MANAGEMENT OF AFLATOXIN CONTAMINATION IN GROUNDNUT THROUGH BIOLOGICAL CONTROL, HOST PLANT RESISTANCE AND BOTANICALS

By

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CERTIFICATE

This is to certify that the thesis entitled "**MANAGEMENT OF AFLATOXIN CONTAMINATION IN GROUNDNUT THROUGH BIOLOGICAL CONTROL, HOST PLANT RESISTANCE AND BOTANICALS**" submitted in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** of the Acharya N.G. Ranga Agricultural University, Hyderabad is a record of the bonafide research work carried out by **Mr. HARED ABDULLAHI NUR** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma or has been published. The published part has been fully acknowledged. All the assistance and help received during the course of investigation have been duly acknowledged by the author of the thesis.

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DECLARATION

I, **Mr. HARED ABDULLAHI NUR**, hereby declare that the thesis entitled "**MANAGEMENT OF AFLATOXIN CONTAMINATION IN GROUNDNUT THROUGH BIOLOGICAL CONTROL, HOST PLANT RESISTANCE AND BOTANICALS**" submitted to Acharya N.G. Ranga Agricultural University for the degree of **DOCTOR OF PHILOSOPHY,** is a result of the original research work done by me. I also declare that the thesis or part thereof has not been published earlier elsewhere in any manner.

Date: (**HARED ABDULLAHI NUR**)

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LIST OF ABBREVIATIONS

µg	:	Micro gram
µl	:	Micro liters
AF	:	*Aspergillus flavus*
AFB1	:	Aflatoxin B1
AFPA	:	*Aspergillus flavus* and *Parasiticus* agar
BCAs	:	Biocontrol agents
BSA	:	Bovine serum albumin
CDA	:	Czapex dox agar
Cfu g^{-1}	:	Colony forming units per gram of soil
cm	:	Centimeter
CMC	:	Carboxy methyl cellulose
DAS	:	Days after sowing
ELISA	:	Enzyme linked immosorbent assay
FYM	:	Farm yard manure
g	:	Gram
GCY	:	Glucose casamino acid yeast extract
ICRISAT	:	International crops research institute for the semi arid tropics
IDM	:	Integrated disease management
Kg	:	Kilogram
LBA	:	Luria betarni agar
LBB	:	Luria betarni broth
MEA	:	Malt extract agar
mg	:	Milligrams
PBS	:	phosphate buffered saline
PDA	:	Potato dextrose agar
PDB	:	Potato dextrose broth
PGPR	:	Plant growth promoting rhizobacterium
TSM	:	*Trichoderma* specific medium
UV	:	Ultraviolet

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ABSTRACT

Aflatoxins, produced by *Aspergillus flavus* Link, Fr. and *A. parasiticus* Speare, are toxic, carcinogenic and immunosuppressive. These contaminate a large number of agricultural commodities, including groundnut, and pose serious threat to human and animal health.

In the present study, efforts were made to find out ecofriendly management approach of preharvest aflatoxin contamination in groundnut.

Forty-nine isolates of *Trichoderma* and seventy-seven bacterial isolates were isolated from groundnut rhizosphere soils. These were screened in the laboratory and most effective five *Trichoderma* isolates and six bacterial isolates were selected, and evaluated under greenhouse and field conditions. Two *Trichoderma* isolates (*Trichoderma. viride* Pers, ex S.F. Gray (T47) and *T. harzianum* Rifai (T23)) and two bacterial isolates (*Pseudomonas cepacia* Burkholder, Pelleroni and Holmes and *Pseudomonas fluorescens* (Trevisan) Migula were found to be most effective in reducing

A. flavus population in the groundnut rhizosphere, preharvest seed infection and aflatoxin content in the kernels throughout the study.

Four neem commercial formulations tested, significantly inhibited the *A. flavus* mycelial growth *in vitro*. Two selected neem formulations reduced the *A. flavus* population though did not reduce seed infection and aflatoxin content significantly under field conditions.

Forty-five groundnut genotypes from different sources were evaluated for resistance to seed infection and *in vitro* seed colonization by *A. flavus*. ICGV 91114 was identified as resistant to seed infection and seed colonization by *A. flavus*. One-hundred eighty-four groundnut core collection from ICRISAT gene bank were also tested for resistance to *in vitro* seed colonization by *A. flavus*. Among these, 49 genotypes were resistant to *in vitro* seed colonization by *A. flavus* recording constantly colonization severity of less than 2 on 1-4 scale. Eighteen genotypes were found resistant to aflatoxin production and their resistance was comparable to that of resistant genotypes like U-4-7-5 and VRR 245.

An IDM field experiment was conducted at ICRISAT, Patancheru A.P. and at Anantapur using most effective biocontrol agents (*Trichoderma viride* (T47) and *Pseudomonas cepacia*), resistant genotype (ICGV 91114) and selected neem commercial formulation (Sasyaneem). Resistant cultivar + *T. viride* (T47) + *P. cepacia* + Sasyaneem was the most effective treatment in reducing, *A. flavus* population in the groundnut rhizosphere (46.52%), seed infection (35.30%) and aflatoxin content in the kernels (34.75%).

Plant growth promoting potential of 11 biocontrol agents was tested under greenhouse conditions. Maximum increase in root length (32%), root weight (59%), shoot length (18%) and shoot weight (34%) was obtained from different biocontrol agents.

CHAPTER I

INTRODUCTION

The cultivated groundnut or peanut (*Arachis hypogaea* L.), an annual oilseed legume native to South America, is grown in diverse environments in six continents between latitudes 40°N and 40°S (McDonald *et al.* 1998). It was introduced into Africa where, along with bananas, it forms a large part of the diet of the peoples of East Central Africa. From Africa the peanut was taken to India, China and United States of America during colonial times (Hartmann *et al.* 1981).

Groundnut is an annual legume grown primarily for high quality edible oil (36 to 54% on dry matter basis) and easily digestible protein (12 to 36 %) in its seeds. It is cultivated in 107 countries in tropical, sub-tropical and warm temperate regions of the world (Upadhyaya *et al.* 2003). The crop is grown on an area of 25.50 m ha world- wide with an estimated total production of 35.10 m tons in shell and an average productivity of 1.4t ha^{-1} (FAO, 2002).

India ranks first in area under groundnut production (7.30 m ha) and second in production (6.20 m tons) followed by China. In Andhra Padesh the area under groundnut cultivation is 1.601 m ha and production is 1.622 m tons. Anantapur district contributes 0.8 m ha of the area and 0.9 m tons of production (Damodaram and Hegde, 2002).

In several countries of Asia, which accounts for 57.4 per cent of world area and 66.7 per cent of production and Africa, which accounts for

37.9 per cent area and 24.5 per cent of production, groundnut is an important cash crop (Upadhyaya *et al.*, 2003). Groundnut is consumed as whole seed or processed as traditional dishes or snack foods. The oil may be extracted and used for cooking, and the residual cake used in production of food or, more commonly, in animal feeds. The haulms are used for feeding livestock (McDonald *et al.*, 1998).

The groundnut plant is unusual because flowers are formed and fertilized above the soil and subsequent fruit development takes place in the soil. The subterranean pod is, therefore, in close contact with soil microorganisms for an extended period, and many species of soil fungi, including *Aspergillus flavus* have been isolated from healthy and damaged seeds (Bilgrami and Choudhary, 1998). *A. flavus* and *A. parasiticus* Spear produce aflatoxins in groundnut seeds and other products.

Aflatoxins are considered a major public health problem worldwide, especially in developing countries where facilities for long term storage of food and food products are often inadequate, and high temperature and high humidity encourage the growth of molds.

Aflatoxins are toxic and carcinogenic to man and animals. Some groundnut producing countries are loosing export earnings because they are not able to achieve the permissible limits of aflatoxin set by importing countries (Gowda and Ramakrishna, 1997).

Aflatoxin contamination is a major problem in many groundnut-producing countries. Thus the presence of aflatoxins in groundnut products

has attracted the attention of the research investigators all over the world owing to a serious health problem caused by these mycotoxins.

Aflatoxin formation in certain products before or after crop harvest cannot be prevented in any known practical way, but it can sometimes be reduced by appropriate management practices (Bhatnagar *et al.*, 1998).

Comprehensive efforts on the part of academia, governments, and food and feed industry to identify and implement control and management strategies to alleviate the mycotoxin problems are highly desirable (Mehan and Jand, 2002).

One of the possible means of reducing aflatoxin contamination of groundnut is the use of cultivars resistant to seed invasion by aflatoxin producing fungi or to aflatoxin production. These cultivars will be of great value to the farmers in both developed and developing countries as there is no cost input (Upadhyay *et al.*, 2002). In the absence of acceptable levels of host plant resistance, use of biocontrol agents could be a promising alternative for the management of aflatoxin contamination (Desai *et al.*, 2000).

An integrated approach through combining chemicals, cultural, and biological management options could be a viable option for reducing preharvest contamination of seeds in groundnut production systems. The efforts to subdue preharvest aflatoxin problem should be based on the principles of greater ecological sustainability in the long run keeping in view the minimal use of pesticides (Vijay Krishna Kumar *et al.*, 2002).

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The solution of this complex problem lies in integrated management, including biocontrol (Thakur and Rao, 2001) and host plant resistance (Rao *et al.*, 2003).

This study was carried out to develop an eco-friendly management practice including biological control, host plant resistance and botanicals for management of preharvest aflatoxin contamination in groundnut. The objectives of the study were:

1. To isolate and characterize *Trichoderma spp.* and bacterial strains from groundnut rhizosphere soils.
2. To evaluate of *Trichoderma spp.* and bacterial strains for *in vitro* antagonistic activity against *A. flavus.*
3. To enhance the biocontrol efficacy of *Trichoderma spp.* by selection and irradiation.
4. To test the efficacy of botanicals against *A. flavus in vitro* and *in vivo.*
5. To identify germplasm accessions, advance breeding lines and varieties for resistance to *in vitro* seed colonization and seed infection by *A.flavus.*
6. To evaluate the combined effects of host plant resistance, biocontrol agents and botanicals as an integrated management practice to reduce aflatoxin contamination in groundnut.
7. To evaluate plant growth promoting potential of the biocontrol agents on groundnut in greenhouse.

CHAPTER II

REVIEW OF LITERATURE

2.1 AFLATOXINS : HISTORY AND BACKGROUND

Fungal-induced food toxicity has existed since early civilization. But mycotoxins and mycotoxicoses were relatively obscure and neglected in the scientific literature until the discovery of aflatoxins as the causative agent of Turkey-x disease in 1960 in England. The aflatoxin story began with the report of an outbreak of disease in turkey poults in England. Since the etiology of the disease was obscure it was called Turkey X disease (Blount, 1961). Affected birds lost appetite, became lethargic, and died within 7 days after the onset of symptoms. Livers of diseased turkeys were severely damaged. A similar disease of ducklings and young pheasants was reported from England (Asplin and Carnaghan, 1961). A common factor in all disease outbreaks was the inclusion of Brazilian groundnut meal in the affected birds' diets (Blount, 1961, Asplin and Carnaghan, 1961). A similar disease of ducklings was reported from Kenya. The ducklings' feed ration contained a groundnut meal produced in Eastern Africa, indicating that the problem was not solely associated with Brazialian groundnut meal (Allcroft and Carnaghan, 1962). Later in 1960, outbreaks of disease occurred in pigs and calves, apparently caused

from England (Asplin and Carnaghan, 1961). A common factor in all disease outbreaks was the inclusion of Brazilian groundnut meal in the affected birds' diets (Blount, 1961, Asplin and Carnaghan, 1961). A similar disease of ducklings was reported from Kenya. The ducklings' feed ration contained a groundnut meal produced in Eastern Africa, indicating that the problem was not solely associated with Brazialian groundnut meal (Allcroft and Carnaghan, 1962). Later in 1960, outbreaks of disease occurred in pigs and calves, apparently caused by an unknown toxic factor in Brazilian groundnut meal contained in animal rations (Loosmore and Harding, 1961).

Sargeant *et al.* (1961) demonstrated that an isolate of the common mold *Aspergillus flavus* Link ex Fries was in fact the responsible agent. The disease was caused by toxins produced by strains of the fungus *A. flavus* when growing on the meal, and hence these toxins, in view of their origin, were named aflatoxins. There were some reports of aflatoxicosis in farm animals from other European countries (Allcroft and Carnaghan, 1962).

Several outbreaks of aflatoxicoses in poultry have been reported from India (Char *et al.*, 1982). In 1962, a heavy mortality occurred among ducklings in Tamil Nadu State (Bhat *et al.*, 1978). The feed used for the ducklings contained groundnut meal with a total aflatoxin content of 6200 $\mu g\ kg^{-1}$.

The aflatoxins (AF) are a group of difuranocoumarin compounds produced as secondary metabolites by the mold *A. flavus* and *A. parasiticus*. The major AF types found in plant food products are aflatoxin B1, B2, G1, G2, M1 and M2. Mycotoxin contamination in groundnut can occur in the field

during pre-harvest, harvest and during postharvest handling (Nahdi, 1997). In many countries they are able to control the entry of contaminated groundnut in food chain by following strict regulatory programs. The maximum permissible limit of these mycotoxins varies from 0 – 100 $\mu g\ kg^{-1}$ depending on the country and food stuff and also whether the commodity is for human or animal consumption (Nahdi, 2000).

2.2 EFFECTS OF AFLATOXINS

Losses due to mycotoxins can be significant, depending on the amount of mycotoxin produced (Jones, 1979). Losses are expressed in any one or a combination of the following (Agarwal and Sinclair, 1997).

2.2. 1. Aflatoxicosis in Animals and Humans

Mycotoxin is derived from the Greek word "mykes," meaning fungus and the Latin word "toxicum" meaning poison (Forgaes and Carll, 1962). Mycotoxins are secondary fungal metabolites that cause pathological or undesirable physiological responses in human and other animals. Mycotoxicoses are diseases caused by the ingestion of foods or feeds contaminated by mycotoxins (Goto, 1990). Aflatoxins have received greater attention than other mycotoxins because of their established carcinogenic effect in various animals and their acute toxicological effects in humans (Mehan, 2002)

The term aflatoxin is derived from *Aspergillus flavus*, A for *Aspergillus*, *fla* for *flavus*, and toxin as a descriptor of the substance.

Mycotoxins result in both acute and chronic toxicities. Acute effects result in rapid, readily noticeable fatal diseases. Some mycotoxins

are acutely toxic to the liver, while others attack the kidneys, central nervous system, or circulatory system. For example aflatoxin B1 is a potent liver toxin, and less than 20ug has a lethal effect on ducklings. The chronic effect results from the carcinogenicity of aflatoxin B1. A diet containing only 0.1 $\mu g/kg^{-1}$ aflatoxin B1 resulted in liver tumors in rainbow trout (Coker, 1984).

Mycotoxins produce toxic reactions in animals by contact or inhalation. Intake of low mycotoxin concentrations reduce mental alertness, physical abilities, and feed intake. Intake of moderate concentrations reduced the activity of the immune system and increased susceptibility to other diseases (Agarwal and Sinclair, 1997).

Physiological damage by mycotoxins varies with individual species, their age, state of health, degree of exposure, and other factors (Agarwal and Sinclair, 1997).

Occurrence of mycotoxicoses is governed by the quantity of toxic food ingested, time lag in symptom development, toxic concentration in the food, sensitivity and age of the individual, sex, nutritional status, season of harvesting, weather and altitude of the crop production area (Joffe *et al*, 1978).

Mycotoxicosis has four clinical symptoms: (a) it is non communicable (b) drugs and antibiotics are ineffective (c) outbreaks are

associated with a specific food or feed (d) foodstuffs show active fungal growth upon examination (Detroy *et al.*, 1971).

Animal health: Aflatoxicosis caused by the presence of aflatoxins in feeds is a serious disease syndrome in poultry. Several species of molds are involved in spoilage of both raw and processed poultry feeds. The commonly occurring storage molds – *Aspergillus parasiticus* and *A. flavus* can invade maize, groundnut and fish meal-based feeds and consequently produce highly toxic and cancer–causing aflatoxins (Narsapur, 2002). The adverse effects of different mycotoxins vary widely due to their diverse chemical structure. High concentrations of mycotoxins produced immediate overt mycotoxicoses while low concentrations of mycotoxins are predominantly associated with immunosupressive activity in domestic livestock and poultry flocks where active immunization schedules are an important part of the animal health programme. Aflatoxin consumption has been found associated with increased susceptibility to coccidiosis and faciolosis in domestic animals. Immunosuppression due to mycotoxins often causes vaccine failures in livestock and poultry (Juyal, 2002).

Aflatoxins are capable of producing liver cancer in the most sensitive animal species when fed at a concentration of only one part per billion (ppb). Ducklings, chicks, calves, guinea pigs and pigs trout are very sensitive to the hepatotoxin effects of aflatoxin B1, whereas rat,

goat, sheep and mouse are relatively less sensitive to the acute effects of this toxin (Mor and Singh, 1998).

Animals which consume sub-lethal quantities of aflatoxin for several days or weeks develop a subacute toxicity syndrome which commonly includes moderate to severe liver damage. Lethal dietary aflatoxin levels in domestic animals range from 0.3mg kg-1 in the ducklings to 2.2mg kg-1 in calves (Allcroft, 1965). Similar results have been obtained in laboratory animals, including the guinea-pig and rat, which develop toxicity symptoms at 0.7 and 3-4 mg kg^{-1}, respectively (Butler, 1964b, 1966a).

Early signs of aflatoxicosis in livestock are reduction in feed intake, and weight loss, this is often rapidly followed by death. The most important pathological effect is liver damage.

Human health: One of the most impressive, aflatoxin-related episodes reported in the scientific literature is an acute poisoning in an area in India in 1974 involving some 400 people and resulting in 106 deaths (Van Rensburg, 1977). The circumstances were typical of those highly conducive to excessive mycotoxin exposure. They consumed aflatoxin contaminated corn kernels.

Epidemiological studies have been carried out to see if aflatoxin ingestion might be a factor in the high incidence of liver cancer in some

areas of Africa, Asia, and the USA. Several studies in Indonesia, Kenya, Mozambique, South Africa, Swaziland, Thailand, and Uganda found a positive correlation between aflatoxin ingestion by humans and liver cancer incidence (Husaini *et al.*, 1974; Peers *et al.*, 1976; Van Rensburg *et al.*, 1974; Keen and Martin, 1971; Shank *et al.*, 1972).

Some researchers have highlighted the strong relationship between hepatitis B virus infection and incidence of liver cancer (Beasley *et al.*, 1981; Beasley, 1982). All epidemiological studies of aflatoxin and liver cancer conducted in Africa and Asia involved populations subjected to hepatitis B virus infection.

Workers engaged in harvesting, shelling, bagging, storage, marketing, and transport of groundnuts may be exposed to aflatoxin through the respiratory route. A chemical engineer involving in sterilizing Brazilian groundnut meal contaminated by *Aspergillus flavus* developed alveolar cell carcinoma and died within a year. Two reports of aflatoxicosis in humans involve consumption of maize heavily contaminated with aflatoxins (Krishnamachari *et al.*, 1975a, b; Nagindu *et al.*, 1982). In one of the incidents, in India, there were 272 hospital admissions with clinical symptoms of aflatoxicosis and a 27% mortality. In the second incident, in Kenya, there were 20 hospital admissions with a 60% death. The presence of aflatoxin in dairy milk is of great public health significance because they are potent toxins, carcinogens and

mutagens. These toxins cause serious health hazards to humans including acute toxicity, liver cancer, and immuno-suppression. Infants and children are highly susceptible to the toxic effects of aflatoxins. Dairy milk is a major component of the diet of children and there are reports that children who consumed aflatoxin contaminated milk developed Indian Childhood Cirrhosis (an inflammation of liver). It should also be noted that women consuming aflatoxin contaminated food can have aflatoxin in their breast milk; a direct threat to the health of the infants. The amount of aflatoxin M_1 excreted in milk is in direct proportion to the in take of aflatoxin B_1. Quantitatively, about 1.5 per cent of the aflatoxin B_1 ingested by a cow appears in the milk as M1 (Jand and Dhand, 2002).

Amla *et al.* (1970, 1971) presented circumstantial evidence to indicate that children exposed to aflatoxin through breast milk and dietary items such as unrefined groundnut oil and parboiled rice may develop cirrhosis. They detected aflatoxin B_1 in 7% of urine samples from cirrhotic children.

2.2.2 Effect on Trade

The application of different permissible levels in different countries (e.g., European Union Countries, the USA, and developing countries) has tremendous impact on international trade in various agricultural commodities such as groundnut, maize, cashewnuts, pistachios, copra, and chillies. The harmful effects of mycotoxins on

animal productivity and on human and animal health justify stricter control of mycotoxin levels in foods and feeds (Mehan and Jand, 2002).

The significance of mycotoxins in international trade is increasingly being recognized by both developed and developing countries. The export of agricultural commodities, such as copra, cottonseed, peanuts and pistachio nuts or their derivatives have been affected. Often, and especially in developing countries, the best quality of these commodities, which are free from mycotoxins, are exported, while the substandard products are distributed and sold within the country. This practice has the potential of unfavorable consequences in either the health of the local population or productivity of the animals fed with contaminated or substandard feed (Dawson, 1991). Thai maize has been noted for its bright yellow color and high protein content. However, samples have been found to contain unacceptably high aflatoxin levels and are therefore discounted or rejected by foreign buyers (Cutler, 1991). Hence, aflatoxin contamination has posed serious problems in commerce and international trade because of stringent quality standards on aflatoxin contamination by importing countries (Bhat, 1988).

Mycotoxin concentration may exceed government standards for trade and be unfit for human consumption.

Table 1: The table below shows the current European Union legislative limits (EU Commission Regulations 446/2001, 257/2002 and 472/2002).

Product Type	Aflatoxins : Maximum Admissible Levels (μg/kg)	
	B1	Total Aflatoxins (B1+B2+G1+G2)
Groundnuts, nuts, dried fruit and processed products for direct human consumption or as a food ingredient.	2	4
Groundnuts to be subjected to sorting, or other physical treatment, before human consumption or as a food ingredient.	8	15
Nuts and dried fruit to be subjected to sorting, or other physical treatment, before human consumption or as a food ingredient.	5	10
Cereals and processed products thereof for direct human consumption or as a food ingredient.	2	4
Chilies, chili powder, cayenne pepper, paprika, white & black pepper, nutmeg, ginger, turmeric.	5	10

2.2.5 Extra Cost for Seed Analysis

The mycotoxin problem in seeds requires additional cost for seed analysis to determine mycotoxin levels prior to marketing. If seeds are infested by fungi and contaminated by mycotoxins, they can be dried so that mycotoxin production is inhibited. Mycotoxin-contaminated seeds require detoxification prior to release for marketing.

2.3 THE CAUSAL ORGANISM

The genus *Aspergillus* dates from Micheli's Nova Plantarum Genera of 1729, but it was not until the middle of the nineteenth century that these fungi began to be recognized as active agents in decay

processes, as causes of human and animal diseases, and as fermenting agents capable of producing valuable metabolic products. Micheli was the fist to distinguish stalks and spore heads. He noted that the spore chains radiated from a central structure to produce a pattern that suggested the aspergillium, hence he applied the name *Aspergillus* to molds he observed (Raper and Fennell, 1965).

Sargeant *et al.* (1961) first reported toxin production by *A. flavus* link ex fres grown on sterile peanut and in Czapek's solution agar. Both fungal differentiation and metabolite formation occur after the period of rapid vegetative growth has ceased (Cotty, 1988).

A. flavus often produces aflatoxins B1 and B2 as well as cyclopiazonic acid, where as *A. parasiticus* typically produces, in addition to the B aflatoxins, aflatoxins G1 and G2 but not cyclopiazonic acid (Pitt, 1988). *A. tamari* Kita, another species that invades peanut seeds, does not produce aflatoxins, though isolates usually produce cyclopiazonic acid (Dorner, 1983). In general AFB1 was the toxin produced in the highest quantity by the aflatoxicogenic strains (Magnoli, 1998).

On the basis of physiological, morphological, and genetic criteria, *A. flavus* can be divided into two strains, S and L (Bayman and Cotty, 1993). Isolates of the S strain produce numerous small sclerotia (<400 μm in diameter) and fewer conidia than L strains. Strain S isolates

produce, on an average, more aflatoxin than L strain isolates both in culture and within developing cotton seed (Cotty, 1989). Many L strain isolates produce little or no aflatoxins. In Arizona, where aflatoxin contamination of cottonseed is severe, the S strain is often dominant. Although several characteristics of the S strain suggest soil adaptation, little data on the divergent ecologies of the S and L strains are available (Garber and Cotty, 1997).

2.4 PREHARVEST AFLATOXIN CONTAMINATION

Aflatoxin research was initially focused on post harvest mycology, pathology, and animal toxicology. However, in the mid 1970s, aflatoxin was discovered before harvest in the U.S. and Indian corn (Bilgrami *et al.*, 1978; Anderson *et al.*, 1975). In the present years aflatoxin has become a major problem because of its extensive preharvest contamination of maize, peanut, cottonseed, tree nuts, mustard, linseed, and sorghum (Sinha and Bhatnagar, 1998).

In groundnut, field contamination is considered to be a significant source of inoculum while storage contamination is of comparatively lesser magnitude (Cole *et al.*, 1989). Preharvest invasion of groundnut seeds by *A. flavus* was earlier thought to depend on physical and/ biological damage to pods by parasitic fungi and insects (McDonald and Harkness, 1964; Sellschop, 1965). It is now established that groundnut without obvious damage can also be invaded by *A. flavus* and

A. parasiticus and contaminated with aflatoxin in field before harvest. Sometimes the mycelium of the fungus remains viable when the seed is sown and may contribute to either seed rot or seedling disease (aflarot). In aflarot disease, the radicle does not develop in secondary root.

There are two types of preharvest aflatoxin contamination in groundnut. Groundnuts mechanically or biologically damaged in the soil are predisposed to invasion by fungi. The saprophytic fungus will live predominantly on dead or dying tissue, therefore it may infect the maturing kernel if the pod is damaged while still in the ground, especially when the growth rate of the plant is in decline during the later growing phase. Another type of preharvest invasion can occur with no obvious kernel damage (Smartt, 1994).

In a standing groundnut crop, *A. flavus* invasion can occur in soil during pod development and maturation. However, the exact mode of infection of groundnut fruit has not been fully demonstrated. Some researchers (Lindsew, 1970; Wells *et al.*, 1972; Styer *et al.*, 1983) have suggested that *A.flavus* may invade the flowers, penetrate down the pegs, and subsequently establish in the developing seeds. Groundnut flowers inoculated with washed conidia of *A. flavus* were readily colonized by the fungus (Wells *et al.*, 1972*)*. Some investigations have been carried out to assess the possibilities of infection via pegs. Aerial pegs of groundnut plants, grown under gnotobiotic conditions after flowers inoculation with

conidia of *A. flavus*, were readily colonized by the fungus without apparent damage to the developing embryo (Wells *et al.*, 1972). However, further studies in Australia (Pitt, 1989) have failed to establish a definite link between flower and peg invasion and between peg and fruit invasion.

Cole *et al.* (1986) have suggested that preharvest *A. flavus* infection and subsequent aflatoxin contamination originate mainly from the soil. Direct invasion of the developing groundnut fruit by *A. flavus* in soil in the geocarposphere has generally been assumed as likely route for eventual contamination of the kernel with aflatoxin after penetration of the pod wall and tastae and infection of the kernels by the fungus. However, *A. flavus* may be present in the developing ovary of peanuts at the tip of the peg even before it is pushed into the soil.

Dorner *et al.* (1989) also showed that kernel moisture loss was accelerated at 29°C, compared with 25°C, and that this higher soil temperature promoted fungal growth and aflatoxin production once phytoalexin producing capacity was lost. In support of this, Diener and Davis (1968) reported that old, over mature groundnut tissue have considerably less KMC and increased susceptibility to *A. flavus*. Under stress conditions immature kernels become contaminated more easily and quickly than the larger, mature kernels that have passed beyond a certain developmental stage. However, under normal conditions the phytoalexin-producing capacities of the mature kernels have been shown to be lower

than those of immature kernels. This suggests that they possess some additional form of resistance, a theory which is supported by their resistance to preharvest contamination, even under conditions of severe and prolonged drought.

In agricultural fields, during hot, dry conditions *A. flavus* populations increase on crop debris, on senescent or dormant tissues and on damaged or weakened crops (Stephenson and Russel, 1974). Crop grown in these fields become associated with large populations of *A. flavus* that may remain associated with the crop throughout crop maturation, harvest and storage. Crop components damaged prior to maturity are susceptible to toxin contamination (Cotty *et* al., 1994).

Several researchers studied fungal infection of groundnut fruits from the early stages of fruit development until harvest. Garren (1966) reported a well-defined endogeocarpic mycoflora with in the fruit as it developed in the soil.

2.5 FACTORS PREDISPOSING A. FLAVUS INFECTION AND AFLATOXIN CONTAMINATION.

Some preconditions are essential for preharvest *A. flavus* invasion and Aflatoxin production. The foremost are the (1) presence of toxigenic strain of the fungus, (2) susceptible host and (3) favorable agroclimatic conditions. Drought and temperature stress at critical

stages in the life-cycle of the crop is one of the dominant factors. Agronomic practices and insects play major role in *A. flavus* infection and aflatoxin contamination in field conditions (Diener , 1989).

2.5.1 Presence of Toxigenic Strain of *A. flavus*

Isolates of *A. flavus* and *A. parasiticus* vary widely in the amount of aflatoxin produced on groundnut, and in their capacity to produce different aflatoxins. Some isolates produce no aflatoxins (Diener and Davis, 1966). Therefore, the presence of aflatoxigenic strain of *A. flavus* and/or *A. parasiticus* in the soil is necessary for preharvest aflatoxin contamination in groundnut.

2.5.2 Host Species

Seeds invasion of groundnut occurs under favorable conditions by direct or indirect pod penetration. However, seeds of cultivars whose pods are penetrated by *A. flavus* may escape with difficulty. Resistant groundnut pod tissues contain compact sclerotized cells with a high lignin content, and resistant seed coats contain tightly packed cells with an outer layer of wax platelets (Pettit *et al.*, 1978). The hilum of resistant cultivars is small and closed; in susceptible ones it is large and open, thus allowing for a higher level of infection (Rodricks, 1976). Since the resistance is due to anatomical features, any damage to the seed coat can render a resistant type susceptible. All cultivars, both resistant and susceptible, should be stored under humidity and temperature conditions unfavorable

for fungal deterioration, because under conditions favorable for fungal development, resistant genotypes may also become contaminated with aflatoxin (Wilson *et al.*, 1977).

Seed surface lipids (SSL) play a key role in supporting aflatoxin formation in oily and starchy seeds following infection by *A. parasiticus*. Fungal growth and aflatoxin production occur on oily seeds when SSL levels are greater than 0.15% of the total oil content of seeds, with a ratio between triglycerides and free-fatty acids of SSL > 1 (Luca *et al.*, 1989).

2.5.3 End Season Drought

In groundnut, late-season drought stress is a major factor associated with aflatoxin contamination (Blaney, 1985; Mehan, 1987). The relationship between late-season drought and increased *A. flavus* invasion and aflatoxin contamination was observed in different countries such as South Africa, (Sellschop, 1965), Nigeria (McDonald and Harkness, 1967) and U.S.A (Sanders *et al.*, 1981; Dickens *et al.*, 1973; Pettit *et al.*, 1971). Aflatoxin levels in kernels harvested from rain-fed plots 120 and 130 days after sowing was in a range of 694 to 10240 $\mu g\ kg^{-1}$, however, zero to trace amount of aflatoxins were detected in kernels from irrigated plots (Mehan *et al.*, 1986; Mehan *et al.*, 1988). Drought stress has a vital role in accelerating *A. flavus* infection and aflatoxin production in sound mature kernels (Davidson *et al.*, 1983). Contamination with aflatoxin in kernels from three growers fields which had no, moderate, and severe drought stress

averaged 6, 73 and 444 µg kg^{-1}, respectively. More than twenty to thirty days of drought stress with optimum soil temperature for aflatoxin production is required for preharvest aflatoxin contamination (Sanders *et al.*, 1983). Pods maturing under fluctuating soil moisture conditions in a season of irregular rainfall are prone to pod splitting. Seeds in split pods are frequently invaded by *A. flavus* and subsequently contaminated with aflatoxins (Graham, 1982).

2.5.4. Soil Temperature

Several studies have provided information about the influence of soil temperature on the extent of *A. flavus* infection and aflatoxin contamination in groundnuts (Cole *et al.*, 1989; Blankenship *et al.*, 1989; Sanders *et al.*, 1985). Kernels from undamaged pods grown under drought conditions at mean soil temperature of 24.8°C or lower were not contaminated with aflatoxin. Under similar conditions, kernels grown at 25.7, 26.3 and 27.8°C had lower concentrations of aflatoxins. However, at 29, 29.6 and 30.5°C, kernels were heavily contaminated with aflatoxin. Kernels from pods grown at 31.3°C were free from aflatoxin, as were kernels from pods grown under irrigation and irrigated heated plots with a mean temperature of 34.5°C. It has been demonstrated that 45°C temperature prevents the growth of *A. flavus*, and 2 to 4 hours at 50°C suppressed growth for nearly 24 hours (Burrell *et al.*, 1964; Hussein *et al.* 1986). Optimum mean pod zone temperature has been determined in a range of 28 to 30°C for aflatoxin production in drought conditions during the last

30 to 50 days of the growing season.

Cole *et al.* (1985) studied the relationship between soil temperature and aflatoxin production by *A. flavus*. They did not record any aflatoxin contamination in kernels from undamaged pods in plots with adequate irrigation or from drought stressed plots when the mean of soil temperature during the last 30 to 50 days of crop life cycle remained less than 25°C or more than 32°C (Sanders *et al.*, 1983; Cole *et al.*, 1985). Their results revealed that even slight change of soil temperature may significantly influence aflatoxin production in drought stressed groundnut. Cole *et al.* (1985) suggested that a drought stressed mean of 31.3°C was too high for aflatoxin production even though kernels were heavily infected by A. *flavus*. This may be high or low temperature on fungus metabolism rather than level of irrigation (Sinha and Bhatnagar, 1998).

2.5.5 Insect Damage

Insects play a major role in aflatoxin contamination of groundnut (Diener, 1989). Insect damaged pods have much higher aflatoxin content than sound, mature kernels. Mites and the lesser corn stalk borer larvae (*Elasmopalpus lingosellus* Zeller) are known vectors of *A. flavus* and *A. parasiticus* (Aucamp, 1969). Internal and external pod damage by the lesser corn stalk borer have been shown to increase the percentage of kernels infected by *A. flavus*. Even microscopic damage to the groundnut pods increases infection by *A. flavus*. A number of soil-inhabiting pests, including pod borer, millipedes, mites white grubs,

termites and nematodes have been implicated in *A.flavus* infection of groundnuts before harvest. Another serious pest of groundnut, southern corn rootworm (*Diabrotica undecimpunctata howardi* Barbar), has been commonly associated with increased fungal infection. Termites (*Microtermes* spp. and *Odontotermes* spp.) are associated with pod scarification and facilitating invasion by *A. flavus* (Sellschop, 1965; Johnson and Gumel, 1981). Potential involvement of mites and nematodes has been implicated in aflatoxin problem in field groundnut crops (Aucamp, 1969; Bell *et al.*, 1971). Mites penetrate ground pods, feed on kernels, and disseminate spores of *A. flauvs.*

Varieties and /or pesticides treatments with less termites damage had significantly lower *Aspergillus spp* infections. These results imply that the application of suitable insecticides at appropriate periods or the use of resistant varieties will reduce termite damage and thus minimize *Aspergillus* infection and aflatoxin contamination (Umeh *et al.*, 2000).

2.5.6 Kernel Moisture Content

Moisture content and relative humidity surrounding a substrate are the most important factors for aflatoxin production. Invasion of groundnut pods and seeds by *A. flavus* in the field occurs rapidly when kernel moisture content is 12 to 20%. After harvesting. *A. flavus* invasion was most rapid when seed moisture content was 14 to 30%. Aflatoxin production in mature. sound seeds was limited by 83% relative

humidity (Diener, 1973). Aflatoxin can be formed in groundnut seeds at moisture contents in equilibrium with relative humidity as low as 85%, but significant quantities are produced at 88, 90 and 99% (Diener *et al.*, 1987).

2.5.7 Pod and Kernel Damage

Seed from groundnut pods with cracks or other such damage are prone to infection and aflatoxin production. The level of aflatoxin in sound groundnut seeds and in those from broken pods was < 0.005 and > 2 mg/ml, respectively (Schroeder and Ashworth, 1965). Invasion of pods by *A. flavus* prior to digging was associated with biological and physical damage to pod shells. Seeds inside broken pods had extensive fungal growth compared to those from nondamaged pods (Diener, 1973).

Whole maize seeds have lower levels of external and internal aflatoxin B1 than wounded kernels, indicating that the pericarp and aleurone layers contribute to the defense of the seed against the fungus (Wallin, 1986). No aflatoxin B1 was produced on sound, viable cottonseeds inoculated with *A. flavus*, whereas cracked viable seeds gave levels similar to those produced on autoclaved seeds (El-Naghy *et al.*, 1991). *A. flavus* spores inoculated onto viable and nonviable soybean seeds showed no difference in growth or sporulation. However, the rate of aflatoxin accumulation in viable seeds was lower than that in nonviable seeds (Agarwal and Sinclair, 1997).

2.5.10 Mycoflora Competition

Microbial competition influences mycotoxin production. *A. flavus* has been associated with several other microorganisms in stored seeds. Microbial competition or microbial break down products lowers aflatoxin levels in groundnut seeds. These microorganisms either breakdown aflatoxin or restrict the development of *A. flavus*. *A. niger* prevented penetration of groundnut pods by *A. flavus* and several other fungi (Diener, 1973).

2.5.11 Planting Time

Planting date directly affected aflatoxin production by influencing maturity time. Late -planted maize had a greater chance for preharvest aflatoxin production due to *A. flavus* than an early-planted crop (Jones and Duncan, 1981). Late harvesting of peanuts resulted in an increase in aflatoxin (Diener, 1973).

2.5.12 Soil Type

There was a lower risk of preharvest *A. flavus* infection and aflatoxin contamination in groundnut grown in vertisols than in alfisols (Mehan *et al.*, 1991).

A. flavus infection and aflatoxin contamination are lower in groundnut seeds of all genotypes harvested from vertisols (silty clay loam) than in those from alfisols (light sandy and red sandy loam). Vertisols also have significantly lower populations of *A. flavus* than

alfisols (Mehan *et al.*, 1991). Different soils may have significant different levels of seed infection by *A. flavus* and *A. parasitucus* (Graham, 1982). Sandy soils and alfisols favor rapid proliferation of the toxigenic fungi, particularly under dry conditions. Vertisols have high water-holding capacity and this may be partly responsible for the lower levels of aflatoxin contamination in groundnuts grown on such heavy soils (Mehan *et al.*, 1991).

2.5.13 Fertilizer

Plant stress associated with reduced fertilization increased the incidence of aflatoxin (Aderson *et al.* 1975 and Lillehoj and Zuber, 1975).

2.6 MANAGEMENT OF *A. FLAVUS* INFECTION AND AFLATOXIN CONTAMINATION

Plant protection through synthetic chemicals has contributed significantly in reducing losses due to diseases and thereby increased agricultural production. However, chemicals share to environmental pollution through air, water, soil and by induction of resistance amongst pathogens currently caused wide spread concern. Hence, the need to look for an alternative safer means for plant disease is felt greater now than ever before (Sunil *et al.*, 2003).

2.6.1 Cultural Control

Prevention of aflatoxin contamination in peanut seed is of vital concern to all segments of the peanut industry. Some production practices effectively reduce incidence of aflatoxin in peanuts. These include: rotation with non-legume crops or planting on fallowed land, deep burial of surface litter, avoidance of drought stress and proper harvesting and curing (Mixon *et al.*, 1984).

Management practices, such as optimum fertilization, avoidance of drought stress through supplementary irrigation, and effective insect pest control can be helpful in reducing stress to the crop and thus lowering mycotoxin contamination (Mehan, 2002).

Cultural control of aflatoxin contamination of groundnuts must take into consideration all the varied environmental and agronomic factors that influence pod and seed infection by the aflatoxin producing fungi, and aflatoxin production (Mehan *et al.*, 1991). These factors can vary considerably from one location to another, and between seasons at the same location. Growing groundnuts continuously on the same land may lead to a build-up of high populations of *A. flavus* and *A. parasitucus* in the soil, which in turn increases seed infection and aflatoxin contamination (Joffe and Lisker, 1970; Subrahmanyam and Rao, 1974). Different soils may have significant different levels of seed infection by *A. flavus* and *A. parasitucus* (Graham, 1982). Sandy soils and alfisols favor rapid proliferation of the toxigenic fungi, particularly under dry

conditions. Vertisols have high water-holding capacity and this may be partly responsible for the lower levels of aflatoxin contamination in groundnuts grown on such heavy soils (Mehan *et al.*, 1991).

Davidson *et al.* (1983) reported that application of gypsum to a soil in Georgia, reduced aflatoxin contamination, but Cole *et al.* (1985) did not observe any such effect.

Waliyar *et al.* (2002) reported that application of lime reduces seed contamination by *A. flavus* by 47%, manure by 33%, crop residues by 24% and combination of manure and crop residues by 56%. Application of lime reduces aflatoxin content in seed by 48%, manure by 32%, crop residues by 27%, combination of manure and lime by 72%, combination of lime and crop residues by 71%, combination of manure and crop residues by 56% and combination of manure, lime, and crop residues by 83%.

2.6.2 Use of Botanicals (Natural Plant Products)

During recent years, use of plant products for the control of plant diseases is gaining importance (Anandaraj and Leela, 1996). The eco-friendly approaches do not encourage the use of chemical fungicides, which pose problems of residual toxicity, environmental pollution and development of resistance in plant pathogens (Babu *et al.* 2001). Due to economical and ecological reasons, use of botanical

extracts for the management of plant diseases is desirable (Karade and Sawant, 1999). Many plant extracts are reported to specifically inhibit the germination of fungal spores.

Neem is one of the versatile trees, and has found multiple uses in medicine and agriculture. Neem has become cynosure of worldwide research effort today (Srinivasan *et al.*, 2001). According to an estimate by *Khadi* and Village Industries Commission there are about 140 to 180 lakh neem trees all over India. Today it grows in tropical and subtropical areas of Africa, America and Australia (Srinivasan *et al.*, 2001).

The inhibition of growth and aflatoxin production by *A. flavus* and *A. parasiticus* by spice oils and their active components has frequently been reported (Farag *et al.*, 1989) but reports of the minimum concentrations required to inhibit growth differ widely (Patkar *et al.*, 1993). Sometimes, toxin production may be inhibited without fungal growth being affected (Bullerman, 1974). Food grade spice oils are of special interest as food preservatives, as they do not cause any health problems (Patkar *et al.*, 1993).

Mahmoud (1999) studied the inhibition of growth and aflatoxin biosynthesis of *A. flavus* by extracts of some Egyptian plants. The results showed that both growth and aflatoxin biosynthesis by *A. flavus* were suppressed by aqueous extracts of the tested plants. The inhibitory effect of these extracts was proportional to their concentrations. Among the

plants he tested, *Lupinus albus* L. appeared to be the most effective. The application of its extracts at concentration of 10mg ml^{-1} reduced both fungal growth and aflatoxin production by 45.3 and 60% respectively, concluded his study that *Lupinus albus* L., *Ammi visnaga* (L) Lam. and *Xanthium pungens* Wallr. are significant inhibitors to *A. flavus* growth and resultant aflatoxins and if inhibitory factors could be examined at biosynthetic level, these plants could be used in controlling aflatoxin formation in food and feed.

Patkar *et al.* (1993) reported that, the addition of 0 .5µl ml^{-1} of cinnamon oil completely inhibited the growth on agar but allowed slight growth in broth, while 1.25µl ml^{-1} of clove oil inhibited growth on both agar medium and broth. Cinnamon oil completely inhibited AFB_1 production with the addition of 0.75µl ml^{-1} to liquid medium while 1.25µl ml^{-1} clove oil was necessary to produce the same effect. They concluded that the inhibitory activity of cinnamon and clove oils against growth and aflatoxin production by *A. flavus* suggests that their active principles, might have potential as grain preservatives but they are unsuitable for use with seed grain since germination may be inhibited.

The growth of *A. flavus* was insignificantly stimulated by clove oil (50 and 100µg/ml) and cinnamon oil (50µg/ml) treatment. Above 100µg/ml treatment, significant reduction in the mycelial growth was recorded due to the inhibitory action of both compounds. With the

suppression of *A. flavus* growth, the synthesis of aflatoxin was also decreased significantly above 100μg ml^{-1} of clove and cinnamon oils in a concentration – dependent manner. At the concentration of 250μg ml^{-1} reduction in the aflatoxin formation was 67 and 73% by clove oil and cinnamon oil respectively. When the maize kernels were treated with test oils , toxin production is reduced. The toxin formation also decreased with the increase in the concentration of test compounds, and at 1000 mg ml^{-1} treatment inhibition was 76 and 78% by clove oil and cinnamon oil, respectively (Sinha *et al.*, 1993).

Kumar and Prasad (1992) reported that the growth of *A. flavus* and the consequent aflatoxin production were inhibited to substantial level by treatment with *Andrographis peniculata* L. extract. The maximum percent aflatoxin inhibition was at 10 mg ml^{-1} concentration of extract (78.6% aft. B1 and 42.5 aft. B2). Growth of *A. flavus* was also correspondingly decreased by increasing concentrations of extract. Reduction of growth of the *A. flavus* and of aflatoxin production by treatment with *Andrographis peniculata* was possibly due to interference by active principles of the extract.

Kshemkalyani *et al.* (1990) studied the effect of allicine and extracts of garlic on *A. flavus* and *A. parasiticus* and their results revealed that total garlic juice and allicin showed antifungal activity at and above 20μg ml^{-1} concentration while antifungal activity of aqueous phase

obtained after ethyl acetate extraction was observed at and above 50µg/ml concentration. They concluded that total garlic juice and its components separated by ethyl acetate can inhibit the growth of aflatoxin producing fungi and this may be the reason for using garlic in certain food preparations, which might show the growth of aflatoxin producing strains of *A. flavus* and *A. pasiticus*.

Masood *et al.* (1994) reported the influence of colouring and pungent agents of red chilli (Capsicum annum) on growth and aflatoxin production by *Aspergillus flavus*. For capsanthin (the coloring agent of red chilli) treatment, aflatoxin production and growth of the *Aspergillus flavus* were completely checked up to the fourth day of incubation. The inhibitory effect on toxin biosynthesis was higher compared to the growth of the fungus, thus resulting in a decreased specific production of aflatoxin. A decrease in the ratio of B1: G1 was recorded at all the incubations and concentration levels of capsanthin. Capsaicin (the pungent agent of red chilli) was effective only up to the fourth day of incubation.

Ansari and Shrivastava (1991) reported the effect of eucalyptus oil on growth inhibition of *A flavus* and aflatoxin production at 6 day of incubation, however, at 12 days of incubation acceleration in toxin production was noticed without any further effect on growth.

2.6.3 Biological Control

In the 1960s, it became apparent that agricultural chemicals were responsible for environmental pollution, were present in the food chain, and were capable of inducing pest resistance. Pesticides also became very expensive to produce and register for use (Lewis and Papavizas, 1990).

In the absence of an immune system to combat pathogenic microorganisms, plants rely primarily on chemical protection. The excessive use of synthetic chemicals for the past few decades has led to problems like development of disease resistance, toxic hazards to man, plants domestic animals and wild life (Narasimhan and Masilamani, 2003). Last two decades scientists are looking for environmentally and toxicologically safe and more effective method to control phytopathogens. Alternative to chemical method is biological control (Narasimhan and Masilamani, 2003).

Cook (1985) defined biological control as the use of natural or modified organisms, genes or gene products to reduce the effects of undesired organisms (pests) and to favour desirable organisms such as crops, trees, animals and beneficial insects and microorganisms. In other terms biological control is the reduction of inoculum density or disease producing activities of a pathogen or parasite in it's active or dormant state, by one or more organisms.

There are three general strategies in considering biological control with introduced microorganisms (a) reduce the population of the pathogen and/or regulate it below some economic threshold. (b) prevent the pathogen from infecting the plant and (c) limit disease development after infection

Understanding the mechanisms through which the biocontrol of plant diseases occurs is critical to the eventual improvement and wider use of biocontrol methods. These mechanisms are generally classified as competition, parasitism and antibiosis (Fravel, 1988).

There are different ways by which the biological control agents can suppress the pathogens:

(a) Hyperparasitism/ Mycoparasitism: One of the mechanisms of biocontrol of plant pathogens with *Trichoderma* is known as mycoparasitism where *Trichoderma* recognizes and attaches to the pathogenic fungus and begins to excrete extracellular hydrolytic enzymes, such as chitinases, β-1, 3-glucanases, proteases, and lipases. These enzymes act on the cell walls of the fungi and thus cause lysis (Anjaiah *et al.*, 2001).

There are several means by which mycoparasites attack fungus structures. They may penetrate mycelia directly, the parasitic hypha growing within the host mycelium, as does Rhizoctonia solani in various

phycomycetes. The mycoparasite may coil around the mycelium of the host, with or without penetration, as does Trichoderma viride (Weindling, 1932). Enzymes may be produced that digest the mycelial walls, or antibiotics may be formed that inhibit growth or cause endolysis, as does *Trichoderma viride* (Weindling, 1934).

While assessing the production of non-volatile chemicals initially, there was very slow growth of *A. flavus*. Even after 10 days of incubation, a maximum of only 27 mm colony diameter of *A. flavus* was recorded with *T. Harzianum* (TH–1) as compared with 85 mm in the control indicating the production of non–volatile chemicals inhibitory to *A. flavus* growth by all *Trichoderma* isolates (Desai *et al.*, 2000).

Twenty-one of the 39 *Trichoderma* isolates showed the inhibition of Af 11-4 colony by producing volatile antibiotics compared with the control. In the control plate, the colony diameter of Af 11-4 was 60 mm whereas in other plates it was 10-45 mm. Fifteen of the 39 *Trichoderma* isolates showed inhibition of Af 11-4 colony by producing diffusible antibiotics compared with the control. Colony diameter of Af 11-4 in the control plate was 55 mm compared with 10-50 mm in plates with *Trichoderma* isolates (Srilakshmi *et al.* 2001).

Molecular analysis of genomic DNA from these *Trichoderma* isolates also revealed the presence of chitinase gene in polymerase chain reaction (PCR). Some of these *Trichoderma* isolates were shown to be

effective not only for reduction of seed and peg infection by *A. flavus* but also reduced *A. flavus* population in the rhizosphere of groundnut (Anjaiah *et al.*, 2001).

Antibiosis has certain *advantages* over other forms of antagonism i.e. the toxic substances produced may diffuse in water films and water-filled pores through soil or on substrates, or air-filled pores in the case of a volatile and thus actual physical contact between antagonist and the pathogen is not required. The antibiotics are commonly more rapid and effective, than that of competitors or hyperparasites. Furthermore, antibiosis may continue for a while after growth of the antagonists ceases, because antibiotic release continues briefly after colony death. In fact, antibiotic release from a living colony apparently comes largely from senescent cells within that colony, but as senescence sets in, permeability changes to allow it to flow out. Therefore, antibiotic action will provide more of a steady-state form of antagonism than will hyperparasitism or competition.

Examples of Biocontrol By Fungal Antagonists

Thakur *et al.* (2003) evaluated six *Trichoderma* isolates and three isolates of *Pseudomonas fluorescens* for their biocontrol potential against *A. flavus* groundnut kernel infection in field condition. The results showed that all the BCAs tested reduced the kernel infection by *A. flavus* (Af-11) significantly over the control. Two isolates of

T. viridae (T-17 and T-20) and one of *T. harzianum* were relatively more effective in reducing kernel infection than others. Among the Pseudomonads, isolates Pf 2 and Pf 76 were also equally effective in reducing kernel infection.

Aflatoxigenic *A. flavus* L strain isolate AF36 reduced formation of both sclerotia and aflatoxin when co-inoculated with S strain isolates. AF36 formed no sclerotia in developing bolls and was more effective at preventing S strain isolates than L strain isolates from contaminating developing cotton seed with aflatoxins. The use of atoxigenic L strain isolates to prevent contamination through competitive exclusion may be particularly effective where S strain isolates are common. In addition to aflatoxin reduction, competitive exclusion of S strain isolates by L strain isolates may result in reduced over wintering by S strain isolates and lower toxicity resulting from sclerotial metabolites (Garber and Cotty 1997).

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

Simultaneous inoculation of wounded 28- to 32-day old cotton bolls with toxigenic and atoxigenic strains of *A. flavus* led to lower levels of aflatoxin B_1 in the cotton seed at maturity than in bolls inoculated with

the toxigenic strain alone. Six of seven atoxigenic strains tested reduced the level of contamination produced by toxigenic strains. Less B_1 was detected when the atoxigenic strain was introduced into the wound 1 day before inoculation with a toxigenic strain than when atoxigenic and toxigenic strains were coinoculated. In contrast, toxin levels at maturity were not reduced when the atoxigenic strain was introduced 1 day after the toxigenic strain. Use of an atoxigenic strain at 10-fold higher spore concentration led to the significant reduction in B_1 if the atoxigenic strain was introduced within 16hr after the toxigenic strain. Atoxigenic strains of *A. flavus* may be useful in biological control of aflatoxin contamination (Cotty, 1990).

The influence of inoculum size in the production of aflatoxin B1 was determined when *Aspergillus parasitucus* NRRL 3000 and *Fusarium graminearum* ITEM 124 were cultured alone and in pairs on irradiated corn kernels at 28°C and 0.97 water activity (Etcheverry *et al.*, 1998).

2.6.3.5 Bacteria as biocontrol agent

Mickler *et al.* (1995) screened geocarposphere bacteria as potential biological control agents against *A. flavus* invasion and Aflatoxin contamination. All 17 geocarposphere strains tested delayed invasion of young groundnut roots and reduced colonization by the fungus in a root-radicle assay used as a rapid laboratory prescreen. In a greenhouse study, seven bacterial strains significantly reduced pod

colonization by *A. flavus* compared to the control. In a field trial, conducted similarly to the greenhouse assay, pods sampled at mid-peg from plants seed-treated with suspensions of either strain 91A-539 or 91A-550 were not colonized by *A. flavus*, and the incidence of pods invaded from plants treated with either 91A-539 or 91A-539 was consistently lower than non-bacterized plants at each of five sampling dates. At harvest, 8 geocarposphere bacterial strains significantly lowered the percentage of pods colonized (>51%) compared to the control.

Munimbazi and Bullerman (1998) reported the inhibition of aflatoxin production of *A. parasiticus* NRRL 2999 by *Bacillus pumilus*. Their results indicated that each of the 6 *B. pumilus* isolates inhibited aflatoxin production and mycelial growth of *A. parasiticus* when both organisms were grown simultaneously in YES broth. Percentage of production ranged between 98.4 (isolate 1) and 99.9 (isolate 2). Mycelium production was less inhibited with percentages of inhibition ranging between 34.4 % (isolate 1) and 56.4% (isolate 2). *B. pumilus* Inhibited aflatoxin production of *A. parasiticus* NRRL 2999 in both simultaneous and deferred antagonism assays. The inhibitory activity was likely due to extracellular metabolites produced by the bacterium in the growth medium.

When *A. flavus* was grown with different bacteria in dual culture on groundnut, aflatoxin production increased in case of all bacteria under

experimental conditions, except in case of *Flavobacterium odoratum*, as compared to *A. flavus* in a single culture. The highest aflatoxin production was noticed when *A. flavus* and *Bacillus megaterium* were grown together at 30°C (2175 µg/kg), 25°C (2005 µg/kg) and 0.98 water activity, there by amounting to a two-fold increase over the single culture. A 10-fold increase in aflatoxin level was found at 30°C (1005 µg/kg) and 0.90 water activity with the same bacterium (Chourasia, 1995).

Misaghi *et al.* (1995) screened 892 indigenous bacterial isolates, including 11 that were endophytic to cotton, for their ability to inhibit the growth of *Aspergillus flavus* on cotton seed in an *in vitro* bioassay. Only six isolates partially or totally inhibited fungal growth.

Yeole and Dube (2000) reported siderophore-mediated antibiosis of rhizobacterial fluorescent pseudomonads against certain soil borne fungal plant pathogens. Twelve rhizobacterial fluorescent pseudomonas isolates obtained from chilli, cotton, groundnut, and soybean inhibited the growth of 12 test soil-borne fungal plant pathogens in iron deficient kings medium B, that varied from lysis (100% inhibition) to "no effect", but in more cases the inhibition ranged 3.3 to 15%.

Chourasia (1995) studied kernel infection and aflatoxin production in peanut by *A. flavus* in presence of geocarposphere bacteria. Almost all the bacterial isolates tested, stimulated growth and aflatoxin production by *A. flavus* on peanut kernels, especially at 30°C and 0.95

and 0.98 water activity. No aflatoxin was produced at 20°C and 0.90 water activity. *Flavobacterium odoratum*, showed inhibition of aflatoxin biosynthesis by *A. flavus*, as compared to culture of *A. flavus* alone. *A. flavus* inhibited some of the bacterial isolates tested on contact at 30°C and 0.95 water activity in peanut extract agar. However, at 25°C and 0.95 water activity the reverse was true.

2.6.3.6 Improvement of *trichoderma* for biocontrol potential (irradiation)

Industrial microbiologists and mycologists, employing mutations and selections, coupled with parasexual hybridizations, developed biotypes of industrial fungi to increase antibiotic production.

Improved strains of *T. reesei* were obtained by mutations and these produced more cellulase than did the wild-type strains (Montenecourt and Eveleigh, 1979).

The first such programme with biocontrol fungi was initiated in the BPDL to explore ultraviolet (UV) mutagenesis for *T. harzianum* and *T. viride*. Papavizas, *et. al* (1982) used the inability of *Trichoderma spp.* to tolerate benomyl as a marker in a selection system following induced mutation with u.v. light. A few of the benomyl-tolerant biotypes of *T. harzianum* and *T. viride* differed from their respective wild-type strains in growth characteristics and ability to

sporulate, survive in soil, and suppress the saprophytic activity of the pathogen *R. solani* in soil. Several u.v. induced biotypes were consistently more effective than the wild-type strains in suppressing *P. ultimum* on peas, *R. solani* on cotton and radish, *S. rolfsii* on beans, and *S. cepivorum* on onion. One of the new benomyl-resistant biotypes of *T. viride* (T-1-R9) developed as a result of this research is effective against Fusarium wilt of chrysanthemum (Locke *et al.* 1985) and Rhizoctonia scurf of potato (Beagle-Ristaino and Papavizas, 1985).

Mukherjee and Mukhopadhyay, (1993) studied the possibility of induction of stable mutants of *Gliocladium virens* by using gamma-irradiation. The mutants differed from the wild type strain in phenotype, growth rate, sporulation and antagonistic potential. Periodical observations on the radial growth of the wild type and the mutants of *Gliocladium virens* on PDA indicated that three mutants were having the same rate of growth as the wild type, while three mutants were of intermediate type, and one was the slowest growing isolate. In dual culture technique, the mutants differed from the wild type and from each other in their ability to antagonize the test pathogens. On *R. solani*, the wild type and three mutants were equally effective by completely overgrowing the pathogen by 18 h. Only one mutant could fully colonize the *S. rolfsii* colony by 170 h in dual culture technique.

Graeme-Cook and Faull (1991) studied the effect of ultraviolet-induced mutants of *Trichoderma harzianum* with altered antibiotic production on selected pathogens *in vitro*. Characterization of extracellular metabolites of these strains showed that the strains with high activity produced only elevated levels of a 6-n-pentyl pyrone, the antibiotic produced by the parental strain, but two new antifungal compounds. One of these has been identified as an isonitrile antibiotic. High antibiotic production by two *T. harzianum* strains, BC10 and BC63, did increase inhibition of hyphal growth of *R. solani* and *P. ultimum*, but there was no correlation between increased antibiotic production and colonization ability.

2.6.4 Host Plant Resistance

Combating plant diseases through host resistance is an economic, ecologically safe and a viable proposition for disease management.

Plant breeding traditionally has been used to control plant diseases and likely will play a role in preharvest control of aflatoxin. However, aflatoxin contamination is unique in that fungi that produce the toxin do not colonize developing tissue without a port of entry and source of resistance appear to be limited or difficult to identify (Cotty *et al.*, 1994; Payne, 1992).

The development of groundnut cultivar with reduced aflatoxin contamination when grown under heat and drought-stressed conditions

would be a valuable tool in alleviating preharvest aflatoxin problem. Aflatoxin contamination is an expensive characteristic to measure and is subject to extreme variability (Holbrook *et al.*, 1997).

Rao and Tulpule (1967) first reported varietal resistance in groundnut to aflatoxin production. In laboratory inoculation tests they found that the cultivar US 26 (PI 246388) did not support aflatoxin production when seeds were colonized by aflatoxin producing strains of *A. flavus*. This finding was not confirmed by other workers, but did stimulate research on possible varietal resistance to aflatoxin production (Mehan *et al.*, 1991).

A laboratory method to screen live groundnuts for resistance to aflatoxin production was used at ICRIAT (Mehan and McDonald, 1980) to test 502 genotypes. None was totally resistant to aflatoxin production but highly significant differences in aflatoxin production were found (Mehan *et al.*, 1986).

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

Mixon and Rogers (1973a) reported that two Valencia type genotypes, PI 337394F and PI 337409, had high levels of resistance to *in vitro* seed colonization by *A. flavus* and *A. parasiticus*.

2.6.4.1 Types of resistance

In groundnut, based on the site at which it is tested or cultivated, resistance to aflatoxin-producing fungi may be of three types: (a) resistance to pod wall infection (b) resistance to seed coat invasion and colonization (c) and resistance to cotyledons aflatoxin production . The fungi have to penetrate the pod wall and the seed coat to reach the cotyledons from which they derive their sustenance. Resistance to pod infection is attributed to pod-shell structure, while resistance to seed invasion and colonization is mostly physical, and has been correlated with thickness, density of palisade cell layers, absence of fissures and cavities, and presence of wax layers. There are conflicting reports regarding the role of fungistatic phenolic compounds in imparting resistance to seed colonization (Upadhyay *et al.*, 2002).

The groundnut shell has logically been considered a barrier to penetration by *A. flavus*, as seeds from pods with damaged shells are more frequently contaminated with aflatoxin than those from undamaged pods (McDonald and Harkness, 1967).

Groundnut seed resistance is due to cuticular wax accumulation, seed coat structure, presence of cracks or detachment of the epidermal foundation, concentration of low molecular weight peptide-like compounds, and tannin concentrations.

Peanut cultivars with seeds resistant to insect attack, a rapid drying rate, and resistance to harvest and handling damage, fungal attack and penetration, or mycotoxin production may not have mycotoxin production problems (Zuber and Lillehoj, 1979; Tuite and Foster, 1979). Some of methanol-extracted and water soluble tannins from peanut testa and cotyledons significantly inhibited *A. parasiticus* and reduced aflatoxin (Azaizeh *et al.*, 1990). Resistant seeds of pulses to aflatoxin production after infection with *A. flavus* showed that total phenol and protein were greater in resistant cultivars, while total sugar was greater in susceptible ones (Singh *et al.*, 1990).

Sources of all the three types of resistance have been reported (Mehan,1989). These include Shulamit and Darou IV for resistance to pod infection, PI 337394 F, PI 337409, GFA 1, GFA 2, UF 71513, Ah 7223, J 11, Var 27, U 4-47-7, Faizpur, and Monir 240-30 for resistance to *in vitro* seed colonization by *A. flavus* (IVSCAF); and U 4-7-5 and VRR 245 for resistance to aflatoxin production.

The value of a resistant source depends upon the level and stability of its resistance. Resistance to pod infection has been reported to

be highly variable and of a low level. Similarly, IVSCAF-resistance is not absolute and even the best sources show up to 15% seed colonization; only a few lines (J 11, PI 337394 F and PI 337409) have shown stable resistance.

After three years of testing in two environments, 16 resistant groundnut genotypes were identified from a core collection representing the entire groundnut germplasm collection (Holbrook *et al.*, 1995). A possible link between low linoleic acid content in peanut and low preharvest aflatoxin production was indicated (Holbrook *et al.* 1995). The significant correlations observed between leaf temperature and aflatoxin levels and/or visual stress ratings and aflatoxin levels may provide the basis for a useful and inexpensive approach to preliminarily screen groundnut cultivars. A system of evaluating peanuts in the field through the manipulation drought stress was successfully tested, demonstrating water stress responses in peanuts similar to field responses, and variations in peanut phytoalexins and aflatoxin levels (Mehan *et al.*, 1988; Basha *et al.*, 1994).

Holbrook *et al.* (1997) studied *A. flavus* colonization and aflatoxin contamination in peanut genotypes with resistance to other fungal pathogens. Their results revealed that none of the genotypes with resistance to late leaf spot or white mold exhibited less colonization of shell or kernels by *A. flavus* group fungi than Florunner when tested in

Georgia. Five out of the nine genotypes tested were more susceptible than florunner to colonization of shells by *A. flavus* group fungi, and one (PI 210831) was more susceptible to kernel colonization. These results indicate that the mechanisms of resistance to other fungi operating in these genotypes are not effective in providing resistance to colonization by *A. flavus*. None of the genotypes examined in this study exhibited a reduced level of preharvest aflatoxin contamination compared to Florunner when tested in Georgia or Arizona. One of the genotypes (PI 196660) exhibited significantly higher aflatoxin contamination in Arizona.

Holbrook *et al.* (2000) evaluated preharvest aflatoxin contamination in drought - tolerant and drought-intolerant peanut genotypes. Twenty genotypes with different levels of drought tolerance were evaluated. Seven of the genotypes exhibited at least a 92% reduction in mean aflatoxin contamination in comparison to susceptible check cultivar (Florunner). Two drought tolerant genotypes (PI 145681 and Tifton 8) showed significant reduction in preharvest aflatoxin contamination compared to Florunner

Waliyar *et al.* (1994) tested 25 groundnut lines for resistance to *A. flavus* colonization and aflatoxin contamination. Average seed infection varied with site and year from 5 to 37%. Cultivars 55-437, J11, and PI 337394 F were the least infected. Among the ICRISAT advanced breeding lines involving parents resistant to *A. flavus*, ICGV 87084,

ICGV 87094 and ICGV 8711 were resistant. The results showed that some breeding lines possessed a good level of resistance to *A. flavus*, reflecting the presence of genes for resistance. *A. flavus* infection was significantly correlated with aflatoxin content, ranging from 1 to 450ppb. Only one line, showed a high percentage of infection by *A. flavus* but a low level of aflatoxin, suggesting that this line may be resistant to aflatoxin production in west Africa. Among the ICRISAT breeding lines, ICGV 87110 had the lowest level of aflatoxin. None of the lines reported as resistant possessed a high level of resistance to *A. flavus*. Waliyar and Ntare (2000) screened some groundnut cultivars for resistance to *A. flavus* infection and aflatoxin contamination in West Africa. Results of such screening have shown that several varieties or genotypes identified as resistance in India are also resistant in Africa. The varieties J 11, UF71513-1, U 447-7, AH 7223 etc, are among the stable ones. Among the West African varieties, 55-437 continues to be the most resistant, followed by 73-30 and 73-33. Several advanced progenies from ICRISAT breeding programs have been screened and many of them have found to be resistant. Among them are ICGV 87084, ICGV 87094, ICGV 87110, ICGV 91276, and ICGV 91289.

Thakur *et al.* (2000) evaluated wild *Arachis* germplasm accessions for *in vitro* seed colonization and aflatoxin production by *A. flavus*. Large variation was recorded in both seed colonization severity (1 to 4 scale) and aflatoxin production high (>5000µg kg^{-1} seed) to

negligible (<100μg kg^{-1}seed) among accessions belonging to different sections and species. Accessions ICG 13212 (*A. pusilla*), ICG 11560 (*A. chiquintana*), and ICG 8131 and ICG 14875 (*A. triseminata*) recorded low colonization severity and relatively low aflatoxin content compared with those of control susceptible cultivars J 11 and JL 24.

2.6.5 Integrated Disease Management (IDM)

The concept of integrated disease management has caught the imagination of the plant pathologists over a period of time and has been found to be very useful. In fact, integrated disease management is an ecological approach among all beneficial, biological and physical form in the eco-system to maintain plant health equilibrium. This involves the simultaneous manipulation of a number of available strategies of reducing the plant diseases with minimum damage to the environment. It includes the study of crop, its pathogens, environmental conditions, ecosystem relationship etc and is thus a part of agroeco-system (Gupta, 2002).

The strategy of integrated disease management comprises of the cultivation of resistant/tolerant cultivars, adoption of agronomic practices resulting in less disease, preserving and promoting the activities of natural biocontrol agents and the use of chemical pesticides wherever necessary to reduce pathogen population to known damaging levels. Farmers had been practicing IDM knowingly or unknowingly over the ages which include modification in cultural practices like planting dates,

fertilizer application, water management, organic amendments, crop rotation and use of quality planting material (Gupta, 2002).

The potential of *Trichoderma* and *Gliocladium* for biocontrol has been studied largely as an end in itself rather than as a synergistic or additive component in integrated pest management systems. Such approaches can be successful only if *Trichoderma* or *Gliocladium* are compatible with pesticides or other control practices (Papavizas, 1985).

The biocontrol agents, especially *Trichoderma*, have been used in experimental combinations with various management practices, for example, satisfactory control of cucumber fruit rot (*Rhizoctonia solani*) in the field was achieved by a combination of plowing and the addition of *T. harzianum* (Lewis and Papavizas, 1980). The indicators for the effectiveness of integrated management of aflatoxin contamination were fungal infection and aflatoxin content in the seed and *A. flavus* population in the soil. Despite the similar initial population levels in both the plots (integrated aflatoxin management package and farmers practice), cumulative gain in cfu was observed in the plot where farmers' practice was followed. Seed infection studies revealed predominance of *A. flavus* infection in plot with farmers' practice (10%) over improved package (2%). This could be because of inhibition of initial rhizosphere soil population build up of *A. flavus* by seed treatment with systemic fungicide and application of biocontrol agent in the improved package (Vijay *et al.*, 2002).

Control of *Almillaria mellea* with a combination of methyl bromide and *Trichoderma* (Ohr *et al.*, 1973) is a classical example of integrated pest management involving the use of a pesticide with a biocontrol agent. Other examples of a pesticide and a biocontrol agent include the use of combined *T. harzianum* and PCNB against *Rhizoctonia* damping-off of several vegetables (Elad *et al.*, 1980; Henis *et al.*, 1978) and methyl bromide and *T. harzianum* combined for the control of *R. solani* on strawberry (Elad *et al.*, 1981) and *R. solani* and *Sclerotium rolfsii* on tomatoes and peanut (Elad *et al.*, 1982).

Kraft and Papavizas (1983) have shown that the highest seed yields in the field with a pea cultivar susceptible to *Pythium ultimum* are obtained with a seed treatment combining metalaxyl and *T. harzianum.*

Shanmugam *et al.* (2001) reported Fluorescent pseudomonad strain Pf 1, which effectively inhibited the mycelial growth of *Macrphomina phaseolina* under *in vitro* condition was compatible with the biofertilizer bacterium *Rhizobium.* The combined application of Pf 1 with *Rhizobium* in different ways was found to be the next best treatment to seed treatment and soil application of Pf 1.

CHAPTER III

MATERIALS AND METHODS

3.1 ISOLATION AND CHARACTERIZATION OF *TRICHODERMA* AND BACTERIAL ISOLATES FROM GROUNDNUT RHIZOSPHERE

Soil samples were collected from geocarposphere soil of groundnut in major groundnut growing areas of Andhra Pradesh and groundnut fields at ICRISAT (International Crop Research Institute for the Semi-Arid Tropics) Patancheru, A.P, India, in the rainy 2000 and post rainy 2000-2001. Serial dilution method was followed (Aneja, 1996) to isolate *Trichoderma* and bacterial isolates. Each sample was made into fine powder and sieved mixed and 10 g of fine soil was added to 90 ml of sterilized distilled water in 250 ml flask to get 10^{-1} dilution. One ml of that dilution was transferred to 9 ml sterile distilled water in test tube to obtain 10^{-2} dilution. In the same way serial dilution was done up to 10^{-4}. The dilutions were vortexed well for 1min. Soil suspension of 500µl of dilutions of 10^{-3} and 10^{-4} were plated on each plate of *Trichoderma* specific medium (TSM). Two replications were maintained for each dilution in each sample. The plates were incubated at 28^{0} C for 7days. The typical *Trichoderma* colonies were counted and population was expressed as colony forming units/g of soil (cfu g^{-1} soil).

For the isolation of bacterial strains dilutions were made up to 10^{-8}. Then 500µl of 10^{-7} and 10^{-8} dilutions were plated on Glucose Casamino acid Yeast extract (GCY) medium (Ajaiah *et al.* 1988) and incubated for 24 hours at 28^0 C. The bacterial colonies developed on the plates were counted and population was expressed as cfu g^{-1} of soil.

The *Trichoderma* and bacterial colonies developed were subcultured on their respective media (TSM and GCY) and used for screening them against *Aspergillus flavus*. The morphological characters like colony colour, growth pattern, growth rate and sporulation were recorded. The *Trichoderma* cultures were preserved in silica gel and kept in the refrigerator and bacterial cultures were preserved on Luria Betarni Broth (LBB) medium plus glycerol at -10°C.

The following media were used during the study:

Czapex Dox Agar (CDA) medium:

Sucrose		30.0 g
Sodium nitrate	$NaNO_3$	2.0 g
Magnesium phosphate	Mg SO4, $7H_2O$	0.5 g
Potassium chloride	KCl	0.5 g
Dipotassium sulphate	K_2HPO_4, $7H_2O$	0.35 g
Ferrous sulphate	$FeSO_4$, 7 H_2O	0.01 g
Agar		15.0 g
Rose Bengal		0.030 g
Streptomycin		1.0 g
Distilled water		1L

Composition of *Aspergillus flavus* and *parasiticus* Agar (AFPA):

Peptone	10.0 g
Yeast extract	20.0 g
Ferric Ammonium Citrate	0.5 g
Agar	15.0 g
Dichloron	2.0 mg
Chloramphenicol	0.2 g
Distilled water	1 L

Luria Betarni Broth medium (LBB):

Caseinenzymatic hydrolysate	10.0 g
Yeast extract	5.0 g
Sodium chloride	10.0 g
Distilled water	1L

Miller Luria Betarni Agar (LBA):

Casein enzymic	10.0 g
Hydrolysate	5 g
Yeast extract	10 g
Sodium chloride NaCl	10 g
Agar	15.0 g

Composition of Glucose Casamino acid Yeast extract (GCY) medium:

Glucose		15.0 g
Casamino acids		1.5 g
Yeast extract		1.0 g
Potassium dihydrogen phosphate	KH_2PO_4	1.5 g
Magnesium sulphate	$MgSO_4$	1.0 g
Water	H_2O	1L

***Trichoderma* Specific Medium (TSM):**		
Glucose		3 0 g
Ammonium nitrate	$NH_4 NO_3$	1 0 g
Dipotassium phosphate	K_2HPO_4	0 9g
Magnesium sulphate	$MgSO_4 7H_2O$	0 2 g
Potassium chloride	KCl	0 15 g
Ferrous sulphate	$FeSO_4 7 H_2O$	20 0 mg
Manganese sulphate	$MnSO_4 7H_2O$	20 0 mg
Zinc sulphate	$ZnSO_4 H_2O$	20 0 mg
Rose Bengal		30 0 mg
Agar		15 0 g
PCNB (75% a i)		0 1 g
Metalaxyl (25% a i)		10 0 mg
Chloromphenicol		50 mg
Streptomycin sulphate		50 mg
Distilled water		1L
Potato Dextrose Agar (PDA):		
Potatoes infusion from		200 0 g
Dextrose		20 0 g
Agar		15 0 g
Distilled water		1L
Potato Dexrose Broth (PBB):		
Potato infusion from		200 0 g
Dextrose		20 0 g
Distilled water		1L
Malt extract Agar (MEA):		
Malt grain extract powder		2%

3.2 EVALUATION OF *TRICHODERMA SPP* AND BACTERIAL ISOLATES FOR *IN VITRO* AND *IN VIVO* ANTAGONISTIC ACTIVITY AGAINST *A. FLAVUS*

In vitro:

Trichoderma spp and Bacterial isolates were screened for their biocontrol efficacy against *A. flavus* by using dual culture technique (Aneja 1996). One disc (5mm dia.) from 7-day-old culture of *Trichoderma* was inoculated on one corner of 90 mm petridish containing PDA medium and on the other corner one disc from 7 days old *A. flavus* (Af 11-4) culture. Three replications were maintained for each test isolate. Plates inoculated with *A. flavus* (strain 11-4) alone served as control.

For screening of bacterial isolates same procedure was followed. Instead of *Trichoderma* disc, a loopful of 48 hrs old bacterial culture to be tested was streaked on the PDA opposite *A. flavus* disc.

Then the inoculated plates were incubated for 7 days at 28^0C. The growth of *A. flavus* in all the plates was measured at 3^{rd}, 5^{th} and 7^{th} day after inoculation. The percentage of growth inhibition by *Trichoderma spp* and bacterial isolates were calculated using the following formula:

$$\text{Inhibition \%} = \frac{\text{Growth in test plate - Growth in control}}{\text{Growth in control plate}} \times 100$$

In vivo:

Glasshouse Biocontrol Experiment:

Five *Trichoderma* isolates (*Trichoderma viride* (T21), *Trichoderma viride* (T38), *Trichoderma viride* (T47), *Trichodrma viride* (T48 and *T. harzianum* (T23) and six bacterial isolates (B_6, B_{18}, B_{33}, B_{50}, B_{58} and *Pseodomonas fluorescens* (PF-2) Which were antagonistic to a toxigenic strain of *Aspergillus flavus* (AF 11-4) *in vitro*, were evaluated in greenhouse and field conditions for their biocontrol potential against *A. flavus* infection and aflatoxin contamination in groundnut at ICRISAT, Patancheru. The experiment was conducted in postrainy 2001-2002 and the 2002 rainy season.

***Trichoderma* Inoculum preparation**

Biocontrol agents (*Trichoderma* isolates) were multiplied on pearl millet grains. Pearl millet grains were soaked in water overnight and washed with tap water to clean it. Eighty grams of soaked pearl millet grains were put in 250 ml conical flask and the required number of flasks containing pearl millet grains were prepared for both greenhouse and field experiments. The flasks containing pearl millet grains were autoclaved at 121°C and 15 psi for 30 min. with two cycles. After the autoclaved grains cool down, one disc from 7 days old *Trichoderma* cultures was inoculated in each flask. The inoculated flasks were incubated at 28°C in dark for 10 days while shaking the

inoculated flasks at 2 days interval to have a uniform mixing of the inoculum in the grains. Fifty ml of sterilized distilled water was poured in each flask containing the infested grains and shaked well to detach the spores in to the water. This was filtered through double-layered muslin cloth and the spore suspensions were collected in sterilized beakers and adjusted to 10^9 spores/ml using haemecytometer.

Seed treatment with bioagent:

Seventy seeds of groundnut cultivar ICGS 11 were poured in 3L beaker containing 50 ml specific *Trichoderma* isolate spore suspension/bacterial cell suspension. About 5 ml of 0.5% suspension of carboxy methyl cellulose (CMC) was used as sticker and bentonite powder as filler and the seeds coated with sterile water, CMC and benonite powder served as control.

Soil preparation:

Red soil, sand and farmyard manure (FYM) were sieved and mixed in a proportion of 3:2:1 by volume. The soil mixture was autoclaved at 121°C and 15 psi for 60 min. with two cycles with an interval of 24 hrs. The autoclaved soil was filled in 12.5" dia. pots and the pots were arranged them randomly on the glasshouse benches The pots were labeled with different treatments of the experiment. The treated seeds were sown in respective pots at 6 seeds/pot. Each

treatment was replicated 4 times with two pots per replication. The pots were irrigated as required. After two weeks the seedlings were thinned to 4 plants/pot. One month after sowing plant heights were recorded by using scale from the soil level to the last growing leaf.

A. flavus inoculum Preparation and Application :

The highly toxigenic strain of *A. flavus* (Af 11-4) was multiplied on autoclaved pearl millet grains as described in *Trichodema* inoculum preparation. Ten grams of ten days old *A. flavus* inoculum grown on pearl millet grains was applied in each pot at flowering stage (40 days after sowing) and covered with a thin layer of sterilized soil. Spore load was adjusted approximately 10^9 spores/g of millet grains by diluting with sterilized millet grains. Light irrigation was provided to the pots to ensure sufficient moisture for the rapid infestation of the pot soil.

Soil sampling:

Soil samples were collected at three stages to monitor the *A. flavus* and biocontrol agents (BCAs) populations in different treatments. First sampling was done at 40 days after sowing (just before *A. flavus* inoculum application) and second and third sampling were done at 80 days after sowing and at harvesting, respectively. The collected samples were processed by using serial dilution method. To estimate the *A. flavus* and *Trichoderma* populations, 10^{-3} and 10^{-4}

dilutions (500 µl) were plated on *A. flavus* and *parasiticus* Agar (AFPA) medium (Pitt *et al.* 1983) and on *Trichoderma* specific medium (TSM). The inoculated plates were incubated at 28° C in dark for 3 days in case of *A. flavus* and for 6 days in case of *Trichoderma*. The number of *A. flavus* and *Trichoderma* colonies developed in each plate were recorded and population density was expressed as cfu g^{-1} of soil.

Harvesting:

Water stress was imposed on the crop at pod formation stage (80 DAS) to facilitate the entry of the fungus into pods. The pots were harvested 120 DAS and number of plants per pot were counted before uprooting. Then threshing was done and the pods were kept in cloth bags and sun dried. Pod weight was recorded. Biomass weight was recorded after drying it in an oven for 2 days at 60°C. The pods were shelled by hand and seed weight was recorded.

Seed infection:

Fifty seeds were counted from each replication of each treatment. The seeds were surface sterilized with 1% sodium hypochlorite (NaOCl) and plated on Czapex Dox Agar (CDA) fortified with rose bengal (30mg L-1) and Streptomycin (1g L^{-1}) and plates were incubated at 28° C for five days in dark. Number of seeds colonized by typical *A. flavus* were counted and percent seed infection was calculated.

Aflatoxin Estimation by ELISA

Preparation of groundnut seed extracts:

Seed samples were ground to make powder using blender and the powder was triturated in 70% methanol containing 0.5% KCl. (5 ml for 1 g seed powder) in a blender, until the seed powder is thoroughly ground. The extract was transferred into conical flasks and thoroughly mixed in a shaker at 300 rpm for 30 min. The extracts were filtered through whatman No.41 filter paper and stored in the cool room for ELISA test.

ELISA (Indirect competitive ELISA):

The ELISA plates were coated with AFB_1-BSA conjugate in carbonate coating buffer at 100 ng/ml by dispensing 150 µl of the diluted toxin-BSA to each well of ELISA plate The coated plates were incubated in refrigerator (4°C) overnight The plates were thoroughly washed in three changes of Phosphate Buffered Saline with Tween (PBS-Tween) allowing 3 min for each wash. Bovin Serum Albumen (BSA) 0.2%, prepared in PBS–Tween was added to the plates at 200 µl per well and incubated at 37° C for 1 h. The plates were washed in three changes of PBS–Tween, allowing 3 min for each wash Antiserum dilution was prepared (in a tube) in PBS–Tween containing 0 2% BSA and incubated at 37°C for 45 min. AFB_1 standards were also prepared

separately by using healthy groundnut extract (1:10 diluted groundnut extract) at concentrations ranging from 100 ng to 10 picogram in 100 µl volume. Antiserum (50 µl) was added to each of the dilution aflatoxin standards (100 µl) and groundnut seed extract (100 µl) intended for analysis. The plates containing the mixture were incubated at 37° C for 1 h to facilitate the reaction between the toxin present in the sample with antibody. The plates were washed as mentioned in previous steps. 1:1000 dilution of goat anti-rabit IgG, lebelled with alkaline phosphatase, in PBS–Tween containing 0.2% BSA was prepared and added into the plates at 150 µl per well. The plates were incubated at 37°C for 1 h and washed like in previous steps. 150 µl of substrate solution (P-nitrophenyl phosphate prepared in 10% diethanolamine buffer, pH 9.8) was added to each well. The plates were incubated at room temperature (25°C) for 1 h or for shorter time, depending on the yellow colour development (in wells where low aflatoxin concentrations were used for competition). The absorbance was measured at 405 nm by ELISA reader. Using the values obtained for aflatoxin B_1 standards, curve was prepared with the help of computer (Sigma Plot, soft ware) taking aflatoxin concentrations on the "X" axis and optical density values on the "Y" axis.

Biocontrol field experiment:

The biocontrol agents (five *Trichoderma* isolates and six bacterial isolates) used for greenhouse experiment were also tested in field conditions. The experiment was conducted in postrainy 2001-2002) and rainy season 2002 at ICRISAT-Patancheru. Six hundred healthy and sound seeds/treatment, of groundnut cultivar ICGS 11 were coated with spore suspension and bacterial cell suspension of different biocontrol agents (BCA) in test using bentonite powder as filler and carboxy methyl cellulose (0.5%) as sticker. Multiplication of biocontrol agents (BCAs) and seed dressing with BCAs were carried out as mentioned elsewhere. Dried BCAs– treated seeds were sown in *A.flavus* sick plot at ICRISAT alfisols. The experiments were conducted in a randomized complete block (RCB) design with 12 treatments including control (seeds coated without BCA) with six replications/treatment. The plots consisted of 2 rows of 4m long with spacing of 60 cm between rows and 10 cm between plants. Dead seedlings were counted, 30 days after sowing and mortality percentage was calculated using total and died plants. The inoculum of the toxigenic strain of *A. flavus* (Af II-4) was multiplied on autoclaved pearl millet seed as described earlier. The spore load was approximately 10^9 spores g^{-1} of millet seed. Twenty grams of the inoculum/row of 4m length was applied at flowering stage (40 days after sowing) in furrows opened adjacent to the plants and

covered with soil. Irrigation was provided by overhead sprinklers. Water stress was imposed on the crop from 80 days after sowing and light irrigation was provided by running the sprinklers for ½ h at an interval of 15-18 days.

Assay of *A. flavus* and biocontrol agents' populations:

Soil samples were collected (one sample from each plot) at 40 DAS (just before *A. flavus* application), 80 DAS, and at harvesting of the crop to monitor the populations of *A. flavus* and biocontrol agents in different treatments. Soil samples were analyzed by using serial dilution method as mentioned earlier. Two dilutions (10^{-3} and 10^{-4}) were simultaneously plated on TSM and AFPA media for the estimation of *Trichoderma* and *A. flavus* respectively. The last two dilutions (10^{-7} and 10^{-8}) were plated on Glucose casamino acid Yeast extract (GCY) medium to estimate bacterial populations in the soil. AFPA medium plates were incubated at 28°C for 3 days and typical *A. flavus* colonies were counted and population was expressed as cfu g^{-1} soil. *Trichoderma* specific medium plates were incubated for 6 days and LBA plates were incubated for 48 hrs at 28°C. *Trichoderma* and bacterial colonies were counted and populations were expressed as cfu g^{-1} of soil.

Seed infection and Aflatoxin Estimation:

The pods were harvested at pod maturity and threshed and dried. Biomass yield $plot^{-1}$ was recorded after drying it in hot air oven for 2 days at 60°C. Pods were sun dried and pod yield $plot^{-1}$ (kg) was recorded. Pods were hand shelled and kernel yield $plot^{-1}$ (kg) was recorded. Hundred seeds from each plot were surface sterilized as mentioned earlier and plated on Czapex Dox Agar (CDA) medium supplemented with rose bengal (30 mg L^{-1}) and streptomycin (1g L^{-1}). Ten seeds were kept in each plate of medium. The plates were incubated at 28°C in dark for five days. Kernels showing *A. flavus* infection were recorded and percent seed infection was calculated.

Twenty grams of seeds were weighed from each plot, surface sterilized and incubated for 3 days. Then the seeds were ground and aflatoxin were extracted in 70% methanol containing 0.5% KCl. The aflatoxin content of the samples was estimated by indirect competitive ELISA. The extraction and estimation of aflatoxin content were done as per the procedure mentioned elsewhere.

1. Improvement of Biocontrol Efficacy of *Trichoderma* spp:

Trichoderma viride (T47), which showed biocontrol efficacy against *A. flavus* under field and greenhouse conditions was used for ultraviolet (UV) irradiation experiment to improve it's biocontrol

efficacy. UV irradiation was done following the method used by Papavizaz *et al.* (1982) with slight modification.

Irradiation: Spore suspension of *Trichoderma viride* (T47) was prepared. One ml of the spore suspension was transferred with the help of micropipette (Finn pipette) into a sterilized petriplate containing *Trichoderma* specific medium (TSM) and spread with the help of cell spreader. The plates were immediately exposed to UV irradiation at the distance of 30 cm between UV lamp and surface of the medium in the plates. Five plates were maintained for irradiation and the experiment was repeated thrice.

After two days of incubation, the developing colonies were subcultured in fresh TSM plates and incubated for 7 days at 28^0C. The mutants were grouped into 3 groups based on their growth rate and subgroups based on the colour and mode of sporulation.

Evaluation of mutants of T47 for antagonism against Af 11-4:

The biocontrol potential of the mutants against *A. flavus* (Af 11-4) was tested for antagonistic effect using dual culture technique mentioned elsewhere. Fourteen mutants were selected from the three groups for this test. Four plates were maintained for each test mutant and the whole experiment was repeated twice. Plates inoculated with *A. flavus* alone served as control.

3.4 EVALUATION OF BOTANICALS FOR THEIR EFFICACY AGAINST *A. FLAVUS IN VITRO* AND *IN VIVO*

***In vitro* test**

Four different Neem commercial formulations were obtained from Hyderguda Pesticide shops, Hyderabad. Three of them Nivaar EC (Shri Disha Biotech. PVT, LTD, 4-69, canara Nagar, Peerzadiguda, Uppal (m), Hyderabad-39), Sasya Neem EC (Sasya shyamala Agri inputs PVT, LTD, G 11, Emerald Apts, Panjagutta, Hyd-82) and Sunny EC (Sunny Neem Extracts PVT, LTD, 136, Dwarakapuri, Panjagutta, Hyderabad-500082) are Neem kernel extract formulations while other one, Starneem EC (Biostar Agri Tech. PVT, LTD. 1-1-570/A Gandhinagar, Hyderabad-500020) is neem oil based formulation. All the formulations contained 0.15% azadirachtin. The formulations were tested against *A. Flavus* (Af 11-4) *in vitro* by following poison food technique (Aneja, 1996). Potato Dextrose Agar (PDA) medium was prepared in conical flasks and added required quantity of 4 formulations to prepare different concentrations (100, 200, 300, 400 and 500 ppm) before pouring the medium into the petriplates. A disc of 3 day old culture of *A. Flavus* strain (Af 11-4) was inoculated at the centre of each plate. *A. flavus* inoculated on PDA medium alone (without Neem formulations) surved as control. The inoculated plates were incubated at 28°C for 7 days. Then the mycelial growth of *A. flavus* was measured

and percent growth inhibition was calculated by using the formula mentioned in second objective in this chapter.

***In vivo* test:**

Two neem formulations viz., Nivaar and SasyaNeem (Neem kernel extract formulations) that were found effective against *Af* 11-4 *in vitro*, were selected for greenhouse and field experiments. The soil preparation and pot (12.5") filling was done as per the procedure mentioned earlier elsewhere. The seeds of groundnut cultivar ICGS 11 were sown in the pots arranged randomly on greenhouse benches, two pots / rep/treatment and the experiment was replicated four times. Six seeds per pot were sown and thinned into 4 plants pot^{-1}. Forty days after sowing, the *A. flavus* (Af 11-4) inoculum multiplied on peal millet grains as mentioned earlier was applied on the pots (10g pot^{-1}). Five days later (45 days after sowing) 1% Neem solutions were applied on the top soil of pots and thin layer of sterilizer soil was covered. Light irrigation was provided to the pots. 80 days after sowing, water stress was imposed on the pots till harvesting and irrigation was provided whenever wilting symptom was observed on the plants. The crop was harvested at maturity and biomass weight (kg), pod weight (kg) were recorded after drying them as mentioned earlier. Then the pods were shelled and seed weight (kg) was also recorded.

Seed Infection and Aflatoxin Estimation:

Fifty seeds were counted from each replication of each treatment, surface sterilized with 1% sodium hypochlorite and plated on Czapex Dox Agar (CDA) medium and incubated at 28°C for five days. Number of seeds showing typical *A. flavus* growth was counted and percent seed infection was calculated. Ten grams of seeds from each replication of each treatment were weighed, surface sterilized and soaked in water for 1 h, later dried and incubated for 3 days at 28°C. Aflatoxin extraction and estimation by ELISA was done as discussed earlier in this chapter.

3.5 SCREENING OF GROUNDNUT GENOTYPES AND ADVANCED BREEDING LINES FOR RESISTANCE TO *A. FLAVUS* SEED COLONIZATION AND SEED INFECTION IN THE FIELD

Genotypes. Forty-five genotypes were selected from different source (Table 3) from ACIAR-ICRISAT collaborative project for evaluation under field conditions for preharvest seed infection, and *in vitro* seed colonization under laboratory conditions at ICRISAT, Patancheru.

Evaluation for preharvest seed infection by *Aspergillus flavus*.

Forty-five genotypes and four controls (Table 3) were planted in the field during the 2001 rainy season in randomized complete block

Table 1: Sources of 49 entries used for screening *Aspergillus flavus* resistance

Genotype ID	Source	Genotype ID	Source
ICGS 11	Medium-duration	ICGV 98163	Medium-duration
ICGV 86158	Dormancy	ICGV 98170	Medium-duration
ICGV 86590	Foliar diseases resistant	ICGV 98383	Foliar diseases resistant
ICGV 86699	Foliar diseases resistant	ICGV 99029	Foliar diseases resistant
ICGV 88145	*A. Flavus* resistant	ICGV 99032	Foliar diseases resistant
ICGV 89104	*A. Flavus* resistant	ICGV 99054	Foliar diseases resistant
ICGV 91114	*A. Flavus* resistant	J 11	*A. Flavus* resistant
ICGV 91278	*A. Flavus* resistant	NC Ac 343	Termite resistant
ICGV 91279	*A. Flavu:;* resistant	WUE (7) ICR 48	Water use efficient
ICGV 91283	*A. Flavus* resistant	WUE (40) JAL 17	Water use efficient
ICGV 91284	*A. Flavus* resistant	WUE (116) TIR 31	Water use efficient
ICGV 92206	Short-duration	WUE (159) ICR 43	Water use efficient
ICGV 93280	*A. Flavus* resistant	WUE (187) ICR 10	Water use efficient
ICGV 93291	*A. Flavus* resistant	TCGP10	Thick shell
ICGV 94341	Short-duration	TCGP 5	Thick shell
ICGV 94350	Short-duration	TCGP 6	Thick shell
ICGV 94358	Short-duration	TCGS 320	Nematode tolerance
ICGV 94433	*A. Flavus* resistant	TCGS 645	Soil-insects tolerance
ICGV 95322	Short-duration	TCGS 647	Soil-insects tolerance
ICGV 95454	*A. Flavus* resistant	TPT 3	Nematode tolerance
ICGV 95460	*A. Flavus* resistant	Controls	
ICGV 95469	*A. Flavus* resistant	ICGV 86031	
ICGV 95477	*A. Flavus* resistant	ICG 44	
ICGV 95492	*A. Flavus* resistant	ICGS 76	
ICGV 95494	*A. Flavus* resistant	CSMG 84-1	

design with 4 replications. Each genotype was planted in 2 rows of 4 m long in each replication with 60 cm spacing between rows and 10 cm between plants. All the plots were applied with a highly toxigenic strain (Af 11-4) of *A. flavus* multiplied on autoclaved pearl millet grains at flowering stage. About 20 gm of inoculum (ca. $1x10^9$ spores g^{-1} of infested millet grain) applied in each plot adjacent of the plants. The plots were provided with light irrigation to provide sufficient moisture for the increase of fungal spores. Drought was imposed by reducing 60% of irrigation from 80 days till harvesting. The pods were harvested, sun-dried and shelled.

Kernel assay for preharvest infection:

One hundred seeds from each replication/ genotype were surface sterilized with 1% sodium hypochlorite and thoroughly washed with sterile distilled water and plated on Czapex Dox Agar (CDA) medium supplemented with rose Bengal and Streptomycin, and incubated for five days at 28°C. Number of infected seeds was recorded and percentage of infection was calculated using total and infected seeds.

Kernel assay for cotyledonary resistance.

Seed colonization test was done in the laboratory as per the procedure followed by Thakur *et al.* (2000). Twenty-four seeds from each replication/ genotype were surface sterilized with 1% aqueous

solution of sodium hypochlorite (Clorox) for 3 min and washed in three changes of distilled sterilized water. These were uniformly pin- pricked and rolled gently with the spore suspension (ca. $1x10^6$ spore ml^{-1}) of *A flavus* (Af 11-4), placed the individual kernels in each well of the multiwell dishes, and incubated at 28°C for 3 days under humid chambers. Seed colonization severity was recorded using 1-4 scale (Thakur *et al.* 2000) where 1< 5% of seed surface colonized with scanty mycelial growth and no sporulation and 4 > 50% seed surface colonized with heavy sporulation. Colonization severity was calculated using the formula:

$$\text{Colonization severity} = \frac{1(x) + 2(x) + 3(x) + 4(x)}{\text{Total number of seeds inoculated}}$$

where x = no of seeds colonized in each severity class.

Kernel assay for seed coat resistance:

Twenty-four kernels from each genotype/replication were surface sterilized, and inoculated with 1 ml spore suspension ($1x10^6$ spores ml^{-1}) of a highly toxigenic strain (Af 11-4) of *A. flavus* (Thakur *et al.* 2000). These kernels were placed in multiwell dishes and followed the same procedure as mentioned earlier. The surface colonization severity was recorded using the above-mentioned scale and colonization

severity was also calculated for each genotype. Individual apparently healthy seeds of 14 genotypes were selected for screening in the field.

Field experiment:

Fourteen genotypes out of 45 genotypes screened in the laboratory were selected (based on colonization severity) for field experiment in post rainy season 2001-2002. These were sown in *A. flavus* sick plot at ICRISAT –Patancheru. All procedures of earlier mentioned field experiment were followed. After harvesting and shelling the seeds were again subjected to *in vitro* seed colonization test.

Preliminary evaluation of groundnut germplasm from ICRISAT core collection for resistance to *in vitro* seed colonization by *A. flavus*

One hundred eighty four groundnut germplasm collected from different countries (Table 3) were evaluated for resistance to *A. flavus* seed colonization in laboratory at ICRISAT-Patancheru. These genotypes belong to different botanical varieties (Table 3). The seeds were obtained from ICRISAT gene bank. Ninety-six seeds of each genotype were surface sterilized by sodium hypochlorite (1%) and thoroughly washed in three changes of sterilized distilled water. Spore suspension of highly toxigenic strain of *A. flavus* (Af 11-4) was prepared from well-sporulated 7-day-old culture grown on PDA plates. The spore suspension was collected in a sterilized beaker and added Tween-20

Table 3: List of groundnut core collection used for preliminary screening for *A. flavus* resistance+A361

ICG	Botanicle Variety	Country name
36	vulgaris	India
76	hypogaea	India
81	vulgaris	Unknown
111	hypogaea	Unknown
115	fastigiata	India
118	vulgaris	India
163	hypogaea	Unknown
188	hypogaea	India
297	fastigiata	USA
332	fastigiata	Brazil
334	vulgaris	China
397	vulgaris	USA
434	vulgaris	USA
442	vulgaris	USA
513	hypogaea	India
532	hypogaea	Unknown
721	hypogaea	USA
862	hypogaea	India
875	hypogaea	India
928	hypogaea	Unknown
1137	vulgaris	India
1142	fastigiata	Benin
1274	hypogaea	Indonesia
1399	fastigiata	Malawi
1415	fastigiata	Senegal
1519	vulgaris	India
1668	hypogaea	USA
1711	vulgaris	Bolivia
1973	vulgaris	India
2019	vulgaris	India
2106	vulgaris	India
2381	hypogaea	Brazil
2511	hypogaea	India
2772	hypogaea	Nigeria
2773	hypogaea	Tanzania
2777	hypogaea	India
2857	hypogaea	Argentina
2925	hypogaea	India
3027	hypogaea	India
3053	hypogaea	India
3102	vulgaris	India
3240	vulgaris	Uganda
3343	vulgaris	India

ICG	Botanicle Variety	Country name
3584	vulgaris	India
3673	fastigiata	Korea
3681	fastigiata	USA
3746	vulgaris	Argentina
3775	fastigiata	Brazil
3992	hypogaea	India
4156	hypogaea	Unknown
4343	hypogaea	India
4389	hypogaea	India
4412	hypogaea	USA
4527	hypogaea	Uganda
4538	hypogaea	India
4543	vulgaris	Unknown
4598	hypogaea	India
4670	fastigiata	Sudan
4684	vulgaris	USA
4729	vulgaris	China
4746	hypogaea	Israel
4750	vulgaris	Paraguay
4911	vulgaris	Malawi
4955	vulgaris	India
4998	hypogaea	China
5016	hypogaea	USA
5195	vulgaris	Sudan
5221	fastigiata	Argentina
5236	vulgaris	Chile
5286	hypogaea	Zambia
5327	hypogaea	USA
5475	fastigiata	Kenya
5494	vulgaris	Malaysia
5609	fastigiata	Sri Lanka
5662	hypogaea	China
5663	hypogaea	China
5745	hypogaea	Puerto Rico
5779	vulgaris	India
5827	hypogaea	USA
5891	hypogaea	India
6022	fastigiata	Sudan
6057	hypogaea	USA
6201	fastigiata	Cuba
6263	vulgaris	Burkina Faso
6375	vulgaris	Unknown
6402	hypogaea	Unknown
6407	vulgaris	Zimbabwe

ICG	Botanicle Variety	Country name
6646	fastigiata	Unknown
6654	vulgaris	Unknown
6667	hypogaea	USA
6703	vulgaris	Paraguay
6766	hypogaea	USA
6813	hypogaea	Senegal
6888	fastigiata	Brazil
6892	hypogaea	USA
6913	hypogaea	USA
6993	hypogaea	Brazil
7000	hypogaea	USA
7153	hypogaea	India
7181	fastigiata	India
7190	vulgaris	Brazil
7243	hypogaea	USA
7906	vulgaris	Zimbabwe
7969	vulgaris	Zimbabwe
8083	hypogaea	Russia & CISs
8106	fastigiata	Peru
8285	hypogaea	USA
8490	hypogaea	Somalia
8517	fastigiata	Bolivia
8567	vulgaris	Uruguay
8760	hypogaea	Zambia
9037	hypogaea	Cote d'Ivoire
9157	vulgaris	Puerto Rico
9249	vulgaris	Mauritius
9315	fastigiata	USA
9418	vulgaris	Martinique
9507	vulgaris	Philippines
9666	hypogaea	India
9777	hypogaea	Mozambique
9809	vulgaris	Mozambique
9842	hypogaea	Tanzania
9905	hypogaea	Zambia
9961	hypogaea	Unknown
10036	peruviana	Peru
10092	fastigiata	Zimbabwe
10185	hypogaea	USA
10384	vulgaris	Nigeria
10474	fastigiata	Cuba
10479	fastigiata	Uruguay
10554	fastigiata	Argentina
10566	fastigiata	Congo
10890	fastigiata	Peru

ICG	Botanical Variety	Country name
11088	Peruviana	Peru
11109	hypogaea	Taiwan
11144	fastigiata	Argentina
11219	hypogaea	Mexico
11249	vulgaris	Tanzania
11322	hypogaea	India
11426	vulgaris	India
11457	hypogaea	India
11515	vulgaris	China
11651	vulgaris	China
11687	vulgaris	India
11855	hypogaea	Korea
11862	hypogaea	Korea
12000	hypogaea	Mali
12189	vulgaris	Unknown
12276	hypogaea	Bolivia
12370	hypogaea	India
12625	aequatoriana	Ecuador
12672	hypogaea	Bolivia
12682	vulgaris	India
12697	vulgaris	India
12879	vulgaris	Myanmar
12921	vulgaris	Zimbabwe
12988	vulgaris	India
13099	hypogaea	Unknown
13491	vulgaris	Central African Republic
13603	vulgaris	Indonesia
13723	hypogaea	Niger
13787	hypogaea	Niger
13856	fastigiata	Uganda
13858	fastigiata	Uganda
14008	hypogaea	Central African Republic
14106	fastigiata	United Kingdom
14118	fastigiata	United Kingdom
14127	fastigiata	United Kingdom
14466	hypogaea	Nigeria
14475	hypogaea	Nigeria
14482	hypogaea	Nigeria
14523	hypogaea	Unknown
14630	fastigiata	Brazil
14705	fastigiata	Cameroon
14710	fastigiata	Cameroon
14985	vulgaris	Unknown
15042	hypogaea	Unknown
15190	hypogaea	Costa Rica
15287	hypogaea	Brazil
15309	fastigiata	Brazil
20016	hypogaea-hst	Ecuador

@ 1:1000. The spore concentration was adjusted to $1x10^6$ spores ml^{-1}. The sterilized seeds were inoculated with 2 ml of the spore suspension for each genotype. The inoculated seeds were plated in multiwell dishes (24 wells/dish), one seed per well. Four plates were maintained for genotype as four replications. Two resistant and two susceptible genotypes were also inoculated as controls. The dishes were kept in humid chamber consist of two plastic trays lined with wet blotting paper to maintain the high humidity (> 90%) and incubated at 28°C for 5 days. Number of seeds colonized in each severity class was recorded using 1-4 scale and colonization severity was calculated for each genotype following the formula mentioned earlier.

Advanced screening of selected germplasm lines:

Ninety-five genotypes that showed low colonization severity (≤ 2 on 1-4 scale) in the preliminary screening were evaluated again to confirm the preliminary results. Screening procedure was followed as mentioned earlier at preliminary screening. After recoding the colonization severity, the seeds were sprayed with Hexane-n to kill the *A. flavus* spores and dried at room temperature (25°C) for two days. These seeds were used for aflatoxin estimation.

Aflatoxin Estimation:

The seeds used for colonization test were also used for aflatoxin estimation, after spraying them with Hexane n and drying. Twenty grams from each genotype were used for aflatoxin estimation. Aflatoxin extraction and estimation by ELISA was done as per the procedure mentioned earlier in this chapter.

3.6 INTEGRATED DISEASE MANAGEMENT (IDM) IN REDUCING AFLATOXIN CONTAMINATION

Two biocontrol agents (Bacterial isolate (B33) and *Trichoderma* isolate (Tv_{47}) that showed biocontrol efficacy against *A. flavus* in both greenhouse and field conditions were selected for IDM experiment. One Neem Kernel extract formulation (SasyaNeem) that is effective against *A. flavus* and showed compatibility with *Trichoderma viride* (Tv_{47}) was also selected for IDM experiment. The promising groundnut genotype, ICGV 91114 was selected from 45 groundnut genotypes screened for resistance to *A. flavus* seed colonization and infection. The susceptible groundnut cultivar JL24 was used as susceptible check.

The IDM experiment was conducted at ICRISAT, Patancheru and at Agricultural Research Station (Acharya N. G. Ranga Agricultural University), Anantapur simultaneously in the 2002 rainy season and

repeated at ICRISAT Patancheru in the Postrainy season 2002-2003. The experiment consisted of the following Ten Treatments:

T_1 : Resistant genotype alone (ICGV 91114)

T_2 : Resistant genotype +*Trichoderma viride* (Tv_{47})

T_3 : Resistant genotype + Bacteria (B_{33})

T_4 : Resistant genotype + SasyaNeem (Neem kernel extract)

T_5 : Resistant genotype + *Trichoderma* +Bacteria +SasyaNeem

T_6 : Susceptible genotype alone (JL24)

T_7 : Susceptible genotype +*Trichoderma viride* (Tv_{47})

T_8 : Susceptible genotype + Bacteria (B_{33})

T_9 : Susceptible genotype + SasyaNeem (Neem kernel extract)

T_{10} : Susceptible genotype + *Trichodrema* +Bacteria +SasyaNeem

Preparation of biocontrol agents' inoculum and seed coating with the inoculum was done as discussed in biocontrol experimcnt. The experiment was conducted in split block design with resistant and susceptible genotypes as main plots and biocontrol agents and sasyaNeem as sub-plots with 4 replications. The individual plots consisted of four rows of 4m long/treatment with spacing of 60 cm between rows and 10 cm between plants at ICRISAT and 30 cm x 10 cm at Anantapur. Irrigation was provided when required. Mortality of the plants was noted at 30 days after sowing in all the plots. A day of 50% flowering was also recorded.

Application of Bioagents and Sasyaneem in Integrated Treatment:

In T_5 and T_{10}, where the biocontrol agents were integrated with Sayaneem, the bacterial (B33) was treated with the seeds before sowing

as per the procedure mentioned earlier. Sasyaneem was applied at 45 DAS as soil drenching. *Trichoderma viride* (T47) was multiplied on autoclaved pearl millet grains just like in *A. flavus* multiplication and applied in furrows opened adjacent to plants and covered with thin layer of soil. Twenty grams of inoculum ($1x10^7$ spores g^{-1} of millet grain) was applied in each row of 4m long at 30 DAS.

Application of *A. flavus*:

Preparation and application of *A. flavus* inoculum at flowering stage was done as mentioned in biocontrol experiment.

Estimation of populations of *A. flavus* and biocontrol agents:

Soil samples where collected at 40 DAS and 80 DAS after sowing and at harvesting from each plot to monitor the populations of *A. flavus* and biocontrol agents in different treatments. The samples were processed by following serial dilution method as mentioned earlier in this chapter. For the estimation of *A. flavus* and *Trichoderma* populations, 10^{-3} and 10^{-4} dilutions were plated simultaneously on AFPA and TSM respectively. For bacterial estimation 10^{-7} and 10^{-8} dilutions were plated on GCY medium. The inoculated plates were incubated at 28°C for 48 h for Bacteria, 3 days for *A. flavus* and 6 days for *Trichoderma*. Number of colonies was counted and populations were expressed in cfu g^{-1} of soil.

Harvesting:

The crop was harvested at maturity, threshed and sun-dried for 3 days. Both biomass and pods weight (kg) were recorded for each plot. Then the pods were shelled by hand and kernel yield (kg) $plot^{-1}$ was recorded. In the rainy season 2002, only fifty seeds from each plot (due to seed shortage) were plated on CDA Medium supplemented with rose Bengal (25 mg L^{-1} and Streptomycin (1g L^{-1}) and incubated for 5 days at 28°C. Number of seeds infected with *A. flavus* was counted and percent infection was calculated. The remaining seeds from each plot were used for aflatoxin estimation by ELISA as discussed earlier. In the postrainy season 2002-2003, hundred seeds were used for seed infection test and 20 g seed for aflatoxin estimation by ELISA.

3.7 EVALUATION PLANT GROWTH PROMOTING EFFECT OF BIOCONTROL AGENTS

Plant growth promoting potential of biocontrol agents (five *Trichoderma* isolates and six bacterial isolates) used in biocontrol experiment of this study was evaluated in greenhouse condition. The experiment consisted of the following treatments:

T_1	*Trichoderma viride* (T21)
T_2	*Trichoderma harzianum*(T23)
T_3	*Trichoderma viride*(T38)
T_4	*Trichoderma viride* (T47)
T_5	*T. viride* (T47)
T_6	Bacterial isolate (B6)

T_7 : Bacterial isolate (B18)

T_8 : Bacterial isolate (B33)

T_9 : Bacterial isolate (B50)

T_{10} : Bacterial isolate (B58)

T_{11} : *Pseudomonas flourescens* (PF 2)

T_{12} : Control (without BCA)

Preparation of inocula of biocontrol agents and seed coating:

Preparation of inocula of different biocontrol agents and seed treatment with them before sowing was done as detailed in biocontrol greenhouse experiment of this chapter.

Soil preparation and sowing:

Sand soil was autoclaved for two consecutive days at 121°C for 30 minutes. The autoclaved sand was filled in 8" pots. Then the pots were arranged randomly on greenhouse benches and labeled with different treatments. The experiment consisted of 12 treatments each one pot per replication with four replications. The groundnut (ICGS 11) seeds treated with different biocontrol agents to be tested were sown in respective pots, 5 seeds pot^{-1} and thinned to 3 plants pot^{-1}. The pots were irrigated as required. Nutrients required for the plant growth were provided in the form of arson solution ones a week. 35 days DAS the plants were gently pulled from the pots (without disturbing the root system) after watering. The roots were separated from the shoot and root and shoot length and root and shoot weight were recorded. Percent increase in root and shoot length and root and shoot weight was calculated.

CHAPTER IV

RESULTS

The results pertaining to the present investigation "Management of aflatoxin contamination in groundnut through biological control, host plant resistance and botanicals" are presented under the following heads :

4.1 Isolation and characterization of *trichoderma isolates* and bacterial strains from groundnut

4.2 Evaluation of *Trichoderma spp* and bacterial strains for *in vitro* and *in vivo* antagonistic activity against *Aspergillus flavus*

4.3 improvement of *t. viride* (t47) by irradiation

4.4 efficacy of neem commercial formulations against *A. flavus in vitro* and *in vivo*

4.5 Identification of germplasm accessions, advance breeding lines and varieties for resistance to *in vitro* seed colonization and seed infection by *A. flavus*

4.6 Evaluation of the effects of host plant resistance, biocontrol agents and botanicals as an integrated management practice to reduce aflatoxin contamination in groundnut

4.7 Evaluation of plant growth promoting potential of biocontrol agents in greenhouse conditions

4.1 ISOLATION AND CHARACTERIZATION OF *TRICHODERMA ISOLATES* AND BACTERIAL STRAINS FROM GROUNDNUT RHIZOSPHERE SOILS

Forty-nine isolates of *Trichoderma* were obtained from the soil samples collected from different groundnut growing areas of Andhra Pradesh using dilution plate technique on the specific medium (TSM). Seventy-seven bacterial isolates were also obtained from the same soil samples on the glucose cyamino acid yeast extract (GCY) medium.

Colony morphology of all the isolates of *Trichoderma* isolates were more or less similar showing sparse to thin cottony mycelial mass. Sporulation started after 72 h of incubation at 28° C on TSM for all the isolates.

4.2 EVALUATION OF *TRICHODERMA SPP* AND BACTERIAL STRAINS FOR *IN VITRO* AND *IN VIVO* ANTAGONISTIC ACTIVITY AGAINST *ASPERGILLUS FLAVUS*

4.2.1 Screening of *Trichoderma* Isolates I*n Vitro*

All the 49 *Trichoderma* isolates were screened for their efficacy to inhibit the mycelial growth of *A. flavus* (Af 11-4) on PDA medium using the dual-culture method. The maximum mycelial growth of *A. flavus* was recorded in control treatment (4.33cm) and the minimum in T48 (1.00cm) followed by T47 (1.03cm) and T38 (1.07 cm) (Table 4). All the *Trichoderma* isolates tested significantly inhibited the mycelial growth of *A. flavus* compared with control.

4.2.2 Screening of Bacterial Isolates *In Vitro*

All the 77 bacterial isolates were evaluated for their biocontrol efficacy against *A. flavus* (Af 11-4). The maximum mycelial growth (4.2cm) of *A. flavus* was recorded in control, B22, B3 and B64 and the minimum mycelial growth was recorded in B33 (2.20) and B6 (2.23), followed by B18 and B58 (2.33) (Table 5). Fifty-two of the 77 bacterial isolates significantly inhibited the *A. flavus* growth while 25 isolates were similar to the control.

4.2.3 Second Screening of 19 Bacterial Isolates

The most effective nineteen bacterial isolates selected from preliminary screening were tested against *A. flavus* (Af 11-4) to confirm the

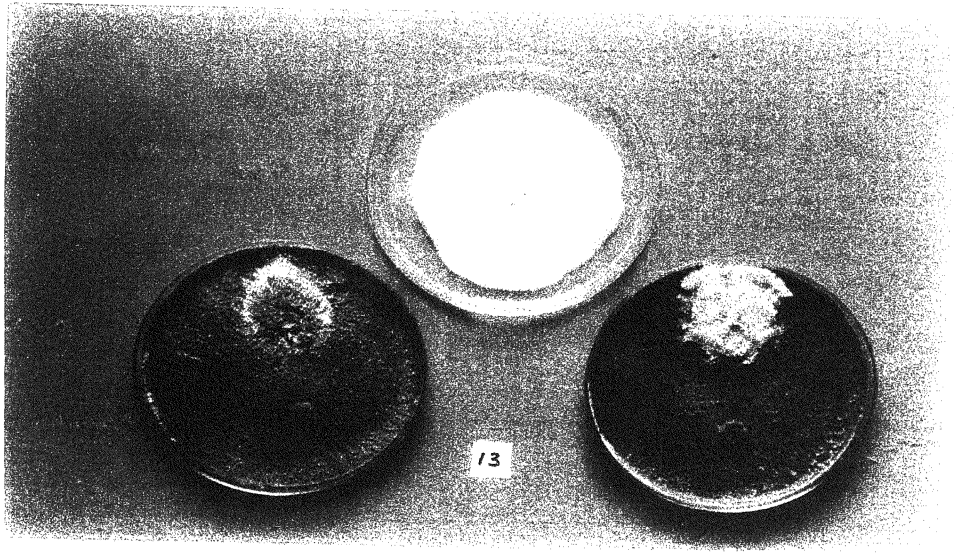

Plate 1: Antagonistic activity of some *Trichoderma* isolates against *Aspergillus flavus*

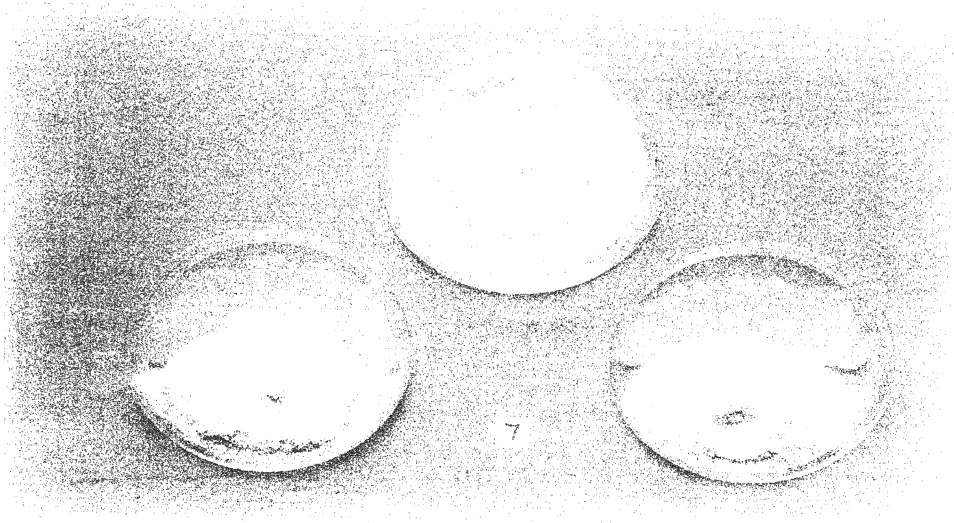

Plate 2: Antagonistic activity of some *Bacterial* isolates against *Aspergillus flavus*

Table 4:*In vitro* antagonistic activity of *Trichdema* isolates against *A. flavus* (Af 11-4)

Trichoderma isolates	Mycelial growth of *A. flavus* (cm) *	Reduction over control %
T1	1.13	73.83
T2	1.30	69.98
T3	1.57	63.82
T4	1.60	63.05
T5	1.30	69.98
T6	1.27	70.75
T7	1.73	59.97
T8	1.40	67.67
T9	1.40	67.67
T10	1.63	62.28
T11	1.30	69.98
T12	1.20	72.29
T13	1.47	66.13
T14	1.23	71.52
T15	1.27	70.75
T16	1.23	71.52
T17	1.23	71.52
T18	1.20	72.29
T19	1.20	72.29
T20	1.30	69.98
T21	1.10	74.60
T22	1.20	72.29
T23	1.30	69.98
T24	1.37	68.44
T25	1.43	66.90
T26	1.90	56.12
T27	1.33	69.21
T28	1.17	73.06
T29	1.43	66.90
T30	1.13	73.83
T31	1.63	62.28
T32	1.33	69.21
T33	1.17	73.06
T34	1.50	65.36
T35	2.33	46.11
T36	2.17	49.96
T37	1.23	71.52
T38	1.07	75.37
T39	1.63	62.28
T40	2.23	48.42
T41	1.23	71.52
T42	1.23	71.52
T43	1.97	54.58
T44	1.17	73.06
T45	2.03	53.04
T46	1.77	59.20
T47	1.03	76.14
T48	1.00	76.91
T49	1.43	66.90
T23	1.13	73.83
Control	4.33	0.00
SEM±	0.12	
LSD (P =0.05)	0.35	
CV%	14.8	

* Mean of four replications

Table 5 : *In vitro* antagonistic activity of 77 bacterial isolates against *A. flavus* (Af 11-4)

Bacterial isolates	Mycelial growth of *A. flavus* (cm)*	Bacterial isolates	Mycelial growth of *A. flavus* (cm)*
B1	3.30	B44	2.97
B2	3.43	B45	2.77
B3	4.20	B46	2.50
B4	3.53	B47	3.10
B5	3.47	B48	3.37
B6	2.23	B49	2.37
B7	3.20	B50	2.40
B8	4.00	B51	2.63
B9	3.63	B52	3.23
B10	2.67	B53	2.60
B11	3.97	B54	3.67
B12	3.70	B55	2.83
B13	3.93	B56	3.83
B14	4.17	B57	3.20
B15	3.77	B58	2.33
B16	4.07	B59	3.63
B17	2.63	B60	3.77
B18	2.33	B61	3.57
B19	4.07	B62	3.50
B20	4.17	B63	3.17
B21	4.13	B64	4.20
B22	4.20	B65	2.83
B23	2.67	B66	3.53
B24	4.00	B67	3.17
B25	4.13	B68	2.80
B26	3.47	B69	2.77
B27	4.20	B70	2.80
B28	2.63	B71	2.80
B29	3.37	B72	3.00
B30	3.63	B73	2.90
B31	2.60	B74	3.03
B32	3.70	B75	2.77
B33	2.20	B76	2.77
B34	2.70	B77	2.93
B35	3.53	Control	4.20
B36	2.83		
B37	2.57	SEM±	0.23
B38	2.97	LSD (P =0.05)	0.65
B39	4.03	CV%	12.4
B40	2.67		
B41	2.63		
B42	2.70		
B43	3.60		

* Means of four replications

Table 6 : Effect of 19 selected bacterial isolates on mycelial growth of *Aspergillus flavus* (Af 11-4) *in vitro*

Bacterial isolates	Mycelial growth of *A. flavus* (cm)*	Reduction over control %
B6	2.07	50.79
B10	2.67	36.51
B17	2.50	40.48
B18	2.40	42.86
B23	2.77	34.13
B28	2.67	36.51
B31	2.60	38.10
B33	2.27	46.03
B34	2.87	31.75
B37	2.70	35.71
B40	2.57	38.89
B41	2.67	36.51
B42	2.80	33.33
B46	2.70	35.71
B49	2.57	38.89
B50	2.43	42.06
B51	2.83	32.54
B53	2.87	31.75
B58	2.37	43.65
P. fluorescens (Pf 2)	2.47	41.27
Control	4.20	0.00
SEM±	0.70	
LSD (P =0.05)	1.99	
CV%	4.50	

* Mean of four replications

results of preliminary screening. Minimum mycelial growth was observed on B6 (2.07) followed by B33 (2.27) and B58 (2.37). All the bacterial isolates tested, differed significantly from the control. Two isolates (B6, B33) were significantly superior over positive control (previously used in controlling *A. flavus*) *Pseudomonas fluorescens* (PF2) and three isolates (B18, B50, B58) were at par with the positive control (PF2) (Table 6).

4.2.4 Evaluation of Selected Biocontrol Agents under Greenhouse Conditions (2001-2002 and 2002)

Highly promising five *Trichoderma* isolates *T. viride* (T21), *T. harzianum* (T23), *T. viride* (T38), *T. viride* (T48) *T. viride* (T47) and six bacterial isolates (B6, B18, B33, B50, B58 and *Pseudomonas fluorescens* (PF2) were selected for *in vivo* evaluation. The experiment was repeated twice in the greenhouse.

4.2.4.1 Effect of biocontrol agents on seedling establishment and yield parameters

Seedling establishments in the pots were similar in all the treatments and there was no significant difference between treatments and the control in both the experiments. The total number of plants ranged from 3.5 to 4 in all the treatments (Table 7).

Most treatments with biocontrol agents gave better pod yield than control. Treatments with *T. viride* (T21) and *P. fluorescens* (PF2), gave significantly higher pod yield than control and the yield increased by 30.88 and 29.03%, respectively, over control. Treatments with *T. harzianum* (T23), B18 and B33 also increased the pod yield by 22.65, 22.19 and 20.42% respectively. However, T. *viride* (T38) and *T. viride*

(T47) were at par with the control (Table 7) In the second experiment, all the biocontrol agents gave increase in pod yield over the control The increase in pod yield ranged from 5 49 to 25 63% *T harzianum* (T23) increased the pod yield significantly (25 63%) followed by *T viride* (T38), *T viride* (T21), B33 and B18 which recorded 22 99, 20.29, 19.90 and 19 65%, respectively (Table 7)

All the biocontrol agents gave better kernel yield except *T viride* (T38) and *T viride* (T47), which were at par with the control *Pseudomonas fluorescens* (PF2), *T viride* (T21) and *T harzianum* (T23) gave 23.74, 23 32 and 22 20 % increase in kernel yield, respectively over the control (Table 7) In the second experiment, *T viride* (T38) gave the highest seed yield and significantly increased the seed weight (30.23%) *T harzianum* (T23) and bacterial isolate (B33) increased the yield by 25.03% and 21 45% respectively Least seed yield increase (3.44) was recorded on bacterial isolate (B6) (Table 7)

All the biocontrol agents were better than the control in increasing the crop biomass yield The increase in biomass yield over control ranged from 2.80 to 28 05% The maximum increase (28.05 %) was recorded by *T viride* (T38) followed by B50 (25.28%) and B6 (24.07%). Minimum increase in biomass yield (2.80%) was recorded on B58 (Table 7). In the second experiment, the increase ranged from 6.27 to 25.91%. *Trichoderma* isolate (T21) and bacterial isolate (B33)

Table 7 : Effect of biocontrol agents (BCAs) on yield parameters of groundnut cultivar ICGS 11 under greenhouse conditions, Patancheru 2001-2002 and 2002

BCAs	Total No.of plants*		Pod yield/2 pots (g)				Seed yield/2 pots (g)				Biomass yield/2 pots (g)			
	Exp. I	Exp. II	Exp. I	Exp. I reduction %	Exp. II	Exp. II reduction %	Exp. I	Exp. I reduction %	Exp. II	Exp. II reduction %	Exp. I	Exp. I reduction %	Exp. II	Exp. II reduction %
B18	4.00	4.00	121.45	22.19	155.25	19.65	59.62	11.1	99.75	15.54	103	20.02	106.41	14.01
B33	4.00	4.00	118.75	20.42	155.75	19.9	61.12	13.29	107.25	21.45	101.5	18.84	111.50	17.94
B50	4.00	4.00	107.8	12.34	148.75	16.13	62.62	15.36	104.62	19.47	110.25	25.28	97.62	6.27
B58	4.00	4.00	113.97	17.08	139.00	10.25	56.5	6.19	92.75	9.16	84.75	2.8	104.75	12.65
B6	4.00	4.00	107.77	12.31	132.00	5.49	61	13.11	87.25	3.44	108.5	24.07	109.00	16.06
PF2	4.00	4.00	133.15	29.03	147.25	15.28	69.5	23.74	90.75	7.16	96.25	14.41	104.75	12.65
T21	4.00	4.00	136.72	30.88	156.5	20.29	69.12	23.32	98.00	14.03	101.75	19.04	114.00	19.74
T23	4.00	4.00	122.17	22.65	167.75	25.63	68.12	22.2	112.38	25.03	94.75	13.06	101.12	9.51
T38	3.00	4.00	94.8	0.32	162.00	22.99	52.25	-1.44	120.75	30.23	114.5	28.05	123.50	25.91
T47	4.00	4.00	93.82	-0.72	133.75	6.73	53.38	0.71	92.88	9.29	94.88	13.17	98.75	7.34
T48	4.00	4.00	115.77	18.37	142.25	12.30	60.77	12.79	96.25	12.47	100.5	18.03	108.50	15.67
Cont.	4.00	4.00	94.5	0.00	124.75	0.00	53	0.00	84.25	0.00	82.38	0.00	91.50	0.00
SEM±	0.23	0.23	13.04		13.99		6.44		12.41		11.01		8.32	
LSD (P=0.05)	0.67	12.20	37.51		19.03		18.54		25.09		31.67		15.70	
CV%	12.64	0.65	23		40.26		21.27		35.70		22.15		23.93	

* Mean of four replications

recorded 19.74 and 17.94% increase in biomass yield respectively (Table 7).

4.2.4.2 Effect of biocontrol agents on *A. flavus* population at different crop stages

At 40 DAS, all the biocontrol agents tested reduced *A. flavus* population except B58, which was at par with control. Treatments B33, *Pseudomonas fluorescens* (PF2) and *T. viride* (T47) significantly reduced the *A. flavus* population by39.23% while B50 and B6 reduced the *A. flavus* population by33.70% over the control (Table 8). Similarly, in the second experiment most of the biocontrol agents tested, reduced the *A. flavus* population significantly. The reduction ranged from 23.53% to 58.82% (Table 8). The highest reduction (58.82%) was obtained from *T. viride* (T47) and *T. harzianum* (T23) followed by *P. fluorescens* (PF2) and B33 (52.94 and 47.06% respectively). The least reduction (23.53%) was recorded in B6 and B50 (Table 8)

At 80 DAS, two biocontrol agents B33 and *T viride* (T47) significantly reduced the *A. flavus* population, by 61.05 and 52.01% respectively, over the control (Table 8). Considerable reduction in *A. flavus* population (33.44%) was recorded in *P. fluorescens* and the minimum reduction was recorded in B50 (1.02%) and *T. viride* (T21) (2.60%). In the second experiment, all the biocontrol agents reduced *A. flavus* population to different levels. The reduction of the population

ranged from 12.09 to 41.16%. The bacterial isolate (B33) and *T. viride* (T47), reduced the *A. flavus* population significantly (41.16 and 40.93%, respectively). *T. harzianum* (T23) and *P. fluorescens* (PF2) gave considerable reduction of *A. flavus* population (36.51% and 29.30%) respectively. The least reduction was recorded in bacterial isolate (B50) and *T. viride* (T48), (12.09 and 13.26%, respectively) (Table 8).

In soil samples collected at crop harvesting, all the biocontrol agents were significantly effective in reducing the *A. flavus* population except *T. viride* (T48). Maximum reduction (50.89%) in *A. flavus* population was obtained from B33 followed by *T. harzianum* (T23) 42.37%) and *T. viride* (T47) (41.58%). Reduction in *A. flavus* population (39.74%) was also recorded by *P. fluorescens* (PF_2) and the least reduction (11.84%) was recorded with *T. viride* (T48) (Table 8). In the second experiment, all the biocontrol agents reduced the population of *A. flavus* significantly. Maximum reduction of *A. flavus* population was obtained from two *Trichoderma* isolates (*T. harzianum* (T23) and *T. viride* (T47) which recorded 36.51 and 34.02% reduction, respectively. These were followed by bacterial isolate (B33) and *P. fluorescens* (PF2), which gave 32.78 and 31.12 per cent reduction, respectively. The minimum reduction (17.01%) was recorded in bacterial isolate (B6) (Table 8).

Table 8 : Effect of biocontrol agents on *A. flavus* population at different crop stages in the pot soil of groundnut cultivar (ICGS 11) under greenhouse conditions, 2001-2002 and 2002

Biocontrol agents	*A. flavus* population (cfu g^{-1}) at 40 DAS				*A. flavus* population (cfu g^{-1}) at 80 DAS				*A. flavus* population (cfu g^{-1}) at harvest			
	Exp. I	Reduction Over control %	Exp. II	Reduction Over control %	Exp. I	Reduction Over control %	Exp. II	Reduction Over control %	Exp. I	Reduction Over control %	Exp. II	Reduction Over control %
B18	4250	6.08	2500	41.18	243250	23.45	82750	23.02	76250	19.74	45000	25.31
B33	2750	39.23	2250	47.06	123750	61.05	63250	41.16	46650	50.89	40500	32.78
B50	3000	33.70	3250	23.53	314500	1.02	94500	12.09	79250	16.58	45750	24.07
B58	4500	0.55	2750	35.29	263250	17.15	91750	14.65	67750	28.68	46750	22.41
B6	3000	33.70	3250	23.53	262500	17.39	77250	28.14	79750	16.05	50000	17.01
PF2	2750	39.23	2000	52.94	211500	33.44	76000	29.30	57250	39.74	41500	31.12
T21	3500	22.65	2500	41.18	309500	2.60	82750	23.02	80250	15.53	45000	25.31
T23	4000	11.60	1750	58.82	256500	19.28	68250	36.51	54750	42.37	38250	36.51
T38	3250	28.18	3000	29.41	255250	19.67	83500	22.33	73000	23.16	44750	25.73
T47	2750	39.23	1750	58.82	152500	52.01	63500	40.93	55500	41.58	39750	34.02
T48	3750	17.13	3000	29.41	225250	29.11	93250	13.26	83750	11.84	46750	22.41
Control	4525	0.00	4250	0.00	317750	0.00	107500	0.00	95000	0.00	60250	0.00
SEM±	564.78		435.38		40262.04		14646.43		4795.4	0	2873.63	
LSD(P=0.05)	1625.01		1252.68		115843.53		42141.27		13796	.00	8268.11	
CV%	32.25		32.4		32.92		35.71		13.6	0	12.67	

* Mean of four replications

4.2.4.3 Efficacy of biocontrol agents in reducing preharvest seed infection by *A. flavus*

All the test biocontrol agents reduced the seed infection compared to control. *T. harzianum* (T23) significantly reduced the seed infection (58.81%). B33, *P. fluorescens* (PF2) and *T. viride* (T47) reduced the seed infection considerably by 45.38, 45.35 and 44.77% respectively. Least reduction (4.65%) was obtained from B18 (Table 9). In the second experiment, highly significant reduction in seed infection by *A. flavus* was obtained from *T. harzianum* (T23) and *T. viride* (T47), which equally reduced the seed infection by 37.37%. These were followed by B33, B18, and *P. fluorescens* (PF2), which recorded reduction in seed infection by 33.89, 33.40 and 30.41%, respectively. The least (6.05%) seed infection reduction was obtained from the bacterial isolate, B6 (Table 9).

4.2.4.4 Effect of biocontrol agents on aflatoxin content in groundnut kernels

All the biocontrol agents tested were better than the control but did not significantly reduce the aflatoxin content. Maximum reduction of aflatoxin content was obtained from B33 and *T. harzianum* (T23) (15.94 and 15.82%, respectively) followed by *T. viride* (T47), which recorded 13.55% and the least reduction (3.40%) was recorded on B58 (Table 10). Treatments B18 and B50 were at par in reducing the

Table 9 : Efficacy of biocontrol agents in reducing seed infection by *Aspergillus flavus* in groundnut (ICGS 11) under greenhouse conditions (2001-2002 and 2002)

Biocontrol agents	EXPERIMENT I		EXPERIMENT II	
	Seed infection %*	Reduction over control	Seed infection %*	Reduction over control
B18	41.00	4.65	9.57	33.40
B33	23.50	45.35	9.50	33.89
B50	30.00	30.23	12.75	11.27
B58	26.75	37.79	12.50	13.01
B6	26.50	38.37	13.50	6.05
PF2	23.50	45.35	10.00	30.41
T21	32.50	24.42	12.50	13.01
T23	19.00	55.81	9.00	37.37
T38	31.00	27.91	10.25	28.67
T47	23.75	44.77	9.00	37.37
T48	26.00	39.53	12.00	16.49
Control	43.00	0.00	14.37	0.00
SEM±	7.72		1.79	
LSD(P=0.05)	22.20		5.14	
CV%	53.45		31.76	

* Mean of four replications

Experiment I: (2001-2002)

Experiment II (2002)

aflatoxin content. Similarly, none of the biocontrol agents reduced significantly the aflatoxin content of the kernels in the second experiment. The maximum reduction (18.26%) was recorded in *T. viride* (T47) followed by *T. harzianum* (T23), B33 and *P. fluorescens* (PF2), which recorded 16.35, 16.35 and 16.09 per cent reduction of aflatoxin content. The least reduction was obtained from *T. viride* (T47) and bacterial isolate (B50), 2.43 and 2.61 %, respectively (Table 10).

4.2.5 Evaluation of Selected Biocontrol Agents under Field Conditions in Postrainy Season (2001-2002) and Rainy Season (2002)

The selected biocontrol agents tested under greenhouse conditions were also evaluated under field conditions in postrainy season (2001-2002) and rainy season (2002).

4.2.5.1 Effect of biocontrol agents on total number of plants and yield parameters

There was no significant difference in total number of plants between treatments and the control (Table 11). While in the rainy season most of the biocontrol agents tested showed more number of plants compared to control. Six biocontrol agents were significantly superior over control. Maximum plants/plot was obtained in B18 and B50 (55.33 and 54.67, respectively) followed by B58 and *T. harzianum*

Table 10 : Potential of biocontrol agents in reducing aflatoxin content in groundnut (ICGS 11) kernels under greenhouse conditions in 2001-2002 and 2002

Bicontrol agents	EXPERIMENT I		EXPERIMENT II	
	Aflatoxin content (i		i	
B18	16.05	4.18	10.43	9.30
B33	14.08	15.94	9.62	16.35
B50	16	4.48	11.20	2.61
B58	16.18	3.4	10.00	13.04
B6	15.95	4.78	10.17	11.57
PF2	15.00	10.45	9.65	16.09
T21	15.80	5.67	10.80	6.09
T23	14.10	15.82	9.62	16.35
T38	15.08	9.97	10.12	12.00
T47	14.48	13.55	9.40	18.26
T48	15.68	6.39	11.22	2.43
Control	16.75	0.00	11.50	0.00
SEM±	1.24		1.20	
LSD(P=0.05)	3.56		3.44	
CV%	16.02		23.22	

Experiment I: (2001-2002)

Experiment II: (2002)

* Mean of four replications

(T23), which recorded 51.67 and 50.83, respectively. Minimum number of plants/plot was obtained from control (35.50) followed by *T. viride* (T48) and *T. viride* (T21) (38.50) (Table 11).

Incase of pod yield, all the treatments, except *T. viride* (T38) were slightly better than the control. B33 significantly increased the pod yield (by 28.62%) followed by B50 (19.24%) (Table 11). The data in table 11 showed that, pod yield increase ranged from 0.58 to 26.98%. The highest and significant pod yield increase was obtained from B18, B50, *P. fluorescens* (PF2) and *T. harzianum* (T23) (23.04, 23.10, 26.97 and 26.76%) respectively. Minimum pod yield increase (0.58) was recorded on B6 followed by *T. viride* (T48) (2.86%).

Incase of seed yield, significant increase (26.07%) was recorded in B33 followed by B6 (18.42%) and *P. fluorescens* (PF2) (16.19%) (Table 11). The results in Table 11 showed that three biocontrol agents, B33, B50 and *T. harzianum* (T23) gave significantly higher seed yield of 212.42, 204.67 and 200.5 g: , respectively, compared to control (154.67 g:). The seed yield increase ranged from 0.37% to 27.19%. Maximum seed yield increase (27.19) was recorded in bacterial isolate (B33) followed by B50 (24.43%).

Incase of biomass yield, there was no significant increase over control and between the biocontrol agents tested. Maximum increase was obtained from B33 (11.94%) and *T. viride* (T48) (11.28%) (Table

Table 11 : Effect of biocontrol agents (BCAs) on yield parameters of groundnut cultivar ICGS 11 under field conditions, Patancheru 2001-2002 and 2002

BCAs	Total No. of plants*		Pod yield/plot (g)*				Seed yield/plot (g)*				Biomass yield (g)*			
	PR	RS	PR	Reduction Over control%	RS	Reduction Over control%	PR	Reduction Over control%	RS	Reduction Over control%	PR	Reduction Over control%	RS	Reduction Over control%
B18	54.00	55.33	433.00	6.31	331.58	23.04	300.58	6.46	182.42	15.21	1050.00	6.35	768.33	31.24
B33	52.17	48.33	568.33	28.62	320.17	20.30	380.33	26.07	212.42	27.19	1116.67	11.94	693.33	23.8
B50	52.50	54.67	502.33	19.24	331.83	23.10	330.92	15.03	204.67	24.43	1041.67	5.60	630.00	16.14
B58	55.83	51.67	465.22	12.80	310.5	17.82	312.33	9.98	180.5	14.31	1083.33	9.23	635.00	16.8
B6	53.33	39.33	486.58	16.63	256.67	0.58	344.67	18.42	155.25	0.37	1083.33	9.23	586.67	9.94
PF2	51.67	47.50	473.75	14.37	349.42	26.97	335.50	16.19	191.17	19.09	991.67	0.84	695.00	23.98
T21	54.00	38.50	460.75	11.95	270.25	5.58	316.50	11.16	152.83	-1.20	1033.33	4.84	535.00	1.25
T23	53.00	50.83	420.75	3.58	348.5	26.78	306.92	8.39	200.50	22.86	1016.67	3.28	686.67	23.06
T38	54.50	40.67	403.42	-0.56	294.17	13.26	285.58	1.54	172.17	10.16	1025.00	4.07	606.67	12.91
T47	54.33	41.67	458.67	11.55	295.33	13.6	309.67	9.20	175.33	11.78	1045.83	5.98	576.67	8.38
T48	57.00	38.00	478.58	15.23	262.67	2.86	341.50	17.67	162.00	4.52	1108.33	11.28	606.67	12.91
Control	53.50	35.50	405.68	0.00	255.17	0.00	281.17	0.00	154.67	0.00	983.33	0.00	528.33	0
SEM ±	2.80	2.36	52.51		26.14		33.18		15.51		48.14		46.22	
LSD (=0.05)	7.83	6.69	148.83		74.08		94.03		43.95		136.44		130.99	
CV%	12.58	12.80	27.78		21.19		25.36		21.26		11.25		18.00	

* Mean of six replications.

PR: Postrainy season (2001-2002) RS: Rainy season (2002)

11). In the second experiment, four biocontrol agents (B18, B33, *P. fluorescens* (PF2) and *T. harzianum* (T23) gave significant increase in biomass yield over control (31.24, 23.80, 23.98 and 23.06% respectively). The lowest biomass increase (1.25%) was obtained from *T. viride* (T21) followed by *T. viride* (T47) (8.38%) (Table 11).

4.2.5.2 Effect of biocontrol agents on *A. flavus* population at different crop stages

At 40 days after sowing, all the biocontrol agents reduced the *A. flavus* population at different levels. The reduction ranged from 12.32 to 37.44%. The maximum reduction (37.44%) was obtained from B33 followed by *T. harzianum* (T23) and *T. viride* (T47), which recorded 36.97 and 36.02%, respectively over the control. Least reduction percentage was recorded in B50 (12.32%) (Table12). The results (Table 12) showed that four bioagents *T. viride* (T47), *T. harzianum* (T23), B33 and *P. fluorescens* (PF2) significantly reduced the *A. flavus* population at 40 days after sowing. The reduction of *A. flavus* population by the biocontrol agents was ranging from 14.29% to 46.43%. *T. viride* (T47) exhibited the maximum reduction (46.43%) of *A. flavus* population over control followed by *T. harzianum* (T23) (42.86%). B6 and B58 exhibited the least reduction in *A. flavus* population (14.29%) over control.

At 80 days after sowing, the reduction over control ranged from 5.78 to 30.82%. B33, *P. fluorescens* (PF2), *T. viride* (T47) and *T. harzianum* (T23) significantly reduced the *A. flavus* population compared to control (29.49, 24.61, 30.82 and 29.27%, respectively (Table 12). In the 2002 rainy season, three biocontrol agents, *T. viride* (T47), *T. harzianum* (T23) and B33 reduced significantly *A. flavus* population over control. Maximum reduction of *A. flavus* population was exhibited by *T. viride* (T47) and *T. harzianum* (T23) (32.48 and 32.12% respectively) followed by B33 (28.47%). The least reduction of *A. flavus* was recorded by bacterial bioagent, B58 (8.39%) (Table 12).

When the *A. flavus* population was estimated at crop harvesting, all the biocontrol agents reduced the *A. flavus* population. The reduction ranged from 7.69 to 39.89%. All the biocontrol agents tested were significantly superior over the control except B18, B50 and *T. viride* (T48). *T. viride* (T47) was found to be the best in reducing *A. flavus* population (39.89%) followed by *T. harzianum* (T23) (38.18%) and B33 (36.75%). Significant reduction was also recorded (34.47%) in *P. fluorescens* (PF2) (Table 12). In the 2002 rainy season, all the biocontrol agents tested, significantly reduced the *A. flavus* population compared to control. The reduction in *A. flavus* population was ranging from 16.51% to 40.63%. *T. harzianum* (T23) and *T. viride* (T47) exhibited the maximum reduction in *A. flavus* population (40.63 and

Table 12 : Effect of biocontrol agents on *A. flavus* population at different crop stages in rhizosphere of groundnut cultivar (ICGS 11) under field conditions, postrainy (2001-2002) and rainy(2002) seasons

Biocontrol agents	*A. flavus* population (cfu g^{-1}) at 40 DAS				*A. flavus* population (cfu g^{-1}) at 80 DAS				*A. flavus* population (cfu g^{-1}) At harvest			
	Postrainy Season	Reduction Over control %	Rainy season	Reduction Over control%	Postrainy Season	Reduction Over control%	Rainy Season	Reduction Over control %	Postrainy season	Reduction Over control %	Rainy season	Reduction Over control%
B18	29500.00	16.11	7500.00	19.64	64166.67	14.63	34666.67	24.09	51667.00	11.68	38666.67	26.35
B33	22000.00	37.44	5833.33	37.50	53000.00	29.49	32666.67	28.47	37000.00	36.75	33666.67	35.87
B50	30833.33	12.32	7666.67	17.86	70333.33	6.43	39000.00	14.60	51667.00	11.68	40000.00	23.81
B58	29166.67	17.06	8000.00	14.29	62666.67	16.63	41833.33	8.39	49000.00	16.24	43833.33	16.51
B6	24833.33	29.38	8000.00	14.29	70833.33	5.76	36000.00	21.17	48500.00	17.09	39833.33	24.13
PF2	25666.67	27.01	6000.00	35.71	56666.67	24.61	34833.33	23.72	38333.00	34.47	35833.33	31.75
T21	27833.33	20.85	6833.33	26.79	69333.33	7.76	34500.00	24.45	48833.00	16.52	37666.67	28.25
T23	22166.67	36.97	5333.33	42.86	53166.67	29.27	31000.00	32.12	36167.00	38.18	31166.67	40.63
T38	24000.00	31.75	6666.67	28.57	69666.67	7.32	34166.67	25.18	48333.00	17.38	39666.67	24.44
T47	22500.00	36.02	5000.00	46.43	52000.00	30.82	30833.33	32.48	35167.00	39.89	31833.33	39.37
T48	22833.33	35.07	7500.00	19.64	70166.67	6.65	36333.33	20.44	54000.00	7.69	34500.00	34.29
Control	35166.67	0.00	9333.33	0.00	75166.67	0.00	45666.67	0.00	58500.00	0.00	52500.00	0.00
SEM ±	6661.94		1044.79		4553.59		4363.31		2461.40		4037.80	
LSD (=0.05)	18880.92		2961.08		12905.53		12366.27		6975.90		11443.72	
CV%	61.87		36.71		17.45		29.72		13.00		25.85	

* Mean of six replications

Postrainy season (2001-2002) Rainy season (2002)

39.37 respectively) followed by B33 (35.87%) and *P. fluorescens* (PF2) (31.75%). The least reduction in the population (16.51%) was recorded in bacterial isolate, B58 (Table 12).

4.2.5.3 Effect of biocontrol agents on preharvest seed infection by *A. flavus*

The seed infection reduction by the biocontrol agents ranged from 7.29% to 21.46%. Two *Trichoderma* isolates (*T. harzianum* (T23) and *T. viride* (T47) reduced the seed infection significantly (21.46 and 20.08% respectively). Least seed infection reduction was obtained from B18 (7.29%) (Table 13). In the 2002 rainy season, *T. viride* (T47) was found to be the best treatment in reducing seed infection by *A. flavus* (33.52 %) followed by *T. harzianium* (T23) (30.11 %) and B33 (26.70 %). The least effective treatment was B6 providing only 3.41 % reduction in seed infection (Table 13)

4.2.5.4 Efficacy of biocontrol agents in reducing aflatoxin content in groundnut kernels under field conditions

Two *Trichoderma* isolates (*T. harzianum* (T23) and *T. viride* (T47)) reduced the aflatoxin content of the kernels significantly by 14.44 and 15.66%, respectively, followed by B33 (12.96%). The least reduction was recorded in B6 (4.03%) and B50 (3.42%) (Table 14). The results revealed that, no biocontrol agent significantly reduced the

Table 13 : Efficacy of biocontrol agents in reducing seed infection by *A. flavus* in groundnut (ICGS 11) under field conditions in postrainy (2001-2002) and rainy (2002) seasons

Bicontrol Agents	Postrainy season (2001-2002)		Rainy season (2002)	
	Seed Infection %*	Reduction over control %	Seed infection %*	Reduction over control %
B18	78.50	7.29	24.33	17.05
B33	70.83	16.35	21.50	26.70
B50	75.83	10.44	25.17	14.18
B58	78.17	7.68	27.33	6.82
B6	76.67	9.45	28.33	3.41
Pf2	71.17	15.94	22.50	23.29
T21	77.00	9.06	26.67	9.07
T23	66.50	21.46	20.50	30.11
T38	75.33	11.03	22.33	23.87
T47	67.67	20.08	19.50	33.52
T48	77.33	8.67	27.50	6.24
Control	84.67	0.00	29.33	0.00
SEM±	5.91		1.67	
LSD(P=0.05)	16.76		4.75	
CV%	19.32		16.70	

* Mean of six replications

Table 14 : Potential of biocontrol agents in reducing aflatoxin content in groundnut (ICGS 11) under field conditions in the postrainy (2001-2002) and rainy (2002) seasons

Bicontrol agents	Postrainy season (2001-2002)		Rainy season (2002)	
	Aflatoxin Content (l		l	
B18	57.13	8.34	48.05	3.67
B33	54.25	12.96	47.22	5.33
B50	58.67	5.87	49.37	1.02
B58	59.82	4.03	48.02	3.73
B6	60.2	3.42	48.67	2.43
PF2	55.38	11.15	47.37	5.03
T21	54.63	12.35	48.00	3.77
T23	53.33	14.44	43.55	12.69
T38	56.43	9.47	47.32	5.13
T47	52.57	15.66	42.42	14.96
T48	59.03	5.29	48.8	2.17
Control	62.33	0.00	49.88	0.00
SEM±	2.77		2.32	
LSD (P=0.05)	7.84		6.69	
CV%	11.9		9.81	

* Mean of six replications

aflatoxin content of the kernels except *T. viride* (T47) in the 2002 rainy season. The maximum aflatoxin reduction (14.96%) was obtained from *T. viride* (T47) followed by *T. harzianum* (T23) (12.69%). All the remaining bioagents gave less reduction that ranged from 1.02% to 5.33% (Table 14).

4.3 IMPROVEMENT OF *T. VIRIDE* (T47) BY IRRADIATION

A *T.viride* (T47) culture was UV-irradiated to improve its biocontrol efficacy against *A. flavus* . After sub-culturing, the mutants were divided into three groups; slow growth, fast growth and very fast growth based on their growth rate compared to the parental culture. Each group was further divided into sub-groups based on the colony colour and sporulation rate. A total of seven sub groups were obtained from the irradiation culture.

Two mutants were randomly selected from each sub group and tested against *A. flavus* (Af 11-4) in dual culture experiment. All the mutants significantly differed from control (*A. flavus* alone). Maximum mycelial growth of *A. flavus* was recorded on control (4.2cm). Minimum mycelial growth was recorded in M18, M15 and M28 (0.82, 0.85 and 0.85cm, respectively). All the mutants recorded less mycelial growth than parental line T47 (1.0cm) except M40, M42, which recorded more than parental line (1.25cm and 1.10cm, respectively).

Plate 3: Antagonistic activity of fast growing mutants against *A.flavus*

Plate 4: Antagonistic activity of *Penicillium* producing mutants against *Aspergillus*

Plate 5: Antagonistic activity of slow growing mutants against *A.flavus*

Table 15 : Effect of irradiated *T. viride* (T47) mutants on the growth of *A. flavus* (strain 11-4) *in vitro*

T. viride (T47) Mutants	Mycelial growth of *A. flavus* (cm) *	% Reduction over control
M1	0.90	78.57
M15	0.85	79.76
M16	1.05	75.00
M18	0.83	80.36
M2	0.95	77.38
M20	0.90	78.57
M28	0.85	79.76
M3	1.00	76.19
M34	0.95	77.38
M40	1.25	70.24
M41	0.95	77.38
M42	1.10	73.81
M5	1.03	75.60
M6	0.88	79.17
T. viride (T47)	1.00	76.19
Control (*A. flavus* alone)	4.20	0.00
SEM±	0.07	
LSD(P=0.05)	0.18	
CV%	11.10	

* Mean of four replications

M15, M18 and M28 were significantly superior to the parental line (T47) (Table 15).

4.4 EFFICACY OF NEEM COMMERCIAL FORMULATIONS AGAINST *A. FLAVUS IN VITRO* AND *IN VIVO*

4.4.1 *In Vitro*

Four different neem commercial formulations were tested for their potential to reduce the growth of *A. flavus in vitro*, using the standard poisoned food technique.

All the formulations tested i.e., Nivaar, Saysaneem, Starneem and Sunny significantly controlled the growth of *A. flavus* (AF 11-4) when it was grown on PDA medium amended with different concentrations of the neem formulations. Maximum mycelial growth of *A. flavus* (4.2cm) was recorded on control (medium without neem formulation) (Table 16a). In case of neem formulations, maximum *A. flavus* mycelial growth was recorded on 100ppm concentration of Sasyaneem (1.82 cm) and 200ppm of same formulation (1.8 cm). Incase of Nivaar mycelial growth of *A. flavus* was observed only on 100ppm concentration. In Sasyaneem, the growth of the fungus was observed up to 300ppm concentration while in Sunny slight growth of the fungus (0.20cm) was recorded even at 500 $\mu g\ l^{-1}$ concentration (Table 16a). When the same experiment was conducted on PDA broth, the fungal

Table 16 (a) : Effect of four Neem commercial formulations on *Aspergillus flavus* mycelial growth at different concentrations

Neem formulations	Mycelial growth 100 ppm*	Reduction %	Mycelial Growth 200 ppm*	Reduction %	Mycelial Growth 300 ppm*	Reduction %	Mycelial Growth 400 ppm*	Reduction %	Mycelial Growth 500 ppm*	Reduction %
Nivaar	0.95	78.51	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00
Sasyaneem	1.82	58.82	1.80	59.28	1.22	72.40	0.00	100.00	0.00	100.00
Starneem	1.40	68.33	1.02	76.92	0.45	89.82	0.32	92.76	0.00	100.00
Sunny	0.80	81.90	0.80	81.90	0.55	87.56	0.30	93.21	0.20	95.48
Control	4.42	0.00	4.42	0.00	4.42	0.00	4.42	0.00	4.42	0.00
SEM±	0.07		0.07		0.07		0.07		0.07	
LSD(P=0.05)	0.21		0.21		0.21		0.21		0.21	
CV%	11.1		11.1		11.1		11.1		11.1	

Mean of four replications

growth was observed in three treatments only, control (0.6 g), Nivaar 100 ppm (2.4 g) and Sasayaneem 100 ppm (2.7 g). Incase of control thin layer of mycelial growth of the fungus with heavy sporulation spread on the broth medium. Incase of Nivaar and Sasyaneem at 100ppm concentration, thick mycelial growth without sporulation spread on the broth medium.

All the neem formulations tested were statistically significant in reducing *A. flavus* growth in all the concentrations used when compared to control (*A. flavus* on PDA medium without neem formulation). There was significant difference between the different concentrations of each formulation except between 100 and 200ppm of Sasyaneem and between 100 and 200ppm of Sunny.

4.4.1.1 Compatibility test

The compatibility of a selected *Trichoderma* isolate (T47) with four neem commercial formulations, Nivaar, Sasyaneem, Starneem and Sunny was tested using the poisoned food technique. Five different concentrations (100, 200, 300, 400 and 500ppm) of each formulation were used against *T. viride* (T47). All the formulations significantly reduced the mycelial growth of *T. viride* (T47) in all the concentrations. In Nivaar, the fungal growth was observed only at 100ppm concentration. In Sasyaneem the *Trichoderma* growth (1.57cm) was observed even at 500ppm (Table 16b). In Starneem the growth of the test fungus was observed at

Table 16 (b) : Effect of four Neem commercial formulations on *Trichoderma viride* (T47) growth on potato dextrose agar medium

Neem formulations	Mycelial growth 100 ppm*	Reduction %	Mycelial Growth 200 ppm*	Reduction %	Mycelial Growth 300 ppm*	Reduction %	Mycelial Growth 400 ppm*	Reduction %	Mycelial Growth 500 ppm*	Reduction %
Nivaar	0.950	78.07	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00
Sasyaneem	1.820	58.824	1.800	59.276	1.220	72.398	10.00	100.00	0.00	100.00
Starneem	1.40	68.33	1.02	76.92	0.45	89.82	0.32	92.76	0.00	100.00
Sunny	0.80	81.90	0.80	81.90	0.55	87.56	0.30	93.21	0.20	95.48
Control	4.42	0.00	4.42	0.00	4.42	0.00	4.42	0.00	4.42	0.00
SEM±	0.110		0.110		0.110		0.110		0.110	
LSD(P=0.05)	0.330		0.330		0.330		0.330		0.330	
CV%	12.50		12.50		12.50		12.50		12.50	

* Mean of four replications

100 to 300 ppm. In Sunny, the mycelial growth of *Trichoderma* was recorded at 100 and 200 $\mu g\ l^{-1}$ only. Maximum mycelial growth (4.40cm) was recorded in control (medium with neem formulations) followed by Starneem at 100ppm concentration (3.55cm). The reduction in all the formulations ranged from 19.32 to 100%.

4.4.2 Evaluation of Two Neem Commercial Formulations under Greenhouse and Field Conditions (Rainy season, 2002)

Based on the results of the experiments conducted in the laboratory, two neem formulations were selected for greenhouse and field experiments. In both the cases, effect of these formulations on yield parameters, *A. flavus* population, seed infection and aflatoxin content (of seeds after harvesting) was recorded. Both the glasshouse and field experiments were conducted simultaneously in 2002 rainy season.

4.4.2.1 Effect of two neem formulations on total number of plants and yield parameters under greenhouse and field conditions

The data in Table 17a showed that both the neem formulations (Nivaar and Sasyaneem) did not significantly differ from the control (pots, not applied with neem formulations) in case of total number of plants. Similarly there is no significant difference between the means of total number of plants of the two formulations. Data in table 17b

showed that, the two formulations tested, Nivaar and Sasyaneem have no significant effect on total number of plants/plot. In the field experiment, the formulations gave slightly higher number of plants/plot (47.00 in Nivaar and 49.67 in Sasyaneem) compared to control (45.5 plants/plot).

In case of pod yield, the neem formulations did not increase the pod yield under greenhouse conditions. In the field experiment, Sasyaneem showed significant increase (9.77 %) over control whereas Nivaar showed only 1.44 % increase in pod yield (Table 17b).

Regarding seed weight, the two formulations were similar to the control.and did not provide increase in seed weight uder greenhouse conditions (Table 17a). Incase of seed field experiment, Sasyaneem significantly increased the seed weight (13.85%) over control whereas Nivaar provided only 1.18 % increase in seed weight over control (Table 17b).

Regarding biomass yield, the two formulations gave slightly higher yield (97.5 g on Nivaar and 94.25 g on Sasyaneem) compared to control (91.5 g). This difference was not statistically significant at 5% level. Under field condtion, Sasyaneem significantly contributed to biomass yield/plot (5.34 % increase over the control) whereas Nivaar recorded only 1.33 % increase in biomass yield/plot (Table 17b).

Table 17 (a) : Effect of two selected Neem formulations on total number of plants and yield parameters under greenhouse conditions (2002)

Neem formulations	Total No. of plants/pot*	Pod yield/2 pots (gm)*	Seed yield/2 pots (gm)*	Biom yield/2 pots (gm)*
Nivaar	3.5	123.5	84.38	97.50
Sasyaneem	3.75	124.75	85.5	94.25
Control	3.75	119.38	84.25	91.50
SEM±	0.28	11.24	10.3	11.65
LSD(P=0.05)	0.96	38.88	35.65	40.32
CV%	15.08	18.34	24.32	24.68

* Mean of four replications

Table 17 (b) : Effect of two Neem formulations on Total number plants and yield parameters under field conditions in the 2002 rainy season

Neem formulations	Total No. of plants/ plot*	Pod yield/plot (gm)*	Increase over control %	Seed yield/plot (gm)*	Increase over control %	Biom yield/plot (gm)*	Increase over control %
Nivaar	47	323.33	1.44	183.67	1.18	628.33	1.33
Sasyaneem	49.67	353.17	9.77	210.67	13.85	655	5.34
Control	45.5	318.67	0.00	181.50	0.00	620.00	0.00
SEM±	2.21	3.89		5.79		4.69	
LSD(P=0.05)	6.97	12.25		18.24		14.79	
CV%	11.44	2.87		7.39		1.81	

* Mean of six replications

4.4.2.2 Effect of two neem formulations on *A. flavus* population under greenhouse and field conditions

The neem formulations in test were applied at 45 days after sowing. The *A. flavus* inoculum was applied to the pots after collecting the 40 DAS soil samples. The *A. flavus* population at 40 DAS, mentioned in the table 18 is the initial population before inoculum application. At 80 days after sowing, both the neem formulations, Nivaar and Sasyaneem reduced the *A. flavus* population significantly over control. These provided similar reduction in the population (40.47 % on Nivaar and 39.07 % on Sasyaneem) under greenhouse conditions. In field experiment, the *A. flavus* population was very high due to the inoculum application. The two formulations, Nivaar and Sasyaneem reduced the *A. flavus* population significantly providing 27.01% and 24.45% reduction over control respectively (Table 18).

In case of soil samples collected at crop harvesting, the two formulations reduced the *A. flavus* population providing 26.14 and 24.90 % from Nivaar and Sasyaneem, respectively (Table 18). The two test formulations showed significant reduction in *A. flavus* population over control providing (24.44% in Nivaar and 20% in Sasyaneem) under field conditions

Table 18 : Effect of two Neem formulations on *Aspergillus flavus* (Af 11-4) population in the rhizosphere of groundnut (ICGS 11) under greenhouse and field conditions, Patancheru, 2002

Neem formulations	40 Days after sowing		80 Days After Sowing				At harvest			
	Exp. I (GH)	Exp. II (Field)	Exp. I (GH)	Reduction %	Exp. II (Field)	Reduction %	Exp. I (GH)	Reduction %	Exp. II (Field)	Reduction %
Nivaar	3750	9500.00	64000	40.47	33333.33	27.01	44500	26.14	39666.67	24.44
Sasyaneem	4000	9666.67	65500	39.07	34500.00	24.45	45250	24.90	42000.00	20.00
Control	4250	9666.67	107500	0.00	45666.67	0.00	60250	0.00	52500.00	0.00
SEM±	448.76	295.02	4597.70		1787.30		2591.39		177.43	
LSD(P=0.05)	1552.92	929.63	15910.11		5631.9		8967.35		559.09	
CV%	22.44	7.52	11.64		11.57		10.37		0.97	

Exp.I: Greenhouse experiment (2002)

Exp I: Field experiment rainy season (2002)

4.4.2..3 Efficacy of two neem formulations on preharvest seed infection by *A. flavus* under greenhouse and field conditions

The results (Table19) showed that the two formulations, Nivaar and Sasyaneem reduced the preharvest seed infection of groundnut kernels. This reduction was not significant at 5% level. The reduction in seed infection was 14.81 % in Nivaar and 11.34% in Sasyaneem. Both the formulations did not differ significantly from the control treatment under field conditions when means of percent seed infection were compared. The two formulations reduced the percent seed infection over control (7.33% in Nivaar and 4.41% in Sasyaneem) (Table 19).

4.4.2.4 Efficacy of two neem formulations in reducing aflatoxin content in groundnut kernels under greenhouse and field conditions

The aflatoxin content of kernels harvested from Nivaar and Sasyaneem treated pots was slightly less than that of kernels harvested from the control pots (Table 20). The reduction in aflatoxin content was 7.39 % in Nivaar and 5.22 % in Sasyaneem. Similarly the results of the field experiment (Table 20) showed that Nivaar and Sasyaneem did not differ from control significantly when means of aflatoxin content were

Table 19 : Efficacy of two Neem formulations in reducing seed infection by *A. flavus* in groundnut (ICGS 11) kernels under greehouse and field conditions(2002)

Neem formulations	Exp. I Seed infection % *	Reduction over control %	Exp. II Seed infection % *	Reduction over control %
Nivaar	12.25	14.81	10.50	7.33
Sasyaneem	12.75	11.34	10.83	4.41
Control	14.38	0.00	11.33	0.00
SEM±	1.06		0.78	
LSD(P=0.05)	3.67		2.46	
CV%	16.15		17.56	

* Mean of four replications in GH and six in the field

Exp. I: Greenhouse experiment (2002)

Exp. II: Field experiment rainy season (2002)

Table 20 : Efficacy of two Neem formulations in reducing aflatoxin content in groundnut (ICGS 11) under greenhouse and field conditions (2002)

Neem formulations	Experiment I Aflatoxin content (µg/kg)*	Reduction over control %	Experiment II Aflatoxin content (µg/kg)*
Nivaar	10.65	7.39	46.03
Sasyaneem	10.90	5.22	47.50
Control	11.50	0.00	49.88
SEM±	0.84		1.03
LSD(P=0.05)	15.19		3.58
CV%	2.89		4.32

* Mean of six replications

Exp. I: Greenhouse experiment (2002)

Exp. II: Field experiment rainy season (2002)

compared and exhibited only 7.72% and 4.77% reduction in aflatoxin content over the control, respectively.

4.5 IDENTIFICATION OF GERMPLASM ACCESSIONS, ADVANCE BREEDING LINES AND VARIETIES FOR RESISTANCE TO *IN VITRO* SEED COLONIZATION AND SEED INFECTION BY *A. FLAVUS*

4.5.1 Evaluation of 50 Groundnut genotypes for Resistance to *A. flavus*

Forty-five groundnut entries selected from different sources (Table 1) plus 5 controls were tested for their resistance to seed infection and *in vitro* seed colonization by *A. flavus* in the laboratory.

Seed infection

The seeds of 50 genotypes harvested from the *A. flavus* sick plot in rainy season 2001, were examined. All genotypes tested significantly differed from susceptible check (JL24). Ten genotypes gave seed infection percent less than the resistant control (J11) but only three genotypes; ICGV 89104, ICGV 91114 and ICGV98170 recorded seed infection significantly less than the resistant control (J11). The maximum seed infection (34.50%) was recorded on susceptible control (JL24) followed by ICGV 93291 (31.5%). The seed infection of the other test genotypes ranged from 0.5% to 24% (Table 21). Four genotypes TCGP5, TCGP6, TCGS645 and TCGS647 are at par with J11.

Seed colonization

The seeds of each of the 50 groundnut genotypes were divided into two lots to conduct the *in vitro* seed colonization experiment.

Unwounded (non-pin pricked):

Most genotypes tested (42 out of 49) were significantly superior from susceptible control JL 24 and six genotypes were at par with JL 24 and one genotype recorded more colonization severity than JL24. Maximum colonization severity was recorded on ICGV 95492 (3.85) on 1-4 scale followed by the susceptible control JL24 (3.64). When compared to resistant check (J11), 43 genotypes out of 49 recorded colonization severity more than J11 and 6 genotypes were at par with J11 (1.92))(ICGS 76 (1.81), ICGV 91114 (1.94), ICGV 94341 (1.87), ICGV 94350 (1.81), ICGV 94433 (1.85) WUE (40) JAL 17 (1.92) (Table 21)

Wounded (Pin pricked)

When the same genotypes (50) were pin pricked before inoculating with *A. flavus* to test their coteledonary resistance to *A. flavus*, twenty-six genotypes did not differ significantly from susceptible check (JL24) whereas twenty-three genotypes were significantly differing from JL24 (Table 21). When compared with resistant control (J11), forty-six genotypes recorded more colonization

severity (3.44%) than J11. Two genotypes (ICGV 91284 and WUE (7) IC 48 recorded significantly less colonization severity (3.14 and 3.31 respectively) and one genotype (WUE 159) IC 43 was at par with J11. Colonization severity in wounded genotypes ranged from 3.14 to 4 on 1-4 scale (Table 21)

Based on the *in vitro* results, fourteen genotypes that have seeds in colonization severity class I were selected and sown in *A. flavus* sick plot in postrainy season 2001-2002. The harvested seeds were artificially inoculated with *A. flavus* spore suspension in the laboratory. Colonization severity ranged from 1.29 to 3.38 on 1-4 scale. All genotypes significantly differed from JL24. Two genotypes ICGV91114 and ICGV95322 recorded less colonization severity than J11 (Table 21). Minimum colonization severity (1.29) was recorded on ICGV 91114 and ICGV 95322 followed by WUE (116) TIR 31 (1.48) and J11 (1.4). Maximum colonization severity (3.38) was recorded on susceptible check (JL24) (Table 21).

The seeds, which showed less colonization severity (less than 5% of the surface of the seed colonized) of each genotype were again selected and sown in the sick plot in rainy season 2002. The results are given in Table 22. All the genotypes tested were significantly superior over JL24. When compared to resistant control J11, only ICGV91114 gave less colonization severity but not significantly differing from J11.

Plate 6 Entry No 1 & 2 showing resistance to *in vitro* seed colonization by *A flavus*

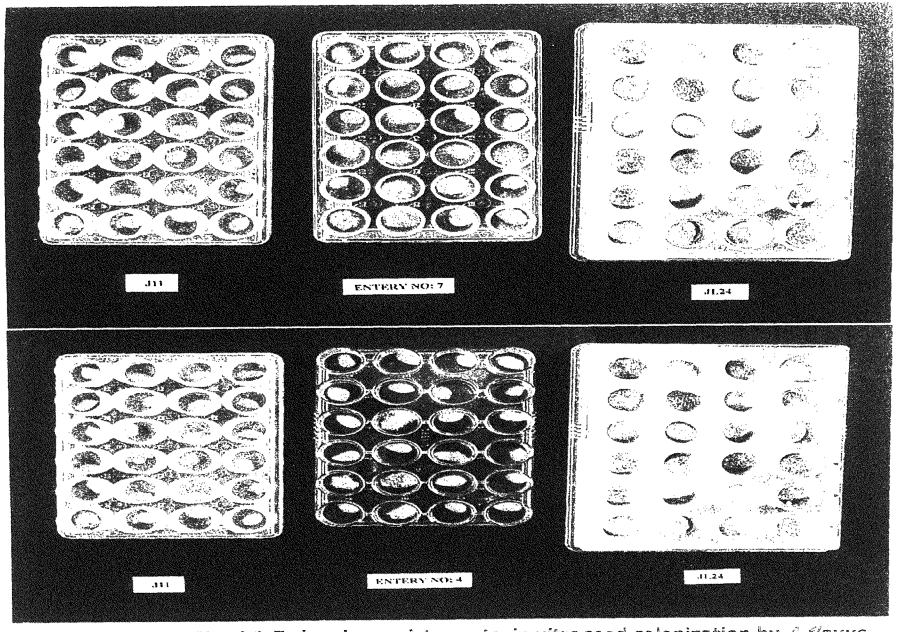

Plate 7: Entry No. 4 & 7 showing resistance to *in vitro* seed colonization by *A.flavus*

Table 21 : Seed infection and *invitro* seed colonization by *A. flavus* of 50 groundnut entries

Entry No	Mean seed Infection %	Colonization severity I (1-4 scale)	Colonization severity II (1-4 scale)
CSMG 84-1	8.50	2.50	4.00
ICGS 44	2.50	2.75	3.90
ICGS 76	12.50	2.25	3.60
ICGS11	5.50	3.65	3.44
ICGS86158	5.50	3.23	4.00
ICGV 86031	12.00	3.17	3.81
ICGV86590	8.00	2.15	3.67
ICGV86699	10.50	3.02	4.00
ICGV88145	12.50	3.44	4.00
ICGV89104	0.50	3.58	4.00
ICGV91114	0.50	3.29	3.92
ICGV91278	11.50	3.69	4.00
ICGV91279	9.25	2.83	4.00
ICGV91283	2.50	2.08	4.00
ICGV91284	2.50	2.48	3.38
ICGV92206	24.00	1.92	3.60
ICGV93280	13.00	2.27	3.94
ICGV93291	31.50	2.63	3.31
ICGV94341	14.25	1.88	3.88
ICGV94350	2.00	1.81	4.00
ICGV94358	13.50	2.63	3.65
ICGV94433	6.50	1.85	3.90
ICGV95322	15.50	2.08	3.92
ICGV95454	11.50	3.29	3.79
ICGV95460	9.25	3.27	4.00
ICGV95469	7.00	3.69	4.00
ICGV95477	7.50	3.67	3.98
ICGV95492	6.50	3.85	4.00
ICGV95494	6.50	3.50	3.98
ICGV98163	7.00	3.21	4.00
ICGV98170	1.00	3.00	3.98
ICGV98383	18.00	3.31	4.00
ICGV99029	12.50	3.38	4.00
ICGV99032	10.50	2.50	3.90
ICGV99054	7.50	2.75	3.60
NCAc343	7.00	3.23	3.81
TCGP10	7.50	3.17	3.67
TCGP5	4.50	2.15	4.00
TCGP6	4.25	3.02	4.00
TCGS 320	8.25	3.44	4.00
TCGS 645	4.50	3.58	3.92
TCGS 647	3.50	3.29	4.00
TPT 3	5.00	3.69	4.00
WUE(116)TIR31	6.00	2.83	4.00
WUE(159)IC 43	5.00	2.08	3.38
WUE(159)IC10	5.00	2.8	3.60
WUE(40)JAL 17	4.50	1.92	3.94
WUE(7)IC48	3.00	2.27	3.31
J11 Control ®	4.50	2.25	3.44
JL 24 Control (S)	34.50	3.65	4.00
SEM±	1.1	0.06	
LSD(P=0.05)	25.3	2.80	
CV%	3.07	0.16	

Colonization severity I: unwounded seeds (non pin pricked)
Colonization severity II: wounded seeds (Pin pricked)

Table 22 : *In vitro* seed colonization of selected entries harvested from the *A. flavus* sick plot in the postrainy season (2001-2002) and rainy season (2002)

Entry Number	Colonization severity I (1-4 scale)	Colonization severity II (1-4 scale)
ICGS76	1.77	2.02
ICGV91114	1.29	1.29
ICGV91279	1.56	1.60
ICGV94341	1.50	1.60
ICGV94350	1.75	1.85
ICGV94433	1.69	1.88
ICGV95322	1.29	1.79
ICGV95477	2.44	2.27
TCGP5	2.04	2.23
WUE (116)TIR31	1.40	1.56
WUE (40)JAL17	1.90	2.02
J11 (Control R)	1.48	1.50
JL24 (Control S)	3.38	3.23
SEM±	0.10	0.09
LSD(P=0.05)	0.31	0.27
CV%	7.90	6.80

Colonization severity I: Postrainy season (2001-2002)
Colonization severity II: Rainy season (2002)

Maximum colonization severity was recorded on JL24 (3.23). The colonization severities recorded on ICGV 91114, ICGV 95477 and ICGP 5 were 1.29, 2.27 and 2.23, respectively.

4.5.2 Preliminary Screening of groundnut core-collection accessions

One-hundred eighty four groundnut core collections from ICRISAT gene bank were screened in the laboratory for *in vitro* seed colonization by *A. flavus*. The genotypes were divided into two sets (Set I and Set II) of 92 genotypes each. The genotypes were artificially inoculated with *A. flavus* spore suspension.

Set I:

All the genotypes in set I, gave colonization severity significantly less than susceptible control (JL24) except three genotypes; ICG 2281, which is at par with JL24, ICG 3673 that recorded more than JL24 and ICG 8517, which gave less than JL24 but not significantly different. Colonization severity ranged from 1.05 to 3.19. Maximum colonization severity (3.39) was recorded on ICG 3673 followed by ICG 2381 and JL24 (3.19 and 3.18) respectively (Table 23). When compared to resistant control (J11), all the test genotypes have got colonization severity higher than the J11 except three genotypes ICG1519, ICG2106 and ICG875 that gave less colonization severity than J11. Minimum

Table 23: *In vitro* seed colonization by *Aspergillus flavus* (Af 11-4) in set I of groundnut core collection (first screening)

ICG No.	Colonization severity (1-4 scale)	ICG No	Colonization severity (1-4 scale)
10036	2 20	4684	2 05
10185	2 56	4729	1 81
10566	2 25	4746	1 81
10890	1 94	4750	2 33
111	1 64	4911	2 11
11219	2 14	1955	2 22
11322	2 03	513	2 00
11426	2 61	5195	2 11
115	1 58	5236	2 33
118	1 89	5286	2 39
13858	2 20	532	2 12
1399	1 39	5475	2 78
14106	1 64	5494	2 28
14127	2 31	5662	2 08
1415	1 31	5663	2 34
1519	1 08	5779	2 14
163	1 55	5827	2 17
20016	2 38	5891	2 06
2106	1 06	6201	2 17
2381	3 19	6263	1 92
2511	2 44	6402	2 50
2772	2 03	6654	2 44
2773	2 22	6813	1 81
2777	1 93	6888	2 14
2857	2 22	6892	2 47
297	1 97	7000	2 19
3053	2 50	7153	2 58
3102	2 00	7181	2 17
3240	1 83	7190	2 44
334	2 28	76	2 31
3343	2 53	7906	2 17
3421	1 58	7969	2 08
3584	1 81	81	1 31
36	1 78	8490	2 50
3673	3 39	8517	2 86
3681	1 97	862	1 31
3746	1 86	875	1 05
3775	2 08	9037	1 32
397	2 78	928	1 45
3992	2 34	9315	1 87
4156	2 22	9666	2 28
434	2 05	9842	2 28
4343	1 92	9961	2 47
4389	1 86	J11 (Control R)	1 11
4412	2 00	JL 24 (Control S)	3 18
4538	2 70		
4543	2 75	SEM ±	0 14
4598	1 94	LSD (=0 05)	0 40
4670	2 17	CV %	11 80

colonization severity was recorded on ICG 875 (1.05) followed by ICG 2106, ICG 1519 and J11 (1.06, 1.08 and 1.11, respectively).

Set II:

Eighty-five genotypes out of 93 have got colonization severity significantly lower than susceptible control (JL24), which recorded 3.18 on 1 to 4 scale of colonization severity. Three genotypes have got less colonization severity than JL24 but not significantly differing. These are ICG 4998, ICG 6993, ICG 8083 that recorded 2.83, 3.05 and 2.93 respectively. Five genotypes, ICG 10554, ICG 14710, ICG 188, ICG 2925 and ICG 8106 have got higher colonization severity than JL24. Two genotypes (ICG 10384, ICG 14523) were slightly better than resistant check (J11) but not significantly differing from it. Three genotypes (ICG 12000, ICG 14705, ICG 13099) were similar to the resistant control. Minimum colonization severity was recorded on 10384 (1.04) and ICG 14523 (1.08) followed by J11 (1.11). Maximum colonization severity was recorded on ICG 8106 (3.76) and ICG 2925 (3.62). The susceptible control recorded 3.18 on 1to 4 colonization severity scale (Table 24).

4.5.3 Advanced Screening of Selected Genotypes from Core Collection

Set I

Forty-five genotypes which gave colonization severity ≤ 2 on 1 to 4 scale in preliminary screening were selected from set I genotypes

Table 24 : *In vitro* seed colonization by *A. flavus* in set II groundnut core collection (first screening)

ICG No.	Colonization severity (1-4 scale)	ICG No	Colonization severity (1-4 scale)
10092	1 92	1668	2 69
10384	1 04	1711	2 22
10474	1 81	188	3 29
10479	2 53	1973	2 29
10554	3 33	2019	1 83
11088	1 39	2925	3 62
11109	1 80	3027	2 42
11144	1 30	332	2 15
11249	2 05	442	1 60
1137	1 66	4527	2 15
1142	2 32	4998	2 83
11457	2 17	5016	1 49
11515	2 30	5221	2 32
11651	1 89	5327	1 69
11687	1 79	5609	2 57
11855	2 31	5745	1 47
11862	1 77	6022	2 73
12000	1 23	6057	2 44
12189	1 54	6375	1 89
12276	2 43	6407	1 57
12370	2 10	6646	2 01
12625	2 51	6667	1 54
12672	2 14	6703	1 32
12682	2 50	6766	2 01
12697	1 51	6913	2 78
1274	2 08	6993	3 05
12879	1 54	721	1 54
12921	1 63	7243	2 62
12988	1 47	8083	2 93
13099	1 18	8106	3 76
13491	1 51	8285	2 05
13603	2 03	8567	1 87
13723	1 93	8760	2 43
13787	1 90	9157	1 66
13856	1 34	9249	1 31
14008	1 57	9418	1 87
14118	1 79	9507	1 82
14466	1 32	9777	2 21
14475	1 73	9809	1 57
14482	1 37	9905	2 04
14523	1 08	Controls	
14630	2 18	JII	1 11
14705	1 29	JL24	3 18
14710	3 26	U4-7-5	1 30
14985	1 57	VRR 245	1 71
15042	1 64		
15190	1 79	SEM±	0 15
15287	2 15	LSD (=0 05)	0 41
15309	1 64	CV%	13

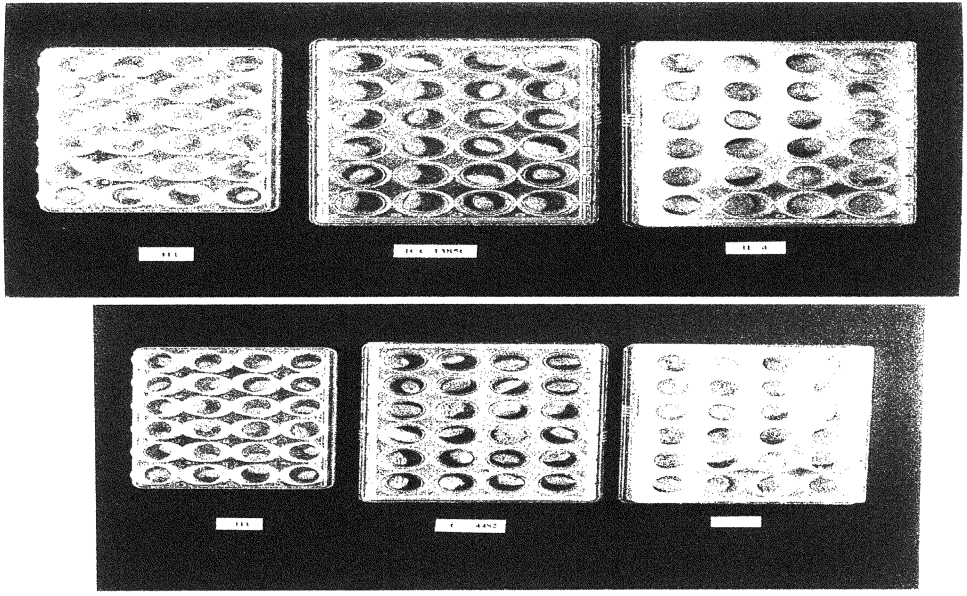

Plate 8: Groundnut genotypes (ICG13856 & 14482) showing highly resistance to *in vitro* seed colonization by *A.flavus*

for advanced screening. Thirty-six genotypes including resistant check gave significantly lower colonization severity than susceptible check (JL24). Six genotypes gave colonization severity lower than JL24 but not significantly differing from it. One genotype (ICG 4412) was similar to JL24. No genotype was significantly superior over resistant check. Maximum colonization severity (3.20) was recorded on ICG 4412 followed by JL24 (3.12) and ICG 4598 (3.00). Minimum colonization severity was recorded on J11 (1.17) followed by ICG 875 (1.30) (Table 25).

Set II:

Fifty-seven genotypes were selected from preliminary screening of Set II core collection, based on their lower colonization severity ≤ 2. Fifty-three genotypes significantly differed from susceptible control. Three genotypes (ICG 12000, ICG 14075 and ICG 6703) gave lower colonization severity than JL24 but not significantly superior over it. Maximum colonization severity was recorded on ICG 11651 (3.31). All the genotypes tested gave higher colonization severity than J11 except U-4-7-5, which gave colonization severity slightly less than J11 but not significantly differing from it. Minimum colonization severity was recorded on U-4-7-5 (1.10) followed by J11 (1.17) (Table 26).

The groundnut genotypes selected from Set I and Set II core collection for second screening against *A. flavus* were used to estimate

Table 25: *In vitro* seed colonization by *A. flavus* and aflatoxin content of selected groundnut core collection (set 1)

ICG No.	Colonization severity (1-4 scale)	Aflatoxin content (µg/kg)
36	2.02	47838.78
81	1.77	25619.35
111	2.35	51362.09
115	2.23	36002.26
118	2.90	44557.16
163	2.04	44557.16
297	1.88	47838.78
434	2.38	34169.26
513	1.67	47838.78
862	1.98	44557.16
875	1.30	38653.81
928	1.60	37562.07
1399	1.77	47838.78
1415	2.00	44557.16
1519	1.67	47838.78
2106	1.96	47838.78
2772	1.96	47838.78
2777	1.85	51362.09
3102	1.63	31075.65
3240	1.52	32746.11
3421	1.42	23917.86
3584	1.96	47838.78
3681	2.63	32214.67
3746	2.21	35190.60
3775	2.42	35075.62
4343	2.08	51362.09
4389	2.44	34983.92
4412	3.20	55144.89
4598	3.00	1089.15
4684	1.94	30173.74
4729	1.83	44557.16
4746	2.85	35537.78
5662	1.85	29976.90
5891	2.92	47838.78
6263	2.42	31588.30
6813	2.58	32109.42
7969	2.23	26558.38
9037	2.60	34619.48
9315	1.50	26996.51
10980	1.90	39460.21
11322	1.46	28262.12
14106	2.31	28822.53
Controls		
J11	1.17	27141.90
JL24	3.12	31152.48
SEM±	0.19	
LSD (=0.05)	0.55	
CV%	13.00	

ate 9: Groundnut genotype (ICG 14985) showing resistance to *in vitro* seed colonization and
(ICG9905) showing high susceptibility to *A.flavus*

the aflatoxin content of the kernels after inoculation and three days of incubation.

Aflatoxin estimation

In set I, five genotypes (ICG 81, ICG 3421, ICG 4598, ICG 7969 and ICG 9315) exhibited less aflatoxin content than J11 (27141.9 $\mu g\ kg^{-1}$). Of these, ICG 4598 was found to be the best genotype recording aflatoxin content (1089 $\mu g\ kg^{-1}$) below one of the resistant controls, VRR 245 (12815.47 $\mu g\ kg^{-1}$). Thirty-one genotypes showed aflatoxin content higher than susceptible control JL24 that recorded 31152.48 $\mu g\ kg^{-1}$. The maximum aflatoxin content was recorded on 4412 (55144.89 μgkg^{-1}) (Table 26).

In set II, the aflatoxin content ranged from 8114.06 µg/kg, which was recorded on ICG 5327 to 49888.16 μgkg^{-1} on ICG 12879. Sixteen genotypes showed aflatoxin content below J11 (27141.9 μgkg^{-1}). Of these, eight genotypes showed aflatoxin content less than the resistant control (VRR 245) (12815.47 $\mu g/kg^{-1}$) and two genotypes, ICG 6646 and ICG 11862 were at par with VRR 245. Thirty-one genotypes showed higher aflatoxin content than susceptible control JL 24 (31152.48 μgkg^{-1}) (Table 26).

Table 26 : *In vitro* seed colonization by *A. flavus* and aflatoxin content of selected groundnut core collection (set II)

ICG No.	Colonization severity (1-4 scale)	Aflatoxin content (µg/kg)	ICG No.	Colonization severity (1-4 scale)	Aflatoxin content (µg/kg)
442	2.56	12005.44	12000	2.69	48265.99
721	1.83	13680.16	12189	1.54	49395.87
1137	1.83	8661.53	12370	1.73	32345.68
2019	2.33	12005.44	12879	2.21	49888.16
5016	2.17	13680.16	12921	1.98	47631.99
5327	1.56	8114.06	12988	2.10	34436.76
5745	1.67	12753.25	13099	1.65	45178.18
6375	1.44	11246.60	13491	2.04	15588.51
6407	1.96	12005.44	13603	2.21	29815.94
6646	1.88	12815.47	13723	2.29	35980.44
6667	1.56	11246.60	13787	1.69	44880.47
6703	2.83	33584.58	13856	1.33	31545.24
6766	2.29	46083.18	14008	1.73	42709.36
8285	2.38	35532.46	14118	2.04	12005.44
8567	1.73	30191.85	14466	2.08	33584.58
9157	2.38	35090.06	14475	2.56	33584.58
9249	2.54	45178.18	14482	1.56	14603.20
9418	2.23	31348.25	14523	2.23	42287.90
9507	2.63	14603.20	14705	2.83	32143.69
9809	2.35	34653.16	14985	1.77	14603.20
9905	3.75	33374.85	15042	1.85	43999.09
10092	1.40	13842.12	15190	2.46	49395.87
10384	1.81	47789.70	15309	2.08	12815.47
10474	2.08	13680.16	**Controls**		
11088	2.06	42427.92	U-4-7-5	1.10	15327.76
11109	1.83	46696.57	J11	1.17	27141.90
11144	1.96	43421.13	VRR 245	1.73	12815.47
11249	1.31	39977.03	JL 24	2.35	31152.48
11651	3.31	34221.71	SEM±	0.16	
11687	1.60	38933.68	LSD (=0.05)	0.45	
11862	1.60	12815.47	CV %	11.1	

4.6 EVALUATION OF THE EFFECTS OF HOST PLANT RESISTANCE, BIOCONTROL AGENTS AND BOTANICALS AS AN INTEGRATED MANAGEMENT PRACTICE TO REDUCE AFLATOXIN CONTAMINATION IN GROUNDNUT

Two biocontrol agents (B33 and *T. viride* (T47) selected from biocontrol experiments, one resistant genotype (ICGV 91114), selected from screening of groundnut genotypes and a neem commercial formulation (Sasyaneem) were combined in an experiment to evaluate their effects on preharvest aflatoxin contamination in groundnut. Groundnut cultivar JL24 was used as susceptible check in all IDM experiments. In the rainy season 2002, this experiment was conducted at ICRISAT, Patancheru and Agricultural Research Station, Anantapur. The same experiment was repeated in the postrainy season 2002-2003 at ICRISAT, Patancheru.

4.6.1 Effect of Treatment Combinations on Total Number of Plants and Yield Parameters Under Field Condition

The data (Table 27) showed that all the IDM treatments were significantly superior to the susceptible control incase of total number of plants per plot in the 2002 rainy season at Patancheru. However, no treatment was significantly superior over the resistant control. The maximum number of plants (72.75) was obtained from ICGV 91114 + *T. viride* followed by J24 + *T. viride* (T47). Minimum number of plants

was recorded in Jl24, susceptible control (44.25), followed by Jl24 + Sasysneem (55.25). In the same season at Anantapur, no treatment was significantly superior over both susceptible and resistant controls. The number of plants / plot ranged from 61 to 68.75 (Table 27). Similarly in the postrainy season (2002-2003) at Patancheru, no significant difference over controls and between the treatments was observed (Table 27).

In case of yield parameters, all combinations with resistant cultivar were compared with resistant control and all combinations with susceptible cultivar were compared with susceptible control. In resistant cultivar combinations no treatment significantly increased the pod yield in the 2002 rainy season at Patancheru and only one treatment (ICGV 91114+ B33) considerably increased the pod yield over the resistant control (21.27%). Incase of susceptible cultivar combinations, susceptible + IDM gave significant increase in pod yield (35.38%). Least percent increase in pod yield was observed on JL 24 + Sasyaneem in 2002 at Patancheru (Table 27). In Anantapur, only one treatment, ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem gave significant pod yield increase (11.35%) over the resistant control (ICGV 91114 alone). In JL24 combinations, three treatments, JL24 + *T. viride* (T47) + B33+Sasyaneem, JL24 + B33 and JL24 + *T. viride* (T47) were found to be significant in increasing pod yield compared to control (JL24 alone).

The maximum yield increase was obtained from JL24 + *T. viride* (T47) + B33+Sasyaneem (24.64%) followed by JL24 + B33 (18.88%) (Table 27). The results (Table 27) showed that two treatments, ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem, ICGV 91114 + B33, gave significant increase over ICGV 91114 and JL24 + *T. viride* (T47) + B33 + Sasyaneem and Jl24 + B33 gave significant increase in pod yield over JL24 alone in the postrainy season (2002-2003). ICGV 91114 + *T. viride* (T47) and ICGV 91114 + Sasyaneem did not differ significantly from resistant cultivar alone. JL24 + *T. viride* (T47) and JL 24 + Sasyaneem did not differ significantly from susceptible check (Jl24 alone). The maximum pod yield increase (11.37 %) was found in ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem followed by JL24 + *T.viride* (T47) + B33 + Sasyaneem (9.24 %).

The results (Table 27) revealed that two treatments, ICGV 91114 + B33 and ICGV 91114 +*T. viride* + B33 + Sasyaneem increased the seed weight significantly over control giving 31.27 and 27.35% respectively in the 2002 at Patancheru. Least seed weight increase was obtained from ICGV 91114 + saysaneem (7.28%). JL 24 + B33 and JL 24 + *T. viride* +B33 + Sasyaneem gave considerable increase in seed weight recording 29.95% and 35.50% increase over JL24 followed by JL 24 *T. viride* (T47). In JL24 + Sasyaneem, the seed yield was at par with JL24 alone (Table 27). Similarly, ICGV 91114 + *T. viride* (T47) +

B33 + Sasyaneem and ICGV 91114 + B33 were significant in increasing the seed yield compared to ICGV 91114 alone in Anantapur. These two treatments provided 32.08% and 18.92% seed yield increase respectively (Table 27). In susceptible control combinations, two treatments, JL24 + *T. viride* (T47) + B33+Sasyaneem and JL24 + B33 gave significant increase in seed weight (33.06% and 22.72% respectively) compared to JL24 alone (Table 27). In the postrainy season, no treatment gave significantly higher seed yield than the controls in both resistant and susceptible combinations when compared to resistant and susceptible checks respectively. In resistant cultivar combinations involving ICGV 91114 maximum seed yield increase (13.79 %) was recorded in ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem followed by ICGV 91114 + B33 (11.35 %). In combinations involving JL24, JL24 + *T. viride* (T 47) + B33 + Sasyaneem was found to be the best in increasing the seed yield (11.66 %) over JL24 alone. JL24 + Sasyaneem (4.30 %) recorded least increase in seed yield over JL24 alone. ICGV 91114 + *T. viride* (T47) and ICGV 91114 + Sasyaneem were at par (4.82 %) in increasing seed yield (Table 27).

In the 2002 rainy season at Patancheru, ICGV 91114 + B33 and ICGV 91114 + *T. viride* + B33 + Sasyaneem gave significant biomass weight increase over ICGV 91114 exhibiting 9.75% and 11.35% increase respectively. JL24 + *T. viride* + B33 + Sasyaneem, JL24 + B33

Table 27 : Effect of IDM on total number of plants and yield parameters under field conditions in ICRISAT (rainy season 2002 and postrainy season 2002-2003) and at Anantapur (rainy season 2002)

Treat-ments	Total Plants / plot*			Pod yield / plot (gm) *						Seed yield / plot (gm)*						Bio-mass yield / plot (gm)*			
	Exp. I	Exp. II	Exp. III	Exp. I	Exp. I % reduction	Exp. II	Exp. II % reduction	Exp. III	Exp. III % reduction	Exp. I	Exp. I % reduction	Exp. II	Exp. II % reduction	Exp. III	Exp. III % reduction	Exp. I	Exp. I % reduction	Exp. III	Exp. III % reduction
R+B33	63.75	65.50	121.75	137.5	21.27	159.50	8.15	1625.00	8.62	90.38	31.27	111.00	18.92	1057.50	11.35	692.5	9.75	2200.00	9.09
R+IDM	65.25	64.25	119.00	127.9	15.36	165.25	11.35	1675.50	11.37	85.5	27.35	132.50	32.08	1087.50	13.79	705	11.35	2200.00	9.09
R	63.25	65.00	121.00	108.25	0.00	146.50	0.00	1485.00	0.00	62.12	0.00	90.00	0.00	937.50	0.00	625	0.00	2000.00	0.00
R+Sasyn	66.25	61.00	122.75	109.62	1.25	147.00	0.34	1525.00	2.62	67	7.28	91.50	1.64	985.00	4.82	627.5	0.40	2100.00	4.76
R+T47	72.75	65.00	122.25	112.5	3.78	154.00	4.87	1590.00	6.60	68.38	9.15	101.00	10.89	985.00	4.82	650	3.85	2190.00	8.68
S+P33	60.75	64.00	121.00	90.5	33.15	147.00	18.88	1545.00	7.77	46.75	29.95	106.75	22.72	980.00	9.18	550	18.18	2120.00	6.25
S+IDM	61.75	68.75	122.25	93.63	35.38	158.25	24.64	1570.00	9.24	50.62	35.30	123.25	33.06	1007.50	11.66	612.5	26.53	2155.00	7.77
S	44.25	64.75	121.25	60.5	0.00	119.25	0.00	1425.00	0.00	32.75	0.00	82.50	0.00	890.00	0.00	450	0.00	1987.50	0.00
S+Sasyn	55.25	63.00	122.5	61.63	1.83	123.50	3.44	1490.00	4.36	33.12	1.12	84.75	2.65	930.00	4.30	485	7.22	2000.00	0.63
S+T47	68.5	64.25	123.25	81.75	25.99	134.75	11.50	1500.00	5.00	43.25	24.28	90.25	8.59	940.00	5.32	547.5	17.81	2025.00	1.85
SEM ±	4.7	6.00	2.04	12.03		4.53		38.74		7.15		4.33		71.69		15.55		87.17	
LSD (=0.05)	13.72	17.51	5.95	36.64		13.21		113.07		20.88		12.63		209.24		45.39		254.43	
CV%	15.12	18.59	3.35	20.7		6.22		5.02		24.67		8.54		14.63		5.25		8.31	

* Mean of four replications

Exp. I : Rainy season 2002 at ICRISAT; Exp. II : Rainy season 2002 at Anantapur; Exp. III : Post rainy season 2002-2003 at ICRISAT

and JL24 + *T. viride* (T47) increased the biomass yield significantly compared to JL24 alone. The maximum biomass yield increase (26.53%) was obtained from JL24 + *T. viride* + B33 + Sasyaneem followed by JL24+B33 (18.18%) and JL24 + *T. viride* (T47) (17.81%) (Table 27). In the postrainy season (2002-2003), there was no significant difference over control and between the treatments in both resistant and susceptible cultivar combinations. ICGV 91114 + *T. viride* T47) + B33 + Sasyaneem and ICGV 91114 + B33 were at par in increasing biomass yield (9.09 %) over resistant cultivar alone. In JL24 combinations, JL24 + *T. viride* (T47) + B33 + Sasyaneem was found to be the best (7.77 % increase in biomass yield over JL24 alone) followed by JL24 + B33 (6.25 %) (Table 27)

4.6.2 Effect of Treatment Combinations on *A. Flavus* Population at Different Crop Stages

At 40 days after sowing, ICGV 91114 + B33, ICGV 91114 + *T. viride* + B33 + Sasyaneem and ICGV 91114 + *T. viride* (T47) showed significant reduction in *A. flavus* population over both resistant and susceptible controls. JL 24 + B33, JL 24 + *T. viride* + B33 + Sasyaneem and JL 24 + *T. viride* were also significantly effective in reducing the *A. flavus* population compared to both the controls. The treatment, ICG 91114 + B33 + *T. viride* (T47) exhibited the maximum reduction in *A. flavus* population (36.84%) followed by ICGV 91114 + *T. viride*

(31.58%). ICGV 91114 + B33 and JL24 + *T. viride* + B33 reduced the *A. flavus* population to an extent of 28.95 and 26.32% respectively (Table 28).

In Anantapur experiment, all combinations involving ICGV 91114 were found to be significant in reducing *A. flavus* population compared to susceptible control (JL24) (Table 28). All JL24 combinations were also significant over JL24 alone except JL24 + Sasyaneem which was applied after 40 DAS samples. Four treatments; ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem, ICGV 91114 + B33, ICGV 91114 + *T. viride* (T47) and JL24 + *T. viride* (T47) + B33 + Sasyaneem were found to be significant in reducing *A. flavus* population when compared to resistant control (ICGV 91114) alone. The maximum reduction in *A. flavus* population (56.76%) was obtained from ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem followed by ICGV 91114 + *T. viride* (T47) (48.65%) and ICGV 91114 + B33 (44.59%). In postrainy season (2002-2003), three treatments, ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem, ICGV 91114 + *T. viride* (T47) and JL24 + *T. viride* (T47) + B33 + Sasyaneem were found to be significant in reducing *A. flavus* population over both resistant and susceptible checks. These provided 46.15%, 30.77% and 30.77% reduction in *A. flavus* populations respectively over JL24 (susceptible) (Table 28).

At 80 days after sowing, all the treatments were significantly effective in reducing *A. flavus* population over both susceptible and resistant controls except JL24 alone in the 2002 rainy season at Patancheru. ICGV 91114 + *T. viride* (T47) + B33+Sasyaneem exhibited the maximum reduction of *A. flavus* population (43.31%) over susceptible control followed by ICGV91114 + *T. viride* (T47) which gave 39.37% reduction over susceptible control. ICGV 91114 B33 was at par with JL 24 *T. viride* (T47) + B33+Sasyaneem in reducing the population of *A. flavus* and both recorded 35.43%. The least percent reduction of *A. flavus* (12.60%) over (JL24) was observed on ICGV91114 alone (Table 28). Similarly in Anantapur experiment, all the treatments except JL24 + Sasyaneem, significantly reduced the *A. flavus* population compared to susceptible control (JL24) alone. All treatments integrated with resistant control and JL24 + *T. viride* (T47) + B33 + Sasyaneem were also significantly effective in reducing *A. flavus* population when compared to resistant control (ICGV 91114). ICGV 91114 + *T. viride* (T47) + B33+Sasyaneem and ICGV 91114 + *T. viride* (T 47) were found to be the best in reducing *A. flavus* population at 80 days after sowing. These two treatments provided 32.45 and 27.13 % reduction respectively over susceptible control (Table 28). The least reduction in the population (10.11 %) over susceptible control was recorded on ICGV 91114 alone and JL24 + B33 (10.64%) followed by JL24 + Sasyaneem (11.70%). In case of postrainy season (2002-2003),

no treatment was significantly differing from both resistant and susceptible cultivars in reducing *A. flavus* population. Considerable reduction in *A. flavus* population (29.41%) was found in ICGV 9114 + *T. viride* (T47) + B33 + Sasyaneem followed by ICGV 91114 + *T. viride* (T47) and ICGV 91114 + Sasyaneem which were at par (23.53%) in reducing *A. flavus* population over JL24 alone. The least reduction was recorded by resistant cultivar alone (9.80%) over susceptible cultivar followed by JL24 + B33 (11.76%) (Table 28).

At crop harvesting, none of the treatments was significant in reducing *A. flavus* population over the controls in the 2002 rainy season. The maximum reduction (39.13%) over susceptible control was obtained from ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem followed by JL24 + *T. viride* (T47) + B33 + Sasyaneem (35.51%). Considerable reduction of *A. flavus* population was observed in ICGV 91114 + *T. viride* (T47) and JL 24 + *T. viride* (T47), (32.61 and 29.71% respectively). The least reduction (8.70%) was recorded by ICGV 91114 alone compared to JL24 (Table 28). Incase of Anantapur samples, all the integrated treatments were found to be significantly effective in reducing *A. flavus* population when compared to both susceptible and resistant control. The highest reduction was obtained from ICGV 91114 + *T. viride* (T47) + B33+Sasyaneem (33.12%) followed by JL24 + *T. viride* (T47) + B33+Sasyaneem (29.87%). The

Table 28 : Efficacy of IDM in reducing *A. flavus* population in the groundnut rhizosphere under field conditions at ICRISAT (rainy season 2002 and postrainy season (2002-2003) and Anantapur (rainy season 2002)

Treat-ments	40 Days After Sowing						80 Days After Sowing						At harvest					
	Exp. I	Reduction %	Exp. II	Reduction %	Exp. III	Reduction %	Exp. I	Reduction %	Exp. II	Reduction %	Exp. III	Reduction %	Exp. I	Reduction %	Exp. II	Reduction %	Exp. III	Reduction %
R+B33	6750	28.95	10250	44.59	2500	23.08	20500	35.43	36500	22.34	21250	16.67	24750	28.26	29250	24.03	6250	51.92
R+IDM	6000	36.84	8000	56.76	1750	46.15	18000	43.31	31750	32.45	18000	29.41	21000	39.13	25750	33.12	4250	67.31
R	8750	7.89	14500	21.62	3250	0.00	27750	12.60	42250	10.11	23000	9.80	31500	8.70	35500	7.79	7250	44.23
R+Sasyn	8750	7.89	14250	22.97	3200	1.54	21250	33.07	36750	21.81	19500	23.53	28000	18.84	31250	18.83	6750	48.08
R+T47	6500	31.58	9500	48.65	2250	30.77	19250	39.37	34250	27.13	19500	23.53	23250	32.61	28000	27.27	5000	61.54
S+P33	7750	18.42	15250	17.57	2750	15.38	23000	27.56	42000	10.64	22500	11.76	25250	26.81	31750	17.53	10500	19.23
S+IDM	7000	26.32	12750	31.08	2250	30.77	20500	35.43	37000	21.28	19750	22.55	22250	35.51	27000	29.87	7250	44.23
S	9500	0.00	18500	0.00	3250	0.00	31750	0.00	47000	0.00	25500	0.00	34500	0.00	38500	0.00	1300	0.00
S+Sasyn	9500	0.00	18250	1.35	3250	0.00	23750	25.20	41500	11.70	21000	17.65	30250	12.32	33750	12.34	1150	11.54
S+T47	7500	21.05	14250	22.97	2500	23.08	21750	31.50	40250	14.36	20500	19.61	24250	29.71	28750	25.32	8500	34.62
SEM ±	290.10		333.85		297.56		270.03		672.37		3882.92		6222.69		476.53		580.95	
LSD (=0.05)	841.90		974.45		868.51		788.16		1962.51		11333.45		18162.75		1390.90		1695.67	
CV%	7.90		4.91		21.84		2.37		3.45		36.89		46.96		3.08		14.48	

* Mean of four replications

Exp. I : Rainy season 2002 at ICRISAT; Exp. II : Rainy season 2002 at Anantapur; Exp. III : Post rainy season 2002-2003 at ICRISAT

lowest reduction in *A. flavus* population was recorded on ICGV91114 alone (7.79%) followed by JL24 + Sasyaneem (12.34%) (Table 28). The results of the postrainy season (2002-2003) soil samples showed that all resistant cultivar combinations gave significant reduction in *A. flavus* population over susceptible control (Table 28). All susceptible cultivar combinations also gave significant reduction in the pathogen population except JL24 + Sasyaneem. Two treatments, ICGV 91114 + *T. viride* + B33 + Sasyaneem and ICGV 91114 + *T. viride* (T47) significantly reduced the pathogen population when compared to resistant cultivar. JL24 + *T. viride* (T47) + B33 + Sasyaneem was at par with resistant cultivar alone (44.23%) when compared to JL24 alone. The highest *A. flavus* population reduction was recorded by ICGV 91114 + *T. viride* +B33+Sasyaneem (67.31%) followed by ICGV 91114 + *T. viride* (T47), which provided 61.54 % reduction over susceptible control (JL24). The least reduction of *A. flavus* population was recorded in JL24 + Sasyaneem (11.54%) compared to JL24 alone (Table 28).

4.6.3 Effect of IDM on preharvest seed infection by *A. flavus*

The results (Table 29) revealed that all the combinations involving ICGV 91114 significantly reduced the seed infection by *A. flavus* compared to susceptible control. ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem exhibited the maximum reduction (62.07%) over susceptible control (JL24) followed by ICGV 91114 + *T. viride* (T47)

which provided 59.36% reduction. ICGV 91114 + B33 + Sasyaneem and ICGV 91114 alone provided significant reduction in seed infection over JL24 (Table29). In case of combination treatments involving JL24, JL24 + *T. viride* + B33 + Sasyaneem exhibited 14.31% seed infection reduction over JL24 alone. There is no significant difference between the treatments involving ICGV 91114. As the case in Patancheru, all treatments integrated with resistant cultivar (ICGV 91114) were significant in reducing seed infection by *A. flavus* compared to susceptible control (JL24) alone in Anantapur experiment (Table 29). Two treatments of JL24 integrations were also significant in the reduction of seed infection; JL24 + *T. viride* (T47) + B33+Sasyaneem and JL24 + *T. viride* (T47) compared to JL24 alone. Only ICGV 91114 *T. viride* (T47) + B33+Sasyaneem and ICGV 91114 + *T. viride* were significantly superior over resistant cultivar. JL 24 + *T. viride* (T47) + B33+Sasyaneem was found to be the best treatment in reducing seed infection by *A. flavus* (51.46%) followed by ICGV 91114 + *T. viride* (T47) (48.06%) (Table 29). The least percent reduction was recorded on JL24 + Sasyaneem (5.83%). The results (Table 29) showed that all treatments in resistant and susceptible cultivar combinations showed significant reduction in seed infection when compared to susceptible cultivar (JL24) alone except JL24 + Sasyaneem. All the combinations are also significant over resistant control except JL24 alone and JL 24 + Sasyaneem. The highest percent reduction in seed infection was

Table 29 : Efficacy of IDM in reducing preharvest seed infection by *A. flavus* in groundnut under field conditions at ICRISAT, Patancheru (rainy season, 2002 and postrainy,2002-2003) and at Anantapur (rainy season, 2002)

Treatments	EXPERIMENT I		EXPERIMENT II		EXPERIMENT III	
	Seed infection %*	Reduction over control %	Seed infection %*	Reduction over control %	Seed infection %*	Reduction over control %
ICGV 1114+B33	13.78	3.50	28.75	44.17	40.50	22.12
ICGV 91114+IDM	11.93	16.46	25.00	51.46	32.25	37.98
ICGV 91114	14.28	0.00	31.75	38.35	49.50	4.81
ICGV 1114+Sasyaneem	14.25	0.21	30.75	40.29	45.50	12.50
ICGV 91114+*T.viride* (T47)	12.78	10.50	26.75	48.06	36.00	30.77
JL24+B33	30.74	2.26	46.75	9.22	45.75	12.02
JL24+IDM	26.95	14.31	39.25	23.79	39.00	25.00
JL24	31.45	0.00	51.50	0.00	52.00	0.00
JL24+Sasyaneem	31.38	0.22	48.50	5.83	50.50	2.88
JL24+*T.viride* (T47)	28.53	9.28	44.75	13.11	42.25	18.75
SEM±			1.73		0.89	
LSD(P=0.05)			5.05		2.59	
CV%			9.26		4.09	

* Mean four replication
Experiment I: Rainy season (2002) at ICRISAT
Experiment II: Rainy season (2002) at Anantapur
Experiment III: Postrainy season (2002-2003) at ICRISAT

recorded in ICGV 91114 + *T. viride* (T47) + B33+Sasyaneem (37.98%) followed by ICGV 91114 + *T. viride* (T47) (30.77%) compared to JL24. The minimum reduction (2.88%) was observed on JL24 + Sasyaneem followed by resistant cultivar alone (4.81%) compared to susceptible control (Table 29).

4.6.4 Effect of Treatment Combinations on Aflatoxin Content in Groundnut Kernels Under Field Conditions

The Data in Table 30 showed that all combination treatments involving ICGV 91114 were significantly superior over susceptible control but not differing from resistant check significantly except ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem which recorded maximum reduction (38.39%) over JL24. This was followed by ICGV 91114 + *T. viride* (T47) (33.93%) and ICGV 91114 + B33 (31.25%). In the combinations involving JL24, only JL24 + *T. viride* (T47) + B33 + Sasyaneem gave significant reduction (23.21%) compared to JL24 alone. In Anantapur experiment, only two treatments, ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem and ICGV 91114 + *T. viride* (T47) were significantly superior to JL24. No treatment is significantly different from resistant cultivar. The highest reduction in aflatoxin content over the susceptible control was found in ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem (26.90%) followed by ICGV 91114 + *T. viride* (T47) (24.96%) (Table 51). JL24 + Sasyaneem did not

Table 30 : Effect of IDM on aflatoxin content in groundnut kernels at ICRISAT, Patancheru (rainy season, 2002 and postrainy season 2002-2003) and at Anantapur (rainy season, 2002)

Treatments	EXPERIMENT I		EXPERIMENT II		EXPERIMENT III	
	Seed infection %*	Reduction over control %	Seed infection %*	Reduction over control %	Seed infection %*	Reduction over control %
ICGV 91114+B33	19.25	31.25	21.25	22.22	16.68	31.92
ICGV 91114+IDM	17.25	38.39	19.97	26.90	14.95	38.98
ICGV 91114	23.00	17.86	22.75	16.73	18.65	23.88
ICGV 91114+Sasyaneem	20.75	25.89	22.00	19.47	18.48	24.57
ICGV 91114+*T.viride* (T47)	18.50	33.93	20.50	24.96	15.98	34.78
JL24+B33	24.50	12.50	26.70	2.27	22.65	7.55
JL24+IDM	21.50	23.21	23.25	14.90	17.73	27.63
JL24	28.00	0.00	27.32	0.00	24.5	0.00
JL24+Sasyaneem	26.50	5.36	28.15	-3.04	23.75	3.06
JL24+*T.viride* (T47)	23.50	16.07	25.75	5.75	20.98	14.37
SEM±	1.59		2.25		1.36	
LSD(P=0.05)	4.65		6.56		3.97	
CV%	14.30		18.92		14.01	

* Mean four replication
Experiment I: Rainy season (2002) at ICRISAT
Experiment II: Rainy season (2002) at Anantapur
Experiment III: Postrainy season (2002-2003

reduce the aflatoxin content in the kernels (Table 40). In postrainy season (2002-2003), all the resistant cultivar combinations gave significant reduction in aflatoxin content compared to susceptible control (JL24) whereas in susceptible cultivar combinations only JL24 + *T. viride* (T47) + B33 + Sasyaneem gave significant reduction over JL24. The maximum percent reduction (38.98%) was obtained from ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem followed by ICGV 91114 + *T. viride* (T47) (34.78%). The least percent reduction (3.06 %) was recorded on JL24 + Sasyaneem (Table 30).

4.7 EVALUATION OF PLANT GROWTH PROMOTING POTENTIAL OF BIOCONTROL AGENTS IN GREENHOUSE CONDITIONS

The plant growth promoting potential of five *Trichoderma* isolates; *Trichoderma viride* (T21), *T. harzianum* (T23), *T. viride* (T38), *T. viride* (T47) and *T. viride* (T48) and six bacterial isolates (B6, B18, B33, B50, B58 and *Pseudomonas fluorescens* (PF2)) used as biocontrol agents were evaluated under greenhouse conditions. Four growth parameters (root length, root weight, shoot length and shoot weight) were recorded 35 days after sowing. All the biocontrol agents were applied to the seeds of cultivar ICGS11 before sowing.

Incase of root length, two bacterial isolates (B18 and B50) gave significantly more root growth over the control. All other

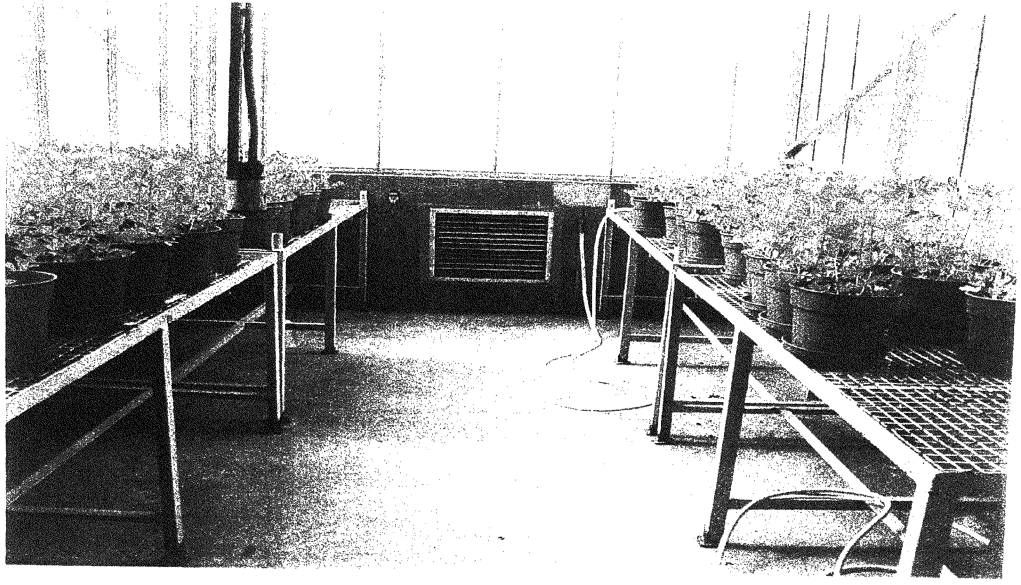

Plate 10: Overview of greenhouse experiment plant growth promoting potential of biocontrol agents

biocontrol agents showed superiority over control but did not differ significantly from it. Maximum root length (25.84 cm) was recorded on B50 followed by B18 (23.63 cm). The least root length was recorded in control and T21 (17.33 and 17.92, respectively) (Table 31).

Incase of root weight, three bacterial isolates (B50, B58, PF2) gave higher root weight over the control. Maximum root weight (2.42 g) was recorded on *Psudomonas fluorescens* (PF2) followed by B50 (2.20 g). Control treatment got the minimum roots weight (0.99 g) (Table 31).

For shoot length growth, seven biocontrol agents out of eleven were significantly superior over control. The maximum shoot length was recorded on B33 (29.92 cm), T47 (29.75 cm) and PF2 (29.04) followed by B50 (28.83) and *T. viride* (T.V 38) (28.83). Minimum shoot length (24.43) was recorded in control followed by B6 (25.50) (Table 32).

Incase of shoot weight, all the biocontrol agents significantly promoted the shoot weight except two biocontrol B18 and *T. viride* (T48). Maximum shoot weight was recorded on T23 (8.49 g), B50 (8.40g), B33 (8.36) and T38 (8.15 g) and the least shoot weight was obtained from control (5.6 g) followed by B18 (6.97 g) (Table 32).

Plate 11: T 23 showing plant growth promoting effect in groundnut under greenhouse conditions

Plate 12: T 38 showing slight growth promoting effect in groundnut under greenhouse conditions

Table 32 : Effect of Biocontrol agents on shoot length and shoot weight of groundnut under greenhouse conditions (2003)

Biocontrol Agents	Shoot length (cm)*	Increase over control %	Shoot weight (gm) *	Increase over control %
B18	26.75	8.67	6.97	19.66
B33	29.92	18.35	8.36	33.01
B50	28.83	15.26	8.40	33.33
B58	28.92	15.53	7.19	22.11
B6	25.50	4.20	7.15	21.68
P. fluorescens (Pf_2)	29.04	15.87	7.56	25.93
T.viride (T21)	26.45	7.64	7.18	22.01
T.harzianium (T23)	28.00	12.75	8.49	34.04
T.viride (T38)	28.83	15.26	8.15	31.29
T.viride (T47)	29.75	17.88	7.62	26.51
T.viride (T48)	27.17	10.08	7.06	20.68
Control (No BCA)	24.43	0.00	5.60	0.00
SEM±	1.22		0.56	
LSD(P=0.05)	3.50		1.61	
CV%	8.80		15.00	

* Mean of four replications

Plate 13: B 33 showing growth promoting effect in groundnut under greenhouse conditions

Plate 14: B 50 showing growth promoting effect in groundnut under greenhouse conditions

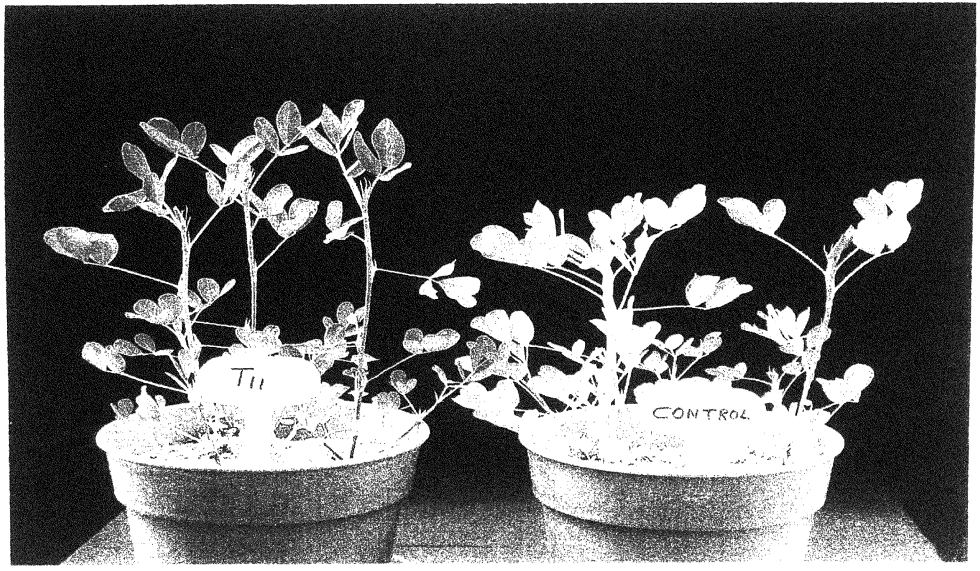

Plate 15: PF 2 showing growth promoting effect in groundnut under greenhouse conditions

CHAPTER V

DISCUSSION

In this chapter the results from the investigation have been discussed under the following heads :

5.1 Isolation of *Trichoderma spp* and bacterial strains from groundnut rhizosphere soils

5.2 evaluation of *Trichoderma spp* and bacterial strains for *in vitro* and *in vivo* antagonistic activity against *A. flavus*

5.3 improvement of *T. viride* (T47) by irradiation

5.4 efficacy of neem commercial formulations against *A. flavus in vitro* and *in vivo*

5.5 identification of germaplasm accessions, advance breeding lines and varieties for resistance to *in vitro* seed colonization and seed infection by *A. flavus*

5.6 Integrated management practice to reduce aflatoxin contamination in groundnut

5.7 evaluation of plant growth promoting potential of biocontrol agents in greenhouse conditions

5.1 ISOLATION OF *Trichoderma Spp* AND BACTERIAL STRAINS FROM GROUNDNUT RHIZOSPHERE SOILS

The rhizosphere microbes have great significance in maintaining the fertility of the soil and in suppressing the soil borne plant pathogens. The fungistatic effect in the rhizosphere against soil borne plant pathogens and root pathogens is in part due to the microbes, which exude certain antibiotics. Keeping this in view, *Trichoderma spp.* and bacterial stains were isolated from groundnut rhizosphere soils to test their potential to control *A. flavus* and aflatoxin contamination in groundnut.

In the present study, forty-nine *Trichoderma* isolates and seventy-seven bacterial strains were isolated from soil samples collected from different groundnut growing areas of Andhra Pradesh using dilution plate method.

Various *Trichoderma* spp. have been isolated from rhizosphere soils of several crops on specific media for their characterization and utilization in biological control (Hadar *et al.*, 1984; Anitha Chowdary, 1997).

Saralamma (2000) also obtained 12 microorganisms including *T. harzianum* from groundnut rhizosphere soils by dilution plate method.

5.2 EVALUATION OF *TRICHODERMA SPP* AND BACTERIAL STRAINS FOR *IN VITRO* AND *IN VIVO* ANTAGONISTIC ACTIVITY AGAINST *A. FLAVUS*

5.2.1 Evaluation of *Trichoderma* Isolates *In Vitro*

In the present study, forty-nine *Trichoderma* isolates were evaluated for *in vitro* antagonistic potential against toxigenic *A. flavus* (Strain 11 – 4). All the *Trichoderma* isolates tested, significantly inhibited the mycelial growth of *A. flavus in vitro*. The most effective isolates were *T. viride* (T48), *T. viride* (T47) and *T. viride* (T38) allowing minimum mycelial growth of the test fungus, 1.00, 1.03 and 1.06 cm respectively. Percent inhibition of *A. flavus* mycelial growth ranged from 46.11 to 76.91 %. This inhibition is due to mechanical suppression mainly or mechanical suppression plus antibiosis. *Trichoderma spp*, though produce antibiotics and cell wall degrading enzymes mainly act as mycoparasites on other fungi (Lewis and Papavizas, 1991). Desai *et al.*. (2000) reported very slow growth of

A. flavus due to production of non-volatile chemicals inhibitory to *A. flavus* growth by *Trichoderma* isolates.

5.2.2 Screening of Bacterial Isolates Against *A. flavus In Vitro*

Seventy-seven bacterial isolates were screened for biocontrol efficacy against toxigenic *A. flavus* (Strain 11-4) *in vitro*. More than 50% of the bacterial isolates tested (52 isolates) suppressed the growth of *A. flavus* significantly whereas 25 isolates did not. In B22, B37, B3, and B64, the *A. flavus* growth was at par with the control (*A. flavus* alone, without bioagent).

Percent inhibition of *A. flavus* mycelial growth ranged from 0 to 47.62%. Munimbazi and Bullerman (1998) reported that six *Bacillus pumilus* isolates inhibited the mycelial growth and aflatoxin production by *A parasiticus*. In the present study, the inhibition of *A. flavus* growth by bacterial isolates was less than that by the *Trichoderma* isolates. He also reported similar results in *B. pumilus* when tested against *A. parasiticus*, which showed less inhibition of mycelium production with percentage of inhibition ranging from 34.40% to 56.40 %. The inhibitory activity was likely due to extracellular metabolites produced by the bacterium in the growth medium. Misaghi *et al.* (1995) screened 892 bacterial isolates for their ability to inhibit the growth of *A. flavus* on cotton seed *in vitro*, out of which only six isolates partially or totally inhibited the fungal growth.

The best 19 bacterial isolates were selected from the preliminary screening of 77 bacterial isolates to confirm the efficacy they showed against *A. flavus*. All the isolates gave more or less similar results of the first screening. All the isolates significantly inhibited the mycelial growth of *A. flavus* in dual culture experiment. In second screening, the percent inhibition of *A. flavus* growth by bacterial isolates ranged from 31.75 to 50.79%. Among the bacterial isolates tested, B6, B18, B33, B50 and B58 were found to be the most effective in inhibiting *A. flavus* growth *in vitro*.

5.2.3 Efficacy of Biocontrol Agents Under Greenhouse Conditions (2001-2002 And 2002)

The most effective five *Trichoderma* isolates, *T. viride* (T21), *T. harzianum* (T23), *T. viride* (T38), *T. viride* (T47) and *T. viride* (T48) were selected from *in vitro* screening of 49 *Trichoderma* isolates. Similarly six bacterial isolates, B6, B18, B33, B50, B58 and PF2 were selected from *in vitro* screening of 77 bacterial isolates. These *Trichoderma* and bacterial isolates were tested in greenhouse experiment to evaluate their efficacy against *A. flavus in vivo*.

In case of total number of plants/pot, no significant difference was noticed among the treatments including control (without BCA). Similarly in the second greenhouse experiment, none of the biocontrol agents tested gave significant increase in total number of

plants. The total number of plants/pot ranged from 3.5 - 4 plants/pot in all the treatments including control.

Most of the biocontrol agents exhibited better pod yield compared to control. However, some of the *Trichoderma* isolates were at par with control in pod yield. *T. viride* (T21) and *P. fluorescens* (PF2) significantly increased the pod yield by 30.88 and 29.03 % over control respectively. *T. harzianum* (Th23), B18 and B33 increased the pod yield by 22.65, 22.19 and 20.42 %, respectively. Similarly in second experiment, all the biocontrol agents gave more than 10% increase in pod yield except B6 and *T. viride* (T47). *T. harzianum* (T23) gave significant increase in pod yield (25.63%) followed by *T. viride* (T38) (22.99%) and *T. viride* (T21) (20.29%). Among the bacterial isolates B33 and B18 were the best treatments providing pod yield increase of 19.90 and 19.65% increase over control. Baig *et al.* (2002) reported that rhizobacteria isolates tested as plant growth promoters of groundnut in pot culture experiment, increased the pod yield compared to untreated control. Plant growth promoting bacteria treated plants produced 9.27 g $plant^{-1}$ pod yield whereas untreated control plants produced 5.5 g/plant. Similarly, Dey *et al.* (1999) reported increase in yield of groundnut by the seed coating with the noncynogenic isolates C185 (24.69%) and C397 (22.53%), which were significantly higher than the control. Raguchander *et al.* (1997) reported that *T. viride* supported higher plant growth, better nodulation and grain yield (323.6 kg/ha) in mungbean.

Saralamma (2000) reported that *T. harzianum* increased the pod yield in groundnut (1373.3 kg ha^{-1}) compared to control (826.7 kg ha^{-1}) when the spore suspension of the bioagent was used as seed treatment in greenhouse experiment.

In case of seed yield, *P. fluorescens* (PF2), *T. viride* (T21) and *T. harzianum* (T23) were the best among the biocontrol agents tested in increasing seed yield providing 23.74, 23.32 and 22.20 percent increase over control respectively. In the second experiment, majority of the biocontrol agents showed less per cent increase in seed yield. However *T. viride* (T38) recorded significant increase in seed yield (3023%) over control followed by *T. harzianum* (Th23) (25.03%) and bacterial isolates B33 (21.45%). *Trichoderma* spp. and *P. fluorescens* are known to increase the yield in different crops. Shetty and Raj (2002) reported that isolates of *Bacillus pumilus* promoted the growth and increased the yield from 27 to 30% in pearl millet under greenhouse and field conditions and *P. fluorescens* isolate (UOM SAR 14) provided higher levels of vegetative and reproductive growth parameters, most important of all being the 22% increase in grain yield.

On the other hand, the biocontrol agents increased the biomass yield. Among the biocontrol agents, *T. viride* (T38), B50 and B6 were found to be best bioagents (in first experiment) in increasing biomass yield recording 28.05, 25.28 and 24.07% increase over control

respectively. In second experiment, the mean biomass weight ranged from 91.5 g to 123.5 g. Majority of the biocontrol agents tested showed more than 10% increase in biomass weight over control. *T. viride* (T38) increased the biomass weight significantly (25.91%) followed by *T. viride* (T21) (19.74%). Growth stimulation by *Trichoderma* spp could be the result of production of plant hormones by the fungus, increased uptake of nutrients by the plant or the control of one or more subclinical pathogens (Chet, 1990).

The ability of the biocontrol agents to reduce the *A. flavus* population was also tested. The *A. flavus* population at 40DAS samples was natural infestation of the pot soil after sterilization. All the biocontrol agents showed some level of reduction in the population of the test fungus. Three biocontrol agents, B33, *P. flurescens* (PF2) and *T. viride* (T47) significantly reduced the *A. flavus* population providing 39.23% reduction over control. Considerable reduction over control (33.70%) was also obtained from two bacterial isolates B6 and B50 followed by *T. viride* (T38) (28.18%). In the second experiment, the *A. flavus* population at 40 DAS ranged from 1750 in *T. viride* (T47) and *T. harzianum* (T23) to 4250 cfu g^{-1} of soil in control pots. Seven out of eleven biocontrol agents tested significantly reduced the *A. flavus* population. The reduction over control ranged from 23.53% observed in two bacterial isolates (B6 and B50) to 58.52% recorded in *T. viride* (T47) and *T. harzianum* (T23). In general, the efficacy of all biocontrol

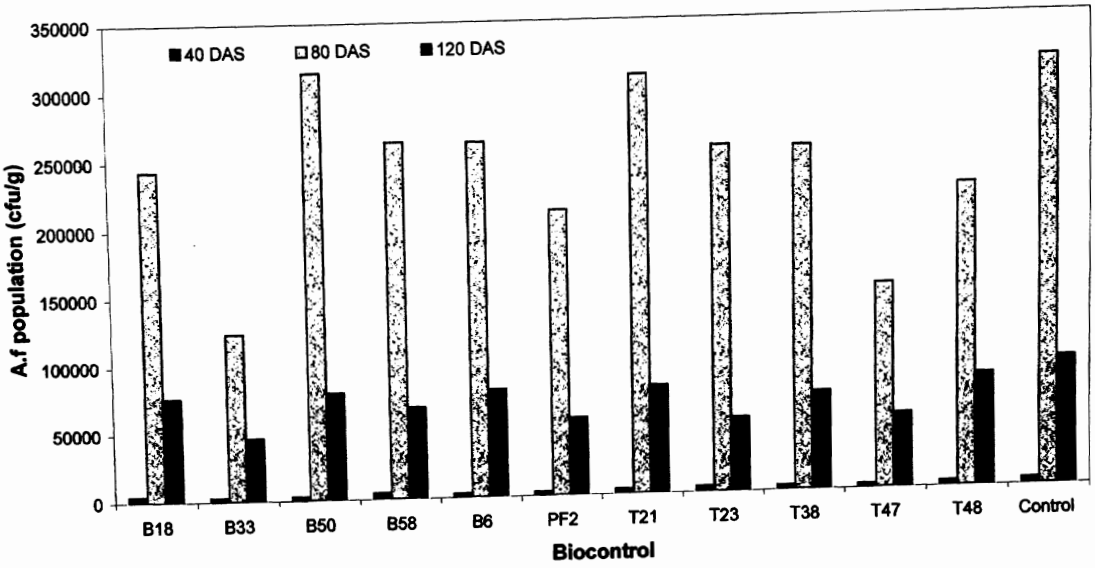

Fig.1: Influence of biochemical agents on *A. flavus* population under green house conditions (2001-2002)

agents in reducing the *A. flavus* population was satisfactory at 40 days after sowing. The reason may be the low inoculum density in the pot soil.

In case of soil samples collected at 80 DAS (40 days after *A. flavus* inoculum application), the *A. flavus* population was very high in the pot soil due to the application of *A. flavus* inoculum. The population was ranging from 123750 to 317750 cfu g^{-1} of soil in different treatments. The biocontrol agents behaved differently in reducing *A. flavus* providing reduction per cent ranging from 1.02 to 61%. Three bioagents, B33, *P. fluorescens* (PF2) and *T. viride* (T47) significantly reduced the *A. flavus* population by 61.05, 33.44 and 52.01% over control respectively. In second experiment, the mean *A. flavus* population ranged from 63250 cfu g^{-1} soil in B33 treated pots to 107500 cfu g^{-1} soil in untreated control. All the biocontrol agents provided more than 10% reduction over the control. However, only two bioagents, B33 and *T. viride* (T47) gave statistically significant reduction over control (41.18 and 40.93% respectively). *T. harzianum* (T23) and *P. fluorescens* (PF2) also reduced the population satisfactorily (36.51 and 29.30 % respectively). In general, the reduction in *A. flavus* population was low at 80 days in all the biocontrol agents except bacterial isolate (B6) compared to 40 days percent reduction. This may be due to high pressure from the high *A. flavus* population.

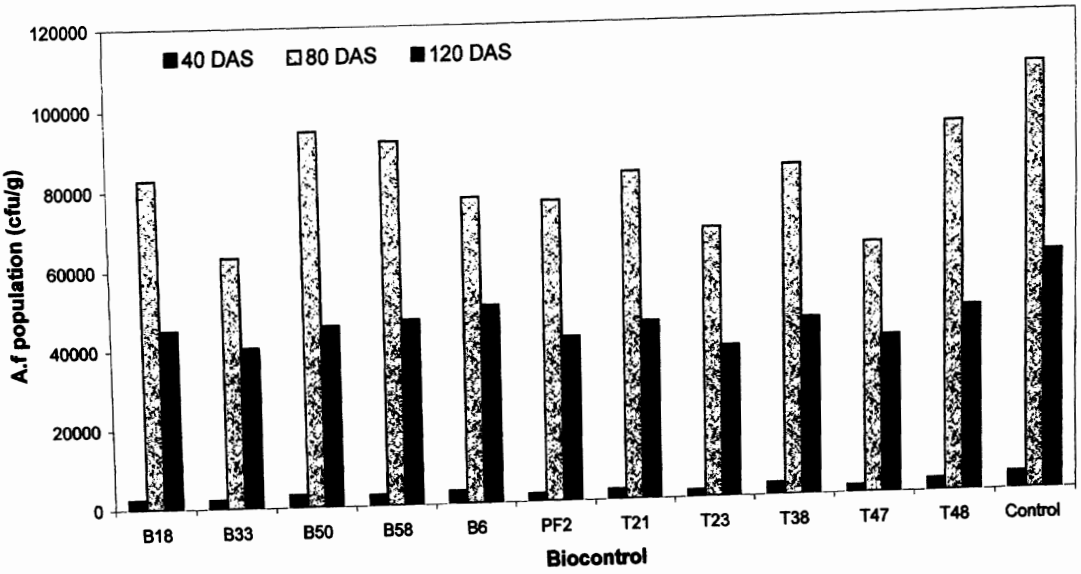

Fig.2: Influence of biochemical agents on *A. flavus* population under green house conditions (2002)

The efficacy of biocontrol agents in reducing seed infection by *A. flavus* was also evaluated under greenhouse conditions. The seed infection was relatively high ranging from 19 to 43% in the first experiment (2001-2002) compared to the same experiment conducted in (2002) in the greenhouse. *T. harzianum* (T23) was the most effective biocontrol agent in reducing seed infection (55.81%) followed by B33 and *P. fluorescens* (45.35 %) and *T. viride* (T47) (44.77%). Seed infection by *A. flavus* was low in the 2002 greenhouse experiment compared to 2001-2002 greenhouse experiment. Majority of the biocontrol agents did not give significant difference over control. However, two *Trichoderma* isolates, *T. harzianum* (T23) and *T. viride* (T47) gave significant reduction over control, providing 37.37% reduction in seed infection. Among the bacterial isolates, B33, B18 and *P. fluorescens* (PF2) were effective in reducing seed infection over control. The reason for the low seed infection percentage in these biocontrol agents could be the reduced *A. flavus* population in their pot soils. Tewari and Mukhopadhyay (2001) reported that *G. virens* alone provided 29.9% protection whereas *G. virens* + carboxymethyl cellulose (CMC) provided maximum protection (70.7%) in chickpea from wilt causing pathogens in pot, possibly because CMC provides better coating and food base for the antagonist. Dasgupta and Raj (1998) reported that seed dressing with *T. harzianum* significantly reduced the seed and collar rot incidence caused by *A. niger* in groundnut under greenhouse

condition. Similar results were obtained by Mickler *et al.* (1995) when they tested bacterial biocontrol agents against pod infection by *A. flavus* in greenhouse conditions. Seven bacterial strains significantly reduced pod colonization by *A. flavus* in groundnut compared to control.

The aflatoxin content of different treatments including control, ranged from 14.08 to 16.75 μg kg^{-1}. None of the biocontrol agents reduced the aflatoxin content of the kernels significantly. However, B33, *T. harzianum* (T23) and *T. viride* (T47) provided 15.94, 15.82 and 13.55% reduction in aflatoxin content over control. Similarly in the second experiment, the mean of aflatoxin content in all the treatments was not significantly differing from the control. The mean aflatoxin content ranged from 9.4 μg kg^{-1} in *T. viride* (T47) to 11.5 μg kg^{-1} in untreated control. None of the bioagents reduced the aflatoxin content significantly. However, two *Trichoderma* isolates (*T. viride* (T47) and *T. harzianum* (T23) and two bacterial bioagents (B33 and *Pseudomonas* (PF2) reduced the aflatoxin content considerably providing 18.26, 16.35, 16.35 and 16.09% reduction over control respectively. When *A. parasiticus* was co-inoculated with *Fusarium graminearum* on corn kernels AFB_1 inhibition percentage were 60, 72 and 56% at 10, 20 and 35 days of incubation respectively. Garber and Cotty (1997) reported that atoxigenic strain of *A. flavus* L strain reduced formation of both sclerotia and aflatoxin when co-inoculated with S strain in developing cottonseed. When *A. flavus* was grown with different bacteria in dual

culture on groundnut, aflatoxin production increased incase of all bacteria except *Flavobacterium odoratum* (Chourasia, 1995).

5.2.4 Efficacy of Biocontrol Agents Under Field Conditions

The biocontrol agents did not show significant increase in total number of plants/plot in postrainy season whereas in rainy season (2002), more than fifty percent of the biocontrol agents significantly increased the total plants/plot. The highest number of plants/plot was obtained from bacterial isolates, *Pseudomonas capacia* (B33) (55.33) and B50 (54.67) followed by B58 (51.67) and *T. harzianum* (T23) (50.67). The reason could be the improved germination and seedling emergence by the biocontrol agents. *G. virens* seed treatment increased the seedling emergence (47.2%) and plant stand (69.7%) under field conditions (Tewari and Mukhopadhyay, 2001).

Most of the biocontrol agents did not show significant increase in pod yield under field conditions. The bacterial isolate *Pseudomonas capacia* (B33) showed significant increase in pod yield (28.62%) over control. Whereas, in rainy season the majority of the biocontrol agents tested increased the pod yield (more than 13%). Four biocontrol agents, *T. harzianum* (T23). *P. fluorescens* (PF2), B18 and B50 were found to be the best treatments in increasing pod yield in this particular season. These bioagents exhibited 26.78, 26.97, 23.04 and 23.1% increase in pod yield over control respectively. Similar results were reported by Dey *et al.* (2000).

They reported that bacterization of groundnut with some plant growth promoting rhizobacteria (PGPR) resulted in significantly higher pod yield (14.7 to 25.5%) in groundnut under field conditions though some of the PGPR they tested were at par with the control in pod yield. The reason may be the solubilization of iron by microbial siderophores and phosphore have been found to increase crop yield substantially (Wani, 1980; Kloepper *et al.*, 1988; Glick, 1995).

As in pod yield, majority of the biocontrol agents tested, did not exhibit significant increase in seed yield. Bacterial isolates (B33) significantly increased the seed yield (26.07%) over control. Considerable increase in seed yield was also observed in two bacterial bioagents (B6) (18.42%) and *P. fluorescens* (PF2) (16.19%) over the control. In the rainy season, majority of the biocontrol agents tested, increased the seed yield more than 10 percent. Two bacterial isolates and one *Trichoderma* isolate (B33, B50 and *T. harzianum* (T23) significantly increased the seed yield recording percent increase over control of 27.19, 24.43 and 22.86. Several workers reported increase in yield by biocontrol agents. Thakur *et al.* (2003) reported that *T. harzianum* (T23) treated plots yielded 263 g $plot^{-1}$ kernel yield and *P. fluorescens* recorded 209 g $plot^{-1}$ in groundnut under field conditions compared to control (No BCA) (214 g $plot^{-1}$). Seed treatment with *P. fluorescens* increased the yield in groundnut (1800.48 kg ha^{-1}) as compared to control (1557.14 kg ha^{-1}) (Sheela and Packiaraj, 2000).

They also reported that seed treatment with *T. viride* and *T. harzianum* increased slightly the pod yield in groundnut (1696.95 and 1678.62 kg ha^{-1} respectively) compared to control (1557.14 kg ha^{-1}). Dey *et al.* (2000) reported that the P content of shoot and seed gave significant increase over control in groundnut due to seed bacterization with three plant growth promoting rhizobacteria.

None of the biocontrol agents tested showed significant increase in biomass yield. Two biocontrol agents, bacterial isolate (B33) and *T. viride* (T48) exhibited increase in biomass yield by 11.94 and 11.28 % over control whereas in the rainy season (2002), eight out of eleven bioagents tested gave more than 10% increase in biomass yield/plot out of these, four biocontrol agents, B18, B33, *P. fluorescens* (PF2) and *T. harzianum* (T23) increased the biomass yield significantly (31.24, 23.80, 23.98 and 23.06 % respectively). This shows that these bioagents have plant growth promoting potential. Manoranjitham *et al.* (2001) reported that *T. viride* and *P. fluorescens* increased the dry matter production in tomato (57.76 and 58.67 % over control respectively). Similar results were also obtained by Dey *et al.* (2000) where inoculation of three PGPR gave significantly higher plant biomass in groundnut as compared to that of the control while other treatments recorded biomass yield at par with that of control.

The *A. flavus* population at 40 days after sowing was relatively high ranging from 22000 to 35166.67 cfu g^{-1} soil in postrainy season (2001-2002). This is due to the inoculation of *A. flavus* inoculum in the previous rainy season 2001. Since, the biocontrol agents were treated on the seed before sowing, they showed different levels of reduction in *A. flavus* population at 40 days after sowing. Most of the bioagents tested, showed considerable reduction (>20%) over control. Among all, B33, *T. viride* (T38), *T. viride* (T47), *T. viride* (T48) and *T. harzianum* (T23) were most effective providing 31.75 to 37.44 % reduction in *A. flavus* population over control. In the rainy season (2002), low *A. flavus* population was observed in the sick plot. The decline of *A. flavus* population observed at the end of previous postrainy season could be continued due to high temperatures in May month (after harvesting the crop). All the biocontrol agents reduced the *A. flavus* population to different levels ranging from 14.29 to 46.43% reduction over control. Four biocontrol agents, *T. viride* (T47), *T. harzianum* (T23), B33 and *P. fluorescens* (PF2) significantly reduced the *A. flavus* population recording 46.43, 42.66, 37.50 and 35.71 % reduction over control respectively.

At 80 days after sowing (40 days after *A. flavus* inoculum application), the population was very high ranging from 52000 to 751667 cfu g^{-1} of soil due to inoculum application. In general, the reduction in *A. flavus* population by bioagents at 80 DAS declined

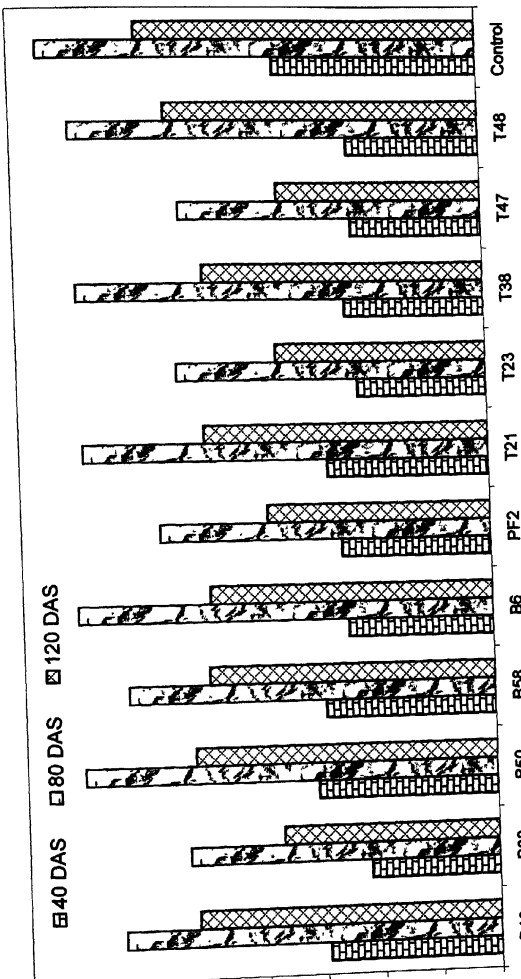

Fig.3: Influence of biochemical agents on *Aspergillus flavus* population under field conditions postrainy season (2001-2002)

compared to reduction at 40 DAS. This may be due to the increased population of the test fungus. However, four biocontrol agents, *T. viride* (T47), *T. harzianum* (T23), bacterial isolate (B33) and *P. fluorescens* (PF2) maintained their effectiveness in reducing *A. flavus* population recording 30.82, 29.27, 29.49 and 24.61% reduction over control. In the rainy season (2002), the population ranged from 30833.33 cfu g^{-1} soil treatment to 45666.67 cfu g^{-1} soil. Nine biocontrol agents out of 11 bioagents tested provided more than 20% reduction in *A. flavus* population over control. *T. viride* (T47), *T. harzianum* (T23) and bacterial isolate (B33) were the most effective bioagents in reducing *A. flavus* population recording 32.48, 32.12 and 28.47 % reduction over control respectively.

At crop harvesting, the *A. flavus* population declined compared to that at 80 DAS. The population ranged from 35167 cfu g^{-1} soil in *T. viride* (T47) treated plot to 58500 cfu g^{-1} soil in control plot. All the biocontrol agents tested gave > 11% reduction in *A. flavus* population over the control except *T. viride* (T48), which gave only 7.69% reduction. *T. viride* (T47), *T. harzianum* (T23), B33 and *P. fluorescens* (PF2) provided highly significant reduction in *A. flavus* population even at crop harvesting stage. These four bioagents showed 39.89, 38.18, 36.75 and 34.47% reduction in *A. flavus* population over control. In the rainy season (2002), the results showed that the *A. flavus* population increased from 80 DAS to crop harvesting. This may be due to the

availability of moisture in the soil and favorable temperature for the growth of the fungus in rainy season. The mean of *A. flavus* population ranged from 31166.67 cfu g^{-1} soil in *T. harzianum* treated plots to 52500 cfu g^{-1} soil in untreated control. However, all the biocontrol agents showed statistically significant reduction in *A. flavus* population at crop harvesting. The most effective biocontrol agents among all were *T. harzianum* (T23), *T. viride* (T47) and bacterial isolate B33, which recorded 40.63, 39.37 and 35.87% reduction over control respectively. These three bioagents gave significant reduction in *A. flavus* population in all the three crop stages (40, 80 days after sowing and at harvesting). This indicates their effectiveness against *A. flavus* even under field conditions. The reduction in population of fungal pathogen in the soil has been reported by several workers. Thakur *et al.* (2003) reported that *Trichoderma* sp. (T28) reduced the *A. flavus* population in groundnut rhizosphere by 78% and *T. harzianum* (T23) *P. fluorescens* (Pf 2) reduced 42 and 55% respectively. Manoranjitham *et al.* (2001) reported that *T. viride* and *Pseudomonas flourescens* reduced the population of *Pythium aphanidermatum* in the soil (4.60×10^2 and 5.2×10^2 cfu g^{-1} soil respectively) compared to control population (8.8×10^2 cfu g^{-1}) soil in tomato field. Srivastava *et al.* (2001) reported that the *Fusarium* propagules in the rhizosphere of VAM treated guava plants varied between 2×10^2, 6×10^2 and 8×10^2 cfu g^{-1} soil (depending on the quantity of VAM applied) compared to 10×10^2 cfu g^{-1} soil in control.

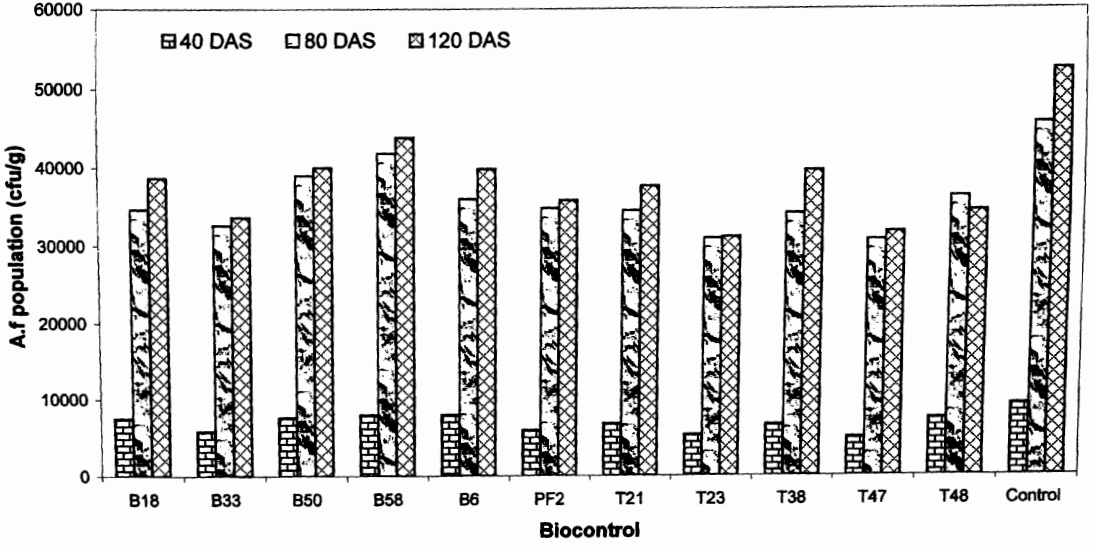

Fig.4: Influence of biochemical agents on *Aspergillus flavus* population under field conditions rainy season (2002)

Strashnow *et al.* (1985) reported that *T. harzianum* reduced the inoculum density of *R. solani* by 85% when the former was mixed with naturally infested soil in the field.

The efficacy of the biocontrol agents in reducing seed infection by *A. flavus* was estimated by recording seed infection after the crop was harvested. The seed infection was very high in the postrainy season (2001-2002) ranging from 66.5% recorded on *T. harzianum* (T23) treated plots to 84.67% recorded in control treatment (No BCA). This could be due to high temperatures in the season and end season drought imposed on the crop, which renders the crop more susceptible. Cracks were found on the pods of many plants at the end of the season that serves entry point for the fungus. Most of the biocontrol agents did not give significant reduction in seed infection. However, *T. harzianum* (T23) and *T. viride* (T47) provided significant reduction in seed infection (21.46 and 20.06 % respectively). B33 and PF2 provided 16.35 and 15.94% reduction respectively. The seed infection data showed more or less a similar trend to that of the *A. flavus* population. This indicates that the reduction in seed infection may be due to reduction in *A. flavus* population in the rhizosphere of the plants in those treatments. Seed infection by *A. flavus* was low in all the treatments including control in the 2002 rainy season compared to seed infection in the postrainy season 2001-2002. The mean seed infection ranged from 19.5 in *T. viride* (T47) to 29.33% infection in control. Seven out of

eleven bioagents tested reduced the seed infection from 14.18 to 33.52%. Among all *T. viride* (T47), *T. harzianum* (T23) and bacterial isolate (B33) were most effective in reducing seed infection by *A. flavus* by providing significant reduction (33.52, 30.11 and 26.70% over control respectively. Rao *et al.* (2003) reported that *Trichoderma* isolate (T28) and *P. fluorescens* (PF2) significantly reduced the seed infection (34 – 39%) compared to control (80%).

Thakur *et al.* (2003) reported that *T. viride* (T23) and *P. fluorescens* (PF 153) were the best biocontrol agents in reducing seed infection by *A. flavus* in groundnut when the biocontrol agents were tested under field conditions. These provided 65 and 73% reduction in seed infection respectively.

The aflatoxin content ranged from 52.57 μg kg^{-1} in *T. viride* (T47) treated plots to 62.33 μg kg^{-1} in control treatment. Majority of the biocontrol agents did not give significant reduction in aflatoxin content of the kernels and percent reduction ranged from 3.42 to 15.66% in all the biocontrol agents. *T. harzianum* (T23) and *T. viride* (T47) significantly reduced the aflatoxin content providing 14.44 and 15.66% reduction over control respectively. The aflatoxin content data showed a similar trend to that of the seed infection. This shows that the reduction in seed infection is one of the reasons of less aflatoxin content in some particular treatments. Another reason could be the inhibition of

aflatoxin production of *A. flavus* by biocontrol agents. The results showed that aflatoxin content was high in all the treatments in the 2002 rainy season compared to the aflatoxin content in the 2001-2002 postrainy season. The possible reason for low aflatoxin content in postrainy season might be due to dry weather during postrainy season and no possibility of wetting pods during or after harvesting of the crop due to rain (Reddy *et al.* 2003). This result of the present investigation is in agreement with the results of Reddy *et al.* (2003) who reported aflatoxin content of rabi (postrainy season) samples and kharif (rainy season) samples as 0-535.4 μg kg^{-1} and 0-8172.3 μg kg^{-1}) respectively. Similarly Desai *et al.* (2004) reported low aflatoxin content in postrainy samples (0.24 - 154.34 ppb) compared to the rainy season samples (0.037-8735.8) and concluded that summer groundnut could be diverted for food purpose with a probability of getting produce that could be within tolerance limits.

All the treatments recorded similar means of aflatoxin content and so the percent reduction of aflatoxin content in most of the biocontrol agents was less. However, one *Trichoderma* isolate (*T. viride* (T47)) was most effective in reducing aflatoxin content significantly providing 14.96 % reduction over control. Munimbazi and Bullerman (1998) reported the inhibition of aflatoxin production in *A. parasiticus* by *Bacillus pumilus* in broth medium due to extracellular metabolites.

5.3 IMPROVEMENT OF *T. VIRIDE* (T47) BY IRRADIATION

T. viride (T47), which showed significant control of *A. flavus in vitro* and *in vivo* was irradiated to improve its biocontrol efficacy against the same fungus. When the mutants were tested against *A. flavus* (Strain 11-4) in dual culture technique, all the mutants significantly controlled the *A. flavus* growth compared to control. All the mutants recorded less mycelial growth of *A. flavus* than that of parental line (T47) (1.00 cm) except two mutants (M40 and M42). Three mutants, M18, M15 and M28 were the best among all in inhibiting mycelial growth of *A. flavus.* These recorded (0.82, 0.85 and 0.85 cm respectively) and they were significantly superior to the parental line (T47).

Mukherjee and Mukhopadhyay (1993) reported that gamma-irradiated *G. virens* and mutants were differing from the wild type in phenotype, growth rate, sporulation and antagonistic potential. Graeme-Cook and Faull (1991) reported that ultraviolet mutants of *Trichoderma* differed from the wild type by producing two new antifungal compounds.

Haggag and Mohamed (2002) reported that gamma-irradiated mutants of *T. harzianum, T. viride* and *T. koningii* displayed variation in growth and antagonistic activity.

5.4 EFFICACY OF NEEM COMMERCIAL FORMULATIONS AGAINST *A. FLAVUS IN VITRO* AND *IN VIVO*

5.4.1 Neem Formulations *In Vitro*

All the formulations tested (Nivaar, Sasyaneem, Starneem and Sunny) significantly inhibited the growth of the fungus. Nivaar was found to be the most effective formulation against *A. flavus* allowing slight growth of the fungus only in 100 ppm concentration. On the other hand Sunny allowed slight growth of the fungus even at 500 ppm concentration. Significant difference was also observed between the different concentrations of each formulation except between 100 ppm and 200 ppm of Sasyaneem and Sunny. The percent inhibition of *A. flavus* growth by different concentrations of the formulations ranged from 58.82 to 100% over control.

When the same experiment was conducted on PDA broth, the growth of *A. flavus* was observed only in 100 ppm concentration of Nivaar and Sasyaneem and the control treatment (without neem formulations). The mycelial weight of these three treatments were 2.4, 2.7 and 0.6 g, respectively. The higher mycelial weight in Nivaar and Sasyaneem compared to control may be due to addition of nutrient in the medium by the formulations. On the other hand, Nivaar and Sasyaneem (100 ppm) inhibited the fungal sporulation whereas in the control treatment heavy sporulation of the fungus was observed. This could be

due to the partial toxicity of the formulations in lower concentration. Sinha *et al.* (1993) reported the stimulation of *A. flavus* growth by the lower concentrations of clove (50 and 100 $\mu g\ ml^{-1}$) and Cinnamon oil (50 $\mu g\ ml^{-1}$). Several workers reported the inhibition of growth and aflatoxin production of *A. flavus* by different plant products.

Capsanthin and Capsaincin, the clouring and pungent principles of red chilli respectively checked the growth and aflatoxin production by *A. flavus* up to the fourth day of incubation. The inhibitory effect of capsanthin was continued (Masood *et al.*, 1994). Sinha *et al.* (1993) reported the significant reduction in the mycelial growth of *A. flavus* due to the inhibitory action of clove and cinnamon oils. Kshemkalyani *et al.* (1990) reported that total garlic juice and its components can inhibit the growth of aflatoxin producing fungi (*A. flavus* and *A. parasiticus*).

The compatibility of selected *Trichoderma viride* (T47) with the neem formulations (Nivaar, Sasyaneem, Starneem and Sunny) was tested *in vitrro*. All the formulations significantly inhibited the mycelial growth of *T. viride* (T47) in all the five concentrations. Nivaar was the most effective formulation in inhibiting the growth of *T. viride* (T47) and it allowed the *Trichoderma* growth at 100 ppm concentration only as the case in *A. flavus*. The growth reduction in all the formulations ranged from 19.32 to 100% over control. Antifungal activity of

different plant products particularly neem has been reported by many workers. Many plant extracts are reported to specially inhibit the germination of fungal spores. Babu *et al.* (2001) reported that neem leaf extract inhibited the mycelial growth of *Alternaria solani* by 33.7%.

5.4.2 Efficacy of Neem Formulations in Greenhouse and Field Conditions

Based on the results of *in vitro* experiment, two neem formulations were selected for greenhouse and field experiment. The reason for selection of these two formulations was, one of them (Nivaar) was very effective in inhibiting the *A. flavus* growth while other formulation (Sasyaneem) was compatible with biocontrol agent (*T. viride* (T47)). Both the formulations did not show any effect on total number of plants. The reason could be the limited number of plants allowed to grow in each pot in all the treatments. Similarly, the two formulations have no significant effect on total number of plants/plot under field conditions.

The formulations did not increase the pod yield in this greenhouse experiment. Sasyaneem, increased the pod yield (9.77%) over control whereas Nivaar showed only 1.44% increase. Under field conditions, Sasyaneem, increased the pod yield (9.77%) over control whereas Nivaar showed only 1.44% increase.

Similar means of seed weight was recorded in the two formulations and the control in greenhouse conditions. In the field experiment, Sasyaneem increased the seed weight significantly (13.85%) over control. Narasimhan *et al.* (1998) reported that the grain yield from neem oil formulations treated plots was higher than that of carbendazim-treated plots in rice under field conditions

Regarding biomass yield, the two formulations gave slightly higher biomass yield (97.5 g in Nivaar and 94.25 g in Sasyaneem) compared to that of the control (91.5 g). But, this difference was not significant at 5% level. Sasyaneem contributed to the biomass yield / plot providing more than 5% increase under field conditions whereas Nivaar recorded less than 2% increase.

Both the neem formulations (Nivaar and Sasyaneem) reduced the *A. flavus* population at 80 days after sowing providing significant reduction of 40.47 and 39.07% reduction over control respectively. Similarly, the two formulations showed considerable reduction in *A. flavus* population (26.14 and 24.90% respectively) at crop harvesting. In both the crop stages, the two formulations recorded similar percent reduction in *A. flavus* population, which shows that they are equally effective against *A. flavus* in greenhouse conditions. The initial population of *A. flavus* in the sick plot ranged from 9500 to 9666.67 cfu g^{-1} of soil before *A. flavus* inoculum application in the field. Both Nivaar

and Sasyaneem controlled the *A. flavus* population significantly at 80 DAS providing 27.01 and 24.45 % reduction over control respectively. Similarly the two formulations significantly reduced the *A. flavus* population at harvesting recording 24.44 % reduction in Nivaar and 20% in Sasyaneem. Both Nivaar and Sasyaneem did not provide significant protection from *A. flavus* seed infection under field conditions. The reduction of *A. flavus* population at crop harvesting was less compared to that of 80 DAS. The reason could be that the formulations may decrease their effectiveness as the crop season closing to end.

The two test formulations Nivaar and Sasyaneem reduced the seed infection by *A. flavus* in groundnut kernels harvested from treated pots compared to control pots though the reduction (14.81 and 11.34 respectively) was not statistically significant. The performance of the two formulations in reducing the seed infection was very low (7.33% in Nivaar and 4.41% in Sasyaneem) under field conditions. This may be due to decreased efficacy of the formulations at the end of crop season since the formulations were applied only once in the growing season (at 45 DAS). One more application of the formulations at 80 DAS could provide better protection to the kernels from *A. flavus* infection. Pasini *et al.* (1997) reported satisfactory control of rose powdery mildew (*Sphaerotheca pannosa*) by neem extract formulations in glasshouse conditions. They used more number of sprays at weekly interval. Narasimhan *et al.* (1998) reported that EC formulations of neem oil

effectively controlled rice sheath rot under field conditions. They used repeated sprays of the formulations with 10-day intervals.

When the aflatoxin content of groundnut kernels harvested from the *A. flavus* inoculated pots was estimated, the results showed that, the two formulations did not reduce the aflatoxin content satisfactorily. The percent reduction over control was 7.39 % in Nivaar and 5.22% in Sasyaneem. Similarly the two formulations did not show significant reduction in aflatoxin content of groundnut kernels harvested from field. This shows that the formulations decreased their efficacy at the end of the crop season.

5.5 IDENTIFICATION OF GERMAPLASM ACCESSIONS, ADVANCE BREEDING LINES AND VARIETIES FOR RESISTANCE TO *IN VITRO* SEED COLONIZATION AND SEED INFECTION BY *A. FLAVUS*

The development of groundnut cultivar with reduced aflatoxin contamination in drought stressed conditions would be very useful in reducing aflatoxin risk. In the present study, efforts were made to identify groundnut genotype, which is resistant to *in vitro* seed colonization and seed infection by *A. flavus*.

All the genotypes (49) tested were superior to susceptible control (JL24) when percent seed infection was compared in all the genotypes. Ten genotypes showed resistance comparable to resistant control (J11). Among these, three genotypes (ICGV 89104, ICGV

91114 and ICGV 98170) exihibited seed infection less than that of the resistant control (J11). The infection of the tested genotypes ranged from 0.5 to 34.5%.

When the unwounded seeds of the 50 entries were artificially inoculated with *A. flavus* spore suspension, 42 out of 49 entries were significantly better than the susceptible check (JL24). Of these, six genotypes showed resistance to *in vitro* seed colonization comparable to that of the resistant control (J11). These recorded similar colonization severity that ranged from 1.81 to 1.94. When the pin-pricked seeds of the 50 entries were inoculated with *A. flavus* spore suspension, more than 50% of the entries (26 entries) did not differ significantly from the susceptible control (JL24) whereas 23 entries showed some superiority to the susceptible cultivar (JL24). Three entries, ICGV 91284, WUE (7) IC 48 and WUE 159 IC 43 were found to be the best genotypes recording colonization severity which is at par or slightly less than that of resistant cultivar (J11). In the pin-prinked inoculated genotypes, the colonization severity ranged from 3.14 to 4 on 1 – 4 scale. This result showed that none of the groundnut entries tested has remarkable cotyledonary resistance to *in vitro* seed colonization by *A. flavus*.

Thakur *et al.* (2000) evaluated wild species of groundnut for *in vitro* seed colonization and only few accessions showed low colonization severity compared to control (JL24).

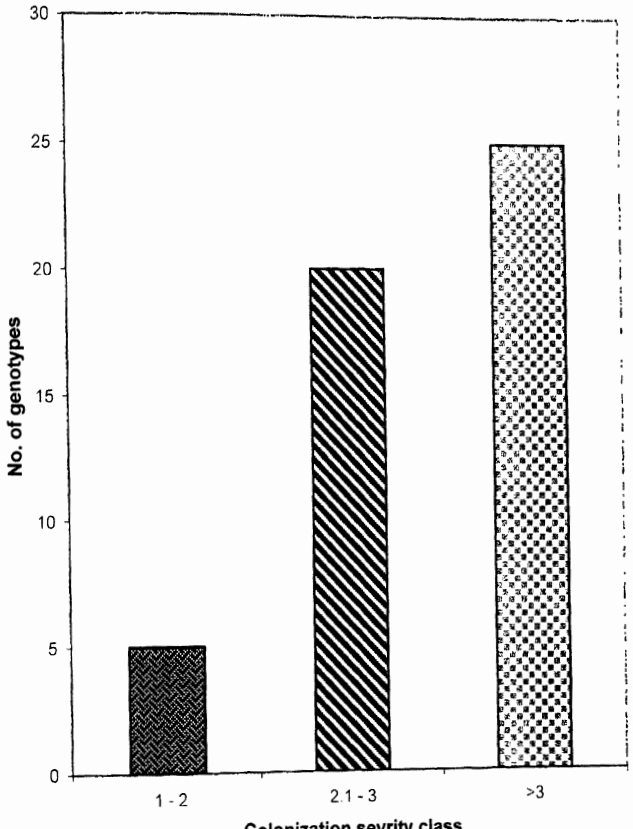

Fig. 5: ***Invitro*** **seed colonization by** ***A. flavus*** **of 50 groundnut entries**

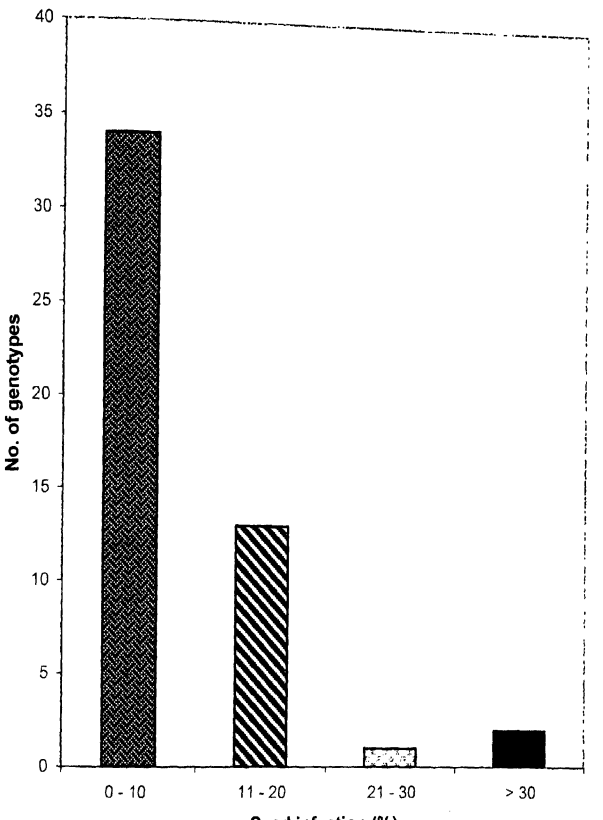

Fig. 6: Seed infection by *A. flavus* of 50 groundnut genotypes

Based on the *in vitro* results, fourteen entries were selected for field evaluation. These entries showed resistance to i*n vitro* seed colonization by *A. flavus* when artificially inoculated and have got number of seeds under class I colonization severity (< 5% of the surface of the seed colonized). The individual seeds selected were sown in *A. flavus* sick plot. After harvesting, the seeds were inoculated. The results showed that all the genotypes were significantly superior to JL24. ICGV 91114, ICGV 95322 and WUE (116) TIR 31 were the best genotypes recording low colonization severity more or less comparable to that of the J11. In the rainy season 2002, the result was similar to the previous season. The colonization severity of the tested genotypes ranged from 1.29 to 2.27. ICGV 91114 recorded lower colonization severity than J11 (resistant control) though the difference was not statistically significant. ICGV 91114 showed consistent resistance to *in vitro* seed colonization in all the experiments.

5.5.2 Evaluation of ICRISAT Groundnut Core Collection for Resistance to *A. Flavus* Contamination

All the genotypes screened under set I core collection were superior to JL24 by recording colonization severity lower than that of JL24 except one genotype (ICG 3673). Colonization severity of all tested genotypes ranged from 1.05 to 3.19. Only three genotypes, ICG

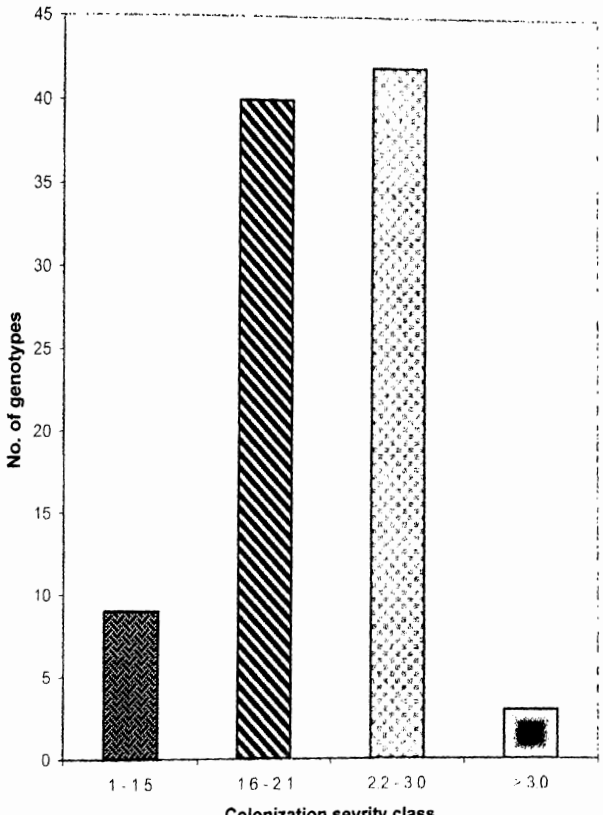

Fig. 7: ***Invitro*** **seed colonization by** ***A. flavus*** **of groundnut core collection (Set I)**

1519, ICG 2106 and ICG 875 recorded colonization severity lower than that of the resistant control (J11).

In preliminary screening of set II core collection, more than 90% of the genotypes were significantly superior to the susceptible control (JL24). When compared to resistant cultivar, three genotypes (ICG 1200, ICG 14705 and ICG 13099) were at par with resistant cultivar (J11) whereas two genotypes (ICG 10384, ICG 14523) showed slightly better resistance than J11. The colonization severity ranged from 1.04 to 3.76. This shows that there is a large variation among the genotypes in resistance to *in vitro* seed colonization by *A. flavus*.

Forty-five genotypes which recorded colonization severity (≤ 2) on 1 to 4 scale were selected from set I for advanced screening. All the genotypes tested were superior to susceptible control except one genotype (ICG 4412), which was at par with JL24. None of the genotypes was significantly superior to the resistant check (J11). Majority of the genotypes maintained their consistence in *A. flavus* resistance in both preliminary and advanced screening recording ≤ 2 colonization severities. Among these, were ICG 875, J11, ICG 3421, and ICG 928. Some of the genotypes showed inconsistent resistance recording different colonization severities in the two screenings. Among these, ICG 4412, which recorded colonization severity (2) in first screening and recorded 3.20 in second screening.

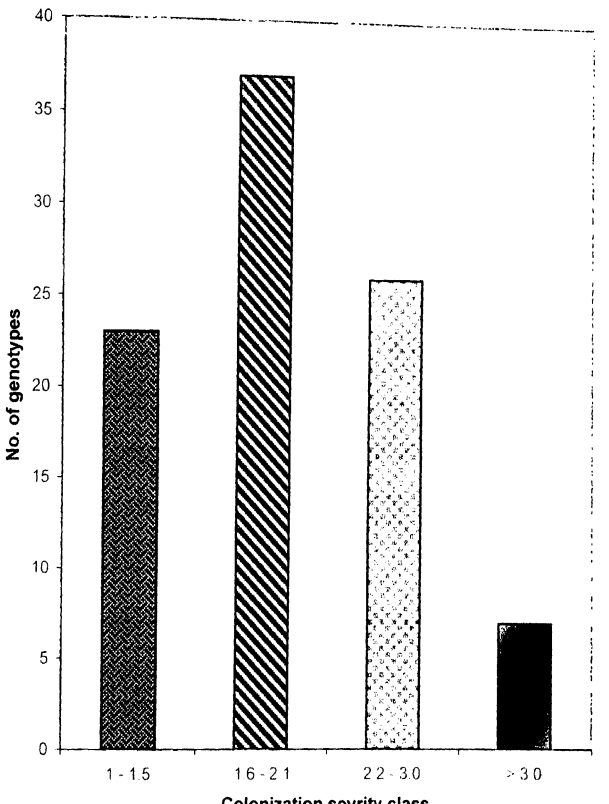

Fig. 8: ***Invitro*** **seed colonization by** ***A. flavus*** **of groundnut core collection (Set.II)**

In Set 2 core collection, fifty-seven genotypes were selected for second screening. Fifty-three out of 57 genotypes were significantly superior over susceptible control (JL24) while three genotypes were at par with JL24. When compared to resistant cultivar (J11) no genotype showed better resistance than J11 except U-4-7-5, which recorded slightly less colonization severity (1.10) than that of J11 (1.17). Cultivar J11 has been reported by Mehan (1989) as resistant to *in vitro* seed colonization by *A. flavus* and cultivars U-4-7-5 and VRR 245 for resistant to aflatoxin production. The results of the present investigation showed that there are some *A. flavus* resistant lines in the groundnut germplasm. The confirmation of *in vitro* results by field trials under high *A. flavus* inoculum pressure is required.

The aflatoxin content in the inoculated kernels of the genotypes selected for second screening was estimated.

The majority of the genotypes tested under set 1 recorded high aflatoxin content. Thirty-one genotypes showed aflatoxin content higher than that of susceptible check (JL24). However, five genotypes, ICG 81, ICG 3421, ICG 4598, ICG 7969 and ICG 9315 exhibited relatively low aflatoxin content than resistant check (J11) that recorded 27141.9 µg/kg. Of these the genotype ICG 4598 was the best recording 1089 µg/kg (Fig. 1).

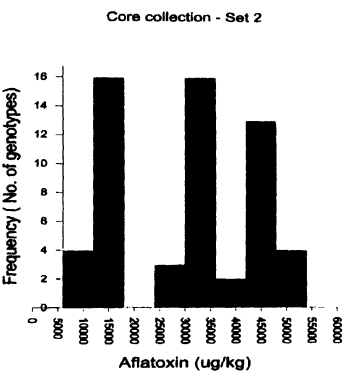

Fig.10: Aflatoxin content of selected groundnut core collection (Set.II)

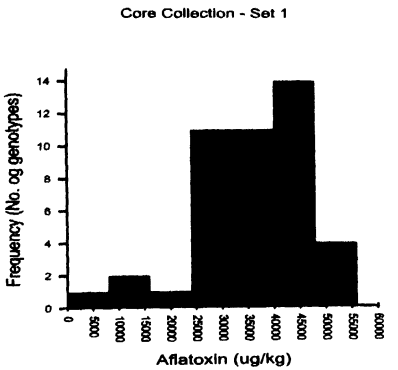

Fig.9: Aflatoxin content of selected groundnut core collection (Set.I)

In set 2 also the aflatoxin content was high in the majority of the genotypes tested. The aflatoxin content of all the genotypes ranged from 8114.06 to 49888.16 μg kg^{-1}. However sixteen genotypes recorded aflatoxin content lower than J11 (27141.9 μg kg^{-1}). Of these, eight genotypes recorded aflatoxin content lower than VRR 245 (12815.47 μg kg^{-1}). This cultivar has been reported as aflatoxin production resistant. The genotype ICG 5327 recorded the minimum aflatoxin content among all the genotypes.

5.5 INTEGRATED MANAGEMENT PRACTICE TO REDUCE AFLATOXIN CONTAMINATION IN GROUNDNUT

Based on the laboratory, greenhouse and field experiments two biocontrol agents (*Pseudomonas cepacia*, *T. viride* (T47), one resistant genotype (ICGV 91114) and one neem formulation (Sasyaneem) which is compatible with selected *Trichoderma* isolate) were selected to evaluate their pooled effects on *A. flavus* and aflatoxin contamination in groundnut under field conditions. In this regard three experiments were conducted in two locations (ICRISAT, Patancheru and Anantapur). The cultivar JL24 was used as susceptible check in all the IDM experiments.

The results revealed that all the IDM treatments increased significantly the total number of plants/plot when compared to susceptible control (JL24 alone). However no treatment was

significantly superior over resistant control (ICGV 91114). ICGV 91114 + *T. viride* (T47) and JL24 + *T. viride* (T47) were the best treatments recording the highest number of plants per plot (72.75 and 68.5, respectively). The biocontrol agents might have increased the germination and improved the plant stand. Tewari and Mukhopadhyay (2001) reported that different formulations of *Gliocladium virens* significantly improved seedling emergence and plant stand. The highest increase in seedling emergence (47.2%) and plant stand (69.7%) was obtained from Gv + CMC as compared to *G. virens* alone (17.1 and 13.6 %, respectively).

Some of the treatments showed considerable pod yield increase. Among the resistant control (ICGV 91114) combinations, ICGV 91114 + B33 was the best treatment, increasing the pod yield by 21.27% over resistant control followed by ICGV 91114 + B33 + *T. viride* (T47) Sasyaneem (15.36%). In susceptible control (JL24) combinations, JL 24 + B33 + *T. viride* (T47) + Sasyaneem was the best combination, increasing the pod yield by 35.38% over JL24 alone followed by JL24+B33 (33.15%). These results showed that the biocontrol agents tested, particularly bacterial isolate (B33) contributed significantly to the increased pod yield. Dey *et al.* (2000) reported that the bacterization of groundnut with PGPR resulted in significant higher pod yield (14.7 to 25.5%). Sheela and Pakiaraj (2000) reported that *T. viride* (ST) + neem cake, gave higher pod yield (1849.49 kg ha^{-1})

followed by *P. fluorescens* (ST) (1800.48 kg ha^{-1}) as compared to control (1557.14 kg ha^{-1}).

The seed yield data showed similar trend to that of pod yield. Some treatments, ICGV 91114 + B33, ICGV 91114 + B33 + *T. viride* (T47) + Sasyaneem, JL24 + B33 + *T. viride* (T47) + Sasyaneem and JL24 + B33 were the best treatments in increasing the seed yield considerably recording 31.27, 27.35, 35.30 and 29.95 % over respective controls respectively. Similarly, these four treatments increased the biomass yield though the increase in biomass yield was less in general. The results of Dey *et al.* (2000) when they tested PGPR under field conditions showed that some of the PGPR increased the plant biomass while others recorded biomass yield at par with that of control.

The data reveals the *A. flavus* population and percent reduction by different treatments at different crop stages. ICGV 91114 + B33, ICGV 91114 + B33 + *T. viride* (T47) and ICGV 91114 + *T. viride* (T47) significantly reduced the *A. flavus* population at 40 DAS compared to both controls. Similarly in susceptible combination, JL24 + B33, JL24 + B33 + *T. viride* (T47) and JL24 + *T. viride* (T47) controlled the *A. flavus* population significantly compared to both resistant and susceptible controls. Among all, ICGV 91114 + B33 + *T. viride* (T47) and ICGV 91114 + *T. viride* (T47) were the best

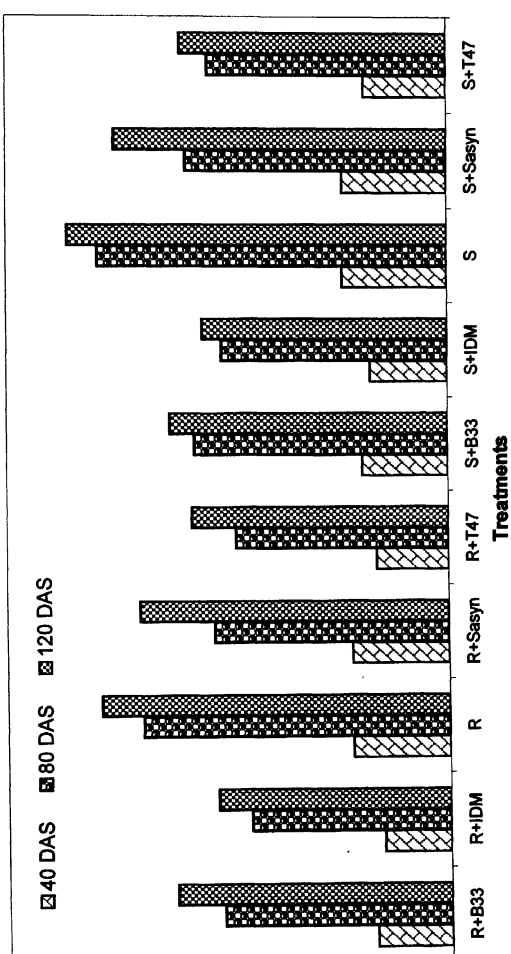

Fig.11: Efficacy of treatment combinations on *Aspergillus flavus* population under field conditions at ICRISAT (Rainy season, 2002)

treatments in reducing *A. flavus* population recording 36.84 and 31.58 %, respectively.

At 80 DAS (40 days after *A. flavus* inoculum application) the *A. flavus* population increased in all the treatments ranging from 20500 to 31750 cfu g^{-1} of soil. However, all the treatments controlled the *A. flavus* population to different levels. ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem and ICGV 91114 + *T. viride* (T47) controlled the *A. flavus* (43.31 and 39.37% respectively) over susceptible control. ICGV 91114 + B33 was at par with JL24 + *T. viride* (T47) + B33 + Sasyaneem in controlling *A. flavus* population (35.43%). At crop harvesting, the *A. flavus* population increased in all the treatments compared to 80-day population. However, all the treatments maintained some level of reduction though in general the percent reduction declined compared to that of 80 DAS. ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem and JL24 + *T. viride* (T47) + B33 + Sasyaneem were found to be the best treatments in reducing *A. flavus* population at harvesting. These results revealed that the biocontrol agents (*T. viride* (T47) and B33) have potential to control the *A. flavus* population under field conditions. The neem formulation (Sasyaneem) provided remarkable reduction of *A. flavus* population (33.07% when combined with resistant control and 25.20% when combined with susceptible control at 80 DAS. But, this decreased drastically to 18.84 and 12.32 % respectively at crop

harvesting. This shows that the formulation was effective after application and its efficacy declines with time.

The data showed that all the treatments involving resistant cultivar (ICGV 91114) significantly controlled the seed infection by *A. flavus* compared to susceptible control. ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem and ICGV 91114 + *T. viride* (T47) provided the maximum and significant reduction in seed infection. In case of treatments involving susceptible control combinations, JL24 + *T. viride* (T47) + B33 + Sasyaneem was the best treatment in reducing seed infection. This shows that the integrated treatments can control the preharvest seed infection by *A. flavus*.

Data showed that when the biocontrol agents were combined with resistant control (ICGV 91114) significant reduction in aflatoxin content in the groundnut kernels was achieved compared to susceptible control (JL24). Resistant cultivar (ICGV 91114) + *T. viride* (T47) + B33 + Sasyaneem was the most effective treatment providing 38.39% reduction in aflatoxin content followed by ICGV 91114 + *T. viride* (T47) (33.93%).

IDM in Anantapur

The results showed that all IDM treatments did not increase the total number of plants significantly. This may be due to the prolonged drought spells at Anantapur in the 2002 rainy season. The total number

of plants per plot was low in all the treatment ranging from 61 to 68.75 plants/plot. In general the pod yield was low in all the treatments. The reason could be the drought and high temperatures and drought that coincided with the critical stages of the crop. However, some of the treatments showed more than 10% increase in pod yield. ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem provided significant pod yield increase (11.35%) over resistant control (ICGV 91114 alone). JL24 + B33 + *T. viride* (T47) + Sasyaneem and JL24 + B33 were found to be the best combination in increasing pod yield among the JL24 combinations compared to JL24 alone. These two treatments provided 24.64 and 18.88% pod yield increase over JL24 alone respectively.

Saralamma (2000) obtained increase in pod yield of groundnut from IDM experiment. The pod yield was more in biocontrol agent + neem cake when applied to the soil infested with *Sclerotium rolfsii.*

Due to the reasons mentioned above, the seed yield/plot was very low. However, the majority of IDM treatments provided more than 10% seed yield increase. ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem and ICGV 91114 + B33 were the best treatments among the resistant control combinations providing 32.08 and 18.92% seed yield increase respectively. Similarly among JL24 combinations, JL24 + *T. viride* (T47) + B33 + Sasyaneem and JL24 + B33 were the best in increasing the seed yield (33.08 and 22.72 % respectively).

At 40 DAS, all the IDM treatments significantly reduced the *A. flavus* population at 40 DAS compared to JL24 alone except JL24 + Sasyaneem. Because Sasyaneem was applied at 45 days after sowing. The highest percent reduction was recorded in ICGV 91114 + *T. viride* (T47) + B33 (56.76%) followed by ICGV 91114 + *T. viride* (T47) (48.65%). Similarly in case of JL24 combinations, JL24 + *T. viride* (T47) + B33 and JL24 + *T. viride* (T47) were the best treatments in reducing the population of the fungus (31.08 and 22.97% over JL24 alone, respectively).

At 80 DAS (40 days after inoculation), the *A. flavus* population was very high ranging from 31750 to 47000 cfu g^{-1} of soil. All the treatments controlled the *A. flavus* population more than 10% under the high pressure of inoculum. The highest control of *A. flavus* population was provided by ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem (32.45%) followed by ICGV 91114 + *T. viride* (T47) (27.13%).

At crop harvesting, decline in *A. flavus* population observed in all the treatments might be due to high temperatures and low soil moisture particularly during the last 40 days of the season when the crop was imposed to moisture stress. Majority of the treatments controlled the population of the fungus more than 15%. Two combinations, ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem and JL24 + *T. viride* + B33

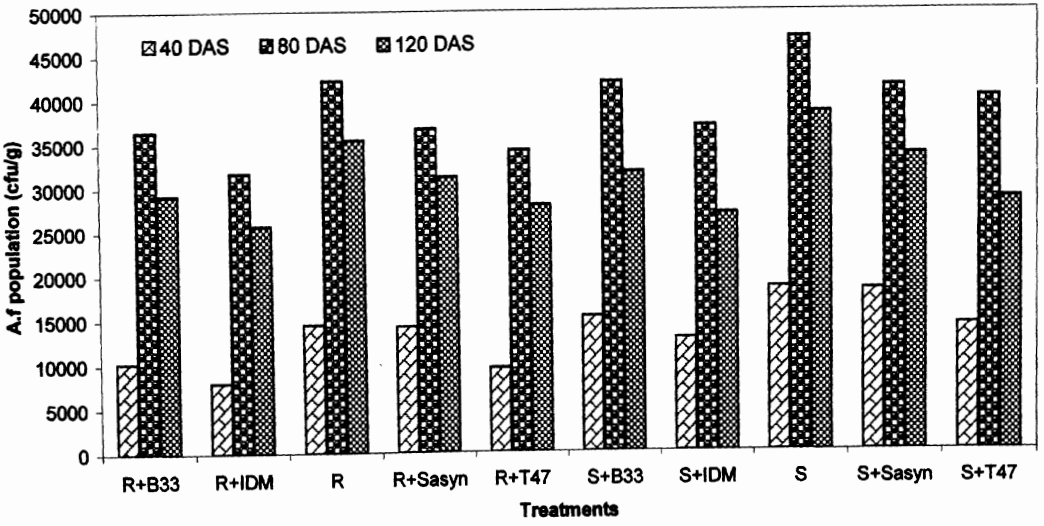

Fig.12: Efficacy of treatment combinations on *Aspergillus flavus* population under field conditions at Anantapur (Rainy season, 2002)

+ Sasyaneem controlled the *A. flavus* population by 33.12 and 29.87 % respectively.

The seed infection was relatively high in Anantapur experiment compared to same experiment conducted at ICRISAT, Patancheru in the same rainy season. The reason could be drought prevailed at the end of season in Anantapur, which rendered the crop more susceptible to the *A. flavus* infection. The seed infection ranged from 25 to 51.5%. Majority of the treatments significantly controlled the seed infection providing more than 20% protection. The most effective treatments in reducing seed infection were ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem (51.46%) and ICGV 91114 + *T. viride* (T47) (48.06%).

The results showed that only two treatments significantly reduced the aflatoxin content in the kernels. These were ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem and ICGV 91114 + *T. viride* (T47) providing 26.90 and 24.96 % respectively over JL24. One treatment, JL24 + Sasyaneem did not control the aflatoxin content of the kernels, which shows the ineffectiveness of the formulation (Sasyaneem) against aflatoxin contamination in these particular conditions.

IDM experiment in ICRISAT,Patancheru postrainy season (2002-2003)

The results showed that the IDM treatments did not increase the total number of plants and all the treatments including control

recorded similar means in total number of plants per plot. The means ranged from 119 to 123.25 number of plants / plot.

In case of pod yield, there was difference between the treatments when the mean of pod yield was compared. However, per cent pod yield increase was not high ranging from 2.62 to 11.37%. The best treatments were ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem (11.37%) and JL24 + *T. viride* (T47) + B33 + Sasyaneem (9.24%).

None of the treatments provided significantly higher seed yield over the controls. In resistant cultivar combinations, the maximum seed yield increase (13.79%) was obtained from ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem followed by ICGV 91114 + B33 (11.35%). Similarly in the JL24 combinations, JL24 + *T. viride* (T47) + B33 + Sasyaneem was the best treatment recording 11.66% increase in seed yield followed by JL24 + B33 (9.18%).

The IDM treatments did not show significant increase in biomass yield over control. Some of the treatments contributed slightly to the biomass yield. ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem increased the biomass yield (9.19%) over ICGV 91114 alone. JL24 + *T. viride* (T47) + B33 + Sasyaneem contributed to the biomass yield by 7.77% over JL24 alone.

At 40 DAS, the *A. flavus* population was low in the sick plot ranging from 1750 to 3250 cfu g^{-1} of soil. Significant reduction of *A.*

flavus population was obtained from three treatments, ICGV 91114 + *T. viride* (T47) + B33, ICGV 91114 + *T. viride* (T47) and JL24 + *T. viride* (T47) + B33 recording 46.15, 30.77 and 30.77 % reduction over JL24 alone.

At 80 DAS (40 days after *A. flavus* inoculation), the *A. flavus* population in the sick plot drastically increased ranging from 18000 to 25500 cfu g^{-1} of soil. Majority of the treatments provided reduction in *A. flavus* population of not less than 15%. ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem provided the highest significant reduction (29.41 %) in the pathogen population followed by ICGV 91114 + *T. viride* (T47) (23.53%) and ICGV 91114 + Sasyaneem (23.53%). This shows that the neem formulations (Sasyaneem) were effective in reducing pathogen population at least in the first 35 days after its application.

At crop harvesting, the *A. flavus* population decline in all the treatments might be due to higher temperatures of the summer and the end of season drought imposed on the crop. In addition to that, majority of the treatments controlled the *A. flavus* population (11.54 to 67.31%). The highest percent reduction was obtained from ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem (67.31%) followed by ICGV 91114 + *T. viride* (T47) (61.54).

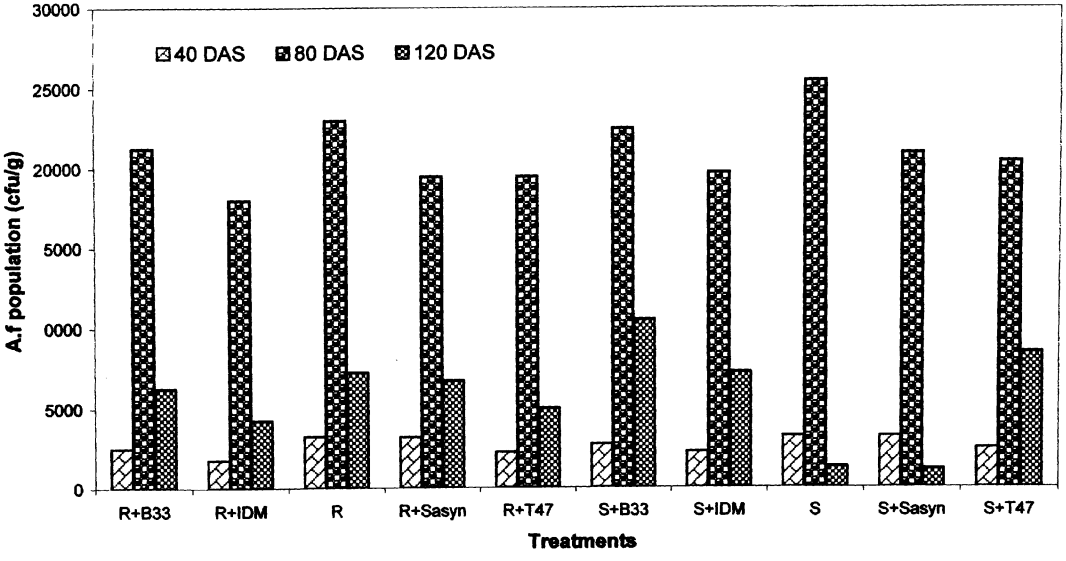

Fig.13: Efficacy of treatment combinations on *Aspergillus flavus* population under field conditions at ICRISAT (Posrtrainy season, 2002-2003)

Majority of the IDM treatments tested reduced the seed infection significantly. The reduction in seed infection followed similar trend to that of pathogen population reduction. ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem was the best treatment in reducing seed infection providing 37.98% reduction followed by ICGV 91114 + *T. viride* (T47) (30.77). In general, the treatments, which reduced the *A. flavus* population significantly, recorded less seed infection by *A. flavus*. Seed infection was high in all the treatments compared to previous rainy season (2002) due to high temperatures.

All the treatments involving resistant cultivar significantly reduced the aflatoxin content in the kernels compared to susceptible control (JL24 alone). The most effective treatment was ICGV 91114 + *T. viride* (T47) + B33 + Sasyaneem, which provided 38.98 % reduction in aflatoxin content followed by ICGV 91114 + *T. viride* (T47) (34.78). Significant reduction in aflatoxin content was also provided by ICGV 91114 + B33 and JL24 + *T. viride* (T47) + B33 + Sasyaneem (31.92 and 27.63 % respectively).

5.7 EVALUATION OF PLANT GROWTH PROMOTING POTENTIAL OF BIOCONTROL AGENTS IN GREENHOUSE CONDITIONS

The plant growth promoting potential of five *Trichoderma* isolates and six bacterial isolates used in the biocontrol experiments was evaluated under greenhouse conditions.

All the biocontrol agents tested showed increase in root length compared to control though they did not differ significantly. Two bacterial bioagents (B18 and B50) increased the root growth significantly over control (without BCA). These two recorded 23.6 and 25.84 cm respectively whereas control treatment recorded 17.33 cm. Seed treatment of *T. harzianum* and *T. viride* increased the root length (17.1 and 17.0 cm respectively) compared to control (11.2 cm). In case of root weight, most of the biocontrol agents did not differ significantly from control mean. However, three bacterial bioagents (B50, B58, PF2) increased the root weight significantly over the control. These recorded 2.20, 1.78 and 2.42 g against control (0.99 g). The bacterial isolate B50 showed significant increase in both root length and root weight.

All the biocontrol agents tested showed better shoot length growth than the control though some of them did not differ significantly from the mean of the control. However, seven out of eleven biocontrol agents tested significantly increased the shoot length. B33, PF2 and *T. viride* (T47) were found to be the best treatments in increasing shoot length. These recorded 29.92, 29.04 and 29.75 cm shoot length against 24.43 cm of the control. Results are in agreement with results of Saralamma (2000) who reported that *T. harzianum* and *T. viride* increased the shoot length (26.5 and 25.7cm respectively) against control (18.0cm) in groundnut.

A. flavus under field conditions seems to be important for the use of this formulation separately or with other management practice.

3. Field screening of groundnut genotypes that are resistant to *in vitro* seed colonization is desirable to find out groundnut genotypes resistant to preharvest seed infection by *A. flavus* under field conditions.

4. Since the aflatoxin contamination is a complex problem, study on the integration of different management approaches and their compatibility will be very useful in reducing the aflatoxin risk in groundnut.

5. Research on the identification of aflatoxin resistant gene and transferring it into groundnut genotype with desirable agronomic traits could provide one of the best solutions in minimizing aflatoxin problem.

6. The IDM practice could be tried on large scale in farmers' fields to effectively manage the aflatoxin contamination problem in groundnut.

CHAPTER VI

SUMMARY

The present study on the management of aflatoxin contamination in groundnut was undertaken to isolate and evaluate the efficacy of biocontrol agents (*Trichoderma* isolates and bacterial strains) in controlling *A flavus* infection and subsequent aflatoxin contamination A large number of groundnut genotypes were screened to test their resistance *in vitro* for seed colonization and seed infection by *A flavus* The efficacy of four commercial neem formulations (Nivaar, Sasyaneem, stairneem and sunny) was evaluated against *A flavus in vitro* and *in vivo* The selected biocontrol agents, resistant cultivar and promising neem formulations were put together and evaluated as integrated management of aflatoxin in groundnut The results of these investigations are summarized hereunder

Forty-nine *Trichoderma* isolates and 77 bacterial isolates were isolated from ground groundnut rhizosphere soil of major groundnut growing areas of Andhra Pradesh All the biocontrol agents were screened *in vitro* against *Aspergillus flavus* All the *Trichoderma* isolates significantly inhibited the mycelial growth of *A flavus* and gave percent inhibition ranging from 46 11 to 76 91 per cent Among the *Trichoderma* isolates, *T viride* (T47), *T viride* (T48) and *T viride* (T38) were most effective against *A flavus in vitro* Fifty-two of the 77 bacterial

isolates suppressed the mycelial growth of *A. flavus* significantly whereas, 25 isolates did not significantly controlled the test fungus. The percent inhibition of *A. flavus* mycelial growth by bacterial isolates ranged from 0 to 47.62%.

When the efficacy of selected biocontrol agents tested under greenhouse conditions, they increased the pod yield (30%), seed yield (25%) and biomass yield (28%). Among the biocontrol agents tested, B33, *T. viride* (T21), *T. harzianum* (T23) *T. viride* (T38), B50 and B6 were the best bioagents in increasing yield parameters.

The biocontrol agents reduced the *A. flavus* population at different stages of crop growth under greenhouse conditions. B33, *T. viride* (T47) and *P. fluorescens* (PF2) were the most effective in reducing the *A. flavus* population at all the crop stages. Similarly, significant reduction in seed infection was obtained from the effective biocontrol agents, like *T. viride* (T47), *T. harzianum* (T23) and bacterial isolates *P.seudomonas* fluorescens (PF2) and B33. Some biocontrol agents showed superiority over control in reducing aflatoxin content in the groundnut kernels though they did not show significant reduction over control.

When these biocontrol agents tested under field conditions, they increased the yield levels particularly in the rainy season. The most efficient bioagents were B33, B58, B50, *T. harzianum* (T23)

and *P fluorescens* (Pf2) The biocontrol agents reduced the *A flavus* population in the field Four biocontrol agents (*T viride* (147), *T harzianum* (T23), B33 and *P fluorescens* (Pf2)) consistently reduced the *A flavus* population at different crop stages through the seasons providing upto 46 per cent reduction of the population Similarly, the biocontrol agents reduced the seed infection by *A flavus* and aflatoxin content in the kernels Hence, *T viride* (T47) and *T harszianum* were the most effective bioagents

The UV irradiated mutants of *T viride* (T47) inhibited the *A flavus* mycelial growth significantly over control and were superior to the parental isolates except two mutants Three mutants M18, M15 and M28 were the most effective among the evaluated in suppressing the mycelial growth of *A flavus*

Four neem formulations tested (Nivaar, Sasyaneem, Starneem and Sunny) significantly inhibited the growth of *A flavus in vitro* The most effective formulation was Nivaar, which allowed the growth of the fungus only at 100 ppm concentration Sasyaneem was compatible with *T viride* (T47) permitting its growth upto 400 ppm concentration

The selected neem formulations (Nivaar and Sasyaneem) reduced the *A flavus* population under greenhouse conditions (up to 40%) and under field conditions (27%) Similarly, the two formulations reduced the seed infection and aflatoxin content in the kernels both

under green-house and field conditions though the reduction was not statistically significant over the control The two formulations showed similar efficacy under greenhouse and field conditions

Screening of 50 groundnut genotypes for resistance to infection by *A flavus* resulted the selection of 14 genotypes, which were resistant to *in vitro* seed colonization by *A flavus* These were tested under field conditions and most of the genotypes maintained their resistance to *in vitro* seed colonization Among all ICGV 91114 was the most resistant and was selected for use in the IDM experiment

On the other hand evaluation of 184 groundnut genotypes from ICRISAT groundnut core collection for *in vitro* seed colonization by *A flavus* resulted in the identification of some genotypes resistant to *in vitro* seed colonization though most of them showed high aflatoxin content Some genotypes viz , ICG 1519, ICG 2106, ICG 875, ICG 10384, ICG 14523 showed resistance comparable or slightly higher than the resistant check (J11) in the first screening In the second screening, some of the selected genotypes from the first screen maintained their resistance, for example ICG 875 The aflatoxin estimation of inoculated kernels of the selected genotypes revealed that most of the genotypes were not resistant to aflatoxin production by *A flavus* Some the genotypes were resistant to aflatoxin production and recorded lower aflatoxin content than resistant cultivar (J11) Of these, the genotypes

ICG 4598 was the best recording 1089 μgkg^{-1} in set I In set II core collection, eight genotypes recorded aflatoxin content lower than resistant cultivars (J11 and VRR 245) Among these ICG 5327 was the most resistant to aflatoxin production recording 8114 06 μg kg^{-1}

The IDM treatments increased the total number of plants, pod yield and seed yield particular in the 2002 rainy season at Patancheru whereas in the post rainy season at Patancheru and rainy season 2002 at Anantapur, the increase in yield parameters was low JL24 + *T viride* (T47) + B33 + Sasyaneem, increased the pod and seed yield (35%) and biomass yield (26%) in the rainy season 2002

The IDM treatments reduced the *A flavus* population at different stages of the crop growth Some of the treatments consistently reduced the *A flavus* population in all the three IDM experiments and at all the crop growth stages The best treatments were ICGV 91114 + *T viride* (T47) + B33 + Sasyaneem, ICGV 91114 + *T viride* (T47), ICGV 91114 + B33 and JL 24 + *T viride* (T47) + B33 + Sasyaneem The most effective treatment in all the three experiment was ICGV 91114 + *T viride* (T47) + B33 + Sasyaneem This treatment reduced the *A flavus* population significantly (43 31%) of 80 DAS in the rainy season 2002 at Patancheru and (56%) at 40 DAS in Anantapur The same treatment controlled the *A flavus* population at 40 and 80 DAS and at harvesting (46 15, 29 41 and 67 31%, respectively)

Two IDM treatments viz, ICGV 91114 + *T viride* (T47) + B33 + Sasyaneem and ICGV 91114 + *T viride* (T47) were most effective in reducing seed infection by *A flavus* in all the three experiments These treatments recorded reduction of 62 07% and 59 36% in the 2002 rainy season at Patancheru, 51% and 48% at Anantapur and 37 98% and 30 77 % in the postrainy season at Patancheru, respectively Similarly, the most affective treatments were ICGV 91114 + *T viride* (T47) + B33 + Sasyaneem and ICGV 91114 + *T viride* (T47) These two treatments gave significant reduction in aflatoxin content in most experiments The two treatments reduced the aflatoxin content (38 39% and 33 93% at Patancheru and 26 90% and 24 96% at Anantapur in the 2002 rainy season and 38 98% and 34 78% in the post rainy season at Patancheru, respectively

Most of the biocontrol agents increased the root length Two bacterial isolates (B18 and B50) significantly increased the root length Similarly, B50, B58 and *P fluorescens* (Pf2) significantly increased the root weight whereas remaining bioagents did not increase the root weight significantly

Most of the biocontrol agents significantly promoted the shoot growth contributing the shoot length and shoot weight B33, *P fluorescens* (Pf2) and *T viride* (T47) were the most potential bioagents in increasing shoot length (29 22, 29 04 and 29 75 cm,

respectively) against 24 43 cm of control Incase of shoot weight B33, B50, *T harzianum, T viride* (T38) were the most efficient treatments in increasing shoot weight

CONCLUSIONS

1 All the *Trichoderma* isolates tested controlled the *A flavus in vitro* and some of them were effective in reducing aflatoxin contamination under field conditions Similarly some of the bacterial isolates were effective in reducing aflatoxin contamination even under field conditions Hence, these biocontrol agents can be used in the management of *A flavus* and aflatoxin contaminations to groundnut kernels

2 Commercial neem formulation, Nivaar (neem kernel extract) can be used in controlling *A flavus* separately whereas Sasyaneem (neem kernel extract) can be used in IDM with *Trichoderma* These formulations have limited efficacy *in vivo* and can be used in the management of aflatoxin contamination, if the number of applications increased to twice per season

3 The groundnut cultivar ICGV 91114 is resistant to seed and *in vitro* seed colonization by *A flavus* and could be used in integrated management of aflatoxin contamination

4 There is a variation among the groundnut genotypes (ICRISAT groundnut core collection) for *in vitro* seed colonization by *A*

flavus. Some of the genotypes are highly resistant to *in vitro* seed colonization though most of them are not resistant to aflatoxin production.

5. Combination of biocontrol agents (*Pseudomonas cepacia* and *T. viride* (T47)) with resistant cultivar (ICGV 91114) reduced the *A. flavus* population in the soil, seed infection and to some extent aflatoxin content in the kernels. This could be used as integrated management to reduce preharvest aflatoxin contamination in groundnut.

6. Some of the biocontrol agents used in the present study exhibited plant growth promoting potential in groundnut contributing to enhanced root length and weight, shoot length and weight and increased the yield. Such biocontrol agents (like *Pseudomonas cepacia*) can be used as dual-purpose bioagents.

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