INVESTIGATIONS ON AFLATOXIN CONTAMINATION IN GROUNDNUT CAUSED BY *Aspergillus flavus* (Link ex. Fries) AND ITS MANAGEMENT USING PGPR (PLANT GROWTH-PROMOTING RHIZOBACTERIA)

MAMIDALA RAVITEJA

B. Sc. (Ag.)

MASTER OF SCIENCE IN AGRICULTURE

(PLANT PATHOLOGY)

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BY

MAMIDALA RAVITEJA
B.Sc. (Ag.)

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CHAIRPERSON: Dr. K. VIJAY KRISHNA KUMAR

DEPARTMENT OF PLANT PATHOLOGY
COLLEGE OF AGRICULTURE
RAJENDRANAGAR, HYDERABAD – 500 030
PROFESSOR JAYASHANKAR TELANGANA STATE AGRICULTURAL UNIVERSITY

2015

DECLARATION

I**, Mr. M. RAVITEJA,** hereby declare that the thesis entitled **"INVESTIGATIONS ON AFLATOXIN CONTAMINATION IN GROUNDNUT CAUSED BY *Aspergillus flavus* (Link ex. Fries) AND ITS MANAGEMENT USING PGPR (PLANT GROWTH-PROMOTING RHIZOBACTERIA)"** 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: **(M. RAVITEJA)**

Place: Hyderabad **I. D. No. RAM/13-56**

CERTIFICATE

Mr. M. RAVITEJA has satisfactorily prosecuted the course of research and that the thesis entitled **"INVESTIGATIONS ON AFLATOXIN CONTAMINATION IN GROUNDNUT CAUSED BY *Aspergillus flavus* (Link ex. Fries) AND ITS MANAGEMENT USING PGPR (PLANT GROWTH-PROMOTING RHIZOBACTERIA)"** 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: **(K. VIJAY KRISHNA KUMAR)**
Place: Hyderabad Chairperson

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This is to certify that the thesis entitled **"INVESTIGATIONS ON AFLATOXIN CONTAMINATION IN GROUNDNUT CAUSED BY *Aspergillus flavus* (Link ex. Fries) AND ITS MANAGEMENT USING PGPR (PLANT GROWTH- PROMOTING RHIZOBACTERIA)"** submitted in partial fulfillment 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. M. RAVITEJA** 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.

CHAIRMAN

ADVISORY COMMITTEE

Thesis approved by the Student Advisory Committee

Chairperson: **Dr. K. VIJAY KRISHNA KUMAR**

Assistant Professor, Department of Plant Pathology,
College of Agriculture, PJTSAU,
Rajendranagar, Hyderabad-500 030 &
Visiting Scientist, Groundnut Pathology,
Grain Legumes ----------
ICRISAT, Patancheru, Hyderabad – 502 324

Member: **Dr. HARI KISHAN SUDINI**

Senior Scientist
Groundnut Pathology, Grain Legumes ----------
ICRISAT, Patancheru,
Hyderabad – 502 324

Member: **Dr. S. R. KOTESWARA RAO**

Professor
Department of Entomology, ----------
College of Agriculture, PJTSAU,
Rajendranagar, Hyderabad-500 030.

Date of final viva-voce:

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LIST OF ABBREVIATIONS

AF	:	*Aspergillus flavus*
AP	:	Andhra Pradesh
CAM	:	Coconut Agar Medium
CDA	:	Czapek Dox Agar
Cfu	:	Colony forming units
Cm	:	Centimeter
CRD	:	Completely Randomized Design
ELISA	:	Enzyme-linked immunosorbent assay
et al.	:	and other
etc.	:	and so on
EU	:	European Union
Fig.	:	Figure
g	:	gram
g^{-1}	:	Per gram
GIS	:	Geographical Information System
gl^{-1}	:	Grams per litre
h	:	hours
HPLC	:	HighPerformance Liquid Chromatography
i.e.	:	that is
IVSC	:	*In vitro* seed colonization
Kg	:	Kilogram
$KMNO_4$	:	Potassium permanganate
L	:	litre
m	:	Metre
M	:	Molar
M.ha	:	Million hectares
mg	:	milligram
ml	:	millilitre
mm	:	millimetre
Mt	:	Million tonnes
N	:	Normal
NA	:	Nutrient Agar
ng g^{-1}	:	nanogram per gram
ng ml^{-1}	:	nanogram per millilitre

ng	:	nano gram
nm	:	nano metre
No.	:	Number
^{o}C	:	Degree Centigrade
PDA	:	Potato Dextrose Agar
Pf	:	*Pseudomonas fluorescens*
Pg	:	Picogram
PGPR	:	Plant Growth-Promoting Rhizobacteria
pH	:	Hydrogen ion concentration
psi	:	pounds per square inch
Rpm	:	Revolutions per minute
SEM	:	Scanning electron microscope
Spp.	:	Species
TLC	:	Thin Layer Chromatography
UV	:	Ultra Violet
v/v	:	volume by volume
viz.,	:	namely
YES	:	Yeast Extract Sucrose
µg Kg^{-1}	:	Micrograms per kilogram
- ve	:	Negative
%	:	per cent
@	:	At the rate of
+ ve	:	Positive
±	:	plus or minus
µg	:	Microgram (s)
µL	:	Microlitre

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ABSTRACT

Aflatoxin contamination is a qualitative problem in groundnut (*Arachis hypogaea* L.) occurring at both pre-and post-harvest stages. These aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus* and have carcinogenic, hepatotoxic, teratogenic and immuno-suppressive effects. Use of plant growth-promoting rhizobacteria (PGPR) is a viable and sustainable option in managing aflatoxin problem in groundnut. The objectives of our present study were to evaluate the prevalence of *A. flavus* infection and aflatoxin contamination in groundnut oil mills/traders of Telangana and Andhra Pradesh (AP); differentiate toxigenic and atoxigenic strains using cultural methods; identify a superior PGPR (*Pseudomonas fluorescens*) isolate and determine its mode of inhibition on *A. flavus* and aflatoxin contamination. Pod samples were collected from eight selected oil mills/traders in Mahaboobnagar, Rangareddy, Nizamabad, Karimnagar (Telangana); and Anantapur (AP) districts and *A. flavus* infection was enumerated. Further, aflatoxin contamination in kernels was estimated by indirect competitive ELISA. A total of 24 *A. flavus* isolates were obtained from the collected pod samples. These isolates were identified as toxigenic/atoxigenic using cultural detection methods on Yeast extract sucrose (YES) media and coconut agar medium (CAM). Ten native *P. fluorescens* isolates from groundnut rhizosphere were isolated and screened against *A. flavus* by dual culture and *in vitro* seed colonization (IVSC) assays. Superior PGPR isolate (*Pf7*) against mold infection and aflatoxin contamination was screened for determining its mode of inhibition using scanning electron microscopy (SEM). In Telangana, kernel infection by *A. flavus* ranged from 42 (Mahaboobnagar) to 90.7% (Nizamabad). After Nizamabad, samples from Rangareddy district recorded up to 90% kernel infection. In AP, Tadimarri mandal recorded kernel infection up to 29.3%, whereas Tadipatri recorded up to 59.3%. Aflatoxins in kernels from these mills in Telangana were highest in Rangareddy (1205.2 µg kg^{-1}) followed by Karimnagar (365.5 µg kg^{-1}). Oil mills of

Nizamabad and Mahaboobnagar have recorded aflatoxins to a tune of 4.9 and 11.5 µg kg^{-1}in Telangana. In AP, aflatoxins in pod samples were 2.8 µg kg^{-1}(Tadipatri) and 6148.4 µg kg^{-1} (Tadimarri). Based on cultural methods, it was confirmed that there were18 toxigenic, five atoxigenic and one false positive/negative isolate out of the 24 *A. flavus* isolates obtained from surveyed oil mills. Atoxigenic isolates were obtained from Karimnagar and Nizamabad districts of Telangana. In dual culture and IVSC studies, *Pf*7 exhibited higher degree of antagonism on *A. flavus* (54% inhibition), inhibited its colonization and reduced aflatoxin contamination (27.8 µg kg^{-1}) in kernels. In SEM, *Pf*7 exhibited both antibiosis and hyperparasitism. In antibiosis, deformation and coiling of hyphae of test fungus was noticed. Further, the mycelium appeared thread like, wrinkled and flaccid. Conidiophores are twisted near the zone of inhibition. In hyperparasitism, a white slimy growth of bacterial cells was seen on mycelia of test fungus. Further, structural disintegration of conidiophores and conidia of *A. flavus* was noticed. Overall, our results indicated prevalence of *A. flavus* infection and aflatoxin contamination in groundnut oil mills. Future studies on mode of action of potential PGPR isolate (*Pf*7) in preventing *A. flavus* invasion into groundnut seed and its field efficacy in reducing aflatoxin contamination are to be carried out.

Chapter I

INTRODUCTION

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INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important grain legume and oilseed crop with huge revenue potential. The crop occupies about 25.4 Mha worldwide with an annual production of 45.2 Mt and a productivity of 1.77 tonnes ha^{-1} (FAOSTAT, 2013). In India, the crop is grown to an extent of 5.25 Mha with a production of 9.47 Mt and productivity of 1.80 tonnes ha^{-1} (FAOSTAT, 2013). Groundnut is a rich source of protein, dietary fiber, minerals, and vitamins (Ntare *et al*., 2008). Groundnut production is hampered by several biotic stresses that result in severe yield reduction all over the world (Subrahmanyam *et al*., 1989; Waliyar, 1991). Early leaf spot (ELS), late leaf spot (LLS) and rust are the economically significant foliar diseases (McDonald *et al*., 1985; Grichar *et al*., 1998; Subrahmanyam *et al*., 1980; Johnson *et al*., 1987; Smith and Littrell, 1980) in groundnut. Of different soilborne diseases affecting its production, collar rot (*Aspergillus niger*) (Bakhetia, 1983); stem rot (*Sclerotium rolfsii*) (Mayee and Datar, 1988); and bacterial wilt (*Ralstonia solanacearum*) (Liao, 2001) are the devastating diseases in all crop growing areas of the world. Another important biotic stress in groundnut cultivation is aflatoxin contamination which occurs at both pre-and post-harvest stages of the crop. It is a qualitative problem affecting grain quality and trade (Waliyar *et al*., 2008).

Aflatoxins are a group of twenty secondary metabolites produced by *Aspergillus flavus* (Link ex. Fries) and *Aspergillus parasiticus* (Speare) (Liu and Wu, 2010; Snigdha *et al*., 2013). These toxins are difuranocoumarin derivatives produced by a polyketide pathway. These aflatoxins were isolated and characterized after the death of >100,000 turkey poults (turkey X disease) due to consumption of mold-contaminated groundnut meal (Blout, 1961). Major aflatoxins are categorized as B_1, B_2, G_1 and G_2 based on their fluorescence under UV light and their relative chromatographic mobility during thin layer chromatography (TLC). The aflatoxins M_1 and M_2 are the metabolically biotransformed and hydroxylated forms of B_1 and B_2 respectively in milk when cows consume aflatoxin-contaminated feed (D'Mello and MacDonald, 1997; vanEgmond, 1989). Of these toxins, aflatoxin B_1 (AFB_1) is the most potent natural carcinogen and also the major aflatoxin produced by the toxigenic strains (Squire, 1981).

These aflatoxin producing fungi are air-borne, soil-inhabitants and are frequent contaminants of crops, food stuffs including storage (Waliyar *et al*., 1994; Jaime-Garcia and Cotty, 2004; Williams *et al*., 2004). Predominantly, these toxins affect cereals and millets (maize, sorghum, pearl millet, rice, wheat); oilseeds (groundnut, soybean, rapeseed, sunflower, cotton); spices (chillies, black-pepper, coriander, turmeric, zinger); tree nuts (almond, pistachio, walnut, cashewnut, hazelnut, brazilnut, tigernut, coconut); pulses (pigeonpea, horsegram, greengram, mungbean, lentil, cowpea, haricot bean); figs; meats; dairy products; and fruit juices (apple, guava) (Abdin *et al*., 2010).

In groundnut, environmental conditions play a major role in the attack of these molds, and the crop is affected at various stages such as pre, post-harvest and during storage (Waliyar *et al*., 2008). Ascertaining the extent of aflatoxin contamination at groundnut oil mills/traders' level gives an indication on the prevalence of this qualitative problem. Several reports are available on the extent of *A. flavus* infection of pods and aflatoxin contamination at oil mills/traders' level (Vijay Krishna Kumar *et al*., 2001). Such information can be useful for mapping of risk and sensitive areas with respect to aflatoxin contamination of food and animal feed. Further, for timely interventions to better manage this problem, identifying the actual stage at which aflatoxin contamination is flared up is essential. Earlier studies have ascertained the aflatoxin contamination in groundnut at pre-and post-harvest stages (Cole *et al*., 1982). However, a comprehensive mapping of the risk and sensitive areas with respect to toxin accumulation during storage at oil mills/traders is not documented. Further, in several of the documented cases, there was no direct correlation between the quantum of *A. flavus* infection and kernel aflatoxin contamination (Desai *et al*., 1991). One of the important factors for lack of correlation is the presence of non-aflatoxigenic/atoxigenic *A. flavus* strains in groundnut crop soils and their invasion to pods. It is precisely at this juncture, differentiation of toxigenic and atoxigenic *A. flavus* isolates assumes significance.

Co-existence of toxigenic and atoxigenic strains of *A. flavus* in groundnut crop ecosystem is common in different crop soils. Further, both aflatoxigenic and atoxigenic strains are commonly seen infecting the food commodities (Diener and Davis, 1966; Joffe, 1969). For example, in the recent acute aflatoxin poisonings in Kenya due to contaminated maize, presence of different morphotypes such as L strain, S strain (both toxigenics) and atoxigenic strains were reported (Probst *et al*., 2007). Differentiation of these toxigenics from atoxigenics through use of cultural, molecular (Sudini *et al*.,

2015) and analytical approaches (Reddy *et al*., 2001) will enable to estimate the actual threat due to aflatoxins in a particular food commodity. However, in view of the frequent encounters with false positives and false negatives, a polyphasic approach would be an ideal method for precise detection (Almoammar *et al*., 2013). But maintainance of molecular and analytical laboratories which is expensive and expertise oriented, it is the cultural methods of detection of aflatoxigenic strains that is gaining momentum in many areas of the world (Abbas *et al*., 2004). These cultural methods rely either on quantification of purified extracts (Filtenborg and Frisvad, 1980; Shotwell *et al*., 1966) or on qualitative assessments through fluorescence (Bennett and Goldblatt, 1973; de Vogel *et al*., 1965; Hara *et al*., 1974; Lin and Dianese, 1976) or UV absorption (Yabe *et al*., 1987). A broad understanding on the ratio of co-existence of toxigenic and atoxigenic strains in a particular crop growing area will help predict the risk and sensitive areas with respect to pre-and post-harvest contamination. Further, the atoxigenic strains can be used as bioagents in the management of pre-harvest aflatoxin contamination in maize (Jane *et al*., 2014) and groundnuts (Dorner and Lamb, 2006). Commercial formulation of non-aflatoxigenic *A. flavus* strains (Aflaguard^{TM}) was effective in bringing down the pre-harvest aflatoxin contamination as well as the population levels of toxigenic strains in groundnut (Dorner and Lamb, 2006).

Several management strategies have been attempted to minimize the aflatoxin problem. Important of them are development of resistant lines (Nigam *et al*., 2009), development of transgenics or enhancing host plant resistance (Brown *et al*., 2003; Cleveland *et al*., 2003). Strong sources of genetic resistance are however not available in the cultivable germplasm of groundnut. Of different management strategies, biological control of aflatoxin producing *A. flavus* is a viable option and is sustainable over long run. Of different biocontrol agents, use of plant growth-promoting rhizobacteria (PGPR) is gaining momentum. Several PGPR genera have been reported to suppress *A. flavus* besides producing plant growth-promoting effects (Dey *et al*., 2004). Of different PGPR, *Pseudomonas* spp. is one of the widely used bacteria against major plant pathogens in groundnut (Sreedevi and Charitha Devi, 2012). Earlier reports indicated the use of PGPR in groundnut for controlling soil and foliar diseases besides yield enhancement (Dey *et al*., 2004). Identification of a superior PGPR isolate with high degree of antagonism against *A. flavus* is necessary prior to conducting of greenhouse and field studies. In view of this, screening of the *P. fluorescens* isolate against *A. flavus* under *in vitro* and *in vivo* conditions is a pre-requisite. In particular,

the extent of inhibition of *A. flavus* infection by a PGPR isolate on groundnut seed need to be ascertained through *in vitro* seed colonization assays (Thakur *et al*., 2000). In addition to inhibition of pathogen growth and multiplication, the PGPR isolates also contribute to increased yields. Plant growth-promoting effects and enhancement of pod yields by *P. fluorescens* have been reported in groundnut (Dey *et al*., 2004).

For a PGPR isolate to be an effective candidate bioagent against pre-harvest aflatoxin contamination, understanding its mechanism of action against *A. flavus* is mandatory. It is precisely at this stage, ultra-structural studies on the interaction between *P. fluorescens* and aflatoxin producing molds is required. Though, few reports on the interaction between these two microbes are available, comprehensive studies on the effect of antibiosis and hyperparasitism of a PGPR strain on *A. flavus* are still lacking. The present study therefore focused on documenting the effectiveness of elite PGPR isolate against *A. flavus* through scanning electron microscopy (SEM). Keeping the above criteria in view, the current research is proposed with the following objectives.

OBJECTIVES OF INVESTIGATION

1. To evaluate the extent of *Aspergillus flavus* infection and aflatoxin contamination in pods at selected groundnut mills of Andhra Pradesh and Telangana
2. To detect the toxigenic *A. flavus* strains using cultural methods
3. To identify the superior PGPR isolates against *A. flavus*
4. To determine the mode of action of PGPR against *A. flavus* using scanning electron microscopy

Chapter II

REVIEW OF LITERATURE

Chapter II

REVIEW OF LITERATURE

A brief review of literature pertaining to the "Investigations on aflatoxin contamination in groundnut caused by *Aspergillus flavus* (Link ex. Fries) and its management using PGPR (Plant Growth-Promoting Rhizobacteria)" has been presented in this section under the following headings.

2.1 Aflatoxin producing fungi

2.2 Economic importance of aflatoxin problem

2.3 Health hazards due to aflatoxin contamination

2.4 *Aspergillus flavus* infection in groundnut

2.5 Aflatoxin contamination in groundnut

2.6 Cultural methods for detection of toxigenic and atoxigenic strains of *A. flavus*

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2.1 Aflatoxin producing fungi

Aflatoxins are a group of twenty secondary metabolites produced by *Aspergillus* section *Flavi* group of fungi. Two groups of species were included under the *Aspergillus* section *Flavi*. The first group comprises of *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii* and *A. bombycis*. Among these species, *A. flavus* and *A. parasiticus* are the major aflatoxin producers (Cary and Ehrlich, 2006; Ehrlich *et al*., 2007). The other aflatoxigenic *Aspergillus* spp. in this section are *A. pseudocaelatus* (B_1, B_2 and G_1, G_2 aflatoxins), *A. pseudonomius* (B_1 and not G-type aflatoxins) (Varga *et al*., 2011) and *A. parvisclerotigenus* (Godet and Munaut, 2010). In the second group, atoxigenic molds such as *A. sojae*, *A. tamari* and *A. oryzae* are included (Kumeda and Asao, 2001). These atoxigenic molds such as *A. sojae* and *A. oryzae* are generally used in fermentation of foods (Chang *et al*., 2007). Besides, *A.*

oryzae and *A. sojae* are considered as atoxigenic variants of *A. parasiticus* and *A. flavus* respectively (Klich and Pitt, 1988). Species outside the section *Flavi* are also reported to be aflatoxigenic (Chang *et al*., 2007). From the section *Ochraceroesi*, the mold, *A. ochraceoroseus*; the ascomycete fungi (*Emericella astellata*) and one species from *Aspergillus* section *Nidulantes* (*E. venezuelensis*) are also capable of producing AFB_1 (Cary *et al*., 2005; Klich *et al*., 2000; Frisvad *et al*., 1999, 2005).

2.2 Economic importance of aflatoxin problem

The impact due to aflatoxin contamination can be in the following areas (Wu, 2009). According to the Food and Agricultural Organization (FAO), 25% of the world's food crops are significantly contaminated with mycotoxins (Boutrif and Canet, 1998). Approximately, there will be $ 450 million annual loss to all food exporters if all nations harmonized to EU aflatoxin standard (Wu, 2004). Further, $ 670 million annual loss is incurred to African food exporters from attempting to meet EU aflatoxin standard (Otsuki *et al*., 2001). Only 15 countries in sub-Saharan Africa have regulation governing aflatoxins, thus making the trade challenging. More than 4.5 billion people in developing countries are chronically exposed to aflatoxins in their diets according to Centre for Disease Control. These aflatoxins are considered to be having significant negative impact on health, food and nutritional security and incomes at the household, community and national levels (Coulibaly *et al*., 2008). Health risks associated with aflatoxin consumption will decrease 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 Cardwell (2001), many people in developing countries are being exposed chronically to high levels of aflatoxin in their diet. Kwashiorkor, a protein related malnutritious syndrome is often associated with consumption of aflatoxin tainted food in children (Ramjee *et al*., 1992).

2.3 Health hazards due to aflatoxin contamination

As a result of aflatoxin contamination in food and feed stuffs, safety to human and animal health is a concern. Aflatoxins pose a major qualitative problem affecting human and animal health due to their wide array of actions (Shephard, 2003; Strosnider *et al*., 2006). These aflatoxins have carcinogenic, hepatotoxic, teratogenic and immunosuppressive effects when contaminated food and feed is consumed (Machida and Gomi, 2010). Other important health related hazards include liver cancer especially in persons with Hepatitis C (Liu and Wu, 2010). The International Agency for Research

on Cancer (IARC) of the World Health Organization (WHO) has classified AFB_1 as Group I carcinogen (established carcinogens to humans) (IARC, 1982 & 2002). Consumption of high aflatoxin dose often results in acute aflatoxicosis, while chronic exposure leads to cancer and cirrhosis of liver (Williams *et al*., 2004; Strosnider *et al*., 2006). Acute toxicity results in death and is characterized by haemorrhage, acute liver damage and edema. Recent outbreaks on acute aflatoxicosis were reported from Kenya due to consumption of contaminated home-grown maize during the year 2004 (Probst *et al*., 2007). Chronic toxicity have much wider health effects such as hepatocellular carcinoma (HCC), immune-suppression (Sahoo and Mukherjee, 2001), growth retardation especially in children (Gong *et al*., 2004), and micro-nutrient deficiencies in animals.

2.4 *Aspergillus flavus* infection in groundnut

Mohammed and Chala (2014) conducted a study to identify *Aspergillus* species associated with groundnut and determine the frequency of seed contamination. They reported heavy infestation of groundnut samples by various molds including *Aspergillus niger, A. flavus, A. ochraceus, A. parasiticus* and *Penicillium* species. Among the 270 groundnut samples collected from three districts of Eastern Ethiopia, the incidence of infected groundnut kernels ranged from 50 to 80% at the district level. Within the surveyed districts, the kernel infection varied between 36.3 and 100%.

Schroeder and Boller (1973) isolated aflatoxin producing fungi, from peanuts, cotton seed, rice, and sorghum over a period of 3-years from Texas, USA. They reported that the aflatoxins were found each year in peanut and cotton seed for 2 of 3 years in rice and sorghum. *Aspergillus flavus* was much more prevalent in peanut and rice than in cotton seed and sorghum. Among the isolates of *A. flavus*, production of aflatoxins was 96% from peanuts, 79% from cotton seed, 49% from sorghum, and 35% from rice.

Rajarajan *et al*. (2013) collected naturally infected peanuts from traders' godown and evaluated the levels of aflatoxin from the isolates of *Aspergillus* infecting peanuts which were separately grown on modified Czapek's medium at 28º C by HPLC. It was observed that the peanuts were contaminated with aflatoxin B_1, after several months' storage.

Ginting and Rahmianna (2015) conducted a study to analyze the physical quality and *A. flavus* infection in sixteen peanut samples collected from farmers, collectors, retailers and food processors. They reported that the moisture content, damaged kernels and *A. flavus* infection were 8.8%, 46.7% and 45.1% respectively in the peanut samples. Further, there was a positive correlation existed between the *A. flavus* infection and kernel damage.

The incidence of mycotoxigenic fungi associated with 300 peanut seed, samples collected from different places of Warangal district in Andhra Pradesh (now Telangana), India during 2011-12 were evaluated by Kalyani *et al*. (2014). Results indicated that 45 fungal species belonging to 20 genera isolated were *Aspergillus* (50%), *Fusarium* (40.3%), *Cladosporium* (16.4%), *Alternaria* (17.1%), *Curvularia lunata* (15.7%) and *Penicillium* sps (23.8%) were the dominant fungal genera in peanut seeds. Further, the mycotoxins detected in the collected samples were aflatoxins, sterigmatocystin, ochratoxin A, citrinin and zearalenone.

2.5 Aflatoxin contamination in groundnut

Mutegi *et al*. (2009) conducted a survey to obtain baseline data on aflatoxin levels in peanuts from major production regions of western Kenya. The aflatoxin levels in peanut samples collected from Busia (384 samples) and Homabay (385 samples) districts were estimated with an indirect competitive ELISA. The estimated levels of aflatoxins in Busia and Homabay districts were up to 2688 and 7525 μg kg^{-1} respectively. Among the surveyed samples, 87% contained <4 μg kg^{-1} of aflatoxin, 5% were in the range of ≥ 4 to 20 μg kg^{-1}, and 7.5% exceeded the Kenya's regulatory limit (20 μg kg^{-1}).

Further, Mutegi *et al*. (2013) also investigated the peanut market characteristics and their association with aflatoxin levels in peanuts from Kenya. They obtained data from 1263 vendors in various market outlets. The peanuts and peanut products from each vendor and these samples were analyzed for aflatoxin levels. About 37% of the samples exceeded the regulatory limit for aflatoxin levels (10 μg kg^{-1}) set by the Kenya Bureau of Standards (KEBS). The most aflatoxin-contaminated products were peanut butter (69%) and spoilt peanuts (75%) which exceeded10 μg kg^{-1}.

Kaaya *et al*. (2006) determined the aflatoxin content of peanut from farmers and dealers in three districts of Uganda, and from three busiest peanut markets in Kampala.

They reported that the mean aflatoxin levels at farm level (7.3 to 12.4 μg kg^{-1}) were lower than the FDA/WHO regulatory limit of 20 μg kg^{-1}. In most samples, the aflatoxin content exceeded 20 μg kg^{-1} when peanuts were processed and stored both at wholesale and retail levels. They also reported that the levels of aflatoxins in all forms of peanut obtained from retailers in all markets were significantly higher than corresponding samples obtained from wholesalers.

To determine the aflatoxin contamination under market conditions pod samples (n=42) were collected from Madhugiri (11 samples) and Pavagada (31 samples) market yards of Tumkur district of Karnataka, India (Vijay Krishna Kumar *et al*., 2001). Aflatoxins from collected samples were estimated using enzyme-linked immunosorbent assay (ELISA). Their results revealed that the amount of aflatoxin contamination in all the collected samples was below 20 μg kg^{-1} seed.

Ndung'u *et al*. (2013) conducted a study to determine the status of aflatoxin contamination in peanuts and peanut butter in Nairobi and Nyanza regions. Competitive ELISA technique was used for the estimation of aflatoxin levels in the 82 samples comprising raw and roasted peanuts and peanut butter from market outlets and cottage processors. The aflatoxin levels in all the samples ranged from 0 to 2377.1 μg kg^{-1}. Further, the raw samples from Nairobi and Nyanza have shown highest aflatoxin levels.

Ramakrishna and Mehan (1993) optimized direct and indirect competitive enzyme-linked immunosorbent assays for the determination of aflatoxin B_1 in groundnut using a specific monoclonal antibody developed at the University of Strathclyde, UK. For direct competitive assay, the monoclonal antibody was conjugated to horseradish peroxidase (HRP). For indirect competitive ELISA, a commercially available goat-antimouse IgG-HRP conjugate was employed. The sensitivities of both the ELISAs were as low as 20 pg/well and were useful for routine analysis of aflatoxin B_1 in groundnuts.

Groundnut samples were collected from 21 markets in 10 regions of Ghana, and the level of aflatoxigenic fungus, *Aspergillus flavus* on half strength PDA was recorded. In the damaged kernels, the *A. flavus* infection was 31.7% and aflatoxin contamination ranged from 5.7 to 22,168 μg kg^{-1}. In the undamaged kernels, *A. flavus* infection was however 12.8%. Aflatoxin contamination was not observed in 50% of

undamaged kernels. Further, the positively tested undamaged kernels recorded aflatoxins in the range of 0.1 to 12.2 μg kg^{-1} (Awuah and Kpodo, 1996).

Chala *et al*. (2013) estimated aflatoxin contamination in groundnut using ELISA. A total of 124 groundnut samples were collected from farmers' stores and markets, of which 93 samples were tested positive for aflatoxin contamination. In the positive samples, the aflatoxin levels ranged from15 to11,900 μg kg^{-1}.

For the estimation of groundnut aflatoxin contamination, a total of 1397 groundnut samples were collected from farm homesteads, local markets, warehouses and shops during 2008 and 2009. These samples were analyzed for AFB_1 contamination using ELISA. The results indicated that 46% and 23% of the total samples collected have shown aflatoxin contamination of more than 4 μg kg^{-1}. Further, aflatoxin contamination of above 20 μg kg^{-1} was found in 21% of samples in 2008; and 8% of total samples in 2009 (Monyo *et al*., 2012).

Leong *et al*. (2010) monitored the aflatoxin contamination in a total of 196 nuts collected from the products marketed in Penang, Malaysia by enzyme-linked immunosorbent assay (ELISA). Reversed phase high performance liquid chromatography (HPLC) was used for quantification and confirmation of aflatoxin contamination in the nuts. Among the 196 samples examined, 32 samples (16.3%) were contaminated with aflatoxins, ranging from 16.6 to 711 μg kg^{-1} (mean 17.2–350 μg kg^{-1}) for total aflatoxins.

Navya *et al*. (2013) conducted a survey across different agro-climatic regions of India to determine the *A. flavus* infection and aflatoxin contamination. A total of 38 groundnut seed samples were collected from various sources. Their results indicated that all samples were found infected with *Aspergillus flavus* with a range of 2 to 50% incidence and the aflatoxin content of 0.0 to 28 μg kg^{-1}. Seeds were predominantly contaminated with aflatoxin B_1 followed by aflatoxin B_2. When the *A. flavus* isolates were analyzed through cultural, thin layer chromatography (TLC), competitive direct enzyme-linked immunosorbent assay and multiplex polymerase chain reaction; 31 isolates were found aflatoxigenic and seven were non-aflatoxigenic.

Kishore *et al*. (2002) evaluated the presence of mycotoxins and toxigenic fungi in a total of 182 groundnut samples collected at harvesting stage from farmers' fields in five districts of Andhra Pradesh (Anantapur, Chittoor, Cuddapah, Kurnool, and Mahaboobnagar) during rainy seasons of 1999 and 2000. In each district, average seed

infection by *A. flavus* was 11.9-18.3% and 9.5-14.1% in 1999 and 2000 respectively. Further, the aflatoxin contamination in the collected samples was 20.3% and 16.5% during 1999 and 2000 respectively. Moreover, in 11.4% and 8.7% of the seed samples collected in two seasons, the aflatoxin content was >30 μg kg^{-1}. Highest aflatoxin content (851.9 μg kg^{-1}) was found in samples collected from Anantapur district during the rainy season of 1999.

Aldao *et al*. (1995) developed an indirect ELISA to quantify aflatoxin B_1 (AFB_1) with a detection limit of 0.025 ng ml^{-1}. Polyvinyl chloride (PVC) plates were used for the test which was activated with AFB_1 bound to bovine serum albumin (BSA). Polyclonal antibodies were raised in rabbits against AFB_1-BSA, and the specific anti-AFB_1 antibodies were recovered from the crude antiserum. For the detection of rabbit IgG anti-AFB_1 antibodies bound to PVC plates, goat anti-rabbit IgG antibodies bound to peroxidase were used. Spectrophotometry was used for the detection of colour developed by the subsequent enzyme conversion of the substrate. The developed colour yielded clear absorbance differences at varying doses of AFB_1.

Sixty samples of stored groundnut kernels were collected from four different locations in Sudan *viz.*, Mayo city, Umbaddah city in Khartoum state, Al-Helalia city, and Al-Managel city in Al-Jazeera state. The kernels were later examined for aflatoxin contamination. Thirty five samples (58.33%) gave positive readings with TLC technique, and *Aspergillus flavus* was isolated from twenty six samples (43.33%). The concentration of aflatoxin B_1 in these samples ranged from low (17.5μg kg^{-1} kernel) to very high, (404 μg kg^{-1} kernel) (Bakhiet and Musa, 2011).

Latha *et al*. (2011) conducted a survey to obtain information on aflatoxin and to create awareness among farmers and traders in groundnut kernels of both *Kharif* and *Rabi* seasons. The produce was collected from groundnut pods from different delivery chains and also from different storage points in Eastern and Western mandals of Chittoor district of Andhra Pradesh. Among the surveyed samples, two kinds of aflatoxins (B_1 and B_2) were detected. Aflatoxin B_1 was highest in the groundnut kernels collected from farmers in *Kharif* (33-40 μg kg^{-1}) than in *Rabi* season (20-32 μg kg^{-1}) and lowest from retailer samples (8-10 μg kg^{-1} in *Kharif* and 5-10 μg kg^{-1} in *Rabi*).

Chun *et al.* (2007) assessed the aflatoxin levels in a total of 85 nuts and their products marketed in South Korea. The monitoring scheme consisted of ELISA for rapid screening, HPLC for quantification and LC–mass spectrometry (MS) for

confirmation. Among the 85 samples screened, 31 samples gave positive results with ELISA readings above 0.06. Aflatoxin contents of possible positive samples were determined using HPLC with a detection limit of 0.08–1.25 μg kg^{-1} and a quantification limit of 0.15–2.50 μg kg^{-1}. Results indicated that nine samples were contaminated with aflatoxins (10.6% of incidence), ranging in various levels up to 28.2 μg kg^{-1}.

Devi *et al*. (2002) standardized an indirect competitive enzyme-linked immunosorbent assay for estimating AFM_1 in milk and milk products. A total of 280 milk samples collected from rural and peri-urban areas in Andhra Pradesh, India were tested for the AFM_1, in which 146 were found to contain <0.5 ng mL^{-1}. But in the rest of the samples, a variance was observed. In 80 samples, it varied from 0.6 to 15 ng mL^{-1}, where in 42 samples it ranged 16 to 30 ng mL^{-1}, and in 12 samples it ranged from 31 to 48 ng mL^{-1}. It was reported that most of the milk samples that contained high AFM_1 concentrations were obtained from peri-urban locations and there was a significant exposure of humans to AFM_1 levels in India.

An antiserum was produced in rabbits using the commercially available hapten, afla B_1-oxime-bovine serum albumin. For the detection of aflatoxin B_1 using competitive direct ELISA, the same hapten was coupled with alkaline phosphatase (hapten-BSA-ALP) (Anjaiah *et al.,* 1989)**.** It was further reported that the competitive direct ELISA method was more rapid and less expensive than the physico-chemical methods of aflatoxin analysis and it can detect levels of aflatoxin B_1 as low as 50 picograms.

Ayejuyo *et al*. (2011) evaluated the levels of aflatoxin B_1 in groundnut and cereal grains using ELISA technique. They reported that the aflatoxin content of groundnut ranged from 6.25 to 7.80 ng g^{-1}, whereas in millet samples the levels ranged from 4.18 to 28.50 ng g^{-1}. In maize samples, aflatoxin levels ranged between 2.51ng g^{-1} and 3.94 ng g^{-1} and in sorghum it was 5.20 ng g^{-1}. Among the 99 samples investigated, about 51% incidence of aflatoxin was found.

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. Of the 182 chilli samples tested, 59% were contaminated with AFB_1 and 18% recorded the toxin at above permissible levels. 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}).

A study was conducted in Northern India to detect the presence of aflatoxigenic *A. flavus* and *A. parasiticus* strains and also the aflatoxin contamination in 256 feed samples (Dutta and Das, 2000). Out of 198 *A. flavus* and 15 *A. parasiticus* strains isolated, 76% and 86% respectively, were found to be toxigenic. The aflatoxin B_1 content of these feeds as estimated by TLC and ELISA indicated very high (average 0.412 ± 0.154 ppm) in comparison to the permissible Indian regulation level (0.03 ppm).

2.6 Cultural methods for detection of toxigenic and atoxigenic strains of *Aspergillus flavus*

Davis *et al*. (1987) used cultural methods for the screening of nine isolates of *Aspergillus flavus* and *A. parasiticus* for the aflatoxin production on coconut agar medium (CAM). When the fungal cultures were observed under long wave UV light (365 nm) after 2 to 5 days of growth, aflatoxigenic strains produced blue fluorescence on the reverse side of the culture plate. Further, the presence of aflatoxin in blue-fluorescing agar plates were confirmed by chemical analysis (Thin layer chromatography) and its absence in non-fluorescing agar plates.

Twenty-five isolates of *Aspergillus flavus* were tested by ammonia vapour test, a cultural method for the aflatoxin detection. Four isolates were found to be highly toxigenic which showed dark red colour development on the reverse side of the culture plate. Different media *viz.,* yeast extract sucrose (YES) agar, potato dextrose agar (PDA) and czapekdox agar (CDA) were tested for dark red colour development by ammonia vapour test. The YES agar media has shown maximum colour change. The results of analytical methods using thin layer chromatography for the detection of aflatoxin were 92% in agreement with the results of ammonia vapour test (Kumar *et al*., 2007).

The presence of aflatoxins in a sample was determined by cultural based approaches which include 1) presence of aflatoxin B_1, detected by its blue fluorescence in the culture medium, 2) yellow pigment production by the yellow anthraquinone biosynthetic intermediates in the aflatoxin pathway and 3) colour change on exposure

to ammonium hydroxide vapor. When a total of 517 *A. flavus* isolates from the Mississippi Delta, were tested for the aflatoxin production using cultural methods, the combined assay reduced false positives for aflatoxigenicity to 0%, and false negatives to 7% (Abbas *et al*., 2004).

Production of aflatoxins by *A. flavus* on coconut agar medium (CAM) can be detected by the presence of blue fluorescence ring surrounding the colonies, after an incubation period of 3 days (Lin and Dianese, 1976). The presence of yellow pigmentation on the lower side of the colonies was useful for the detection of toxigenic strains of *A. flavus* without the use of UV light. From the 27 Brazilian isolates of *A. flavus*, 7 isolates were proved to be toxigenic on CAM. Later, the toxigenic isolates were confirmed by thin-layer chromatography (TLC) assay of culture broths.

Fente *et al*. (2001) developed a new reliable, fast, and simple method for detecting the aflatoxigenic *Aspergillus* strains. They reported that the addition of cyclodextrin (a methylated β-cyclodextrin derivative) to the fungal culture media enhanced the natural fluorescence of aflatoxins. When the fungal colonies were observed under long wavelength (365 nm) UV light, the production of aflatoxin coincided with the presence of bright blue or blue-green fluorescent area surrounding colonies. Later, the presence of aflatoxins was confirmed by extracting the medium with chloroform and the extracts were examined by high-pressure liquid chromatography (HPLC) with fluorescence detection.

Rodrigues *et al*. (2009) conducted a polyphasic approach to characterize and identify aflatoxigenic and non-aflatoxigenic strains of 31 isolates of *A. flavus* by morphological, chemical and molecular methods. The aflatoxin production was screened on Coconut Agar Medium (CAM), and the results were consistent with the HPLC analysis.

Ordaz *et al.* (2003) reported a simple, rapid and reliable method for screening of aflatoxin production by *Aspergillus flavus* strains. These strains were cultivated on yeast extract agar (YES) with the addition of methylated β-cyclodextrin derivative plus sodium deoxycholate and incubated for a period of 3 days at 28º C. Aflatoxin production was readily detected by direct visualization of a beige ring surrounding the colonies. The beige ring exhibited blue fluorescence, when examined under UV light. Further, these results were confirmed by HPLC with fluorescence detection.

Atoxigenic *A. flavus* isolates from Iranian pistachio orchards were identified using cultural, analytical and molecular methods. Two rapid aflatoxin assays *viz.*, ammonium vapour (AV) and fluorescence detection (FD), using various media preparations were directly compared to screen 524 isolates of *A. flavus* obtained from the selected orchards. The per cent of false negatives were high for all media preparations, which ranged from 13 to 15% when FD assays were used. In AV assays, incidences of false negatives ranged from 0 % (on coconut agar medium) to 7.2 % (on potato dextrose agar). Thin layer- and high-performance liquid chromatographies were used to confirm the aflatoxin producing ability of all isolates of *A. flavus* (Fani *et al*., 2014).

Concentration of aflatoxins in agar medium was estimated using a direct technique that quantifies the fluorescence of agar containing aflatoxins. Spores of aflatoxigenic *Aspergillus* isolates were inoculated in tubes containing 5 ml of an agar medium and incubated for 3 days at 30º C. When the culture tubes were observed under UV light, fluorescence (450 nm and above) was elicited which can be measured photometrically. Agar fluorescence was in direct correlation with the concentration of aflatoxin within the range 0 to 18.7 μg kg^{-1}. The lowest aflatoxin concentration detected was 50 ng g^{-1} (Cotty, 1988).

Peanut samples from five regions (Alexandria, El-Beheira, El-Sharqiya, El-Daqahelaya in northern Egypt and Asyut, southern Egypt) and in two seasons (2007 and 2008) were collected to understand the importance of mycotoxigenic fungi in Egyptian peanuts. The potential mycotoxin production by strains isolated from peanuts were analyzed using qualitative (coconut cream agar) and quantitative (HPLC) methods. The results of fluorescence method on coconut cream agar medium have revealed that almost all strains except two strains produced a blue fluorescence. The comparability of the results between coconut agar method and HPLC was approximately 90% (Sultan and Magan, 2010).

When the cultures of *A. flavus* and *A. parasiticus* were exposed to ammonia vapour, the reverse side of aflatoxigenic strains on petridishes will turn to pink color on different culture media *viz* potato dextrose, coconut and yeast extract sucrose agar. Among the 120 strains of *A. flavus, A. parasiticus* and two related species in *A. flavus* group: *A. oryzae* and *A. sojae* tested by ammonia vapour, only the aflatoxin producing strains of *A. flavus* and *A. parasiticus* have shown the pink pigmentation. The colour

change was observed immediately after the colony came in contact with the ammonia vapour and was useful for the rapid screening of the aflatoxin producing strains of *A. flavus* and *A. parasiticus* (Saito and Machida, 1999).

For the detection of aflatoxin production by isolates of *Aspergillus flavus* and related species, a new coconut cream agar medium was developed. The detection of isolates of *A. flavus* was more effective on coconut cream agar, which comprised coconut cream (50%) and agar (1.5%), than the synthetic media tested. The differentiation of *A. flavus* from *A. parasiticus* and *A. nomius* was done based on the fluorescence of colonies grown on coconut cream agar (Dyer and McCammon, 1994).

Sheir *et al*. (2005) evaluated two tests for the production of aflatoxins by *Aspergillus* strains *i.e.* (i) yellow pigment production, and (ii) change in the colour of the colony to plum-red colour when exposed to ammonium hydroxide vapour. Seven pigments were isolated using colour changes when chromatography spots were exposed to ammonium hydroxide vapour to guide fractionation. The structures of isolated pigments were known to be anthraquinone pigments that associated with the aflatoxin biosynthetic pathway in *Aspergillus* spp. It was reported that the detection of excess aflatoxin biosynthetic intermediates production was the basis of both empirical tests for aflatoxigenicity.

Rojas-Duran *et al*. (2007) described a novel screening method for the visual detection of aflatoxigenic strains from *Aspergillus* genus based on room temperature phosphorescence (RTP). Strains of *Aspergillus* were cultured on media to which methyl-β-cyclodextrin plus bile salts (0.6% sodium deoxycholate) were added and incubated for three days at 28º C. When the cultures were observed after exposure to UV light, aflatoxin production was readily detectable from the mycelium of aflatoxigenic strains by RTP emission.

Yabe *et al*. (1987) conducted a study to monitor the UV absorption by aflatoxins in GY (glucose yeast extract) agar medium by UV photography. They reported that the aflatoxin-producing molds were identified as gray or black colonies, whereas atoxigenic molds appeared as white colonies in the UV photographs. It was found that the products absorbing UV light in cellophane transplantation experiments and silica gel thin-layer chromatography were found to be mainly aflatoxins B_1 and G_1 excreted from the mold mycelium into the agar medium.

Aflatoxin producing strains of *Aspergillus flavus* and related species were detected by fluorescence method which utilizes the ultraviolet-induced fluorescence of aflatoxin produced in a modified czapek's solution agar containing corn steep liquor, $HgCl_2$, and $(NH_4)H_2PO_4$ instead of $NaNO_3$ (Hara *et al.,* 1974). The results of thin-layer chromatography of $CHCl_3$ extracts of the fluorescing agar were used to confirm the presence of aflatoxin.

Yousefi *et al*. (2009) examined the ability of aflatoxin production by 43 isolates of *Aspergillus flavus* from cultured green tiger shrimps of Persian Gulf. To detect the fluorescence under UV light, aflatoxin producing ability medium and coconut agar medium were used. The presence of aflatoxin in culture extract was confirmed and quantified by high pressure liquid chromatography (HPLC). Of all the isolates, only 4.6% have fluoresced on aflatoxin producing ability medium and coconut agar medium under UV light.

Ritter *et al*. (2011) evaluated the aflatoxin producing capacity of three isolates of *Aspergillus flavus* under different culture conditions. Their results indicated that YES agar was an alternative medium for detecting the toxigenic potential of *Aspergillus flavus* at a pH of 5.2, temperature of 25º C, and incubation time of 11 days, producing 206.05 ng CFU^{-1} of aflatoxin B_1.

Almoammar *et al*. (2013) used polyphasic taxonomic techniques to identify 21 isolates of *Aspergillus flavus* and *A. niger* from Saudi Arabia camel feeds, using six aflatoxin producing culture media. The non-aflatoxigenic isolates did not shoe blue fluorescent ring when observed under UV light, which indicates the inability to produce aflatoxins.

Yazdani *et al*. (2010) studied several screening methods for direct visual determination of aflatoxins and ochratoxin A (OTA) production, using different types of coconut culture media, methylated β-cyclodextrin and ammonium hydroxide vapour tests. Their results indicated that use of these techniques were not sufficiently sensitive for all *Aspergillus* species. Further, they suggested simple thin layer chromatography (TLC) as a sensitive and reliable technique for detection of aflatoxins and OTA produced by *Aspergillus* and *Eurotium* species.

2.7 Identification of superior PGPR (*Pseudomonas fluorescens*) isolates against *Aspergillus flavus*

In a study conducted by Reddy *et al.* (2009) on the identification of an effective bioagent against *A. flavus* growth and aflatoxin reduction in rice, it was reported that PGPR such as *P. fluorescens* and *B. subtilis* inhibited *A. flavus* growth up to 93% and 68% respectively. The fungal bioagent, *Trichoderma virens* showed an inhibition of 80% of test fungus. The research also highlighted the efficacy of certain plant extracts like *Curcuma longa, Allium sativum* and *Ocimum sanctum* in inhibiting *A. flavus* growth and aflatoxin contamination. Further, it was reported that the culture filtrate of *Rhodococcus erythropolis* was completely inhibitory to AFB_1 production at 25 ml kg^{-1} concentration.

Sudha *et al.* (2013) evaluated the efficacy of fungicides, bioagents and plant extracts against *A. flavus* on chilli under both *in vitro* and field conditions. Among different bioagents tested, *Pseudomonas fluorescens* inhibited up to 74% and *Trichoderma harzianum* inhibited up to 70.4% of *A. flavus* growth. The fungicide 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), nimbicidin and pongamia. In the field experiment, least incidence of *A. flavus* was recorded in NSKE sprayed chilli plot (1.6%), followed by captan (2.2%), *P. fluorescens* (2.4%) and *T. harzianum* (2.6%) compared to control (7.4%).

The potential use of biocontrol agents in the reduction of toxigenic *Aspergillus flavus* growth and subsequent aflatoxin B_1 (AFB_1) production in sorghum were evaluated by Reddy *et al*. (2010). They reported that the culture filtrate of *Rhodococcus erythropolis* has shown complete inhibition of *A. flavus* growth and AFB_1 production at 25 ml kg^{-1} concentration. The other biocontrol agents like *Pseudomonas fluorescens, Bacillus subtilis* and *Trichoderma viride* showed 74%, 72% and 65% inhibition of *A. flavus* growth, and 54%, 62.6% and 39% reductions of AFB_1 respectively.

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

Baig *et al*. (2012) investigated the mycoflora associated with selected oil seeds, the reasons of deterioration and its control by integrated methods. Several biocontrol agents like *Trichoderma viride, T. harzianum, Pseudomonas fluorescens* and *Bacillus subtilis* were tested for their antagonistic activity against dominant fungi like *Aspergillus flavus, A. niger, Fusarium oxysporum* and *Alternaria alternata*. Results indicated that *P. fluorescens* inhibited the growth of *A. flavus* effectively (67.6 %) as compared to *B. subtilis* (57.3 %).

Mushtaq *et al*. (2010) assessed the antagonistic potential of soil bacterial strains (*Escherichia coli, Baccillus fortis, B. faragiris, Pseudomonas fluorescens* and *P. malophilia*) against some food pathogenic fungi, *i.e. Aspergillus flavus, A. niger, Penicillium italicum* and *P. simplicissimum* by the dual culture technique. They reported that *E. coli* showed almost complete inhibition against *A. niger* and *A. flavus* whereas *B. fortis* was least effective. The bacterial bioagent, *P. fluorescens* showed highest antifungal activity against *P. italicum* (94%) and was least effective against *A. flavus* (46%).

Bhushan *et al*. (2013) conducted *in vitro* experiments (dual culture technique and blotter test method) to study the antagonistic activity of two bacteria, *Bacillus subtilis* and *Pseudomonas fluorescens* against *Aspergillus flavus, A. fumigatus, A. niger, A. terreus and Fusarium oxysporum* of *Pennisetum americanum*. It was evident that *B. subtilis* was highly effective in reducing the seed-borne mycoflora than *P. fluorescens*.

Pandey *et al*. (2014) studied the antagonistic effect of *Pseudomonas* on six fungi *viz. Pyricularia oryzae, Fusarium oxysporum, Aspergillus niger, Aspergillus flavus, Alternaria alternata* and *Erysiphe cruciferarum.* Overall, the results indicated that *Pseudomonas* showed the maximum inhibition on the growth of *P. oryzae* (89%) followed by *A. niger* (80%), *A. alternata* (77%), *F. oxysporum* (76%) and *A. flavus* (71%) and *E. cruciferarum* (64%) over control.

The potential of bacterial isolates from maize field soil and maize rhizosphere samples were evaluated for their biocontrol activity against *Aspergillus flavus* and *Fusarium verticillioides.* It was reported that the strains of *P. chlororaphis* and *P. fluorescens* consistently inhibited growth of *A. flavus* and *F. verticillioides* in different media (Palumbo *et al*., 2007).

Six *Trichoderma* and three *Pseudomonas* strains which were identified as highly antagonistic to AF 11-4, a highly toxigenic *Aspergillus flavus* strain (Thakur *et al*., 2003). In *A. flavus*-sick plots, the antagonists were applied as seed dressing and soil application at flowering stage. Among the biocontrol agents used, 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 than others.

To evaluate the antifungal activity against aflatoxin-producing *A. flavus*, bacteria were isolated from California almond orchard samples. A total of 171 bacteria isolated from almond flowers, immature nut fruits, and mature nut fruits have shown inhibition of *A. flavus*, using solid and liquid media in co-culture assays. The predominant genera isolated from the almond samples were *Bacillus*, *Pseudomonas*, *Ralstonia*, and *Burkholderia*, as well as several plant-associated enteric and non-enteric bacteria. Based on the results obtained, it was clear that a number of bacterial strains completely inhibited the growth of *A. flavus* in three different media (Palumbo *et al*., 2006).

In a study conducted by Sanskriti *et al*. (2015), the antifungal activity of *Pseudomonas fluorescens* against *A. flavus* in groundnut was examined in laboratory and field trials. Their results indicated that, there was a significant reduction of seed infection by *A. flavus* by the inoculation of *P. fluorescens* on groundnut. They reported that the utilization of *P. fluorescens* as the antagonist of *A. flavus*, have improved the health of the groundnut plant.

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

For the control of pre-harvest groundnut seed infection by *A. flavus*, potential antagonistic agents like, fluorescent *Pseudomonads*, *Bacillus* and *Trichoderma* spp. were isolated from the geocarposphere (pod-zone) of groundnut (Anjaiah *et al*., 2006). The results of greenhouse and field experiments indicated that the inoculation of selected antagonistic strains 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 was observed.

2.8 Determining the mode of action of PGPR against *Aspergillus flavus* using Scanning Electron Microscopy

Ultra-structural studies using scanning electron microscopy (SEM) were mostly confined to areas relating to identification and characterization of *Aspergillus flavus* and aflatoxins. Rodrigues *et al*. (2007) studied the isolates of *A. flavus* and *A. parasiticus* isolated from material on sugarcane using light and SEM and found that the conidia had two distinct ornamentations. The SEM studies confirmed that conidia of *A. flavus* have relatively thin walls which were finely to moderately roughened. Further, their shape varied from spherical to elliptical. In contrast, conidia of *A. parasiticus* were more spherical and noticeably echinulate or spinulose.

Both morphological and structural characterization of fungal pellets and biofilms can be carried out using SEM. The growth of *Aspergillus niger* varied in the form of pellets to biofilm. Verma *et al.* (2011) observed that polyester sheet acted as a better solid support for development of thick biofilm having lesser interstitial voids and more structured channel, that provided conditions for improved application of *A. niger* in industrial and environmental management process.

Further, SEM studies on *A. flavus* were also used in characterization for production of nanoparticles. The cell free filtrate of *Aspergillus* sp when incubated with 1mM silver nitrate on a rotary shaker at 120 rpm, biological synthesis of nano silver particles was confirmed using SEM as appeared within the size range of 40-70 nm. These nanoparticles such as silver (Ag) have antibacterial activity that was advantageous over conventional antibiotics against *Pseudomonas aeruginosa*. (Subha Rajam *et al*., 2013).

The infection of *A. flavus* on groundnut kernels was also studied by Achar *et al.* (2009) using SEM to establish the seed borne nature of *A. flavus* in groundnut. Mycelium of fungus with continuous branching of young hyphae was seen established inside the host tissues both intercellularly and intracellularly. Mycelium of *A. flavus* was also detected in xylem vessels using SEM thereby indicating the systemic nature of infection.

However the research on understanding the interaction of PGPR and *A. flavus* was scanty. Akocak *et al.* (2015) worked on the antagonistic effect of chitinolytic

Pseudomonas and *Bacillus* on the growth of fungal hyphae and spores of aflatoxigenic *A. flavus*. SEM observations indicated that the chitinases of these antagonists induced numerous ultrastructural morphological changes during spore germination and mycelial growth of *A. flavus*.

Huang *et al.* (2012) established the biocontrol abilities of the PGPR strain, *Bacillus pumilus* SQR-N43 on *Rhizoctonia solani* Q1 (damping-off of cucumber) using SEM. SEM studies have confirmed that SQR-N43 caused hyphal deformation and enlargement of cytoplasmic vacuoles. Further, cytoplasm leakage was the final manifestation of antagonism by N43 strain on the damping-off pathogen. However, healthy hyphae (control treatment) of *R. solani* did not exhibit any of these phenomena.

In another study using SEM, the anti-microbial activity of glycolipids produced by *Rhodococcus erythropolis* was established. On *Aspergillus niger*, *A. flavus* besides, *Escherichia coli* and *P. aeruginosa* these glycolipids have shown significant inhibitory effects. SEM analysis revealed total deformity and severe destruction of microbial bodies. Further, many of the pathogen cells were enlarged, elongated and fragmented indicating low viability (Abdel-Megeed *et al*., 2011).

The PGPR isolate, fluorescent *Pseudomonad* CW2 was found inhibitory to root pathogens such as *Pythium ultimum* and *Rhizoctonia solani*. SEM confirmatory studies revealed that CW2 effectively colonized cucumber roots infested with root pathogens than on healthy plants. Further, the CW2 isolate caused irregular and abnormal fungal growth. Swellings and shrinkages of hyphae of these root pathogens were also noticed when cucumber roots were drenched with CW2 (Salman *et al*., 2013).

The mode of action of PGPR strains on other soilborne pathogens in different crops was determined using SEM by Vijay Krishna Kumar *et al.* (2013) on rice sheath blight disease (*Rhizoctonia solani*) and its biological control using *Bacillus subtilis* MBI 600 strain revealed antibiosis and hyperparasitim. SEM studies on the effect of MBI 600 on mycelia and sclerotia revealed abnormal coiling, shriveling and breakdown of pathogen hyphae. Further, MBI 600 caused maceration and fragmentation of inner walls of sclerotia, when they were dipped and incubated in commercial formulation of MBI 600 (Integral®).

Chapter III

MATERIAL AND METHODS

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3.1 Location of work

The present investigation was carried out with the facilities available at Department of Plant Pathology, College of Agriculture, Professor Jayashankar Telangana State Agricultural University (PJTSAU), Rajendranagar, Hyderabad and International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, India. The scanning electron microscopy (SEM) work pertaining to the mode of action of plant growth-promoting rhizobacteria (PGPR) (specifically, *Pseudomonas fluorescens*) against *Aspergillus flavus* was carried out at Ruska Laboratories, College of Veterinary Science, Rajendranagar, Hyderabad of Sri Venkateswara Veterinary University.

3.2 Glassware

Glassware of Borosil make were used throughout the present investigation. The glassware used were petri plates (90 mm diameter), conical flasks (250 ml, 500 ml, 1000 ml), measuring cylinders (25 ml, 250 ml, and 500 ml), test tubes, pipettes (0.1 ml,1.0 ml, 2.0 ml, 5.0 ml and 10 ml), glass slides, glass rods, and cover slips etc.

3.2.1 Cleaning of glassware

The glassware were first cleaned with a detergent, followed by thorough washing in tap water. The cleaned glassware were later placed in potassium dichromate ($K_2Cr_2O_7$) solution for 24 hrs and finally rinsed with distilled water for 3-4 times. Then, the glassware were air-dried prior to use.

The following are the ingredients of $K_2Cr_2O_7$ solution.

Potassium dichromate ($K_2Cr_2O_7$)	:	60 g
Concentrated sulphuric acid (H_2SO_4)	:	60 ml
Distilled water	:	1000 ml

3.3 Chemicals

Chemicals of Analytical Reagent (AR) and Guaranteed Reagent (GR) grades of standard make were used. The pH of the media was adjusted by using either 0.1 N hydrochloric acid (HCl) or 0.1 N sodium hydroxide (NaOH) as the case may be.

3.4 Equipments and Instruments

Compound microscope (OLYMPUS BH 2 make) (10x, 40x magnifications) was used for microscopic examination of fungi. The size of the specimen image for observation was obtained by multiplying the eyepiece magnification by the magnification of the objective. Hot air oven and autoclave were used for sterilization of glassware and media respectively. Incubators (Sanyo) were used for incubating test microbes at different temperatures. The cultures were stored in a refrigerator (Kelvinator Scientific) for short-term storage. Weighments were carried out on a single pan electronic balance (SDFCL- EA 3000- Sd fine Chem -limited) with a sensitivity of 0.001 g. Other tools which were used in the present investigation for various purposes include multi-well plates, vortex (VORTEX GENIE-2, Scientific Industries), pH meter, camel brush, plastic trays, and inoculation needle etc.

3.5 Common nutrient media and their preparation

The nutrient media that were used for isolation, culturing and maintenance of fungi and bacteria in the laboratory are the commercial ready made products of Potato dextrose agar (PDA), Nutrient agar (NA), Czapek dox agar, King's B, Coconut agar media, and Yeast extract agar. All the media were supplied from HiMedia Laboratories Pvt. Ltd, Mumbai.

3.5.1 Potato dextrose agar (PDA)

Potato dextrose agar (PDA) medium was used for culturing and maintenance of fungi. PDA powder was dissolved at the rate of 39 g in 1000 ml of distilled water. The pH of the medium was adjusted to 6.8 with 1 N NaOH or 1 N HC1 as the case may be with a pH meter. The medium was distributed to culture tubes and conical flasks. Later, the tubes and flasks along with the medium were sterilized in an autoclave at 15 *psi* (120.6º C) for 15 minutes.

3.5.2 Nutrient agar (NA)

Nutrient agar (NA) medium was used for culturing and maintenance of bacteria. Nutrient agar powder was dissolved at the rate of 28 g in 1000 ml of distilled water. The pH of the medium was adjusted to 6.8 with 1 N NaOH or 1 N HC1 as the case may be with a pH meter. The medium was distributed to culture tubes and conical flasks and then sterilized in an autoclave at 15 *psi* (120.6º C) for 15 minutes.

3.5.3 Czapek dox agar (CDA)

Czapek dox agar (CDA) medium was used for the evaluating the seed infection by *A. flavus* in the collected groundnut samples. Czapek dox agar powder was dissolved at the rate of 49 g in 1000 ml of distilled water and 25 mg of rose bengal was added. The pH of the medium was adjusted to 6.8 either with 1 N NaOH or 1 N HC1. The medium was distributed to culture tubes and conical flasks and then sterilized in an autoclave at 15 *psi* (120.6º C) for 15 minutes.

3.5.4 Yeast extract sucrose (YES)

Yeast extract sucrose (YES) medium was used for the detection of toxigenic isolates of *Aspergillus flavus* by exposing to “Ammonia vapour”. Ten grams of yeast extract, 75 g of sucrose and 7.5 g of agar were dissolved in 1000 ml of distilled water. The pH of the medium was adjusted to 6.8 with either 1 N NaOH or 1 N HC1 as the case may be with pH meter. The medium was distributed to culture tubes and conical flasks and then sterilized in an autoclave at 15 *psi* (120.6º C) for 15 minutes.

3.5.5 Coconut agar medium (CAM)

Coconut agar medium (CAM) was used for the detection of toxigenic isolates of *A. flavus* under UV light. Fresh coconut milk was prepared and mixed with water in 1:1 ratio. Fifteen grams of agar was added to one litre of medium. The pH was adjusted to 6.8 with 10 N NaOH. The medium was distributed to culture tubes and conical flasks and later sterilized in an autoclave at 15 *psi* (120.6º C) for 15 minutes.

3.5.6 King’s B medium

King’s B medium was used for the identification of *Pseudomonas fluorescens* strains under UV light. King’s medium B base was dissolved in 1000 ml of water at the rate of 42.23 g and 15 ml of glycerol was added. The pH of the medium was adjusted to

6.8 with either 1 N NaOH or 1 N HC1 as the case may be with a pH meter. The medium was distributed to culture tubes and conical flasks and then sterilized in an autoclave at 15 *psi* (120.6º C) for 15 minutes.

3.5.7 Preparation of PDA/ NA Slants

PDA/ NA slants were prepared by transferring eight ml of the medium to culture tubes. The tubes were plugged with non-absorbent cotton and sterilized in an autoclave. After sterilization, the tubes were removed from the autoclave when they were still in hot condition (approximately 40º C) and kept in a slanting position for the medium to solidify. After solidification, the slants were kept in refrigerator at 4º C for further use.

3.5.8 Plating of medium

The sterilized medium was melted and distributed in Petri dishes of nine cm diameter at the rate of 20 ml per plate aseptically in a laminar air flow chamber and allowed to solidify. The Petri dishes containing the medium were used for culturing and maintenance of fungi or streaking with bacteria as the case may be.

3.6 To evaluate the extent of *Aspergillus flavus* infection and aflatoxin contamination in pods at selected groundnut mills of Andhra Pradesh and Telangana

3.6.1 Selection of oil mills/Traders

Eight oil mills from different locations of Andhra Pradesh (Anantapur district) and Telangana were selected and three pod samples from each oil mill were collected. Among the eight oil mills, five mills were selected from four districts (Mahaboobnagar, Rangareddy, Nizamabad and Karimnagar) of Telangana. Three mills were selected in Andhra Pradesh from Anantapur district. A total of 24 pod samples were collected from these selected sites with a sample size of one kg each. The samples collected from Telangana were designated as T-1a to T-5c, and the samples from Andhra Pradesh were designated as A-6a to A-8c. The details of the pod samples collected from the oil mills are given in Table 3.1 and 3.2.

3.6.2 Enumeration of *Aspergillus flavus* infection in groundnut kernels

The pod samples collected from oil mills/traders were brought to the groundnut pathology laboratory, ICRISAT to determine the extent of *Aspergillus flavus* infection.

Pods were shelled and kernels were surface sterilized before plating them on Czapek dox agar (CDA) fortified with rose bengal. Fifty seeds per sample were plated to ascertain the *A. flavus* infection. For each sample, apparently healthy seeds were plated. The plates were incubated under dark at 28º C for seven days. Data on number of seeds colonized by typical *A. flavus* colonies were counted and per cent seed infection was determined.

3.6.3 Isolation and confirmation of *Aspergillus flavus*

Conidia from the typical *A. flavus* colonies in seed infection assays were picked using a sterile needle and placed in Petri dishes containing PDA. The inoculated plates were kept in the incubator at 28º C. A total of 24 *A. flavus* isolates were picked up representing one isolate from each pod sample collected. The *A. flavus* cultures were confirmed up to species based on standard protocols (Raper and Fennel, 1965; Okuda *et al*., 2000; Klich, 2002). The *A. flavus* isolates from Telangana were designated as AFT1a; AFT1b; AFT1c; AFT2a; AFT2b; AFT2c; AFT3a; AFT3b; AFT3c; AFT4a; AFT4b; AFT4c; AFT5a; AFT5b; and AFT5c. The isolates from Andhra Pradesh were designated as AFA6a; AFA6b; AFA6c; AFA7a; AFA7b; AFA7c; AFA8a; AFA8b; and AFA8c.

3.6.4 Aflatoxin estimation by indirect competitive enzyme-linked immunosorbent assay (indirect competitive ELISA) (Reddy *et al*., 2001)

3.6.4.1 Required materials

ELISA Plate Reader (Bio-Rad) (Plate 3.1 A)

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 (Plate 3.1 B).

Others

IPCV-H Polyclonal antibodies

Mortar and pestle, muslin cloth, pH meter, incubator, refrigerator

Aflatoxin B_1 (Sigma A6636)

Aflatoxin B_1 -BSA conjugate (Sigma A6655)

Bovine Serum Albumin (Sigma A6793)

200 mg in 100 ml of PBS-Tween (0.2%)

3.6.4.2 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 buffered 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

3.6.4.3 Substrate buffer

Substrate buffer for alkaline phosphatase system

P-nitrophenyl phosphate (PNPP) was stored at –20º C. It was a chemical in tablet form (5, 15 or 40 mg tablets are available). Ten percent diethanol amine (v/v) was prepared in distilled water; pH was adjusted to 9.8 with concentrated HCl. This solution was stored and pH was adjusted to 9.8 prior to use. Para nitro phenyl phosphate (PNPP) (0.5 mg ml^{-1}) was prepared in 10% diethanol amine, pH 9.8 (for each 15 mg tablet 30 ml solution was required). PNPP solution was not allowed to turn yellow. This may sometimes happen because of alkaline phosphatase (ALP) contamination from skin.

3.6.4.4 Preparation of groundnut seed extracts

Groundnut seed (100 g) was blend 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. The extract was filtered through Whatman No. 1 filter paper (Plate 3.2) and diluted at 1:10 in PBS-Tween (1 ml extract and 9 ml of buffer). To estimate lower levels of AFB_1 (<10 µg Kg^{-1}), prior to ELISA, a simple liquid-liquid cleanup and concentration (5:1) procedure was adopted. Twenty ml of methanol extract, 10 ml of distilled water and 20 ml chloroform were mixed in a separating funnel and used for cleanup. After vigorous shaking for one minute, the lower chloroform layer was collected and evaporated to near desiccation in water bath at 60º 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^{-1} 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 or at 37º 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%) prepared in PBS-Tween was added at 170 µl per each well of ELISA plate and incubated at 37º C for 1h. The plates were washed in three changes of PBS-Tween, allowing 3 min between each wash.

3.6.4.5 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.

3.6.4.6 Procedure of ELISA

Fifty µl of antiserum was added to each dilution of aflatoxin standards (100 µl) and groundnut seed extract (100 µl) intended for analysis.To facilitate reaction between the toxin present in the sample with antibody, the plate containing the mixture of

aflatoxin samples (100 µl) and antiserum (50 µl) was incubated for 1 h at 37º C. Entire process was carried out in ELISA plate and there was no need to pre incubates the toxin and antibody mixture in separate tubes.

The plate was washed in three changes of PBS-tween allowing for 3min for each wash. Goat anti-rabbit IgG (1:4000 dilution) was prepared and labeled with alkaline phosphatase, in PBS-Tween containing 0.2% BSA. To each well, 150 µl was added and incubated for 1h at 37º C. The plate was washed in three changes of PBS-Tween allowing for 3 min for each wash. Substrate solution (p-nitro phenyl phosphate prepared in 10% diethanolamine buffer, pH 9.8) (150µl) was added and incubated for 1h at room temperature (Plate 3.1C). Absorbance was measured at 405 nm in an ELISA reader.

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. Amount of aflatoxin present in the sample was calculated using the formula below

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

A = AFB_1 concentration in diluted or concentrated sample extract (ng ml^{-1})

D = Time dilution with buffer

C = Times concentration after clean up

E = Extraction solvent volume used (ml)

G = Sample weight (g)

3.6.5 Mapping of risk and sensitive areas with respect to kernel infection by *Aspergillus flavus* and aflatoxin contamination

The mean kernel infection by *A. flavus* and aflatoxin levels in the pod samples collected at oil mills/traders' in Telangana and Andhra Pradesh were mapped using Geographical Information System (GIS) at ICRISAT, Patancheru, India. Accordingly, the surveyed areas were categorized as follows.

Areas with kernel aflatoxins (µg kg^{-1}): ≤30 =safe zone; and >30=risk zone

Kernel infection by *A.flavus* (%) : ≤30 = safe zone; and >30= risk zone

3.7 To detect the toxigenic *Aspergillus flavus* strains using cultural methods

All the 24 *A. flavus* isolates obtained from the groundnut samples of oil mills/traders were evaluated for their toxigenicity using cultural methods as follows.

3.7.1 Coconut agar medium (CAM)

Coconut agar medium (CAM) was prepared based on Dyer and McCammon (1994) with slight modifications. Spore suspension of *Aspergillus flavus* isolates was inoculated at the center of the Petri dishes containing CAM. The Petri dishes were later incubated in dark at 28º C for seven days and then the fluorescence around the fungal colonies was evaluated. Presence of fluorescence surrounding the fungal colonies under UV light indicates the toxigenicity of an isolate, whereas absence of fluorescence indicates atoxigenic nature. Based on the presence or absence of fluorescence, the *A. flavus* isolates were categorized as toxigenics and atoxigenics.

Further, the ioslates were observed for the yellow pigmentation on the lower side of the CAM plates. The isolates, that have shown yellow pigmentation were categorized as toxigenics, whereas those without any yellow pigmentation were categorized as atoxigenics.

3.7.2 Yeast extract sucrose (YES) medium and Ammonia vapour test

Single spore cultures of *A. flavus* were inoculated on to Petri dishes containing YES media and incubated in dark at 28º C (Kumar *et al.,* 2007). After 3-4 days, the Petri dishes were kept inverted over 0.5ml ammonium hydroxide (Sigma-Aldrich). Within five minutes, aflatoxigenic isolates turns pink to red in colour. Whereas no colour change was observed for atoxigenic isolates.

3.8 Identification of superior PGPR isolates against *Aspergillus flavus*

3.8.1 Isolation of *Pseudomonas fluorescens*

Soil samples were collected from groundnut fields at ICRISAT, Patancheru, Telangana**,** India. Serial dilution method was followed (Aneja, 2001) to isolate PGPR (*P. fluorescens*). Each soil sample was grinded into fine powder and sieved. Ten grams of soil was added to 90 ml of sterilized distilled water in 250 ml flask to arrive at 10^{-1} dilution. One ml of this 10^{-1} dilution was transferred to nine ml sterile distilled water (SDW) in test tube to obtain 10^{-2} dilution. In the same way, serial dilutions were carried

out up to 10^{-6}. The dilutions were vortexed for one minute. Soil suspensions of 500 µl of dilutions of 10^{-4}, 10^{-5} and 10^{-6} were plated on each Petri dishes containing King's B medium. Three replications were maintained for each dilution. The Petri dishes were later incubated at 28º C for seven days. Bacterial colonies that developed were counted and observed under UV light. The population was expressed as colony forming units (CFU g^{-1}soil). The bacteriological tests for confirming the *P. fluorescens* isolates were conducted as per laboratory guide for "Identification of Plant Pathogenic Bacteria" published by the American Phytopathological Society (Schaad, 1992). Ten isolates of *P. fluorescens* were isolated and designated as *Pf*1 through *Pf*10. These PGPR isolates were then maintained on nutrient agar (NA) for further studies.

3.8.2 Dual culture studies

Ten *P. fluorescens* isolates were used in the present study. The antagonistic activity of *P. fluorescens* on *A. flavus* was tested by dual culture technique (Dennis and Webster, 1971). The toxigenic isolate of *A. flavus*, AFT5b was used in the present study. PGPR isolates were streaked at one side of Petri dish (one cm away from the edge) containing PDA. A mycelial disc from seven days old PDA culture of *A. flavus* was placed at the opposite side of Petri dishes perpendicular to the bacterial streak and incubated at 28±2º C for seven days. Petri dishes with PDA inoculated with fungal discs alone served as control. Altogether, there were 10 treatments plus a control. Three replications were maintained for each treatment. Observations on radial growth of test fungus were recorded and per cent inhibition was calculated by using the formula proposed by Vincent (1927).

Per cent inhibition (I) = 100(C-T)/C

Where, C= radial growth of *A. flavus* in control (in cm)

T= radial growth of *A. flavus* in treatment (in cm)

The experiment was executed in a completely randomized design (CRD).

3.8.3 *In vitro* seed colonization assay

The efficacy of *P. fluorescens* isolates in reducing aflatoxin production by *A. flavus* was studied by using the procedure of *in vitro* seed colonization (IVSC) according to Thakur *et al.* (2000). Multi-well plates were used for this purpose (Plate 3.3 A). Healthy and undamaged groundnut kernels (JL24) were surface sterilized and then treated with PGPR inoculum @ 1 x10^9 CFU ml^{-1} for one minute. Kernels dipped in

SDW serves as control. Later, the seeds were sprayed with an aflatoxigenic *A. flavus* strain- AFT5b @ 1 x10^8CFU ml^{-1} (Plate 3.3 B) and then the multi-well plates were kept in plastic trays with wetted blotting papers to provide moisture (Plate 3.3 C). Later, the plastic trays with multi-well plates were incubated for one week in dark at 28º C. The experiment was executed in a completely randomized design (CRD). Altogether, there were 11 treatments including control. Each treatment was replicated thrice. After incubation, the seeds were rated for colonization severity by *A. flavus* on severity scale of 1-4 (Thakur *et al.*, 2000) and details presented in Table 3.3. Further, the same seeds used for IVSC experimentation, were later used for estimating aflatoxin content through indirect ELISA (Reddy *et al.*, 2001).

3.9 To determine the mode of action of PGPR against *Aspergillus flavus* using scanning electron microscopy

3.9.1 Antibiosis of *Pseudomonas fluorescens* on *Aspergillus flavus*

The potential isolate of *P. fluorescens* (*Pf*7) identified in dual culture and IVSC assay was used against the toxigenic *A. flavus* isolate, AFT5b for establishing its mode of action. The antagonistic properties of *P. fluorescens* isolate were studied using SEM (Gupta *et al*., 2001; Weidenborner *et al*., 1989; Gopalakrishnan *et al*., 2015). The SEM facilities of Ruska laboratories of Sri Venkateswara Veterinary University at Rajendranagar, Hyderabad were utilized in the present study (Plate 3.4). Plugs of mycelium (5 mm diameter) were cut from the edge of an actively growing fungal colony on PDA with a No. 2 Cork borer, and one plug was placed in the center of each PDA plate (100 x 15 mm). Two parallel 3.5 cm long streaks of PGPR were then made 2 cm apart on opposite sides of the plug. The test fungus which was not co-inoculated with PGPR isolate served as control. Later, the Petri dishes were incubated at 25º C for five days in the dark. Fungal mycelia growing towards the inhibition zone (Plate 3.5) was processed for SEM by the following procedure. Agar discs of 1mm thickness were cut from mycelial growth of *A. flavus* near the inhibition zone and placed on cover glasses. For the fungal control, the 1 mm agar discs were sampled from the leading edge. The separated discs containing mycelia were washed with phosphate buffer, post fixed in 2% osmium tetroxide for 4 h and dehydrated using a graded series of ethanol. The dehydrated samples were dried with critical-point liquid carbon dioxide as a transition fluid. The dried materials were adhered on to aluminum specimen mounts with double stick adhesive tape. The samples were later coated with gold-palladium in

an automated sputter coater (JEOL JFC-1600) and examined with a scanning electron microscope (JEOL-JSM 5600) as per the standardized protocols at Ruska laboratories. Mycelial growth of *A. flavus* in control plates was also observed. The hyphal deformities near the zone of inhibition were recorded and compared with that of control plates.

3.9.2 Hyperparasitism of *Pseudomonas fluorescens* and *Aspergillus flavus*

The *A. flavus* culture was multiplied on PDA at 28º C for 36 h under dark conditions. Eight mm diameter plugs that were cut from the leading edge of *A. flavus* culture were later sprayed with *P. fluorescens* (*Pf7*) at a concentration of 1 x 10^8 CFU ml^{-1} and incubated for three days at 28º C (Plate 3.6). Fungal discs sprayed with sterile distilled water served as the controls. Discs of fungal mycelium were prepared for SEM (Ziedan and El-Mohamedy, 2008). Samples were washed with phosphate buffer, post fixed in 2% osmium tetroxide for 4 h and dehydrated using a graded series of ethanol. The dehydrated samples were dried with critical- point liquid carbon dioxide as a transition fluid. The dried materials were adhered on to aluminum specimen mounts with double stick adhesive tape. The samples were later coated with gold-palladium in an automated sputter coater (JEOL JFC-1600) and examined with a scanning electron microscope (JEOL-JSM 5600) as per the standardized protocols at Ruska laboratories. The occurrence of morphological changes in the hyphae of *A. flavus* was recorded and compared with that of healthy hyphae in control plates.

3.10 STATISTICAL ANALYSES

The statistical analysis carried out for different experiments were as follows.

3.10.1 Evaluation of the extent of *Aspergillus flavus* infection and aflatoxin contamination in pods at selected groundnut mills of Andhra Pradesh and Telangana

The data pertaining to the *A. flavus* infection and aflatoxin contamination were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the treatment means were differentiated by a least significant difference (LSD) at P=0.05 using PROC-GLM. Data on extent of kernel infection by *A. flavus* were square root transformed, whereas, the data on aflatoxin contamination were log transformed.

3.10.2 Identification of the superior PGPR (*Pseudomonas fluorescens*) isolates against *Aspergillus flavus*

3.10.2.1 Dual culture studies

The dual culture experiment was executed in a Completely Randomized Block Design (CRD), and the data were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the treatment means were differentiated by a least significant difference (LSD) at P=0.05 using PROC- GLM.

3.10.2.2 *In vitro* seed colonization assay (IVSC)

The data pertaining to the IVSC results of seed colonization by *A. flavus* were analysed using a non-parametric approach. Kruskal-Wallis test was used for converting the measured observations and ranks were assigned. The treatments means were differentiated based on Wilcoxon ranks.

3.10.2.3 Efficacy of *Pseudomonas fluorescens* in reducing the aflatoxin contamination in groundnut kernels

The current IVSC experiment was executed in a Completely Randomized Design (CRD). The data were square root transformed and analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the treatment means were differentiated by a least significant difference (LSD) at P=0.05 using PROC- GLM.

Chapter IV

RESULTS AND DISCUSSION

Chapter IV

RESULTS AND DISCUSSION

The results and discussions pertaining to the current investigations are presented under the following headings

4.1 Evaluation of the extent of *Aspergillus flavus* infection and aflatoxin contamination in pods at selected groundnut mills of Andhra Pradesh and Telangana

4.1.1 Kernel infection in surveyed oil mills

There was a significant difference with respect to kernel infection by *A. flavus* ($P<0.0001$). Of different oilmills surveyed in Telangana and Andhra Pradesh, district-wise, the kernel infection in groundnut was highest (96%) in samples (A-7) collected from Tadipatri mandal of Anantapur district (Table 4.1) (Plate 4.1), followed by kernel infections in samples collected from Nizamabad (T-3) (90.7%) and Rangareddy (T-2) (90%) of Telangana. No significant differences in kernel infection were found among these samples. For the remaining samples, the kernel infections ranged from 22.7 to 54.0%. The kernel infections in Karimnagar (T-4 & T-5) and Mahaboobnagar (T-1) were significantly inferior to samples from Nizamabad (T-3) and one sample from Anantapur district (A-7). The other two samples from Anantapur district, A-6 (Tadipatri) and A-8 (Tadimarri) have recorded least *A. flavus* infection levels of 22.7% and 29.3% respectively (Table 4.1).

4.1.1.1 Kernel infection in Telangana

District-wise, kernel infection ranged from 42 to 90.7% in samples collected from oil mills of Telangana (Fig 4.1) and mean kernel infection by *A. flavus* was highest in Nizamabad (90.7%). This was followed by samples from Rangareddy district that recorded 90% kernel infection. No significant differences were noticed between the kernel infections for samples from Nizamabad and Rangareddy (Table 4.1). The kernel infection in samples from Nizamabad and Rangareddy were significantly superior over that of Mahaboobnagar (42%) and Karimnagar (54%). Further, no significant differences were noticed between samples of Mahaboobnagar and Karimnagar with

respect to kernel infection. Overall, our results indicated higher *A. flavus* infections at oil mills of Nizamabad and Rangareddy (Table 4.1).

4.1.1.2 Kernel infection in Andhra Pradesh

In Andhra Pradesh, in the surveyed mandals of Anantapur district, there were marked differences in the kernel infections (Fig 4.2). In the two mandals surveyed, Tadimarri recorded a mean kernel infection of 29.3%. However, in the other mandal, Tadipatri, kernel infections were up to 59.3%. Though, the mean kernel infection in Tadipatri was 59.3%, individually, the two locations in this mandal have shown *A. flavus* infection levels as 22.7% (A-6) and 96% (A-7) kernel infections (Table 4.1).

4.1.2 Isolation of *Aspergillus flavus* from the infected groundnut seed

The groundnut kernels that were used in the seed infection assay were used as a source to isolate *A. flavus*. One *A. flavus* isolate from each sample was isolated. Altogether, 24 *A. flavus* isolates were obtained from the surveyed groundnut mills. The isolates were designated based on the state of origin. The *A. flavus* isolates from Telangana were designated as AFT1a to AFT5c, whereas the *A. flavus* isolates from Andhra Pradesh were designated as AFA6a to AFA8c (Plate 4.2).

4.1.3 Aflatoxin contamination in the surveyed oil mills

Of different oil mills surveyed in Telangana and Andhra Pradesh, there was a significant difference with respect to kernel aflatoxin content ($P<0.0035$). District-wise, aflatoxin contamination was highest (6148.4 µg kg^{-1}) in kernels collected from Anantapur (Tadimarri) (A-8), followed by T-2 sample from Rangareddy (1205.5 µg kg^{-1}) and T-5 sample from Karimnagar (724.03 µg kg^{-1}) (Table 4.2). Overall, the aflatoxin levels in surveyed oil mills in Telangana and Andhra Pradesh ranged from 2.16 µg kg^{-1} to 6148.4 µg kg^{-1}. The pod samples from Tadipatri of Anantapur had recorded low aflatoxin levels (up to 2.2 µg kg^{-1}). Aflatoxin levels were also less in pod samples collected from Nizamabad (4.9 µg kg^{-1}), T-4 sample of Karimnagar (7.06 µg kg^{-1}) and Mahaboobnagar (11.5 µg kg^{-1}).

4.1.3.1 Aflatoxin contamination in Telangana

District-wise in Telangana, pod samples from oil mills of Rangareddy recorded highest mean aflatoxin levels (1205.2 µg kg^{-1}) (Fig 4.3). This was followed by oil mills from Karimnagar with mean kernel aflatoxin levels up to 365.55 µg kg^{-1}. Samples from

the oil mills of Nizamabad and Mahaboobnagar have recorded aflatoxins to a tune of 4.9 and 11.5 µg kg^{-1}.

4.1.3.2 Aflatoxin contamination in Andhra Pradesh

In the surveyed mandals of Anantapur district of Andhra Pradesh, oil mills of Tadipatri have recorded mean aflatoxin levels of 2.8 µg kg^{-1}. However, pod samples from Tadimarri mandal have shown highest aflatoxin levels (6148.4 µg kg^{-1}) (Fig 4.4).

4.1.4 Mapping of risk and sensitive areas with respect to kernel infection and aflatoxin contamination of groundnut by *Aspergillus flavus*

Based on the results obtained in the kernel infection and aflatoxin contamination in pods at selected groundnut mills of Andhra Pradesh and Telangana, GIS mapping was carried out to depict the risk and sensitive areas.

4.1.4.1 Risk and sensitive areas in Telangana

In Telangana, all the surveyed oil mills were categorized under risk zone for kernel infection (%) by *A. flavus*. Based on the aflatoxin contamination in the pod samples, oil mills sampled in Mahaboobnagar and Nizamabad districts were categorized as safe zone. The other oil mills that fall in Rangareddy and Karimnagar districts were categorized as sensitive areas (Fig 4.5).

4.1.4.2 Risk and sensitive areas in Andhra Pradesh

In Anantapur district of Andhra Pradesh, based on kernel infection by *A. flavus*, Tadipatri mandal was categorized as risk zone. However, based on aflatoxin levels in pod samples, oil mills from Tadipatri mandal was categorized as safe zone (Fig 4.6). In Tadimarri mandal, oil mills were categorized as safe zone based on *A. flavus* infection and as risk zone based on aflatoxin contamination (Fig 4.6).

Our studies indicated that both Telangana and Andhra Pradesh have significant levels of *A. flavus* infection and aflatoxin contamination in kernels at oil mills. Post-harvest contamination of groundnut pods during storage at traders/markets/oil mills in the present study is attributed to either improper storage conditions or the carry over inoculum of *A. flavus* from field to farmers' storage and ultimately to markets/oil mills. Improper storage practices are the major factors for aflatoxin build up during storage. Pod storage at high moisture levels increases post-harvest molding and aflatoxin

contamination (Heathcote and Hibbert, 1978). Besides, storing the pods at optimum moisture levels, pod drying by farmers immediately after harvest also helps in reducing the *A. flavus* infection and aflatoxin contamination during storage. Inverted windrowing is an ideal drying procedure of pods after harvest. It helps in proper drying of pods with adequate exposure to sunlight. Dick (1987) reported that $\geq$7% moisture levels in grains is ideal for preventing mold growth including aflatoxigenic strains. Proper drying practices such as windrowing and immediate stripping of pods is recommended for minimizing aflatoxin contamination during storage below acceptable limits (Richard, 2000). Based on our results, we can assume that groundnut pods/kernels from oil mills of Telangana and Andhra Pradesh are at increased risk to aflatoxin contamination.

In our present study, there seemed to be no correlation between kernel *A. flavus* infection and aflatoxin contamination for the pods sampled from oil mills. Our studies reported that safe zones with respect to *A. flavus* infection had higher levels of aflatoxin contamination. For example, pod samples from oil mills of Nizamabad (T-3) district of Telangana had higher *A. flavus* infection (90.7%), whereas the aflatoxin content was at significantly lower levels (4.9 µg kg^{-1}). Similarly, pod samples from oil mills in Mahaboobnagar (Telangana) (T-1) also had higher kernel *A. flavus* infection (42%) and low aflatoxin levels (11.5 µg kg^{-1}). In Tadipatri mandal, (Anantapur district) of Andhra Pradesh, the pod samples from oil mills had mean higher *A. flavus* infections (59.3%) and mean low aflatoxin levels (2.18 µg kg^{-1}) (Fig4.2 and 4.4). Higher *A. flavus* infections and low aflatoxin levels in these samples colud be attributed to the co-existence of toxigenic and atoxigenic *A. flavus* strains. Another reason could be the storage practices at these oil mills could be relatively safer that prevent aflatoxin production by the contaminated toxigenic molds.

Also the pod samples from oil mills of Tadimarri (A-8) of Anantapur district in Andhra Pradesh had higher aflatoxin levels (6148.4 µg kg^{-1}), but with lower *A. flavus* infection (29.3%). Higher toxin levels with low mold infections in Tadimarri samples is attributed to the fact that the infection of pods by potent toxigenic strains. Another important reason could be that pre-harvest aflatoxin contamination at farmers' fields in Tadimarri mandal could be at higher levels. However, proper drying practices in these areas by the farmers before selling them to traders/oil mills might have prevented the mold growth at oil mills during storage. In our studies, the pod samples from oil mills/traders of Rangareddy and Karimnagar districts had higher aflatoxin levels and *A.*

flavus infections. Thus there is a potential threat to human and animal health in areas with more aflatoxin contamination and *A. flavus* infections.

Aflatoxin contamination in groundnut and peanut butter is a major post-harvest problem in Kenya. Earlier studies on the prevalence and potential of aflatoxin contamination in Nairobi and Nyanza provinces of Kenya indicated that sources of groundnut and presence of defective nuts were the major factors influencing increased aflatoxin contamination in these food commodities. Further, the predominant fungal species in the groundnut samples collected from farmers and traders in these areas were *A. flavus* (L and S strains), *A. parasiticus*, *A. niger*, *A. tamari*, *A. alliaceus*, *A. caeletus* and *Penicillium* spp (Ndung'u *et al*., 2013). In another study at Eldoret and Kericho towns of Kenya, it was observed that the levels of total aflatoxins were higher in groundnut samples from informal (97.1 µg kg^{-1}) than formal (55.5 µg kg^{-1}) market outlets (Nyirahakizimana *et al*., 2013). Studies in Andhra Pradesh, India have reported alarming levels of post-harvest aflatoxins in Anantapur district. Aflatoxin levels in insect damaged samples collected from farmers of Andhra Pradesh were above 500 µg kg^{-1} (Waliyar *et al*., 2003). Significant measures at both pre-harvest and post-harvest stages are to be advocated to farmers in these areas to prevent pre-harvest *A. flavus* infection and aflatoxin contamination. Further, importance of ideal grain storage practices are to be explained to farmers as well as traders to curb the aflatoxin problem from reaching alarming levels. Since, groundnut and oil cakes are the major products from oil mills, constant monitoring at these places has to be taken up to prevent the aflatoxins from reaching the food chain. Aflatoxin contamination in oilcake is a major problem (Banu and Muthumary, 2008), and is potentially hazardous to animal health. Further, groundnut oil (unrefined) from aflatoxin contaminated kernels also has significant levels of aflatoxins (Idris *et al*., 2010). Good agricultural practices at both pre-and post-harvest stages of groundnut including storage are available to prevent aflatoxin build up in groundnut. Ascertaining the actual reasons for post-harvest buildup of aflatoxins will therefore help in identifying areas of intervention to overcome the problem. Based on this, different management tools for reducing this aflatoxin contamination such as proper post-harvest grain handling, role of post-harvest machinery, physical separation of grains, proper storage methods and conditions, disinfestation, detoxification, inactivation, filtration, use of binding agents and antifungal compounds can be advocated situation-wise to overcome this problem (Waliyar *et al*., 2015). Adequate drying of pods to safe moisture levels and proper

storing in containers of jute bags is therefore essential for stored groundnut that prevent critical increases in fungal populations and thereby aflatoxin contamination (Wagacha *et al*., 2013).

4.2 Detection of the toxigenic *Aspergillus flavus* strains using cultural methods

A total of 24 isolates of *A. flavus* made from the pod/kernel samples collected from groundnut oil mills/traders' of Telangana and Andhra Pradesh were used in the present study. The toxigenic isolates were differentiated from the atoxigenics based on cultural methods such as fluorescence test and yellow pigmentation on coconut agar medium (CAM); and ammonia vapour test on yeast extract sucrose (YES) medium. The *A. flavus* isolates that have shown both yellow pigmentation and fluorescence or either one of the reactions on CAM were considered as toxigenic strains. In case of YES medium, development of plum red colour was considered as positive for detecting toxigenic strains.

The results obtained from these tests indicate that, out of the 24 isolates of *A. flavus*, 18 (AFT1a; AFT1b; AFT1c; AFT2a; AFT2b; AFT2c; AFT4c; AFT5b; AFT5c; AFA6a; AFA6b; AFA6c; AFA7a; AFA7b; AFA7c; AFA8a; AFA8b; and AFA8c) have shown positive reaction to toxigenicity in both the tests (on CAM and YES media) under study (Table 4.3 and Table 4.4). Further, five *A. flavus* isolates, AFT3a; AFT3b; AFT3c; AFT4a; and AFT4b were proved to be atoxigenic in both the tests (Plate 4.3, 4.4, 4.5, 4.6, 4.7 and 4.8). The remaining one isolate, AFT5a was categorized into false (+ve/-ve) which may be false +ve with respect to the coconut agar medium tests or may be false –ve with respect to the ammonia vapour test on YES medium. The details of the toxigenic and atoxigenic isolates of *A. flavus* in each surveyed district were discussed in Table 4.3 and Table 4.4.

4.2.1 Prevalence of toxigenic/atoxigenic *Aspergillus flavus* isolates in Telangana

The prevalence of toxigenic and atoxigenic isolates of *A. flavus* from the groundnut pods/kernels collected from oil mills/traders of Telangana were as follows (Table 4.3).

4.2.1.1 Mahaboobnagar district

All the three isolates of *A. flavus*, AFT1a; AFT1b; and AFT1c isolated from groundnut mills of Mahaboobnagar district were tested positive (toxigenic) on both YES and CAM media. On CAM, AFT1a and AFT1b isolates have shown both fluorescence and yellow pigmentation. The third isolate, AFT1c has exhibited fluorescence but with no yellow pigmentation on the reverse side (Table 4.3).

4.2.1.2 Rangareddy district

Three isolates of *A. flavus*, AFT2a; AFT2b; and AFT2c isolated from groundnut mills of Rangareddy district were tested positive (toxigenic) on both YES and CAM media. On CAM, all the isolates have shown fluorescence; however, only two of three isolates, AFT2b and AFT2c have shown yellow pigmentation. The other isolate, AFT2a has shown fluorescence with no yellow pigmentation (Table 4.3).

4.2.1.3 Nizamabad district

All the three *A. flavus* isolates, AFT3a; AFT3b; and AFT3c (Plate 4.9) isolated from the pod samples collected from Agarwal Agro Industries, Khanapur village of Nizamabad district, were detected as atoxigenics based on their negative reactions on YES and CAM (Table 4.3).

4.2.1.4 Karimnagar district

Out of the six *A. flavus* isolates from the pod samples collected from Karimnagar district, three isolates, AFT4c; AFT5b and AFT5c were toxigenic with positive reaction on both YES and CAM media. Further, two isolates, AFT4a and AFT4b were proved to be atoxigenic. The remaining one *A. flavus* isolate, AFT5a was categorized as false (+ve/-ve). This isolate has shown toxigenic reaction with respect to fluorescence test, but atoxigenic reaction based on fluorescence on CAM. However, on YES media, the isolate was atoxigenic with no plum red color development. Hence, the isolate AFT5a may be false +ve with respect to fluorescence test on CAM or false –ve with respect to ammonia vapour test on YES medium (Table 4.3).

Overall, of all the 15 isolates of *A. flavus* from pods/kernels from oil mills from Telangana, there were nine toxigenic isolates, five atoxigenic isolates and one isolate being either a false positive or false negative. Few of the toxigenic isolates detected from oil mills of Telangana were shown in Plate 4.10.

4.2.2 Prevalence of toxigenic/atoxigenic *Aspergillus flavus* isolates in Anantapur district of Andhra Pradesh

The prevalence of toxigenic isolates of *A. flavus* in the surveyed mandals of Anantapur district of Andhra Pradesh was as follows (Table 4.4).

4.2.2.1 Tadipatri mandal of Anantapur district

Out of the six isolates from the groundnut pod samples collected from M/S. Sri Buggaramalingeshwara oil mills and Sri Sai Ram oil mills, Terannapalli village of Tadipatri mandal, all the isolates were proved to be toxigenic with respect to both the cultural methods (Table 4.4). On YES media, all the six isolates, AFA6a; AFA6b; AFA6c; AFA7a; AFA7b; AFA7c have shown positive reaction (plum red colour). On CAM, all these six isolates have shown fluorescence. However, on CAM, except AFA7a and AFA7b, the remaining four toxigenic isolates exhibited yellow pigmentation on the reverse side of colonies.

4.2.2.2 Tadimarri mandal of Anantapur district

All the three isolates from the groundnut pod samples collected from M/S New Sreenivasa Baby decorticators, Tadimarri village of Tadimarri mandal were proved to be toxigenic in both the cultural methods (Table 4.4). However, the isolate AFA8b was the only toxigenic isolate without yellow pigmentation on CAM.

Overall, all the six isolates from Tadipatri mandal (Plate 4.11) and three isolates from Tadimarri mandal (Plate 4.12) of Anantapur district in Andhra Pradesh were categorized as toxigenic isolates.

Earlier researchers have used cultural methods for detecting toxigenic/atoxigenic strains of *A. flavus* (Hara *et al*., 1974; Cotty, 1988; Dyer and McCammon, 1994). These cultural techniques rely on either quantification of purified extracts (Filtenborg and Frisvad, 1980; Shotwell *et al*., 1966) or on qualitative assessment of fluorescence (Bennett and Goldblatt, 1973; Hara *et al*., 1974). However, a polyphasic approach is essential for precise differentiation of toxigenics and atoxigenics. This is in view of the frequent occurrence of false positives and false negatives in detection through cultural and other methods of detection. Researchers have earlier used polyphasic detection of toxigenic/atoxigenic *A. flavus* strains using cultural, analytical and molecular methods (Almoammar *et al*., 2013).

In the present study, cultural methods were used to differentiate the isolates of *A. flavus* into toxigenic and atoxigenic, by testing them on CAM and YES media. Based on the results, it was evident that the oil mills isolates collected from Mahaboobnagar, Rangareddy (districts from Telangana) and Tadipatri, Tadimarri mandals of Anantapur district (Andhra Pradesh) were toxigenic, while in Karimnagar district, there were co-existence of mold infections from both toxigenic and atoxigenic isolates (Table 4.3). Further, a false positive/false negative *A. flavus* isolate (AF5a) was noticed in kernel samples from oil mills of Karimnagar (Bachu Veera Mallaiah & Sons). This AF5a isolate might be a false negative according to YES; and false positive according to CAM (Table 4.3). In view of the frequent false positives/false negatives in detection through a single method, polyphasic detection is an ideal approach to differentiate *A. flavus* strains. Hence, further investigations on the aflatoxigenicity of this AF5a isolate needs to be reconfirmed based on other cultural, analytical and molecular techniques using a polyphasic approach.

In our studies, atoxigenic isolates were detected in groundnut samples from oil mills Nizamabad besides Karimnagar district (Table 4.3). Co-existence of atoxigenic strains of *A. flavus* along with toxigenic strains is a common phenomenon in crop soils (Probst *et al*., 2007). These atoxigenic isolates of *A. flavus* in the present study as contaminants at oil mills can be originated from groundnut soils in the respective districts and adjoining areas as pre-harvest contaminants or during storage at farmers' end. Antagonistic activities of atoxigenic *A. flavus* strains in mitigating pre-harvest aflatoxin contamination in maize (Abbas *et al*., 2006) and groundnut (Dorner and Lamb, 2006) are reported. However, before exploring the antagonistic potential of these atoxigenic *A. flavus* isolates, their atoxigenicity need to be reconfirmed using polyphasic studies. Further, there are metabolites produced by *A. flavus* other than aflatoxins that are toxic in nature (Liu and Wu, 2010; Snigdha *et al*., 2013). Screening of these atoxigenic isolates in the present study for production of these other toxic metabolites is a pre-requisite before exploring their antagonistic activities on toxigenic *A. flavus* at fields.

4.3 Identification of the superior PGPR (*Pseudomonas fluorescens*) isolates against *Aspergillus flavus*

Ten isolates of *Pseudomonas fluorescens* (*Pf*) (designated as *Pf*1 to *Pf*10) (Plate 4.13) were isolated by serial dilution technique from the soil samples collected from

groundnut fields of ICRISAT, Patancheru. These ten isolates were used in the present study to test the efficacy of *P. fluorescens* in reducing the *A. flavus* infection and aflatoxin contamination in groundnut kernels. The toxigenic *A. flavus* isolate AFT5b, isolated from groundnut kernels collected from Karimnagar district (Bachu Veera Mallaiah & Sons oil mill) of Telangana was used as test fungus in the present study.

4.3.1 Dual culture technique under *in vitro* conditions

The efficacy of *P. fluorescens* in inhibiting the mycelial growth of *A. flavus* was studied using dual culture technique and the results were presented in Table 4.5. There was a significant difference among the treatments evaluated (P<0.0001). In general, all the *Pf* isolates under study inhibited *A. flavus*. Of different treatments, highest inhibition of *A. flavus* was obtained with *Pf*7 (54.8%) (Plate 4.14) followed by *Pf*2 (48.7%) and *Pf*6 (48.2%). However, significant differences were not observed among the three isolates. Next best inhibitions of test fungus were obtained with *Pf*4 (46.2%) and *Pf*9 (44.6%) with no significant differences between them. Further, these two isolates were statistically at par with *Pf*2 and *Pf*6. For the remaining *Pf* isolates, the per cent inhibition was up to 35.9 (*Pf*8). The inhibitions of *A. flavus* by *Pf*1, *Pf*10 and *Pf*3 were about 31.7%, 32.3% and 33.3% respectively. Least inhibition of *A. flavus* was obtained with *Pf*5 (28.2%).

4.3.2 *In vitro* seed colonization assay (IVSC)

4.3.2.1 Efficacy of *Pseudomonas fluorescens* in reducing the *Aspergillus flavus* infection in groundnut kernels

The isolates of *P. fluorescens* (*Pf*) evaluated by IVSC indicated that the colonization severity of *A. flavus* was significantly reduced over control when seeds were treated with bioagents, *Pf*2, *Pf*6, *Pf*7 and *Pf*9. Of these, seed treatment with *Pf*7 has resulted in least colonization severity of *A. flavus* (6.2 wilcoxon score) (Plate 4.15). This is followed by *Pf*2 and *Pf*9 (9.3 wilcoxon score each) and *Pf*6 (13.5 wilcoxon score). The remaining six *Pf* isolates (*Pf*1, *Pf*3, *Pf*4, *Pf*5, *Pf*8 and *Pf*10) failed to show significantly less colonization severity over control. The seeds in control have recorded maximum colonization severity by *A. flavus* (32 wilcoxon score) (Plate 4.16). The difference in colonization severity over control was highest with *Pf*7 (25.8), followed by *Pf*2 and *Pf*9 (22.7) and *Pf*6 (18.5). Overall, the *Pf* isolates, *Pf*7, *Pf*2, *Pf*9 and *Pf*6 were effective in reducing *A. flavus* infection on groundnut seeds (Table 4.6).

4.3.2.2 Efficacy of *Pseudomonas fluorescens* in reducing the aflatoxin contamination in groundnut kernels

The data on aflatoxin levels in groundnut kernel in different treatments were non-significant at P=0.05% (Pr=0.24). Further, the Type I and Type III error sum of squares also had shown non-significance. However, based on the toxin levels in kernels in various treatments, the results were summarized as follows. Of different treatments, the aflatoxin content was least in seeds treated with *Pf7* (27.8 µg kg^{-1}). The efficacy of *Pf*7 was significantly superior over other *Pf* isolates. This was followed by seeds treated with *Pf*1 (754.7 µg kg^{-1}), *Pf*8 (1051.6 µg kg^{-1}) and *Pf*2(1151.9 µg kg^{-1}) with no significant differences among them (Table 4.7). For the remaining *Pf* isolates (*Pf*s3, 4, 5, 6, 9 and *Pf*10), the aflatoxin content ranged from 1218.6 to 1512.7 µg kg^{-1}. The performances of six *Pf* isolates were not significantly superior over control. Seeds in control have recorded highest aflatoxin content of 1521.1 µg kg^{-1} (Table 4.7). Overall, the PGPR isolate, *Pf*7 was highly effective in reducing kernel aflatoxin contamination in groundnut through IVSC assays.

PGPR is one of the commonly used antagonists in managing soilborne diseases of several crops (Vijay Krishna Kumar *et al*., 2012). Of different PGPR, *P. fluorescens* is widely used in controlling several plant pathogens (Abeysinghe, 2009). However limited success in combating aflatoxin problem in groundnut was achieved with PGPR. In our studies, the *Pf*7 isolate was found to be superior among other *P. fluorescens* isolates. Plant growth-promotion by PGPR is due to direct and indirect mechanisms (Ahemad and Kibret, 2014). Direct mechanisms involve either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels. Indirect mechanisms of plant growth-promotion are by decreasing the inhibitory effects of various pathogens on plant growth and development (Glick, 2012).

In our present study, the *Pf*7 isolate exhibited superior activity in inhibiting mycelial growth of *A. flavus*, its colonization on groundnut seeds and aflatoxin production. Antifungal activity of *P. fluorescens* was due to the production of siderophores (Jahanian *et al*., 2012; Tian *et al*., 2009); HCN (Michelsen and Stougaard, 2012); competition for space and nutrients and also by production of antibiotics (Mavrodi *et al*., 2007). A wide range of antifungal metabolites (antibiotics) are produced by *P. fluorescens* strains against plant pathogens. For example, certain strains of *P. fluorescens* produce 2, 4-diacetylphloroglucinol (2, 4-DAPG) that has antifungal and antihelminthic activity (Mavrodi *et al*., 2007). Similarly, reports on the production

of other antibiotics by *P. fluorescens* are also available (Yang *et al*., 2011). In our studies, an inhibition zone between test fungus (*A. flavus*) and *Pf*7 appeared in dual culture studies. Further, this zone of inhibition prevailed up to 15 days in Petri dishes. Based on this, we can attribute the inhibitory activity of *Pf*7 on *A. flavus* to the production of antifungal antibiotics.

In our studies, the *Pf*7 isolate also showed significant effect on groundnut seeds in reducing colonization of *A. flavus* in an IVSC assay. IVSC assays were earlier used in groundnut to assess the resistance among germplasm to *A. flavus* infection (Thakur *et al*., 2000). Inhibition of *A. flavus* in IVSC in the present study by *Pf*7 is attributed to both antibiosis and hyperparasitism. Reduction in aflatoxin content in *Pf*7 treated seeds in IVSC assay is also attributed to the fact that the bioagent occupied the groundnut spermoplane (seed surface) and thereby prevented the significant invasion of *A. flavus* and subsequent aflatoxin production. Overall, *Pf*7 was highly effective in reducing aflatoxin contamination.

The isolates of PGPR (*P. fluorescens*) were effective bioagents at field level, inhibiting the soil population of *A. flavus*, known to possess certain growth-promoting and specific pathogen inhibitory traits. Characterization of the identified PGPR isolate is therefore necessary to understand the exact trait possessed and its role in plant growth-promotion, pod yield enhancement besides reducing *A. flavus* populations in soil and also the aflatoxin contamination. In this context, it is essential to characterize *Pf*7, identify the potential antibiotic produced, and investigating its efficacy under greenhouse and field conditions against pre-harvest aflatoxin contamination.

4.4 Mode of action of PGPR (*Pseudomonas fluorescens*) against *Aspergillus flavus* using Scanning Electron Microscopy

The results of dual culture studies have shown that, among the ten isolates of *P. fluorescens*, *Pf*7 was highly effective in reducing the mycelial growth of *A. flavus*. Further, in IVSC assays, the *Pf*7 isolate inhibited the seed colonization and aflatoxin contamination by *A. flavus*. Hence, this potential isolate, *Pf*7 was selected to study its mode of action on the toxigenic *A. flavus* isolate (AFT5b). The scanning electron microscopic image of the *P. fluorescens* (*Pf*7) is depicted in Plate 4.17 (a&b).

4.4.1 Antibiosis of *Pseudomonas fluorescens* on *Aspergillus flavus*

SEM studies on the antibiosis of *Pf*7 isolate on toxigenic *A. flavus* (AFT5b) revealed that there was a definite inhibition of *A. flavus* hyphae. Clear deformation and coiling of hyphae near the zone of inhibition between test fungus and bioagent was noticed. Marked differences were noticed in the structure of mycelia, conidiophore and conidia near the zone of inhibition and in control plates (healthy). The mycelial structure of *A. flavus* in the control plates was tubular, turgid, and the hyphae were linearly elongated. Further, the mycelium grew densely in various directions, with numerous conidia on their surface (Plate 4.18 a&b). In contrast, the mycelium in the treatment (near the zone of inhibition) appeared thread like, wrinkled and flaccid. Further, shriveling of the hyphae with a clear reduction in the hyphal branching was observed (Plate 4.19 a&b). The conidiophores of *A. flavus* near the zone of inhibition in treatment were seen twisted and distorted (Plate 4.20 a&b). However, such coilings and distortions of conidiophores of healthy *A. flavus* in control plates were not seen. (Plate 4.21 a&b) Overall, the SEM studies revealed the effect of antibiosis of *P. fluorescens* (*Pf*7) on *A. flavus*.

4.4.2 Interaction between *Pseudomonas fluorescens* and mycelia of *Aspergillus flavus* (Hyperparasitism)

The studies on effect of hyperparasitism of *P. fluorescens* (*Pf*7) on the mycelia of *A. flavus* (AFT5b) revealed the presence of white slimy growth of bacterial cells on the *A. flavus* mycelia (Plate 4.22). Further, the bacterial cells were seen engulfing the conidia of *A. flavus* (Plate 4.23). Observations also revealed coiling and shriveling of conidiophores, deformities in conidia and conidiophores of *A. flavus* due to hyperparasitism (Plate 4.24). Besides, there was also structural disintegration of conidiophores of *A. flavus* due to hyperparasitism. Rarely, breakdown of conidiophores were also noticed due to hyperparasitism (Plate 4.25). On the other hand, conidiophore and conidia were normal, without any shrinking, shriveling and deformities in control plates (Plates 4.22 a&b).

Ultrastructural studies on interaction between bioagent and a test pathogen assumes significance in order to understand the nature of antagonism that is exhibited by bioagent. However, SEM studies between PGPR and the aflatoxin producing mold, *A. flavus* are scanty. Mostly, SEM studies focused on understanding the structure of these aflatoxigenic molds and their identification up to species level and

characterization (Rodriguez *et al*., 2007). Further, these SEM studies also are used to confirm the seed borne nature of *A. flavus* in groundnut. For example, in a study by Achar *et al*. (2009), it was observed that mycelium of *A. flavus* was seen established in the host tissues both intercellularly and intracellularly. Further, continuous branching of young hyphae was seen. Mycelial structures were also detected in xylem vessels of roots, confirming its systemic infection. Other SEM studies are focused on confirming the biological synthesis of nano-particles such as silver by *A. flavus* (Subha Rajam *et al*., 2013). Colonization of rhizosphere of crop plants by PGPR strains and thereby plant disease control is another area where SEM studies are used. For example, in a study on the effects of fluorescent *Pseudomonads* (CW2 isolate) in cucumber rhizosphere, SEM results indicated that the frequency of PGPR (CW2) colonization was higher in cucumber roots infested with fungi than in healthy roots (Salman *et al*., 2013). In another study by SEM, it was reported that the endophytic PGPR isolates such as *Bacillus subtilis* and *P. fluorescens* were able to colonize cortex tissue of groundnut roots. Further, the ultrastructural studies using transmission electron microscopy (TEM) of groundnut root tissues revealed that when these endophytic PGPR strains were inoculated onto root surfaces, the plant defense reactions were activated (Ziedan, 2006).

Only few reports are available on establishing the antifungal activity of microbes against *A. flavus*. For example, SEM studies have confirmed the antifungal activity of glycolipids produced by *Rhodococcus erythropolis* on *A. flavus* (Abdel-Megeed *et al*., 2011). Specific studies on the antagonism of PGPR strains on *A. flavus* are focused on post-harvest losses in agricultural commodities. For example, use of fungal cell wall-degrading enzymes produced by biological control agents against *A. flavus* is one area that is confirmed using SEM studies. In a study by Akocak *et al* (2015), the antagonistic effects of PGPR strains such as *P. fluorescens* (PB27) and *Bacillus cereus* (B1) against aflatoxigenic *A. flavus* were investigated. The SEM results indicated that, when the test fungus was exposed to chitinolytic PGPR strains, PB27 and B1, the test fungus, *A. flavus* has shown numerous ultrastructural morphological changes during spore germination and mycelial growth.

However, several reports have established the antagonism of PGPR strains on other plant pathogens. The antagonistic effects of *P. fluorescens* (CW2 isolate) in cucumber rhizosphere on important root pathogens such as *Pythium ultimum* and *Rhizoctonia solani* are well established. When cucumber roots were drenched with

CW2 strain, it caused irregular and abnormal fungal growth. The SEM studies further confirmed the swellings and shrinkages of *P. ultimum* and *R. solani* hyphae (Salman *et al*., 2013). In a study by Vijay Krishna Kumar *et al.* (2013), it was observed that the PGPR strain, *B. subtilis* MBI 600 (commercially available as Integral®) was highly antagonistic to soilborne pathogen of rice, *Rhizoctonia solani*, the causal agent of sheath blight disease. Ultrastructural studies on the interaction between sheath blight pathogen and MBI 600 indicated that the bioagent has exhibited both antibiosis and hyperparasitism. The bioagent caused loss of structural integrity, shriveling, abnormal coiling and lysis of *R. solani* hyphae due to antibiosis and hyperparasitism. Further, maceration and fragmentation of inner walls of sclerotia of sheath blight pathogen were observed when the sclerotia were treated with commercial formulation of MBI 600.

In our studies, the *Pf*7 isolate exhibited higher degree of antibiosis on *A. flavus*. Further, with one week of incubation after spraying of *Pf*7 on test fungus, the conidiophores were structurally disintigrated, coiled and shriveled with deformities. Further, break down of conidiophores due to engulfing by bacterial slimy growth was noticed, indicating hyperparasitism. Future investigations through SEM are necessitated to investigate the mode of action of *Pf*7 in inhibiting *A. flavus* entry on seeds. Further, SEM on spermoplane and rhizosphere colonization of *Pf*7, besides its endophytic nature in groundnut if any are to be investigated.

Chapter V

SUMMARY AND CONCLUSIONS

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Groundnut (*Arachis hypogaea* L.) is an important grain legume and is a rich source of protein for human and animals. Besides severe biotic stresses that affect groundnut production worldwide, aflatoxin contamination is a serious food safety issue affecting human and animal health besides trade. Groundnut crop is affected with aflatoxin contamination at both pre-and post-harvest stages thereby resulting in quality deterioration. These aflatoxins are a group of 20 secondary metabolites produced by *Aspergillus* section *Flavi* type of fungi (*Aspergillus flavus* and *A. parasiticus*) and have carcinogenic, hepatotoxic, teratogenic and immuno-suppressive effects. In view of identifying a sustainable option to manage the aflatoxin problem in groundnut, the present study was carried out at ICRISAT, Patancheru. The immediate goals of the study were to evaluate the *A. flavus* infection and aflatoxin contamination in pods at oil mills/traders' level in Andhra Pradesh and Telangana; detect toxigenic/atoxigenic *A. flavus* strains using cultural methods; identify the superior PGPR isolates against *A. flavus*; and determine the mode of action of PGPR against *A. flavus* using scanning electron microscopy.

Groundnut pod samples were collected from oil mills/traders' in Mahaboobnagar, Rangareddy, Nizamabad and Karimnagar districts of Telangana; Anantapur district of Andhra Pradesh (Tadipatri and Tadimarri mandals). Eight oil mills were selected altogether, and *A. flavus* infection was assessed by plating the kernels in Petri dishes containing Czapek's dox agar medium, followed by incubation for 7 days at 28º C. Aflatoxin content was estimated using indirect competitive ELISA. There was significant difference in kernel infection in the surveyed oil mills. Highest infection was observed in samples collected from Tadipatri mandal of Anantapur district (A-7) (96%), followed by samples collected from Nizamabad (T-3) (90.7%) and Rangareddy (T-2) (90%). Further, the kernel infection in samples from Nizamabad and Rangareddy were significantly superior over that of Mahaboobnagar (42%) and Karimnagar (54%). Overall, our results indicated higher infections at oil mills of Rangareddy and Nizamabad of Telangana. In AP, kernel infections were up to 29.3% (Tadimarri) and 59.3% (Tadipatri). Aflatoxin levels in Telangana were highest in pod samples from Rangareddy (1205.2 µg kg^{-1}), followed by Karimnagar (365.55 µg kg^{-1}). Samples from the oil mills of Nizamabad and Mahaboobnagar have recorded aflatoxins

to a tune of 4.9 and 11.5 µg kg^{-1}. In AP, oil mills of Tadipatri have recorded aflatoxin levels of 2.8 µg kg^{-1}, whereas Tadimarri mandal have recorded highest aflatoxin levels (6148.4 µg kg^{-1}). In Telangana, all the surveyed oil mills were categorized under risk zone for kernel infection (%) by *A. flavus.* Based on the aflatoxin contamination, oil mills sampled in Mahaboobnagar and Nizamabad districts were categorized as safe zone, while oil mills from Rangareddy and Karimnagar districts were categorized as sensitive areas. In AP, based on kernel infection, Tadipatri mandal was categorized as risk zone. Based on aflatoxin levels, oil mills from Tadipatri mandal was categorized as safe zone (Fig 4.6). In Tadimarri mandal, oil mills were categorized as safe zone based on *A. flavus* infection and as risk zone based on aflatoxin contamination.

A total of 24 *A. flavus* isolates were obtained from kernels of oil mills and these were differentiated into toxigenic and atoxigenic strains using cultural methods. Fluorescence test and yellow pigmentation on coconut agar medium (CAM); and ammonia vapour test on yeast extract sucrose (YES) medium associated with plum red colour development for toxigenic strains. The *A. flavus* isolates from oil mills of Mahaboobnagar and Rangareddy were identified as toxigenic, whereas atoxigenics (AFT3a; AFT3b; and AFT3c) were obtained from Nizamabad. Two (AFT4a &AFT4b) of six isolates from Karimnagar were atoxigenic. Further, the isolate, AFT5a from Karimnagar was categorized as false (+ve/-ve). Overall, there were nine toxigenic, five atoxigenic isolates in Telangana with one isolate being false +ve/-ve. In AP, all the six isolates from Tadipatri and three isolates from Tadimarri mandal are proved to be toxigenic under *in vitro* conditions.

Ten *Pseudomonas fluorescens* (PGPR) (*Pf*1 to *Pf*10) isolates were obtained from groundnut rhizospheric soils of ICRISAT, Patancheru and screened against toxigenic *A. flavus* isolate (AFT5b) using dual culture and *in vitro* seed colonization (IVSC) assays using standard protocols. All the *Pf* isolates were effective in dual culture assays and highest inhibition of *A. flavus* was obtained with *Pf*7 (54.8%), followed by *Pf*2 (48.7%) and *Pf*6 (48.2%) with no significant differences. Next best inhibitions of test fungus were obtained with *Pf*4 (46.2%) and *Pf*9 (44.6%) The inhibitions of *A. flavus* by *Pf*1, *Pf*10 and *Pf*3 were about 31.7%, 32.3% and 33.3% respectively. Least inhibition of *A. flavus* was obtained with *Pf*5 (28.2%). In IVSC experiments, the *Pf*7 has resulted in least colonization severity of *A. flavus* (6.2 Wilcoxon score). This was followed by *Pf*2 and *Pf*9 (9.3 Wilcoxon score each) and *Pf*6 (13.5 Wilcoxon score). Overall, the *Pf* isolates, *Pf*7, *Pf*2, *Pf*9 and *Pf*6 were effective in

reducing *A. flavus* infection on groundnut seeds (Table 4.6). Results on aflatoxin inhibition by *Pf* isolates in IVSC studies indicated that the toxin content was least in seeds treated with *Pf7* (27.8 µg kg^{-1}) and was significantly superior over other *Pf* isolates. This was followed by seeds treated with *Pf*1 (754.7 µg kg^{-1}), *Pf*8 (1051.6 µg kg^{-1}) and *Pf*2 (1151.9 µg kg^{-1}) with no significant differences among them (Table 4.7). Seeds in control have recorded highest aflatoxin content of 1521.1 µg kg^{-1} (Table 4.7). Overall, the PGPR isolate, *Pf*7 was highly effective in reducing kernel aflatoxin contamination in groundnut through IVSC assays.

Of the ten *Pf* isolates, *Pf*7 was highly effective in reducing the mycelial growth of *A. flavus* and in inhibiting the seed colonization and aflatoxin contamination by *A. flavus* in IVSC studies. The *Pf*7 isolate was further selected to study its mode of action on the toxigenic *A. flavus* isolate (AFT5b) using scanning electron microscopy (SEM). The effect of antibiosis and hyperparasitism of *Pf*7 on the growth of *A. flavus* was determined using standard protocols. Deformation and coiling of hyphae of *A. flavus* near the zone of inhibition obtained in dual culture assays was noticed. Further, the mycelium appeared thread like, wrinkled and flaccid. And the hyphae were seen shriveled, with a clear reduction in the hyphal branching. The conidiophores of *A. flavus* near the zone of inhibition in treatment were seen twisted and distorted. In hyperparasitic reaction, a white slimy growth of bacterial cells on the *A. flavus* mycelia was observed. Further, the bacterial cells were seen engulfing the conidia of *A. flavus* (Plate 4.27). Observations also revealed that coiling, shriveling and deformities in conidiophores of *A. flavus* due to hyperparasitism.

CONCLUSIONS

- Groundnut pod samples at specified oil mills of Telangana and Andhra Pradesh have *A. flavus* infections and aflatoxin contamination and can pose potential health hazards.
- Risk and sensitive areas with respect to kernel *A. flavus* infection and aflatoxin contamination at groundnut oil mills identified.
- Precise detection and differentiation of toxigenic and atoxigenic *A. flavus* strains was verified.
- Scope of using polyphasic detection in precise detection of toxigenic strains emphasized in view of the false positives/false negatives in detection

- Native rhizospheric isolates of *P. fluorescens* were antagonistic to *A. flavus* (based on *in vitro* dual culture studies)
- The PGPR isolate, *Pf*7 was highly effective in reducing mycelial growth of toxigenic *A. flavus*
- The *Pf*7 isolate was effective in inhibiting the colonization severity of *A. flavus* on groundnut seeds
- The *Pf*7 isolate was effective in reducing aflatoxin contamination due to *A. flavus* infection when applied to groundnut seeds
- Based on ultra-structural studies through SEM, the *Pf*7 strain was found to exhibit antibiosis and hyperparasitism kinds of antagonism on *A. flavus*.

FUTURE LINES OF RESEARCH

- Characterization of *Pf*7 (*P. fluorescens*) isolate for plant growth-promoting and pathogen inhibiting traits in groundnut
- Specific antibiotic producing ability of *Pf*7 isolate may be verified using genomic approaches with specific primers
- Role of *Pf*7 in inducing systemic resistance (ISR) in groundnut may be established.
- Greenhouse and field evaluation of *Pf*7 in reducing kernel infection and aflatoxin contamination may be tested
- Mode of action of *Pf*7 in preventing the mode of entry/reducing *A. flavus* invasion need to be determined through SEM
- Rhizospheric competency of *Pf*7 isolate need to be verified through studies on spermoplane and rhizospheric colonization capacity

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