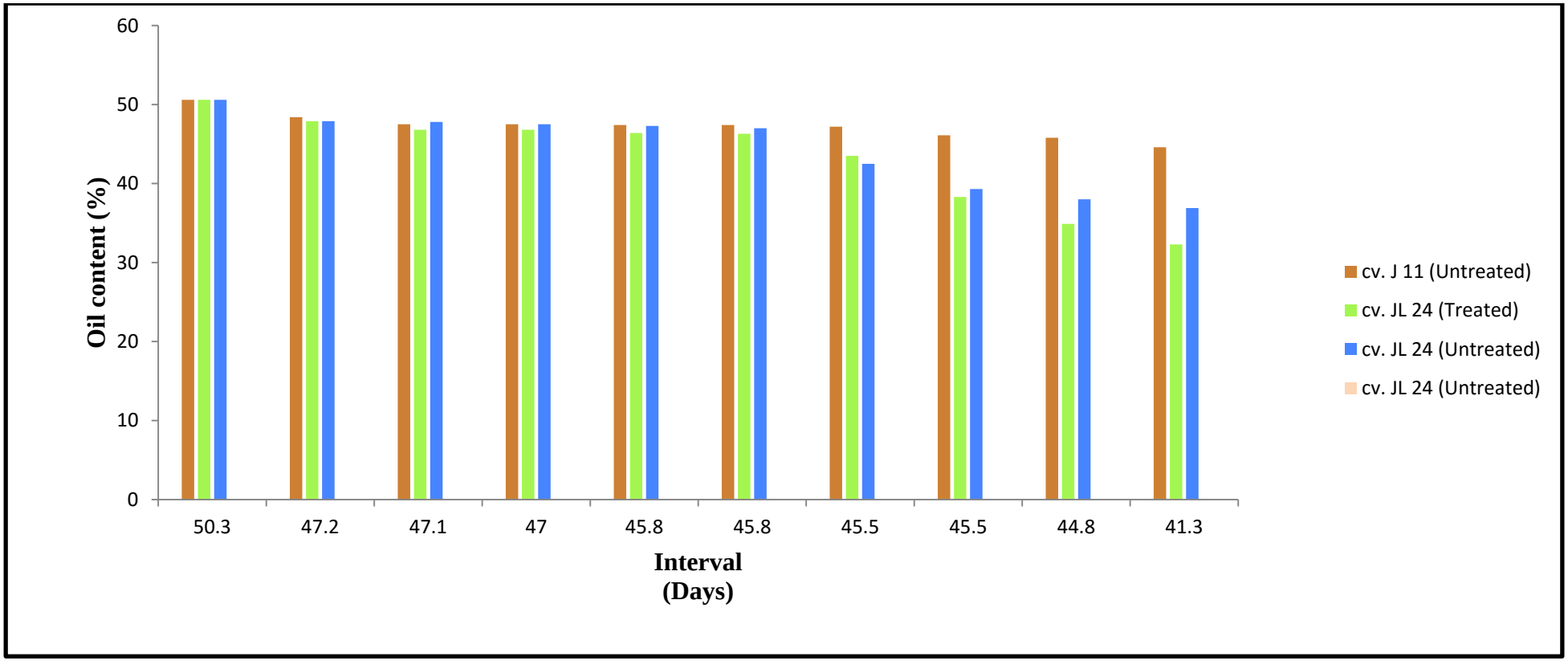

Fig 4.3. Oil content in treated and untreated seed samples of groundnut cultivars

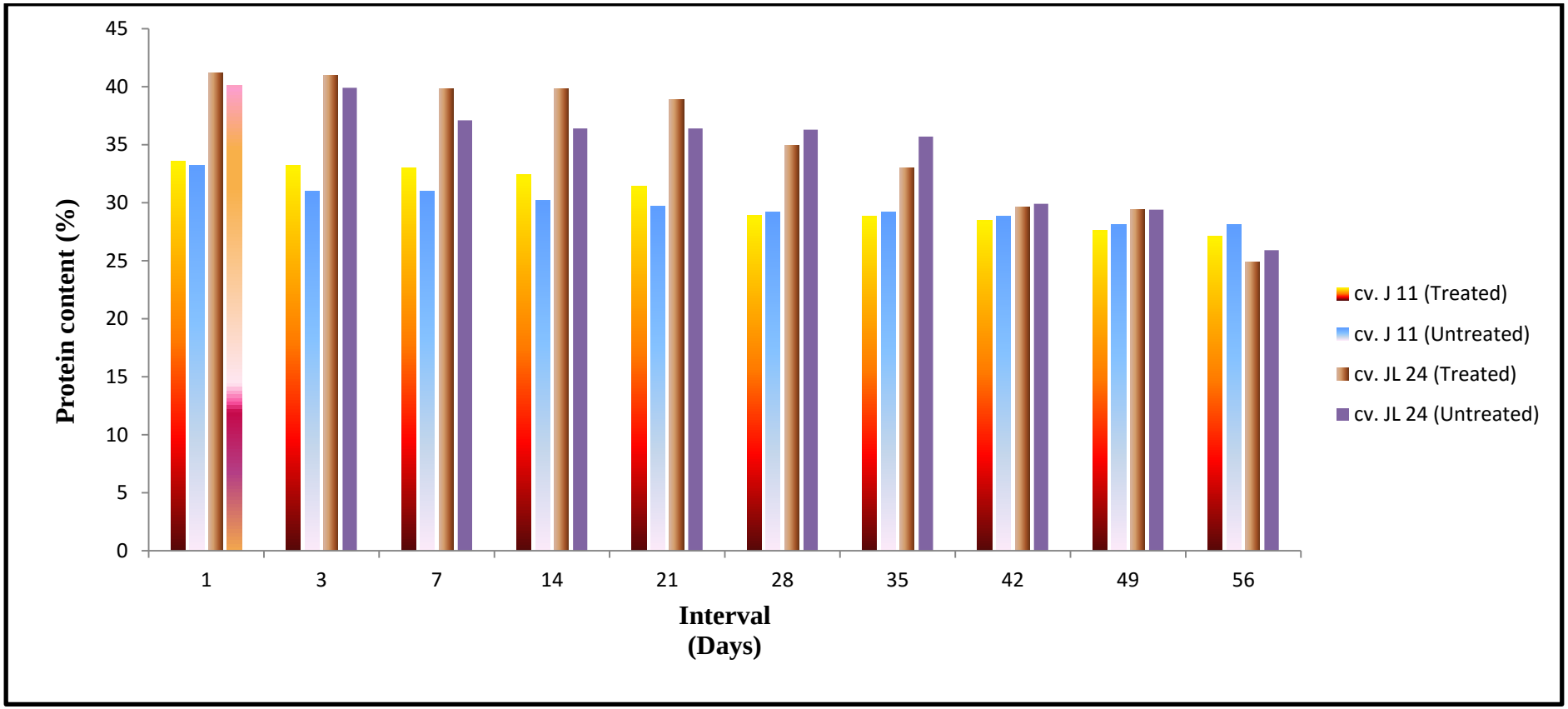

Fig 4.4. Protein content in treated and untreated seed samples of groundnut cultivars

4.6 EFFECT OF *A. flavus* ON PROTEIN CONTENT IN GROUNDNUT RESISTANT cv. J 11

The effect of *A. flavus* infection on protein content in groundnut cv. J11 was recorded. Protein content was significantly differed in treated and untreated seed samples analyzed. The protein content was gradually reduced at 1 to 56 days after incubation. It was ranged from 33.6 % to 27.1 % in the treated seeds and untreated seeds (33.2 % to 28.1 %) (Table 4.6).

4.6.1 Effect of *A. flavus* on protein content in groundnut susceptible cv. JL 24

The effect of *A. flavus* infection on protein content of groundnut cv. JL 24 was recorded. Protein content was significantly differed in treated and untreated seed samples analyzed. The protein content was gradually reduced from 1 to 56 days after incubation and ranging from 41.2 % to 24.9 % in the seeds treated with *A. flavus* where-as in the untreated seeds (control) recorded less reduction in protein content 40.1 % to 25.9 % (Table 4.6) (Fig 4.4).

Overall the per cent reduction in the protein content was found high in susceptible groundnut cv. JL 24 (16.3 %) as compared to resistant groundnut cv. J 11 (6.5 %). While the reduction in protein content was low in the untreated seeds of groundnut cvs. JL 24 and J 11 (14.2 % & 5.1%). The present results revealed that the rate of depletion in total protein was significantly differed. It might be attributed that protein served as a primary source of readily available carbon and nitrogen for growth and metabolism of the invading fungi. Loss in protein content during the early phase of invasion and incubation indicated that proteolysis and formation of simpler compounds such as amino acids which were utilized by the fungi. Similar trend of reduction in protein content in the groundnut due to storage fungi was reported earlier by Narayanswamy (2003), Adiver *et al*. (2015), Rammorthy and Karivarataraju (1989), Braccini *et al.* (2000), Ushamalini *et al*. (1998), Kakde and Chavan (2011) and Bilgrami *et al*. (1976).

4.7 EFFECT OF *A. flavus* ON SATURATED FATTY ACIDS (PALMITIC AND) IN GROUNDNUT RESISTANT cv. J 11 AND SUSCEPTIBLE cv. JL 24

The effect of *A. flavus* infection on palmitic acid content of groundnut seeds were recorded. The palmitic acid content in resistant cv. J 11 was gradually increased from 1 to 56 days after incubation. In the treated seeds the increase in palmitic acid content was high (11 % to 14.9 %) as compared with untreated seeds (control) (11 % to 13.9 %). Where as in susceptible cv. JL 24 the

Table 4.7. Effect of *A. flavus* infection on saturated fatty acids (Palmitic acid) content (%) in treated and untreated seeds of groundnut cvs. J 11 and JL 24

S. No	Interval (Days)	Palmitic acid (%)			
		cv. J 11		cv. JL 24	
		Treated	Untreated	Treated	Untreated
1	1	11.0	11.0	11.0	11.0
2	3	11.4	11.5	11.5	11.3
3	7	11.6	11.6	11.5	11.5
4	14	12.5	11.7	11.8	11.5
5	21	12.5	11.7	11.9	11.9
6	28	12.9	12.5	11.9	11.9
7	35	13.5	12.5	12.3	13.0
8	42	13.5	12.5	12.8	13.0
9	49	14.3	13.8	13.0	13.5
10	56	14.9	13.9	15.5	13.5
	SE (m) ±	0.31	0.39	0.56	0.55
	CD at 5 %	0.92	1.17	1.66	1.64

Table 4.8. Effect of *A. flavus* infection on saturated fatty acids (Stearic acid) content (%) in treated and untreated seeds of groundnut cvs. J 11 and JL 24

S. No	Interval (Days)	Stearic acid (%)			
		cv. J 11		cv. JL 24	
		Treated	Untreated	Treated	Untreated
1	1	0.72	0.74	0.72	0.71
2	3	0.79	0.83	0.96	0.72
3	7	1.36	0.95	0.95	1.36
4	14	1.67	0.96	1.19	1.67
5	21	1.91	1.68	2.48	1.91
6	28	2.20	2.17	2.84	2.30
7	35	2.37	2.30	3.35	2.31
8	42	2.40	2.48	3.45	2.40
9	49	2.63	2.56	4.89	2.63
10	56	3.65	2.68	5.31	2.71
	SE (m) ±	0.20	0.30	0.39	0.19
	CD at 5 %	0.61	0.90	1.16	0.57

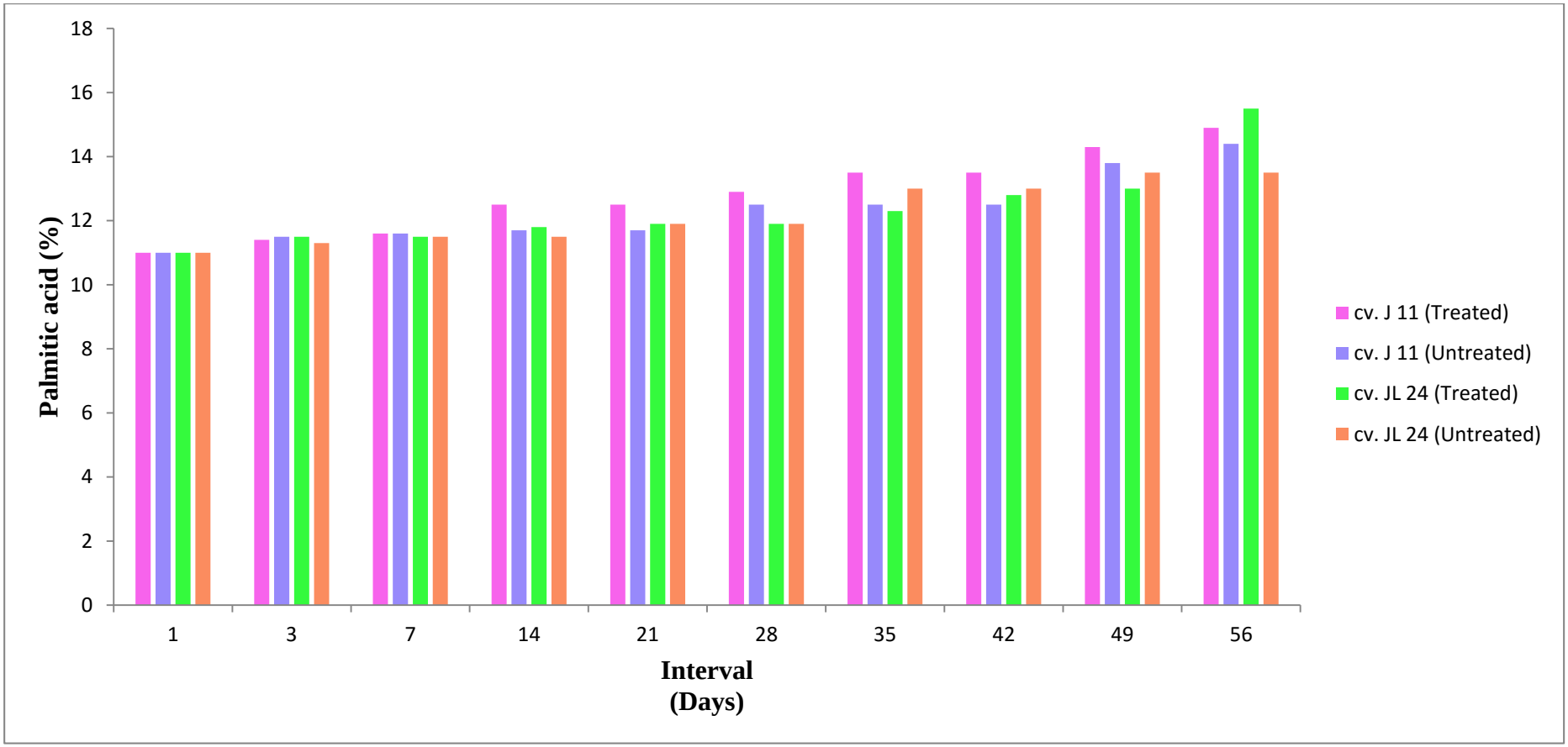

Fig 4.5. Palmitic acid content in treated and untreated seed samples of groundnut cultivars

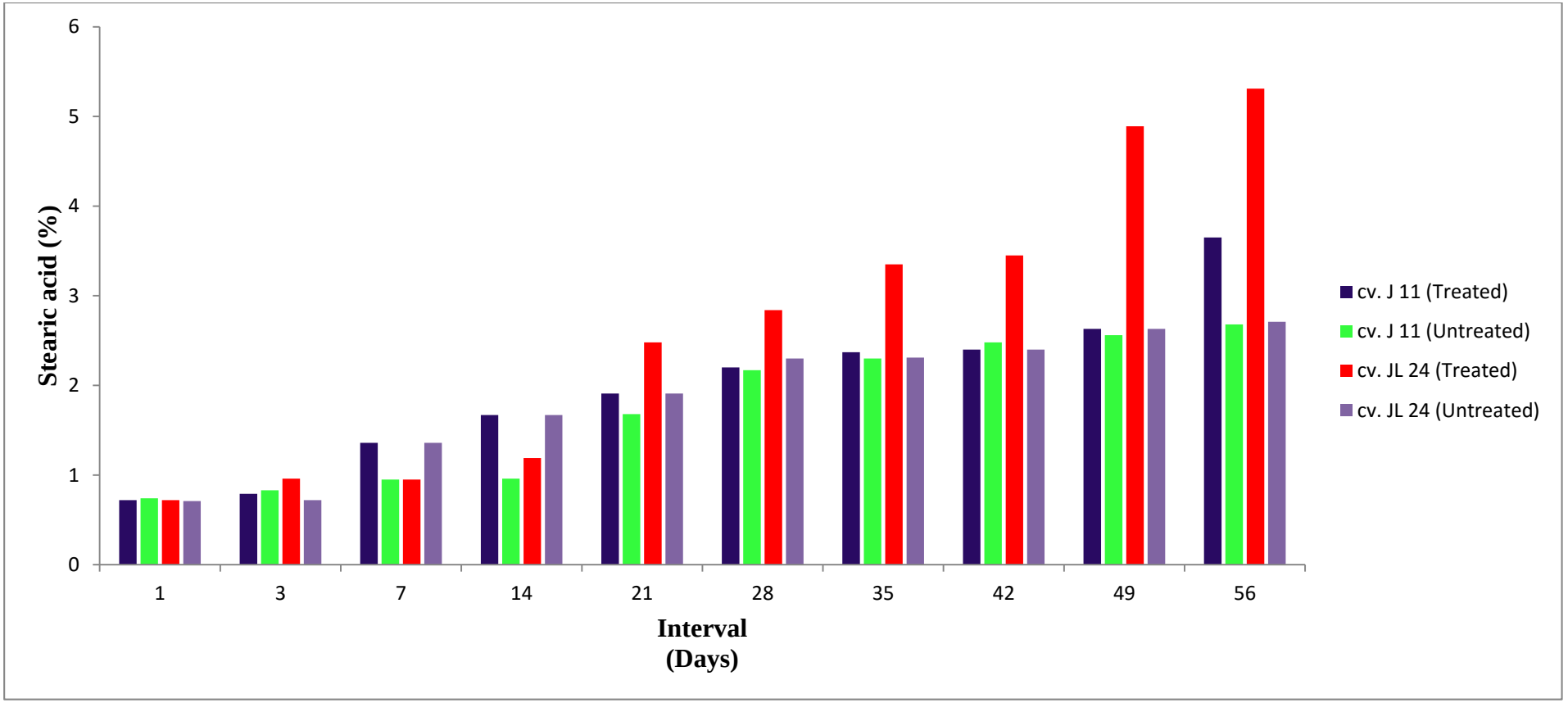

Fig 4.6. Stearic acid content in treated and untreated seed samples of groundnut cultivars

increase in palmitic acid content of 11 % to 15.5 % in the treated seeds where as in the untreated seeds there was slow increase of 11.0 % to 13.5 % (Table 4.7) (Fig 4.5).

4.7.1 Effect of *A. flavus* on saturated fatty acids (stearic acid) in groundnut resistant cv. J 11 and susceptible cv. JL 24

The effects of *A. flavus* on stearic acid content of groundnut seeds were recorded. The increased levels of stearic acid content in resistant cv. J 11 was observed at 1 to 56 days after incubation in treated seeds (0.72 % to 3.65 %) and untreated seeds (0.74 % to 2.68 %). Whereas, in susceptible cv. JL 24 the stearic acid content was gradually increased from 1 to 56 days after incubation. The per cent increase in stearic acid content was high (0.72 % to 5.31 %) in the treated seeds as compared with the untreated seeds (0.71 % to 2.71%) (Table 4.8) (Fig 4.6).

Many of the fungi have been reported to cause physical and biochemical changes in crops during storage as well as in releasing toxic substances which tend to limit their use and general acceptability. In general, when the seeds were stored at higher moisture content the activity of *Aspergilli* were found high which releases toxic metabolites into seeds. Presence of these toxic substances in the seeds mainly affects seed quality and adversely making the seeds unfit for consumption. An increased levels of free fatty acid contents in the groundnut cultivars over a period of storage indicates the breakdown of triglycerides in groundnut oil leading to an eventual deterioration of the seed quality and production of hydrolytic rancidity.

The present results are in conformity with Rammorthy and Karivarataraju (1989) who reported a progressive increase in free fatty acid levels in the stored kernels than pods because there was invasion of storage fungi. Jain (2008) also reported the increased levels of free fatty acid content in the damaged seeds by fungal invasion. Mutegi *et al*. (2013) showed that rancidity was significantly increased during storage. Storage fungi can change fat quality of groundnuts by hydrolytic enzymes producing free fatty acids and glycerol. There was decrease in crude fat because fungi might have degraded the lipids by lipase enzyme.

4.8 EFFECT OF *A. flavus* INFECTION ON UNSATURATED FATTY ACIDS (LINOLEIC ACID) IN GROUNDNUT RESISTANT cv. J 11 AND SUSCEPTIBLE cv. JL 24

The effect of *A. flavus* infection on linoleic acid content of groundnut seeds were recorded. The linoleic acid content was gradually reduced at 1 to 56 days after incubation. The reduction in linoleic acid was high in the treated seeds (42.1 % - 27.1 %) as compared with untreated seeds (43

Table 4.9. Effect of *A. flavus* infection on unsaturated fatty acids (Linoleic acid) content (%) in treated and untreated seeds of groundnut cvs. J 11 and JL 24

S. No	Interval (Days)	Linoleic acid (%)			
		cv. J 11		cv. JL 24	
		Treated	Untreated	Treated	Untreated
1	1	42.1	43.0	40.9	40.8
2	3	41.4	41.5	39.3	37.9
3	7	40.6	38.9	37.3	37.8
4	14	36.6	36.9	32.6	33.8
5	21	36.1	36.8	30.9	30.3
6	28	34.4	33.8	30.2	29.4
7	35	33.9	33.6	29.8	29.3
8	42	32.3	33.2	25.4	29.3
9	49	31.3	32.1	24.1	28.1
10	56	27.1	31.7	23.4	27.5
	SE (m) ±	2.15	1.70	4.04	2.43
	CD at 5 %	6.34	5.03	11.9	7.18

Table 4.10. Effect of *A. flavus* infection on unsaturated fatty acids (Oleic acid) content (%) in treated and untreated seeds of groundnut cvs. J 11 and JL 24

S. No	Interval (Days)	Oleic acid (%)			
		cv. J 11		cv. JL 24	
		Treated	Untreated	Treated	Untreated
1	1	43.9	43.9	41.7	42.0
2	3	42.8	42.9	37.8	41.1
3	7	40.3	41.3	36.9	40.6
4	14	40.2	41.2	33.6	38.0
5	21	40.1	40.6	30.3	36.9
6	28	40.1	40.2	29.4	36.8
7	35	39.7	40.1	29.0	36.8
8	42	38.9	39.7	27.7	35.9
9	49	38.6	38.7	27.5	34.6
10	56	29.9	37.9	25.1	33.8
	SE (m) ±	1.70	1.02	1.98	1.82
	CD at 5 %	5.01	3.03	5.85	5.38

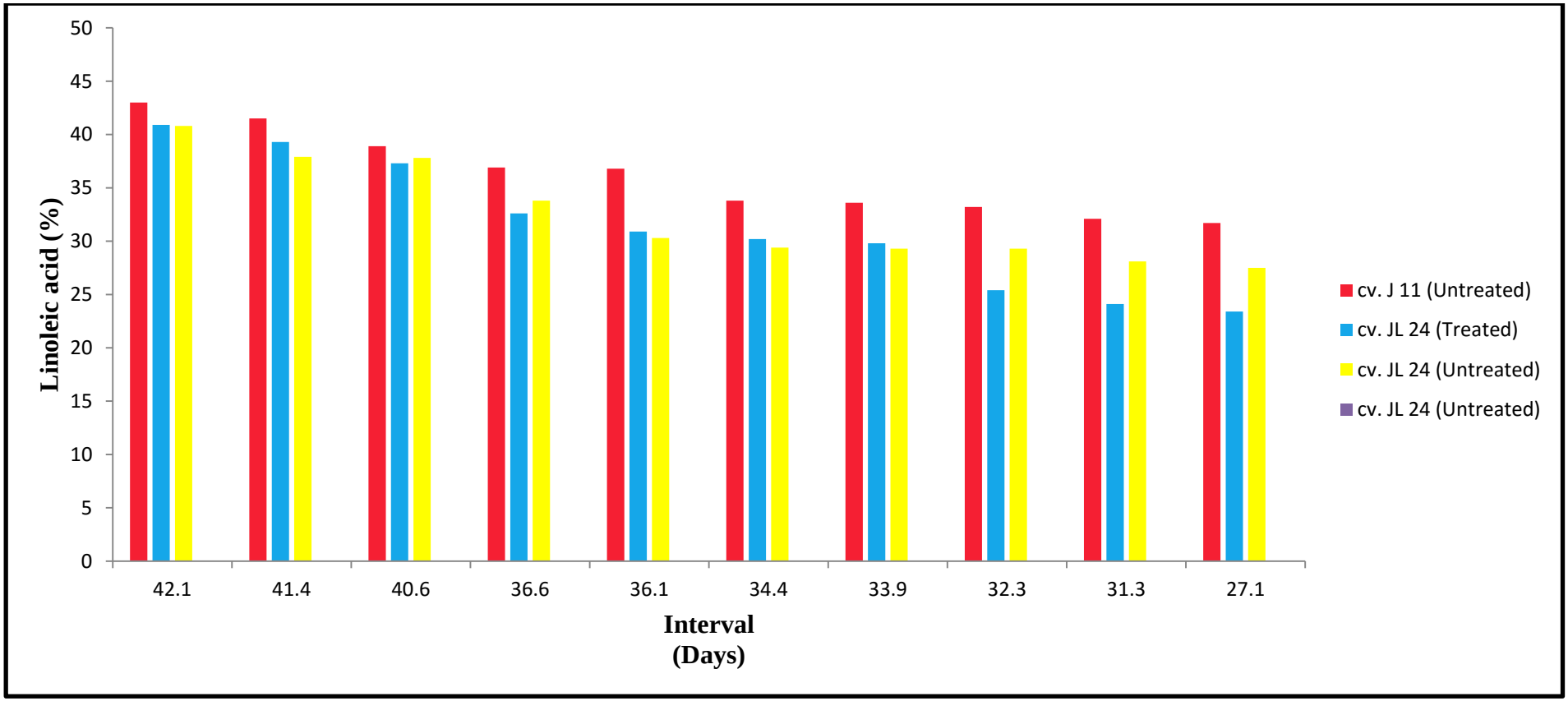

Fig 4.7. Linoleic acid content in treated and untreated seed samples of groundnut cultivars

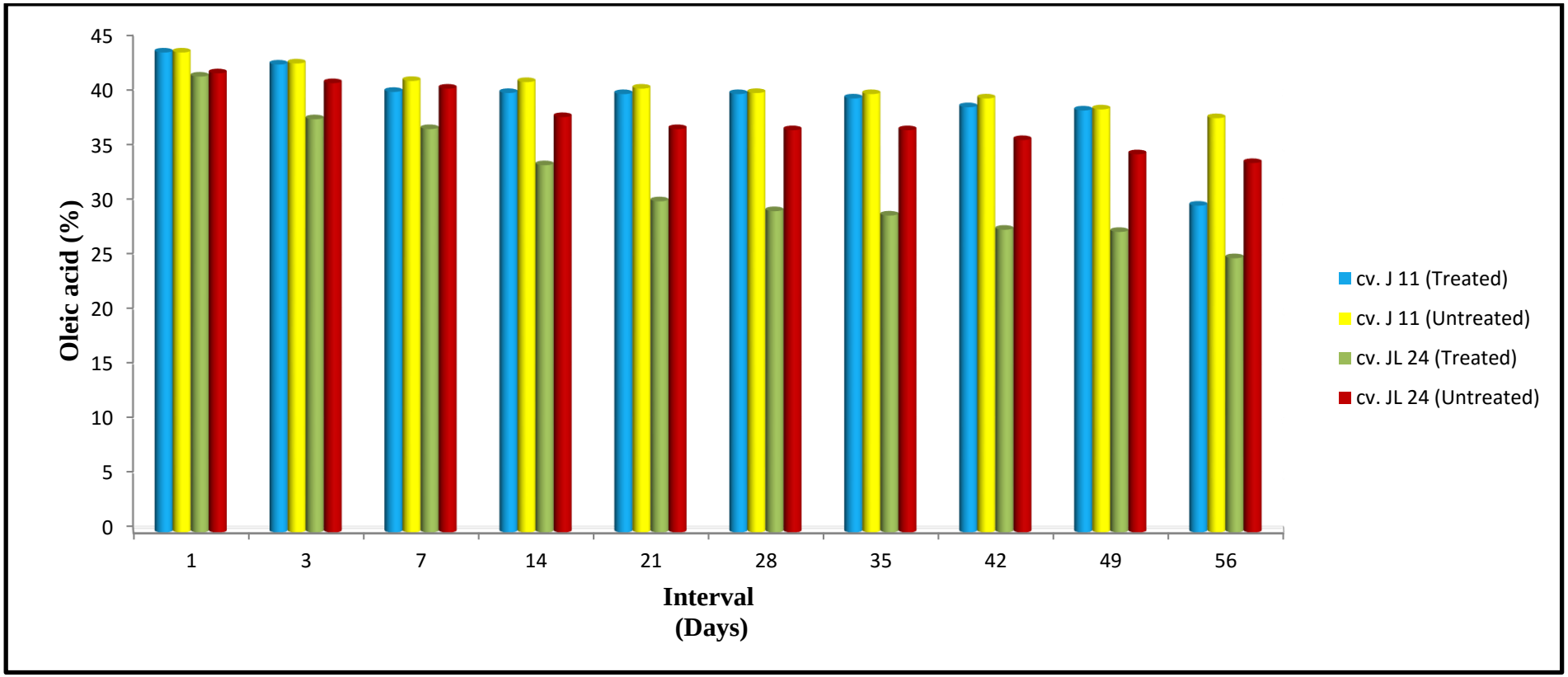

Fig 4.8. Oleic acid content in treated and untreated seed samples of groundnut cultivars

% to 31.7 %). Whereas, in susceptible cv. JL 24 the linoleic acid content was gradually reduced at 1 to 56 days after incubation. The rate of decrease was high in the treated seeds (40.9 % - 23.4 %) as compared with untreated seeds (40.8 % - 27.5 %) (Table 4.9, Fig 4.7).

4.8.1 Effect of *A. flavus* on unsaturated fatty acids (oleic acid) in groundnut resistant cv. J 11 and susceptible cv. JL 24

The effect of *A. flavus* infection on oleic acid content in groundnut cv. J 11 was recorded. The reduction in oleic acid content was observed in the treated seeds (43.9 % to 29.9 %) as compared with untreated seeds (43.9 % to 37.9 %). Similar trend of reduction in oleic acid content were observed in treated seeds of susceptible cv. JL 24 (41.7 % to 25.1 %) and untreated seeds (42.0 % to 33.8 %) (Table 4.10, Fig 4.8).

The reduction in linoleic and oleic acid contents were high in the treated seeds as compared to the untreated seeds.

The per cent reduction in the unsaturated fatty acids like linoleic and oleic acids were high in susceptible groundnut cv. JL 24 (17.5 % and 16.6 %) as compared to resistant groundnut cv. J 11 (15 % and 14 %). Whereas, in the untreated seeds, the per cent reduction in linoleic and oleic acids were found low (11.3 % and 6 %) in groundnut cv. J 11 and 13.3 % and 8.2 % in groundnut cv. JL 24, respectively.

These results are in conformity with Braccini *et al*. (2000) who reported that reduction in unsaturated fatty acids, protein and lipid content of soybean.

4.9 EFFECT OF *A. flavus* ON AFLATOXIN CONTET OF GROUNDNUT RESISTANT cv. J 11 AND SUSCEPTIBLE cv. JL 24

The level of aflatoxin contents was increased at 1 to 56 days after incubation in the treated seeds of groundnut cv. J 11 and cv. JL 24. Aflatoxin content of 2.15µg/kg to 2861.3 µg/kg in the treated seeds and in the untreated seeds 2.15 µg/kg to 14.7 µg/kg were recorded in resistant cv. J 11. The aflatoxin content was found high in susceptible cv. JL 24 (63.4 µg/kg to 4077.1 µg/kg) in the treated seeds as compared to untreated seeds (2.15 µg/kg to 21.1 µg/kg) were recorded at different days of incubation period.

In the present study, susceptible groundnut cv. JL 24 recorded increased aflatoxin levels (63.4 µg/kg to 4077.1 µg/kg) as compared to groundnut cv. J 11 (2.15 µg/kg to 2861.3 µg/kg) (Fig

Table 4.11. Effect of *A. flavus* infection on aflatoxin content (µg kg^{-1}) in treated and untreated seeds of groundnut cvs. J 11 and JL 24 at different days of incubation period.

S. No	Interval (Days)	cv. J 11		cv. JL 24	
		Treated	Untreated	Treated	Untreated
1	1	2.15 (1.62)	2.15 (1.76)	63.4 (6.03)	2.15 (1.76)
2	3	37.9 (6.10)	3.61 (2.14)	89.9 (9.40)	3.94 (2.22)
3	7	74.7 (8.60)	6.04 (2.64)	186.0 (13.2)	6.37 (2.70)
4	14	87.7 (8.43)	6.76 (2.75)	639.5 (20.4)	6.76 (2.75)
5	21	220.4 (12.0)	8.77 (2.98)	635.0 (23.7)	9.43 (3.06)
6	28	278.2 (16.5)	11.5 (3.44)	963.9 (29.9)	9.84 (3.23)
7	35	338.4 (17.5)	12.9 (3.72)	1111.7 (33.0)	12.9 (3.72)
8	42	850.9 (28.4)	13.8 (3.67)	1303.7 (34.4)	13.8 (3.67)
9	49	2182.6 (37.3)	14.0 (3.86)	2367.3 (48.5)	15.6 (4.07)
10	56	2861.3 (45.4)	14.7 (3.97)	4077.1 (63.8)	21.1 (4.66)
	SE (m) ±	9.37	0.49	5.26	1.27
	CD at 5 %	27.6	1.23	15.5	0.42

Figures in the parenthesis are square root transformed values. Each value is means of three replications.

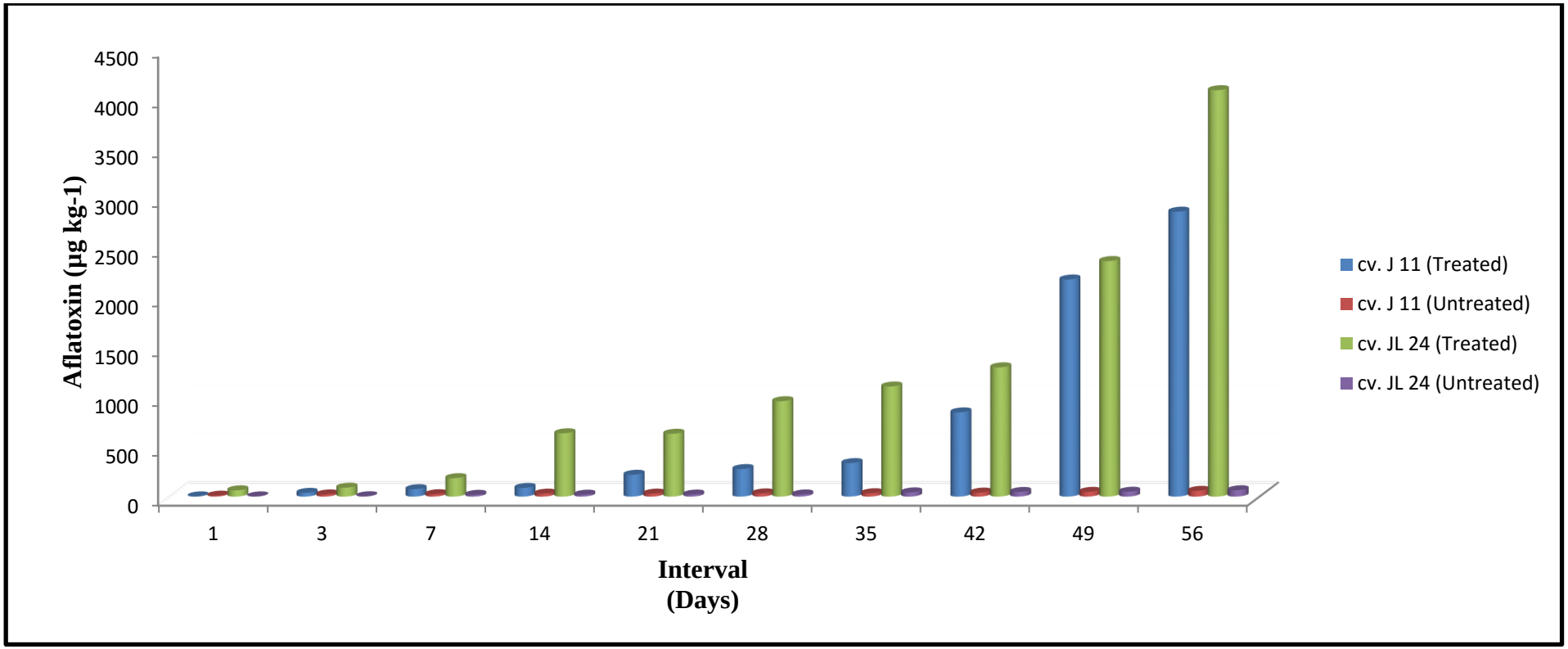

Fig 4.9. Effect of *A. flavus* infection on aflatoxin content in treated and untreated seeds of groundnut cvs. J 11 and JL 24 at different days of incubation period

4.9). Whereas, in the untreated seeds the detected aflatoxin content was within the permissible levels in both resistant cv. J 11 (2.15 µg/kg to 14.7 µg/kg) and susceptible cv. JL 24 (2.15 µg/kg to 21.1 µg/kg).

The present results are in agreement with the findings of Yu *et al.* (2004), Hameeda *et al*. (2006), Alemayehu *et al*. (2012) and Wartu *et al*. (2015) who reported that *A. flavus* was the most predominant species responsible for aflatoxin contamination of crops prior to harvest or during storage. The findings of Fabri *et al.* (1983), Passi *et al*. (1984), Doehlert *et al.* (1993) and Burrow *et al.* (1997) indicated that fatty acid composition might directly or indirectly affects aflatoxin biosynthesis.

4.10 EVALUATION OF SEED TREATMENTS WITH BIOAGENTS AND FUNGICIDES AGAINST *A. flavus* IN GROUNDNUT SUSCEPTIBLE cv.JL 24 UNDER GLASSHOUSE CONDITIONS

The results revealed that significant differences in different seed treatments were observed as compared to untreated and pathogen treated seeds (Table 4.13). The germination percentage was significantly improved in all the seed treatments as compared to untreated seeds. Among the seed treatments, groundnut seeds treated with *T. harzianum* recorded higher seed germination (96 %) followed by seed treatment with *T. viride* (91 %) and it was found on par with seeds treated with *P. fluorescens* (88.2 %). The other seed treatments viz., carbendazim (81 %) and mancozeb (73.5 %) were also found effective in increasing the seed germination over untreated seeds (65 %) and pathogen treated seeds (54.5 %) (Fig 4.10) (Table 4.13).

Significant differences in plant height due to different seed treatments in groundnut seeds were observed when compared with untreated and pathogen treated seeds. However, seed treatment with *T. harzianum* recorded higher plant height (4.75, 12.9 and 14.1 cm) followed by seed treatment with *T. viride* (4.10, 11.5 and 13.5 cm) and it was found on par with seeds treated with *P. fluorescens* (3.40, 10.2 and 10.8) at 15, 30 and 45 DAS. The other seed treatments, carbendazim (2.77, 8.02 and 9.21 cm) and mancozeb (2.72, 6.92 and 8.43 cm) were also found effective in increasing plant height over untreated seeds (2.67, 6.62 and 7.37 cm) and pathogen treated seeds (2.57, 5.65, 6.41 cm) at @ 15, 30 and 45 DAS (Fig 4.11) (Table 4.13).

Yield per plant was significantly improved in all the seed treatments as compared to treated and untreated seeds. Among the seed treatments, groundnut seeds treated with *T. harzianum* recorded higher pod yield per plant (4.6) followed by seed treatment with *T. viride* (4.2) and it was found on par with seeds treated with *P. fluorescens* (4.1). Seeds treated with carbendazim (3.65)

Table 4.13 Evaluation of seed treatments with bioagents and fungicides against *A. flavus* under glasshouse conditions

S. No	Treatments	Dosage (g/kg)	Germination (%)	Plant height 15 DAS (cm)	Plant height 30 DAS (cm)	Plant height 45 DAS (cm)	Pod yield per plant (g)
1	*Trichoderma harzianum*	10	96.0 (79.7)	4.75	12.9	14.1	4.60
2	*Trichoderma viride*	10	91.0 (73.4)	4.10	11.5	13.5	4.20
3	*Pseudomonas flourescens*	10	88.2 (69.9)	3.40	10.2	10.8	4.10
4	Mancozeb	2.5	73.5 (59.0)	2.72	6.92	8.43	3.37
5	Carbendazim	2.0	81.0 (64.1)	2.77	8.02	9.21	3.65
6	Inoculated control/Treated seeds		54.5 (47.5)	2.57	5.65	6.41	2.55
7	Uninoculated control/untreated seeds		65.0 (53.7)	2.67	6.62	7.37	2.92
	SEm ±		1.49	0.34	0.80	0.78	0.09
	CD at 5 %		4.43	1.01	2.36	2.31	0.27

Figures in parenthesis are angular transformed values. Each value is mean of four replications.

.

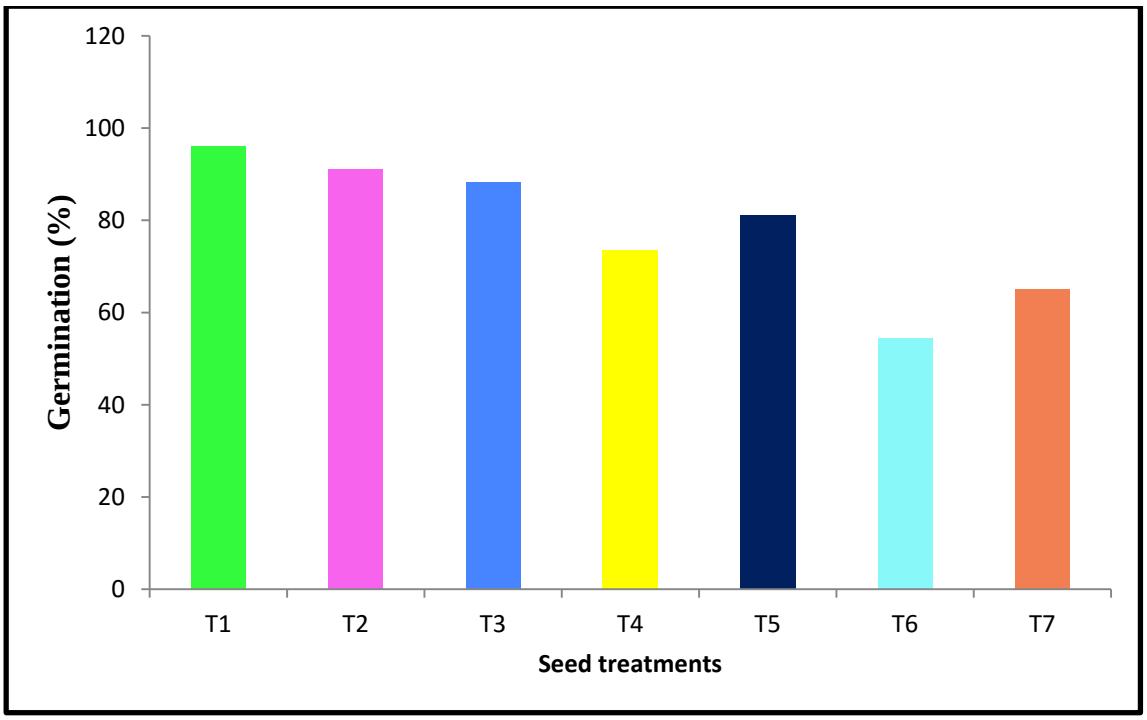

Fig. 4.10. Evaluation of seed treatments with bioagents and fungicides against *A. flavus* on germination

T_1 - *Trichoderma harzianum*

T_2 - *Trichoderma viride*

T_3 - *Pseudomonas flourescens*

T_4 - Mancozeb

T_5 - Carbendazim

T_6 - Inoculated control/Treated seeds

T_7 - Uninoculated control/untreated seeds

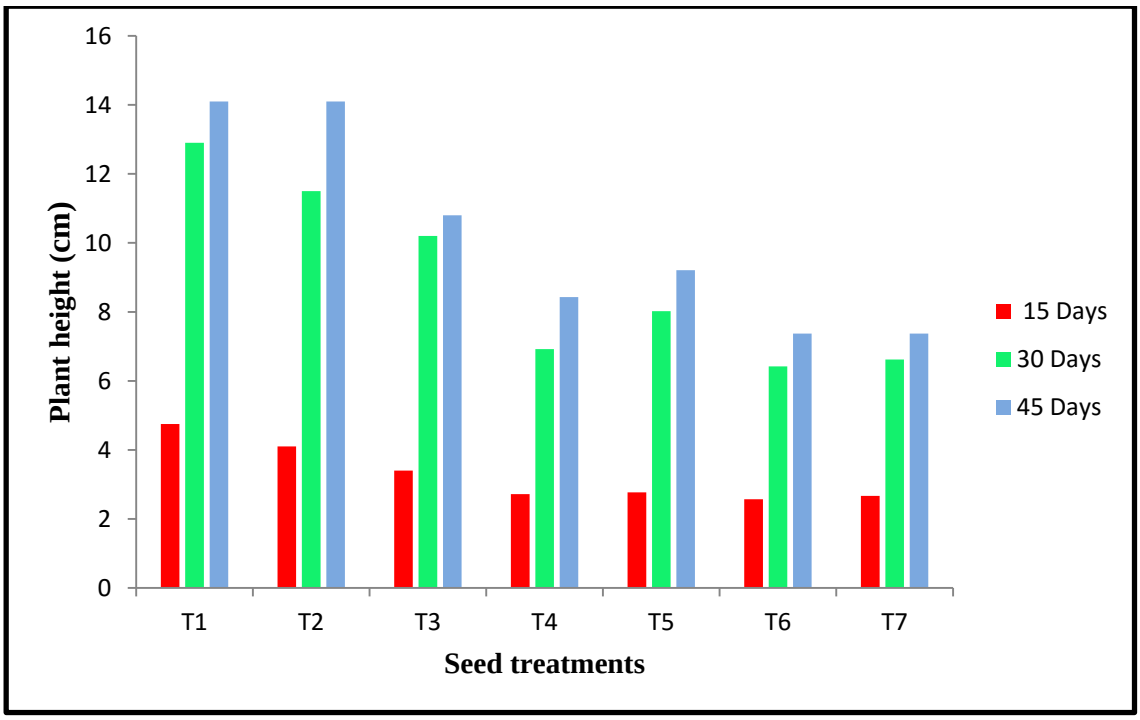

Fig. 4.11. Evaluation of seed treatments with bioagents and fungicides against *A. flavus* on plant height

T_1 - *Trichoderma harzianum*

T_2 - *Trichoderma viride*

T_3 - *Pseudomonas flourescens*

T_4 - Mancozeb

T_5 - Carbendazim

T_6 - Inoculated control/Treated seeds

T_7 - Uninoculated control/untreated seeds

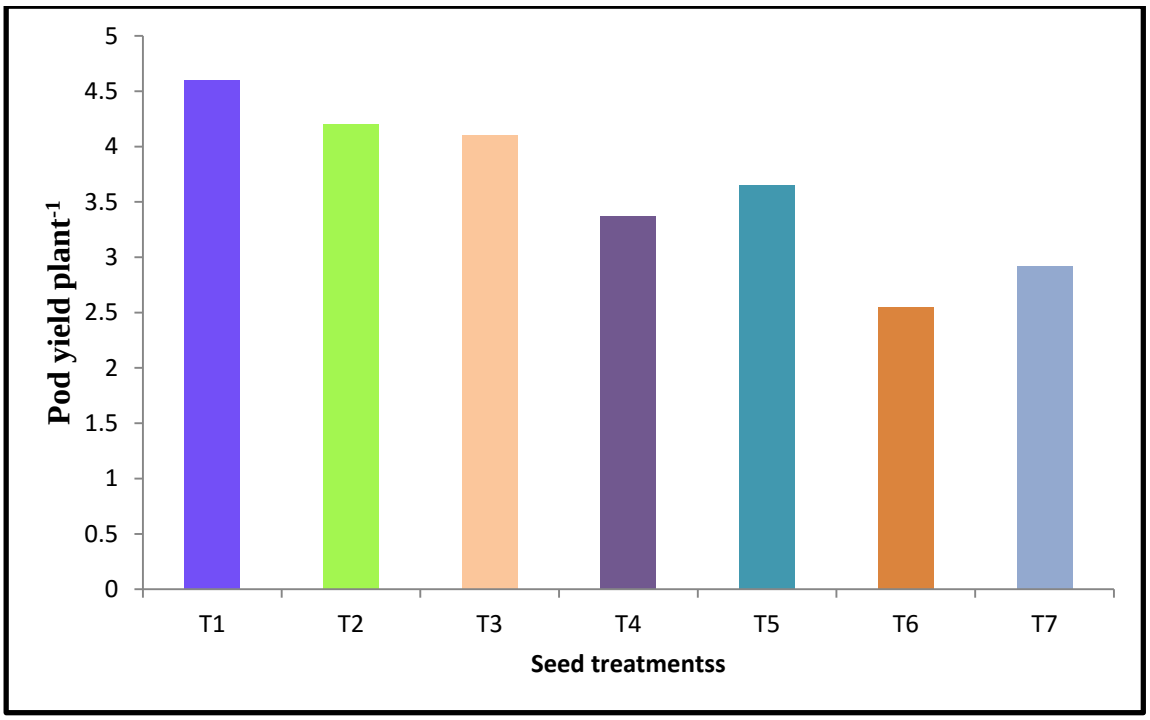

Fig. 4.12. Evaluation of seed treatments with bioagents and fungicides against *A. flavus* on yield

T_1 - *Trichoderma harzianum*

T_2 - *Trichoderma viride*

T_3 - *Pseudomonas flourescens*

T_4 - Mancozeb

T_5 - Carbendazim

T_6 - Inoculated control/Treated seeds

T_7 - Uninoculated control/untreated seeds

Plate 4.7. Evaluation of seed treatments using bioagents and fungicides in groundnut susceptible cv. JL 24 under glasshouse conditions

and mancozeb (3.37) were also found effective in increasing pod yield over untreated seeds (2.92) and pathogen treated seeds (2.55) (Fig 4.12).

The results are in conformity with the earlier findings of Divakara *et al.* (2014) who reported that seed treatment with talc based powder formulations of antagonist rhizobacteria and *Trichoderma* sp. improved crop yield and reduced aflatoxin production in sorghum and ensuring high economic returns. Dey *et al*. (2004) also observed that improvement in the growth, yield and nutrient uptake in groundnut cultivar JL 24 in pots prior inoculated with PGPR isolates. Raju *et al*. (1999) reported that formulations of *P. fluorescens* were effective in reducing *F. moniliforme* infection and also increasing the germination, vigour index and field emergence.

4.10.1 Estimation of aflatoxin content in the harvested seeds of groundnut cv. JL 24

Groundnut seeds of cv. JL 24 treated with bioagents and fungicides were evaluated under glasshouse conditions. The aflatoxin levels through ELISA method in the harvested produce of different seed treatments were assessed. The presence of aflatoxin was detected in pathogen treated seeds (1.38 µg/kg) and untreated seeds (0.69 µg/kg) which is below permissible levels (Table 4.12).

The present results are in conformity with Ajitkumar *et al.* (2011) who reported that *T. harzianum* inhibited the growth of *A. flavus* to an extent of 70% followed by *T. viride* (68.9 %).The beneficial effects of seed treatments with bioagents and fungicides in minimizing the pre harvest infection in groundnut was reported earlier by Mixon *et al*. (1984) who stated that treatment of groundnut pods with *T. harzianum* reduced the aflatoxin contamination. The present results were also in agreement with Sanskrit *et al.* (2015) who reported that *P. fluorescens* showed inhibitory activity against *A. flavus*. Use of biocontrol agents in aflatoxin management was also suggested by Vijay Krishna Kumar *et al.* (2002). The present results are similar with Rathod *et al,* (2010) who found that carbendazim was found effective against storage rot of groundnut caused by *A. flavus*. Yitayih *et al.* (2013) also proved that seed treatments with carbendazim and mancozeb + carbendazim were found effective in reducing the population densities of *A. flavus*. Alemayehu *et al.* (2012) also reported that the total aflatoxin levels in *Aspergillus flavus* positive samples of groundnut seed varied between 15 and 11865 µg/kg.

4.12. Estimation of Aflatoxin content (µg kg^{-1}) in the harvested produce of groundnut cv. JL 24 treated with bioagents and fungicides

S. No	Treatments	Dosage (g kg^{-1})	Aflatoxin content (µg kg^{-1})
1	*Trichoderma harzianum*	10	0 (1.00)
2	*Trichoderma viride*	10	0 (1.00)
3	*Pseudomonas flourescens*	10	0 (1.00)
4	Mancozeb	2.5	0 (1.00)
5	Carbendazim	2	0 (1.00)
6	Inoculated control/Treated seeds		1.38 (1.29)
7	Uninoculated control/untreated seeds		0.69 (1.53)
	SEm ±		0.12
	CD at 5 %		0.04

Figures in the parenthesis are square root transformed values. Each value is means of four replications.

CHAPTER V

SUMMARY AND CONCLUSIONS

Groundnut (*Arachis hypogaea* L.) is an important oil seed crop in India. It contains oil to an extent of 48 - 51 %. The major problem associated with groundnut is aflatoxin contamination. It is mainly caused by *Aspergillus flavus* and *Aspergillus parasiticus.* Keeping this in view, the present findings pertaining to the investigations was carried out on detection and identification of seed mycoflora, mode of entry of *A. flavus* into groundnut seed, effect of *A. flavus* on seed and oil quality and to study the efficacy of bioagents and fungicides in the management of *A. flavus* in groundnut. The results obtained from the present investigation are summarised as follows:

A total of seventy two groundnut (72) pod samples comprising farmer samples (36) and market samples (36) were collected from major groundnut growing districts of Telangana state during 2015 - 2016. The seed samples were analysed for seed health by agar plate method as per ISTA (1996). Significant differences in occurrence of total number of fungal colonies due to location and source of seed samples were observed. Irrespective of the districts, total per cent occurrence of seed mycoflora was found high in farmer samples (92.4 %) over market samples (45.3 %). Out of four districts, samples of Mahabubnagar district (47.1 % & 26.2 %) followed by Warangal district (43.5 % & 24.6 %) recorded more total number of fungal colonies in farmer and market samples. Irrespective of the samples, occurrence of six fungal flora *viz., A. flavus, A. niger, Fusarium* sp. *Alternaria* sp. *Macrophomina* sp. *Penicillium* sp. Among them, *A. flavus* (43.2 %), *A. niger* (26.7 %) were found predominant in both farmer and market samples.

External seed colonization due to *A. flavus* in groundnut resistant cv. J 11 and susceptible cv. JL 24 were observed at different days of incubation period. Resistant cv. J 11 inoculated with *A. flavus* colonized the seeds with severity score of 1, 2, 3, 4 and susceptible cv. JL 24 inoculated with *A. flavus* colonized the seeds with a severity of 2, 3, 4, 4 at 3, 5, 7 and 9 days of incubation period.

The mode of entry of pathogen into groundnut seed was studied by Scanning Electron Microscopy. Groundnut seeds of resistant and susceptible cultivars cvs. J 11 and JL 24 were inoculated with *A. flavus* toxigenic strain penetration and establishment of the pathogen in case of cv. J 11 was slow as compared to cv. JL 24. Irrespective of the cultivars, presence of pathogenic mycelium on the damaged seed coat with fractured discontinuous epidermis and loose broken cell junctions between epidermal cells were observed. In addition to this discontinuation, disorganized parenchyma, broken and missing cell walls and total depletion of storage protein were observed.

Whereas, in untreated seeds of both the cultivars under SEM exhibited well organized, unruptured cell walls with minimal intercellular space and abundant storage protein. The present results revealed that *A. flavus* was seed borne in nature and contaminated seeds served as an important source of seed infection and spread of the pathogen during storage.

Effect of *A. flavus* infection on oil content, protein content and unsaturated fatty acid profile were reduced and increase in saturated fatty acid profile were observed over a period of incubation in treated seeds and untreated seeds of both resistant and susceptible groundnut cultivars.

The per cent reduction in oil content was high in susceptible groundnut cv. JL 24 (18.3%) as compared to resistant groundnut cv. J 11 (9 %). While the reduction in oil content was less in the untreated seeds of groundnut cv. JL 24 and groundnut cv. J 11 (13.7% and 6%). Overall the per cent reduction in the protein content was found high in susceptible groundnut cv. JL 24 (16.3 %) as compared to resistant groundnut cv. J 11 (6.5 %). While the reduction in protein content was less in the untreated seeds of groundnut cvs. JL 24 and J 11 (14.2 % & 5.1 %).

The increase levels of saturated fatty acids *viz*., palmitic and stearic acids were high in susceptible cv. JL 24 (4.5 % & 4.5 %) as compared to resistant cv. J 11 (3.9 % & 2.93 %). Where as in untreated seeds, the increased levels in palmitic and stearic acids were found low (2.5 & 2 %) in cv. J 11 and 2.9 % and 1.94 % in groundnut cv. JL 24 respectively.

The per cent reduction in the unsaturated fatty acids linoleic and oleic acids were high in susceptible groundnut cv. JL 24 (17.5 % and 16.6 %) as compared to resistant groundnut cv. J 11 (15 % and 14 %). Whereas, in the untreated seeds, the per cent reduction in linoleic and oleic acids were found low (11.3 % and 6 %) in groundnut cv. J 11 and 13.3 % and 8.2 % in groundnut cv. JL 24, respectively.

The aflatoxin content at 1 to 56 days after incubation were increased from 2.15 µg/kg - 2861.3 µg/kg & 63.4 µg/kg - 4077.1 µg/kg in groundnut cv. J 11 and JL 24 respectively. When it was compared with in untreated there was low levels of aflatoxin 2.15 µg/kg – 14.7 µg/kg in cv. J 11 and 2.15 µg/kg - 21.1 µg/kg in cv. JL 24 were recorded.

The efficacy of seed treatments against seed borne *A. flavus* were evaluated under glasshouse conditions. Groundnut seeds treated with *T. harzianum* was significantly superior in recording higher seed germination (96 %), plant height (4.75, 12.9 and 14.1 cm) and yield (4.60 g) followed by *T. viride* (91 %, 4.10, 11.5 and 13.5 cm, 4.20 g) which was on par with *P. fluorescens* (88.2 %, 3.40, 10.2 and 10.8 cm 4.10 g). The remaining seed treatments were also found effective in improving seed germination, plant height and yield in seeds treated with carbendazim (81 %, 2.77, 8.02 and 9.21 cm, 3.65 g), mancozeb (73.5 %, 2.72, 6.92 and 8.43 cm, 3.37 g) over untreated (65 %,

2.67, 6.62 and 7.37 cm, 29.2) and pathogen treated seeds (54.5 %, 2.57, 5.65, 6.41 cm, 2.55 g) at 15, 30, 45 DAS. Aflatoxins were detected in pathogen treated seeds (1.38 µg/kg) and untreated seeds which is better permissible level. While aflatoxin was not observed in the seed treated with pathogen as *T. harzianum, T. viride* and *P. fluorescens.*

The following conclusions have been drawn from the investigations are as follows.

- Groundnut farmer seed samples recorded high incidence of seed mycoflora over market samples.
- Out of four districts of seed samples collected, Mahabubnagar district samples recorded maximum occurrence of more number of total fungal colonies.
- Among the seed mycoflora, *A. flavus* was predominant fungi in farmers and market samples.
- The surface colonization of *A. flavus* was more in susceptible cv. JL 24 as compared to resistant groundnut cv. J 11.
- Groundnut seeds inoculated with *A. flavus* toxigenic strain through SEM showed penetration and establishment of the pathogen in case of J 11 is slow as compared to JL 24.
- Effect of *A. flavus* on oil content, protein content and unsaturated fatty acid profile were reduced and increased levels of saturated fatty acid profile over incubation period was observed in treated and untreated seeds.
- Groundnut seeds treated with *T. harzianum* was found effective in improving seed germination, plant height and yield and also reducing the *A. flavus* infection.

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