Biological Control of *Aspergillus flavus* Invasion in Groundnut

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By

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DECLARATION

I, **Gottumukkula Harini,** Research Scholar, Department of Microbiology, Osmania University, do hereby declare that the research work embodied in this thesis entitled **"Biological Control of *Aspergillus flavus* Invasion in Groundnut" s**ubmitted to Osmania University for award of the degree of Doctor of Philosophy in Microbiology is a bonafide record of work done by me during the period of research carried out under the supervision of Dr. Farid Waliyar, Director-West and Central Africa, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Bamako, Mali and Prof. Gopal Reddy, Head, Department of Microbiology, Osmania University, Hyderabad. This thesis has not formed, in whole or part, the basis for the award of any degree or diploma to any other University prior to this date.

Date: **(Gottumukkula Harini)**
Hyderabad

Dedicated to my parents
and my brothers (Kalyan and Raghu)

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CHAPTER - I

INTRODUCTION

1.1 Introduction

Mycotoxins are fungal metabolites, which are chemically diverse groups and occur in a wide variety of food and fodder. Consumption of mycotoxin contaminated food affects human health and also causes economic loss in livestock by causing diseases and thereby reducing production efficiency (Iheshiulor et al. 2011). Among all the mycotoxins identified till date, aflatoxins form the most important group (Blount 1961). Aflatoxins are produced mainly by a fungus *Aspergillus flavus* and *A. parasiticus* (Kurtzman et al. 1987; CAST 2003). *A. flavus* is an imperfect filamentous fungus that principally obtains nutrients in a saprophytic mode but under favourable conditions it acts as an opportunistic pathogen as well (Mellon et al. 2007).

A. flavus infects a wide variety of agricultural crops, such as maize (corn), cotton, groundnuts (peanuts), chillies and also fruits and vegetables etc. suggesting its broad host range (St Leger et al. 2000). Environmental factors play a major role in the inception of aflatoxin as it infects a wide range of crops and it can do so at various stages of the cropping period including pre-, post-harvest, and storage (Waliyar et al. 2008). Contamination can also occur at other times as well, for example, during transportation, processing and product storage. The wide consumption of groundnuts by humans and feed by livestock make them vulnerable to attack by these toxins leading to a wide range of undesirable effects, viz. mutagenic, immunosuppressive and teratogenic (Machida and Gomi 2010).

Management of aflatoxin in groundnut is very difficult as it shows geocarpy, therefore, many approaches have been developed for all the stages of the cropping period, such as development of resistant lines (Nigam et al. 2009), biological control through various agents which are found naturally in the environment (Prapagdee et

al. 2008), introgressing resistant genes and thereby developing transgenics or increasing host plant resistance etc. (Brown et al. 2003, Cleveland et al. 2003).

Out of all these methods, biological control has evolved into a promising tool as it is also an alternative to chemical methods (both fertilizers and pesticides) which kill many beneficial organisms and have a great negative impact on soil properties (Massart and Jijakli 2007, Li et al. 2008). Biological control is slow but can be long lasting, inexpensive, and harmless to living organisms and ecosystem, thus maintaining biodiversity; it neither eliminates the pathogen nor the disease, but brings them into natural balance (Ramanathan et al. 2002). Sometimes an integrated approach incorporating all the methods mentioned above can also bring about the necessary result and thus help in managing the fungus as well as the subsequent aflatoxin contamination.

Biological agents exist naturally in the environment and can be manipulated for the control of various soil-borne diseases caused by bacteria, fungi and helminths. Rhizosphere resident antagonistic microorganisms are ideal biocontrol agents, as it provides the frontline defense at the root level against infection by pathogens (Whipps 2001; Anjaiah et al. 2006). Mechanisms of biological control by many strains of antagonistic bacteria involve the production of bacterial metabolites that adversely affect the pathogen. These metabolites include antibiotics, siderophores, cell wall degrading enzymes such as chitinase, glucanase, protease and hydrogen cyanide (Benizri et al. 2001).

Among most of the biocontrol agents including bacteria, actinomycetes and fungi reported till date, bacteria belonging to the genera *Pseudomonas* sp. produce many types of antibiotics which bring about antagonism or biocontrol activity (Weller et al. 2002). Soil actinomycetes, especially *Streptomyces* sp. and among fungi

Trichoderma harzianum improves soil fertility and acts against a broad range of soil-borne phytopathogens (Joo et al. 2005; Errakhi et al. 2007; Diby et al. 2005). The metabolites produced by the biocontrol agents are target specific and get naturally degraded in the soil without affecting the physical, chemical and biological properties (Siddiqui and Shaukat 2002). Biocontrol agents induce systemic resistance in host plant defenses, and should be applied through proper carrier materials in order to bring about necessary action.

Microbe mediated formulations improve plant growth and yield, check plant pathogens, activate host plant resistance and they are less expensive as compared to chemical fertilizers and pesticides, and above all they leave no residues in the products (Nakkeeran et al. 2005). Composts form a good carrier for biocontrol agents and they also enhance metabolite production and beneficial microorganisms can multiply well in the compost, which further help in agricultural waste management (Hameeda et al. 2006a, 2006b). Proper formulation, an efficient delivery system, and alternative methods of bacterial application play a crucial role in the success of biocontrol agents in minimizing *A. flavus* invasion of groundnut kernels (Kishore et al. 2005 b; Jeyarajan and Nakeran 2000).

Due to its efficacy, biological control forms a vital part of integrated mycotoxin management (IMM) and is a promising tool for sustainable agriculture that can minimize agricultural losses to an economically viable level, thus rendering a safe environment for humans, animals and plants (Jeger 2000). Integration of biocontrol agents with host plant resistance along with reduced or no application of chemical fungicides or application of a consortium of biocontrol agents can help to manage on-farm aflatoxin contamination of the produce (Mendes et al. 2011).

1.2 OBJECTIVES

The present study, therefore, is focused on characterization of biocontrol bacteria and actinomycetes from a microbial rich source i.e., compost. The emphasis was on selecting potential antagonistic as well as plant growth promoting bacteria and actinomycetes against *A. flavus* invasion and subsequent aflatoxin contamination in groundnut. We screened various strains and found two novel species of bacterial strain *Pseudomonas* sp. CDB 35 and actinomycete strain *Streptomyces* sp. CDA 19. These were evaluated for antagonistic activity against *A. flavus* and further work carried forward with the following objectives:

- Isolation and characterization of bacteria and actinomycetes from rice straw compost against *A. flavus*
- Screening potential isolates for their antagonistic traits and characterize metabolites involved in antagonism
- Screening potential bacteria and actinomycetes for their plant growth promoting traits and evaluate under glasshouse conditions on groundnut JL-24 as host plant
- Study the effect of abiotic factors on the growth of the potentially antagonistic bacterial strain *Pseudomonas* sp. CDB 35 and actinomycete strain *Streptomyces* sp. CDA 19
- Develop appropriate methods for on-farm inoculation of biocontrol agents
- Study the effect of enriched rice straw compost on biocontrol activity and plant growth promotion of groundnut under field conditions
- Identify *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 by 16s rRNA sequencing

1.3 PRESENTATION OF THE THESIS

The thesis is presented in six chapters followed by references. The chapters are as follows: Chapter I – Introduction; Chapter II – Review of Literature; Chapter III – Materials and Methods; Chapter IV – Results; Chapter V – Discussion; and Chapter VI – Summary, Conclusions and Future Prospects.

At the end, the literature studied for this thesis is arranged in alphabetical order of author/s, year of publication, article title, journal name (abbreviated), volume number and both initial and final page numbers according to the guidelines of the American Society for Microbiologists. Reference by the same author(s) are arranged chronologically and if more than one publication by the same author(s) has been published in the same year, then it is distinguished in the text and reference by the letters a, b, c, etc., after the year of publication.

CHAPTER- II

REVIEW OF LITERATURE

2.1 Mycotoxins

Mycotoxins are chemically diverse secondary metabolites produced by various groups of fungi, which are harmful to the health of both human beings and livestock. Moreover, the agricultural economy of less developed groundnut producing countries is also adversely affected as mycotoxin contaminated food cannot be exported to the world market as it has stringent quality control requirements (FAO 2004). The UN Food and Agriculture Organization (FAO), has estimated that up to 25% of the world's foods are contaminated significantly with mycotoxins (Choudhary and Kumari 2010).

Mycotoxins are comprised of Aflatoxins, Ochratoxins, Citrinins, Ergot alkaloids, Patulin, Fusarium toxins (fumonisins, trichothecenes, zearalenone, beauvercin and enniatins, butenolide, equisetin, and fusarins). Mycotoxins have gained notoriety as it leads to a wide array of biological ill-effects; and individual mycotoxins can be mutagenic, carcinogenic, embryo-toxic, teratogenic, oestrogenic or immunosuppressive (Machida and Gomi 2010).

2.2 Aflatoxins

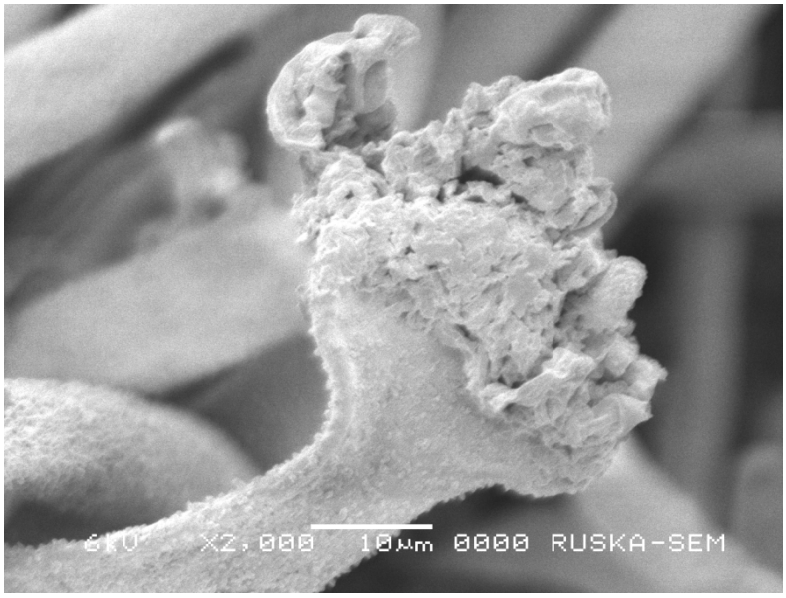
The aflatoxin problem came to light in the 1960's when there was a severe outbreak of a disease, referred to as Turkey X disease in England, in which over 100,000 turkeys and other farm animals died. The cause of the disease was found to be the peanut meal which was contaminated with *A. flavus* that the turkeys had eaten. Among the above mentioned major groups of mycotoxins, aflatoxins are naturally occurring mycotoxins produced by *Aspergillus* species, *viz. Aspergillus flavus* and *A. parasiticus*. The habit as well as the habitat of *Aspergillus,* require a high-humid environment, high temperature and drought condition etc., favors the colonization of fungi and the subsequent contamination of the host crops. The toxin is a highly potent

natural carcinogenic compound, which causes mutation (transversion) at 249 th codon of P53 gene (Kurtzman et al. 1987; CAST 2003). The most commonly found aflatoxins in groundnut, maize and other agricultural produce are aflatoxin B_1, B_2 produced by *A. flavus* and *A. parasiticus* and G_1 and G_2 produced by *A. parasiticus*. Aflatoxin M_1 and M_2 are produced in the milk when cattle fed with aflatoxin B_1 and B_2 contaminated feed. Apart from the above two *A. nomius, A. tamarii* and *A. bombycis* are the only molds which produce aflatoxins (Kurtzman et al. 1987). Nontoxigenic strains of *A. flavus* are very rare in nature, when compared to the rest of the aflatoxinogenic molds *A. parasiticus* is very stable in toxin production (Dorner et al. 2002).

Acute toxicity and long-term carcinogenicity are correlated with intake of aflatoxin contaminated food and feed. Dietary exposure to aflatoxins is a major cause of hepatocellular carcinoma in many third world countries (Williams et al. 2004). In humans, liver is generally the target organ leading to aflatoxicosis, and it can also cause systematic disease in immuno-compromised individuals. After *A. fumigatus*, *A. flavus* is the second leading cause of invasive and non-invasive aspergillosis (Yu et al. 2005).

2.2.1 Causative agent

A. flavus and *A. parasiticus* are the two main causative agents for colonization and production of secondary metabolites *viz.* aflatoxins.



Photograph 2.1. Terminal portion of a conidiophore of *A. flavus* showing the basal portion of the vesicle and distribution of radiation phialides X 2000.

Photograph 2.2. *Aspergillus flavus* toxigenic strain (AF 11-4) isolated in Mycotoxicology lab at ICRISAT, Patancheru.

A. flavus is a soil-borne, filamentous ascomycete, semithermophilic (grows in temperatures between 12°C to 48°C with optimum ranging from 25°C to 42°C) and semi xerophytic (water potential may be as low as – 35 MPa). Optimal conditions for fungal development are 36 to 38°C, with a high humidity of above 85% (Diener et al. 1987). It is a saprophyte living on dead and decaying organic matter, however, under drought stress and high temperatures, the fungus may colonize seeds whether the pods

are injured or not (SASA 2010). The ability of *A. flavus* to attack seeds of both monocots and dicots, and to infect seeds produced both above and below ground (geocarpy), demonstrates that this fungus has evolved a battery of mechanisms to breach the resistance of host, and it must be noted that only a few plant pathogenic fungi have such a broad host range and *A. flavus* when compared with *A. fumigatus* and *A. nidulans,* lacks host specificity (St Leger et al. 2000).

2.2.2 Aflatoxins structure

Aflatoxin normally refers to the group of difuranocoumarins and it is classified into two broad groups according to their chemical structure; the difurocoumarocyclopentenone series (AFB1, AFB2, AFB2A, AFM1, AFM2, AFM2A and aflatoxicol) and the difurocoumarolactone series (AFG1, AFG2, AFG2A, AFGM1, AFGM2, AFGM2A and AFB3) (Maggon et al. 1977). The aflatoxin molecule contains a coumarin nucleus linked to a bifuran and either a pentanone as in AFB_1 and dihydro derivative AFB_2, or a six member lactone, as in AFG_1 and its corresponding derivative AFG_2 (Sanz et al. 1989). Structurally the dihydrofuran moiety, containing double bond, and the constituents linked to the coumarin moiety are of importance in producing biological effects. These four compounds are separated by the colour of their fluorescence under long wave ultraviolet light illumination (ca. 365) (B=blue, G= green) (Dutton and Heathcoate 1966). The subscripts relate to their relative chromatographic mobility and of the four, aflatoxin B_1 is found in highest concentrations followed by M_1 and M_2.

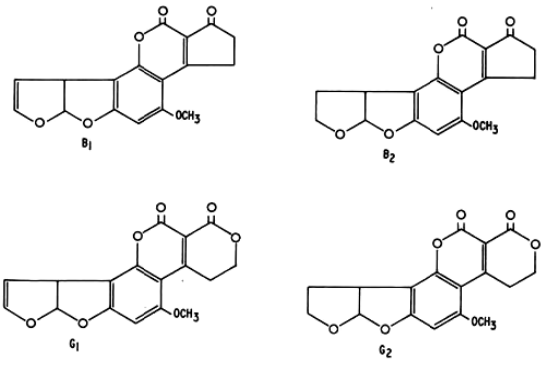


Fig. 1. Structures of aflatoxins B_1, B_2, G_1 and G_2.

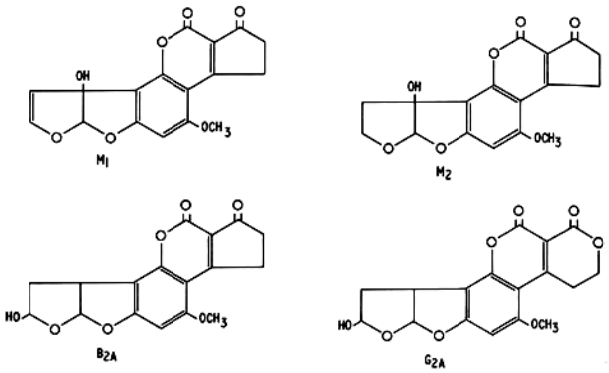


Fig. 2. Structures of aflatoxins M_1, M_2, B_{2A}, and G_{2A}.

2.2.3 Factors affecting aflatoxin production

The factors like species, substrate and environment play a crucial role in aflatoxin production. These can be further divided in to three catagories physical, nutritional and biological, physical factors include temperature, pH, moisture, light, aeration and level of atmospheric gases. The temperature required for aflatoxin production is between 12-42°C and 25-35°C being the optimum (Diener and Davis 1965). The moisture requirement is about 25% at 30°C and the minimum relative humidity varies between 83%-88%. Both CO_2 and O_2 influences mold growth and aflatoxin production and presence of 20% CO_2 in air reduces the aflatoxin production and also effect or reduce the mold growth. Oxygen limitation to 10% in air decreases the aflatoxin production and O_2 levels less than 1% inhibits the mold growth and toxin production completely (Landers et al. 1967). Weak acidic conditions increase the

aflatoxin production and decrease the mold growth considerably (Jarvis 1971). The carbon sources required generally are glucose, sucrose or fructose and micro nutrients like zinc and manganese are essential for aflatoxin biosynthesis where as the mixture of cadmium and iron decreased the growth as well as the toxin production.

2.3 Crops infected by *A. flavus*

A. flavus is one of the notorious soil borne plant pathogens and it is widely distributed throughout the world infecting various crops including both monocots and dicots (St Leger et al. 2000). Crops which are frequently affected include cereals (maize, sorghum, pearl millet, rice, wheat), oilseeds (groundnut, soybean, sunflower, cotton), spices (chilli peppers, black pepper, coriander, turmeric, ginger), and tree nuts (almond, pistachio, walnut, coconut, brazil nut). It infects crops both at pre and post harvest stages after insect or mechanical damage has occurred, as well as during storage (Abnet 2007).

2.4 Consequences of aflatoxin contamination in various agricultural commodities

The toxicity and carcinogenicity of aflatoxins have led to its recognition as a significant health hazard all over the world. *A. flavus* infection and aflatoxin contamination has been a serious agricultural problem for several decades now (Waliyar et al. 2005). The annual crop losses due to aflatoxin contamination and the costs involved in monitoring and disposal of contaminated commodities affect the agricultural economy of a country (Van Egmond 1995, 2003). Conditions that increase the likelihood of aflatoxicosis in humans include limited availability of food, environmental conditions that favour mold growth on food stuffs and the lack of regulatory systems for monitoring and control (Machida and Gomi 2010). Hence, it is difficult to find cost-effective solutions that are useful for both producers and

consumers. This is yet another handicap for farmers in developing countries who want to export their products to international markets.

2.5 Groundnut

Groundnut (*Arachis hypogaea*) is thought of as an "unpredictable legume" due to its unusual underground development of pods *i.e.* geocarpy and its surprising durability. It takes from 90 to 150 days to grow depending on the variety, environment and weather. In India, the sowing season occurs mostly (85% area) during the rainy season, the rest of the area sown is in either the post-rainy season (10% area) or in the summer (5% area). Groundnut grows best in light, sandy loam soil and requires five months of warm weather and an annual rainfall of 500 to 1000 mm, though it can bear up to 50 days of drought and its reproduction is almost exclusively by self pollination (http://www.aflatoxin.info/introduction.asp).

2.6 *A. flavus* infection and subsequent aflatoxin contamination in groundnut

A. flavus infection is often associated with aflatoxin contamination which makes groundnut unfit for consumption as well as reduces its trade value. The infection can colonize and contaminate grain before harvest or during storage (Waliyar et al. 2008). Host crops are particularly susceptible to infection by *A. flavus* following prolonged exposure to a high humidity environment or damage from stressful conditions, such as drought, a condition that paves the way for entry. *A. flavus* infection and subsequent aflatoxin contamination of groundnut occurs not only in post-harvest but also in pre-harvest conditions (Liang et al. 2006; Passone et al. 2008). Since groundnut shows geocarpy, several factors both biotic (soilborn insects) and abiotic (drought and high temperature) are considered to affect pre-harvest aflatoxin contamination. Furthermore mature pods are more likely to have contaminated seeds, while the late season drought (20-40 days before harvest) which

predisposes peanut to aflatoxin contamination is more prominent in the semi-arid tropics (Waliyar et al. 2005).

2.7 Management of aflatoxin contamination in groundnut

Aflatoxin contamination can be minimized by adopting better cultural, produce handling and storage practices (Wilson and Mubatanhema 2001). Management of aflatoxin in groundnut can be broadly divided into two methods:

- Preventing the infection process (host plant resistance, biocontrol);
- Control of environmental factors (temperature, rainfall, relative humidity, evapotranspiration and soil type) including efforts to build predictive models, such as pre-harvest crop management practices, post-harvest management strategies (timely harvesting, proper drying, sorting, proper storage, apt transportation, use of plant extracts and preservatives, good manufacturing practices and finding alternative uses for contaminated grain will reduce the lossess (Clements and White 2004; Kerstin Hell and Charity Mutegi 2011).

Cultural practices include irrigation in late season, which can reduce peanut pre-harvest aflatoxin contamination, but this cultural practice is practically not possible in the arid and semi-arid zones. Nevertheless, some countries have regular monitoring programs for groundnut and its products for aflatoxin management at different stages (farm, processing, markets, and storage) (Upadhyaya et al. 2001). Pre-harvest management includes breeding of resistant lines as it is an important alternative method with very little risk. Another is the enhancement of host resistance by plant breeding and/or genetic engineering of plant varieties to express specific resistant gene for fungal resistance by plants that inhibit fungal entry and subsequent aflatoxin production (Nigam et al. 2009; Sundaresha et al. 2010).

When it comes to the groundnut *A. flavus* system, attempts were made to develop aflatoxin resistant varieties (Waliyar et al. 1994; Upadhyaya et al. 2004), however, resistance in peanuts to aflatoxin contamination under all conditions has still has not been achieved and breeding efforts continue, including the use of microarrays to aid in the identification of genes involved in crop resistance (Guo et al. 2009).

Biological control that aids in crop protection is another effective approach to combat plant pathogens. Biocontrol agents including bacteria, actinomycetes and fungi are extensively studied and usage of biocontrol agents has gained importance as it reduces the application of chemical pesticides, which leads to sustainable agriculture (Choudhary and Kumari 2010). Pre-harvest measures are efficient in reducing aflatoxin levels and, at the same time, enhancing crop yields (Kerstin Hell and Charity Mutegi 2011). The process whereby groundnut pods are invaded by *A. flavus* which then moves into the seeds and the subsequent production of aflatoxin is quite complex and different from any other root or seedling disease where biological control has been successfully investigated (Handelsman and Stabb 1996; Dorner et al. 2002).

2.7.1 Biological control of *A. flavus* and aflatoxin production through plant growth promoting and/biocontrol microorganisms

Beneficial bacteria harbouring rhizosphere are termed as plant growth promoting bacteria (Kloepper and Schroth 1978). Based on their mode of action, plant growth promoting microorganisms are classified as plant growth promotors that directly stimulate plant growth and biocontrol that indirectly stimulates plant growth by reducing deleterious microorganisms in the rhizosphere. Research in this area has gained considerable attention and appears to be promising as a viable alternative to chemical control strategies, and therefore control of soil borne pathogens with

antagonistic bacteria and fungi has been intensively investigated (Prapagdee et al. 2008).

2.7.2 Bacteria

Several bacteria were reported to inhibit *A. flavus* growth and subsequent aflatoxin contamination. Of them include, *Bacillus pumilis* (Munimbazi and Bullerman 1996; Cho et al. 2009), *Bacillus subtilis* (Sommartya 1997), *Streptococcus lactis* (Coallier-Ascah and IDziak 1985), *Flavobacterium aurantiacum* (Line et al. 1995), *Bacillus subtilis* (NK- 330) (Kimura and Hirano 1988), *Xanthomonas maltophila* and *Pseudomonas putida* (Mickler et al. 1995), *Pseudomonas cepacia* (D1) (Misaghi et al. 1995), *Bacillus subtilis* (Cuero et al. 1991), *Bacillus, Pseudomonas, Ralstonia, and Burkholderia* (Palumbo et al. 2006), *Bacillus megaterium* (Kong et al. 2010), and *Pseudomonas fluorescens* (Anjaiah et al. 2006) etc; have been extensively reported.

2.7.3 Actinomycetes

Very few actinomycetes have been reported against *A. flavus* infection and aflatoxin production like *Streptomyces* sp. (Bressan 2003), *Streptomyces* AS1 (Sultan and Magan 2010, 2011), *Streptomyces maritimus* (Al-Bari et al. 2007), *Streptomyces* sp. MRI142 (Kondo et al. 2001).

2.7.4 Fungi

Fungal biocontrol agents against *A. flavus* are also very few in number like non-toxigenic (atoxigenic) strains of *A. flavus and A. parasiticus* (Yin et al. 2008, Dorner 2009), *A. chevalieri* and saprophytic yeasts, such as *Pichia anomala, Candida krusei,* (Hua et al. 1999; 2000), *Trichoderma harzianum*, (Gokul et al. 2000, EL-Katatny et al. 2001), *Trichoderma viridae*, *Fusarium*, *Rhizopus* , *Trichoderma* sp.

(Thakur and Waliyar 2005), etc; have been reported to be effective in inhibiting *A. flavus* growth and aflatoxin production.

2.8 Mechanisms of biocontrol

Mechanisms of biological control by many strains of antagonistic agents include antibiosis, competition for space and nutrients, parasitism or lysis, and degradation of the cell wall of the pathogen; or by inhibiting its secondary metabolite formation (Benizri et al. 2001).

2.8.1 Antibiosis

The most widely studied group of bacteria with respect to the production of antibiotics is the fluorescent Pseudomonads. The first antibiotic described as being implicated in biocontrol were phenazine derivatives produced by fluorescent *Pseudomonas* (Reddy and Reddy 2009). The antibiotics synthesized by the biocontrol *Pseudomonad* include 2,4- diacetyphloroglucinol, herbicolin, oomycin, phenazines, pyoluteorin and pyrrolnitrin. The broad-spectrum antibiotic 2,4-diacetyl phloroglucinol (2,4-DAPG) is a major determinant involved in the biological control of a wide range of plant diseases by fluorescent *Pseudomonas* sp. (Raaijmakers et al. 2002). Pyoluteorin (Plt), a phenolic polyketide, is reported for its bactericidal, herbicidal and antifungal functions produced by *Pseudomonas aeruginosa*, followed by *P. fluorescens* Pf-5 and *P. fluorescens* CHAO (Bender et al. 1999).

Phenazine-1-carboxylic acid (PCA) antibiotic producing *Pseudomonas chlororaphis* (strain PA-23) was effective against *Sclerotia* stem rot of canola (*Brassica napus*) in glasshouse as well as field evaluations (Zhang and Fernando 2004). Effectiveness of Pyrrolnitrin (PRN) is reported against a wide range of fungal pathogens such as, *Rhizoctonia solani, Botrytis cinerea, Verticillium dahlia, Fusarium*

and Sclerotium sp. (Ligon et al. 2000). Cyclic lipopeptides (CLPs) (viscosinamide, tensin and amphisin) produced by *P. fluorescens s*trains suppressed the pathogens, such as *P. ultimum* and *R. solani* (Nielsen et al. 2002). Studies of Sharma and Parihar (2010) revealed that antibiotic extracts obtained from actinomycetes inhibited *Alternaria, Aspergillus niger, A. flavus, Fusarium* and *Rhizopus stolonifer*. Extracellular metabolites produced by *Streptomyces hygroscopius* inhibited various phytopathogenic fungi (Prapagdee et al. 2008). Ono et al. 1997 have reported that aflastatin A, which has been isolated from mycelial extracts of *Streptomyces* effectively inhibits aflatoxin production. These metabolites are target specific and get degraded naturally in the soil without affecting physical, chemical and biological properties of the soil.

2.8.2 Volatile metabolites

Several genera of plant growth promoting microorganisms released volatile antibiotics which inhibit pathogenic fungus. This volatile secondary metabolite includes hydrogen cyanide (HCN), which is formed from glycine is catalysed by HCN synthase and inhibits cytochrome c oxidases of many organisms (Castric 1994). HCN is reported to be produced by *P. fluorescens, P. aeruginosa, Pseudomonas putida, Chromobacterium violaceum* etc. (Faramarzi et al. 2004; Faramarzi and Barand 2006). Actinomycetes belonging to the genera *Streptomyces* sp. are reported to produce diffusible and volatile antibiotics involved in antagonism (Zarandi et al. 2009). A few fungi, such as *Trichoderma harzianum* is reported to produce volatiles which inhibited *A. flavus* biomass accumulation and aflatoxin B1 production in stored maize under laboratory conditions (Aguero et al. 2008). Other volatilic antifungal agents belonging to aldehydes, alcohols, ketones and sulfides identified from *P. chlororaphis* (PA23), a soyabean root bacterium, inhibited *Sclerotinia sclerotiorum*

(Fernando et al. 2005).

2.8.3 Competition

Siderophores are iron chelating, highly electronegative low molecular weight compounds that bind to ferric form of iron in hexacordinated complex. Siderophores by virtue of their ability to sequester most of the available Fe^{+3} creates iron starvation in the rhizosphere, and inhibit the growth of other pathogens in the vicinity of the root system due to lack of iron (Sharma and Johri 2003). Studies by Lagopodi et al. (2002) showed that *Pseudomonas* sp. colonize plant roots and thus create a competitive environment for pathogenic fungus for iron. *Pseudomonads* are known to produce siderophores, such as pyoverdine, pyochelin, pseudobactin and salicylic acids (Loper and Henkels 1999; Yang and Crowley 2000). *Pseudomonas putida strain* DFC 31 produced siderophores along with other metabolites and suppressed the growth of *Colletotricum capsicum, Fusarium oxysporum, A. niger, A. flavus* (Sarode et al. 2007). Studies by Hamdali et al. (2008 a) revealed that actinomycetes, like *S. griseus BH7, YH1, S. cavourensis BH2 and Micromonospora aurantiaca KH7*, produced siderophores along with Indole acetic acid (IAA).

2.8.4 Hyperparasitism

To reduce disease severity in plants biocontrol microorganisms have developed an altered mechanism that hydrolyzes fungal cell and inhibits fungal proliferation (Bashan and de Bashan 2005). Some plant growth promoting microorganisms (PGPM) are reported to produce fungal cell wall degrading enzymes such as chitinases (Dunne et al. 1996), proteases (Ross et al. 2000), cellulases (Chatterjee et al. 1995) and glucanases (Jijakli and Lepoivre 1998) and act synergistically with other biocontrol mechanisms (Fogliane et al. 2002).

Chitin, a linear polymer of β-(1,4)-N-acetylglucosamine is the constitutent in most of the fungal pathogens and chitinases hydrolyze the same. Chitinolytic enzymes produced by *Bacillus cereus, Pantoea agglomerans, Paenibacillus, Serratia marcescens* and fluorescent pseudomonads are reported to be involved in biological control of *F. oxysporum* and *R. solani* (Someya et al. 2000; Singh et al. 2006). Another cell wall component β-1,3-glucan can be degraded by β-1,3-glucanases produced by PGPM. β-1,3-glucanases contribute to the ability of *Lysobacter enzymogenes* to control bipolaris leaf spot (Palumbo et al. 2005). Such enzymes may be involved in bacterial mycoparasitism, a form of antagonism in which bacteria directly colonize the hyphae. *P. cepacia* producing β-1,3-glucanases showed antagonism against various phytopathogens including *Rhizoctania solani*, *S. rolfsii* and *Pythium ultimum* (Fridlender et al. 1993). Studies reveal that actinomycetes, such as *S. griseus* (BH7, YH1), and *Streptomyces sindeneusis,* also facilitate plant growth indirectly by producing chitinases, β-l,3-glucanases, siderophores and antifungal substances (Zarandi et al. 2009). In addition, oxidative enzymes synthesized by such organisms also play a significant role in defense reactions against plant pathogens. Bacterization of betel vine cuttings with *S. marcescens* NBRI1213 induced plant defense enzymes, such as phenylalanine ammonia lyase, peroxidase and polyphenoloxidase (Lavania et al. 2006).

Studies by Gomes et al. (2000) reported that actinomycetes isolated from Brazilian soil under cerrado vegetation showed efficient chitinolytic activity and inhibited several phytopathogenic fungi including *Fusarium* sp., *F. solani*, *Aspergillus parasiticus*, *F. graminerium*, *Colletotrichum gloesporioides* and *F. oxysporum*. Studies on the extracellular metabolites of actinomycetes, isolated from soil, inhibited *Rhizopus stolonifer*, *Aspergillus flavus*, *F. oxysporum*, and *Alternaria sp* (Sharma and

Parihar 2010). Studies by Taechowisan (2003), revealed that *Streptomyces aureofaciens,* an endophytic actinomycete had great potential for chitinase production and was effective in fungal cell wall lysis. Chitinase and β 1,3 glucanase are considered to be important hydrolytic enzymes in the lysis of fungal cell walls of *Fusarium oxysporum, Sclerotinia minor*, and *S. rolfsi*. (El Katatny et al. 2001).

2.8.5 Induced systemic resistance (ISR)

Some biocontrol agents are reported as being able to induce systemic resistance in the host plant thereby enabling the plant to fight against the pathogen by itself. The mechanism of biocontrol agents includes both antagonism directly inhibiting the pathogenic fungus and elicitation of host defence responses (Brown et al. 2003; Cleveland et al. 2003). Plant growth promoting microorganisms capable of establishing such interaction induce metabolic changes in plants that increase resistance to a wide range of plant pathogenic microorganisms and viruses (Harman et al. 2004). In rhizobacteria-induced systemic resistance the systemic response occurs through the Jasmonic acid (JA)/ethylene signaling pathway in plants (Shoresh et al. 2005). Endophytic isolates from sunflower (*Helianthus annuus* L.) have shown bacterial production of abscisic acid (ABA) higher than JA and 12-oxo-phytodienoic acid (OPDA) (Forchetti et al. 2007). Synthesis of these hormones could help alleviate stress on the plants and these isolates assigned to a third category of beneficial bacteria termed “plant stress homeo-regulating rhizobacteria” (PSHR), as proposed by Cassán et al. (2005).

Plant defense responses can be induced, for example, by oligosaccharide elicitors of plant origin (pectin and cellulose or hemicellulose oligomers) and of bacterial origin (chitinases, glucanases, etc). Thus, by knowing the interactions of plants, plant growth promoting bacteria (PGPB) can be more effective for plant

resistance to various biotic and abiotic stresses. Increase in the activity and accumulation of defence-related enzymes, peroxidase, lipoxygenase, and phenylalanine ammonia lyase, depend not only on the inducing agent but also on plant genotype, physiological condition, and the pathogen (Tuzun 2001). An alternative mechanism is induced systemic resistance when biocontrol bacteria stimulate the host plant defenses thereby controlling the entry of the pathogenic fungus (Siddiqui and Shaukat 2002). The reports of Jetiyanon and Kloepper (2002) showed that the application of a consortia of biocontrol organisms with no significant antifungal activity increased host plant resistance and increased disease suppression than individual strains in vegetables. Application of a mixture of plant growth promoting microorganisms brought about plant growth promotion and disease suppression in multiple pathogens of cucumber (Nandakumar et al. 2001).

2.9 Factors pre-requisite for biocontrol agents

2.9.1 Root colonizing ability

For effective plant growth promotion and biological control activity, the inoculated PGPM must colonize the roots by establishing molecular signaling interaction with the host plant (Chin-A-Woeng et al. 2000). PGPM colonized on roots could act as a basin for nutrients available in the rhizosphere, thus reducing the available nutritional status to create starvation, and subsequently the death of fungal pathogens (Fernando et al. 1996). Phyllosphere as well as rhizosphere bacteria are reported to be successful control pathogens via niche exclusion. One such example of niche exclusion is by biocontrol strain *B. subtilis* 6051 that competes through biofilm formation against pathogen *P. syrinae* pv. tomato DC 3000 by colonizing *Arabidopsis* roots (Bais and Vivanco 2004); pseudomonads have also been reported to colonize root surface by biofilm formation thereby rendering antagonism (Ramey et al. 2004).

Plant growth promotion and biocontrol activity can be brought about by either being outside the roots of the plants or inside (Zinniel et al. 2002; Gray and Smith 2005).

Root colonization is affected by a number of abiotic and biotic factors. Important abiotic factors include temperature, pH, and nutrients; and biotic factors include plant species and the physiological stage of the plant and normal fauna of the soil. Some bacterial and fungal populations might benefit more from the nutrients offered by the plant than others which might affect their numerical dominance and activity. Microorganisms utilize specific root exudates become primary communities in the rhizosphere, for example, *Pseudomonas* sp. by virtue of their diverse nutrient utilization and competition for limited carbon sources are believed to be potent root colonizers (Lugtenberg et al. 2001). This specificity implies that the PGPM with the ability to change their metabolism according to environmental conditions will only colonize the rhizosphere (Bais et al. 2004). Further the colonization also depends on other soil microflora for example, some studies revealed that colonization levels of *P. fluorescens* SBW25 were substantially increased by the presence of nematodes than in their absence (Oliver et al. 2004).

2.10 Formulation development

Carrier materials play a vital role for working efficiency of the product developed. The frequently used carrier materials include peat, lignite, talc, turf, kaolinite, pyrophyllite, zeolite, montmorillonite, alginate, pressmud, sawdust, compost and vermiculite etc. Beneficial microorganisms are protected from desiccation in the presence of carrier materials. The shelf life of bacteria depends on the bacterial genera, physical, chemical and biological nature of the carrier materials. Studies by Dandurand et al. (1994) revealed that the survival rate of *P. fluorescence* (2-79RN10, W4F393) increased when inoculated in montmorillonite, zeolite and

vermiculite with smaller particle size than in kaolinite, pyrophyllite, and talc with bigger particle size. The proper formulation, an efficient delivery system and alternative methods of bacterial application play a crucial role in the success of biocontrol agents (Fernando et al. 2009). Application of the desired microorganism at one site may not give proper results, thus application at multiple sites and at different stages in the crop period is required for expected results. Previous studies have revealed that peat can be a good carrier material and has a good shelf life of the inoculated organisms (Kishore et al. 2006; Hameeda et al. 2008). The additional soil application of a talc-based formulation improved yield and showed significant disease control when compared to seed treatment alone (Vidhyasekaran et al. 1997). Rice blast was controlled under field conditions by application of *P. fluorescens* as seed treatment followed by three foliar applications (Krishnamurthy and Gnanamanickam 1998). A talc-based formulation of *B. subtilis* strain Pf1 and Pf2 increased grain yield of pigeonpea besides controlling pigeonpea wilt (Vidhyasekaran et al. 1997). Treatment of groundnut and pigeonpea seeds with peat-based formulation of *B. subtilis* supplemented with 0.5% chitin or with 0.5% of sterilized *Aspergillus* mycelium controlled crown rot of groundnut and wilt of pigeonpea respectively (Manjula and Podlie 2001). Composts can become a form of carrier materials as they are rich sources of nutrients which help the inoculated beneficial microorganisms to multiply and bring about the desired action when applied.

2.10.1 Composts and enriched composts

Composting has gained importance due to their ecofriendly nature and it is one of the best methods for utilizing agricultural wastes. Composts and vermicomposts form a rich source of plant beneficial bacteria, (Alvarez et al. 1995; Abbasi et al. 2002) as such composts have been reported to increase soil fertility and enhance plant

growth as well as suppress the fungal pathogens (Scheuerell et al. 2005). Studies of Haggag and Sedera (2005) suggested that *Trichoderma* survived well in peanut haulms and maintained its population in good number. Composts form a carrier for biocontrol agents as well as enhance the metabolite production and help plants with defense mechanisms (Haggag & Saber 2007). Application of farmyard manure and gypsum reduced aflatoxin contamination in groundnut in the semi-arid tropics (Waliyar et al. 2008).

2.11 Commercialization of the bioproducts

Powdered formulations of plant growth promoting microorganisms (PGPM) have been developed and a number of commercially available gums were tested as suitable substrates for PGPM in comparison to methylcellulose. Methylcellulose powder formulations were most suitable for pelleting onto sugar-beet (Suslow and Schroth1982). Powdered formulations have benefit as it is easy to store, transport, and it is easy to handle along with long shelf life. Also, by pelleting seed with a powder formulation it is possible to get a higher population of PGPR around the seed than by dipping in bacterial suspensions. A few of the reported methods of transferring inoculants include, seed treatment, biopriming, soil application, seedling dip, foliar spray, fruit spray, hive insert, sucker treatment, sett treatment, etc. (Nakkeeran et al. 2005). However, most of these strategies have been shown to be limited in effectiveness; so now the focus has shifted to the integrated approach. Non-toxigenic strains of *A. flavus* are reported to be highly effective against toxigenic strains and research in this area reveals that the application rate of at least 300 kg/hec could reduce the incidence of toxigenic strains of the fungus in the soil to a ratio equal to or greater than 1:1 (non-toxigenic : toxigenic). Non-toxigenic strains of the fungus do not release toxins even under drought stress. In USA the Environmental Protection

Agency (EPA) has started using a similar product, commercially known as Aflaguard. The other commercial products available against *A. flavus* are AF-36, Histick N/T (*Bacillus subtilis* MB 1600) and Kodiak (*Bacillus subtilis* GB03) etc.

2.12 Biocontrol as a part of integrated mycotoxin management (IMM)

A number of approaches have been proposed for the management of aflatoxin contamination, such as growing resistant cultivars, frequent irrigation to avoid drought stress during pod-formation stages, application of gypsum, prevention of damage due to soil pests and soil-borne diseases, proper lifting of plants, rapid drying of pods and kernels, and good storage methods (Waliyar et al. 2006). However, no single approach has been shown to be fully successful in preventing aflatoxin contamination and emphasis is placed on an integrated approach combining various options to prevent aflatoxin contamination (Waliyar et al. 2005). Biological control of *A. flavus* using competitive exclusion of toxigenic strains using non-toxigenic strains, and/or use of bacterial strains to inhibit the fungi is emerging as a promising approach, which can become part of the integrated management options for aflatoxin control (Thakur and Waliyar 2005) along with the resistant breeding lines and transgenics.

CHAPTER-III

MATERIALS and METHODS

3.1 Chemicals and raw materials

The chemicals and raw materials used in this study were obtained from different sources. General chemicals, such as ammonium chloride, ammonium sulphate, boric acid, calcium chloride, citric acid, copper sulphate, ferric chloride, ferrous sulphate, gluconic acid, magnesium sulphate, potassium chloride, potassium iodide, potassium nitrate, potassium phosphate, sodium chloride, sodium phosphate, tartaric acid, tris salt, zinc sulphate, etc., and H_2SO_4, HCl, NaOH, KOH, chloroform, methanol were from Qualigens India. 1-amino-cyclopropane-1-carboxylic acid, chrome azurol S dye, 8- Hydroxy quinoline, hexadecyltrimethyl ammonium bromide, phytic acid, PIPES [Piperazine 1,4 Bis (2-ethane sulphonic acid)] buffer, tryptophan are from Sigma Chemicals, USA. All media components, such as casaminoacid, cellulose, glucose, Luria broth, nutrient broth, peptone, yeast extract, agar, laminarin, ammonium sulphate, sephadex coloumn, DEAE cellulose coloumn, etc., were from Himedia. Crop residues used in the study for the preparation of rice straw compost were obtained from farms at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, AP, India.

ELISA plates: For high binding NUNC-MAXISORP TM surface plates were used, ELISA Plate Reader (Labsystems Multiscan R plus) IPCV-H Polyclonal antibodies, Aflatoxin B1 (Sigma A6636), Aflatoxin B_1 – BSA conjugate (Sigma A6655) Bovine Serum Albumin (Sigma A6793) 200 mg in 100 ml of PBS – Tween (0.2%) Carbonate buffer Na_2CO_3 – 1.59g $NaHCO_3$- 2.93, Distilled water - Phosphate buffer saline (PBS), (pH 7.4) Na_2HPO_4 - 02.38 KH_2PO_4 - 00.40g KCl - 00.40g, NaCl - 16.00, Phosphate buffered saline Tween (PBS-T) PBS - Tween, 0.5ml Antibody buffer PBS-, Polyvinyl Pyrrolidone (PVP) 40, 000 MW 2.0 g Bovine serum albumin.

3.2 Isolation and preservation of bacteria and actinomycetes from rice straw compost

Bacteria, actinomycetes and fungi were isolated from the rice straw compost prepared at the International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru. Ten grams of rice straw compost was suspended in 90mL of sterile distilled water in multiple numbers and left for shaking on a horizontal shaker. Appropriate dilutions of all the suspensions were plated on to Luria agar for bacteria, and actinomycete isolation agar for actinomycetes. The plates were incubated for 72 h at 30^{o}C for growth. Isolated bacteria and actinomycetes with distinct morphologies in each treatment were subcultured and maintained as glycerol stocks for bacteria, mineral oil for actinomycetes and PDA slopes for fungi. All were stored at -70^{o}C as germplasm collection at ICRISAT for future use. Bacterial isolates were given prefix compost degrading bacteria (CDB), actinomycetes (CDA) and fungi (CDF).

3.2.1 Pathogenic fungal isolates used in the study

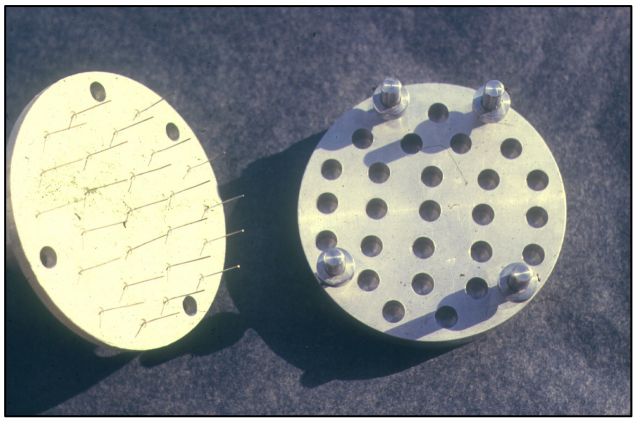
Aspergillus flavus AF 11-4 used in the study was isolated from the soil samples, *Aspergillus niger* was isolated from groundnut seeds, and *Sclerotium rolfsii* from groundnut stem samples from farmers fields of Anantapur. *Macrophomina phaseolina*, *Fusarium udum*, *F. oxysporum*, *F. solani* cultures were obtained from IMTECH, Chandigarh.

3.3 *In vitro* screening of bacteria and actinomycetes against *A. flavus*:

3.3.1 Double layer method

All the bacteria and actinomycetes isolated from rice straw compost were screened against *A. flavus* by double layer method on glucose casamino yeast extract (GCY) medium. A layer of medium is poured on to the Petri plate and allowed to solidify, then on top of this layer fungus of appropriate dilution is spread plated and

allowed to dry in the laminar flow. Once dried, one more layer of medium of appropriate temperature is poured and allowed to solidify. On to this layer mid log phase of bacteria and actinomycetes were inoculated with a pin inoculator and incubated at 28±1° C. The experiment was repeated twice with six replications.



Photograph 3.1. Pin inoculator used in screening of bacteria and actinomycetes against *A. flavus*.

3.3.2 Dual plate method

All the isolates which inhibited *A. flavus* by double layer method were screened against *A. flavus* on PDA, GCY and Kings B medium using dual plate method. In plate assay, a loopful of 24 h old culture of each bacterium and actinomycetes was streaked in a straight line on one edge of a 90-mm diameter Petri plate. Then an agar block (five mm diameter) was cut from an actively growing (five-day-old) fungal culture and placed on the surface of fresh agar medium perpendicular to that of the test organism. Plates inoculated with the same fungus without bacteria were used as control. For each treatment six replicates were maintained and repeated twice. Plates were incubated at 28±2°C and the inhibition zone between the two cultures was measured 5 days after inoculation. The percentage of inhibition of fungi was calculated by using the formula as given below. The distance between the fungus

and the test organism measured as the inhibition distance $I = \frac{100(C-T)}{C}$

where,

I=Inhibition % of mycelial growth (growth reduction over control)

C=radial growth of fungus in the control plate (mm)

T=radial growth of fungus on the plate inoculated with bacteria (mm).

3.4 *In vitro* screening of CDB 35 and CDA 19 against groundnut pathogenic fungi by dual culture method

The two isolates CDB 35 and CDA 19 which suppressed *A.flavus* significantly were screened against *Aspergillus niger, Fusarium udum, Fusarium oxysporum, Fusarium solani, Macrophomina phaseolina,* and *Sclerotium rolfsi* to know their broad range activity. The percent of inhibition of fungi was calculated by using the formula mentioned above.

3.5 Effect of volatile metabolites on *A. flavus* growth

Volatile antibiotic study was done following the method of Dennis and Webster (1971b). The potential isolates of bacteria and actinomycetes were screened for their volatile metabolite production and subsequent radial growth inhibition of *A. flavus*. A fungal plug of 5 mm diameter was inoculated in to potato dextrose agar (PDA), GCY and King's B agar (KB) and was incubated for 24 hours. The bacterial and actinomycete isolates were lawn streaked on the three media mentioned and these plates (GCY- GCY, PDA - PDA and KB – KB) of test fungus and potential isolates were taped together with adhesive tape. The lids of control plates contained only media with no isolates. Test plates and control plates were set up in triplicate and repeated twice. The assembly was opened after five days of incubation and the radial growth inhibition was calculated over control.

3.6 Antagonistic activity of cell culture filtrates (CCF) of CDB 35 and CDA 19 on *A. flavus* radial growth inhibition

The effect of extracellular metabolites of selected antagonistic bacteria on radial growth of *A. flavus* was determined by the poisoned food technique (Nene and Thapliyal 1971). Potential bacteria were inoculated in to Luria broth and actinomycetes in Bennets broth. Bacteria were grown for 24 hours and actinomycetes for 48 hours in an incubator shaker at 180 rpm and 28±2°C. The culture was centrifuged at 7000 rpm at 4°C. Centrifugation was carried out twice depending on pellet formation. The supernatant was carefully transferred to the pre-cooled PDA at final concentrations ranging from 10-50% v/v. A 5mm diameter agar disc from actively growing fungal culture was inoculated at the centre of each plate. The inoculated plate was incubated at 28±2°C. The colony diameter was measured after 7 days of incubation. Fungus inoculated on PDA without any cell-free culture filtrate served as control.

3.7 Inhibition of *A. flavus* under broth culture conditions

The effect of the bacterial and actinomycete cell pellet (CP) and their cellfree culture filtrates (CCF) were tested on the fungal biomass. CDB 35 and CDA 19 were selected based on *in vitro* studies under plate culture conditions against *A. flavus*. The experiment was performed with simultaneous inoculation of the fungal plug and the CP of the isolates and their CCF. Bacteria (CDB 35) and actinomycetes (CDA 19) were grown in their respective media for 24 h and 48 h respectively. This was centrifuged and the pellet and the culture filtrate were used for inoculation. 100µL of the pellet of bacteria and actinomycetes was inoculated. The culture filtrate was inoculated in the ratio of 1:1. The culture was incubated on a rotary shaker (120 rpm, Model G25, New Brunswick, Sci. Inc. NJ, USA) at 30±1°C for five days. For control,

the fungus alone was inoculated. The experiment was repeated twice with six replications each time. After 5 days dual cultures were passed through the pre-weighed Whatman No.1 filter paper and dried for 24 h at 65°C and weights were measured. Biomass reduction (percent reduction over control) of fungi and electron microscopic observation of the mycelium was done.

3.8 Inhibition of *A. flavus* biomass and spore germination under broth culture conditions

CDB 35 culture grown for 24 hours and 48 hour-grown culture of CDA 19 were used for testing spore germination. Both cells and cell culture supernatant were tested for spore germination on a cavity slide. A drop of CCF and CP were taken and spores of *A. flavus* of appropriate dilution was inoculated and incubated for 24 hours and observed under microscope for spore germination, control plate was without CP and CCF.

3.9 Antagonistic traits of the selected potential bacteria and actinomycetes

3.9.1 Production of HCN

The qualitative production of HCN was determined by inoculating the potential isolates on LB agar supplemented with 4.4 g /lit glycine. Inoculated Petri plates were inverted and a piece of filter paper impregnated with 0.5% picric acid in 2% Na_2CO_3 was placed on the lid (Bakker and Schippers 1987). The Petri plates were sealed with parafilm and incubated at 25^{o}C for 96 h. A change of the filter paper colour from yellow to light brown, brown, and reddish brown was recorded as having weak (+), moderate (++), and strong (+++) Cyanogenic potential, respectively. Uninoculated plates were maintained as control.

3.9.2 Production of siderophores

Production of siderophores by the potential isolates of bacteria and actinomycetes was determined using chrome azurol S (CAS) agar medium (Schwyn

and Neilands 1987). To prepare one litre of CAS agar, 60.5 mg of CAS was dissolved in 50 ml of DDW and mixed with 10 ml iron (III) solution (1mM Fecl3.6H2O, 10 mM HCL), while stirring the solution was slowly added and the resulting dark blue-coloured liquid was autoclaved. A mixture of 750 ml DDW, 100 ml of 10X MM9 salts, 15 g agar, 30.24 g PIPES (Sigma) and 12.0 g of 50% (w/v) NaOH solution (to raise the pH to the pKa of PIPES i.e, 6.8) was autoclaved separately. After cooling to 50^{o}C, 30 ml of casaminoacid solution 10% w/v was added as carbon source. The dye solution was finally added along the glass wall with enough agitation to achieve mixing with generation of foam, and dispensed in to the Petri plates. The casamino acid solution was de-ferrated before autoclaving using hydroxyquinoline as a chelating agent. Potential isolates were inoculated on to these plates and incubated for 48 h at 30^{o} C. Siderophore production by the isolates was indicated by the orange halo around the colony. Depending upon the size of the orange halo the isolates were rated as +, ++, +++ for siderophore production.

3.9.3 Production of chitinases

To 400 mL of con. HCL, 10 g of chitin (Sigma Chemical Co., USA) was added, stirred at 4^{o}C until the chitin dissolved and incubated at 37^{o}C until viscosity decreased. The whole mixture was added to 4 L of DDW and left overnight 4^{o}C. The precipitate was collected on a filter paper after decanting the supernatant and washed extensively with DDW to attain neutral pH and then was lyophilized and stored at -20^{o}C. (Berger and Reynolds 1988). This was used as the substrate and the media was prepared, all the potential isolates were inoculated and the zone of hydrolysis was observed for enzyme production every 24 hours for five days. Depending upon the zone size the isolates were rated as +, ++, +++ for chitinase production.

3.9.4 Production of β -1,3 glucanases

Production of β- 1,3 glucanases was determined by spotting the isolates on LB agar supplemented with 4% laminarin (Sigma Chemical Co., USA). The inoculated plates were incubated for 72 h at 30ºC and enzyme production was observed by staining the plates with 1% (w/v) aqueous congored solution. Depending on the diameter of the clear zone around the colonies in the pink background, the isolates were rated as +, ++, +++ for glucanase production.

3.9.5 Production of lipases

Lipase activity was tested in lipase medium where the carbon source was Tween 20. The potential isolates were inoculated and incubated for 24 h and after incubation the Petri plate was flooded with Congo red dye and the zone of hydrolysis indicated the lipase production. Depending upon the zone size the isolates were rated as +, ++, +++ for lipase production.

3.9.6 Production of cellulases

Modified M9 medium (Booth 1971) was used for cellulose utilization. The potential isolates were inoculated and incubated after 48 hours. Zone of hydrolysis indicated cellulase production. Depending upon the zone size the isolates were rated as +, ++, +++ for cellulase production.

3.9.7 Production of proteases

Protease production was detected by testing the isolates on skim milk supplemented agar. The potential isolates were inoculated on to the media and incubated at 30^{o}C for 24 h. The proteolytic activity was observed as clear zone around the colonies. The zone size increased with the time of incubation and accordingly the zone size was rated as +, ++, +++ for protease production.

3.9.8 Acid production

All the potential bacterial isolates were inoculated in to Luria broth and actinomycetes were inoculated in Bennets broth (100mL) in 250 mL conical flasks and the intial pH was maintained neutral. The sample was collected for every 12 hours and the supernatant was tested for pH drop with a pH meter till five days of incubation on an orbital shaker at 180 rpm.

3.9.9 Pigment production

All the potential bacteria were streaked on KB, *Pseudomonas* isolation agar and PDA actinomycetes were streaked on BA and PDA. Pigment production for bacteria was observed under fluorescence (after 24 hours of incubation) and actinomycetes by the colour of the substrate mycelium by inverting the plate after 48 hours of incubation.

3.10 Effect of Iron on *A. flavus* growth and aflatoxin production

Czapecdox medium was supplemented with various concentrations of iron (0, 10, 25, 50, 75 and 100 ppm). Five-days old culture of *A. flavus* was inoculated and incubated. The radial growth and toxin production (ELISA) was measured for 3, 5 and 9 days of incubation.

3.11 Detection of siderophore production (CAS assay)

A loopful culture of bacterial isolate CDB 35 and actinomycete isolate CDA19 was subcultured and separately inoculated in 100 ml of low strength nutrient medium and incubation was carried out for 18 h at 28^{o} C on rotary shaker at 120 rpm. This culture was used as inoculum for studying siderophore production. Production of siderophores occurs only under iron deficient and highly aerobic conditions. Each siderophore positive isolate was separately inoculated in iron deficient succinate medium at the rate of one percent (v/ v) and incubated on rotary shaker at 28^{o} C, 120

rpm. Supernatants for every 24 hours was collected and was tested for siderophore production by using universal chemical assay as per Schwyn and Neilands (1987). Fermented broth was centrifuged (10,000 rpm for 15 min) and supernatant was subjected to estimation of siderophores. Briefly, 0.5 ml aliquot of culture filtrate was mixed with 0.5 ml of CAS reagent. The O.D was read at 630 nm against un-inoculated succinate medium as a reference and percent decolourization was calculated by using the following formula:

$$\frac{Ar - AS}{ArX\ 10}$$

Percent decolourization where, Ar = Absorbance of reference; As = Absorbance of sample at 630 nm.

3.12 Detection of β- 1,3 glucanaces by CDB 35 and CDA 19

3.12.1 β- 1,3 glucanase purification

CDB 35 bacteria was grown in LB for three days and CDA 19 actinomycetes was grown in yeast extract malt broth for five days and then the supernatant of the bacterial culture was obtained after low speed centrifugation. The supernatant was precipitated using ammonium sulphate (75%) in cold room. The resultant was centrifuged and the pellet was collected. This was purified with DEAE cellulose column. After that column was washed with 390 mL of the sodium phosphate buffer (pH 6.5) and the proteins were eluted with 420 mL of a linear 0-0.5 M Nacl gradient collecting 3mL fractions. Fractions containing glucanase were pooled, dialyzed against the same buffer, and concentrated. The concentrated enzyme was applied to a Sephadex G-100 column equilibrated with 50mM Tris-HCL buffer, pH 7.5 and eluted with the same buffer at a flow rate of 6ml per hour collecting 1mL fractions. The active fractions were pooled, concentrated and stored at 4^{o} C.

3.12.2 β- 1, 3 glucanase assay

The amount of reducing sugars released using laminarin as substrate was measured. 10 µL of the crude enzyme was taken and to this 90 µL of 5 mg/mL laminarin in 0.1 M sodium acetate buffer of pH 5.0 was taken and incubated at 40^{o} C with gentle shaking for 10 min. The reaction was stopped by boiling for 5 min and 0.2 mL of 1% DNS and 0.2 mL of sodium acetate buffer were added and boiled for another 5 min, then placed in an ice bath and 0.9 mL of distilled water was added and absorbance was measured at 540 nm. Reducing sugar was measured from standard graph prepared with glucose, and glucanase activity was expressed as M mol glucose equivalents/min. Total protein from culture filtrate, crude enzyme, ammonium sulphate precipitation pellet and DEAE column purified enzyme was measured by Lowry's method.

3.13 Detection of chitinases

3.13.1 Purification of chitinases

Actinomycete CDA 19 was grown in chitin medium with colloidal chitin as substrate for 7 days. This was centrifuged with low speed centrifugation and the culture filtrate was used for detection of chitinase enzyme. The proteins from the culture supernatant were precipitated by ammonium sulfate (75%). The precipitate was collected by centrifuging at 8,000 × g for 20 min, and re-suspended in an acetate buffer (50 mM, pH 5.0). It was dialyzed against the same buffer and freeze-dried. The sample was then loaded on a pre-equilibrated DEAE-cellulose column (2 × 20 cm), and washed with the acetate buffer. The proteins were eluted in a stepwise gradient on NaCl (0-1.0 M) at a flow rate of 24 ml/h and the fractions of 3 ml were collected, and the absorbance was read at 280 nm in a spectrophotometer. The fractions with

chitinase activity were combined, dialyzed against the acetate buffer, and concentration made by lyophilization. The concentrated sample was passed through a Sephadex G-100 column (2 × 40 cm) and eluted with the acetate buffer at the rate of 15 ml/h. The fractions of 3 ml were collected, and the absorbance and chitinase activity were measured.

3.13.2 Chitinase assay

Colloidal chitin was used as a substrate to assay chitinase activity; 0.2 g in a 2 ml acetate buffer (50 mM, pH 5.0) was incubated with 1 ml of enzyme at 30^{o} C for 1 h. The product was measured in 1 ml of filtrate by the dinitrosalicylic acid (DNS) method (Miller1959). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of N- acetylglucosamine (NAG) per ml in 60 min at 405 nm. Total protein from culture filtrate, crude enzyme, ammonium sulphate precipitation pellet and DEAE column purified enzyme was measured by Lowry's method.

3.14 Evaluation of plant growth promoting traits by the potential isolates of bacteria and actinomycetes

3.14.1 Phosphate solubilization

All potential bacterial and actinomycete isolates were screened for phosphate solubilization on rock phosphate buffered medium (Gyaneshwar et al. 1998) at 28$\pm^{o}$ C for 24 hours. Phosphate solubilization was identified as pink zone due to pH reduction around colonies, both colony and zone diameters were measured in mm.

3.14.2 Phytase production

Phytic acid medium was used for the detection of phytase activity by the

selected isolates (Richardson and Hadobas 1997). The potential isolates were inoculated and incubated on the media and were observed for zone of hydrolysis at various time intervals.

3.14.3 Production of IAA

All the selected isolates were screened for IAA production. Bacterial cultures were grown in Luria broth and actinomycetes were grown in Bennets broth with tryptophan amendment (1-10mg/mL). After 24 hours of incubation on a shaker at 180 rpm and 28$\pm$2^{o} C, the culture was centrifuged and the supernatant was used for IAA production by Salwosky's method.

3.14.4 Detection of IAA

Bacterial isolate CDB 35, actinomycete CDA 19 were screened for IAA production, CDB 35 was inoculated in to Luria broth and CDA 19 in to Bennet's broth with L-Tryptophan at the rate of 0, 1, 2, 3, 4, 6, 7, 8, 9 and 10 mg/mL and incubated on a shaker at 28$\pm$2 ^{o}C at 180 rpm. Samples were drawn every 24 hours and centrifuged at 3000 rpm for 30 min. Two mL of the supernatant was mixed with 4 mL of the Solawasky's reagent (50 ml of perchloric acid, 1 mL of 0.5 $FeCl_3$). Development of a pink colour indicates IAA production, O.D was read at 530 nm using spectrophotometer and the observation was taken for every 24 hours for 10 days.

3.15 Effect of abiotic factors on growth of bacterium CDB 35 and actinomycete CDA 19

3.15.1 Effect of various pH on growth of bacterium CDB 35 and actinomycetes CDA 19 at 30± 2° C *in vitro*

At constant temperature of 32±2°C effect of various pH (5,6,7 and 8) was tested under broth culture conditions for both CDB 35 and CDA 19. CDB 35 was grown in Luria broth and CDA 19 was grown in Bennets broth and pH was adjusted with con HCL. The experiment was conducted with 6 replications and repeated twice. The organisms were grown in an incubator shaker at 180 rpm and the sample was collected every 12 h up to 90 hours and OD was recorded at 600nm.

3.15.2 Effect of various salinity levels on growth of bacterium CDB 35 and actinomycetes CDA 19

At constant temperature of 30± 2°C, the effect of various salinity levels was tested under broth culture conditions for both CDB 35 and CDA 19. Bacterium CDB 35 was grown in Luria broth and CDA 19 was grown in Bennets broth. Salinity levels were maintained with Nacl at 50 mM, 100 mM, 150 mM, 200mM and 250mM concentration. The experiment was conducted with 6 replications and repeated twice. The organisms were grown in an incubator shaker at 180 rpm and the sample was collected for every 12 h up to 90 hours and the O.D is recorded at 600nm.

3.15.3 Effect of various temperatures on growth of bacterium sp CDB 35 and actinomycetes CDA 19 at pH 7

Temperature variation on growth of potential bacterium and actinomycete were tested under broth culture conditions. Bacterium CDB 35 was inoculated in Luria broth, actinomycete CDA 19 was inoculated in to Bennets broth, and both were incubated in a rotary shaker at 180 rpm and the temperatures maintained were 15, 30, 36, 48, and 55°C. Culture was harvested at every 12 h of incubation and O.D was

measured at 600 nm. Throughout the experiment the pH was maintained at 7. The experiment was conducted with 6 replications and repeated twice.

3.15.4 Effect of various temperatures on growth of bacterium sp CDB 35 and actinomycetes CDA 19 at 100mM Nacl concentration

Temperature variation on growth of potential bacterium and actinomycete were tested under broth culture conditions. Bacterium CDB 35 was inoculated in Luria broth (LB), actinomycete CDA 19 was inoculated in to Bennets broth and both were incubated in a rotary shaker at 180 rpm, and the temperatures maintained at 15, 30, 36, 48, and 55°C. Culture was harvested at every 12 h of incubation and O.D was tested at 600nm. Throughout the experiment the salinity was maintained at 100mM. The experiment was conducted with 6 replications, repeated twice.

3.16 Compatibility of CDB 35 and CDA 19 with groundnut specific *Rhizobium* IC 7114 (in plate culture conditions)

Bacterium CDB 35 and actinomycete CDA 19 used for biocontrol studies were characterized for their interaction with groundnut specific rhizobium. Groundnut (IC 7114) was obtained from the microbial culture collection at ICRISAT (Rupela et al. 1991). It grew on yeast extract mannitol agar (YEMA) (Dalton 1980). Rhizobium was inoculated 24 h prior to the inoculation of test organisms because of the slow growth of rhizobia. Rhizobium was inoculated as vertical streak and the test organisms were streaked as horizontal streaks. The plates were incubated for 24 hours and observed for “interaction distance” and “spreading capacity”.

Photograph: 3.2. Interaction of CDB 35 and CDA 19 (vertical streak) with rhizobium (horizontal streak).

3.17 Evaluation of plant growth promotion of the selected isolates *in vitro*

All the potential bacteria selected were grown in LB and actinomycetes were grown in Bennet's broth. Surface sterilized groundnut seeds with $Hgcl_2$ for three minutes were soaked in the culture broth for half-an-hour. These were plated on to the soaked sterile filter paper placed in the Petri plates and incubated for five days in the trays lined with the wet blotting paper. For each culture 10 replications with ten seeds each was maintained. Control was maintained without any inoculum. The viable cell count as determined by dilution plating was 10^6-10^7 colony forming unit (CFU) per seed and root length and number of rootlets was recorded.

Photograph: 3.3 Plate assay for plant growth promotion by the potential bacteria and actinomycetes.

3.18 Glasshouse evaluations of the plant growth-promoting bacteria

The plant growth-promoting ability of bacterial and actinomycete isolates of groundnut JL-24 was evaluated under soil culture conditions. Five seeds inoculated with potential bacteria (8) and actinomycetes (3) were planted in 25 cm diameter plastic pots filled with red alfisol and sand (2:1). Seeds treated with 0.5% CMC alone were planted simultaneously, as control. The temperature in glasshouse was maintained at 28 ± 2^{o} C and the pots were adequately watered daily. The emergence of seedlings was recorded 7 days after sowing. The plants were uprooted 30 days after sowing, and the root and shoot lengths were recorded. The plants were washed, oven dried at 100° C for 24 hours and the dry weight was recorded. The viable cell count determined by dilution plating was 10^{6}-10^{7} colony forming unit (CFU) per seed and the experiment was conducted with six replications and repeated twice.

3.19 Development of various formulations of CDB 35 and CDA 19 and shelf life studies

Selected plant growth promoting bacterial isolates viz bacterium CDB 35 and actinomycetes CDA 19 were used to develop various formulations using peat, lignite and talc (Biocare Technology Pvt. Ltd., Australia) as carrier materials. Initial pH of peat was 6.1 and was adjusted to 7.0 by adding $CaCO_3$. Thirty g of neutralized peat, lignite and talc were packed in individual high molecular and high density polyethylene bags, and sterilized by autoclaving at 121^{o}C for 20 min twice with 24 hours of time gap between two sterilizations. Bacterial cells were harvested from mid-log phase cultures grown in Luria broth and actinomycetes grown in Bennet's broth by centrifugation and resuspended in equal volume of 10 mM phosphate buffer, pH 7.0. The cell suspension was diluted 100 fold using the same buffer and 15 ml of the diluted suspension was aseptically injected to the peat, lignite and talc packets, mixed

well and thoroughly kneaded to ensure uniform adsorption of the inoculated organisms in to the carrier materials. Moisture loss from the packets was compensated by adding sterile distilled water at regular intervals, based on the loss of the initial weight. Variability of the formulated organisms were determined at frequent time intervals up to 180 days after inoculation (DAI).

3.19.1 Evaluation of peat-based inoculums of bacterium CDB 35 and actinomycetes CDA 19 on plant growth of groundnut

Isolates which showed significant results under plate culture conditions and glasshouse were further tested with peat as the carrier material as both survived well in peat for 180 days. Bacterium CDB 35 and actinomcyete CDA 19 were coated to groundnut seeds JL 24 as peat-based inoculum with cellulose methyl cellulose (CMC) as adhesive and were evaluated for their growth promotion. The treatments under study were seed coating with CDB 35, seed priming with CDB 35, seed priming with CDB 35+ rhizobium, seed coating with CDA 19, seed priming with CDA 19, seed priming with CDA 19 + rhizobium. The control treatment had seeds with CMC. The viable cell count as determined by dilution plating was 10^6-10^7 colony forming unit (CFU) per seed. The experiment was conducted in random block design with six replications and repeated twice.

3.20 Preparation of enriched compost and its evaluation under glasshouse conditions for plant growth promotion

Vermicompost was prepared under glasshouse conditions using plastic pots (33 cm diameter pots x 29 cm height) by inoculating with bacterial isolate CDB 35 and actinomycete isolate CDA 19. A half-inch layer of gravel was placed at the bottom of the pots followed by 2mm plastic net. A two-inch layer of cow dung was placed above the net. About hundred earthworms (*Eisenia foetida*) of uniform size were released in to the dung and left for 2-3 days for stabilization. On the third or

fourth day, 500g of rice straw soaked for two minutes in 1% dung slurry mixed with 0.1% peat based inoculum of a given bacterial culture (CDA 19 or CDB 35) was placed in the pot. This was repeated once every 15 days up to 90 days. Control pots were without any bacterial culture and each pot was covered with 10-15cm thick layer of dried mango leaves tied in the net to prevent evaporation losses and to keep the surface dark for earthworms. The above set up was for three treatments: a) vermicompost + CDA 19; b) vermicompost + CDB 35; and c) vermicompost (control) and two replications. CDB 35 was enumerated on rock phosphate-buffered antibiotic medium (Hameeda et al 2006) and actinomycetes on actinomycetes isolation agar (AIA) with antifungal Bavistin at 45 and 90 days of composting period.

3.20.1 Glasshouse evaluation of compost amended *Pseudomonas* sp CDB 35 and *Streptomyces* sp CDA 19

The potential isolates of bacteria and actinomycetes were inoculated in the compost and this was tested for plant growth promotion under glasshouse conditions. The treatments being compost + CDB 35, compost + CDA 19, control compost and control. The observation was taken 30 days after inoculation for root and shoot length after washing the roots. The plants were dried in the oven at 100^{o} C for dry weight. The experiment was conducted with six replications and repeated twice.

3.21 Evaluation of various modes of application CDB 35 and CDA 19 under field conditions against *A. flavus*

3.21.1 Seed coating (SC)

Ground seeds were surface sterilized with 3% sodium hypochlorite (Na OCl_2) for five minutes and washed three times with sterilized distilled water. The seeds were treated with peat-based inoculum of bacteria (CDB 35) and actinomycetes (CDA 19) (10^8-10^9 gm^{-1} peat) using 1% carboxy methylcellulose (CMC) as adhesive and dried

in laminar airflow before testing at the field level. The viable cell count as determined by dilution plating was 10^6-10^7 colony forming unit (CFU) per seed.

3.21.2 Seed priming (SP)

Ground nut seeds were soaked in distilled water for eight hours and it was followed by seed coating. The viable cell count as determined by dilution plating was 10^6-10^7 colony forming unit (CFU) per seed. In seed priming along with the organisms of interest (CDB 35 and/or CDA 19) groundnut specific rhizobium IC 59 was used.

3.21.3 Preparation of *A. flavus* inoculum for field application

Sorghum seeds were washed and soaked overnight and were filled in to 500mL conical flasks and sterilized for 20 minutes. After cooling this was inoculated with *A. flavus* 0.1mL/100 gm of seed and this was incubated at 28 ± 2^{o} C for five days in light. This set up was left for five days till rapid sporulation *A. flavus* was observed with frequent mixing by shaking the flasks. This mother inoculums were multiplied with soaked sterilized sorghum seeds and then filled in to plastic bags and kept for three days at room temperature; this was used for inoculation in the field. The viable cell count as determined by dilution plating was 10^5-10^6 colony forming unit (CFU) per gram of seed. *A. flavus* was applied for three times of the cropping period i.e., before sowing, flowering, and before harvesting. About 60 gm of the *A. flavus* infected sorghum seeds were added along the rows of the groundnut plants.

3.21.4 Preparation of composts

A scale-up study to prepare rice straw compost (RSC) in heaps was designed at ICRISAT, unlike the old method of using digesters for compost preparation (Rupela et al. 2003a). Multiple heaps (5 m long x 1.5 m wide x 1.5 m high) of 500 kg

capacity were prepared for composting. Five to 10 kg bundles of air-dried rice-straw were tied up in plastic nets or twine and dipped in water (every 1 kg dry straw soaked in 1.5 L water) for 2 to 3 minutes and then allowed to drain for five minutes. The drippings were captured on a tarpaulin sheet and recycled. Moistened straw was allowed to stay in a heap and covered with polythene sheet to reduce evaporation losses and to prevent it from getting extra moisture from rain during the composting period. Each heap was covered on all sides with at least 10 cm thick layer of moistened rice straw (non-experimental). Care was taken to maintain wetness of contents and water was applied using a specially designed lance. Temperature and relative humidity (RH%) was recorded using Hobo data logger (from Onset Computer Corporation, USA), which helped to decide the time for watering. After maturity of compost, contents of the heap were passed through a pulverizer and used.

Photograph 3.4. Preparation of rice straw compost under field conditions.

Table 3.1. Preparation of rice straw compost under field conditions.

Main material	Rice straw
Additives	Cow dung slurry
Primary fermentation	45-60 days
Maximum temperature	55-60° C

3.21.5 Preparation of enriched rice straw vermicompost in cement tanks under field conditions

Enriched rice straw compost was prepared in cement tanks. Rice straw was soaked in cowdung slurry and after some time for setting was transferred in to the tanks. Earth worms (*Eisenia foetida*) were added to the tanks. Peat based inoculums of bacteria CDB 35 and actinomycete CDA 19 were added to the tanks for every 15 days. Water was sprinkled to maintain the moisture in the tank and the tank was covered with wire mesh, above which dried mango leaves were placed for partial aeration which is required for the aerobic microorganisms. In winters the time period of the entire composting process takes about 50 days to reach the thermophilic stage whereas in summers it takes 40 days.

Photograph 3.5. Preparation of vermicompost in cement tanks.

Photograph: 3.6. Earthworm-enriched vermicompost.

One week before application on to the field peat-based bacteria CDB 35 and

actinomycetes CDA 19 was inoculated and mixed in to the rice straw compost. For 500 kg of compost about 250 g of peat-based inoculums was added and left for settling, whereas control compost did not receive any inoculums. For every three parts of rice straw compost one part of enriched vermicompost with the same organisms was added separately. The population in the compost was tested by dilution plating on to the respective media. In the compost amended field experiments it was applied at the rate of 4.2 kg per plot.

3.21.6 Field inoculation

Seed bacterization was done as mentioned above with peat and was inoculated in the field about 20 seeds per row and 6 rows were maintained per plot and about 200 plants were planted. The viable cell count as determined by dilution plating was 10^6-10^7 colony forming unit (CFU) per seed.

3.21.7 Survival studies of *A. flavus* under field conditions

Soil sample was collected at four stages of the cropping period before application of the *A. flavus* in the field, before sowing, flowering and at harvest. Dilution plating was done on to *A. flavus* and *A. parasciticus* (AfAp) specific medium and was incubated at 28$\pm$ 2^{o}C and the observation recorded.

3.21.8 Survival studies of bacterium CDB 35 under field conditions

Soil was collected after 50 days of planting and was repeated for every 15 days till harvest. This was plated on to rock phosphate buffered antibiotic medium (Hameeda et al. 2007). The colonies which showed pink zones in yellow background were counted for the survival studies of CDB 35.

3.21.9 Preparation of the field

The study was conducted in 2006-2007 growing seasons at ICRISAT, Patancheru. The experiment was designed as a random block design with four treatments and four replications with bacteria CDB 35 and with actinomycetes CDA 19 seed coating. Each plot has six rows with a row space of 30 cm. Each row has 45 seeds with 10 cm space between the seeds. The experiment was conducted with four treatments with four replications as described below.

In the seed application experiments the following treatments were conducted: I. (1) Seed coating with CDB 35; (2) Seed priming with CDB 35; (3) Seed priming with CDB 35+ *Rhizobium*; and control with bacterium CDB 35. II. (1) Seed coating with CDA 19; (2) Seed priming with CDA 19; (3) Seed priming with CDA 19+ *Rhizobium*, and control with actinomycete CDA 19.

In enriched compost amended experiments there were three treatments with CDB 35 and CDA 19 and four replications. The following treatments were conducted: I. (1) Compost + CDB 35; (2) Control compost; and Control with bacterium CDB 35 and II. (1) Compost + CDA 19: (2) Control compost; and (3) Control with actinomycete CDA 19.

Photograph: 3.7 Field evaluation of CDB 35 and CDA 19

3.21.10 Aflatoxin analysis: indirect competitive ELISA

3.21.10.1 Substrate buffer

Substrate buffer for alkaline phosphate system: p- nitrophenyl phosphate (p-npp) was stored at -20^{o} C. Chemical in tablet form was used (5, 15 or 40 mg tablets are available). Ten percent diethanol amine (v/v) was prepared in distilled water, pH was adjusted to 9.8 prior to use, 0.5 mg/ml p-nitro phenyl phosphate (*p*-npp) was prepared in 10% diethanol amine, pH 9.8 (for each 15 mg tablet 30 ml solution was required), p-npp solution was not allowed to turn yellow. This could sometimes happen because of alkaline phosphatase (ALP) contamination from skin.

3.21.10.2 Methodology

Preparation of groundnut seed extracts: The seed was ground into powder using a blender. The seed powder was titrated in 70% methanol (v/v- 70 ml absolute methanol in 30 ml distilled water) containing 0.5% KCL (proportion used in 100 ml for 20g seed) in a blender, until the seed powder was thoroughly ground. The extract was transferred to a conical flask and shaken for 30 min at 300 rpm. The extract was filtered through Whatman No. 41 filter paper and diluted 1:10 in PBS-Twen (1ml extract and 9ml of buffer). To estimate lower levels of AfB1 (<10 μg/kg), prior to ELISA a simple liquid-liquid clean up and concentration (5:1) procedure was adopted. Twenty ml of methanol extract, 10ml of distilled water and 20 ml chloroform were mixed in a separating funnel and used for clean up. After vigorous shaking for one minute, the lower chloroform layer was collected and evaporated to near dryness in water bath at 60^{o} C. To the residue 4ml of PBS – Tween containing 7% methanol was added and used for analysis by ELISA.

AfB1-BSA conjugate was prepared in carbonate coating buffer at 100 ng/ml concentrations and 150 µl of the diluted AfB1 – BSA is dispensed to each well of ELISA plate. The plate was incubated in a refrigerator overnight or at 37º C for at least one-and-a-half hours. The plates were washed in three changes of PBS – Tween, allowing 3 min gap in between for each wash (so as to inhibit non-specific binding of antibodies and thus false positive reaction). BSA (0.2%) prepared in PBS Tween was added at 150 µl per each well of ELISA plate and incubated at 37º C for an hour. The plates were washed in three changes of PBS-Tween, with 3 min between each wash.

3.21.10.3 Preparation of aflatoxin B_1 standards

Healthy groundnut seed extract was prepared as mentioned above. Aflatoxin B1 standards (using 1:10 healthy groundnut seed extract) were diluted at concentrations ranging from 100 ng to 10 picogram in 100 µl volume. Addition of polyclonal antisera raised each polyclonal antiserum; this was predetermined (at ICRISAT) in PBS-Tween against aflatoxin B1-BSA conjugate. A 1: 80,000 dilution of antiserum (for containing 0.2% BSA) was prepared. Fifty µl of antiserum was added to each dilution of aflatoxin standards (100 µl) and groundnut seed extract (100µl) intended for analysis. The plates were incubated for 30 min at room temperature and the mixture containing aflatoxin samples (100 µl) and antiserum (50 µl) to facilitate reaction between the toxins present in the sample with antibody. The entire process was done in ELISA plate and there was no need to pre-incubate the toxin and antibody mixture in separate tubes, and the plate was incubated for 1h at 37º C. The plate was washed in three changes of PBS-Tween allowing 3 min for each wash. Goat anti- rabbit Ig G (1: 1000) was prepared and labeled with alkaline phosphatase, in PBS-Tween containing 0.2% BSA. 150 µl was added to each well and incubated for 1h at 37º C. The plate was washed in three changes of PBS-Tween

allowing for 3 min for each wash. Substrate solution (p- nitro phenyl phosphate prepared in 10% diethanol amine buffer, pH 9.8) was added and incubated for 1 hour at room temperature. After satisfactory development of yellow colour in each ELISA plate (colour development takes place in 40 min to 1 hour) absorbance is measured at 405 nm in an ELISA reader. Using the values obtained for aflatoxin B_1 standards a curve was drawn

$$\text{AfB1 (µg/Kg)} \quad = \frac{AXDXE}{G} \text{ or } \frac{AXE}{CXG}$$

wherein A = AfB_1 concentration in diluted or concentrated sample extract, D = time dilution with buffer, C = times concentration after clean up, E = extraction solvent after clean up, G = Sample weight (gm).

3.22 Morphological and physiological studies of CDB 35 and CDA 19

Various morphological characters, such as size, shape, texture, colour, and grams stain were studied.

3.23 Utilization of various carbon and nitrogen sources by *Pseudomonas* sp CDB 35 and *Streptomyces* sp CDA 19

The Biolog system consists of a microplate containing 95 different carbon sources and a control well, a turbidimeter, and a computer-driven automatic plate reader. CDB 35 and CDA 19 cultures were sub-cultured twice and the inoculums to be used for testing were prepared from the second sub-culture. The inoculum was prepared by rolling a cotton swab over the agar plate and preparing a suspension in 18 to 20 ml of 0.85% saline to establish the appropriate inoculum density relative to that of the milk of magnesia standard specified by the manufacturer (roughly equivalent to a range of 53 to 58% transmittance or 4 x 10^{8} to 6 x 10^{8} organisms per ml). The resulting suspension was poured into a multichannel pipette reservoir (Costar Corp.,

Cambridge, Mass.). By using an eight-channel repeating pipette fitted with 1,500-,µL-capacity sterile tips (Flow Laboratories, McLean, Va.), precisely 150 µL of the suspension was dispensed into each well of the microplate. The lid was replaced, and the microplate was incubated at 28±2°C for 4 h . After this initial incubation period, the plate was placed in the reader and read automatically. The plates were then returned to the incubator and the readings were taken for every 12 h for 48 h.

3.24 Characterization of CDB 35 and CDA 19 by 16S rRNA gene sequencing

3.24.1 Preparation of template DNA

Pure cultivated colonies of bacterial isolate CDB 35 and actinomycete isolate CDA 19 were picked up with a sterilized toothpick and suspended in 0.5 mL eppendorf tube and was centrifuged at 10,000 rpm for 10 min. Supernatant was discarded and the pellet was suspended in 0.5 mL of Insta Gene Matrix (Bio-Rad, USA). Pellet was then incubated at 56° C for 30 min and then heated at 100° C for 10 min. After heating the supernatant obtained was used for carrying out PCR.

3.24.2 PCR

To the PCR reaction solution 1µL of template DNA was added. 27F/1492 R (27 F AGAGTTTGATCMTGGCTCAG; 1492R TACGGYTACCTTGTTACGACTT) primers were used for the bacteria to perform 35 amplification cycles at 94° C for 45 sec, 55° C for 60 sec, and 72° C for 60 sec. DNA fragments were amplified about 1500 bp. A positive control (*E. Coli* genomic DNA) and negative control were included in the PCR.

3.24.3 Purification of PCR products

Unincorporated PCR primers and dNTPs from PCR products were removed using Montage PCR clean up kit (Millipore). The purified PCR product was used further for sequencing.

3.24.4 Sequencing

The purified PCR products of approximately 1500 bp were sequenced by using 2 primers 518F/800R (518FCCagCAgCCgCggTAATACg: 800RTACCAgggTATATAATCC). Sequencing was performed by using Big dye terminator cycle sequencing kit (Applied Biosystems, USA). Sequenced products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Biosystems, USA). The sequence obtained was further analyzed using eZ-Taxon server to find out closely related bacterial species. 16S rRNA gene sequences of closely related bacterial species exhibiting more than 96% similarity were taken and analyzed using Mega 4.1 software. Distance matrix was calculated on the basis of Jukes and Cantor (1969) and topology was inferred using the neighbour-joining (nj) method based on boot strap analysis of 1000 trees.

3.24.5 Molecular characterization of bacterial strain CDB 35and CDA 19 by 16 s RNA gene sequence

16 s rRNA gene sequence is the most conserved sequence in bacteria and hence 16 S rRNA gene sequence analysis was considered as the most reliable method for molecular identification of bacteria at the species level. 16s rRNA gene was amplified from DNA of CDB 35. For this process 27 F (AGAGTTTGATCMTGGTCAG) and 1492R (TACGGYTACCTTACCTTGTTACGACTT) primers were used to amplify the gene about 1500 bp. The resultant PCR products were run on agarose gel electrophoresis

along with a positive control (*E. Coli* genomic DNA). PCR products were purified using MONTAGE PCR clean up kit (Millipore) and were used for sequencing. A sequence of 1493 base pairs was obtained and further it was analysed using eZ- Taxon server to find out the closely related bacteria.

3.25 Statistical analysis

All the experiments were carried out with six replications and repeated twice. Glasshouse and field experiments were arranged in a completely randomized block design and the data was subjected to Genstat Statistical Package 10.

CHAPTER - 4

RESULTS

RESULTS

Plant beneficial microorganisms directly promote plant growth by solubilising the nutrients in the soil, or indirectly by being antagonistic to the various plant pathogenic fungi. Antagonistic microbes inhibit the phytopathogens, colonize the root and promote the plant growth in soil conditions and are known as biocontrol agents. Based on this, we have isolated microorganisms (80 bacteria, 40 actinomycetes, and 40 fungi) from rice straw compost (RSW) and maintained them as germplasm collection at ICRISAT for future use. All these bacterial and actinomycete isolates were screened for their antagonistic ability against *A. flavus.* Out of them the most potential isolates – *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 – were screened against other soil borne pathogenic fungi *Aspergillus niger Fusarium udum, Fusarium oxysporum, Sclerotium rolfsii, and Macrophomina phaseolina* to characterize their broad spectrum anti-fungal activity. Both these potential anti-fungal isolates reduced the fungal biomass under broth culture conditions. The effect of volatile metabolites produced by all the eight potential bacteria and four actinomycetes for inhibition of *A. flavus* was studied. Production of secondary metabolites/enzymes by *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 that may be responsible for antagonism were characterized and quantified so as to know the mode of action of these biocontrol agents. Both the biocontrol agents were evaluated for their plant growth promoting traits *in vitro* and production of IAA was quantified. Glasshouse evaluation of *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 for plant growth promotion of groundnut was evaluated. Field evaluation of *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 against *A. flavus* infection and aflatoxin production was carried out for three seasons (Kharif 2006, Rabi 2006-2007, and Kharif 2007). Utilization of various carbon and nitrogen sources by

Pseudomonas sp. CDB 35 and *Streptomyces* sp. CDA 19 was monitored for 96 hours of incubation. Molecular identification revealed them at their species level as *Pseudomonas aeruginosa* CDB 35 and *Streptomyces cavourensis* CDA 19. The results of these studies are presented in this chapter.

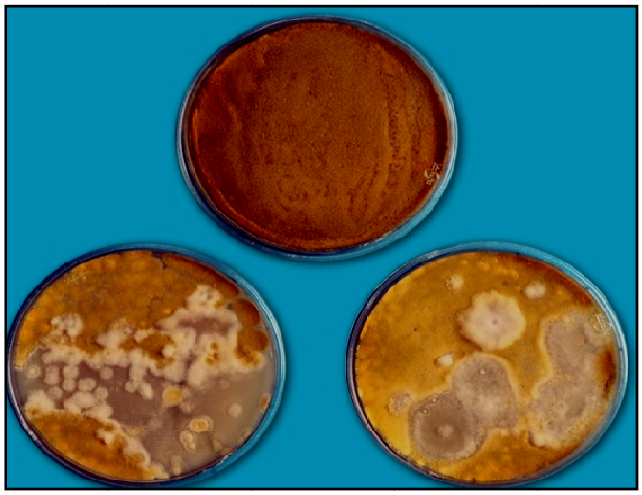
4.1 Isolation of bacteria, fungi and actinomycetes

A total of 80 bacteria, 40 actinomycetes, and 40 fungi were isolated from rice straw compost based on variation in colony morphology. Bacteria were designated as compost degrading bacterium (CDB), fungi as compost degrading fungi (CDF), and actinomycetes as compost degrading actinomycete (CDA). Single colonies of bacterial isolates were sub-cultured and stored as glycerol stocks, and actinomycete isolates were preserved on Bennetts agar and fungi on potato dextrose agar (PDA) slopes, and all of them were stored at -70º C for future use.

4.2 Antifungal activity of the bacterial and actinomycete isolates

4.2.1 Double layer method

Both bacteria and actinomycete isolates were evaluated against *A. flavus* by double layer method on a glucose casaminoacid (GCY) medium. Eight out of 80 bacteria and three out of 40 actinomycete isolates showed maximum inhibition of *A. flavus* under plate culture conditions by double layer method. Based on the inhibition zone shown by CDB 35 (50mm) and CDA 19 (35mm) these two were selected for further studies (Table 4.1, and Photograph 4.1).



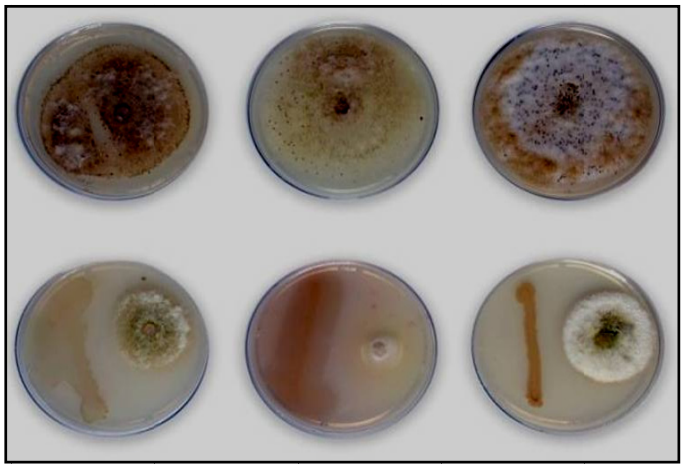
Photograph: 4.1 Screening of bacteria and actinomyetes against *A. flavus* by double layer method.

Table: 4.1 Screening potential bacteria and actinomycetes isolated from rice straw compost by double layer method against *A. flavus*

Isolates	Zone of inhibition (mm)
CDB 15	10
CDB 16	14
CDB 30	15
CDB 31	13
CDB 35	**50**
CDB 41	15
CDB 47	18
CDB 58	16
CDA 16	10
CDA 19	**35**
CDA 26	15

4.2.2 Screening of potential bacteria against *A. flavus* by dual culture method

Antagonism by potential bacteria and actinomycetes against *A. flavus* was evaluated by dual culture method to know the percent inhibition. The bacterial isolate CDB 35 showed significant reduction of *A. flavus* radial growth on Kings B (KB) (89 %) followed by GCY (70%) and potato dextrose agar PDA (67%) (Table 4.2 and Photograph 4.2).



Photograph: 4.2 Screening of CDB 35 against *A. flavus* by dual culture method on (a) GCY, (b) KB and (c) PDA.

Table 4.2: Antagonistic activity of potential bacterial isolates against *A. flavus* on GCY , KB and (PDA) (values are percent inhibition of test fungus)

Isolates	Percent inhibition of fungus on		
	GCY	KB	PDA
CDB 15	56	61	57
CDB 16	50	63	68
CDB 30	48	44	63
CDB 31	54	61	56
CDB 35	**70**	**89**	**67**
CDB 41	58	65	46
CDB 47	44	75	39
CDB 58	44	67	56
LSD(P=0.05)		**7.9**	
CV%		**13.2**	

4.2.2.1 Mean fungal inhibition of A. *flavus* by potential bacteria

Mean fungal inhibition of *A. flavus* by eight bacterial isolates was more on KB (63%) than PDA (52%) and GCY (48%). KB is a nitrogen rich medium and may have enhanced the antagonistic activity of the bacteria against *A. flavus* (Fig. 4.1).

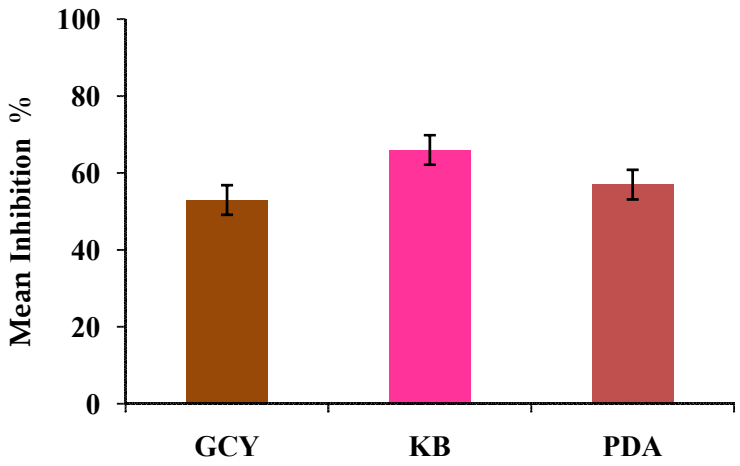
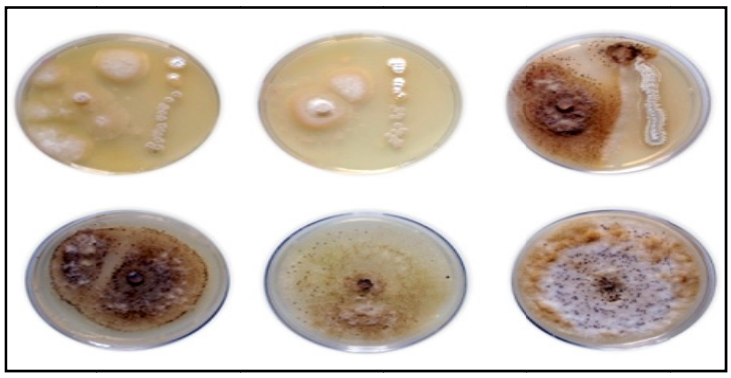


Fig: 4.1 Mean percent inhibition of *A. flavus* by eight bacterial isolates on GCY, KB and PDA media

4.2.3 Screening of potential actinomycetes against *A. flavus* by dual culture method

Among three actinomycetes *Streptomyces* sp. CDA 19 showed significant reduction of *A. flavus* on PDA (62%), followed by GCY (48%) and KB (40%) (Table 4.3, Photograph 4.3).



Photograph : 4.3 Screening of CDA 19 against *A. flavus* by dual culture method on GCY (a), KB and PDA.

Table 4.3. Screening of potential actinomycete isolates against *A. flavus* by dual culture method.

Isolates	(Percent inhibition of fungus on)		
	GCY	KB	PDA
CDA 16	39	27	37
CDA 19	**48**	**40**	**62**
CDA 26	30	19	35
LSD (P=0.05)		**7.2**	
CV%		**16.5**	

4.2.3.1 Mean fungal inhibition of *A. flavus* by potential actinomycete isolates

The potential actinomycete isolates inhibited *A. flavus* on the three different media tested but maximum reduction was observed on PDA (45%), followed by GCY (39%) and KB (29%). This revealed that actinomycetes could use the minimum nutrient sources of PDA medium and bring about inhibition of the fungus (Fig 4. 2).

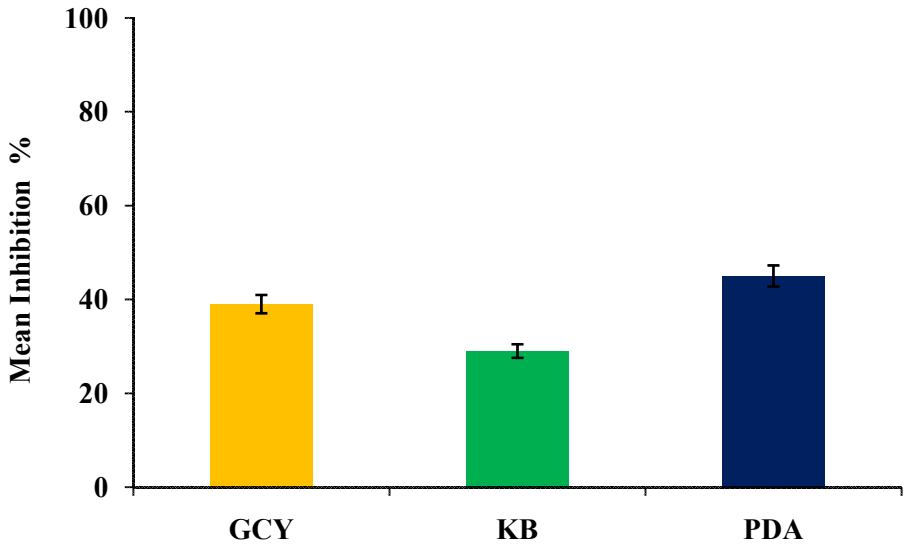
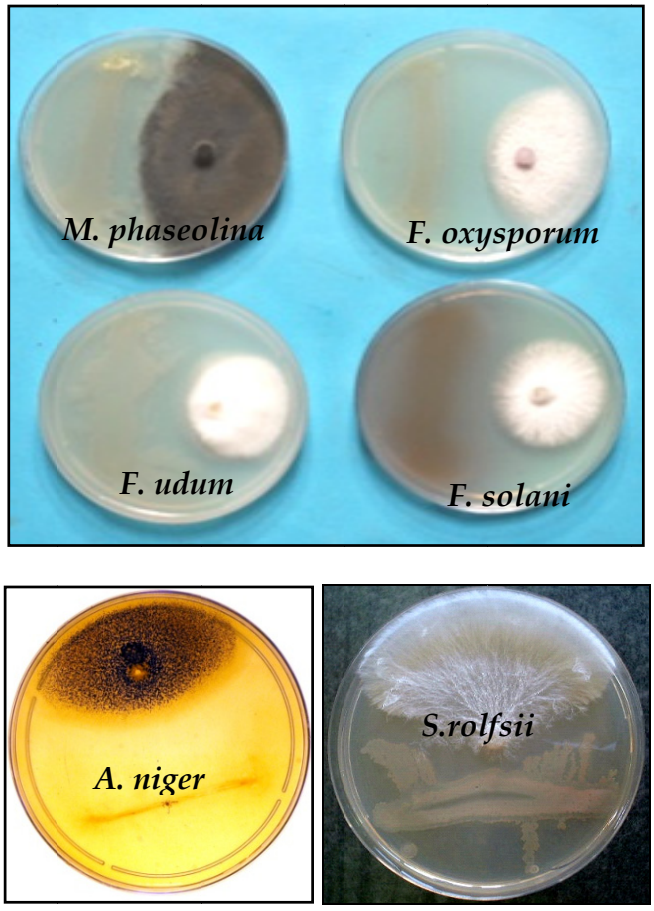


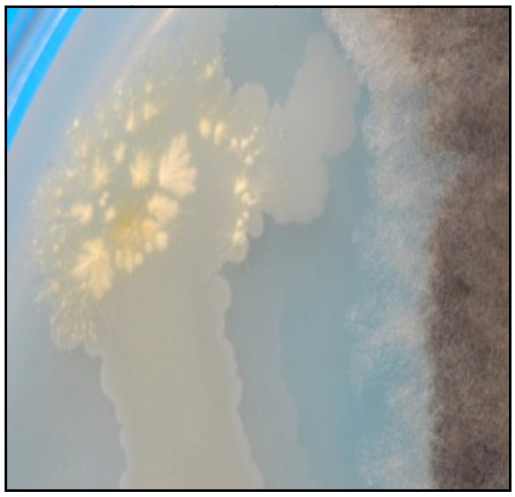
Fig 4.2: Mean percent inhibition of *A. flavus* by potential actinomycetes on GCY, KB and PDA media.

4.3 Evaluation of CDB 35 against various soil borne plant pathogenic fungi by dual culture method

Out of eight bacterial isolates, bacterium CDB 35 was selected based on its effective antagonism towards *A. flavus* on the three media tested. It was further screened against other soil borne plant pathogenic fungi to know its broad spectrum antifungal activity. Among the six fungi (*Aspergillus niger*, *Fusarium oxysporum*, *Fusarium udum*, *Fusarium solani*, *Macrophomina phaseolina*, *Sclerotium rolfsii*) tested, maximum inhibition of *Fusarium oxysporum* was observed which was more on KB (87%), followed by PDA (71%), GCY (70%). All other fungi were inhibited more or less similarly on the three media studied by the bacterium *Pseudomonas* sp. CDB 35 (Table 4.4, Photograph 4.4).



Photograph: 4.4 Broad spectrum antifungal activity of *Pseudomonas* sp. CDB 35 against several plant pathogenic fungi



Photograph: 4.4.1 Antibiotic spikes production by CDB 35 (antibiotic chlororaphin like) when screened against *Macrophomina phaseolina*

Table: 4.4 Screening of CDB 35 against various soil borne fungal plant pathogens.

	(Percent inhibition of fungus)		
Plant pathogenic fungi	**GCY**	**KB**	**PDA**
Aspergillus niger	60	60	59
Fusarium oxysporum	**70**	**87**	**71**
Fusarium solani	65	79	66
Fusarium udum	60	73	62
Macrophomina phaseolina	62	65	60
Sclerotium rolfsii	61	84	54
LSD (P=0.05)		**0.04**	
CV%		**15%**	

4.3.1 Mean fungal inhibition of five fungi by *Pseudomonas* sp. CDB 35

Mean fungal inhibition of five fungi by *Pseudomonas* sp. CDB 35 was more on KB (64%), followed by GCY (57%) and PDA (56%) (Fig. 4.3).

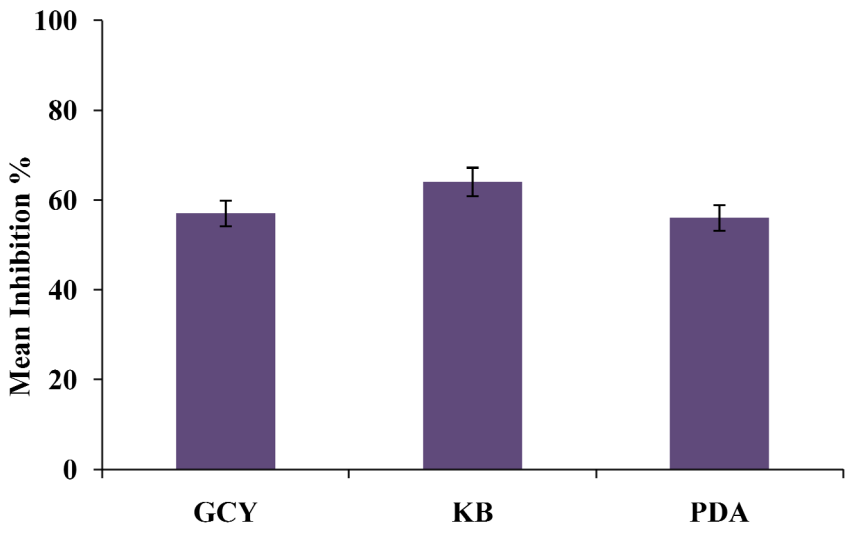
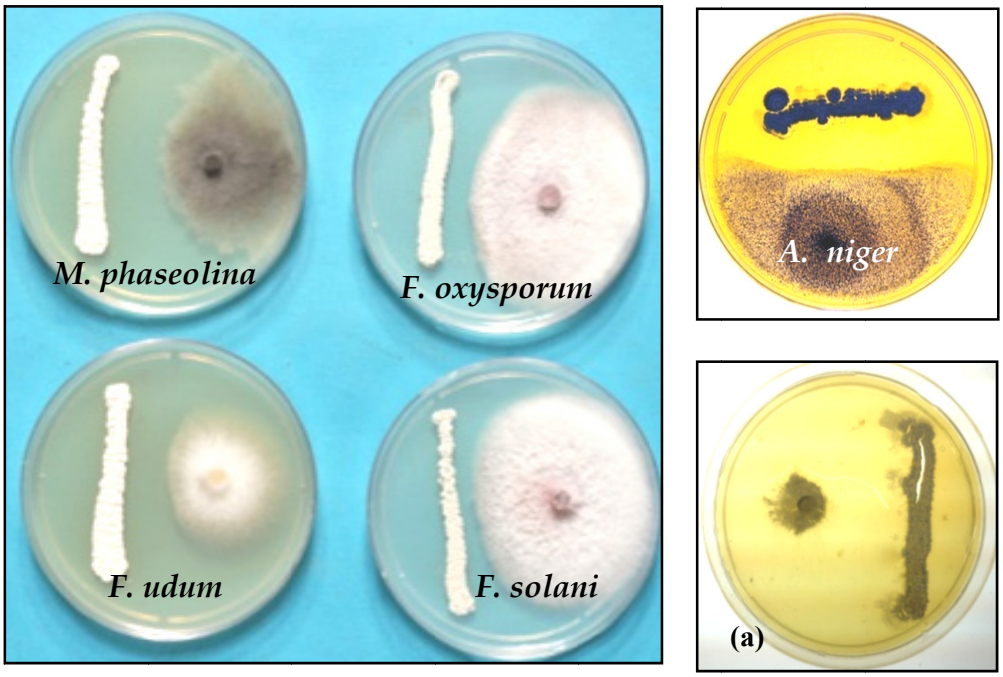


Fig: 4.3 Mean percent inhibition of the six pathogenic fungi by CDB 35 on three different media

4.4 Screening of CDA 19 against various soil borne plant pathogenic fungi by dual culture method

Out of three potential actinomycete isolates CDA 19 was selected based on its significant inhibition of *A. flavus* after testing on three media. It was screened against five soil borne pathogenic fungi – *Aspergillus niger*, *Fusarium solani*, *Fusarium oxysporum*, *Fusarium udum*, *Macrophomina phaseolina*, and *Sclerotium rolfsii* – to know its broad spectrum anti-fungal activity. Of the six fungi tested, maximum inhibition of *Aspergillus niger* was observed on GCY (60%) and PDA (60%), followed by KB (59%). Rest of the fungi were inhibited more or less to a similar extent (Table 4.5, Photograph 4.5), *Streptomyces* sp. CDA 19 did not inhibit *Sclerotium rolfsii*.



Photograph: 4.5 Broad spectrum antifungal activity of CDA 19 against several plant pathogenic fungi. (a) Fungicidal nature of CDA 19

Table: 4.5 Antagonistic activity of CDA 19 on various soil borne plant pathogens

		(Percent inhibition of fungus)	
Plant pathogenic fungi	**GCY**	**KB**	**PDA**
Aspergillus niger	**60**	**59**	**60**
Macrophomina phaseolina	46	49	48
Fusarium solani	46	66	44
Fusarium oxysporum	37	58	34
Fusarium udum	37	54	33
LSD (P=0.05)		**5.1**	
CV%		**9.2**	

4.4.1 Mean fungal inhibition of the four fungi by CDA 19

Mean fungal inhibition of four fungi by three actinomycete isolates was maximum on KB (57%), followed by GCY (45%) and PDA (44%) (Fig. 4.4).

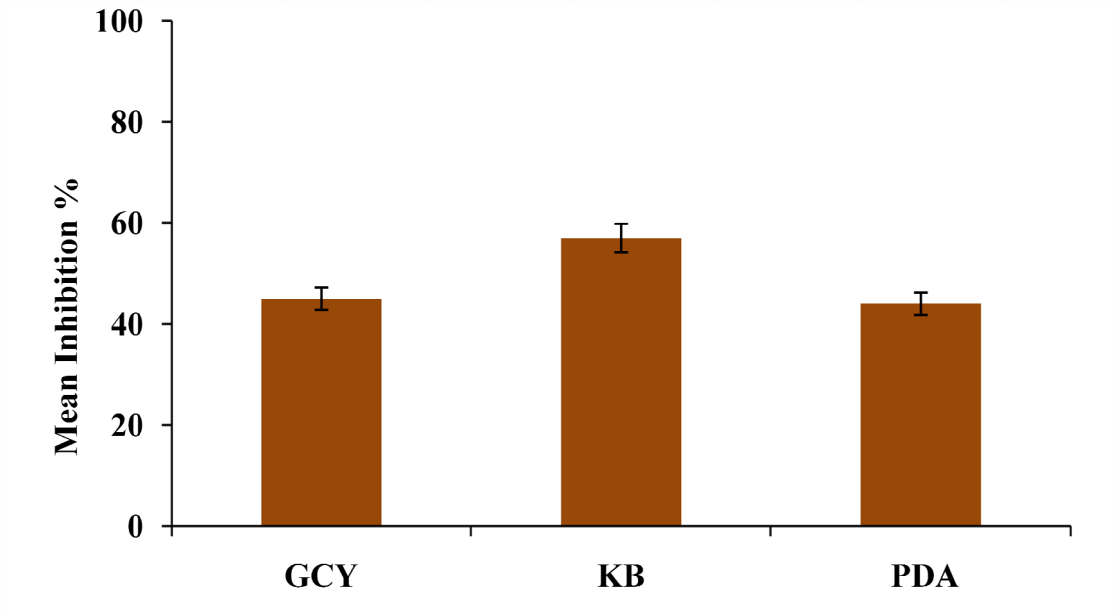
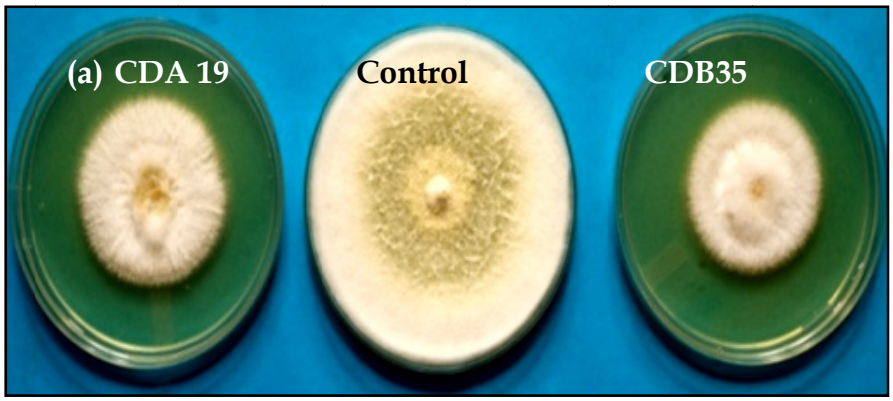
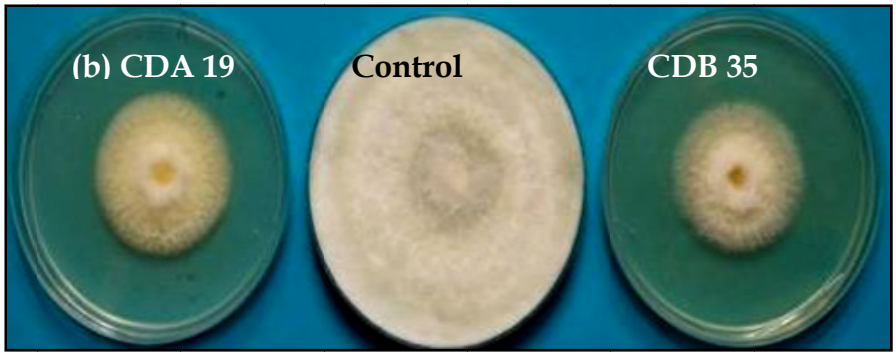


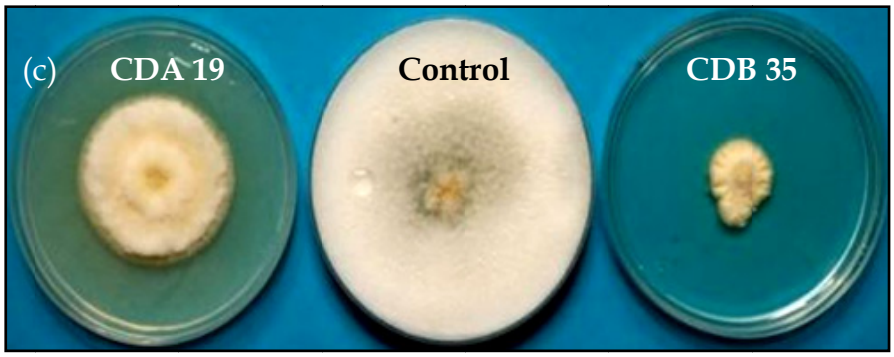
Fig: 4.4 Mean percent inhibition of the five different fungi by CDA 19 on three different media

4.5 Effect of volatile antibiotics of potential bacteria and actinomycetes on *A. flavus* radial growth

Volatile antibiotics released by the potential bacteria and actinomycetes inhibited the fungal radial growth. Out of eight bacteria CDB 35 showed maximum radial growth inhibition on KB (67%), followed by PDA (62%) and GCY (60%) and among the three actinomycetes CDA 19 inhibited *A. flavus* radial growth ranging between 52-54%. (Photograph 4.6, Table 4.6).







Photograph: 4.6 Screening for volatile antibiotics of potential bacteria and actinomycetes on (a) GCY, (b) KB, (c) PDA.

Table: 4.6 Effect of volatile antibiotics of potential bacteria and actinomycetes on *A. flavus* radial growth.

Isolates	(Percent inhibition on)		
	GCY	KB	PDA
CDB 15	33	63	46
CDB16	38	61	28
CDB 30	40	65	36
CDB 31	44	65	35
CDB 35	**60**	**67**	**62**
CDB 41	40	56	40
CDB 47	31	58	33
CDB 58	35	60	35
CDA 16	46	40	45
CDA 19	**53**	**54**	**52**
CDA 26	35	50	52
LSD (P=0.05)		0.04	
CV%		15.1%	

4.6 Effect of cell-free culture filtrate (CCF) on *A. flavus* radial growth inhibition

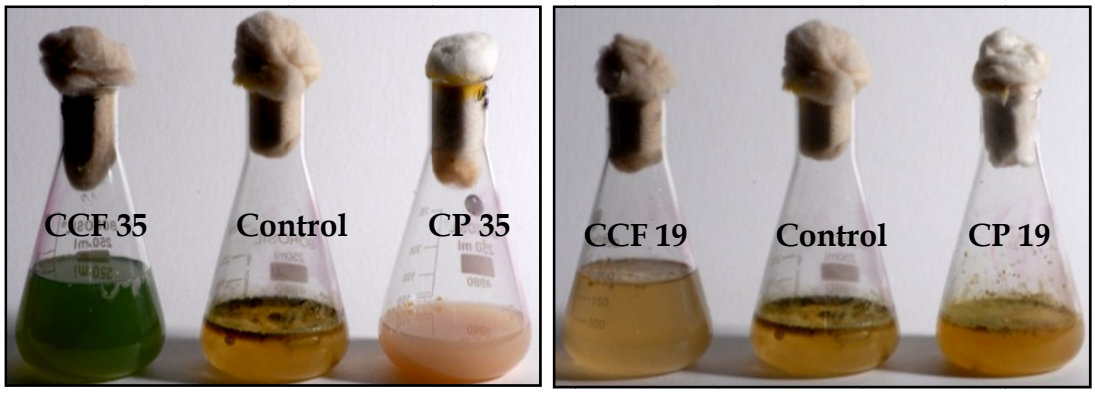
The anti-fungal activity of cell-free culture filtrates (CCF) of bacterium CDB 35 and actinomycetes CDA 19 was determined by testing their effect on radial growth of *A. flavus* by the poisoned food technique. The CCF was added to PDA at various concentrations from 10% - 50% (v/v) of the medium. Both bacterium CDB 35 and actinomycete CDA 19 inhibited *A. flavus* radial growth to the maximum at 50% (v/v) of CCF which was 93% and 82% respectively. Mycelial discs of *A. flavus,* which failed to grow in the presence of CCF of CDB 35 and CDA 19 when transferred to fresh PDA plates, failed to grow even after four days of incubation suggesting the fungicidal nature of CCF (Table 4.7).

Table: 4.7 Effect of cell culture filtrates (CCF) on *A. flavus* radial growth

	Radial growth inhibition of *A. flavus* (cm) in presence of	
CCF (%)	**CDB 35**	**CDA 19**
10%	1.1	2.7
20%	0.9	2.6
30%	0.8	2.6
40%	0.7	2
50%	0.5	1.5
0%	**8.3**	**8.3**
LSD	**0.2**	**0.4**
CV%	**23.5**	**16.2**

4.7 Inhibition of fungal biomass by cell-free culture filtrate (CCF) and cell pellet (CP) of *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19

CCF and the CP of the potential bacterial isolate CDB 35 and actinomycete isolate CDA 19 were tested for *A. flavus* biomass inhibition under broth culture conditions. Since maximum radial growth inhibition was observed at 50% (v/v) concentration of CCF, this concentration was used throughout the experiment. Both the organisms reduced the dry weight of the fungus over control (Table 4.8). Percent *A. flavus* biomass reduction over control by CCF and CP of CDB 35 was 67% and of CDA 19 was 39% and 22% respectively (Fig. 4.5). Inhibition of fungal growth can be clearly observed in Photograph 4.7.



Photograph: 4.7 Effect of CDB 35 and CDA 19 CP and their CCF's on *A. flavus* growth under broth culture conditions.

Table 4.8: Effect of cell culture filtrates and cells of CDA 19 and CDB 35 on biomass of seven day grown *A. flavus* under broth culture conditions.

CCF and CP	Fungal biomass (gm)
CP 19	0.24
CCF 19	0.19
CP 35	0.10
CCF 35	0.08
Control	0.36
LSD(P=0.05)	**0.05**
CV%	**20**

CP= cell pellet, CCF= cell free culture filtrate

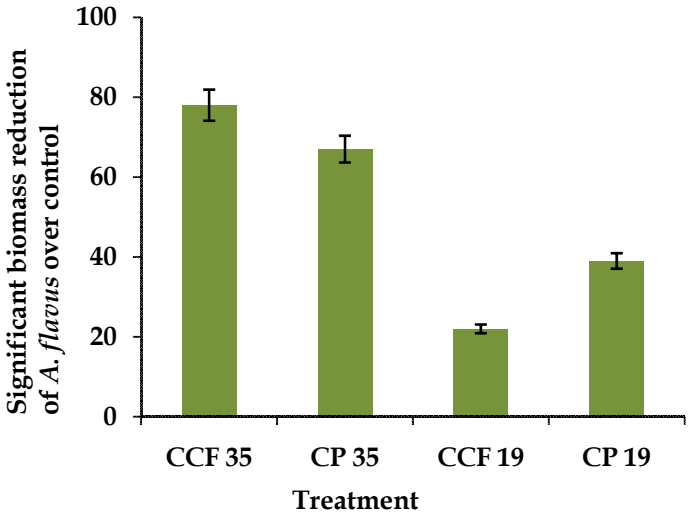
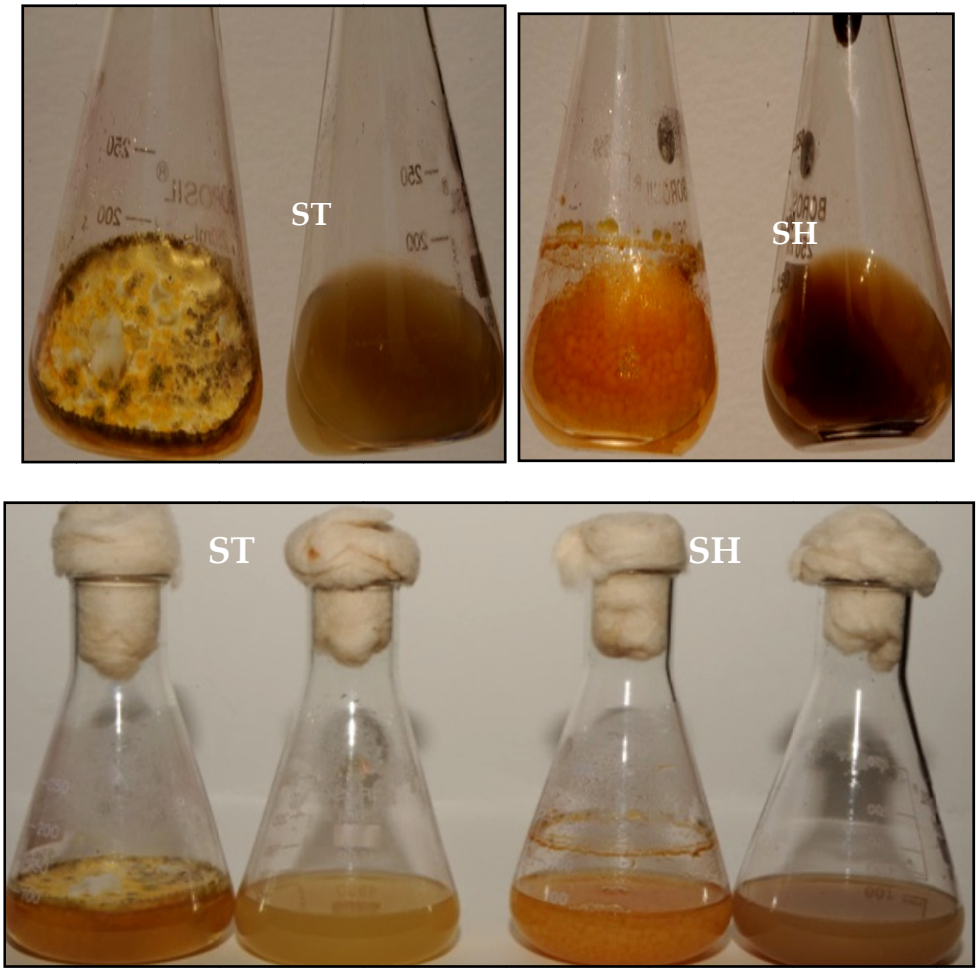


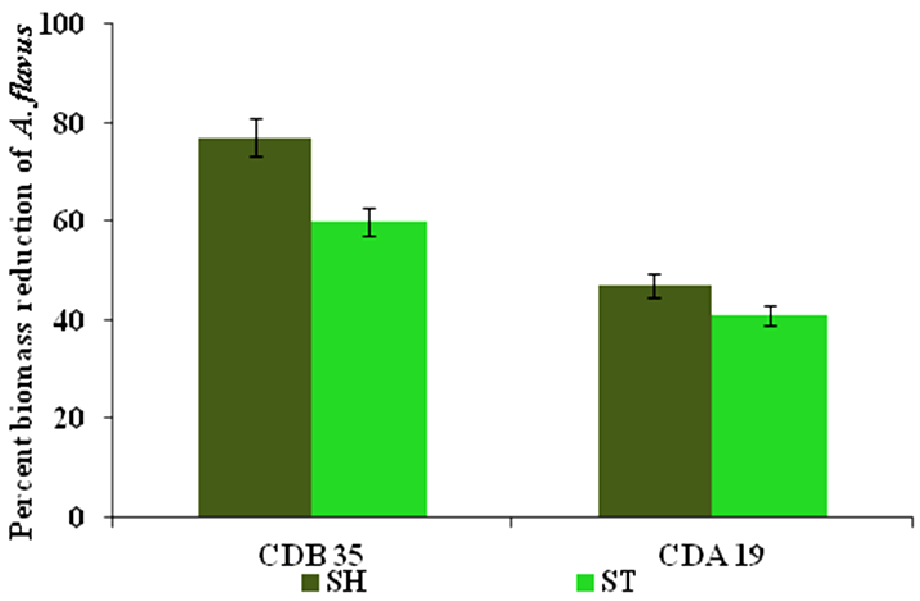
Fig: 4.5 Percent biomass reduction of *A. flavus* in the presence of CCF and CP of CDB 35 and CDA 19 under broth culture conditions.

4.7.1 Effect of aeration on *A. flavus* biomass reduction by potential anti-fungal isolates

Effect of aeration on biomass reduction of *A. flavus* by anti-fungal isolates bacterium CDB 35 and actinomycete CDA 19 was tested by incubating in a rotary shaker for five days. With CDB 35 at stationary culture conditions the reduction was more (77%) compared to the shaking culture conditions (60%). On the other hand, with CDA 19 reduction of the biomass was more under shaking culture conditions (47%) to that of stationary culture conditions (41%) (Photograph 4.8, Fig. 4.6).



Photograph: 4.8 Effect of CDB 35 on *A. flavus* growth under stationary and shaking culture conditions (a) Stationary conditions (b) Shaking culture conditions

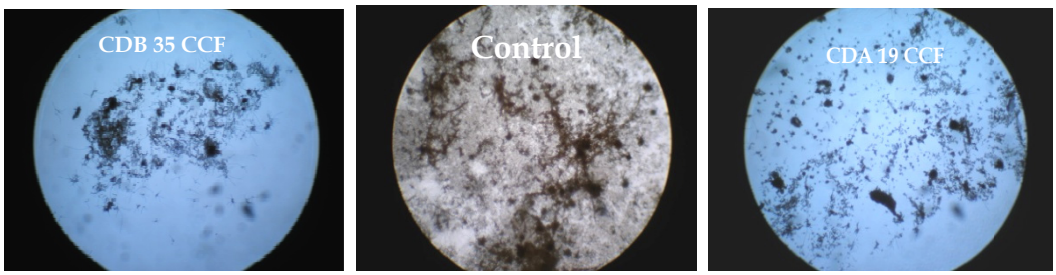


ST=Stationary culture, SH=Shaking culture

Fig: 4. 6 Percent biomass reduction of *A. flavus* by CDB 35 and CDA 19 under stationary and shaking culture conditions.

4.8 Spore germination inhibition by CCF and CP of bacterium CDB 35 and actinomycete CDA 19

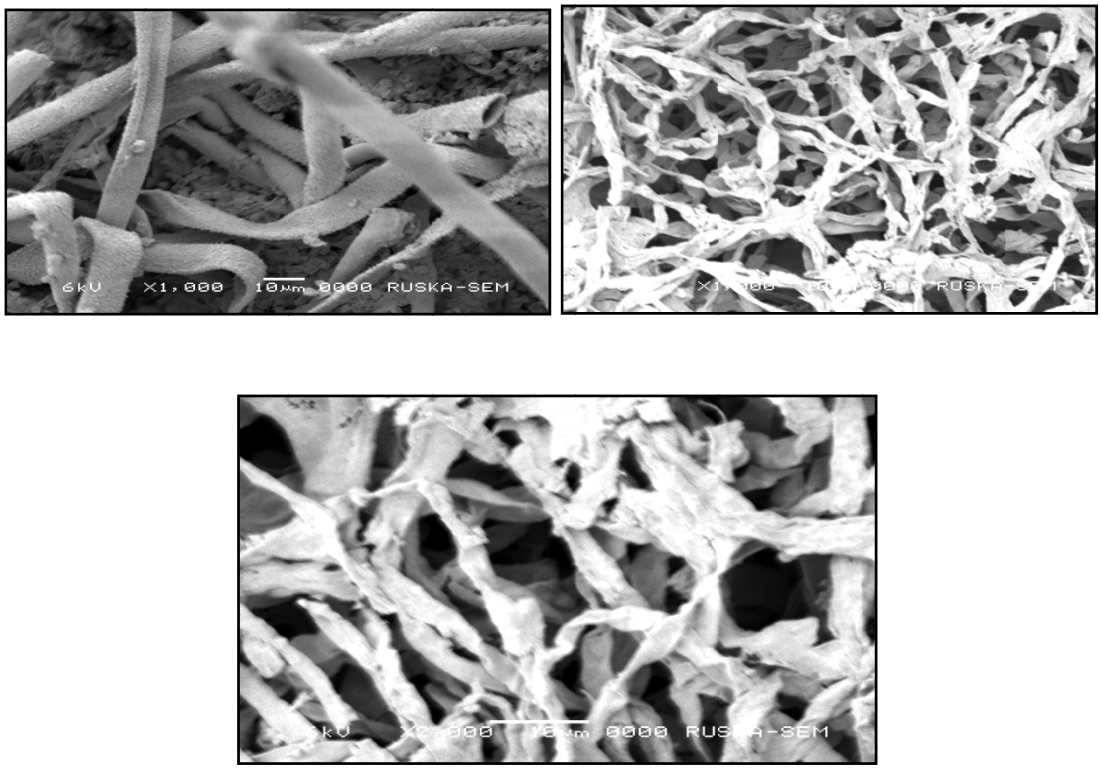
Spore germination inhibition was observed under compound microscope (100X) when inoculated with CP and CCF of CDB 35 and CDA 19, respectively. Considerable reduction of spore germination over control was observed with both the isolates under study. The CCF of CDB 35 could inhibit spore germination compared to CP. In comparison with both the isolates, CDB 35 showed more inhibition than CDA 19 (Photograph 4.9).



Photograph: 4.9 Inhibition of spore germination by CDB 35 and CDA 19 cells and their filtrates under light microscope at 100X.

4.9 Mycelial morphological changes in the presence of CCF of CDB 35 and CDA 19

When inoculated with CCF of CDB 35 and CDA 19 there was maximum reduction of spore formation by *A. flavus*. There were morphological changes observed under electron microscopy, such as mycelia shrinkage and cell deformation when compared to the control (Photograph 4.10)

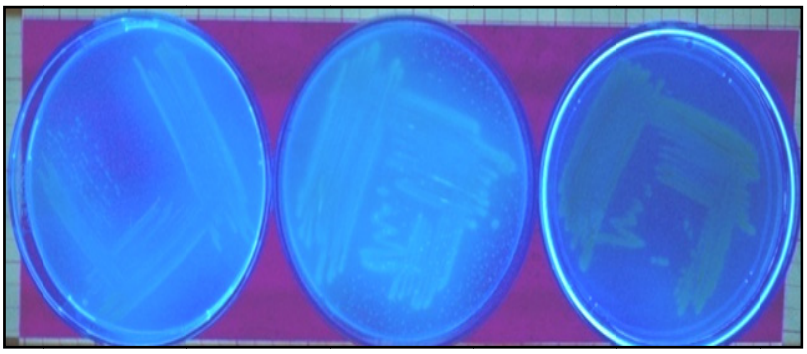


Photograph 4.10: Effect of CCF of CDB 35 (a) and CDA 19 (b) on *A. flavus* mycelium

4.10 Screening potential bacterial and actinomycete isolates for antagonistic traits

The selected bacterial and actinomycete isolates used in the study were screened in terms of their ability for *in vitro* production of extracellular metabolites or enzymes responsible for antagonism, viz HCN, siderophores, chitinases, glucanases, lipases, cellulases, proteases, acid production, and pigment production. Among eight bacteria selected, CDB 15, CDB 16, CDB 22, CDB 30, CDB 31 and CDB 35

produced proteases. CDB 36 produced HCN and siderophores. *Pseudomonas* sp CDB 35 produced all the metabolites mentioned except chitinase. Among actinomycetes CDA 16 and CDA 26 produced siderophores, lipases and proteases. *Streptomyces* sp. CDA 19 produced all the metabolites mentioned except HCN and cellulases (Table 4.9). Fluorescence pigment production was observed for CDB 35 (Photograph 4.11) and a brown pigment was produced by CDA 19 (Photograph 4.12a). Acid production was noticed for CDB 35 (Photograph 4.12b).



Photograph: 4.11 Fluorescence shown by *Pseudomonas* sp. CDB 35 (a) *Pseudomonas* isolation agar (PIA) (b) Kings B agar (c) PDA.

Photograph: 4.12 Pigment production by CDA 19 on Bennets agar and acid production by CDB 35 on rock phosphate buffered media.

Table 4.9: Antagonistic traits of the selected bacteria and actinomycetes

Isolates	HCN	Siderophores	Chitinases	Glucanases	Lipases	Cellulases	Proteases	Acid production	Pigment production
CDB 15	-	-	-	-	-	-	+	-	-
CDB16	-	-	-	-	-	-	+	-	-
CDB 30	-	-	-	-	-	-	+	-	-
CDB 31	-	-	-	-	-	-	+	-	-
CDB 35	+++	+++	-	+++	++	+	++++	+++	++
CDB 41	-	-	-	-	-	-	+	-	-
CDB 47	-	-	-	-	-	-	-	-	-
CDB 58	-	-	-	-	-	-	-	-	-
CDA 16	-	++	-	-	+	-	+++	-	-
CDA 19	-	+++	++++	++++	++	-	+++	++	+
CDA 26	-	++	-	-	+	-	+++	-	-

+ = Slight production, ++ = medium production, +++ = good production, +++ = very good production, - = no production

4.11 Siderophore production

Pseudomonas sp. CDB 35 and *Streptomyces* sp. CDA 19 produced siderophores on chrome azurol succinate (CAS) medium under plate culture conditions. Quantification of siderophores in terms of decolouration was measured in iron-free succinate broth by CAS assay. After six days, the percent decolouration by *Pseudomonas* sp. CDB 35 and actinomycete CDA 19 was 8%. Least percent of decolouration was observed by CDB 35 after one day which was 1% whereas it was 3% with CDA 19 (Fig. 4.7 and Fig. 4.8).

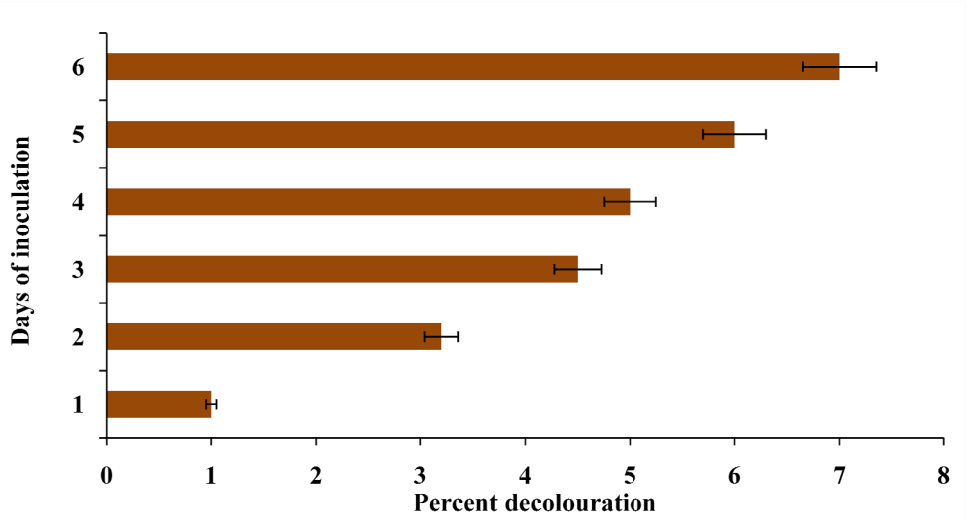


Fig: 4.7 Percent decolouration of cell free supernatant of bacterium CDB 35 by Chromeazurol reagent.

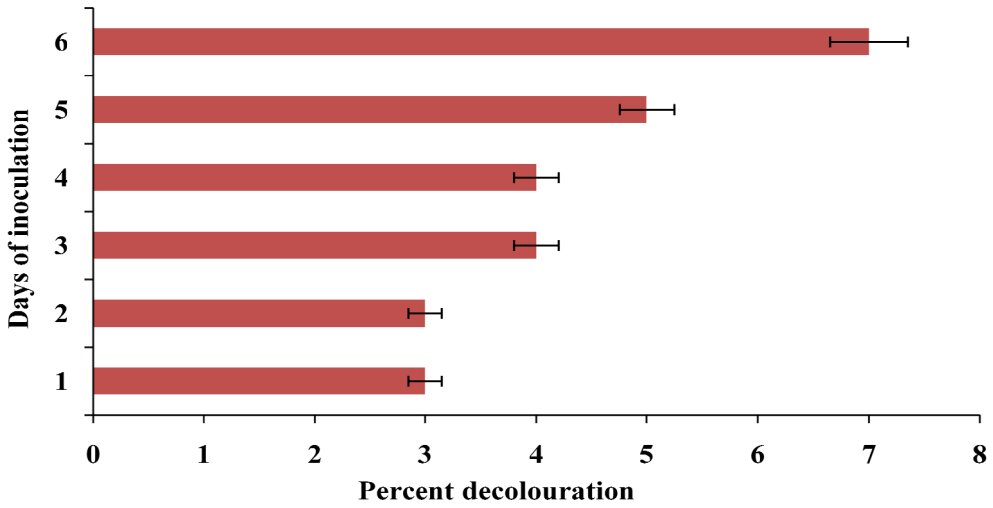


Fig: 4.8 Percent decolouration of cell free supernatant of *Streptomyces* sp. CDA 19 by Chromeazurol reagent.

4.12 Effect of iron supplementation on growth and aflatoxin production by *A. flavus*

Iron supplementation in the growth media had little or no effect on the radial growth of *A. flavus* when observed at 3, 5 and 9 days of incubation (Fig 4.9) but considerable variation in the toxin production was observed. After 3 days of incubation least aflatoxin concentration was noted at 0 ppm iron concentration (3.43 µg/Kg) and highest was at 100ppm of iron (6.25 µg/Kg). After 5 days of incubation aflatoxin

concentration was highest at 100 ppm iron concentration (31.58 µg/Kg) followed by 0 ppm (16.3 µg/Kg). At 9 days of incubation least was observed at 10 ppm (50.58 µg/Kg) and the highest was observed at 100 ppm iron (260.48 µg/Kg) (Fig 4.10).

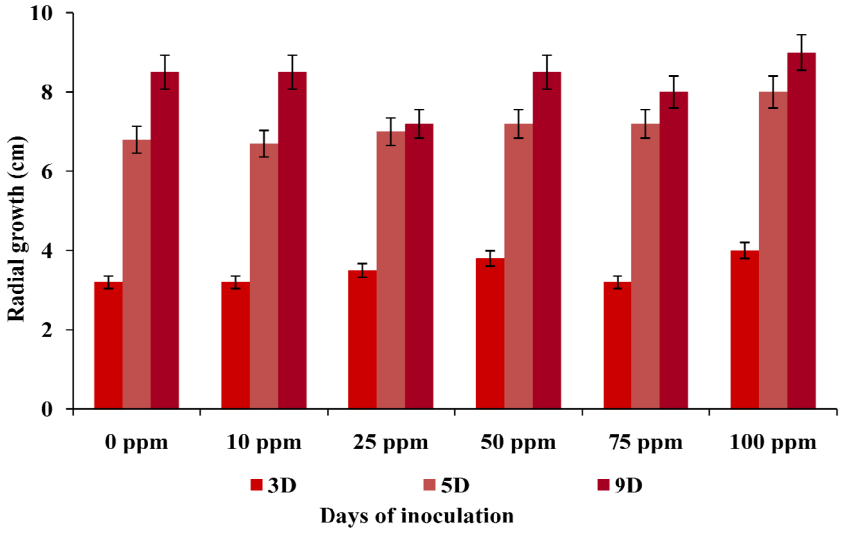


Fig: 4.9 Effect of iron on *A. flavus* radial growth

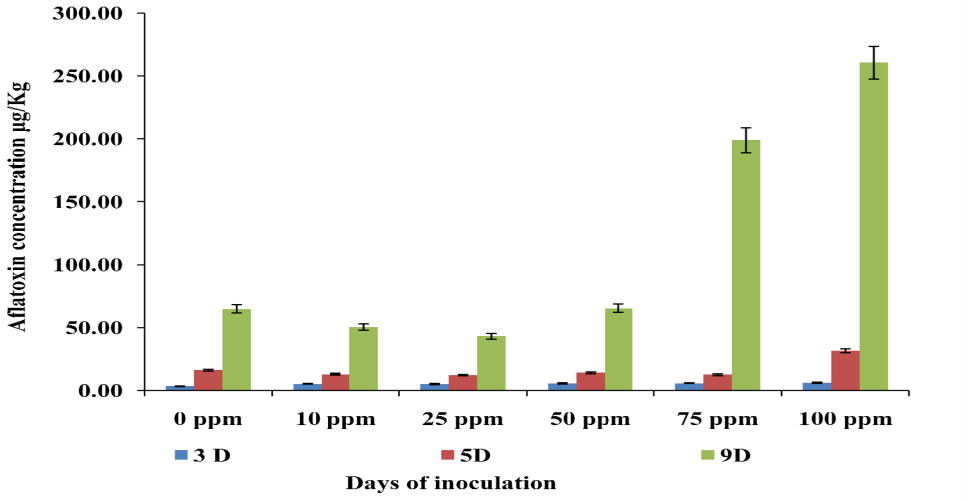
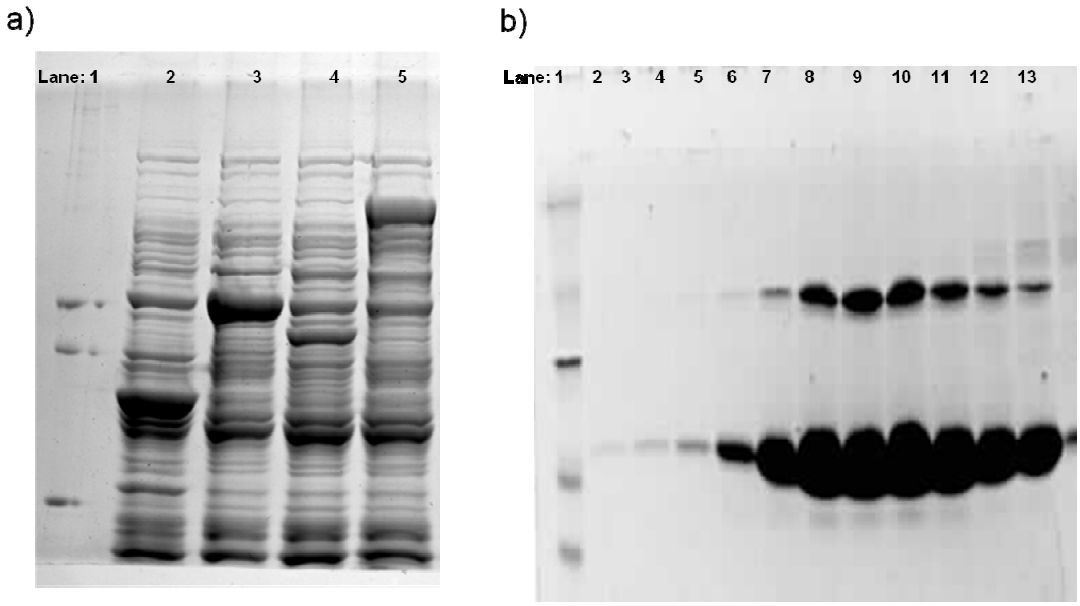


Fig. 4.10 Effect of iron on aflatoxin production by *A. flavus*

4.13 Purification of chitinases from *Streptomyces* sp. CDA 19

The CDA 19 isolate showed chitinase and β-1,3-glucanase activities (Photograph 4.14). The purification strategy adopted yielded highly purified fractions. The specific activity of chitinase and β-1,3-glucanase activity were shown in Table 4.11 and 4.12. Chitinase activity of actinomycete CDA 19 was found to be significantly higher than that of β -1,3-glucanase activity but both the enzymes bring about the hydrolytic action on the fungal cell wall. Quite contrastingly, the CDB 35 isolate showed only β -1,3-glucanase activity (Table 4.10), the yield and the specific activity of the was enzyme was significantly in the higher fold that than the CDA 19.



Photograph: 4.13 Enzyme production by CDB 35 and CDA 19

a) Protein profiles of fractions obtained at different stages of purification of the chitinase (35 kD) and β -1,3-glucanase activity (26 kD) from the active isolates. PAGE was performed on SDS gel. The corresponding lines shown are lane 1, 60 Mg of Biorad Low-molecular weight range Mr markers (13.5 to 60 Mr given in kD); lane 2 crude protein (β -1,3-glucanase) extract from bacterial (CDB 35) isolate, lane 3 and 4 crude protein (chitinase and β -1,3-glucanase) extract from bacterial isolates lane 5

Ammonium sulphate precipitation extract of chitinase and β -1,3-glucanase b) DEAE-cellulose purified proteins were further subjected to Sephadex G-75 for final purification of chitinase shown here and similar strategy was obtained for β -1,3-glucanase (data not shown).

Table: 4.10 Purification steps of glucanase from CDB 35

Step	Total Protein (mg)	Total activity (Unit)	Specific activity (U/mg)
Culture filtrate	301.8±5.8	1,125	18.5
Ammonium sulfate Precipitate	191.6±0.9	876	47.3
DEAE-cellulose	102±3.4	359	68.2
Sephadex G-75	44.2±2.1	176	142

Table: 4.11 Purification steps of glucanase from CDA 19

Step	Total Protein (mg)	Total activity (Unit)	Specific activity (U/mg)
Culture filtrate	179.8±4.7	1,125	10.5
Ammonium sulfate Precipitate	129.6±2.9	876	32.3
DEAE-cellulose	92±1.8	359	61.4
Sephadex G-75	26±0.5	176	139

Table: 4.12 Purification steps of chitinase from CDA 19

Step	Total Protein (mg)	Total activity (Unit)	Specific activity (U/mg)
Culture filtrate	593.6±2.9	4,125	5.35
Ammonium sulfate Precipitate	335.4±7.7	3,576	22.64
DEAE-cellulose	209.6±4.8	2,059	37.8
Sephadex G-75	87.2±3.6	976	125

4.14 Abiotic Factors pH, salinity and temperature affecting growth of biocontrol agents

4.14.1 pH

The effect of various pH levels (5,6,7,8) on the survival of the two potential antagonistic isolates *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 under broth culture conditions was studied. The culture was harvested for every 12 h for 60 hours of incubation and the turbidity was measured at 600nm. With CDB 35 the optimum growth was observed at pH 7 and at 36 hours of incubation and then the growth slowly decreased. The same trend was shown by CDA 19 in Bennets broth under the same experimental conditions (Fig. 4.11), both CDB 35 and CDA 19 grew, at all the pH levels under study.

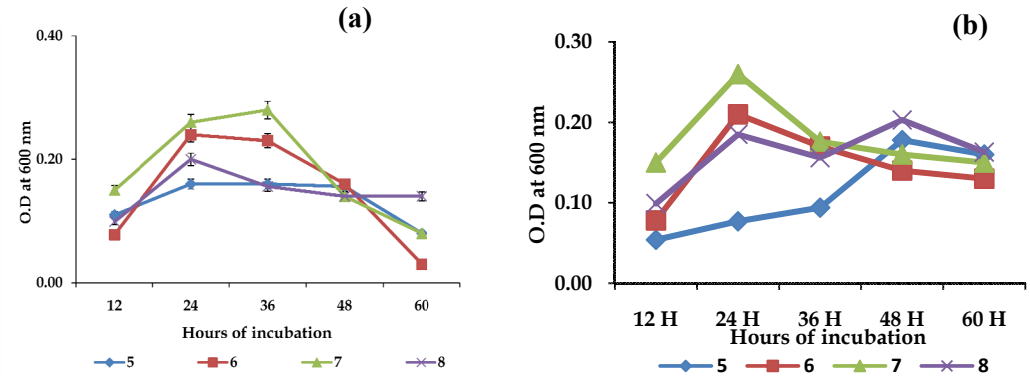


Fig: 4.11 Effect of pH on growth of CDB 35 and CDA 19 at 30 ± 2°C at various time intervals.

4.14.2 Salinity

The effect at various salinity levels (0, 50 mM, 100 mM, 150 mM, 200 mM and 250 mM)for both CDB 35 and CDA 19 was observed and growth was optimum at 100mM salinity level. All the other salinity levels showed a similar trend of maximum growth until 36 hours and slowly decreased after 48 hours of incubation (Fig. 4.12).

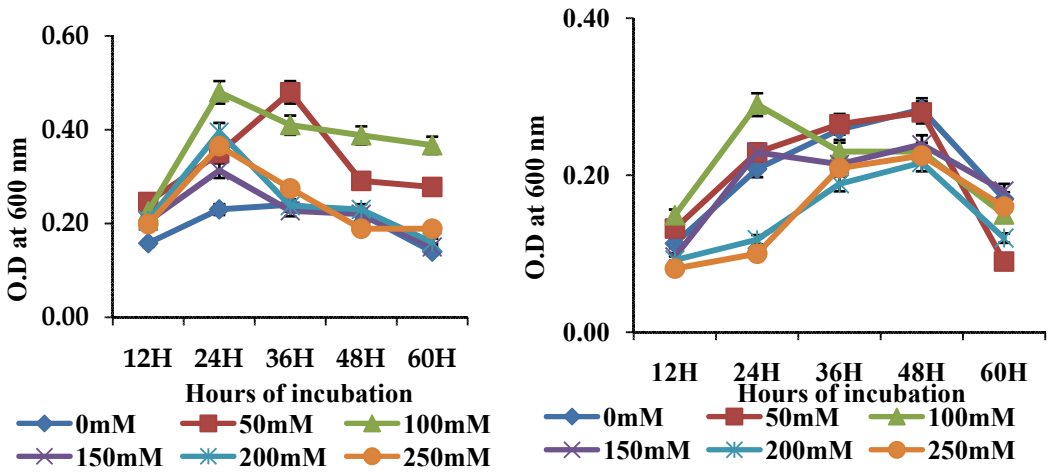


Fig: 4.12 Effect of salinity on growth of CDB 35 and CDA 19 at 28 ± 2°C at various time intervals of incubation

4.14.3 Temperature at constant pH 7

Growth of CDB 35 and CDA 19 were studied at various temperatures at constant pH of 7. Both the isolates grew at all temperatures and it increased until 36 hours of incubation and there was a stationary phase till 48 hours and then there was a decline in growth, though the maximum growth was observed at 28 ± 2° C. (Figs. 4.13)

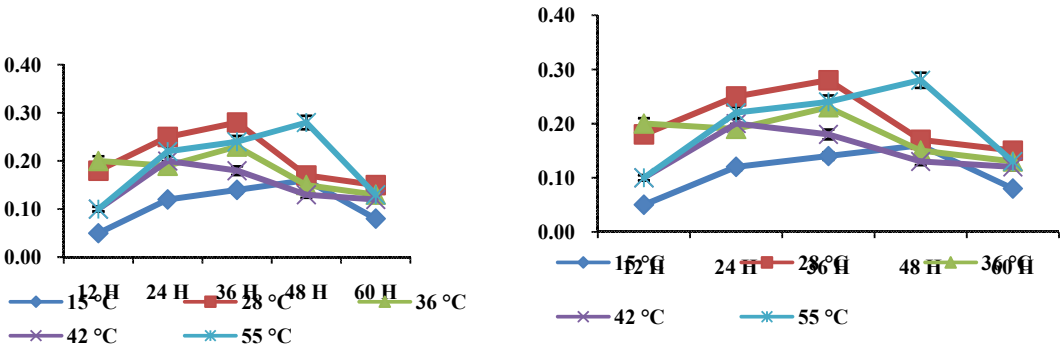


Fig: 4.13 Effect of various temperature on growth of CDB 35 and CDA 19 at pH 7 at various time intervals of incubation.

4.14.4 Temperatures at constant salinity

Growth of CDB 35 and CDA 19 were studied at various temperatures at constant salinity of 100 mM. Both of the isolates grew at all the temperatures, and the

growth of the isolates increased till 24 hours of incubation and there was a constant growth till 48 hours, then the growth decreased. Maximum growth was noticed at 28° C and at 24 hours of incubation for both the isolates (Figs. 4.14)

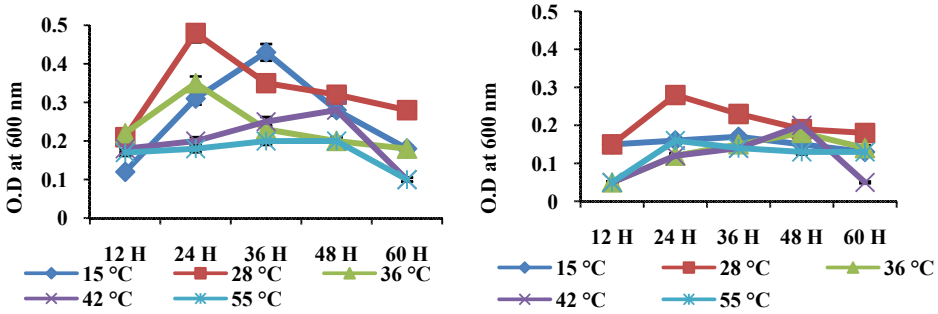


Fig: 4.14 Effect of various temperatures on growth of CDB 35 and CDA 19 at 100mM salinity at various time intervals of incubation.

4.15 Plant Growth Promoting Traits of Potential Bacteria and Actinomycetes

The selected bacterial and actinomycete isolates used in the study were characterized in terms of their ability for *in vitro* production of metabolites responsible for their plant growth promoting ability, viz. rock phosphate solubilization (RP), and phytase activity (indole acetic acid production (IAA). Among the selected potential bacteria CDB 15, CDB 16, CDB 30, CDB 35, and CDB 41 produced IAA; and CDB 16, CDB 31 and CDB 35 showed RP solubilisation. Only CDB 35 showed phytase activity among all the bacteria studied. Among actinomycetes only CDA 19 showed IAA production (Table 4.13).

Table: 4.13 Plant growth promoting traits of CDB 35 and CDA 19

Isolates	Rock phosphate solubilisation	Phytase Activity	IAA
CDB 15	-	-	+
CDB16	+	-	+
CDB 30	-	-	+
CDB 31	+	-	++
CDB 35	+++	+++	++++
CDB 41	-	-	+
CDB 47	++	-	-
CDB 58	-	-	-
CDA 16	-	-	-
CDA 19	-	-	++++
CDA 26	-	-	-

+ = Slight production, ++ = medium production, +++ = good production,
++++ = very good production , - = no production

4.16 IAA production by CDB 35 and CDA 19 in the presence and absence of tryptophan

Both bacterial isolate CDB 35 and actinomycete isolate CDA 19 produced IAA both in the presence and absence of precursor tryptophan (Photograph 4.14). Both produced IAA at all concentrations of tryptophan (1-10mg/mL) and also in its absence, though maximum production was observed at 5mg/mL of tryptophan concentration and at 96 hours of incubation with 595 µg/mL for CDB 35 and 573 µg/mL with CDA 19. The production gradually increased till 5mg/mL and after that it reduced till 10mg/mL, thereby clarifying the optimum concentration required for maximum production. At all concentrations and also in its absence the IAA production increased till 90 hours of incubation and thereafter decreased till 150 hours (Fig. 4.15).

Photograph 4.14: IAA production by CDB 35 and CDA 19

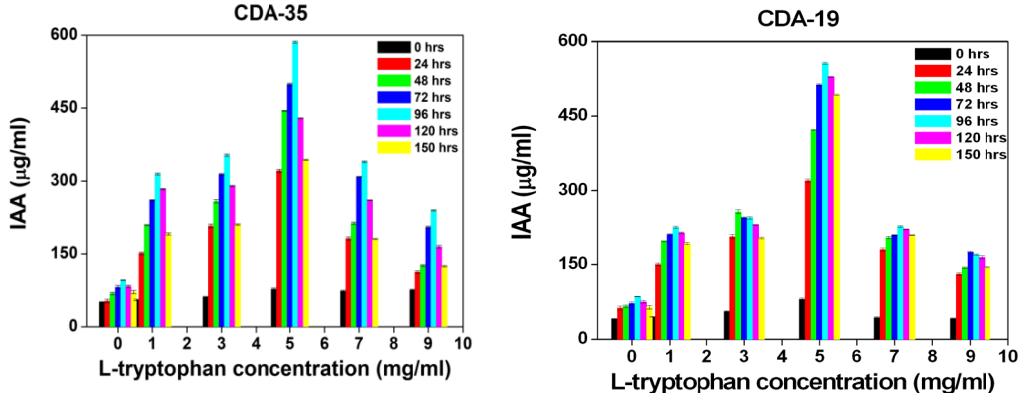
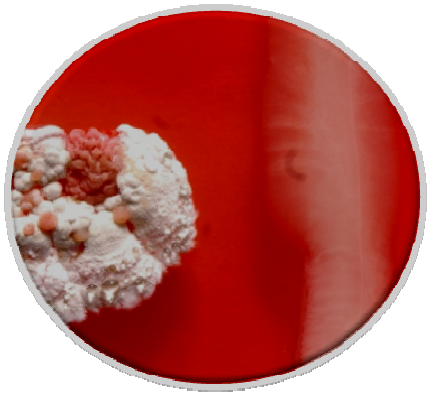


Fig 4.15: IAA production by CDB 35 and CDA 19 at various concentrations of tryptophan

4.17 Compatibility of *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 with groundnut specific rhizobium IC 7114

Bacterial isolate *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 were tested for their interaction with the groundnut specific rhizobial strain IC 7114 (IC - Strain number given for *Rhizobium* isolates from ICRISAT). Their interaction with *Rhizobium* was measured as interacting distance and their growth away from *Rhizobium* was measured as spreading capacity. The interaction distance between rhizobium and *Pseudomonas* sp. CDB 35 is 8mm and *Streptomyces* sp. CDA 19 is 5 mm and spreading capacity of *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 was 22mm and 25 mm, respectively (Photograph 4.16).



Photograph: 4.15 Compatibility of CDB 35 and CDA 19 with groundnut specific rhizobium

4.18 Plant growth promotion (Plate assay) of potential isolates

Potential bacterial and actinomycete isolates which showed plant growth promoting traits (Table 13) and antagonism against *A. flavus* were evaluated for plant growth promotion of groundnut using plate assay method.+ Both bacterium *Pseudomonas* sp. CDB 35 and actinomycete CDA 19 improved the germination percent, thus showed improvement in root length and number of rootlets. Germination percent was 85% with *Pseudomonas* sp. CDB 35 and 76% with CDA 19 which was maximum when compared to the other isolates tested. Increase in root length by *Pseudomonas* sp. CDB 35 was 35% and by CDA 19 was 29% when compared to control. The average number of root lets were 38% and 33% increase over control with *Pseudomonas* sp. CDB 35 and CDA 19 respectively (Table 4.15).

Table: 4.14 Plant growth studies (Plate assay) by the potential bacterial and actinomycete isolates.

Treatment	Percent germination	Root length	Avg number of rootlets
CDB 15	63	2.3 (24)	2.3(27)
CDB 16	66	2 (8)	2.4(33)
CDB 30	61	1.95 (5)	2.1(16)
CDB 31	54	2.2(18)	1.9(6)
CDB 35	**85**	**2.5(35)**	**2.5(38)**
CDB 41	68	2.2(18)	2.3(28)
CDB 47	65	1.9(2)	2(11)
CDB 58	66	1.8()	2(11)
CDA19	**75**	**2.4(29)**	**2.4(33)**
CDA 26	67	2.2(18)	1.9(6)
Control	56	1.85	1.8
LSD (P=0.05)	**1.8**	**1.31**	**1.2**
CV%	**1.6**	**30.2**	**27.9**

(Value in parenthesis indicate SD values)

4.19 Glasshouse evaluations for plant growth studies

All the potential isolates which were screened *in vitro* for growth promotion were tested in soil under glasshouse conditions. Both showed significant improvement in plant growth parameters, increase in rootlength was 66% with *Pseudomonas* sp. CDB 35 and 42% with CDA 19 and shoot length was 27 with *Pseudomonas* sp. CDB 35 and 22% with CDA 19 respectively and dry weight being 68% with *Pseudomonas* sp. CDB 35 and 57% with CDA 19 over control (Table 4.15).

Table: 4.15 Glasshouse evaluation of the selected potential antifungal bacteria and actinomycetes for 30 days.

Treatment	Root length(mm)	Shoot length (cm)	Dry weight (gm)
CDB 15	17.8(42)	11.8(15)	2.7(42)
CDB 16	16.83(34)	10.6(3)	2.8(47)
CDB 30	14.83(18)	11.3(10)	2.5(31)
CDB 31	17.2(37)	10	2.6(36)
CDB 35	**20.83(66)**	**13.2(29)**	**3.2(68)**
CDB 36	17.77(42)	11.6(13)	2.7(42)
CDB 41	16.3(30)	11.5(12)	2.7(42)
CDB 47	15(20)	11.7(14)	2.5(31)
CDB 58	16(20)	11.4(14)	2.2(31)
CDA19	**17.83(42)**	**13(27)**	**3(57)**
CDA 26	17.5(40)	10	2.9(52)
Control	12.5	10.2	1.9
LSD (P=0.05)	**4.12**	**3.16**	**2.61**
CV%	**14.4**	**16.6**	**18.6**

4.20 Survival studies of bacterium CDB 35 and actinomycete CDA 19 in various carrier materials

Population of CDB 35 ranged between 8.4 to 5.9 CFU g^{-1} in peat, 7.2 to 2.3 CFU g^{-1} in talc and 7.2 to 2.2 CFU g^{-1} in lignite on Luria agar (Fig 4.21), and population of CDA 19 ranged between 8.2 to 5.8 CFU g^{-1} in peat, 7.1 to 2.2 CFU g^{-1} in talc and 7.7 to 2.1 CFU g^{-1} in lignite on actinomycetes isolation agar (AIA) from 30 days after inoculation to 180 days. (Fig : 4.16).

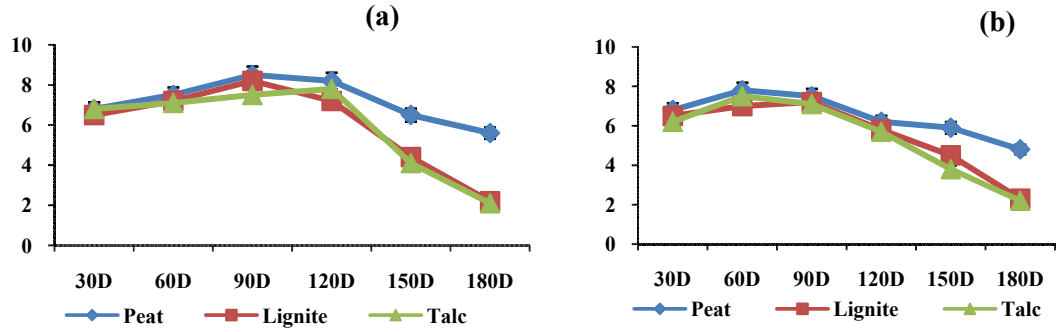


Fig 4.16: Survivability of the inoculated bacteria CDB 35 (a) and CDA 19 (b) in peat, lignite and talc

4.21 Effect of mode of inoculation of bacterium CDB 35 under glasshouse conditions

With bacterial isolate CDB 35 as seed treatments, overall increase in root, shoot and dry weight was observed in all the treatments studied. Seed coating with *Pseudomonas* sp. CDB 35 (SC 35) increased root and shoot length by 27 and 16%, seed priming with *Pseudomonas* sp. CDB 35 (SP 35) increased root and shoot length was 35% and 38% followed by seed priming with CDB 35 + rhizobium by 26% and 29%. Increase in dry weight was significant in SC with *Pseudomonas* sp. 35 (47%) followed by SP with *Pseudomonas* sp. 35 (44%) and SP with *Pseudomonas* sp. 35 + rhizobium (36%) (Table 4.16).

Table: 4.16 Seed coating experiment with CDB 35 under glasshouse conditions for 30 days.

Treatment	BCA	Root length(mm)	Shoot length(cm)	Dry weight(gm)
Seed coating	CDB 35	18.2(27)	11.6(16)	2.8(47)
Seed priming	CDB 35	19.4(35)	13.8(38)	2.7(44)
Seed priming	CDB 35+ rhizobium	18 (26)	12.9(29)	2.6(36)
Control		14.3	10	1.9
LSD (P=0.05)		**6.21**	**5.15**	**0.84**
CV%		**17**	**21.3**	**13.4**

(Value in parenthesis are significane over control)

4.21.1 Effect of mode of inoculation of actinomycete CDA 19 under glasshouse conditions

Overall increase in root, shoot and dry weight was observed in seed coating experiments by CDA 19. But significant root length was in seed priming with CDA 19 (19) followed by seed coating with CDA 19 (14%) and seed priming with CDA 19 + rhizobium (13%). Shoot length was more in SP CDA 19 with rhizobium (43%) followed by SP CDA 19 (35%) and SC CDA 19 (36%) and seed coating with CDA 19 (24%). Dry weight was more in SP CDA 19 (56%) followed by SP CDA 19 + rhizobium (51%) and seed coating CDA 19 (26%) (Table 4.17).

Table: 4.17 Seed coating experiment with CDA 19 under glasshouse conditions for 30 days.

Treatment	BCA	Root length(mm)	Shoot length(cm)	Dry weight(gm)
Seed coating	CDA 19	19.7(14)	12.3(24)	2.58(26)
Seed priming	CDA 19	20.5(19)	13.5(36)	3.2(56)
Seed priming	CDA 19+ rhizobium	19.5(13)	13.2(43)	3.13(51)
Control	Un inoculated	17.2	9.9	2.07
LSD (P=0.05)		**3.1**	**1.8**	**1.1**
CV%		**8.3**	**7.4**	**21.1**

4.22 Survivability of CDB 35 and CDA 19 in rice straw compost

Compost samples were prepared using both the potential bacterial isolate CDB 35 and actinomycete isolate CDA 19. Both these isolates were able to survive and multiply using rice straw as carbon and nutrient source. Growth of CDB 35 ranged between Log $_{(10)}$ 6.5 to 6.2 and CDA 19 Log $_{(10)}$ 5.8 to 5.1 at 45 and 90 days after inoculation (Table 4.18).

Table: 4.18 Survivability of CDB 35 and CDA 19 in compost.

Treatment	BCA	45 days	90 days
Compost	CDB 35	6.56	6.25
Compost	CDA 19	5.63	5.51
LSD (P=0.05)		**0.4**	**0.32**
CV%		**4.5**	**3.8**

Values are Log $_{(10)}$ per gram of compost.

4.23 Evaluation of enriched compost under glasshouse conditions

Both CDB 35 and CDA 19 survived well in the compost and this was evaluated under glass house conditions for plant growth promotion. With compost CDB 35 the increase in the growth parameters were significant, the root and shoot length was 43, 42% and dry weight was 27%. With compost CDA 19 the increase in root and shoot length 34% and 32% and dry weight was 20% followed by compost control where root and shoot length was 21 and 29% and dry weight 7% over control (Table 4.19).

Table : 4.19 Plant growth promotion by CDB 35 and CDA 19 in compost amended experiments.

Treatment	BCA	Root length(mm)	Shoot length(cm)	Dry weight(gm)
Compost	CDB 35	21(43)	15(42)	3.4(27)
Compost	CDA 19	20(34)	14(32)	3.2(20)
Compost	control	18(21)	13(29)	2.9(7)
Control	un inoculated	15	10	2.7
LSD (P=0.05)		**5.1**	**4.9**	**1.8**
CV%		**14**	**19**	**16**

4.24 Field experiments

Field experiment conducted during kharif 2006 revealed significant increase in growth parameters in the presence of *Pseudomonas sp*. CDB 35 over control. Haulm weight was 35% in SP CDB 35 + rhizobium followed by SC CDB 35 (18%) and SP CDB 35 (14%) over control. Pod weight was more significant in SC CDB 35 which was 18% significant followed by SP CDB 35 and SP CDB 35+ rhizobium which was 16 and 15% respectively, and seed weight was more in SP CDB 35 (59%) followed by SC CDB 35 (44%) and SP CDB 35(30%) respectively (Table: 4.20).

Table: 4.20 Effect of *Pseudomonas* sp. CDB 35 on groundnut growth parameters during kharif 2006 (weight measured in tonnes/hectare)

Treatment	BCB	Haulm weight	Pod weight	Seed weight
SC	CDB 35	1.74 (18)	**0.8 (18)**	**0.39 (44)**
SP	CDB 35	**2.02(37)**	0.78 (16)	0.35 (30)
SP	CDB 35 + *Rhizobium*	1.67 (14)	0.77 (15)	**0.43 (59)**
Control	Uninoculated	1.47	0.68	0.27
LSD (P=0.05)		**5.9**	**0.9**	**0.9**
CV%		**28**	**10**	**20**

Field experiment conducted during rabi 2006-2007 revealed that haulm weight was more in SP with rhizobium followed by SC CDB 35 (14%) and SP CDB 35 (10%) and pod weight was more in SC CDB 35 which was 40% followed by SP CDB 35 + rhizobium (32%) and SP CDB 35 (16%) while seed weight was more in SP CDB 35(47%) followed by SC CDB 35 (24%) over control respectively (Table: 4.21).

Table: 4.21 Effect of *Pseudomonas* sp. CDB 35 on groundnut growth parameters during rabi 06-07 (weight measured in tonnes/hectare)

Treatment	BCB	Haulm weight	Pod weight	Seed weight
SC	CDB 35	3.65 (14)	2.25 (**40**)	1.07 (**24**)
SP	CDB 35	3.50 (10)	1.87(16)	1.26 (**47**)
SP	CDB 35 + *Rhizobium*	3.47 (**32**)	2.12 (**32**)	0.86
Control	Uninoculated	3.19	1.61	0.86
LSD (P=0.05)		**4.9**	**4.5**	**1.6**
CV%		**9**	**14**	**10**

Values in the parentheses are significance over control

Field experiment conducted during kharif 2007 revealed that the haulm weight was more in SP CDB 35 + rhizobium followed by SC CDB 35(19%) and SP CDB 35 (16%). Pod weight was more significant in SP CDB 35 which was 31% followed by SP CDB 35 (9%) and SC CDB 35 which was (4%) and seed weight was more in SC CDB 35 (38 %) followed by SP CDB 35 (17 %) and SP CDB 35(13 %) respectively over control. (Table: 4.22)

Table: 4.22 Effect of *Pseudomonas* sp. CDB 35 on growth parameters of groundnut during kharif 2007 (weight measured in tonnes /hectare)

Treatment	BCB	Haulm weight	Pod weight	Seed weight
SC	CDB 35	3.80 (**19**)	0.78 (4)	0.66 (**38**)
SP	CDB 35	3.70 (16)	0.82 (9)	0.56 (17)
SP	CDB 35 + *Rhizobium*	4.00 (**25**)	0.98 (**31**)	0.54 (13)
Control	Uninoculated	3.20	0.75	0.48
LSD (P=0.05)		**4.9**	**2.5**	**1.3**
CV%		**9**	**19**	**15**

SC = Seed coating ., SP= Seed priming., BCA = Biocontrol bacteria

Kharif 2006 experiment with CDA 19 revealed the haulm weight was more in SC CDA 19 (20 %) followed by SP CDA 19 (18 %) and SP CDA 19 + rhizobium (13 %). Pod weight was more in SC CDA 19 (10 %) and seed weight was more in SC CDA 19 (41 %) more followed by SP CDA 19 (3%) over control (Table 4.23).

Table: 4.23 Effect of *Streptomyces* sp. CDA 19 on groundnut growth parameters during Kharif 2006 (weight measured in tonnes/hectare)

Treatment	BCA	Haulm weight	Pod weight	Seed weight
SC	CDA 19	**2.08 (20)**	**0.90 (10)**	**0.45 (41)**
SP	CDA 19	**2.05 (18)**	0.65	0.33 (3)
SP	CDA 19+ *Rhizobium*	1.97 (13)	0.71	0.40
Control	Uninoculated	1.74	0.82	0.32
LSD (P=0.05)		**5.2**	**1.5**	**1.0**
CV%		**24**	**16**	**22**

In the experiment conducted during rabi 06-07 the following results were observed the haulm weight was 26 % more in SP CDA 19 followed by SC CDA 19 (15 %) and SP CDA 19+ rhizobium (14 %) over control. Pod weight was 5% more in SC CDA 19 followed by SP CDA 19 which was 3 % and seed weight was 30 % more followed by SP CDA 19 (10 %) over control (Table: 4.24).

Table: 4.24 Effect of *Streptomyces* sp. CDA 19 on groundnut growth parameters during rabi 06-07 (weight measured in tonnes/hectare

Treatment	BCA	Haulm weight	Pod weight	Seed weight
SC	CDA 19	**3.04 (15)**	**1.97(5)**	**1.07 (30)**
SP	CDA 19	**3.33 (26)**	1.93 (3)	0.73 (10)
SP	CDA 19+ *Rhizobium*	3.01(14)	1.50	1.03
Control	Uninoculated	2.64	1.87	0.82
LSD (P=0.05)		**8.5**	**6.7**	**2.9**
CV%		**18**	**23**	**20**

Haulm weight was more in SP CDA 19 (36 %) followed by SC CDA 19 (27 %) and SP CDA 19 with rhizobium (26 %) over control. Pod weight was more in SP CDA 19 (39 %) followed by SP CDA 19 + rhizobium (36 %) over control and seed weight was more in SC CDA 19 (57 %) followed by SP CDA 19+ rhizobium (32 %) over control (Table: 4.25).

Table: 4.25 Effect of *Streptomyces* sp. CDA 19 on growth parameters of groundnut during Kharif 2007 (weight measured in tonnes /hectare)

Treatment	BCA	Haulm weight	Pod weight	Seed weight
SC	CDA 19	**4.14 (27)**	0.72 (3)	**0.58 (57)**
SP	CDA 19	**4.46 (36)**	**0.97 (39)**	0.60
SP	CDA 19+ *Rhizobium*	4.13 (26)	**0.95 (36)**	**0.49 (32)**
Control	Uninoculated	3.27	0.70	0.37
LSD (P=0.05)		**15.3**	**2.1**	**2.3**
CV%		**24**	**16**	**29**

SC = Seed coating ., SP= Seed priming., BCA = Biocontrol actinomycete

In the compost amended with CDB 35experiment during kharif 2006 revealed the following. The haulm weight was more significant in compost + 35 treatment (35%) followed by control compost (28%) more than control. Pod weight was 42% in compost 35 followed by control compost which was 26% more than control and seed weight was 26% more significant than control (Table: 4.26).

Table: 4.26 Effect of compost amended *Pseudomonas* sp. CDB 35 on growth parameters of groundnut during kharif 2006 (weight measured in tonnes /hectare)

Treatment	BCB	Haulm weight	Pod weight	Seed weight
Compost	CDB 35	3.2 (**35%**)	1.04 (**42%**)	0.61 (**26%**)
Compost	Control	2.9 (**30%**)	0.92 (**26%**)	0.51
Control	Uninoculated	2.3	0.73	0.56
LSD (P=0.05)		2.7	1.7	0.8
CV%		8	14	11

In rabi 06-07 compost experiment pod weight was significant (12%) in compost with 35 followed by control compost which was 6% more than control. Seed weight was more in control compost (9%) followed by compost CDB 35 (6%) (Table: 4.27).

Table: 4.27 Effect of compost amended *Pseudomonas* sp. CDB 35 on growth parameters of groundnut during rabi 06-07 (weight measured in tonnes /hectare)

Treatment	BCB	Haulm weight	Pod weight	Seed weight
Compost	CDB 35	3.46	**2.51 (12)**	1.33 (6)
Compost	Control	3.41	2.38 (6)	1.16 (9)
Control	Uninoculated	3.47	2.25	1.26
LSD (P=0.05)		**4.0**	**10.4**	**2.3**
CV%		**7**	**25**	**11**

In the field experiment conducted in kharif 2007 experiment, haulm weight was 4% more in the compost CDB 35 treatment followed by control compost (1 %). Pod weight was 21% more in compost CDB 35 more than control. Seed weight was 41 % more in control compost followed by compost CDB 35 (37 %) than control (Table: 4.28).

Table: 4.28 Effect of compost amended *Pseudomonas* sp. CDB 35 on growth parameters of groundnut during Kharif 2007 (weight measured in tonnes /hectare)

Treatment	BCB	haulm weight	Pod weight	Seed weight
Compost	CDB 35	**4.27 (4)**	**0.76 (21)**	**0.56 (37)**
Compost	Control	4.14 (1)	0.89	**0.58 (41)**
Control	Uninoculated	4.09	0.63	0.41
LSD (P=0.05)		**15.6**	**1.8**	**1.3**
CV%		**22**	**4**	**14**

In kharif 2006 compost amended experiment the pod weight and seed weight was 27% and 35% in compost CDA 19 treatments followed by compost control which was 17% and 28% over uninoculated control (Table 4.29).

Table: 4.29 Effect of compost amended *Streptomyces* sp. CDA 19 on growth parameters of groundnut during kharif 2006 (weight measured in tonnes /hectare)

Treatment	BCA	Haulm weight	Pod weight	Seed weight
Compost	CDA 19	2.05 (8)	**0.71 (27)**	**0.34 (35)**
Compost	Control	1.96 (3)	0.75 (17)	**0.39 (28)**
Control	Uninoculated	**1.90**	**0.59**	0.29
LSD (P=0.05)		**6.6**	**0.8**	**0.6**
CV%		**26**	**9**	**13**

Pod weight was significant in compost with CDA 19 (31 %) followed by control compost (8%) over control. Seed weight was 48% more significant in compost with CDA 19 followed by control compost (30 %) over control (Table: 4.30)

Table: 4.30 Effect of compost amended *Streptomyces* sp. CDA 19 on growth parameters of groundnut during rabi 06-07 (weight measured in tonnes /hectare)

Treatment	BCA	Haulm weight	Pod weight	Seed weight
Compost	CDA 19	3.40 (3)	**2.49 (31)**	**1.36 (48)**
Compost	Control	**3.30 (31)**	2.05 (8)	1.20 (30)
Control	Uninoculated	3.2.0	1.90	0.92
LSD (P=0.05)		**4.7**	**5.3**	**2.4**
CV%		**8**	**14**	**12**

In kharif 2007 experiment the seed weight was 41 % more in compost CDA 19 followed by control compost 28% over control (Table 4.31).

Table: 4.31 Effect of compost amended *Streptomyces* sp. CDA 19 on growth parameters of groundnut during kharif 2007 (weight measured in tonnes /hectare)

Treatment	BCA	Haulm weight	Pod weight	Seed weight
Compost	CDA 19	3.74	0.85	**0.65 (41)**
Compost	Control	4.64	0.93	0.59 (28)
Control	Uninoculted	3.83	0.63	0.46
LSD (P=0.05)		**9.9**	**2.2**	**1.3**
CV%		**14**	**16**	**13**

BCA= Biocontrol actinomycete

4.24.1 Survival studies of *Pseudomonas* sp. CDB 35 under field conditions

In kharif 2006 experiment the colony forming units (CFU) of the inoculated CDB 35 varied with treatments In seed coating with CDB 35 the population ranged between CFU Log $_{10}$ 6 DAI)- Log $_{10}$ 3 (110 DAI). In seed priming treatment the population ranged between Log $_{10}$ 6.1 (50 DAI) to Log $_{10}$ 3.1 (110 DAI). In seed priming with rhizobium the population ranged between CFU Log $_{10}$ 6.1 to Log $_{10}$ 3.1. (Fig 4. 17).

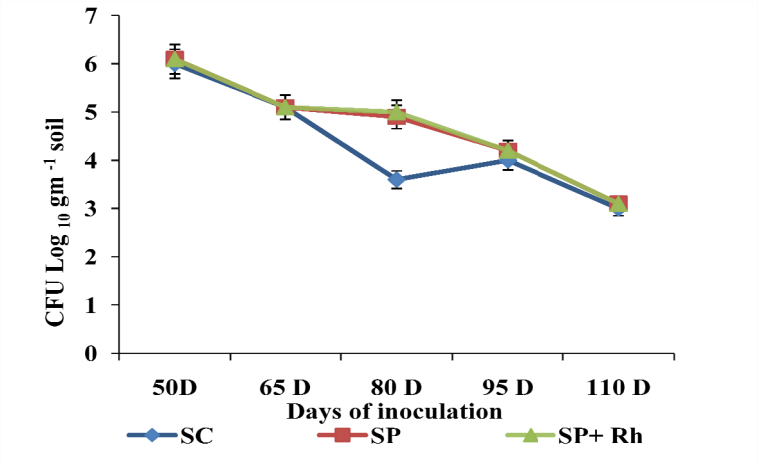


Fig: 4.17 Survival of the inoculated *Pseudomonas* sp. CDB 35 in field in Kharif 2006

In rabi 06-07 experiment the CFU of the inoculated CDB 35 varied with treatments In seed coating with CDB 35 the population ranged between CFU Log $_{(10)}$ 6 DAI)- Log $_{(10)}$ 2.7 (110 DAI). In seed priming treatment the population ranged between Log $_{(10)}$ 5.9 (50 DAI) to Log $_{(10)}$ 2.6 (110 DAI). In seed priming with rhizobium the population ranged between CFU Log $_{(10)}$ 4.5 to Log $_{(10)}$ 2 (Fig 4.22).

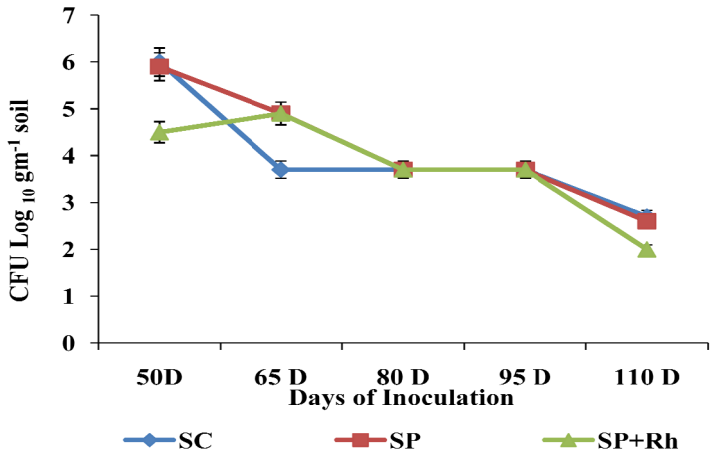


Fig: 4.18 Survival of the inoculated *Pseudomonas* sp. CDB 35 in field in rabi 06-07

In kharif 2007 experiment the CFU of the inoculated CDB 35 varied with treatments, in seed coating with CDB 35 the population ranged between CFU Log $_{(10)}$ 5.4 (DAI)- Log $_{(10)}$ 3 (110 DAI). In seed priming treatment the population ranged between Log $_{(10)}$ 4.9 (50 DAI) to Log $_{(10)}$ 2.5 (110 DAI). In seed priming with rhizobium the population ranged between CFU Log $_{(10)}$ 4.6 to Log $_{(10)}$ 1.8 (Fig 4.23).

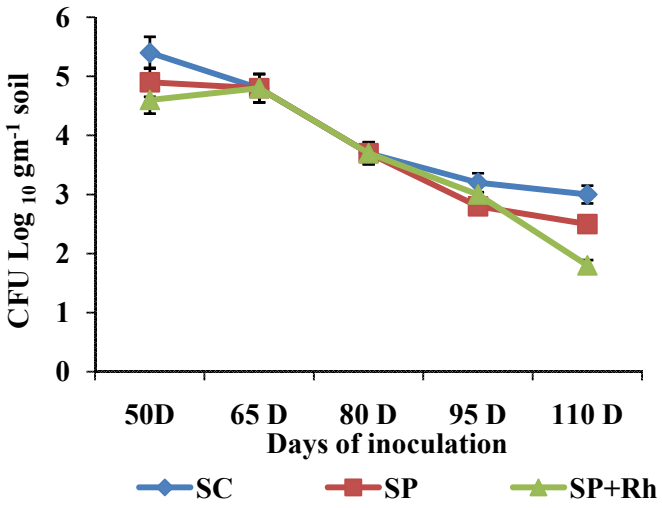


Fig: 4.19 Survival of the inoculated *Pseudomonas* sp. CDB 35 in field in Kharif 2007

In enrihced compost with CDB 35 experiment the mean value of the survivability over a period of three seasons ranged between CFU 6 Log $_{(10)}$ (50 DAI) to 3 Log $_{(10)}$ (110 DAI) (Fig 4.20).

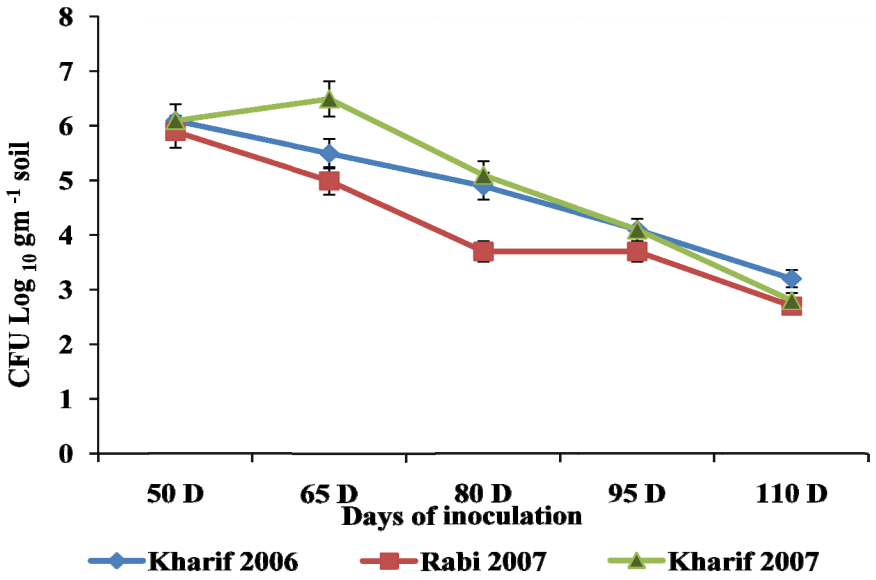
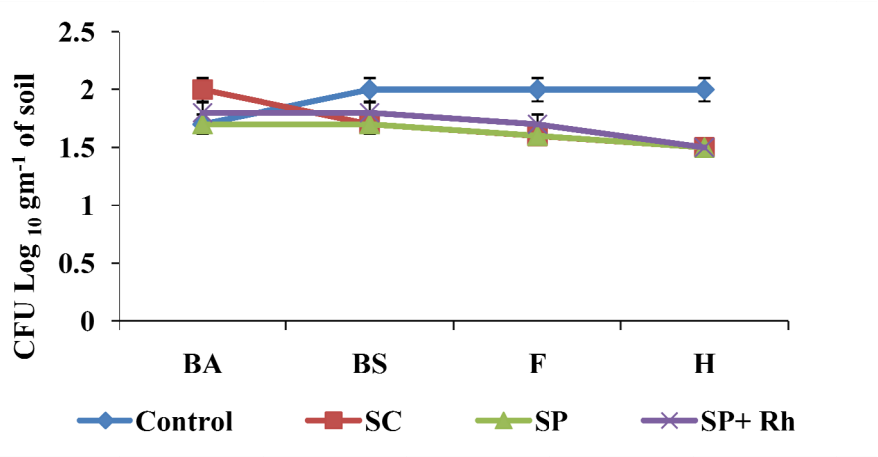


Fig 4.20: Survivability of the inoculated *Pseudomonas* sp. CDB 35 at different stages of cropping period in compost amended experiment.

4.24.2 Survival studies of the inoculated *A. flavus* in bacterial CDB 35 screening under field conditions

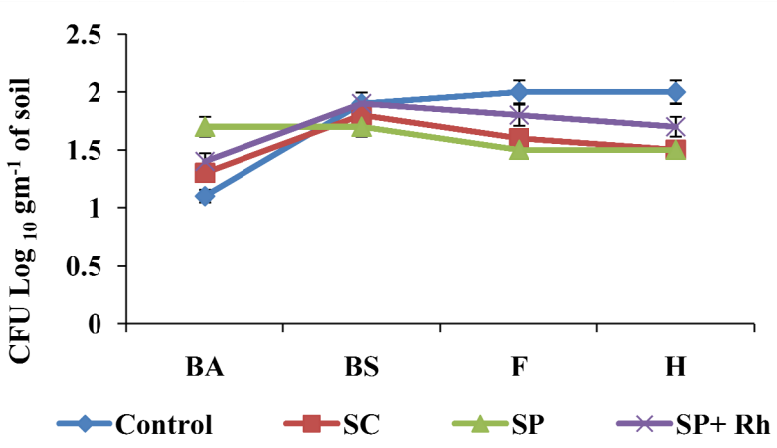
In the field experiments with seed coating with CDB 35 conducted during kharif 2006, rabi 06-07 and kharif 2007. Population of *A. flavus* was studied at various stages of cropping period. During kharif 2006 in the control treatment the native population was CFU Log $_{10}$ 1.7 gm^{-1} soil before application of the *A. flavus* inoculums, before sowing of groundnut and after application of the initial inoculum in the field the population was CFU Log $_{10}$ 2 gm^{-1} soil and this was maintained till harvest. In seed coating treatment with CDB 35 the population was CFU Log $_{10}$ 2 gm^{-1} soil before application of the *A. flavus* and before sowing the population was CFU Log $_{10}$ 1.7 gm^{-1} soil and it decreased till harvest which was CFU Log $_{10}$ 1.5 gm^{-1} soil. The same variation was observed in seed priming with CDB 35 and seed priming with CDB 35+ *Rhizobium* (Fig 4.21).



BA=Before application, BS=Before sowing, F=Flowering, H=Harvest

Fig 4.21: Survivability of inoculated *A. flavus* at different stages of cropping period during kharif 2006 in CDB 35 seed treated experiments.

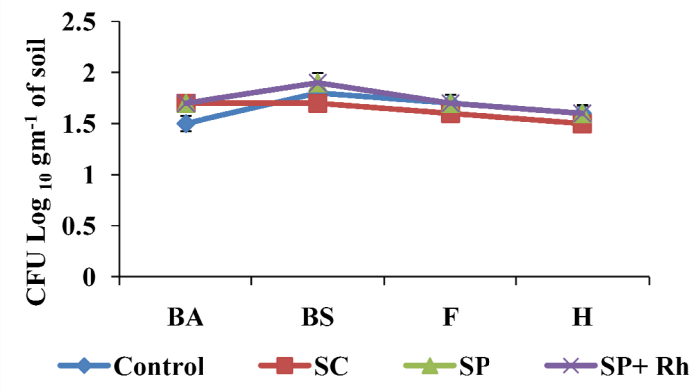
In rabi 06-07 experiment the population dynamics of *A. flavus* revealed the following, in control treatment and in the rest of the treatments the native population before application of the inoculums ranged between CFU Log $_{10}$ 1.1-1.4 gm^{-1} soil. At before sowing period the population across the treatments ranged between CFU Log $_{10}$ 1.7 gm^{-1} – 1.9 gm^{-1} soil. During flowering period the population was CFU Log $_{10}$ 2 gm^{-1} soil in control treatment and it decreased to CFU Log $_{10}$ 1.8 gm^{-1} soil across the treatments. Significant reduction was observed in seed priming with CDB 35 + *Rhizobium* treatment. At harvest stage the population in the control treatment was CFU Log $_{10}$ 2 gm^{-1} soil and it decreased to CFU Log $_{10}$ 1.7 gm^{-1} soil across the treatments. It was observed that population was CFU Log $_{10}$ 1.5 gm^{-1} soil in both seed coating and seed priming with CDB 35 (Table 4.22).



BA=Before application, BS=Before sowing, F=Flowering, H=Harvest

Fig 4.22: Survivability of inoculated *A. flavus* at different stages of cropping period during rabi 06-07 in CDB 35 seed treated experiments.

In kharif 2007 experiment the native population was CFU Log $_{10}$ 1.5-1.9 gm^{-1} soil across the treatments at before application stage of the field. This population was maintained almost same at before sowing period across the treatments. During flowering stage the population decreased from CFU Log $_{10}$ 2 gm^{-1} soil- CFU Log $_{10}$ 1.7 gm^{-1} soil across the treatments. Maximum decrease was noted at seed priming with CDB 35 + *Rhizobium* which was CFU Log $_{10}$ 1.6 gm^{-1} soil. At harvest stage the population reduced from CFU Log $_{10}$ 1.9 – 1.6 gm^{-1} soil. Maximum reduction was observed in seed priming with CDB 35+ *Rhizobium* treatment (Fig 4.27).

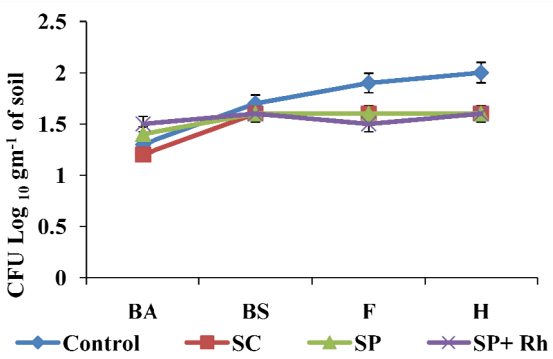


BA=Before application, BS=Before sowing, F=Flowering, H=Harvest

Fig 4.23: Survivability of inoculated *A. flavus* at different stages of cropping period during kharif 2007 in CDB 35 seed treated experiments.

4.24.3 Survival studies of *A. flavus* in actinomycete CDA 19 screening under field conditions.

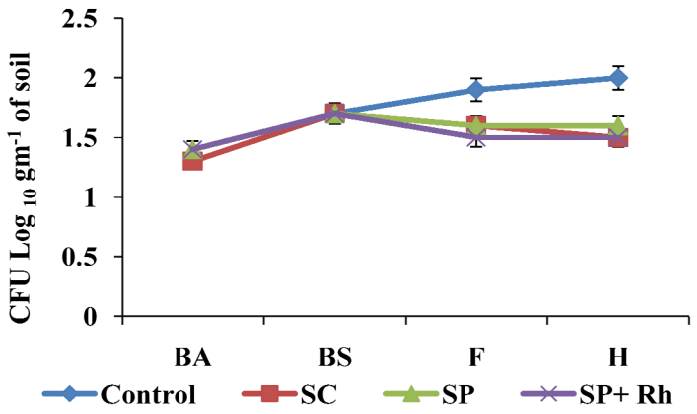
In the field experiments conducted with seed coating treatments with CDA 19 during kharif 2006 the population of *A. flavus* or the native population ranged between CFU Log $_{10}$ 1.3-1.5 gm^{-1} soil before application of the inoculum. Before sowing the population ranged between CFU Log $_{10}$ 1.6-1.7 gm^{-1} soil. At flowering stage the population reduced from CFU Log $_{10}$ 1.9-1.5 gm^{-1} soil with maximum reduction in seed priming with *Rhizobium* treatment. During harvest stage the population varied from CFU Log $_{10}$ 2-1.6 gm^{-1} soil (Fig 4.28).



BA=Before application, BS=Before sowing, F=Flowering, H=Harvest

Fig 4.24: Survivability of inoculated *A. flavus* at different stages of cropping period during kharif 2006 in CDA 19 seed treated experiments.

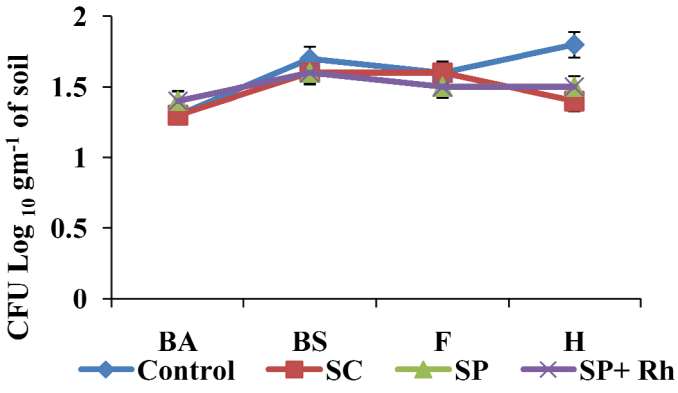
In rabi 06-07 season the native population ranged between CFU Log $_{10}$ 1.3-1.4 gm^{-1} soil across the plots. Before sowing of the groundnut and after application of the *A. flavus* inoculum the population was CFU Log $_{10}$ 1.7 gm^{-1} soil. Before sowing the population was CFU Log $_{10}$ 1.7 gm^{-1} soil throughout the treatments. At flowering the population reduced from CFU Log $_{10}$ 1.9-1.5 gm^{-1} soil. At harvest the population reduced from CFU Log $_{10}$ 2-1.5 gm^{-1} soil (Fig 4.25).



BA=Before application, BS=Before sowing, F=Flowering, H=Harvest

Fig 4.25: Survivability of inoculated *A. flavus* at different stages of cropping period during rabi 06-07 in CDA 19 seed treated experiments.

In kharif experiment the native population was CFU Log $_{10}$ 1.3 gm^{-1} soil across the treatments. Before sowing of groundnut and after application of *A. flavus* the population ranged between CFU Log $_{10}$ 1.7-1.6 gm^{-1} soil across the treatments. At flowering stage the population ranged between CFU Log $_{10}$ 1.9-1.5 gm^{-1} soil for control and across the treatments. At harvesting stage the population ranged between CFU Log $_{10}$ 1.9-1.5 gm^{-1} soil at control and across the treatments (Fig 4.30).

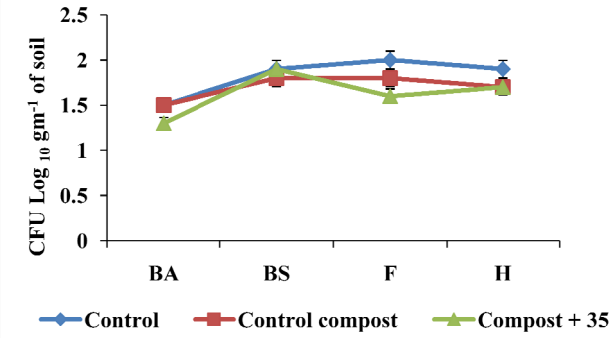


BA=Before application, BS=Before sowing, F=Flowering, H=Harvest

Fig 4.26: Survivability of inoculated *A. flavus* at different stages of cropping period during kharif 2007 in CDA 19 seed treated experiments.

4.24.4 Survival studies of *A. flavus* in compost amended CDB 35 screening under field conditions:

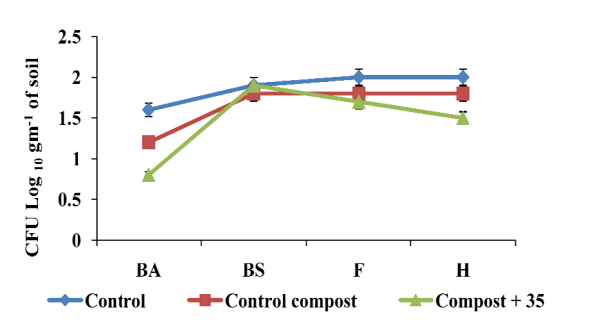
At kharif 2006 in compost amended experiments the native population of *A. flavus* was CFU Log $_{10}$ 1.5 gm^{-1} of soil in all the treatments. The population before sowing of groundnut was CFU Log $_{10}$ 1.9 gm^{-1} of soil in all the treatments. At flowering stage the population reduced from CFU Log $_{10}$ 2 gm^{-1} to CFU Log $_{10}$ 1.6 gm^{-1}. During harvest the population the population decreased from CFU Log $_{10}$ 1.9-1.7 gm^{-1} in all the treatments (Fig 4.31).



BA=Before application, BS=Before sowing, F=Flowering, H=Harvest

Fig 4. 27: Survivability of inoculated *A. flavus* at different stages of cropping period during kharif 2006 in CDB 35 compost amended experiments.

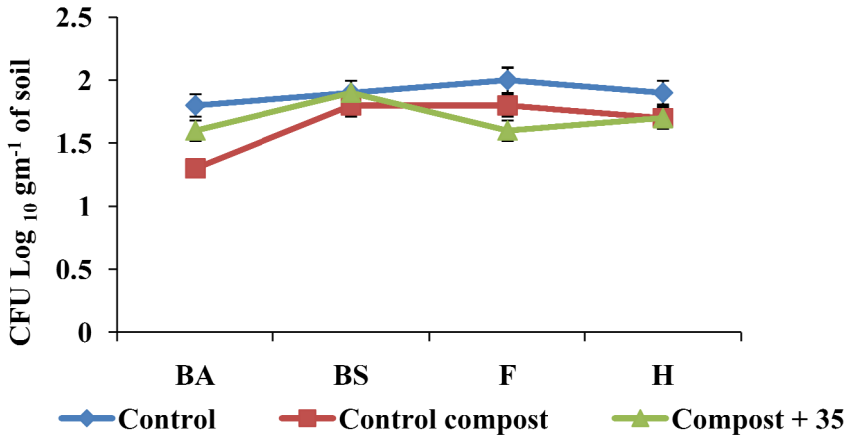
In rabi 06-07 compost amended experiment the native population was CFU Log $_{10}$ 1.6 gm^{-1}. Before sowing and after application of inoculums in to the field the population was CFU Log $_{10}$ 1.9 gm^{-1}. At flowering stage the population decreased from CFU Log $_{10}$ 2 gm^{-1} to CFU Log $_{10}$ 1.7 gm^{-1}. At harvesting stage the population reduced from CFU Log $_{10}$ 2 gm^{-1} to CFU Log $_{10}$ 1.5 gm^{-1} in all the treatments the difference was negligible (Fig 4. 28).



BA=Before application, BS=Before sowing, F=Flowering, H=Harvest

Fig 4.28: Survivability of inoculated *A. flavus* at different stages of cropping period during rabi 06-07 in CDB 35 compost amended experiments.

In kharif 2007 experiment the native population was CFU Log $_{10}$ 1.8 gm^{-1}. Before sowing of groundnut the population was CFU Log $_{10}$ 1.9 gm^{-1}. At flowering stage the population decreased from CFU Log $_{10}$ 2-1.7 gm^{-1}. At harvest the population decreased from CFU Log $_{10}$ 1.9-1.7 gm^{-1} (Fig 4.33).



BA=Before application, BS=Before sowing, F=Flowering, H=Harvest

Fig 4.29: Survivability of inoculated *A. flavus* at different stages of cropping period during kharif 2007 in CDB 35 compost amended experiments.

4.24.5 Survival studies of *A. flavus* in compost amended actinomycete CDA 19 screening under field conditions:

In the compost amended experiments during kharif 2006 along with CDA 19 the native population of *A. flavus* was CFU Log $_{10}$ 1.6 gm^{-1} soil. Before sowing of

groundnut the population ranged between CFU Log $_{10}$ 1.5-1.7 gm^{-1}. At flowering stage of the cropping period the population decreased from CFU Log $_{10}$ 1.9 gm^{-1} to CFU Log $_{10}$ 1.5 gm^{-1}. At harvest the population reduced from CFU Log $_{10}$ 1.9 gm^{-1} (Control) to CFU Log $_{10}$ 1.5 gm^{-1} across the treatments. Though there was a reduction in population in control compost treatment it was not more when compared to compost + CDA 19 treatment (Fig 4.34).

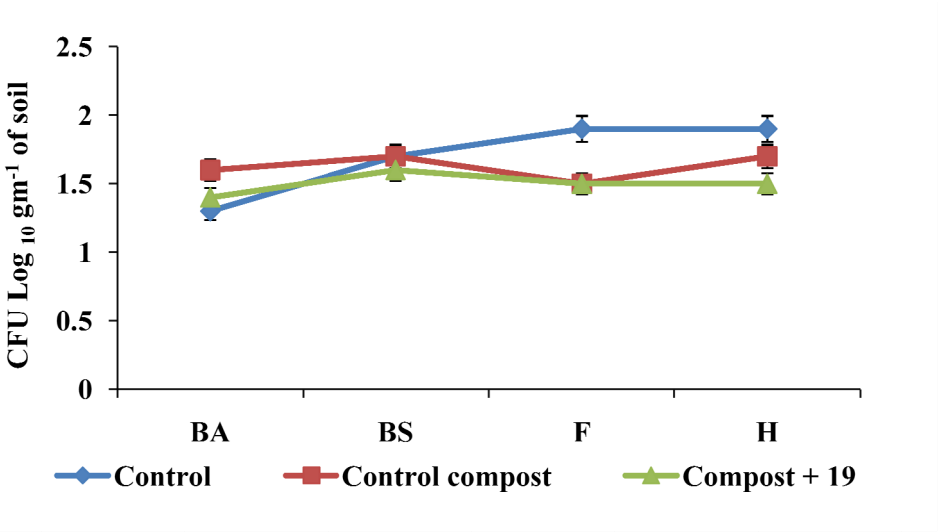


Fig 4.30: Survivability of inoculated *A. flavus* at different stages of cropping period during kharif 2006 in CDA 19 compost amended experiments.

In rabi 06-07 experiment the native population ranged between CFU Log $_{10}$ 1.3-1.6 gm^{-1}. Before sowing of groundnut samples the population was CFU Log $_{10}$ 1.7 gm^{-1}. At flowering stage the population decreased from CFU Log $_{10}$ 19 gm^{-1} to CFU Log $_{10}$ 1.5 gm^{-1}. During harvest the population reduced from CFU Log $_{10}$ 2 gm^{-1} to CFU Log $_{10}$ 1.5 gm^{-1} (Fig 4.31).

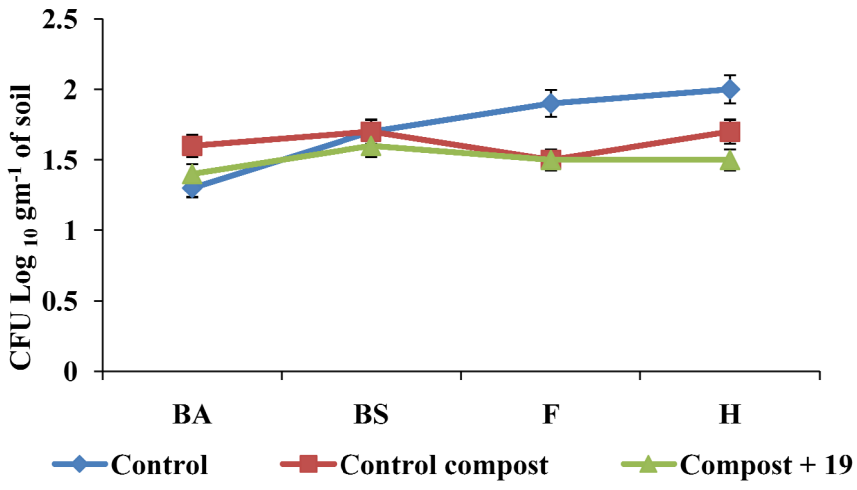


Fig 4.31: Survivability of inoculated *A. flavus* at different stages of cropping period during rabi 06-07 in CDA 19 compost amended experiments.

In kharif 2007 compost amended experiment native population ranged between CFU Log $_{10}$ 1.3-1.6 gm^{-1}. Before sowing of groundnut samples the population was CFU Log $_{10}$ 1.7 gm^{-1}. At flowering stage the population decreased from CFU Log $_{10}$ 19 gm^{-1} to CFU Log $_{10}$ 1.5 gm^{-1}. During harvest the population reduced from CFU Log $_{10}$ 1.9 gm^{-1} to CFU Log $_{10}$ 1.5 gm^{-1} (Fig 4.36).

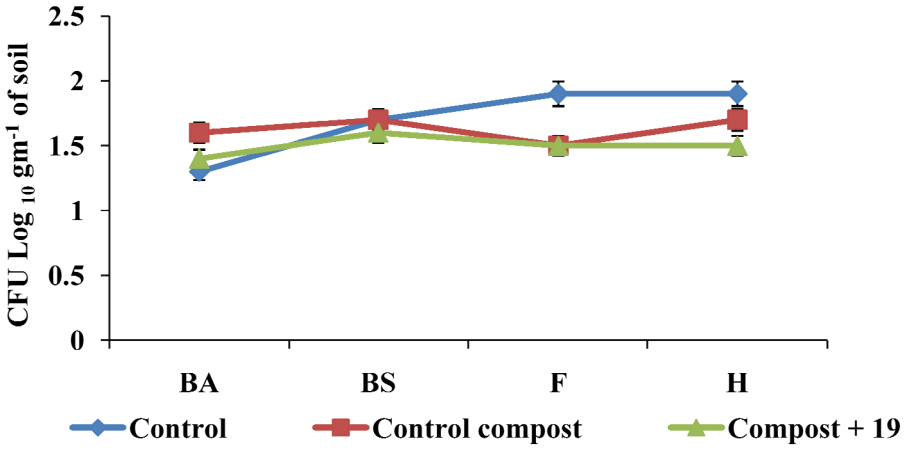


Fig 4.32: Survivability of inoculated *A. flavus* at different stages of cropping period during kharif 2007 in CDA 19 compost amended experiments.

4.24.6 Toxin detection in the groundnut samples from field experiments in *A. flavus* sick fields in *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19

Aflatoxin was measured in the seeds through indirect competitive enzyme linked immunosorbant assay (ELISA) method developed at ICRISAT. After harvest the pods were dried properly and shelled the seeds were ground finely and used for toxin detection by methanol extraction. In kharif 2006 experiment with *Pseudomonas* sp. CDB 35 revealed that percent toxin reduction was more in seed priming treatment with CDB 35 + *Rhizobium* (77%) followed by seed coating with CDB 35 (72%) and seed priming with CDB 35 (61%) over control (Fig: 36). With *Streptomyces* sp. CDA 19 seed treatments percent reduction of aflatoxin observed was 79% in seed priming with CDA 19 + *Rhizobium* followed by seed priming with CDA 19 (74%) and seed coating treatment (52%) over control (Fig 4.37, 4.38).

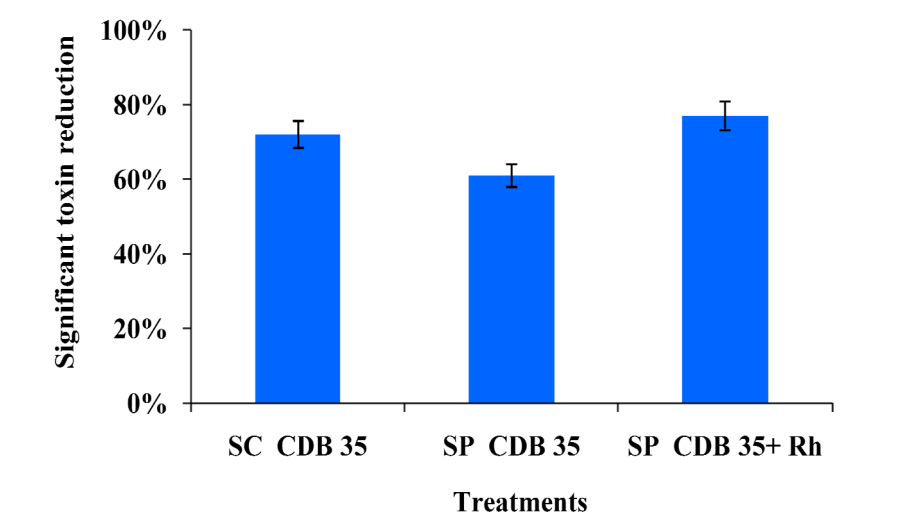


Fig: 4.33 Toxin concentration in various treatments in seed coating experiments with *Pseudomonas* sp. CDB 35 during kharif 2006

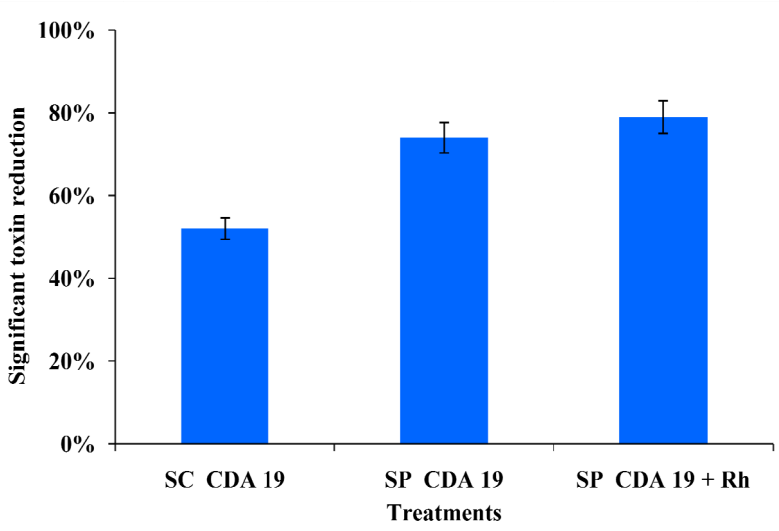


Fig: 4.34 Toxin concentration in various treatments in seed coating experiments with *Streptomyces* sp. CDA 19 during kharif 2006

4.24.6.1 Aflatoxin reduction in the seed coating experiments in rabi 06-07 experiments

Aflatoxin reduction in rabi 06-07 experiment with *Pseudomonas* sp. CDB 35 was 38% in seed priming with CDB 35 treatment followed by seed coating with *Pseudomonas* sp. CDB 35 (37%) and seed priming with *Pseudomonas* sp. CDB 35 + *Rhizobium* (32%) (Fig: 38). With *Streptomyces* sp. CDA 19 the percent aflatoxin reduction was more in seed coating (44%) followed by seed priming with *Streptomyces* sp. CDA 19 (43%) and seed priming with *Streptomyces* sp. CDA 19 + Rh (42%) (Fig: 4.39, 4.40).

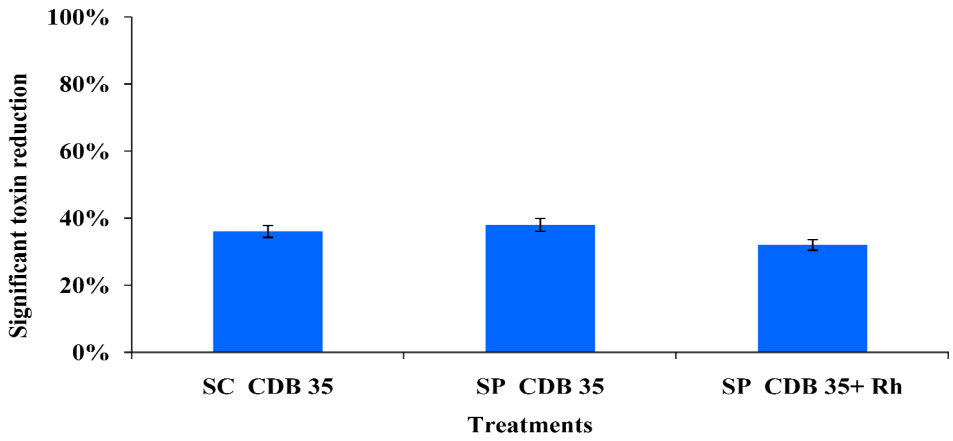


Fig: 4.35 Toxin concentration in *Pseudomonas* sp. CDB 35 seed application experiment conducted during rabi 06-07 experiment.

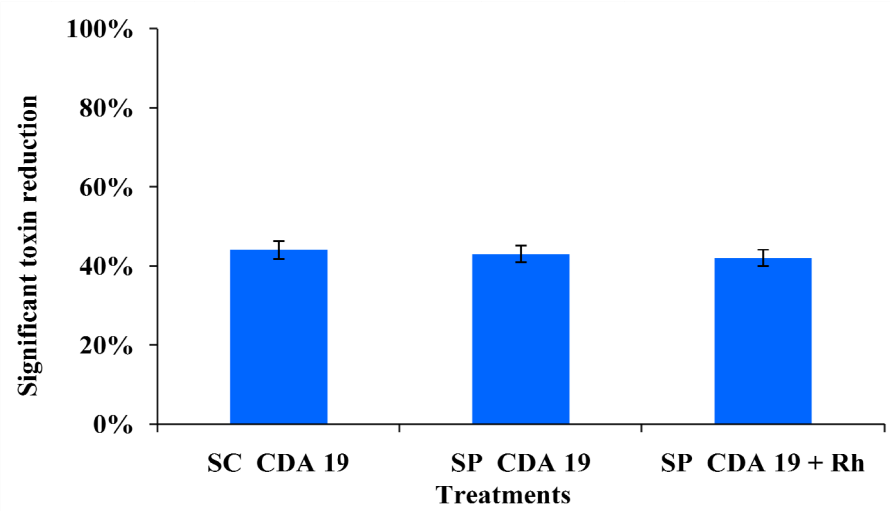


Fig: 4.36 Toxin concentration in the seed application experiment with *Streptomyces* sp. CDA 19 conducted during rabi 06-07 experiment.

4.24.6.2 Aflatoxin reduction in seed coating experiments in kharif 2007 experiments

In kharif 2007 seed coating experiments the toxin reduction was more in seed coating with *Pseudomonas* sp. CDB 35 (66%) followed by seed priming with *Pseudomonas* sp. CDB 35 (48%) and seed priming *Pseudomonas* sp. CDB 35 (34%) (Fig: 40). With *Streptomyces* sp. CDA 19 the aflatoxin percent reduction was more in seed priming with *Streptomyces* sp. CDA 19 and seed priming with *Streptomyces* sp. CDA 19 + *Rhizobium* (56%) followed by seed coating *Streptomyces* sp. CDA 19 (53%) over control (Fig 4.37, 4.38).

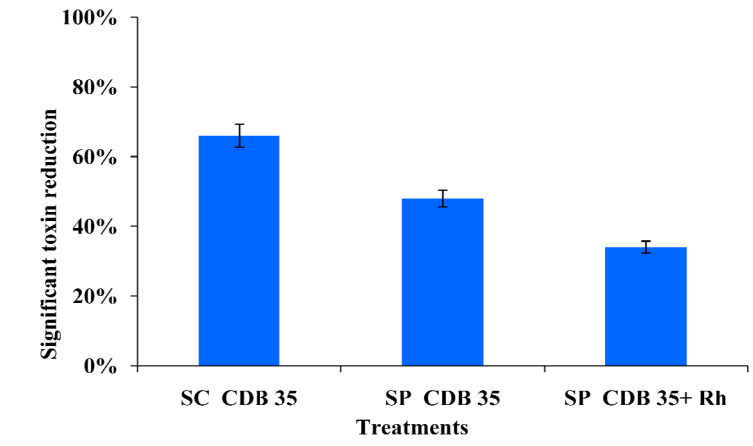


Fig: 4.37 Toxin reduction by bacterium *Pseudomonas* sp. CDB 35 seed experiments in kharif 2007

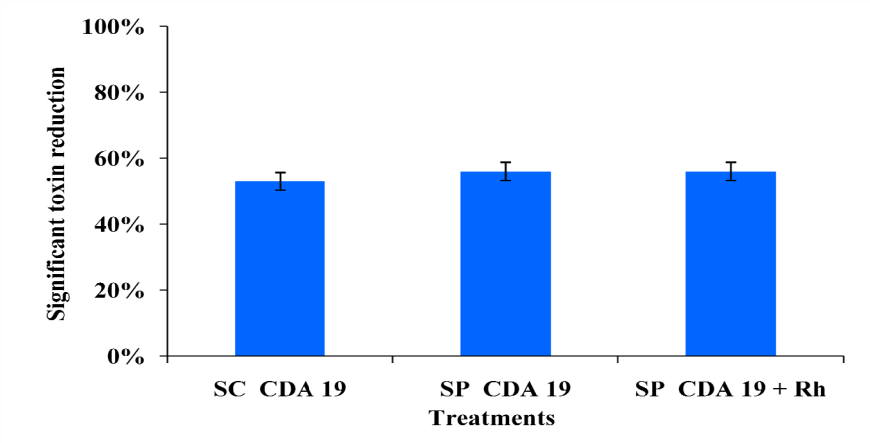


Fig: 4.38 Toxin reduction by *Streptomyces* sp. CDA 19 seed experiments in kharif 2007

4.24.7 Aflatoxin reduction in the enriched compost amended experiments

Aflatoxin reduction in the enriched compost experiments with *Pseudomonas* sp. CDB 35 was more in compost with CDB 35 (74%) followed by control compost (22%) over control. In the enriched compost experiments during rabi 06-07 experiments with *Pseudomonas* sp. CDB 35 the percent aflatoxin reduction was more or less same in compost *Pseudomonas* sp. CDB 35 and control compost which was 35% and 32% over control. In kharif 2007 enriched compost amended experiments, the percent reduction in the aflatoxin was 85% in both *Pseudomonas* sp. CDB 35 and control compost (Fig 46).

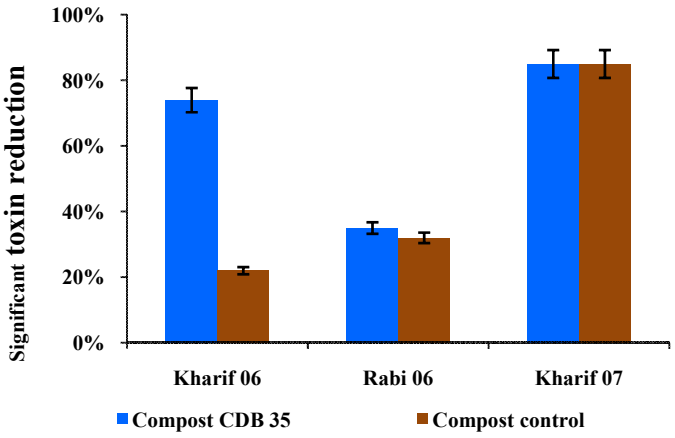


Fig: 4.39 Toxin concentration in the compost amended treatments along with *Pseudomonas* sp. CDB 35 during three seasons.

With *Streptomyces* sp. CDA 19 the percent aflatoxin reduction was more in compost with *Streptomyces* sp. CDA 19 (85%) followed by control compost (79%) over control. In the *Streptomyces* sp. CDA 19 compost and control compost treatments similar trend was observed of 67% and 63% reduction over control. *Streptomyces* sp. CDA 19 the percent toxin reduction was more in compost with *Streptomyces* sp. CDA 19 (91%) followed by control compost 86% (Fig: 4.44).

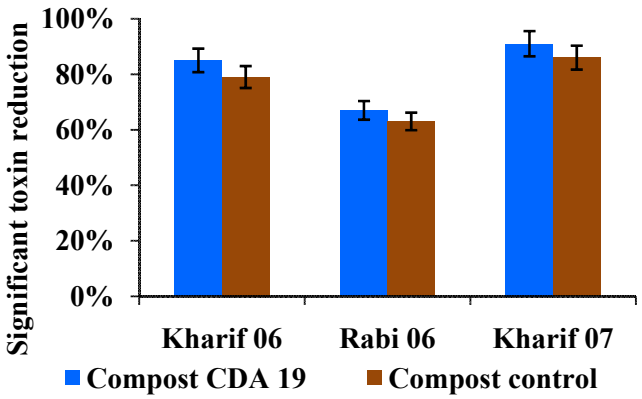


Fig: 4.40 Toxin concentration in the compost amended treatments along with *Streptomyces* sp. CDA 19 during three seasons.

4.25 Morphological, cultural and microscopic studies of the potential isolates of bacteria and actinomycetes:

The characteristics such as morphological including shape, cell shape, density, margin. Elevation, surface texture, and cultural such as pigment production and microscopic studies such as spore shape and gram stain were done for the potential isolates of bacteria and actinomycetes and their characters were given as abbreviation (Table : 4.32).

Table. 4.32: Morphological, cultural and microscopic characteristics of potential bacteria and actinomycetes

Name of the test	CDB 15	CDB 16	CDB 30	CDB 31	CDB 35	CDB 41	CDB 47	CDB 58	CDA 16	CDA 19	CDA 26
GS	+	+	+	+	-	-	+	-	+	+	+
SS	S	S	S	S	NS	NS	S	NS	S	S	S
Cell shape	R	R	R	R	SR	SR	R	R	F	F	F
Density	O	O	O	O	Tl	O	O	O	O	O	O
Elevation	C	C	C	C	C	C	Ra	C	C	C	C
Surface texture	Sm	Sm	Sm	Sm	Sm	Sm	Sm	Sm	R	R	R
Margin	I	W	I	W	Ro	Ro	Ro	Ro	Ro	Ro	Ro
Pigments	NP	NP	NP	NP	GY	NP	NP	NP	B	NP	NP

GS = Gram stain, +Gram positive rods, -Gram negative rods, SS = spore stain, S=sporulating, S=non-sporulating, R = rods, LR = long rods, SR = short rods, O = opacity, Tl = transluscent, C = convex, Ra = raised, Sm = smooth, W = wavy, Ro = round, E = entire, I = irregular, NP=no pigment, GY = Greenish yellow on Luria B agar medium. B= Brown pigment on bennets agar, F= Filamentous

4.26 Carbon source utilization by bacterium CDB 35

Many carbon sources are available in the plant rhizosphere and if the inoculated organism can make use of them it can readily colonise the roots and bring about the necessary action of either direct plant growth promotion or indirectly by rendering antagonism towards plant pathogenic fungi. So various carbon sources and nitrogen sources are used to screen antagonistic and plant growth promoting potential bacteria CDB 35 and actinomycete CDA 19 for their reduction patterns. The observation was made (colour change of the source which indicates the reduction) for every 12 H till 96 H of incubation. Out of 63 carbon sources tested 34 were readily used by bacterium CDB 35 and 29 were not used, the data is represented in the following table 4.33.

Table: 4.33 Carbon sources utilization by bacterium CDB 35

S No.	Carbon Sources	24 H	48 H	72 H	96 H
1	Water (control)	-	-	-	-
2	a Cyclodextrin	-	-	-	-
3	Dextrin	-	-	-	-
4	Glycogen	-	-	-	-
5	Tween 40	±	+	+	+
6	Tween 80	±	+	+	+
7	N-Acetyl- D Galactosamine	-	-	-	-
8	N-Acetyl- D Glucosamine	-	-	-	+
9	Adonitol	-	-	-	-
10	L-Arabinose	+	+	+	+
11	D-Arabitol	-	-	-	-
12	D-Cellobiose	-	-	-	-
13	i-Erythritol	-	-	-	-
14	D-Fructose	+	+	+	+
15	L-Fucose	+	+	+	+
16	D-Galactose	+	+	+	+
17	Gentiobiose	+	+	+	+
18	a-D-lactose	+	+	+	+
19	m-inositol	-	-	-	-
20	a-D-Lactose	-	-	-	-
21	Lactulose	-	-	-	-
22	Maltose	-	-	-	-
23	D-Mannitol	-	-	-	-
24	D-Mannose	+	+	+	+

.....**Table continued**

S No.	Carbon Sources	24 H	48 H	72 H	96 H
26	b-Methyl -D-Glucoside	-	-	-	-
27	D-Psicose	+	+	+	+
28	D-Raffinose	-	-	-	-
29	L-Rhamnose	-	-	-	-
30	D-Sorbitol	-	-	-	-
31	Sucrose	-	-	-	-
32	D-Trehalose	-	-	-	-
33	Turanose	±	±	+	±
34	Xylitol	-	-	-	-
35	Pyruvic Acid methyl Ester	+	+	+	+
36	Succinic Acid Mono-Methyl-Ester -		+	+	+
37	Acetic Acid	±	±	+	±
38	Cis-Aconitic Acid	+	+	+	+
39	Citric Acid	+	+	+	+
40	*Formic Acid*	+	+	+	+
41	D-Galactonic Acid Lactone	-	-	-	-
42	D-Galactouronic Acid	+	+	+	+
43	D-Gluconic Acid	+	+	+	+
44	D-Glucosaminic Acid	-	-	-	-
45	D-Glucuronic Acid	+	+	+	+
46	a-Hydroxybutyric Acid	±	+	+	+
47	b-Hydroxybutyric Acid	±	+	+	+
48	g-Hydroxybutyric Acid	-	-	-	-
49	p-Hydroxy Phenylacetic Acid	+	+	+	+

.....**Table continued**

S.No	Carbon sources	24 H	48 H	72 H	96H
50	Itaconic Acid	+	+	+	+
51	a-Keto Butyric Acid	±	+	+	+
52	a-Keto Glutaric Acid	+	+	+	+
53	a-Keto Valeric Acid	±	+	+	+
54	D,L-Lactic Acid	+	+	+	+
55	Malonic Acid	-	-	-	-
56	Propionic Acid	+	+	+	+
57	Quinic Acid	±	+	+	+
58	D-Saccharic Acid	-	-	-	-
59	Sebacic Acid	-	-	-	-
60	Succinic Acid	+	+	+	+
61	Bromosuccinic Acid	+	+	+	+
62	Succinamic Acid	+	+	+	+
63	Glucuronamide	+	+	+	+

4. 26. 1 Nitrogen source utilization by CDB 35

Among 33 different nitrogen sources used bacterium CDB 35 utilized 26 of them over 96 hours of incubation (Table : 33) which showed it could metabolise most of the sources for nitrogen nutrition and thus can colonise the rhizosphere and bring about the function (Table 4.34).

Table. 4.34 Nitrogen source utilization patterns by CDB 35 at various incubation times

S No.	Nitrogen Sources	24 H	48 H	72 H	96 H
1	L- Alanamide	+	+	+	+
2	D- Alanine	+	+	+	+
3	L-Alanine	+	+	+	+
4	L-Alanylglycine	±	+	+	+
5	L-Asparagine	+	+	+	+
6	L-Apartic Acid	+	+	+	+
7	L-Glutamic Acid	+	+	+	+
8	Glycyl-L-Aspartic Acid	-	-	-	-
9	Glycyl-L-Glutamic Acid	-	-	-	-
10	L-Histidine	-	-	-	-
11	Hydroxy-L-Proline	+	+	+	+
12	L-Leucine	+	±	+	+
13	L-Ornithine	+	+	+	+
14	L-phenylalanine	-	-	-	-
15	L-Proline	+	+	+	+
16	L-Pyroglutamic Acid	+	+	+	+
17	D-Serine	-	±	-	-
18	L-Serine	±	+	+	+
19	L-Threonine	-	±	-	-
20	D,L-Carnitine	±	+	+	+
21	g-Amino Butyric Acid	-	+	+	+

Table continued

S No.	Nitrogen Sources	24 H	48 H	72 H	96 H
22	Urocanic Acid	-	+	+	+
23	Inosine	-	+	+	+
24	Uridine	-	±	-	+
25	Thymidine	-	-	-	-
26	Phenyethylamine	-	-	-	-
27	Putrescine	+	+	+	+
28	2-Aminoethanol	±	+	+	+
29	2,3-Butanediol	-	±	-	-
30	Glycerol	±	+	+	+
31	D,L-a-glycerol Phosphate	±	±	±	±
32	a-D-Glucose-1-Phosphate	-	-	-	-
33	d-Glucose-6-Phosphate	±	±	±	±

4.27 Carbon and nitrogen source utilization by CDA 19

Among 72 carbon sources used for screening CDA 19 only 27 were utilized and among 24 nitrogen sources 5 were used indicating its selectivity (Table 4.35, 4.36).

Table 4.35 : Carbon Source Utililization by CDA 19 at various incubation times

S No.	Carbon Sources	24 H	48 H	72 H	96 H
1	Water	-	-	-	-
2	a-Cyclodextrin	-	-	-	-
3	B-Cyclodextrin	-	-	-	-
4	Dextrin	+	+	+	+
5	Glycogen	-	-	-	-
6	Inulin	-	-	-	-
7	Mannon	-	-	-	-
8	Tween 40	+	+	+	+
9	Tween 80	+	+	+	-
10	N-Acetyl-D-Glucosamine	-	-	+	+
11	N-Acetyl-D-Mannosamine	-	-	-	-
12	a-mygdolin	-	-	-	-
13	L-Arabinose	+	+	+	+
14	D-Arabitol	-	-	-	-
15	Arbutin	-	-	+	+
16	D-Cellobiose	-	-	-	-
17	D-Fructose	-	-	-	-
18	L-Fucose	-	-	-	-
19	D-Galactose	-	-	±	±
20	D-Galacturonic Acid	-	-	±	±
21	Gentiobiose	-	-	+	+
22	D-Gluconic Acid	-	-	-	-
23	a-D-Glucose	-	-	+	-
24	m-Inositol	-	-	±	+
25	a-D-Lactose	-	-	-	-
26	Lactulose	-	-	-	-
27	Maltose	-	-	-	-
28	Maltotriose	-	-	-	-
29	D-Mannitol	-	-	-	-
30	D-Mannose	-	+	+	+
31	D-Melezitose	-	-	-	-
32	D-Melibiose	-	-	-	-
33	a-Methyl-D-Galactoside	-	-	-	-
34	b-Methyl-D-Galactoside	-	-	-	-
35	3-Methyl-D-Glucose	-	-	-	-
36	a-Methyl-D-Glucoside	-	-	-	-

Table continued

Table continued

S No.	Carbon Sources	24 H	48 H	72 H	96 H
37	b-Methyll-d-Glucoside	-	-	-	-
38	a-Methyl- D-Mannoside	-	-	-	-
39	Palatinose	-	-	-	-
40	D-Psicose	-	-	±	-
41	D-Raffinose	-	-	-	-
42	L-Rhamnose	-	-	-	-
43	D-Ribose	+	+	+	+
44	Salicin	-	-	-	-
45	Sedoheptulosan	-	-	-	-
46	D-Sorbitol	-	-	-	-
47	Stachyose	-	-	±	-
48	Sucrose	-	-	-	-
49	D-Tagatose	-	-	-	-
50	Trehalose	+	+	+	+
51	Turanose	-	-	-	±
52	Xylitol	-	-	±	±
53	D-xylose	+	+	+	+
54	Acetic Acid	+	+	+	+
55	a-Hydroxybutyric Acid	-	-	-	±
56	b-Hydroxybutyric Acid	-	-	±	±
57	g-Hydroxybutyric Acid	-	-	-	-
58	p-hydroxybutyric Acid	-	-	-	-
59	a-keto Glutaric Acid	-	-	-	-
60	a-ketoValeric Acid	-	-	-	-
61	Lactamide	-	-	-	-
62	D-Lactic acid Methyl ester	-	-	-	-
63	L-Lactic Acid	-	-	-	-
64	D-Malic Acid	-	-	-	-
65	L-Malic Acid	+	+	±	±
66	Pyruvic acid methyl ester	-	-	-	-
67	Succinic acid monomethyl ester		-	±	±
68	Propionic Acid	+	+	+	+
69	Pyruvic acid	-	-	±	±
70	Succinamic Acid	-	-	-	-
71	Succinic Acid	-	-	±	-
72	N acetyl glutamic acid	-	-	-	-

4.36 Nitrogen source utilization by CDA 19

S No.	Nitrogen Sources	24 H	48 H	72 H	96 H
1	L-Alaninamide	-	-	-	-
2	D-Alanine	-	-	-	-
3	L-Alanine	-	-	-	-
4	L-Alanyl-Glycine	-	-	-	-
5	L-Aspargine	-	-	-	-
6	L-Glutamic Acid	-	-	-	±
7	Glycyl-L-Glutamic Acid	-	-	-	-
8	L-Pyroglutamic Acid	-	-	-	-
9	L-Serine	-	-	-	-
10	Putrescine	-	-	-	-
11	2,3-Butanediol	-	±	-	-
12	Glycerol	+	+	+	+
13	Adenosine	-	-	-	-
14	2,-Deoxy Adenosine	-	-	-	-
15	Inosine	-	-	-	-
16	Thymidine	-	-	-	-
17	Uridine	-	-	-	-
18	Adenosine-5,-Monophosphate	-	-	-	-
19	Thymidine-5,-Monophophate	-	-	±	-
20	Uridine-5,-Monophosphate	-	-	-	-
21	D-Fructose-6-Phosphate	-	-	±	-
22	a-D-Glucose-1-phosphate	-	-	-	-
23	D-Glucose-6-Phosphate	-	-	-	-
24	D,L-a-Glycerolphosphate	-	-	-	-

4.28 Partial 16s rRNA gene sequence (1493 bases) of biocontrol bacterial isolate CDB 35 and CDA 19

16 s rRNA gene sequence is the most conserved sequence in bacteria hence its analysis was considered as the most reliable method for molecular identification of bacteria at species level. 16s rRNA gene was isolated and amplified from DNA of CDB 35. For this process forward 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) primers were used to amplify the gene about 1500bp. The resultant PCR products were run on agarose gel electrophoresis along with positive control (*E. Coli* genomic DNA). PCR products were purified using Montage PCR Clean up kit (Millipore) and were used for sequencing. A

sequence of 1456 base pairs was obtained and further it was analyzed using eZ-Taxon server to find out closely related bacteria.

The sequence analysis for streach of 1493 bases of 16s rRNA gene sequence of the isolate The neighbour joining phylogenitic tree further confirmed that the strain CDB 35 phylogenetically Related to species of *Pseudomonas aeruginosa* and CDA 19 related to *Streptomyces Cavourenses*. The closely related species of *Pseudomonas aeruginosa* is represented in Fig 4.49, Table 4.37 and for *Streptomyces cavourenses* is represented in Fig 4.44, Table 4.38.

TATCGGATTACTGGGCGTAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATG
TGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAG
AGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGA
TATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGA
CACTGAGGTAAGAGTTGTGATCATGGCTCAGATTGAACGCTGGCGGCAGG
CCTAACACATGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAG
CGGCCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATA
ACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTG
GGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTA
GTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGA
GGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGC
CGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGG
AAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAG
CACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCG
TTANAAANAANGCTTTTTTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTT
GAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTAC
GGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTT
GACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGA
CACAGGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AAGTCCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCCTCCG
GGTGGGCACTTCTAGGAGACTGCCGGTGACAAACNGGAGGAGGTGGGGG
ATGACGTCAAGTCATCATGGGCCTTACGCCAGGGCTACCCCCTGCTAAAA
TGGTCGGTACCAAGGGTTGCCAAGCCGNNAGGTGGAGCTAATCCCANAAA
AACGATCGTAGTTCGGAATCGCAGTCTGCACTCACTGCGTGAAGTCGGAA
TCGCTAGTAATCGTGAATCAGAATGTCACGGTGATACGTTTCCNGGNCTTG
TACACACCGCCCCGTCNCACNTGGGAGTGGGTGCTNCAGAAGTAGCTAGT
CNANCCGCAAG

Fig: 4.41 Partial 16s rRNA gene sequence (1456 bases) of biocontrol bacterial isolate CDB 35

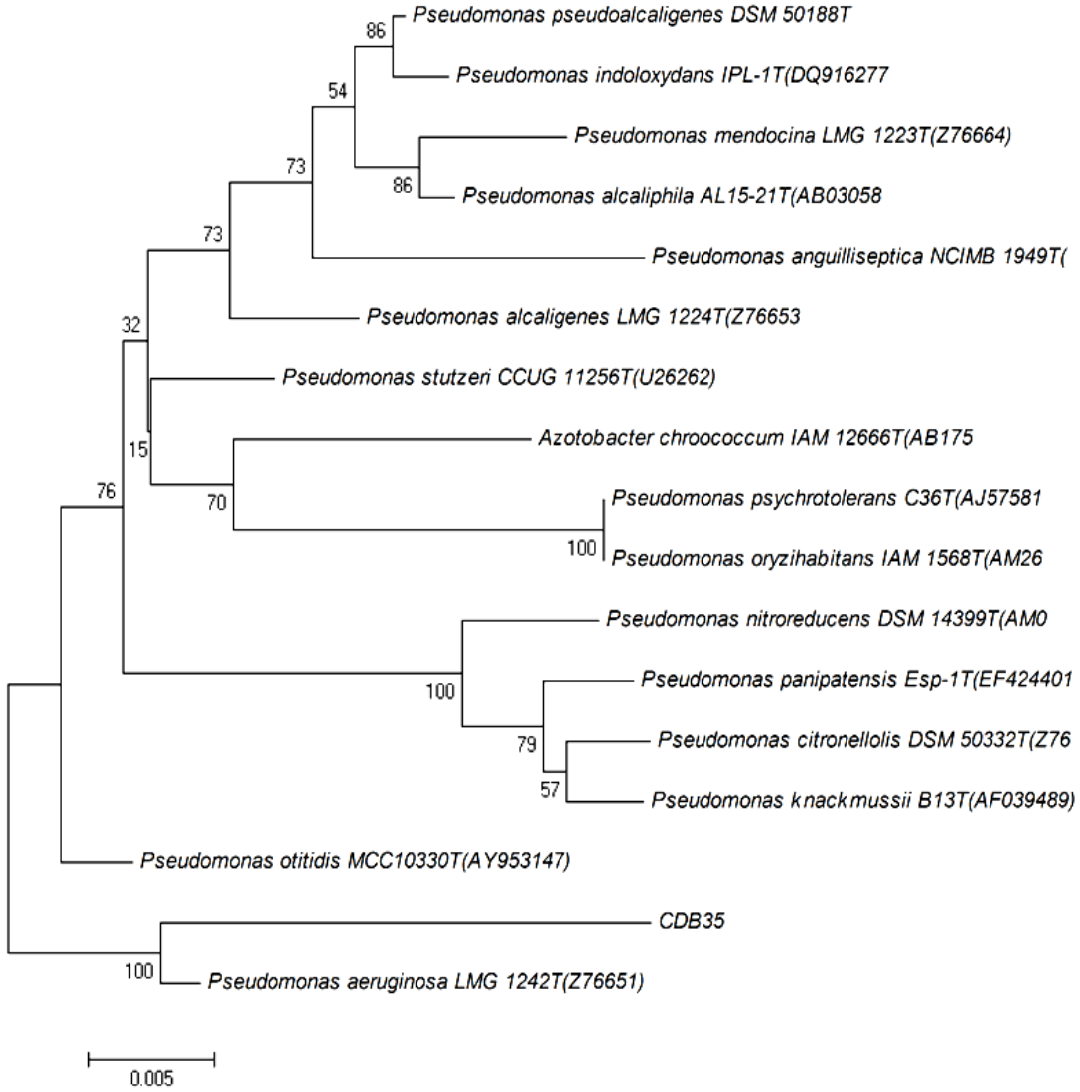


Fig: 4.42 Phylogenitic tree of *Pseudomonas aeruginosa* CDB 35 associated with other members of the *Pseudomonas* genus. Distance matrix was calculated on the basis of Jukes and Cantor (1969) and topology was inferred using the neighbour-joining (nj) method based on boot strap analysis of 1000 trees.

Table: 4.37 List of closely related 16 s RNA partial gene sequences (with accession numbers and % similarity) of *Pseudomonas* sp. CDB 35 partial 16 s rRNA gene sequence obtained by analysing in ez taxon server

Rank	Name/Title	Strain	Accession	Pairwise Similarity	Diff / Total count
1	*Pseudomonas aeruginosa*	LMG 1242m	276651	98.259	21/1206
2	*Pseudomonas otltidis*	MCC10330(T)	AY953147	96.903	38/1227
3	*Pseudomonas stutzeri*	CCUG 11256m	U26262	95.934	49/1205
4	*Pseudomonas alcaligenes*	LMG 1224m	Z76653	95.861	50/1208
5	*Pseudomonas pseudoalcaligenes*	DSM 50188(T)	276675	95.526	54/1207
6	*Pseudomonas citronellolis*	DSN 50332(T)	276659	95.360	56/1207
7	*Pseudomonas indoloxydans*	IPL-1(T)	DQ916277	95.238	57/1197
8	*Pseudomonas nitroreducens*	DSM 14399m	AM088474	95.168	59/1221
9	*Pseudomonas psychrotoierans*	C36(T)	AJ575816	95.033	60/1208
10	*Pseudomonas anguilliseptica*	NCIMB 1949m	X99540	95.009	57/1142
11	*Pseudomonas mendocina*	LMG 1223m	276664	95.004	60/1201
12	*Pseudomonas knackmussii*	B13(T)	AF039489I	94.951	62/1228
13	*Pseudomonas alcaliphila*	AL1S-2im	AB030583	94.950	61/1208
14	*Azotobacter chroococcum*	IAM 12666m	AB175653	94.876	60/1171
15	*Pseudomonas oryzihabitans*	IAM 1568m	AM262973	94.870	61/1189
16	*Pseudomonas panipatensis*	Esp-im	EF424401	94.855	62/1205

GGACTTTGNTTTTTAGAGTTTTGAATCATGGCTCAGGACGAACGCTGGCGG
CGTGCTTAACACATGCAAGTCGAACGATGAAGCTTTCGGGGTGGATTAGT
GCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAG
CCTGGAAACGGGGTCTAATACCGGATAATACTTCTGCCTGCATGGGTGGG
GGTTGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTT
GGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGG
GCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGC
CGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAG
AAGCGCAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGC
GTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCT
TAACCCCGGGTCTGCATTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAG
ATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACAC
CGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGAGCGAAACN
ANGTCTCACGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGC
AGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAA
CTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTA
ATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAAG
CATCAGAGATGGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGGGGACTCACAG
GAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCAT
CATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATG
AGCTGCGATGCCGTGAGGCGGAACGAATCTCAAAAGCCGGTCTCAGTTCG
GATTGGGGTCTGCAACTCGACCCATGAAGTCCGAGTTGCCTAGTAATCGC
AGAATCAGCATTGCTGCNGTGAATACGTTTCCCGGGCCTGTACACACCGC
CCCGTCNCCGTCACAAAGTCGGTAACACCCGAATGGCCCACCCCCTGT

Fig: 4.43 Partial 16s rRNA gene sequence (1460 bases) of biocontrol actinomycete isolate CDA 19.

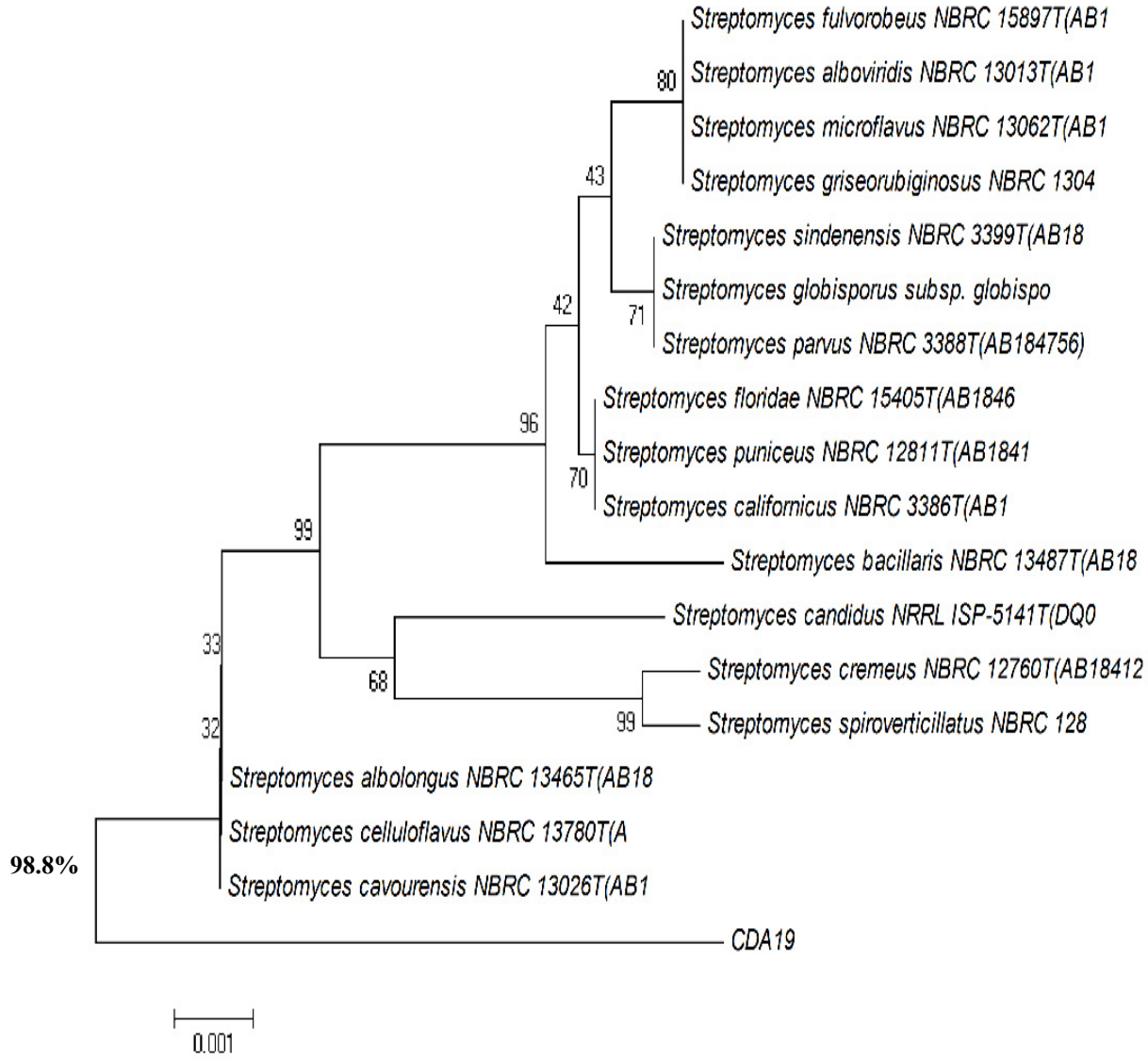


Fig: 4.44 Phylogenitic tree of *Streptomyces cavourensis* CDA 19 associated with other members of the *Streptomyces* genus. Distance matrix was calculated on the basis of Jukes and Cantor (1969) and topology was inferred using the neighbour-joining (nj) method based on boot strap analysis of 1000 trees.

Table: 4.38: List of closely related 16s rRNA partial gene sequences (with accession numbers and % similarity) of *Streptomyces* sp to the *Streptomyces* sp CDA 19 partial 16 s RNA gene sequence obtained by analysing in ez taxon server.

Rank	Name/Title	Strain	Accession	Pairwise Similarity	Diff/Total count
1	*Streptomyces cavourensis*	NBRC 13026(T)	AB184264	98.851	16/1393
2	*Streptomyces puniceus*	NBRC 12811(T)	AB184163	98.492	21/1393
3	*Streptomyces floridae*	NBRC 15405(T)	AB184656	98.492	21/1393
4	*Streptomyces bacillaris*	NBRC 13487(T)	AB184439	98.421	22/1393
5	*Streptomyces californicus*	NBRC 3386(T)	AB184755	98.421	22/1393
6	*Streptomyces fulvorobeus*	NBRC 15897(T)	AB184711	98.418	22/1391
7	*Streptomyces candidus*	NRRL ISP-5141(T)	DQ026663	98.351	23/1395
8	*Streptomyces microflavus*	NBRC 13062(T)	AB184284	98.350	23/1394
9	*Streptomyces alboviridis*	NBRC 13013(T)	AB184256	98.349	23/1393
10	*Streptomyces griseorubiginosus*	NBRC 13047(T)	AB184276	98.349	23/1393
11	*Streptomyces globisporus subsp. globisporus*	NBRC 12867(T)	AB184203	98.348	23/1392

CHAPTER - 5

DISCUSSION

World's economy largely depends on agriculture and this is severely affected by invasion of pests and pathogens leading to serious losses for trade (Wrather and Sweets, 2008). Mycotoxin contamination is one such problem which does not affect crop productivity but it makes the produce unfit for consumption. Mycotoxins such as aflatoxins, fumonisins, trichothecenes, and ochratoxins are contaminants of many agronomic crops worldwide, causing both economic losses and health effects (Plaumbo and Keeffe et al 2008). Though there are many regulations for mycotoxin management in developed countries, risk of mycotoxin exposures continues due to lack of food security, poverty and malnutrition (Williams et al, 2004). Of the various mycotoxins aflatoxins have gained importance due to the intensity of the deleterious effects rendered to humans and animals. Most of the antagonistic microorganisms are found abundantly in nature and of them composts form a rich source where they can be developed into effective biocontrol agents (Hameeda et al. 2006b). Many approaches, including biological control, are being investigated to manage, reduce, and finally eliminate aflatoxin contamination of various crops though field efficacy of these stratagies have been very limited till date (Waliyar et al, 2005). Very few biocontrol organisms like, bacteria yeast, and filamentous fungi have been investigated in several croping systems, for control of mycotoxigenic fungi. Several studies have suggested that some actinomycetes have anti-fungal activity (Boudemagh et al. 2005; Dhanasekaran et al. 2005). However, none of them have been reported for the control of mycotoxigenic fungi.

Post harvest contamination of peanut kernels with *A. flavus* is mainly a result of infection under field conditions (Dorner and Cole, 2002). Atoxigenic *A. flavus* and

A. parasiticus are the only bioagents which have proved till date as effective against their toxigenic counterparts and has reached market level (Dorner 2004).

Thus our study mainly focussed on identifying biocontrol agents other than fungi against *A. flavus* belonging to bacteria and actinomycetes. Composts are a rich source of many biocontrol and plant growth promoting organisms (Hameeda et al. 2006a), in view of this 80 bacteria and 40 actinomycetes were isolated from rice straw compost (RSC) and tested for their ability to suppress *A. flavus*. Among them eight bacteria and three actinomycetes inhibited the fungus significantly in double layer method on GCY medium (Photograph: 4.1). These potential isolates were further screened by dual culture method against *A. flavus* on GCY, KB and PDA media, among them bacterial isolate *Pseudomonas aeruginosa* (CDB 35) and actinomycete isolate *Streptomyces cavourensis* (CDA 19) inhibited *A. flavus* radial growth significantly than rest of the isolates (Table: 4.2 , 4.3) . *Bacillus subtilis* (NK- 330) and *Streptomyces* strain AS1 inhibited radial growth and aflatoxin production of *A. flavus* and *A. parasiticus* in the laboratory conditions (Kimura and Hirano, 1988; Sultan and Magan, 2011). Both CDB 35 and CDA 19 showed broad spectrum antifungal activity inhibiting five major soil borne plant pathogenic fungi that causes yield losses in the semi arid tropics (SAT). The antifungal activity of these isolates was comparable with several other antagonists which are already reported. Most of the isolates which are reported that showed prominent broad spectrum antifungal activity majorly belonged to *Pseudomonas aeruginosa* (Viji et al 2003). Pseudomonads are reported to be distributed in a wide range of agricultural ecosystems due to their wide adaptation and good root colonizing ability (Raj et al, 2004; Tripathi et al, 2005). Of all the actinomycetes reported till date *Streptomyces* sp. are proved to be efficient biocontrol agents suppressing various plant pathogenic

fungi (Nassar et al, 2005; Hamdali et al, 2008a).

The antagonistic nature of eight bacteria and three actinomycetes were screened against five fungi using three different media to know the effect of different nutrients on the inhibition of the fungus (Photograph: 4.2, 4.3). The mean fungal inhibition of *A. flavus* by eight potential bacteria and the inhibition of the five plant pathogenic fungi by CDB 35 was more on KB medium (63% and 64%) when compared to other two media (GCY and PDA) (Fig: 4.1, 4.2) this might be due to the nitrogen source present in it and might have enhanced the antagonistic activity. King's B medium enhances pigment production by *Pseudomonas* sp. favors the siderophores which bring about the inhibition of the fungus (Buyer and Leong 1986). Whereas the mean fungal inhibition of *A. flavus* by three potential actinomycetes was more on PDA, with CDA 19 inhibition of the four fungi was more on KB (Fig: 4.3). Similar results of varied performance on two media were observed when *Burkholderia cepacia* strains were tested against the selected soil borne fungal pathogens (Baligh et al. 1999). Leifert et al 1995 explained the variation in the spectrum of antifungal activity of microorganisms or their metabolites which is not uncommon. The reason for the selection of three medium was to know the effect of nutrient rich and nutrient poor conditions on the biocontrol ability of the selected organisms.

CDB 35 produced needle like shaped structures when screened against *Macrophomina phaseolina* in dual culture method (Photograph: 4.11). *Pseudomonas aeruginosa* are reported to produce several greenish yellow crystalline structures of several mm in size (chlororaphin) which might be responsible for the antagonism (Kanner et al 1978). These substances might be the reason for CDB 35 to have the significant performance *in vitro* against various plant pathogenic fungi. In our study

we observed the colonization of the fungal mycelia plug with the spores of CDA 19 and preventing its growth explaining the fungistatic and fungicidal nature. (Photograph: 4.5(a).

All the potential bacteria and actinomycetes produced volatile antibiotics and reduced radial growth of *A. flavus* but maximum growth reduction was brought about by CDB 35 and CDA 19 (Table: 4.6, Photograph: 4.6). Volatile antibiotics produced by *P. fluorescens, P. aeruginosa, P. putida, Chromobacterium violaceum* and Actinomycetes of both streptomycete and non streptomycete type are reported responsible for fungal inhibition (Faramrazi et al, 2004; EL-Tarabily et al, 2000, 2006). CDB 35 produced HCN *in vitro* and this might be one of the reason for efficient fungal inhibition (Table: 4.9). Volatile metabolites as hydrocyanic acid (HCN) is produced by many bacterial strains and has been considered as important in biocontrol activity (Bano and Musarrat 2003; Fernando et al. 2005).

The antifungal activity of cell culture filtrate (CCF) of the selected CDB 35 and CDA 19 inhibited the radial growth of *A. flavus* showing compact colonies when compared to the control and after five days of incubation when the mycelia plugs were transferred to fresh PDA they did not grow even after three days of incubation explaining the fungicidal nature of the CCF (Table 4.7). Both CCF and cell pellet of CDB 35 and CDA 19 reduced the biomass of *A. flavus* under broth culture conditions, when compared to the CP the CCF of the organisms inhibited the fungus more this might be attributed to the presence of antifungal substances released by both CDB 35 and CDA 19 extracellularly (Table : 4.8).

Further under co-culture conditions when aeration was supplied the inhibitory effect varied indicating the oxygen requirement for the antagonistic activity. The percent biomass reduction by CDB 35 was more under stationary condition and for

CDA 19 it was more under shaking culture conditions. (Fig:4.8), reports suggest that presence of CO_2 and O_2 influences *A. flavus* growth (Landers et al, 1967)

Further the CCF of both CDB 35 and CDA 19 inhibited the *A. flavus* spore germination when observed under compound microscope (Photograph: 4.9). Both CDB 35 and CDA 19 produced many antifungal extracellular metabolites *in vitro* (Table: 4.9) and CDA 19 further produced chitinases which might be involved in spore inhibition. Cell free culture filtrates of *Pseudomonas* sp. GRS 175 and chitinase producing *Streptomyces plicatus* has been shown to inhibit spore germination and spore tube growth of various plant pathogenic fungi (Kishore et al 2005b; Abd-Allah, 2001). Reports suggested that the diffusible metabolites of Streptomyces AS1 and *Streptomyces maritimus* produced were the main mechanism of inhibition of *A. flavus* conidial germination and also spore inhibition (Sultan and Magan 2011; Al-Bari et al. 2007). Kimura and Hirano (1988) isolated a strain of *Bacillus subtilis* (NK-330) that inhibited growth and aflatoxin production by *A. flavus* and *A. parasiticus* in the laboratory. Cho et al. (2009) showed that the purified iturin of *B. pumilus* HY1 at inhibited the fungal biomass by more that 50%, also Palumbo et al. (2006) demonstrated that some strains of Bacillus species isolated from almonds reduced *A. flavus* growth to the extent that no visible mycelia occurred on YES and in 2% almond broth media after 7 days.

Along with spore inhibition many morphological changes in the mycelium of *A. flavus* were noted in the presence of CCF of both CDB 35 and CDA 19 when observed under electron microscopy. The mycelium was thin and placid indicating the draining of the cell contents and there were many morphological changes when compared to the control (Photograph: 4.10). In the presence of CCF of *Bacillus brevis*, hyphal tips of *Fusarium udum* showed characteristic swelling and also cells

were found to be bulbous and swollen with shrunken, granulated cytoplasm compared to hyaline cytoplasm, and rectangular cells were seen in control mycelia (Bapat and Shah, 2000). Reports of Prapagdee et al, (2008) suggested that the cell free culture filtrates of *Streptomyces hygroscopicus* when observed under microscope changed the hyphal morphology, including hypal swelling distortion and aggregation of plant pathogenic fungi. Reports suggest that coagulation of the cytoplasmic contents of *Phytopthora capsici* was observed when co-inoculated with *Pseudomonas fluorescence*, and the active compound from released from *Bacillus pumilus* inhibited spore germination of *A. flavus* and aborted elongating mycelium presumably by inducing a cell wall lesion (Diby et al 2005; Al-Bari et al. 2007).

Antagonism involves various mechanisms like competition for nutrients, antibiosis, hyperparasitism etc. (Bashan and Holguin 2002; Gamalero et al, 2002). All the eight potential bacteria and three actinomycetes were screened for various antagonistic traits like production of HCN, siderophores, chitinases, glucanases, lipases, cellulases, proteases, acid production, pigment production etc. Most of them produced at least one of the metabolite, but CDB 35 and CDA 19 produced most of the metabolites (Table: 4.6) which explains their biocontrol ability over the rest of the isolates. *Pseudomonads* are known to produce siderophores like, pyoverdine, pyochelin, pseudobactin , salicylic acids lipopeptides and HCN which aid in antifungal activity and *in vivo* disease control (Yang and Crowley, 2000, Haas and Keel, 2003). Production of various bioactive compounds by actinomycetes which render antagonism have been widely reported in many studies (Errakhi et al. 2007).

In the present study it was observed that increase in iron supplementation to the growth medium of *A. flavus* increased aflatoxin production explaining the vital role of iron in aflatoxin biosynthesis. With the increase in the concentration of iron

though there was no remarkable difference in *A.flavus* growth (Fig: 4.9) the aflatoxin production increased significantly (Fig: 4.10). This clearly explains the co-relation between the iron nutrition and the toxin production. Both CDB 35 and CDA 19 produced siderophores in iron deficient medium (Fig: 4.8, 4.7) thus might help in creating competition for iron nutrition for *A. flavus* and thereby minimize or inhibit the fungal proliferation. Siderophores may be one or a mixture of two or more antibiotics which bring about iron nutrition and also act as antibiotics inhibiting the fungal growth (Nassar et al. 2005, Geetha et al. 2008).

The lytic action brought about by the hydrolases like chitinases and β-1,3-glucanase (laminarinase) could be a possible reason for the antifungal nature of the biocontrol agents or might act in conjunction to the other metabolites produced by the same isolate. CDB 35 produced β-1,3-glucanase (Table: 4.10), CDA 19 produced chitinases (Table: 4.11) and β-1,3-glucanase (Table: 4.12). Fluorescent pseudomonad are reported to produce chitinase and β-1,3-glucanase and bring about inhibition of *R. Solani* (Arora et al 2007). Chitinolytic enzymes produced by *Bacillus cereus, Pantoea agglomerans, Paenibacillus, Serratia marcescens* and fluorescent pseudomonads are reported to be involved in biological control of *F. oxysporum* and *R. solani* (Someya et al, 2000; Singh et al, 2006). Actinomycetes like *Streptomyces griseus* (BH7, YH1) *Streptomyces sindeneusis,* also facilitate plant growth indirectly by producing chitinases, β-l,3-glucanases, siderophores and antifungal substances (Zarandi et al, 2009). *Streptomyces* sp. have been reported as able to produce chitinase (Gomes et al. 2001) and secondary metabolites (AL-Bari et al. 2007) active against *Aspergillus*. Furthermore, some *Streptomyces* strains produce compounds able to inhibit the aflatoxin production, and these do not affect fungal growth (Sakuda et al. 1996, 1999, 2000a,b; Yoshinari et al. 2007)

Both CDB 35 and CDA 19 were compatible with the groundnut specific rhizobial strains in plate culture conditions (Photograph: 4.16). Both the strains inoculated along with *Rhizobium* showed growth promotion of groundnut in pots using unsterilized soil (Table 4.17, 4.18). Previous studies by Hameeda et al, 2010 revealed that antagonists inoculated by seed priming method showed significant difference in dry matter yield and nitrogenase activity of chickpea and than seed coating method. Seed priming is also done to alleviate stress conditions for in vitro tissue-propagated plants (Nowak and Shulaev 2003). Co-inoculation of fluorescent *Pseudomonas* and *Rhizobium* improved plant growth of *Pisum sativum* (Dileep Kumar et al. 2001). Studies by Parmar and Dadarwa (1999) showed that *Pseudomonas* sp. that produce siderophores could increase the level of flavonoid-like compounds in the root which, increased total plant nitrogen in chickpea. In this study, both the strains were siderophore producers.

Various abiotic factors like different temperature gradient, pH and salinity levels were tested on growth of the two isolates CDB 35 and CDA 19. Both of them survived well at all pH (5, 6, 7 and 8) (Fig: 4.11) salinity levels (0, 50, 100, 150, 200 and 250mM) (Fig: 4.12) and temperatures (15,28,36,42 and 55 ºC) (Fig: 4.13). The reason behind evaluation of abiotic stress tolerance for the current strains was that the stress tolerant strains can be efficiently deployed in extreme environments where they can show better rhizosphere competence and saprophytic competitive ability. Environmental conditions especially high humidity and temperature favour fungal proliferation, but also drought conditions increase risk of aflatoxin contamination (Kerstin Hell and Charity Mutegi, 2011). If the selected biocontrol agent can tolerate to extreme environmental conditions it can compete with the pathogenic fungi in the rhizosphere in the arid and semiarid tropics. Some PGPR also elicit physical or

chemical changes related to plant defense, a aprocess referred to as 'induced systemic resistance' (ISR) (van Loon et al, 2004). ISR elicited by PGPR has suppressed plant diseases caused by a range of pathogens in both the glasshouse and field conditions (Jetiyanon and Kloeppar 2002). Studies of Timmusk et al, 1999 revealed that inoculation with the PGPR *Paenibacillus polymyxa* enhanced the drought tolerance of *Arabidopsis thaliana*. This further explains that the beneficial organisms bring about induced systemic tolerance in the plants.

Though the main emphasis was given on biocontrol aspect in this study, still the plant growth promoting traits were also characterized to know the potentiality of the biocontrol agents in aiding plant growth. Growth promoting traits like IAA production, rock phosphate solubilization and phytase production were evaluated for all the eight bacteria and three actinomycetes (Table: 4.13). CDB 35 showed all the three traits (rock phosphate solubilisation, phytase and IAA production) but CDA 19 showed only IAA production. CDB 35 solubilized rock phosphate under soil conditions by releasing gluconic acid and reducing the pH of the soil solution (Harini, 2005). The bound form of the phosphorus in the soil is released by the microorganisms (Arcand and Schneider, 2006). P-solubilizing microbes (PSMs) belong to *Bacillus, Pseudomonas*, *Micrococcus*, *Actinomycetes* etc. (Mba 1997, Rudresh et al, 2005). Both CDB 35 and CDA 19 produced IAA both in the presence and absence of tryptophan (Fig:4.19, 4.20) (Photograph: 4.15). Plant beneficial microorganisms bring about plant growth directly by producing IAA, gibberlins, etc (Ait Barka et al, 2000; Dong et al, 2004).

All the potential isolates of bacteria and actinomycetes were evaluated for plant growth *in vitro*, by plate assay CDB 35 and CDA 19 increased growth parameters like percent germination, root length and number of root lets significantly

over control (Table: 4.15). Shankar Naik et al, (2008) reported that the of rice, jowar, groundnut and finger millet. *Pseudomonas putida* DFC 31 a siderophore producing bacteria increased percent germination, root length and number of root lets of groundnut (Sarode et al, 2007).

From the inference of plate assay *in vitro* all the isolates were screened for their plant growth promotion of groundnut JL-24 under soil conditions in glasshouse. All the isolates promoted growth by increasing root length, shoot length and biomass weight (dry weight) (Table:4.16).

Both CDB 35 *and* CDA 19 were efficient over the rest of the isolates, this may be attributed to the plant growth promoting characters shown by them like IAA production, rock phosphate solubilisation and siderophore production. Though there was no clear relation between *in vitro* auxin production and plant growth, it was observed that both the isolates CDB 35 and CDA 19 produced auxins even in the absence of precursor L- tryptophan. This may be attributed that these isolates can enhance the plant growth even in the absence of the precursor. Studies of Asghar et al, (2002) revealed that there was a positive correlation between L-tryptophan-derived auxin production by PGPR *in vitro* and grain yield, number of pods and branches per plant of *Brassica juncea.*

Development of a suitable carrier material is very important as it decides the viability of the potential organism and thus helps in bringing about the required effect on application. For a bio control agent to work in field conditions efficiently, proper carrier material and efficient delivery system is very important (Hameeda et al. 2009).

In the present study peat, lignite and talc were selected for development of suitable formulations for the potential antagonistic isolates, CDB 35 and CDA 19. Both of them survived well till 180 days of inoculation (Fig: 4.21, 4.22), the other two

(lignite and talc) tested had less population compared to peat thus we can consider peat as a good carrier material for these isolates. Peat based formulation had viable populations of *Bacillus subtilis* AF 1 which was Log 9.0 CFU g^{-1} (Manjula and Podile, 2005), *Bacillus firmis* GRS 123 and *Bacillus megaterium* GPS 55 which was Log 7.0 CFU g^{-1} (Kishore et al. 2005a), *Serratia marcescens* EB 67 Log 6.2 CFU g^{-1} (Hameeda et al. 2008).

Peat based inoculums of CDB 35 and CDA 19 were tested for growth promotion under glasshouse conditions through two different modes of inoculation as seed coating and seed priming. Overall growth promotion was increased in two modes of application but root length and shoot length increased in seed priming treatment with CDB 35 and biomass weight with seed coating treatment (Table: 4.17,4.18). Reports suggest that hydration for different durations in three cultivar of Bambara groundnut significantly increased the germination per cent, seedling emergence, and dry weight (Massawe et al, 1999). Unsoaked seeds started germination six days after sowing while hydrated seeds started germination on the fourth day. Inoculation of corn seeds with *Azospirillum braziliense* increased dry matter accumulation (Dilfuza, 2007). Seed biopriming of chickpea decreased the disease incidence and promoted plant growth (Hameeda et al 2010)

CDB 35 and CDA 19 survived well in the vermicompost till 90 days (Table: 4.19). Composts depending on the degree of maturity, provide a rich medium supporting a high microbial activity (Chen et al, 1988) and may also contain a diverse microbial population and promote plant growth (McKinley et al, 1984).

Application of enriched vermicompost with CDB 35 and CDA 19 enhanced plant growth of groundnut under green house conditions (Table: 4.20). Dual inoculation of compost with plant growth promoting bacteria (PGPB) is an

uncommon practice but various studies showed increased growth when enriched compost was amended (Tomati and Galli 1995). Composts and vermicomposts are known to promote plant growth when added to soil enhance enzymatic activity and release of phytohormones (Atiyeh et al. 2000a; Arancon et al. 2004b). Composts do not stimulate the growth of microorganisms in the rhizosphere but aid as nutrient source and thereby bring about root colonization of the desirable microorganisms.

Peat based inoculums of CDB 35 and CDA 19 were evaluated under field conditions. All the treatments of seed coating, seed priming and seed priming with rhizobium showed significant results for plant growth parameters like total biomass weight, pod weight and seed weight for three seasons of evaluation under *A.flavus* sick field conditions at ICRISAT. One reason could be as both CDB 35 and CDA 19 released a wide variety metabolites which help in both plant growth and biocontrol. They also metabolised a wide variety of carbon and nitrogen sources *in vitro* which further explains their wide adaptation to different rhizosphere conditions. Interactions occurring in the rhizosphere between plant, soil and microorganisms are important and mainly depends on soil nutrient level and on the microbial effect in rhizosphere (van Veen et al. 1997). Biocontrol agents, applied as seed treatment provide unique benefits for crop protection as the antagonist is in close proximity to the seedling and also reduce cost as the inoculums needed is less compared to that required for soil treatment (Aghighi et al. 2004).

The potential uses of plant associated bacteria as agents stimulating plant growth and managing soil and plant health by rendering antagonism towards the plant pathogens in the rhizosphere (Sturz et al 2000, and Welbaum et al, 2004). Numerous studies have shown a substantial increase in dry matter accumulation and seed yield following inoculation with plant growth promoting microorganisms (Wani et al,

2007). Seed priming with CDB 35 and CDA 19 showed significant increase in the plant growth parameters compared to seed coating of explains that hydration of the seeds have more germination percent over non hydrated. Seed priming with PGPR had significant effects on grain yield, plant height, number of kernels per ear and number of grains per ear row (Seyed Sharifi et al, 2011). seed priming with PGPR increased dry matter accumulation and grain yield of wheat , soy bean (Zaidi and Khan 2005, Basu and Choudhary 2005). Soaking the seeds of three sunflower genotypes in water for 2 hours significantly enhanced germination percentage, field emergence, root length, shoot length and vigour index Khan et al, 2003. Seed bio priming decreased disease incidence in chickpea by seed biopriming with *Pseudomonas* sp. CDB 35 and *Serratia* sp. EB 67 (Hameeda et al, 2010).

When the seeds were tested for aflatoxin levels it was noticed that toxin levels decreased in the treatments compared to control which suggests that these organisms along with plant growth promotion bring about the biocontrol mechanism by inhibiting the metabolite production by *A. flavus*. Bacillus subtilis prevented aflatoxin contamination in corn in field tests when ears were inoculated with the bacterium 48 hours before inoculation with *A. flavus* (Cuero et al., 1991). Several bacterial species were evaluated in glasshouse and field experiments for biological control of A. flavus infection and aflatoxin contamination of peanuts (Mickler et al., 1995). Peanut seeds were treated with the bacteria prior to planting and bacterial suspensions were applied as a soil drench (glasshouse) or sprayed over the row (field) at the mid-peg stage (80–90 days after planting). Only one bacterial strain (Xanthomonas maltophila) produced a significant reduction ($P = 0.05$) in A. flavus infection of pods at harvest in both the glasshouse and field studies. One strain of Pseudomonas putida reduced the incidence of A. flavus on harvested seed. Further development of biological control of aflatoxin

in peanuts using bacteria has not been reported. Six of 892 bacterial isolates indigenous to cotton inhibited *A. flavus* growth in an in vitro assay using inoculated cottonseed (Misaghi et al., 1995). One of those isolates, Pseudomonas cepacia (D1), significantly reduced A. flavus damage to cotton locules by 41–100% when the bacterium was inoculated simultaneously with A. flavus in field studies. Further work showed a reduction in the level of *A. flavus*-infected cottonseed when plants were spray-inoculated with a suspension of D1 (Misaghi, 1995).

Enriched compost prepared by using *Pseudomonas* sp. CDB 35, *Streptomyces* sp. CDA 19 was tested at field level against *A. flavus* and aflatoxin contamination. Survivability of both *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 was studied till 90 days of inoculation and both survived well, Application of this in *A. flavus* sick field improved plant growth parameters over control for all the three years of study. Aflatoxin reduction in the seeds was also significantly higher. Lime application, use of farm yard manure and cereal crop residues as soil amendments have shown to be effective in reducing *A. flavus* contamination as well as aflatoxin levels by 50-90%, as described by Waliyar et al, (2008).

Compost amendment not only increases the organic matter in the soil but also help in root colonization of many other beneficial organisms. Studies revealed that when compost in conjunction with beneficial microorganisms not only plant growth but also colonization VAM in sorghum and maize (Hameeda et al. 2007). Previous studies of Bacilio *et al,* (2003) where *Azospirillum* sp. was added along with composts showed improvement in germination and alleviated the noxious effect of cattle ranch composts. Studies Filip and Kubat 2001 revealed that microorganisms in soil use humic acids bring about degradation, transform and mineralize and thus make the unavailable nutrients available to plants.

Though the potentiality as low-input agents conferring protection to the plants, application of biocontrol agents has been effected by inconsistent performance in field tests (Thomashow et al, 1996), this can be explained to their poor ability to colonize roots not able to maintain required population in the rhizosphere (Schroth et al 1981, Weller et al 1988). The inoculated Pseudomonas sp. CDB 35 survived well both in the seed coating experiments and compost amended experiments. This could explain that *Pseudomonas* sp. CDB 35 survived well in the rhizosphere of the groundnut and colonize the roots and bring about the necessary plant growth promoting and or biocontrol activity. Given the importance of rhizosphere competence as a prerequisite of effective biological control, understanding root-microbe communication (Bais et al. 2004, Ping and Boland 2004) as affected by genetic (Kilic-Ekici and Yuen, 2004; Okubara et al, 2004) and environmental (Pettersson and Baath, 2004) determinants in spatial (Bais et al, 2004) and temporal (Chatterton et al, 2004) contexts, will significantly contribute to improve the efficacy of these biocontrol agents.

Composts when amended in soils facilitate for the multiplication of beneficial microorganisms and thus protect plants from soil-borne pathogens (Grappelli et al, 1987; Ros et al, 2005). Microorganisms associated with farm waste compost that can enhance growth of plant and productivity have been known from past (Brown 1974; Hameeda et al, 2006a).

A wide range of carbon and nitrogen sources were tested for their utilization by the two isolates *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19. As the rhizosphere soil have a varied types of carbon and nitrogen sources and if the potential organism can use a wide range of them then it can bring about the desired action of growth promotion either by inhibiting the pathogenic fungus or release of

growth elicitors.

Molecular identification by 16s RNA sequencing showed CDB 35 as *Pseudomonas aeruginosa* . Most of the reports are focused on *Pseudomonas* sp. as potential plant growth and biocontrol agents as they are widely dispersed in the agricultural system and they are also good colonizers of plant root system due to their competitive nature (Garbeva et al, 2004). Identification of CDA 19 revealed that it belongs to *Streptomyces* sp. Many species of actinomycetes particularly those belonging to the genus *Streptomyces* are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi and have been proven in many agricultural systems (Joo, 2005; Xiao et al 2002).

There have been very few studies using actinomycetes for biocontrol of Aspergillus section Flavi on peanuts, either pre- or post-harvest. Most of the successes to date have been achieved by applying certain non-toxigenic strains of A. flavus and A. parasiticus to the rhizosphere of peanut or maize plants. The applied strains occupy the same niche as the naturally occurring toxigenic strains and competitively exclude them when crops are susceptible to infection (Dorner 2004). Based on the concept that biocontrol agents isolated from the crop will be better adapted, they will potentially be more effective for biocontrol than microorganisms isolated from other sources (Kerry 2000). Nowadays, biological control methods are broadly used for the management of many phytopathogenic fungi. On this account a considerable number of formulations, using a large range of species, have already been used commercially (Fravel 2005). However, relative to the use of *Streptomyces* sp. as a biocontrol agent, there are only two approved commercial products: Mycostop and Actinovate, however, none of them are recommended for the control of *Aspergillus* sp. (Gardener and Fravel 2012).

CHAPTER - 6

Summary, Conclusions and Future Prospects

World's economy depends mostly on agriculture and this is severely effected by pests and pathogens. Of them mycotoxin contamination of the food and feed has gained importance due to their deleterious effects rendered when consumed by humans and animals. Among various mycotoxins aflatoxins produced by *Aspergillus flavus* and *A. parasciticus* has gained importance due to the deleterious effects rendered to both humans and animals as well as international trade. Several approaches have been developed to combat this problem of them biological control has gained importance due to its potentiality of management at preharvest stage of the crop and also due to its beneficial effects in maintaining the biodiversity and ecological balance.

In the present study to identify potential bio-control agents against *A. flavus* and aflatoxin contamination we have screened eighty bacteria, 40 actinomycetes and 40 fungi which were isolated from rice straw compost prepared at ICRISAT.. All bacteria and actinomycetes were studied for their antagonism against *A. flavus* by double layer method on GCY medium. Among them eight bacteria (CDB 15, CDB 16, CDB 30, CDB 31, CDB 35, CDB 41, and CDB 48) and three actinomycetes (CDA 16, CDA 19 and CDA 26) species significantly inhibited *A. flavus.* Among them CDB 35 (50 mm) and actinomycete CDA 19 (35 mm) showed significant zone of inhibition when compared to the rest of the isolates. To further evaluate and characterize the properties of strains all eight bacteria and three actinomycetes were screened against *A. flavus* by dual culture method on PDA, KB and GCY media to have the precise percent inhibition. All the selected strains inhibited *A. flavus* by more than 50% and among them CDB 35 and CDA 19 inhibited more ranging between 67-

89% and 40-62% respectively on the three media . These two (CDB 35 and CDA 19) were further selected to evaluate against other soil borne plant pathogenic fungi (*Aspergillus niger*, *Fusarium solani*, *Fusarium oxysporum*, *Fusarium udum*, *Macrophomina phaseolina* and *Sclerotium rolfsii*) in order to evaluate their broad spectrum antifungal activity. All the above mentioned fungi were inhibited by both CDB 35 and CDA 19 but *Fusarium oxysporum* was inhibited by 76% with CDB 35 and *Aspergillus niger* was inhibited by 60% with CDA 19 significantly. All the eight bacteria and three actinomycetes were screened for the volatile antibiotics where bacterium CDB 35 and actinomycete CDA 19 decreased the fungal diameter by (60-67%) and (52-54%) on the three media tested. Cell culture filtrate (CCF) of CDB 35 and CDA 19 was evaluated against *A. flavus* by poisioned food technique, maximum reduction of fungal diameter was observed at 50% v/v which was 93% with CDB 35 and 82% with CDA 19 respectively. Fungal biomass of *A. flavus* was reduced by 67% and 40% when co-cultured with CDB 35 and CDA 19 in potato dextrose broth. When the aeration was supplied by shaking the co-cultured medium at 180 rpm on a incubator shaker at 28$\pm$ 2 ºC the fungal biomass reduction was more under stationary conditions which was 77% than shaking culture condition being 60% with CDB 35 unlike with CDA 19 where fungal biomass reduction was more under shaking culture conditions (47%) than stationary condition (41%).

All the eight bacteria and three actinomycetes were screened for antagonistic and plant growth promoting traits. . Each isolate showed atleast one trait but CDB 35 and CDA 19 showed the production of HCN, siderophores, glucanases, proteases, acid production and pigment production. CDB 35 further showed the production of cellulases and lipases and CDA 19 showed the production of chitinases. When studied for plant growth promoting traits CDB 35 showed rock phosphate solubilisation,

phytase activity and IAA production and CDA 19 produced IAA. Siderophores which bring about antagonism was estimated for both the isolates in iron free medium for seven days of incubation and the percent decolouration was 8% for both CDB 35 and CDA 19.. Effect of iron nutrition on *A. flavus* growth and aflatoxin synthesis was studied by supplementing iron at various concentrations in to the media. It was observed though there was no visual difference in the growth but a considerable variation was observed in the toxin production where the production was maximum at 100 ppm iron 260.48 µg/Kg and at 9 days of incubation.

Hydrolytic enzymes like chitinases and β-1, 3 glucanases from CDA 19 and β-1,3 glucanases from CDB 35 were purified. . CDA 19 showed the production of chinases of 20 kDa size and β-1, 3 glucanases of 36 kDa size and CDB 35 showed the production of β-1, 3 glucanases of 32 kDa size. IAA which bring about plant growth promotion was estimated in the presence and absence of tryptophan at variuos concentrations and the observation showed that the production was maximum at 5mg/mL which was 580 and 593 µg/mL at 96 hours of incubation with CDB 35 and CDA 19 respectively.

The cell culture filtrate of both CDB 35 and CDA 19 was tested on the spore germination of *A.flavus* on a cavity slide under light microscope, both CDB 35 and CDA 19 inhibited spore germination. Effect of CCF of CDB 35 and CDA 19 on A. *flavus* mycelial morphology was observed under electron microscopy and it showed that the sporulation was rapidly inhibited and also there was mycelial shrinkage and swelling of the tips was noted.

Plant growth promotion by the eight potential bacterial and three actinomycete isolates was evaluated on gropundnut (JL-24) by plate assay *in vitro*. The germination percent, root length and number of rootlets increased significantly over control in all

the treatments. Among them CDB 35 and CDA 19 showed more germination per cent (85%, 76%), root length (63%, 61%) and number of rootlets (34%, 31%) over control. All the isolates were tested for the plant growth promotion under soil conditions in glasshouse, among them CDB 35 and CDA 19 increased the root length (55%, 48) Shoot length (27%, 32%) and dry weight (38%, 25%) significantly over control. Peat, lignite and talc were tested for the development of suitable carrier materials for CDB 35 and CDA 19 up to six months, CDB 35 and CDA 19 survived well and maintained population of Log $_{10}$ 5.9 gm^{-1} CFU and Log $_{10}$ 5.8 gm^{-1} CFU respectively at 180 days after inoculation.

Peat based inoculums of both the isolates were screened under glasshouse conditions for plant growth promotion through various treatments like seed coating and seed priming treatments with groundnut variety JL- 24 as test crop. With CDB 35 the root length and shoot length was 35% and dry weight of 23% and with CDA 19 root length (30%), shoot length (40%) and dry weight (23%) which was more significant than control.

Enriched compost was prepared using CDB 35 and CDA 19 both of the them could maintain the population of Log $_{10}$ 6.2 CFU gm $^{-1}$ and Log $_{10}$ 5.1 gm^{-1} CFU gm^{-1} till 90 days of inoculation. This enriched compost was evaluated under green house conditions and plant growth parameters were studied. With both . CDB 35 and . CDA 19 compost treatments there was significant increase in root length (41% 34), shoot length (42% 32) and dry weight (51%, 45) over control.

CDB 35 and CDA 19 were evaluated under *A. flavus* sick field conditions as peat based inoculums. Both the isolates increased halum weight, seed weight and pod weight in all the three seasons of study. Overall significance over control ranged between 14-37% for halum weight, 4-40% for pod weight and 13-59% for seed

weight across the treatments with CDB 35. With CDA 19, halum weight ranged between 13-36% pod weight being 5-39% and seed weight ranged between 10-57% across the treatments. Aflatoxin detection was done in the seeds by ELISA after harvesting and it was observed that with CDB 35 seed application treatments the toxin reduction ranged between 34-77% and 43-79% with CDA 19 respectively across the treatments.

Enriched compost prepared by usingCDB 35, CDA 19 was tested at field level and it was observed both the organisms along with the compost increased the growth parameters in the three seasons of study where the halum weight ranged between 4-35%, pod weight ranged between 12-27%, and seed weight ranged between 6-41% over control with CDB 35. With CDA 19 the halum weight ranged between 3-31%, pod weight by 8-31% and seed weight by 28-48% over control. Toxin reduction ranged between 35-85% and 32-91% with CDB 35 and CDA 19 compost treatments respectively for three seasons of field study .both CDB 35 and CDA 19 reduced most of carbon sources indicating their wide range of nutrient utilization metabolism. 16 s RNA sequencing revealed CDB 35 as *Pseudomonas aeruginosa* and CDA 19 as *Streptomyces cavourensis*.

Application of the biocontrol organisms to combat plant diseases is an environmentally safe procedure and this further can be brought about by seed application and through compost amendment. Since the organisms are isolated from natural source this when again given back to the field conditions can easily acclimatize the rhizosphere and bring about the action. Thus biological control can form a useful alternative to the chemicals to mitigate the aflatoxin problem and can form a part of integrated mycotoxin management (IMM). .

6.2 CONCLUSIONS

- Overall, 80 bacteria, 40 actinomycetes and 40 fungi were isolated from rice straw compost. All bacteria and actinomycetes were screened for antagonistic traits such as HCN, siderophores, chitinases, glucanases, lipases, cellulases, acid production, pigment production and plant growth promoting traits such as phosphate solubilization, phytase activity.

- Eight bacteria and three actinomycetes were selected based on their plant growth biocontrol ability against *A. falvus*. Among them *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 inhibited *A. flavus* efficiently *in vitro*. They were screened against *A. niger*, *F. udum*, *F. solani, F. oxysporum*, *M. phaseolina*, *S. rolfsii* for broad spectrum antifungal activity.

- The biocontrol mechanisms like siderophore production, volatile antibiotics, hydrolytic enzyme production involved for the antifungal activity of the two isolates *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 were evaluated.

- Two of the eight bacteria and three actinomycetes increased germination percent, root length and number of nodules of groundnut JL-24.

- Peat based inoculums of *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 under glasshouse conditions improved plant growth characters like root length, shoot length and biomass weight of groundnut JL-24.

- Both seed inoculation and through compost amendment of the two potential isolates *Pseudomonas* sp. CDB 35 and *Streptomyces* sp. CDA 19 increased groundnut yield and reduced the aflatoxin in the seeds under field conditions.

- The two strains *S. marcescens* EB 67 and *Pseudomonas* sp. CDB 35 could be useful for preparation of improved composts for crop productivity.

6.4 Biological control – Future prospects

- ✓ Biocontrol approach to combat aflatoxin contamination in food and feed is promising tool and can be included in the integrated approach for mycotoxin management.
- ✓ Isolating and identification of new isolates from natural sources like composts.
- ✓ Understanding the mechanism of biological control
- ✓ More emphasis should be given on pre harvest management as the route of entry of fungus occur in this stage of the crop.
- ✓ Comprehensive understanding the pathogenesis of aflatoxin infections.
- ✓ Development of efficient molecular diagnostics kits for aflatoxin detection
- ✓ Good crop production and post-harvest management technologies. Educating the farmers.

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ABBREVIATIONS

AFPA	*Aspergillus flavus* and *parasiticus* agar
AfB1-BSA	Aflatoxin B1 Bovine serum albumin conjugate
ALP	Alkaline phosphatase
BA	Bennett's Agar
bp	base pairs
CAS	Chrome Azurol S
CCF	cell culture filtrate
CDA	Compost degrading actinomycetes
CDB	Compost degrading bacteria
CDF	Compost degrading fungi
CFU	Colony forming unit
Cm	centi meter
CMC	Cellulose methyl cellulose
Con	Concentration
CP	cell pellet
DAI	Days after inoculation
DDW	Double distilled water
DEAE	Diethylaminoethyl cellulose
DNA	Deoxy ribo nucleic acid
DNS	2-hydroxy-3,5-dinitrobenzoic acid
dNTPs	deoxy nucleotide tri phosphste
ELISA	Enzyme-linked immunosorbent assay
g	gram

GCY	Glucose casamino acid yeast extract agar
IAA	Indole aceticacid
IPCV-H	hapten polyclonal antibodies
KB	Kings B agar
Kg	Kilogram
L	Litre
LB	Luria Bertani
M	Molarity
mg	milli gram
mL	milli litre
Mm	milli metre
mM	milli molar
MW	Molecular weight
NAG	N-acetyl-d- glucosamine
Nm	nano meter
O.D	Optical density
^{o}C	degree centigrade
PASE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline Tween
PCR	Polymerase chain reaction
PDA	Potato dextrose Agar
pH	Power of hydrogen
PIPES	Piperazine 1,4 Bis 2-ethane sulphonic acid
PKa	Dissociation Constant

Pnpp	p- nitrophenyl phosphate
ppm	Parts per million
PVP	Polyvinyl Pyrrolidone
RH%	Relative humidity
RNA	Ribo nucleic acid
rpm	rotations per minute
SDS	sodium dodecyl sulphate
Tris-HCL	hydroxymethyl aminomethane
TWEEN	Polyoxy ethylene sorbitan monolaurate
YEMA	Yeast extract mannitol agar
µL	micro litre