EVALUATION AND CHARACTERIZATION OF ISOLATES OF *TRICHODERMA* SPP. FOR THEIR BIOCONTROL ABILITY AGAINST *ASPERGILLUS FLAVUS* IN GROUNDNUT (*ARACHIS HYPOGAEA* L.).

THESIS SUBMITTED TO THE OSMANIA UNIVERSITY
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BOTANY

P. SRILAKSHMI

DEPARTMENT OF BOTANY
OSMANIA UNIVERSITY
HYDERABAD – 500 007
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Dedicated to my fond husband (sreedhar) and my loving parents (Venkat Reddy & Surya Prabha)

Dr. R.P Thakur
Senior Scientist (Plant Pathology)
Chief PlantQuarantine Officer
ICRISAT, Patancheru PO
Andhra Pradesh 502 324

Dr. K. Satya Prasad
Associate Professor
PG College of Science
Saifabad, Osmania University
Hyderabad 500 004, AndhraPradesh

CERTIFICATE

We certify that the thesis entitled **"Evaluation and characterization of isolates of *Trichoderma* spp. for their biocontrol ability against *Aspergillus flavus* in groundnut (*Arachis hypogaea L.*)"** submitted for the award of Doctor of Philosophy in Botany to the Osmania University by Mrs P Srilakshmi, is the result of bonafied research work carried out by her, at ICRISAT, Patancheru, Hyderabad, under our guidance during the years 2000-2004 and it has not formed the basis for the award of any degree or diploma of this University or elsewhere before

R.P. Thakur

K. Satya Prasad

Dr K Satya Prasad
Associate Professor
Dept of Botany
Post Graduate College of Science
OSMANIA UNIVERSITY
Saifabad HYDERABAD 500 004

DECLARATION

I, P Srilakshmi, declare that this investigation entitled **"Evaluation and characterization of isolates of *Trichoderma* spp. for their biocontrol ability against *Aspergillus flavus* in groundnut (*Arachis hypogaea L.*)"** submitted by me to the Osmania University, for the award of the Degree of Doctor of Philosophy in Botany, is the result of bonafied research work carried out by me under the guidance of Dr K Satya Prasad, Department of Botany, Mycology and Plant Pathology Laboratory, Osmania University, Hyderabad during 2000-2004

I further declare that the results presented here have not been previously submitted by any degree or diploma, either in this or in any other university

P. Srilakshmi
Department of Botany
Osmania University 500 007
Andhra Pradesh
India

Email: SRK2000IN@Yahoo.co.in

Date: 24th July 2004
Place: Hyderabad

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R.P. Thakur

K. Satya Prasad

Dr. K. Satya Prasad
Associate Professor
Dept. of Botany
Post Graduate College of Science
OSMANIA UNIVERSITY
Saifabad HYDERABAD 00 004

Date: 24th July 20004
Place: Hyderabad.

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CONTENTS

Introduction (General)

GENERAL INTRODUCTION

About the host

Groundnut (*Arachis hypogaea* L) is the major oilseed crop in the rain fed farming system in the arid and semi-arid tropics. The major groundnut growing states in India are Andhra Pradesh, Gujarat, Tamil Nadu, Karnataka and Maharastra. Groundnut occupies about 8.2 38% of total oilseeds area and contributes about 41% to the total oilseeds production of our country. Groundnut is the primary source of edible oil and has a high oil and protein content and is a valuable source of vitamins E, K and B. It also helps to enrich poor soils as it leaves behind nitrogen through root nodules and thus contributes to the sustainability of production systems. It is worthy to note that 70% of global groundnut production is in the semi-arid tropics.

About aflatoxin

Aflatoxins, the toxins produced by *Aspergillus flavus* group are known to be hepatotoxic, carcinogenic, and teratogenic. AfB_1 is the most potent toxin. (Fig 1). Aflatoxins have been recognized to be more harmful to malnourished than well-nourished animals and humans. Aflatoxins were initially isolated and identified as the causative toxins in Turkey X disease due to which many died in Turkey in England. Aflatoxins are produced by different species of *Aspergillus*, particularly *flavus* and *parasiticus*. Aflatoxins can contaminate corn, cereals, sorghum, peanuts, and other oil-seed crops. Thus, food contamination by this group of mycotoxins has been implicated in both animal and human aflatoxicoses.

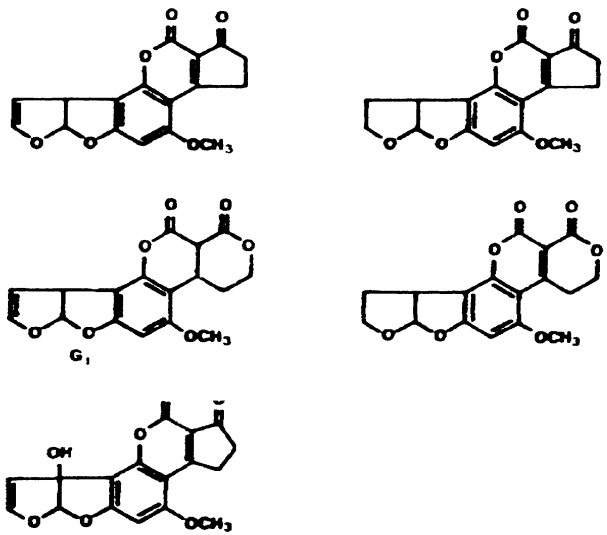

Fig 1: Structure of Aflatoxin B_1 and related aflatoxins.

About the pathogen

In the genus *Aspergillus*, Raper and Fenell (1965) listed close to 200 species and great many varieties.

A flavus is a widely distributed fungus that survives in soil as spores. These germinate to produce hyphae, which invade the pods directly or, more commonly, through injuries. The number of spores may vary from low to extremely high in a single season and probably depend on such factors as nutrient availability and microbial competition. An important attribute is also the ability of *A. flavus* to survive under extremely dry conditions. Thus, it is able to grow at moisture levels only slightly higher than the 12 % used to store nut-in-shell.

Chemical control

Numerous chemicals have been found to destroy or inactivate mycotoxins in naturally contaminated agricultural commodities. Types of chemicals tested for the detoxification of aflatoxins include acids, alkalis, aldehydes, oxidizing agents, and gases like chlorine, sulfur dioxide, ozone and ammonia. Although they destroy aflatoxins, they may decrease significantly the nutritive value of the processed material or produce toxic products or products having undesirable side effects.

Biological control

About biocontrol agents

***Trichoderma* spp.**

The genus *Trichoderma* was introduced by Persoon about 200 years ago. The generic delimitation of *Trichoderma* was given by Harz (1871) taking into consideration the microscopic characters. Species of *Trichoderma* are characterized by rapid growth in culture with sparse aerial mycelium and production of distinctive, white or green, conidiogenous pustules.

The species of *Trichoderma* were investigated as biocontrol agents for over 70 years. Because of their various antagonistic mechanisms and their ability to sustain a wide range of environmental conditions and their remarkable resistance to fungicides make them an ideal choice for biocontrol. They produce extra cellular enzymes, antibiotics and promote plant growth and induce resistance. They have the potential to proliferate abundantly in various soils. Because of their many advantages *Trichoderma* spp. were used in controlling aflatoxin in groundnut.

Mechanism involved in biocontrol

Mode of disease control by biocontrol agents is probably by two methods.

1. Antibiosis is one of the means of Biocontrol in which antibiotics destroy the target fungi either by volatile or diffusible antibiotics. 2. Mycoparasitism in which lytic enzymes destroy the target fungi. Most of the *Trichoderma* spp. control disease through mycoparasitism.

SCOPE OF INVESTIGATION

As biocontrol is an integral part of the integrated pest management (IPM) philosophy, judicious use of *Trichoderma* against soil borne pathogens can serve as a model for the introduction and implementation of other biocontrol means into IPM. Combining two or more types of biocontrol agents can increase biocontrol activity. Proper formulation, efficient delivery system and alternative methods of *Trichoderma* application play a crucial role in the success of the biocontrol agents against soil borne fungi. Chemical pesticides cause health hazards to humans and other non-target organisms while affecting the environment adversely. It is important to develop safer and environment friendly management alternatives by exploiting living organisms from their natural habitat. These organisms provide protection against a wide range of plant pathogenic fungi without any adverse effects to the ecosystem. Potential agents for biological control of soil borne plant pathogens can be obtained from the natural habitat such as rhizosphere. Rhizosphere competent fungi and bacteria with antagonistic activity are capable of promoting growth by either controlling minor pathogens or producing growth-promoting factors (Harman *et al.*, 1989). Biological control through rhizosphere fungi/ bacteria is much safer and non-polluting to the environment compared to the disease management by chemical pesticides. Some rhizosphere organisms may protect the root system of the growing plants from attack by plant pathogens throughout the entire growth period (Ahmed and Baker, 1987; Sivan and Chet, 1989; Harman *et al.*, 1989). When added as seed treatments, the biological control agents enter, establish in the rhizosphere, which leads to the competition and elimination of the pathogens from the rhizosphere throughout the growth period. Further, Paulitz *et al.*, (1990) reported that the combination

of compatible biological control agents produced additional control of soil borne plant pathogens. Other studies have also demonstrated the improved biocontrol activity when *Trichoderma* was combined with other biological control agents (Lynch, 1990).

Aflatoxin in groundnut

The objectives of present investigation are:

1. Isolation and Evaluation of *Trichoderma* isolates for their antagonism against *Aspergillus flavus*.
2. Characterization of *Trichoderma* isolates for morphological and molecular traits.
3. Mechanism of antagonism of *Trichoderma* isolates.
4. Evaluation of selected *Trichoderma* isolates for their biological control potential against *A. flavus*.

Materials and Methods (General)

MATERIALS AND METHODS

(GENERAL)

General laboratory techniques followed in the present investigation were as per Booth (1971).

Glassware

All the glassware used in the research work were of Corning and Borosil.

Chemicals

Chemicals used in the present investigation were from BDH, Merck, Sigma, Qualigens, New England Biolabs, Gibco, Fermentas and were of either GR or AR grade

Cleaning of the glassware

All the glassware was washed thoroughly with tap water, and soaked in 1% clorax or chromic acid (5%) for 15-20 min, and finally rinsed with distilled water.

Sterilization

Sterilization of glassware was done at 180 C for 2 h in the hot air oven (Sanyo)

Media were sterilized in the autoclave at 15 lb p. s. i. And 121 C for 20 min.

Distilled water

Sterile distilled water, and glass distilled water is used for all the experiments

Maintanance of Cultures

Cultures were maintained on Silica gel and stored in refrigerator. Sub-culturing was done at six months interval.

Chapter I
Isolation and Evaluation of *Trichoderma* isolates for their Antagonism against *A. flavus.*

INTRODUCTION

Species of *Trichoderma*, a genus of hypomycetes, are ubiquitous in the environment, especially in soils. They have been encountered in many human activities, including commercial applications in production of enzymes and biological control of plant disease. Contamination of groundnut kernels by aflatoxins, produced by *Aspergillus flavus* group of fungi, is a major problem affecting the quality and trade of the product. Among several management options, biological control can play a significant role in reducing preharvest aflatoxin contamination in groundnut. Control of plant diseases by biological agents is environmentally safe and compatible with sustainable agriculture. *Trichoderma* spp. are well-known biocontrol agents against several plant pathogens (Elad *et al.*, 1982). Quantitative estimation of *Trichoderma* spp. in soil is often difficult because of the relatively rapid growth of other soil fungi on conventional agar media. A semi selective medium was described for the isolation and enumeration of *Trichoderma* spp. from soil (Papavizas and Lumsden, 1982). Strains of the biocontrol fungus *Trichoderm virens* may be separated into glioviridin and gliotoxin producing groups by culture of conidial inocula on agar medium containing selective amendments (Howell., 1999).

Product quality is of utmost importance to the peanut industry in the U.S. Since majority of the peanuts produced are used for human consumption, one of the major quality concerns of the industry is reducing contamination of peanut with aflatoxin, toxins produced by *A. flavus A. parasiticus* (Lynch and Wilson, 1991). Methods were developed and evaluated for removing or reducing aflatoxin-contaminated peanuts via belt screening that removes loose-shelled kernels, immature pods, and foreign material. (Dowell *et al.*, 1990).

The present study was undertaken to isolate *Trichoderma* isolates from rhizosphere soil samples collected from major groundnut growing areas of four districts (Ananthapur, Chittoor, Cuddapah, and Kurnool) of Andhra Pradesh, and two districts (Kolar, and Tumkur) of Karnataka under the National Agricultural Technology Project (NATP) on "Aflatoxin Contamination in Groundnut: Mapping and Management in Gujarat, Andhra Pradesh, and Adjoining Areas".

The objectives of this study were:

1. to Isolate *Trichoderma* isolates from various groundnut-growing areas of Andhra Pradesh, and Karnataka,
2. to identify the isolates up to species level using morphological traits, and
3. to evaluate their antagonism against *A. flavus* with the objective of identifying highly antagonistic isolates that could be used as a biocontrol agents for preventing preharvest aflatoxin contamination of groundnut.

REVIEW OF LITERATURE

Quantitative estimation of *Trichoderma* spp. in soil is often difficult because of the relatively rapid growth of other soil fungi on conventional agar media. A selective medium is a very useful tool for isolating *Trichoderma* from soils as well as for estimating its survival in soil. The role of antagonists in curtailing the growth and pathogenic activity of soil-borne pathogens has been well documented (Nelson and Hoitink ,1983). Elad *et al.* (1980) were able to isolate a isolate of *T. harzianum* capable of lysing mycelia of *Sclerotium rolfsii* and *Rhizoctonia solani* from a soil naturally infested with those pathogens. Elad *et al.* (1981b) developed a *Trichoderma* selective agar medium (TSM) for the quantitative isolation of *Trichoderma* spp. from soil. Selectively was obtained by using chloramphenicol as a bacterial inhibitor and pentachloronitrobenzene, and rose bengal as selective fungal inhibitors. The glucose concentration in the TSM, though low, still allowed relatively rapid growth and sporulation of *Trichoderma*, enabling convenient and rapid identification of *Trichoderma* colonies. Two other effective selective media were developed by (Davet, 1979) and Papavizas and Lumsden (1982). The selectivity of Kayet,s medium is based on the presence of allyl alcohol (0.5ml/l) and vinchlozoline (2.5mg/l). Papavizas and Lumsden (1982) improved the semi-selective medium for *Trichoderma* developed by (Papavizas, 1981), which is ineffective with soils containing Mucorales, by adding alkyl aryl polyether alcohol alone or in combination with sodium propionate. The improved medium contained the following antimicrobial agents. Both or the modified media

allowed *Trichoderma* spp. to develop on the surface of the agar and effectively suppressed rapidly growing fungi such as *Rhizopus*.

Trichoderma spp. are antagonistic to a wide range of phytopathogenic fungi and are able to control economically important diseases in several crop plants. Species of the genus *Trichoderma*, among them *T. viride*, are well known for their production of several lytic enzymes and/or antibiotics.

Smith *et al.* (1990) developed a system to identify isolates of *Trichoderma* and *Gliocladium* with potential for biocontrol of *Phytophthera cactorum*. Mihuta-Grimm and Rowse, (1986) tested 225 isolates of *Trichoderma* obtained from organic and mineral rhizosphere soil in the greenhouse as antagonists for biocontrol of *Rhizoctonia solani* on radish.

Bunker and Kusum Mathur (2001) evaluated local isolates of *Trichoderma* spp. isolated from ginger rhizosphere *in vitro*, to assess their mode of antagonism against chilli root rot pathogen *Rhizoctonia solani*. Godwin-Egein and Arinzae (2001) investigated the antagonism between *T. harzianum Rifai* and *Fusarium oxysporum* Schlecht emend. Sny. And Hans. And the possible mechanisms of antagonism employed by *T. harzianum* when grown on several food wastes.

Elad and Hadar (1981) tested wheat bran culture of *T. harzianum* for control of *Rhizoctonia solani* in carnation in fields treated with methyl bromide. This method is superior to the broadcast application because it required lower rates of application. Biswas and Chitreswar sen (2000) studied the antagonistic properties of *Trichoderma* isolates T8, T10 and T2 through dual plate method against *Sclerotium rolfsii*, the casual agent of stem rot of groundnut and they overgrew the pathogen up to 92%, 82% and 79%

respectively *in vitro*. On seed coated with *Trichoderma* Rajan *et al.* (2002) found that there was reduction *Phytophthora* foot rot disease incidence in pepper when five efficient *Trichoderma* isolates tested both *in vitro* and *in vivo* were applied in the field over the years of study. Alvira *et al.* (2001) isolated eight different isolates (T j, T 1, T 2, T 3, T 5, T 7, T 12, T 16) of *T. harziamum* from soils of different betel vine plantations of West Bengal by dilution plate technique using *Trichoderma* selective medium. The isolates were studied for their cultural, morphological characters and antagonistic potential against four major fungal pathogens of betelvine (*Phytophthora parasitica*, *Colletotrichum capsici*, *Sclerotium rolfsii* and *Rhizoctonia solani*). Isolate T 3 had highest length: breadth ratio of phialospores and that of phialides highest in isolate T 7. Isolates T 1, T 2 and T 3 were more antagonistic under *in vitro* conditions by overgrowing the pathogens within 5-6 days.

MATERIALS AND METHODS

Isolation of *Trichoderma* isolates

Groundnut rhizosphere soil samples collected from various groundnut-growing areas in Andhra pradesh and Karnataka under a National Agricultural Technology Project (NATP) on "Aflatoxin Contamination in Groundnut: Mapping and Management in Gujarat, Andhra Pradesh, and Adjoining Areas are used for isolating *Trichoderma* isolates".

Five hundred µl of each soil sample at 10^{-3} and 10^{-4} aqueous dilutions was spread on plates containing *Trichoderma* specific medium (TSM: glucose 3 g, NH_4NO_3 1 g, $Na_2H_2PO_4$ 0.9 g, $MgSO_4.7H_2O$ 0.2 g, KCl 0.15 g, $FeSO_4.7H_20$ 20 mg, $ZnSO_4.7H_2O$ 20 mg, $MnSO_4.H_2O$ 20 mg, Rose Bengal 30 µg, agar 10g, and distilled water 1000 ml). After autoclaving at 121 C for 20 min the medium was cooled to 50 C and added with streptomycin sulfate 50 mg, chloramphenicol 50 mg, metalaxyl 10 mg, and PCNB (pentachloronitro benzene) 10 mg). Two plates were maintained for each dilution. The plates were incubated for 4 days in dark at 28 C and typical *Trichoderma* colonies (white or whitish-green to green, conidiophores long and thick, with or without sterile branches, side branches mostly thick bearing short and plump phialides, phialospores are globose or ellipsoidal rough or smooth walled) were isolated (Srilakshmi *et al.*, 2001).

In vitro antagonistic studies

The biocontrol ability of 212 isolates of *Trichoderma* spp. was studied against the pathogen *A. flavus*, using dual culture technique (Dennis and Webster 1971c). The mycelial discs (5 mm dia) of *Trichoderma* spp. and *A. flavus* from a four-day-old culture on 2% agar were placed on PDA plates on one side, 1 cm away from the edge of the

plate. The disc of the *A. flavus* was placed on the opposite side 6 cm apart. The plates were incubated at 24 ± 2 C for one week. The plates were observed at regular intervals.

Hyphal interaction

This study was done using the dual culture method (Dennis and Webster 1971c). Potato dextrose- agar medium was prepared with cellophane paper disc laid over the medium. A block of cellophane (10 × 20 cm) was cut from the juncture of the two colonies and mounted in trypan blue-lacto phenol, and examined under microscope for hyphal interactions.

RESULTS

Two hundred and twelve *Trichoderma* isolates were obtained from the groundnut rhizosphere soil samples collected from four districts of Andhra pradesh (Ananthapur, Chittoor, Cuddapah and Kurnool) and Karnataka (Kolar and Tumkur) (Table 1).

Of the 386 soil samples analyzed, 156 (40.4%) yielded *Trichoderma*. Maximum of 64.1%. *Trichoderma* isolates were obtained from the soil samples of Anantapur and minimum of 11.1% from the samples of Kolar (Fig 3). *Trichoderma* population density was maximum in Chittoor soil samples (Table 2).

All the isolates of *Trichoderma* were tested for antagonism or mycoparasitism *in vitro* against *A. flavus* (Af 11-4). Mycoparasitic interaction was assessed following Bell's ranking (Bell *et al*., 1982) (Fig 2).

From a preliminary screening of the total 212 *Trichoderma* isolates, 150 antagonistic isolates were selected. Among the 150 isolates antagonistic to *A. flavus* (Af 11-4), 38 were just in contact with *A. flavus* colony, 48-showed inhibition zone against *A. flavus*, and 64 parasitized *A. flavus*. For further screening 117 isolates that showed inhibition zone and parasitized *A. flavus* were selected and of these only 48 isolates showed clear inhibition zone against Af 11-4. These were further evaluated for mechanisms of antagonism involving production of volatile and non-volatile production of antibiotics.

Among 48 isolates, *T. viride* (T 16 and T 188) and *T. harzianum* (T 109) showed clear hyphal coiling with Af 11-4 mycelia (Fig 4).

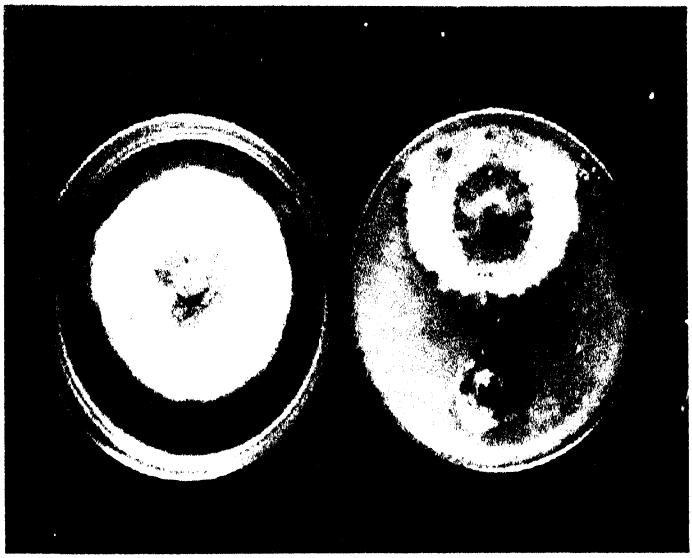

Fig.2. *In vitro* antagonism of *Trichoderma viride* (T 179) against *Aspergillus flavus (Af 11-4) Left: control plate(A. flavus) Right : A. flavus and T. viride showing mycoparasitism by restricted growth of A. flavus.*

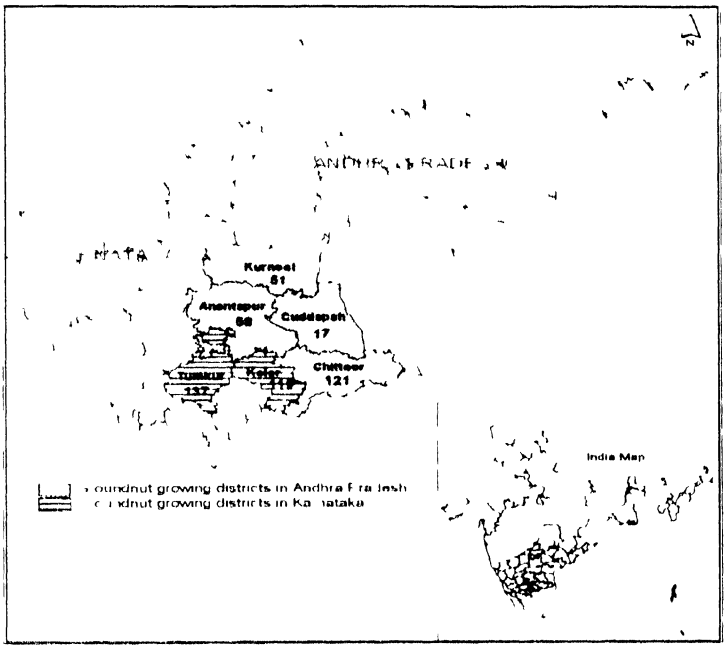

Fig 3 Major groundnut growing districts of Andhra Pradesh (Kurnool, Cuddapah, Anantapur and Chittor) and Karnataka (Kolar and Tumkur)

Fig. 4 Hyphal coiling of *Trichoderma viride* (T 188) over *A. flavus*

Table 1 Population dynamics of *Trichoderma* isolates in four major groundnut-growing districts of Andhra Pradesh and two districts of Karnataka

State	District	No of soil samples analyzed	No of soil samples yielded *Trichoderma* isolates	No of *Trichoderma* isolates obtained	Percentage of soil samples yielded *Trichoderma*
Andhra Pradesh	Ananthapur	53	34	40	64
	Chittoor	97	48	54	49
	Cuddapah	23	11	19	47
	Kurnool	26	15	20	57
Karnataka	Kolar	72	08	35	11
	Tumkur	115	40	44	34
Total		386	156	212	04

Table 2 *Trichoderma* population range in the soil samples collected from six different groundnut- growing districts of Andhra Pradesh and Karnataka

District	Total soil samples	Soil samples analysed	*Trichoderma* population ($Cfug^{-1}$)
Anantapur	58	49	0-25
Chittoor	121	118	0-230
Cuddapah	17	11	0-37
Kurnool	51	38	0-34
Kolar	119	8	0-30
Tumkur	137	56	0-83

DISCUSSION

Quantitative estimation of *Trichoderma* spp in soil is often difficult because of the relatively rapid growth of other soil fungi on conventional agar media

We isolated 212 *Trichoderma* isolates using *Trichoderma* selective medium (Elad *et al.* 1981b) from groundnut rhizosphere soil samples collected from various groundnut-growing areas in Andhra pradesh and Karnataka under a National Agricultural Technology Project (NATP) on "Aflatoxin Contamination in Groundnut: Mapping and Management in Gujarat, Andhra Pradesh, and Adjoining Areas" Selectivity was obtained by using chloramphenicol as a bacterial inhibitor and pentachloronitrobenzene, and rose bengal as selective fungal inhibitors The glucose concentration in the TSM, though low, still allowed relatively rapid growth and sporulation of *Trichoderma*, enabling convenient and rapid identification of *Trichoderma* colonies

Papavizas and Lumsden (1982) improved the semi-selective medium for *Trichoderma* developed earlier by Papavizas (1981), which is ineffective with soils containing Mucorales, by adding alkylaryl polyether alcohol alone or in combination with sodium propionate. The modified medium allowed *Trichoderma* spp to develop on the surface of the agar and effectively suppressed rapidly growing fungi such as *Rhizopus*. *Trichoderma* spp. is antagonistic to a wide range of phytopathogenic fungi and is able to control economically important diseases in several crop plants Species of the genus *Trichoderma*, among them *T. viride*, are well known for their production of several lytic enzymes and/or antibiotics

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Chapter II
Characterization of *Trichoderma* isolates for Morphological and Molecular traits

INTRODUCTION

The genus *Trichoderma* was introduced by Persoon almost 200 years ago, which consisted of anamorphic fungi isolated primarily from soil and decomposing organic matter (Rifai, 1969) Isolates of *Trichoderma* are ubiquitous and are relatively easy to isolate and culture In addition, *Trichoderma* isolates grow quickly on many substrates, produce metabolites with demonstrable antibiotic activity Rifai (1969) divided *Trichoderma* into nine species aggregates on the basis of morphological features Recently, Bisset (1991) revised the genus and included some Hypocrea anamorphs in the genus, resulting in the establishment of five new sections Despite the evident economic importance of these fungi, the taxonomy of *Trichoderma* is still problematic and morphology-based studies have not resulted in a satisfactory comprehensive revision of the genus (Samuels, 1996) Taxonomy of *Trichoderma* is currently based largely on morphological characters (Rifai, 1969, Bissett, 1984, 1991a,b,c & 1992), such as conidial form, size, color and ornamentation, branching pattern and frequency of branching, and the formation of sterile or fertile hyphal elongations from conidiophores However, most species descriptions are based on examination of a limited number of strains where the morphological differences are clear but these differences become less clear as more strains are studied These results suggest that there are not enough morphological and cultural characters to reliably define species In recent years, molecular biological techniques have proven to be valuable tools in fungal taxonomy, and their application has led to the reconsideration of several genera (Sheriff *et al*, 1994) Identification of the respective isolates at the species level has proved difficult, due to the degree of

morphological similarities between them As a solution, Rifai (1969) adopted the concept of aggregate species, and distinguished nine aggregates, some of which comprised two or more morphologically indistinguishable species However, the DNA-based molecular markers have been increasingly used for the characterization of pathogen races, as these are sensitive and precise AFLP (Amplified fragment length polymorphism) analysis is based on selective amplification of DNA restriction fragments (Vos *et al*, 1995) AFLP technology is quite useful for fingerprinting genomic DNA These fingerprints may be used as a tool for determining the identity of a specific DNA sample or to assess the relatedness between samples Fingerprints are also used as a source for genetic markers to generate linkage maps or to identify molecular markers linked to phenotypic traits and/or genetic loci

AFLP technology combines both hybridization-based fingerprinting and PCR (polymerase chain reaction)-based fingerprinting It is based on the selective amplification of a subset of genomic restriction fragments using PCR The advantage of AFLP over other techniques is that multiple bands are derived from all over the genome This prevents over interpretation or misinterpretation due to point mutations or single-locus recombination, which may affect other genotypic characteristics (Vos *et al.*, 1995) PCR has been successful in detecting plant-pathogenic fungi as well as bacteria (Majer *et al.*, 1996, Restrepo *et al*, 1999) The commercial use of *Trichoderma* biocontrol agents must be preceded by precise identification, adequate formulation, and studies about the synergistic effects of their mechanisms of biocontrol (Hermosa *et al.*, 2000)

The AFLP technique has been used in studies on bacteria (Janssen *et al* 1997), fungi (Mueller and Wolfenbarger (1999), and plants (Lin *et al*, 1996) In this study, we used

AFLP with a range of primer pairs and found sufficient variation to draw conclusions about the genetic relationships among 48 *Trichoderma* isolates

Moreover, there is evidence that morphologically defined taxa are polyphyletic e g , *T. harzianum*, (Muthumeenakshi *et al* , 1994) Kindermann *et al* (1998) attempted a first phylogenetic analysis of the whole genus, using sequence analysis of the ITS1 (expand ITS) region of the rDNA However, the use of phylogenies based on single gene sequences is now generally discredited, especially as regards the use of ITS1 and/or ITS2, as some fungi and plants have been shown to contain paralogous copies (Lieckfeldt *et al* , 2000)

Silver staining originally was described for ultra sensitive detection of polypeptides separated by polyacrylamide gel electrophoresis (Merril *et al* , 1981) and later adapted for nucleic acid detection (Blum *et al* , 1987) Beidler *et al* (1982) devised a photochemical silver staining method for nucleic acid detection with increased sensitivity Since then, various small adaptations of these original silver staining methods have been proposed to increase sensitivity, to reduce steps of the procedure, to eliminate toxic staining components, and to better suit the DNA assay or method of fragment separation (Bassam *et al*., 1991), (Sanguinetti *et al*., 1994) Creste *et al*., (2001) compared sensitivity of a new low cost method of silver staining adapted from (Beidler *et al* , 1982) with 2 other commonly used procedures (Bassam *et al* , 1991), (Sanguinetti *et al*., 1994) and optimized the conditions for detection of SSRs using denaturing polyacrylamide gels bound to glass plate The objectives of the present study were to characterize *Trichoderma* species using morphological features and molecular techniques and identify the species

REVIEW OF LITERATURE

The genus *Trichoderma* (Ascomycetes, Hypocreales) contains species that are of great economic importance because they produce enzymes, antibiotics, or are utilized as biocontrol agents Kubicek, (1992) Taxonomy of *Trichoderma* is currently based largely on morphological characters (Rifai, 1969, Bissett, 1984, 1991a,b,c, 1992), such as conidial form, size, color and ornamentation, branching pattern and frequency of branching, and the formation of sterile or fertile hyphal elongations from conidiophores In *Trichoderma*, isoenzyme analysis (Stasz *et al*, 1986, Samuels *et al*, 1994, Leuchtmann *et al*, 1995, Kuhls *et al*, 1995, Bowen *et al*., 1996, Turoczi *et al*, 1996), and rDNA sequencing (Muthumeenakshi *et al*, 1994, Kuhls *et al*, 1996, 1997) have been used to distinguish species specific groups of strains within the species that were defined either by common morphology or ecology Recently rDNA was used in comprehensive survey of two of the sections of the genus that were accepted by (Bissett 1984, 1991a), the rDNA sequence information provided support for merging the two sections Moreover, the expanded section *Longibrachiatum* included five *Hypocrea* species and that the sexual (*Hypocrea)* and asexual (*Trichoderma)* strains did not represent separate lineages but rather represented a single lineage within the ascomycete genus *Hypocrea.* Consequently, we refer to *Trichoderma* sect *Longibrachiatum* as the *Hypocrea schweinitzii* complex (Samuels *et al*, 1998) AFLP technology is a DNA fingerprinting technique that combines both hybridization-based fingerprinting and PCR-based fingerprinting It is based on the selective amplification of a subset of genomic restriction fragments using PCR Reader and Baroda (1985) described a general, simple and inexpensive method for isolation of DNA from filamentous fungi Starting from freeze-

dried mycelium 0 1 - 0.15% by weight can be isolated as high molecular weight DNA suitable for restriction and ligation in 2 h The preparation can be done in eppendorf tubes and allows the processing of many samples in parallel They have used the method with the basidiomycetes, *Phanerochaete chrysosporium*, *Coprinus cinereus* and the ascomycete *Aspergillus nidulans* and others have used it with *Trichoderma reesei*, *A. niger* and for the isolation of DNA from tomato plants Vos *et al* (1995) described a novel DNA fingerprinting technique called AFLP based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA Using this method, sets of restriction fragments may be visualized by PCR without knowledge of nucleotide sequence The method allows the specific co-amplification of high numbers of restriction fragments and provides a novel and very powerful DNA fingerprinting technique of DNAs of any origin or complexity

Nan Ren and Michael Timko (2001) used AFLP analysis to determine the degree of intra- and inter-specific genetic variation in the genus *Nicotiana* Sivaramakrishan *et al* (2002) studied genetic variability among 43 isolates of *Fusarium oxysporum* f sp *ciceri*, the chickpea wilt pathogen, using AFLP markers. Abdel-satar *et al* (2003) analysed the genetic relationships within and between natural populations of five *Fusarium* spp. using AFLP Nucleotide sequence determination of rDNA internal transcribed spacer (ITS) 1 revealed three distinct ITS types, 1,2 and 3, possessed by isolates from the respective groups 1,2, and 3 (Muthumeenakshi *et al* , 1994) Based on these molecular data, group 2 isolates, which were aggressive colonizers of mushroom compost, could be clearly distinguished from the isolates belonging to the other two groups Katrin Kuhls *et al* (1997) studied the variation within ITS-1 and ITS-2 regions of ribosomal DNA of 103

strains to examine relationships within *Trichoderma* sect. *Longibrachiatum* and related teleomorphs, *Hypocrea schweinitii* and *H. jecorina*. Grondona *et al* (1997) reported that monoconidial cultures of 15 isolates of *Trichoderma harzianum* were characterized on the basis of 82 morphological, physiological, and biochemical features and 99 isoezyme bands from seven enzyme systems and identified four distinct groups Representative sequences of the ITS 1 and ITS 2 region in the ribosomal DNA gene cluster were compared between the groups confirming this distribution The utility of the groupings generated from the morphological, physiological, and including an additional environmental isolate in the electrophoretic analysis assessed biochemical data The *in vitro* antibiotic activity of the *T. harzianum* isolates was assayed against 10 isolates of five different soil borne fungal plant pathogens Ospina-Giraldo *et al* (1998) examined nucleotide sequences of ITS-1 and ITS-2 and the 5 8 S region of the ribosomal DNA repeat in 15 lines of *Trichoderma* spp Six lines representing four biotypes of *T. harzianum* associated with mushroom compost or the raw materials used in the compost could be separated into two distinct groups. Ospina-Giraldo *et al* (1999) sequenced a polymerase chain reaction-amplified DNA containing the internal transcribed spacer ITS-1 and ITS-2 regions of the nuclear ribosomal DNA transcriptional unit for 81 isolates of *Trichoderma* spp. associated with mushroom culture Phylogenetic analysis revealed that the biocontrol isolates were more closely related to an isolate of *T. harzianum* biotype 1 than to the aggressive biotypes 2 and 4 Finally, considering the wide range in sequence distribution of our samples, we propose that the consensus sequence found in this investigation be used as the reference sequence for further studies involving the identification and taxonomy of *T. harzianum* (Lieckfeldt *et al.*, 1999) based on combined

molecular data, morphology, physiology and colony characteristics distinguished type I and type II *Trichoderma viride* as two different species. Hermosa *et al.*, (2000) reported that the most common biological control agents (BCAs) of the genus *Trichoderma* have been reported to be strains of *Trichoderma virens*, *T. harziamum*, and *T. viride*. Since *Trichoderma* BCAs use different mechanisms of biocontrol, it is very important to explore the synergistic effects expressed by different genotypes for their practical use in agriculture.

MATERIALS AND METHODS

Morphological Characterization.

Morphological characterization was done of 212 *Trichoderma* cultures obtained from groundnut rhizosphere soil samples from major groundnut growing areas of four districts (Anantapur, Chittoor, Cuddapah, and Kurnool) districts of Andhra Pradesh and two districts (Kolar and Tumkur) of Karnataka, The morphological characters used were growth type, growth rate, colony colour, shape of margins, colony diameter, sporulation and sporulation initiation rate

Species identification:

Microscopic Observation:

Slides were prepared from the place of contact of pathogen and the antagonist and observed for interaction under the microscope

Identification of *Trichoderma* spp

Trichoderma spp were grown on 1 5% malt agar for four days and the slides prepared from the growing edges Conidia were taken from one-week-old PDA culture Identification was done with the help of species identification keys (Rifai 1969, Bisset 1984,1991a,b,c, 1992, Samuels (1996) and Nagamani *et al.*, (2002) Fourty eight antagonistic *Trichoderma* species were attempted to identify to based on branching of conidiophores, shape of the phialides, emergence of phialospores and shape of the phialospores (Table 3)

Table 3. Fourty eight *Trichoderma* isolates antagonistic with *A. flavus*.

Isolate no.	*Trichoderma* spp.	District
T-2	*T. harzianum*	Chittoor
T-10	*T. harzianum*	Chittoor
T-11	*T. harzianum*	Anantapur
T-20	*T. harzianum*	Chittoor
T-42	*T. harzianum*	Chittoor
T-53	*T. harzianum*	Tumkur
T-58	*T. harzianum*	Chittoor
T-72	*T. harzianum*	Anantapur
T-109	*T. harzianum*	Chittoor
T-129	*T. harzianum*	Tumkur
T-170	*T. harzianum*	Chittoor
T-47	*T. fertile*	Tumkur
T-16	*T. viride*	Chittoor
T-24	*T. viride*	Chittoor
T-50	*T. viride*	Chittoor
T-51	*T. viride*	Tumkur
T-60	*T. viride*	Tumkur
T-62	*T. viride*	Chittoor
T-179	*T. viride*	Chittoor
T-188	*T. viride*	Tumkur
T-205	*T. viride*	Kolar
T-6	*T. longibrachiatum*	Chittoor
T-34	*T. longibrachiatum*	Tumkur
T-56	*T. ongibrachiatum*	Tumkur
T-102	*T. longibrachiatum*	Chittoor
T-110	*T. longibrachiatum*	Chittoor
T-12	*T. koningii*	Chittoor
T-13	*T. koningii*	Chittoor
T-21	*T. koningii*	Chittoor
T-33	*T. koningii*	Chittoor
T-49	*T. koningii*	Chittoor
T-70	*T. koningii*	Chittoor
T-83	*T. koningii*	Anantapur
T-143	*T. koningii*	Chittoor
T-161	*T. koningii*	Tumkur
T-29	*T. pseudokoningii*	Chittoor
T-37	*T. pseudokoningii*	Kolar
T-206	*T. pseudokoningii*	Anantapur
T-73	*T. atroviride*	Tumkur
T-74	*T. harzianum*	Tumkur
T-86	*T. inhamatum*	Tumkur
T-1	*T. longibrachiatum*	Chittoor
T-14	*T. aureoviride*	Chittoor
T-142	*T. longibrachiatum*	Chittoor
T-117	*T. aureoviride*	Tumkur
T-46	*T. harzianum*	Chittoor
T-134	*T. pseudokoningii*	Anantapur
T-35	*T. fasiculatum*	Chittoor

Single-spore culture of *Trichoderma* spp.

The spore suspension of *Trichoderma* spp was prepared by taking small amount of spore mass in sterile water, dilutions were made in such a way that final dilution had approximately 5 to 10 conidia per ml One ml of the above dilution was poured into a Petriplate and to this, melted but cool 2% agar was added After 10 to 12 h the plates were observed under the microscope in the laminar flow cabinet for a germinating spore. After it was located, the single germinating spore was picked up with the help of a sterile small cork borer (3 mm) attached to the tip of a hollow objective The single spore thus isolated was inoculated on to a PDA plate

Culture Growth:

Trichoderma was grown on potato dextrose agar medium, (PDA, Himedia, Mumbai, India) for isolating DNA. Sterile cellophane paper disc was placed over the medium and *Trichoderma* culture disc was placed at the center of the plate. The plates were incubated at 28 C for 5 days and mycelium was scraped to isolate DNA

DNA Extraction:

Genomic DNA was extracted from each isolate by the CTAB method (Murray and Thompson, 1980). Mycelial mat (3g) was scooped and ground under liquid nitrogen

extracted with 15 ml of extraction buffer (Tris-HCl p^H 8 0, 20 mM EDTA, 1 4 M NaCl and 2.5% (w/v) CTAB) The contents were taken in 30 ml centrifuge tubes and incubated in a water bath for 5 min at 65 C along with 0 03% β Mercaptoethanol The tubes were incubated in a water bath at 65 C for 30 min samples are mixed for every 5 min Fifteen ml of chloroform isoamyl alcohol (24 1) was added to each tube, mixed and centrifuged at 6000 rpm for 10 min Top layer was separated with filler and 0 7 vol of cold isopropanol was added and incubated at 4 C for 30 min prior to centrifugation at 6000 rpm for 15 min The pellet was transferred to 2 ml eppendorfs and washed with 2 ml of 70% ethanol, after discarding ethanol pellets were air-dried before suspending in 1ml of TE buffer (50 mM Tris-HCl p^H 8 0, 10mM EDTA) and DNA was transferred into fresh eppendorfs containing 150 µl of 50 mg/ml Rnase The tubes were incubated overnight at 37 C 1.5 ml of phenol:chloroform isoamyl alcohol in 25 24 1 ratio was added and kept for centrifugation for 5 min at 14,000 rpm After thorough mixing, the top layer was separated and collected into fresh eppendorfs and 600 µl of ice-cold chloroform isoamyl alcohol (24.1) was added. The tubes were kept for centrifugation at 14,000 rpm for 5min and the top layer was separated again and $1/10^{th}$ vol of sodium acetate (50 µl /500 µl) and 2 vol. of absolute ethanol were added, mixed gently and centrifuged at 14,000 rpm for 5min at the room temperature

The DNA was washed twice with 250 µl of 70% ethanol The pellet was fan-dried for 5min and suspended in varying volumes of T E (10mM Tris-HCl, p^H 8 0, 0 1mM EDTA) buffer depending on pellet size The DNA was quantified using two methods

Estimation and Assessment of DNA

Spectrophotometric method

Quantification of DNA was done using spectrophotometer (UV- 160 A, Shimadzu) by diluting 0 5 µl of DNA with 995 µl of sterile distilled water Concentration of DNA was calculated using the formula $A_1 \times 50/5$ µl of DNA taken, where A_1 is 260 nm and 280 nm wavelength at which O D values were taken DNA concentration was compared with the known concentration of Lambda DNA

Agarose Gel Electrophoresis

The DNA was also quantified using agrose gel electroporesis Thirty ml of 0 8% Agarose gel (0 24 g Agarose, and 30 ml 1X TBE) was prepared by boiling for 5 min and allowed to cool when the temperature of agarose solution reaches 45 C Two µl of Ethidium bromide (2 µg/µl) was added mixed well before the gel was poured into a preset agarose gel unit with two 26-well combs one below each other and allowed to solidify After solidifying, the gel was transferred to gel running unit and tank was filled with 0 5 X TBE and combs were removed The DNA sample (2 µl) was diluted with 2 µl of orange dye (10 ml 0 5 M EDTA, 1 ml 5 M NaCl, 50 mL glycerol, 39 ml distilled water and sufficient orange dye powder in 100 ml distilled water) and 5 µl of 0 5 X TBE buffer and loaded into the gel and λ uncut DNA (0 5 µl 1 µl, 2 µl and 3 µl) is added to 1st, 26th 27th and 52nd wells at 50 ng/µl, 100 ng/µl, 200 ng/µl, and 400 ng/µl concentration The gel was run at 80 V for 30 min and visualized under a U V Transilluminator for DNA Gel

electrophoresis method was preferable than spectrophotometric method since the DNA visualized by this method was more pure than the other method

AFLP-PCR amplification conditions

AFLP core reagent and starter primer kits were purchased from (Life Technologies Invitrogen, Gatherburg, and U S A) The analysis was done as per the manufacturer's protocol with slight modifications

Restriction Digestion of Genomic DNA

Component	Starting conc.	Final conc.	Vol. added per sample (µl)
*Eco*RI/*Mse*I (Invitrogen)	1 25 U/µl	1 25 U//µl	1
Restriction enzyme buffer	1 X	10 X	2
Genomic DNA	50ng	250-600 ng/µl	8
Total			11

1 10 µl of cocktail was distributed in each labeled tube

2 Vortex and briefly centrifuged

3 Digestion was carried out in a final volume of 10 µl at 37 C for 2 h

Ligation of Adapters.

Component	Initial	Volume added Per sample (µl)
T4 DNA ligase 10 X buffer	1U/µl	1
Adapter ligation solution		4

1. 5 ul of ligation mixture was added to 10 µl of digested DNA
2. Vortex and briefly centrifuged
3. Incubated at 20 C ± 2 C for 2 h in liquid scintillation room
4. After terminating the reaction, the ligation mixture was diluted 10-fold with TE (10mM Tris-HCl, p^H 8 0, 0 1mM EDTA) buffer

Pre amplification Reaction

Component	**Volume added Per sample (µl)**
Pre-amp mix I	08 00
10 X PCR buffer	01 00
Taq polymerase	00 16
Template DNA from restriction/ligation	02 00
Total	11 16

Thermal cycling for pre-amplification

94 C	30 sec	
56 C	60 sec	
72 C	60 sec	30 cycles
4 C	hold	

Digested-ligated DNA fragments were used as templates for the Pre selective amplification The specific DNA fragments were amplified by PCR using primers that contained the common sequences of the adapters and 1-2 arbitrary nucleotides as

selective sequence PCR reactions were performed in a PTC-100 Programmable Thermal Controller (MJ Research, USA)

After terminating the reaction pre-amplified DNA was diluted 50 fold with TE (10 mM Tris-HCl, p^H 8 0, 0 1mM EDTA) buffer.

Selective amplification

Component	**Volume added per sample (µl)**
*Eco*RI	00 50
*Mse*I	00 50
10 X PCR buffer	10 00
Taq polymerase	00 16
Distilled H_2O	50 00
Dilute template DNA from pre-selective PCR	30 00
Total	10 00

Thermal cycling for selective amplification

94 C	5 min	
65 C	30 sec	12 cycles, decrease annealing temp by 0 7 each
72 C	1 min	
94 C	5 min	12 cycles
94 C	1 min	
56 C	30 sec	
72 C	1 min	
94 C	5 min	22 cycles
4 C	hold	

The six *Eco*RI (E-AC, E-AT, E-TG, E-AG, E-TA, and E-TA), and 6 *Mse*I (M-CTG, M-CAG, M-CTA, M-CTG, M-CAC and M-CTC) primers were used in six combinations for amplification. Selective amplification was done as per the manufacturer's protocol (Invitrogen, U.S.A). This second amplification was carried out by programming a touch-down cycle profile in a PTC-100 Programmable Thermal Controller (MJ Research, U.S.A) .The *Eco*RI prime, was labeled with [γ- ^{32} P]ATP (3000 Ci/mmol) as per the protocol of Invitrogen (U.S.A).

Gel Analysis

PAGE

Cleaning and assembling the glass plate sandwich

The glass plates were cleaned first with distilled water and alcohol The IPC (Precision Castor) flat was placed on the bench with glass plate facing upward The front glass plate was placed onto the IPC and spacers with the siliconized or coated surface facing down The clamps were slided over the IPC assembly The glass plate was rinsed with cold Bind silane (Amersham, U S A) and IPC was rinsed with repel silane (Amersham, U S A)

Gel Casting

The gel was cast with the glass plate assembly in the horizontal position Two full-length clamps secure the assembly and allow attachment of the precision caster base to the bottom of the glass plate sandwich The gel matrix was prepared using 5% acrylamide, 0 25% bisacryl, and 7 5 M urea in 50 mM Tris/50 mM boric acid/1mM EDTA Sambrook *et al*, (1989) The gel solution was degassed for 5-15 min under a strong vacuum (≥ 26 in /Hg) to insure reproducible gel porosity to remove dissolved oxygen After degassing 450 µl of 10% Ammonium persulphate (APS) (100 mg/ml) and 100 µl of Tetramethylenediamine (TEMED) was added to 100 ml of gel solution and gel was cast The gel matrix was injected from the bottom of glass plate sandwich Flat edge of comb was inserted not more than 5 mm past the short glass plate After the gel was cast the assembly was leveled for polymerization

The gel was pre-run in 0 5 x TBE, freshly prepared from a 10 x TBE (100 mM Tris/ 100-mM boric acid/2 mM EDTA) stock solution Once the solution reaches the top of glass

plates the comb was placed in such a way that tooth of the comb faces upwards The gel polymerizes within 30-60 min Polymerized gel was subjected to electrophoresis

Gel Loading

Amplification products were mixed with one volume of formamide dye (10 mM EDTA p^H 8, 98% formamide, 0 025% xylene cyanol FF, and 0 022% orange dye) and denatured for 4 minutes at 94 C Aliquots (5 ng) of each sample were visualized by electrophoresis on 5% denaturing (sequencing) PAGE The samples were loaded on the gel using 5 µl Finnpippet

The gel was run using a sequencing gel, (Biorad 38 x 50cm) The key electrophoresis parameters include voltage set at 1,500 V, current at 40 mA, power at 100 W, and temperature at 60 C 48 well Vinyl sharks tooth Comb (0 4 mm thick) was placed in such a way that tooth of the comb are immersed into the buffer The gel was run with 0 5 X TBE buffer and stained by a modification of the silver staining method of (Creste *et al*, 2001)

Gel Electrophoresis Disassembly

When the desired dye front mobility was achieved, power supply was turn off and safety covers were removed The clamps from IPC assembly were also removed

Gel Drying and Auto radiography

The gel was run for 2-3 h and the gel was removed along with glass plate Sequencing gels were transferred to a fresh sheet of filter paper The gel was made wet slightly by misting the gel with deionized water The dry filter paper was laid on top of the gel and firmly pressed The gel sticking to the paper was slowly removed by lifting the filter paper carefully from one end The sequencing gel was wrapped with plastic wrap and air

bubbles were removed by running with a paper towel and edges were trimmed to fit the slab gel dryer (Biorad Gel dryer model 583). The gels were dried at for 30 min. The dry gels were placed in contact with Kodak X-Omat films labeled with [γ- ^{32} P]ATP (3000 Ci/mmol) and incubated at –80 C overnight in a black cover. To obtain Autoradiograms the X- ray film was developed with developer and fixer prepared as per manufacturers protocol (Kodak X-Omat films) and every experiment was repeated a minimum of 2 times to establish the consistency of the bands.

Data analysis

Polymorphic AFLP markers were manually scored as binary data with presence as "1" and absence as "0". Monomorphic markers were not scored. Polymorphic bands that were very thin and faint, were excluded from the analysis because these can arise artifactually from differences in genomic DNA quality and other factors (Lin and Kuo (1995), Schondelmaier *et al.*, (1996). The resulting 48 × 250 binary data matrix from 6 primer combinations was used to estimate pair wise genetic similarities among the 48 isolates based on Jaccard's coefficient. The 48 × 48 similarity matrix was subjected to sequential agglomerative hierarchical nested (SHAN) clustering using UPGMA (unweighted pair-group method analysis) in the NTSYS-pc software version 2.0. Multidimensional scaling (MDS) was used to obtain an independent validation of UPGMA clustering results. Cluster analysis was performed on the similarity matrix employing UPGMA algorithm provided in the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) Version 2.0 (Exeter Software Co., New York).

ITS Protocol

PCR amplification:

The sequences of the primers used for amplifying ITS sequences of *Trichoderma* are the following

S. no	ITS Primers	Sequence
1	ITS 1	(5' TCTGTAGGTGAACCTGCGG 3')
2	ITS 2	(5' GCTGCGTTCTTCATCGATGC 3')
3	ITS 3	(5' GCATCGATGAAGAACGCAGC 3')
4	ITS 4	(5' TCCTCCGCTTATTGATATGC 3')

Diluted genomic DNA (10 ng) was amplified in a total volume of 20 µl PCR reaction mixtures containing 0 2 µM concentrations of (each) primer, the reaction was done with four combinations of ITS primers *viz* , 1&2, 1&4, 2&3 and 3&4 200 µM concentrations of each deoxynucleoside triphosphate, and 0 4 U of Taq DNA polymerase (Invitrogen, U.S A) in 1 X PCR Buffer (100 mM Tris-HCl, p^{H} 8 3, 500 mM KCl) PTC-100 Programmable Thermal Controller (MJ Research, USA) These reactions were subjected to an initial denaturation of 2 min at 94 C, followed by

Thermal cycling programme

94 C	3 min
58 C	45 sec
72 C	1 min
94 C	29 min
72 C	5 min

The reaction was carried out in a PTC-100 Programmable Thermal Controller (MJ Research, USA) Aliquots (2 µl) were electrophorised in 1 2% (w/v) agarose gels using TBE (electrophoresis buffer, 40 mM Tris boric acid 2 mM EDTA, P^{H} 8) containing ethidium bromide (0 5 µg/ml) and visualized over a Transilluminator (U V Tech Gel Documentation System, UK) PCR reaction performed with ITS primers 1&4 showed amplification of DNA hence genomic DNA of all the isolates was amplified with ITS Primers 1&4

Restriction

The amplified samples were digested with the following restriction enzymes initially for standardization

S.no	Restriction enzyme	Source	Recognition Sequence
1	*Dra*I	Amersham	3' TTT ↓ AAA 5'
2	*Hae*III	New England Biolabs	3' GG ↓ CC 5'
3	*Eco*RI	Amersham	3' G ↓ AATTC 5'
4	*Pst*I	Amersham	3' CTGCA ↓ G 5'
5	*Msp*I	New England Biolabs	3' C ↓ CGG 5'
6	*Hind*III	Amersham	3' A ↓ AGCTT 5'
7	*Mse*I	New England Biolabs	3' TT ↓ AA 5'
8	*Eco*RV	Invitrogen	3' GAT ↓ ATC 5'

Amplified genomic DNA was restricted with *Hae*III and *Eco*RI

Component	Volume added per sample (µl)
Genomic DNA	05
Restriction enzyme	01
Gene amp buffer (Invitrogen)	01
Sterile distilled water	13
Total	20

Two master mixtures with two restriction enzymes *Hae*III (2-10 U) and *Eco*RI (10 U) were made and the samples were incubated at 37 C overnight with suitable buffers according to manufacturers protocol

*Eco*RI buffer was used for *Eco*RI enzyme (Amersham) and NEB 2 buffer was used for *Hae*III enzyme (New England Biolabs)

Two µl of digest was run on a 6% Non-denaturing PAGE gel The gel was cast in a similar manner as done in AFLP Here urea was not added in the gel matrix and gel was run in a sequencing gel, Biorad (38 x 30 cm) The length of the gel unit was smaller when compared to denaturing gel The voltage was set at 500 V, current at 40 mA, power at 25 W, and temperature at 45 C The gel was run for 2 h with 0 5 X TBE buffer and stained by a modification of the silver staining method (Creste *et al* , 2001)

Silver staining procedure:

An improved method adapted from (Creste *et al* , 2001) was optimized All the chemicals used for staining were analytical grade, either from Amersham or Merck All solutions were prepared using ultra pure distilled water The gel plates were agitated gently in a

shaker throughout the staining processes The gel apparatus was disassembled carefully, and glass plates were separated The glass plate with bound gel was placed onto plastic tray. One thousand ml of fixing solution (10% ethanol, 1% acetic acid) was applied and gently shaken The gel was washed with distilled water for 1 min The gel was pretreated with 1000 ml of 1 5% nitric acid for 3 min, and gently shaken The gel was rinsed with 2000 ml of distilled water for 3-5 min and then impregnated with 1000 ml if 0 1% CTAB solution for 20 min, and gently shaken

CTAB solution was discarded and 25% liquid ammonia solution was added to the gel with gentle shaking for 15 min Ammonia solution was discarded and the gel was rinsed with distilled water The gel was developed by applying, initially 250 ml of developing solution (30 g $Na_2 CO_3$ and 0 4 ml formaldehyde in 2 L distilled water) and gently shaken until the solution became dark The solution was replaced with 750 ml of fresh cold solution, for 4 - 7 min, until the bands appeared with desirable intensity The developing solution was removed The gel was rinsed with water for 1 min to stop staining and transferred to fixer solution (30 ml glycerol in 2 L distilled water) for 15 min The gel was washed in distilled water air dried and scanned

Data analysis

The presence or the absence of each band in the gel was scored as 1 or 0, respectively The dendrogram construction, principal coordinate analysis and multi dimensional scaling on the similarity indices between isolates based on the molecular data was carried out using NT SYS software Reproducible bands or Major bands, which are the thick bands, were scored Minor polymorphic DNA bands. which are very thin and faint, were excluded as explained before. Genetic similarities based on Jaccard's coefficient were

calculated using SIMQUAL program of the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) Version 2 0 (Exeter Software Co , New York) The resulting 25 × 17 binary data matrix from ITS Primer combinations was used to estimate pair wise genetic similarities among the 48 isolates based on Jaccard's coefficient The 25 × 25 similarity matrix was subjected to sequential agglomerative hierarchical nested (SHAN) clustering using UPGMA (unweighted pair-group method analysis) in the NTSYS-pc software version 2.0 Multidimensional scaling (MDS) was used to obtain an independent validation of UPGMA clustering results Cluster analysis was performed on the similarity matrix employing UPGMA algorithm provided in the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) Version 2 0 (Exeter Software Co , New York)

RESULTS

Forty-eight antagonistic *Trichoderma* isolates were identified as *T harzianum* (14), *T fertile* (1), *T. fasiculatum* (1), *and T. koningii* (9), *T. viride* (9), *T. atroviрid* (1), *T inhamatum* (1), *T. aureoviride* (2), *T. pseudokoningii* (4), and *T. longibrachiatum* (7) based on the morphological characteristics such as growth type, growth rate, colony color, shape of margins, sporulation, and sporulation initiation time, using standard manuals (Rifai, 1969, Bissett, 1984, 1991a,b,c, 1992) (Fig 4) These isolates were divided into ten groups (Table 4)

The first group of *T. harzianum* (Fig 5) consisted 14 isolates, second group had only 1 isolate of *T. fertile* (Fig 6), third group also had only 1 isolate of *T. fasiculatum*, fourth group had 9 isolates of *T. koningii* (Fig 9), fifth group also had 9 isolate of *T. viride* (Fig 7), sixth group had only 1 isolate of *T. atroviride*, seventh group also had only 1 isolate of *T. inhamatum*, eighth group had 2 isolates of *T. aureoviride*, ninth group had only 4 *T pseudokoningii* isolates (Fig 10), tenth group had 7 isolates of *T. longibrachiatum* (Fig 8)

1) Macroscopic and microscopic characteristics of *T. harzianum* are Colonies raised, smooth-surfaced and dull- green, hyphae septate, branched, smooth-walled, and colourless, conidiophore branching simple with side branches, arising singly mostly, phialides terminating conidiophores and also arising singly, Phialides terminating with a globose, smooth walled phialospore, measuring 2-3 3 µm and the sporulation initiation time was measured as 24 h

2) ***T. fertile*** of second group has the following characteristics, Colonies raised, cottony, smooth-surfaced and grayish-green, hyphae septate, sterile, branched, smooth-walled, and colourless; Conidiophores branching was arising in compact tufts with side branches Phialides were arising singly and irregularly from the conidiophores and were short, plump and pear-shaped with a narrow neck Phialides were terminating with a globose, smooth walled and pale green phialospore, measuring 9 3-2 8 µm in size and the sporulation initiation time was measured as 48 h

3) *T. fasiculatum* of third group has the following characteristics, Colonies submerged, and dark green in colour; hyphae hyaline, branched, smooth-walled, and colorless, Conidiophores branching was irregular and branches arising singly or in groups, upper part of the conidiophore is sterile, Phialides were arising singly or in groups, ampulliform, narrow at the apec Phialides were terminating with a ellipsoidal, smooth walled and pale green phialospore, measuring 2-3 µm in size and the sporulation initiation time was measured as 48 h

4) T. koningii group has the following characteristics T. koningii group has the following characteristics: Colonies smooth walled, raised and dark green in colour, hyphae septate, smooth walled and hyaline; Conidiophores branching was arising in tufts forming ring like zones; Phialides pin shaped and narrow at the base, terminating the conidiophores and terminal phialide was longer than the other phialides; Phialospores oblong, elliptic, smooth walled and pale green in colour, produced singly at the tip of phialides, and measuring 1-4 µm in size and the sporulation initiation time was measured as 72 h

5) *T. viride* of fifth group has the following characteristics. Colonies submerged, irregular and dark bluish green; hyphae septate, smooth walled, hyaline and giving coconut odour

Conidiophore branching was arising in loose tufts and form continuous ring-like zones and show profuse branching. Phialides slightly curved, pin-shaped, and narrow at the base and the neck was long and were arising at apex of the conidiophores, singly or in groups; Phialospores were globose, rough walled and green coloured, measuring 4-4.5 µm in size and the sporulation initiation time was measured as 48 h.

6) *T. atroviride* group has the following characteristics: Colonies were submerged, smooth walled and blue green in colour; hyphae septate, smooth walled and hyaline; Conidiophores branching was profuse,lateral branches arising at right angles and whole conidiophore structure appears like a pyramid; Phialides lageniform, constricted at the base and attenuate at apex arising in pairs or in groups, often curved, Phialospores single celled, sub-globose, smooth walled and dark green in colour, produced at the tip of each phialide and measuring 2-3 µm in size and the sporulation initiation time was measured as 48 h.

7) *T. inhamatum* group has the following characteristics: Colonies submerged, and bluish green; hyphae septate, smooth walled, hyaline and giving coconut odour. Conidiophore is narrow, flexuous and branching was arising in loose tufts and form continuous ring-like zones and show profuse branching. Phialides slightly curved, and narrow at the base and the neck was long and were arising at apex of the conidiophores, singly or in groups; Phialospores were globose, smooth walled and green coloured and spores were darkening in age and measuring 3-4.5 µm in size and the sporulation initiation time was measured as 48 h.

8) *T. aureoviride* had the following characteristics: Colonies were raised, smooth walled and grass yellowish green in colour, develops needle shaped golden yellow crystals; hyphae septate, branched and hyaline; Conidiophores branching was profuse, lateral branches arising at right angles and whole conidiophore structure appears like a conifer; Phialides horn-shaped slender constricted at the base and attenuate at apex arising in groups of 2-3 or rarely singly Phialospores smooth walled, obovoid, and dark yellowish green in colour, produced singly a the tip of each phialide and measuring 2.5-4 μm in size and the sporulation initiation time was measured as 72 h.

9) *T. pseudokoningii* which is a representitive isolate of sixth group has the following characteristics: Colonies smooth walled, raised and yellowish-green; hyphae septate branched, hyaline and smooth walled; Conidiophores branching was arising in compact tufts terminated by phialedes; Phialides long, bottle-shaped with a narrow base and attenuated towards the tip; Phialospores globose, smooth-walled and pale green in colour and were arising at the apex of phialides, measuring 3-5 μm in size and the sporulation initiation time was measured as 24 h.

10) *T. longibrachiatum of tenth group* has the following characters: Colonies were submerged, smooth walled and bright green in colour; hyphae septate, smooth walled and hyaline; Conidiophores branching was profuse arising in loose tufts and were forming ring like zones; Phialides pin shaped and narrow at the base, arising singly and directly on the conidiophores at the upper portion, lower portion of the conidiophore was showing profuse branching with phialides arising directly on the side branches; Phialospores oblong,

ellipsoidal , smooth walled and pale green in colour, produced singly at the tip of each phialide and measuring 3-4 μm in size and the sporulation initiation time was measured as 72 h.

To estimate the inter-relationships among the 48 isolates of *Trichoderma* morphological parameters such as growth type, growth rate, colony color, shape of margins, sporulation and sporulation initiation time unweighted pair-group method analysis (UPGMA) was done. The UPGMA grouping (Fig 11) of the morphology data revealed the hierarchical nature of the different clusters. Pair–wise similarities between isolates ranged from 0.45 to 1.00.Indeed, one isolate (T-10) was separated from all others at the 0.28% similarity level in the UPGMA dendrogram. Fourty eight isolates were broadly segregated into five clusters representing, *Pachybasium*, *Trichoderma* and *Longibrachiatum* sections of Bisset.

There were five basic groups. Group I comprised of the members of five species of the genus *Trichoderma viz.*, *T. harzianum*, *T. viride*, *T. koningii*, *T. longibrachiatum*, and *T. pseudokoningii* species, which were distributed in several, sub groups.

Group II consists of six species of the genus *Trichoderma viz.*, *T. harzianum*, *T. viride*, and *T. koningii*, *T. fertile*, *T. aureoviride*, *T. longibrachiatum*, and *T. pseudokoningii*. Group III comprised of the members of five species of the genus *Trichoderma viz.*, *T. harzianum*, *T. viride*, *T. koningii*, *T. aureoviride*, *T. pseudokoningii* and *T. atroviride*. while group IV also comprised of six species of *Trichoderma viz.*, *T. harzianum*, *T. viride*, and *T. koningii*, *T. fasiculatum*, *T. aureoviride*, *T. longibrachiatum*, and *T. pseudokoningii*. All the species of four groups belong to *Pachybasium*, *Trichoderma* and *Longibrachiatum* sections of Bissett.

Group V includes three species of the genus *Trichoderma viz*., *T harzianum T viride, and T koningii* Members of group V belong to *Pachybasium* and *Trichoderma* sections of Bissett

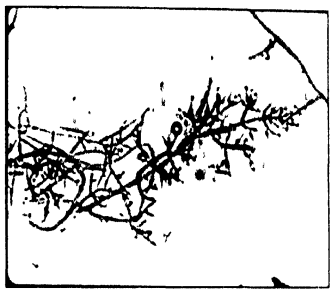
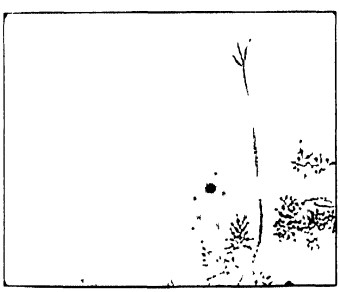


Fig 5 *Trichoderma harzianum* (T 109) showing pyramidal appearance of conidiophore branching



ig 6 *Trichoderma fertile* (T 47) showing
te conidiophore apex terminating with 2-3 phialides

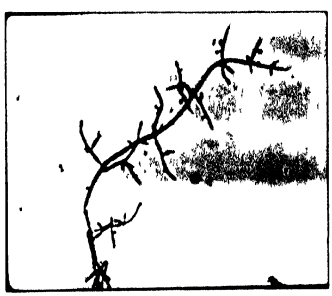


Fig 7 *Trichoderma viride* (T 179) showing typical curved phialides

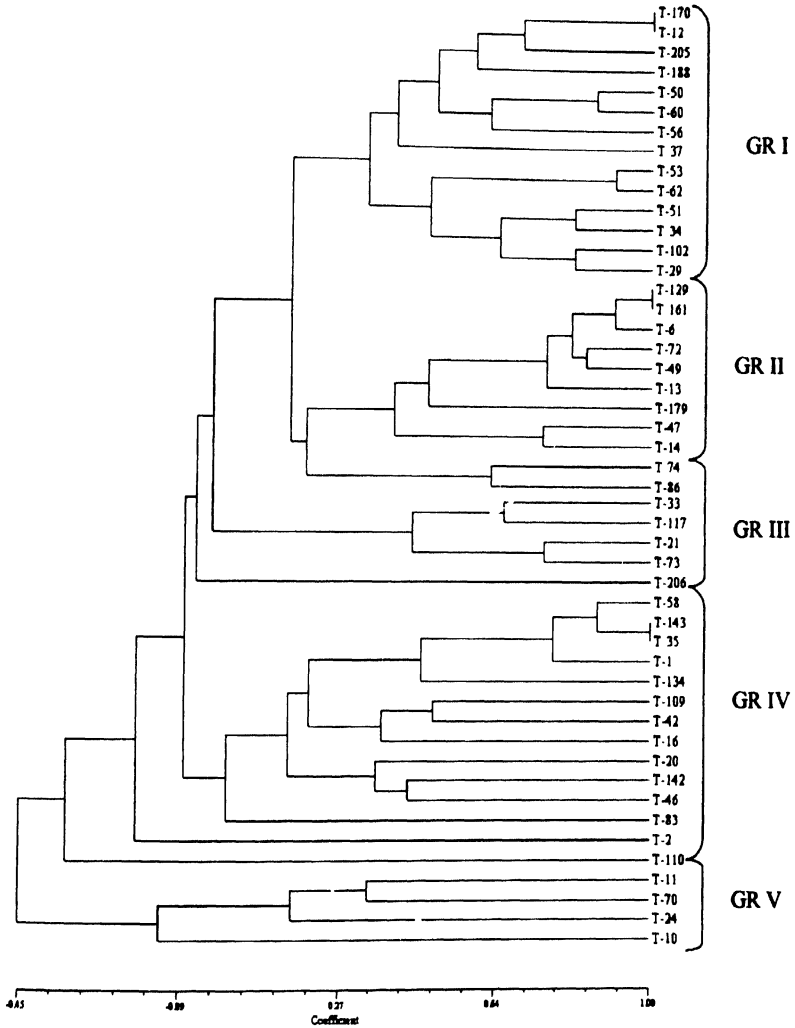


Fig. 11. UPGMA Dendrogram using morphology data of 48 *Trichoderma* isolates

Table 4 Identification of *Trichoderma* isolates into ten species

Trichoderma spp.	*Trichoderma* isolâtes	Bisset sections
T. harzianum	T 2, T 10, T 11, T 20, T 42, T 53, T 58, T 72, T 109, T 129, T 170, T 46, T74	*Pachybasium*
T. fertile	T 47	
T. fasiculatum	T 35	
T. viride	T 16, T 24, T 50, T 51, T 60, T 62, T 179, T 188, T 205,	*Trichoderma*
T. koningii	T 12, T 13, T21, T 33, T 49, T 70, T 83, T 143, T 161	
T. atroviride	T 73	
T. inhamatum	T 86	
T. aureoviride	T 14, T117	
T. pseudokoningii	T 29, T 37, T 206,T 134	*Longibrachiatum*
T. longibrachiatum	T 6, T 34, T 56, T 102, T 110,T 1, T 142	

AFLP Results:

Molecular characterization of the 48 *Trichoderma* isolates was done to compare the diversity study of *Trichoderma* isolates done based on morphological characteristics. AFLP was done, as it is able to simultaneously survey many more points on the genome than any other molecular assay.

Primary screening of AFLP primers combination

Thirty-six primer combinations were tested on four isolates for selection of primer combinations producing highest number of polymorphic fragments. The generated fingerprints were evaluated for overall clearness of the banding pattern and the number of polymorphic bands present is recorded. Six primer combinations were chosen for further screening on 48 *Trichoderma* spp. isolates. *Eco* RI+TC primer combination with any of the *Mse*I primers resulted in not-scorable fingerprints due to the amplification of too many and/or faint bands. Finally, six primer combinations were chosen for the diversity screening: they are *Eco*RI+AC/*Mse*I+CTG, *Eco*RI+AT/*Mse*I+CTA, *Eco*RI+TG/*Mse*I+CTA, *Eco*RI+ AG/*Mse*I+CTG, *Eco*RI+ TA/*Mse*I+CAC, *Eco*RI+ TA/*Mse*I+CTC.

Genetic diversity as defined by AFLP fingerprinting

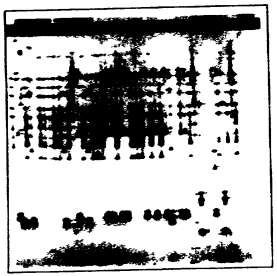
A total of 250 bands were obtained from six primer combinations (Fig 12), of which were polymorphic, with an average of 20 polymorphic bands per primer combinations. The number of polymorphic bands per primer varied as follows: *Eco*RI+AC/*Mse*I+CTG, 64 for *Eco*RI+AT/*Mse*I+CTA, 40 for *Eco*RI+TG/*Mse*I+CTA, 48 for *Eco*RI+

AG/*Mse*I+CTG, 28 *Eco*RI+ TA/*Mse*I+CAC, 46 for *Eco*RI+TA/*Mse*I+CTC. The genetic similarity coefficients obtained using the Jaccard algorithm were used for generating an UPGMA dendrogram (Fig 13) Pair–wise similarities between isolates ranged from 0.21 to 0.97. Indeed, one isolate (T 24) was separated from all others at the 21% similarity level in the UPGMA dendrogram. There were five basic groups. First group consists of only isolates of *T. harzianum*, which belong to the *Pachybasium* section of Bissett. Second group consists of isolates of *T. viride* and *T. koningii* belonging to section *Trichoderma* of Bissett. Third group consists isolates of six species of the genus *Trichoderma viz.*, *T. harzianum*, *T. pseudokoningii*, *T. longibrachiatum*, *T. koningii*, *T. viride* and *T. fasiculatum*. Some isolates of, *T. koningii*, *T. pseudokoningii*, *T. atroviride.*, *T. aureoviride and T. inhamatum* fall in to fourth group. Isolates of fourth group belong to *Pachybasium*, *Trichoderma* and *Longibrachiatum* sections of Bissett. Fifth group consists of *T. harzianum*, *T. pseudokoningii*, *T. longibrachiatum*, *T. koningii*, *T. viride, and T. fertile* and *T. aureoviride species* belonging to *Trichoderma* and *Longibrachiatum* sections of Bissett.

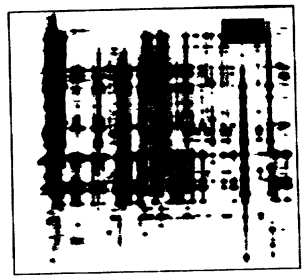
Inter- Relationships among Isolates

Based on MDS analysis of the AFLP dataset it was possible to separate isolates into 5 different groups with thirteen outliers (T 10, T 20, T 134, T 86, T 129, T 117, T 53, T 60, T 11, T 14, T 12, T 47 and T 50) (Fig 14). Group I consists of isolates of only *Trichoderma* section *viz.*, *T. atroviride*, *T. viride*, and *T. koningii*. Group II consists of *T. koningii*, *T. pseudokoningii* and *T. harzianum* species of *Pachybasium*, *Trichoderma* and *Longibrachiatum* sections. Group III consists species of only *Longibrachiatum* section

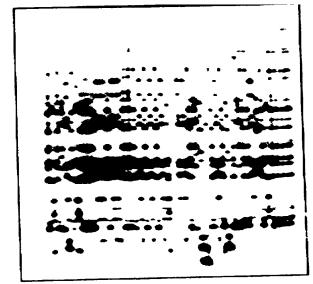
viz., *T. longibrachiatum* and *T. pseudokoningii. Group* IV consists of *harzianum* and *longibrachiatum* species of *Trichoderma* belonging to *Pachybasium* and *Longibrachiatum* sections. Group V consists of species of *Trichoderma viz*, *T. harzianum, T. viride, T. koningii, T. longibrachiatum,* and *T. pseudokoningii* belonging to all the three sections.



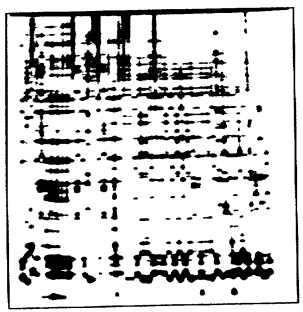
(a)



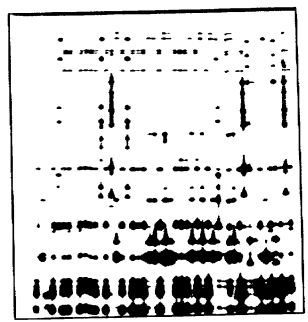
(b)



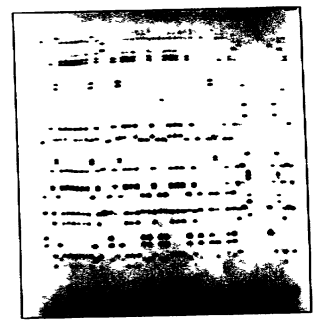
(c)



(d)



(e)



(f)

Fig 12 Autoradiogram showing the AFLP analysis of the 48 *Trichoderma* isolates Carried out using the following primer combinations a) *Eco*RI+AC/*Mse*I+CTG, b) *Eco*RI+AT/*Mse*I+CTA, c) *Eco*RI+TG/*Mse*I+CTA, d) *Eco*RI+ AG/*Mse*I+CTG, e) *Eco*RI+ TA/*Mse*I+CAC f) *Eco*RI+ TA/*Mse*I+CTC Order of loading of 48 DNA samples in the lanes is as given in Table 1

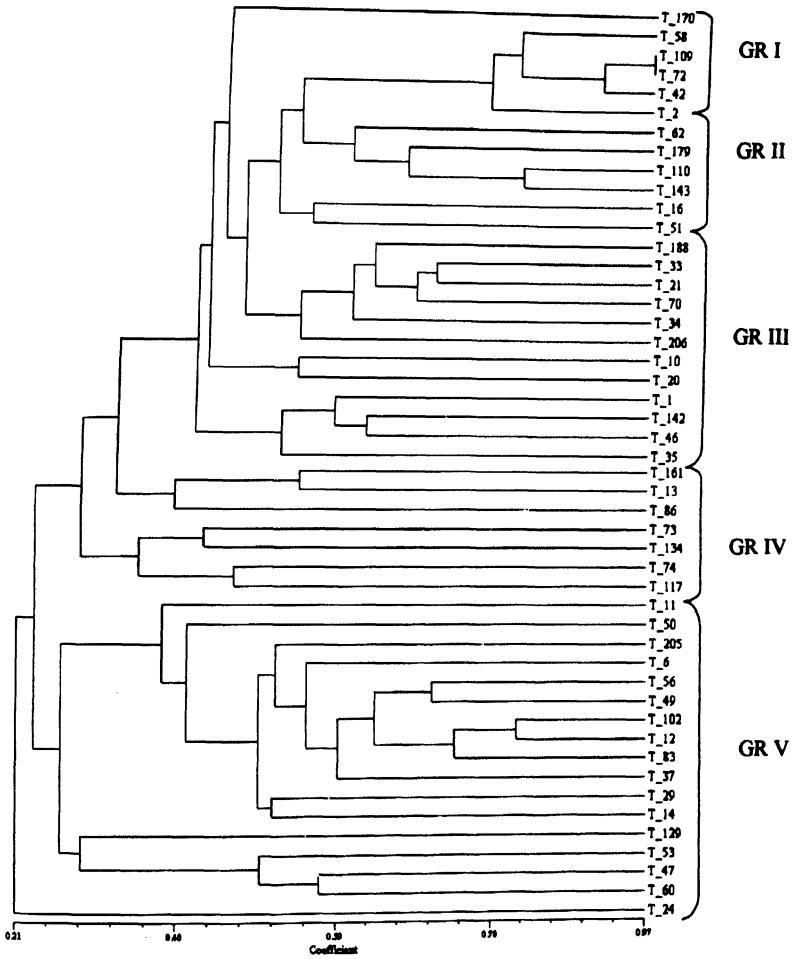


Fig. 13. UPGMA dendrogram obtained from AFLP analysis data of 48 *Trichoderma* isolates using 6 AFLP Primers.

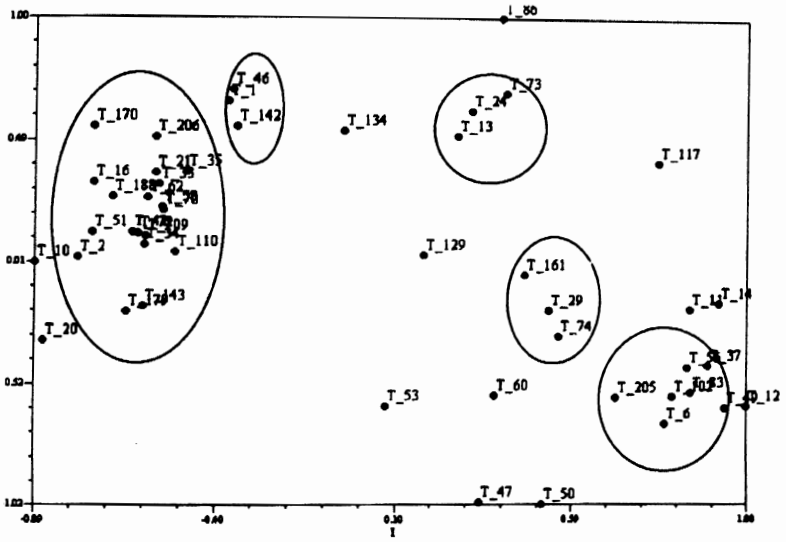


Fig. 14. Multidimensional scaling of 48 *Trichoderma* isolates using AFLP data.

ITS Results:

Apart from AFLP marker ITS primers were also used in pairs to compare the diversity study of *Trichoderma* isolates. PCR reaction was performed with ITS primers 1&4 showed amplification of DNA without adding extra $MgCl_2$ the reaction. ITS primers were used as they concentrate on smaller part of the genome. ITS primers 1, and 4 were used to identify fungi where there is prior knowledge about the genome. Twenty-five *Trichoderma* isolates only showed amplification producing 17 polymorphic bands (Fig 15). A single product of approximately 350 to 550 bp was obtained from all the PCR amplifications with ITS 1 and ITS 4 for 25 biocontrol isolates of *Trichoderma* spp. when restricted with *Hae*III and *Eco*RI restricted enzymes except for T 19, which showed a 650 bp fragment. This may be due to lack of restriction sites in that ITS region or it might be only amplified product and not the restricted product. Restriction with *Hae*III produced only 6 polymorphic bands as only nine restriction sites were there among 25 isolates for *Hae*III enzyme. Restriction with *Eco*RI produced 11 polymorphic bands as only 11 restriction sites were there among 25 isolates. Monomorphic bands were not seen. Dendrogram clusters correlated with species designations based on morphological characteristics and geographical collection site.

The genetic similarity coefficients obtained using the Jaccard algorithm were used for generating an UPGMA dendrogram (Fig 16). Pair–wise similarities between isolates ranged from 0 to 1.

All the 25 isolates amplified with ITS primers are broadly divided into two major clusters. The first major cluster consists of only *T. harzianum* isolates belonging to

Pachybasium section. The second major cluster is sub-divided into three sub clusters. The first sub-cluster consists of *T. harzianum*, *T. viride*, *T. koningii*, *T. fertile*, *T. longibrachiatum* and *T. pseudokoningii* species which belong to *Trichoderma* , *Pachybasium* and *Longibrachiatum* sections of Bissett. Second sub-cluster consists of *T. viride*, *T. koningii*, *T. harzianum*, *T. aureoviride*, *T. longibrachiatum*, *T. atroviride*, and *T. fasiculatum* species belonging to *Pachybasium*, *Trichoderma* and *Longibrachiatum* sections. Third sub-cluster consists of *pseudokoningii* and *aureoviride* species of *Trichoderma* belonging to *Trichoderma* and *Longibrachiatum* sections.

Based on MDS analysis of ITS dataset it was possible to separate the isolates into four groups with seven outliers (T 73, T 206, T 14, T 58, T 24, T 134 and t 170), (Fig 17). First group consists of *T. viride* and *T. longibrachiatum* species. Second group consists of species of *Trichoderma viz.*, *T. viride* and *T. longibrachiatum*. Third group consists of *koningii* and *pseudokoningii* species of *Trichoderma*. Species of all the three groups belong to the sections *Trichoderma* and *Longibrachiatum*. Fourth group consists of *T. koningii* species belonging to *Trichoderma* section.

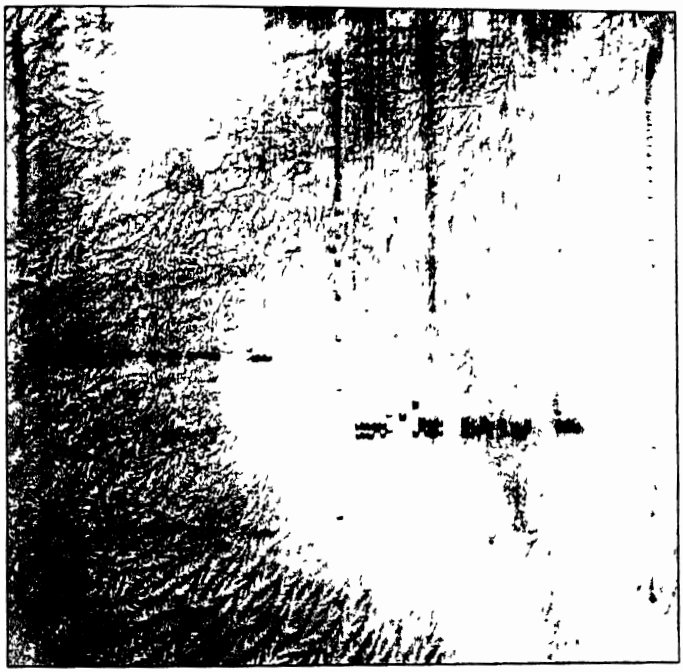


Fig 15 Polyacrylamide gel electrophoresis of amplified DNA of 25 *Trichoderma* isolates using ITS 1 and ITS 4 Primers restricted with *Hae*III and *Eco*RI restriction enzymes

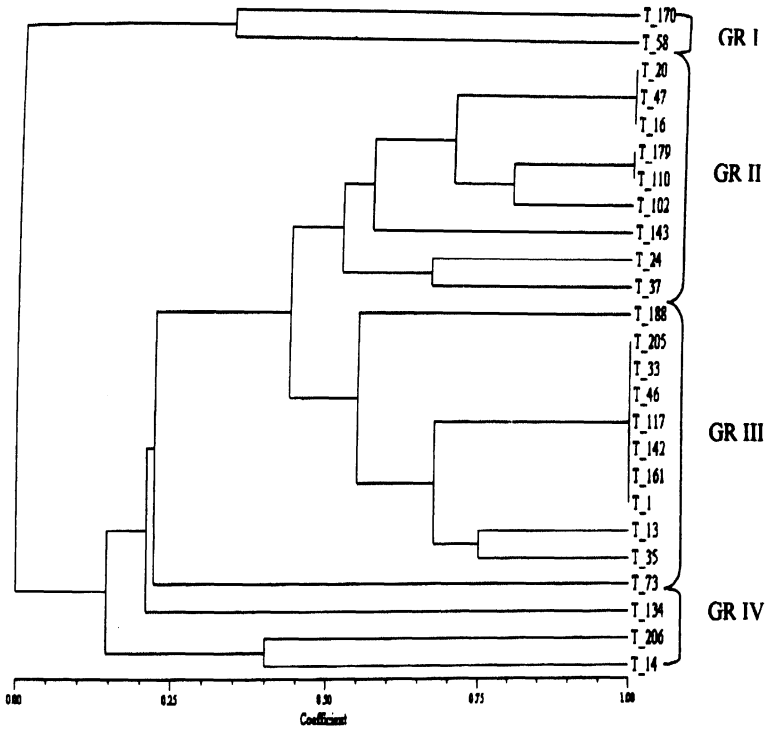


Fig. 16. UPGMA dendrogram of 25 *Trichoderma* isolates based on the similarity index data with ITS 1 and ITS 2 Primer data on 25 *Trichoderma* isolates was carried out under the Materials and methods.

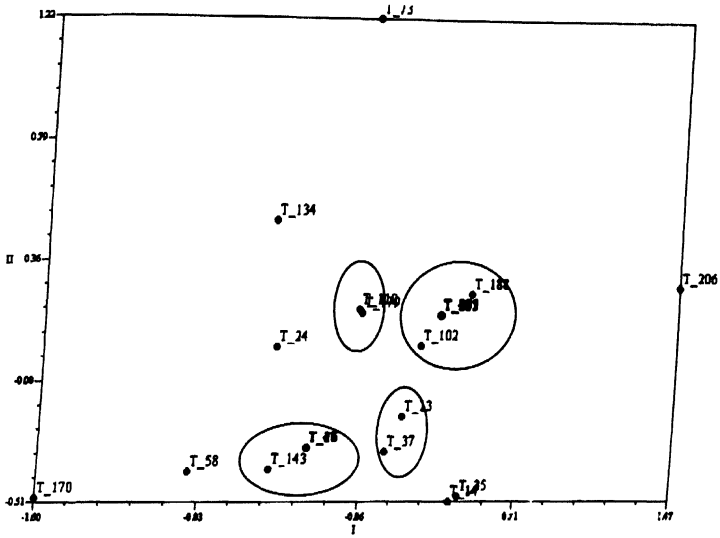


Fig. 17. Multidimensional scaling of ITS Primer data on the similarity index data from with ITS 1 and ITS 2 Primer data on 25 *Trichoderma* isolates was carried out under the Materials and methods.

DISCUSSION

Taxonomy of *Trichoderma* is complex, problematic and ever changing. Rifai, (1969) considered his system to be preliminary and, a way of grouping morphologically similar strains. Despite the artificiality of the approach, Rifai's system was immediately adopted and is still today the most generally used *taxonomy* of *Trichoderma*. Unfortunately, it can be argued that the users of the system have lost sight of its artificiality. The increasing number of users of *Trichoderma* species in biological control and biotechnology applications, as well as the involvement of *Trichoderma* as agricultural pests, and in toxin production, and human disease has emphasized the need for a predictive taxonomy for the genus.

Bissett's (1984, 1991a,b, c, 1992) rearrangement of the genus was a response to the need to recognize biological species in *Trichoderma*. However, this system that is based on morphological characters is difficult to use because of the paucity of morphological characters and their seeming plasticity. It should be noted, however, that difficulty in using the system does not invalidate it, but it leaves the user wishing for corroboration of determinations. Isoenzymes and nucleic acids especially haven proven to be a source of additional information. The *Hypocrea schweinitzii* complex, with the inclusion of section *Saturnisporum*, was shown to be monophyletic (Khuls *et al.*, 1997) and thus the morphological basis upon which individual species were defined are taxonomically predictive. Data from the six morphological characteristics (Srilakshmi *et al.*, 2001) was separated into quantitative and qualitative parameters and subjected to UPGMA diversity analysis. We have selected 48 isolates of *Trichoderma* based on their antagonistic activity

and identified up to species level. To estimate the inter-relationships among the 48 isolates of *Trichoderma* morphological parameters such as growth type, growth rate, colony color, shape of margins, sporulation and sporulation initiation time UPGMA grouping was done. Forty-eight isolates were broadly segregated into five clusters representing *Longibrachiatum*, *Pachybasium* and *Trichoderma* sections of Bisset in the present study. Our results broadly conform with the taxonomic system of Bissett and the isolates fall under *Longibrachiatum*, *Pachybasium* and *Trichoderma* sections of Bissett.

For a wide range of taxa, including plants, fungi and bacteria, AFLP markers have been used to uncover cryptic genetic variation of strains, or closely related species, that have been impossible to resolve with morphological or other molecular systematic characters. Therefore, AFLP have broad taxonomic applicability and have been used effectively in a variety of taxa including bacteria and fungi (Majer *et al.*, 1998). Complex AFLP patterns were obtained in the present study using six different primer pairs whose taxa had been uncertain based on morphological criteria. AFLP markers are useful in revealing genetic variation of *Trichoderma* isolates. Using six primer combinations with *Eco*RI (E) + 2 and *Mse*I (M) + 3 at the 3' –end of the primers on 48 isolates, a total of 250 bands were amplified with 250 polymorphic bands and there were no monomorphic bands.

The UPGMA analysis grouped the isolates into five different clusters, which consists of *Trichoderma* spp. belonging to the sections *Pachybasium*, *Trichoderma* and *Longibrachiatum*. AFLP analysis clearly separated the isolates into a sub-group *Pachybasium*. *Trichoderma* section was diversified into four groups among which one group is solely consisted of isolates of *Trichoderma* section while this section dominated in another group. Similarly in MDS analysis AFLP distinguished the isolates into five

groups with *Trichoderma* section distributed in all except in one group. Janssen *et al.*, (1996) have showed that the choice of the restriction enzymes and the length and composition of selective nucleotide will determine the complexity of the final AFLP fingerprint. The present finding is consistent with the work of Majer *et al.* (1996) in the AFLP analysis of pathogenic isolates of *Cladosporium fulvum* where they used E +2 and M +3 nucleotides. Gonzalez *et al.* (1998) also used two instead of three selective nucleotides (E +2 and M +3) in order to generate adequate number of fragments for AFLP analysis of *Colletotrichum lindemuthianum* isolates. Primer with one or two selective nucleotides are good in simple genomes such as fungi, bacteria and some plants, although selectivity is still acceptable with primers having three selective nucleotides, but it is lost with addition of the nucleotide (Vos *et al.*, 1995).

The exact characterization and identification of strains to the species level is the first step in utilizing the full potential of fungi in specific applications (Leickfeldt *et al.*, 2001).

Use of the AFLP fingerprinting method resulted in a high degree of discrimination and identification of *Trichoderma* spp. isolates and was found to be useful and practical.

However, evaluation of 40 or more morphological traits (Leickfeldt, 2001) presents a major bottleneck for the rapid identification of useful isolates. The similarity matrices generated by each of six primer pairs were highly correlated and were combined to determine the genetic relationships among the *Trichoderma* species and isolates. A similarity of 21% was found between 48 isolates of six *Trichoderma* spp. One isolate (T-24) was separated from all others at the 21% similarity level in the UPGMA dendrogram.

AFLP results revealed the usefulness of the primer pairs in segregating them into clear sub-groupings belonging to different sections of *Trichoderma* as propounded by Bisset

(1991a&b). AFLP analyses also sub grouped similar isolates of same species into separate groups validating the morphological identification.

Recently rDNA was used in comprehensive survey of section II and section III of the genus that were accepted by Bissett, (1984, 1991a), the rDNA sequence information provided support for merging the two sections. Moreover, the expanded sect. *Longibrachiatum* included five *Hypocrea* species and that the sexual (*Hypocrea)* and asexual (*Trichoderma)* strains did not represent separate lineages but rather represented a single lineage within the ascomycete genus *Hypocrea.* Consequently, they refered *Trichoderma* sect. *Longibrachiatum* as the *Hypocrea schweinitzii* complex (Samuels *et al.*, 1998). Only Twenty-one *Trichoderma* isolates showed amplification producing 17 polymorphic bands (Fig 16) when restricted with *Hae*III and *Eco*RI enzymes. The rDNA regions amplified by ITS 1&4 clearly divided the isolates into two major groups which showed distinct distribution of species. The rDNA ITS data segregates the isolates into *Pachybasium*, *Trichoderma* and *Longibrachiatum* sections. A close correlation of molecular characters with morphological identification is seen in this work. Molecular analysis of rDNA region of the genome is also supportive of the relationship with geographical origin of isolates. Ospina-Giraldo *et al.* (1998) examined nucleotide sequences of internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S gene of the ribosomal DNA repeat in 15 lines of *Trichoderma* spp. In the present study based on MDS analysis of the ITS dataset 4 different groups are separated. The present results clearly indicate the polymorphic nature of all 25 antagonistic *Trichoderma* isolates.

It is widely reported that *T. harzianum- T. inhamatum* is the most abundant taxon in virtually all habitats (Kubicek *et al.*, 2002). However, of the isolates investigated in this

study pre-selected on the basis of antagonism to *Aspergillus*, we found that the predominant taxon was *T. koningii* (T 83) followed by *T. longibrachiatum* (T 102) and *T. viride* (T 179). Abundance of *A. flavus* antagonistic *Trichoderma* species in groundnut fields could provide an effective biocontrol strategy for reducing the *A. flavus* population and consequently the aflatoxin contamination of groundnut kernels. The global *Trichoderma* biodiversity initiative has reported the identification of a high proportion of new species from collections in Asia and Europe (Bisset *et al.*, 2003). AFLP analysis has differentiated virtually all isolates tested in the present study in contrast to all previous used single assays (Lieckfeldt *et al.*, 2001). One of the main advantages of AFLP analysis is that it is able to simultaneously survey many more points on the genome than any other molecular assay. Unfortunately, this very high level of detectable genetic polymorphism across the genome (250 polymorphic bands in this study from just 6 primer combinations) generated highly complex diversity patterns.

We have investigated the possibility of using *Trichoderma* spp. derived from soils in major groundnut production areas in Southern India, as Biocontrol agent for the control of toxigenic *Aspergillus* spp. This endeavor would be significantly enhanced by the development of rapid and precise diagnostics for *Trichoderma* species and diversity groups with high levels of antagonistic behavior against *Aspergillus* species. To this end we have shown that candidate AFLP bands can be identified for conversion of simple PCR based markers, which may differentiate between the five major groups identified in this study. On the other hand species-specific metabolic properties was found associated with the different *Trichoderma* spp. but they were also not able to differentiate all species. (Kubicek *et al.*, 2002).

Chapter III
Mechanism of antagonism of *Trichoderma* isolates

INTRODUCTION

Biological control of pathogenic fungi in nature may be achieved by modifying the indigenous microbial community leading to the elimination of the pathogen, probably through a preferential stimulation of microbes capable of degrading cell walls of pathogenic fungi. Biological control of plant pathogenic fungi by *Trichoderma* mainly takes place either by antagonism or through cell lysis. The antagonistic principle may be diffusive or sometimes by the production of volatile substances. Antagonistic principle may vary from species to species or between the strains of the same species.

Enzyme induced lysis of fungal cell walls through extra cellular chitinases has been implicated as one of the chief mechanisms of biological control by the microorganisms Mycoparasitic activity is one of the major mechanisms proposed to explain the antagonistic activtity of *Trichoderma* spp. against many soil-borne plant pathogenic fungi (Elad *et al.*, 1982, Ridout *et al.*, 1986, Lynch, 1990).

Chitin, a linear polymer of β-1,3-glucanase, is an abundant polysaccharide in nature after cellulose, which is a major component of the cell walls of many fungi Peberdy (1990). In mycoparasitic interactions performed by *Trichoderma* spp. the production of a very complex cocktail of cell wall degrading enzymes is an important tool to destroy and feed on the host cell wall. Within the complex cocktail of enzymes, principal role has been attributed to chitinases and glucanases. The lytic activity of fungal and bacterial antagonists is mainly due to the lytic enzymes like β-1,3-glucanase and chitinase. The enzymes degrade the cell walls of target fungi allowing the mycoparasitic biocontrol agent to enter the lumen of the hyphae (Chet *et al.*, 1981, Chet, 1987). Most of the fungal and bacterial biocontrol agents (BCAs) are capable of producing chitinases, which. is an

inducible enzyme secreted by many microorganisms in cultures containing chitin or its oligomers as sole carbon source. β-1,3-glucanase is a semi- constitutive enzyme induced by laminarin, starch, xylose, mannitol and glycerol (Reese and Mendels, 1959).

Chitinolytic enzymes have been receiving attention in recent times in developing biopesticides, chemical defense proteins in transgenic plants and biocontrol agents. Being an important biocontrol agent and producer of lytic enzymes, *Trichoderma* spp. have become a safe and ecofriendly biological choice in the management of soil borne plant pathogens. Therefore the ability of an organism to secrete chitinases and β-1,3-glucanases is a desirable trait for a successful biocontrol agent. The enzyme producing capability may vary from species to species and even strain to strain within a species. In view of the diversity of strains among the species of *Trichoderma* this study was undertaken to estimate the production of lytic enzymes, and production of volatile and non-volatile principles by *T. harzianum* and *T. viride* effective against *A. flavus* (Af 11-4)

The objectives of this study were:

1. To estimate the nature of inhibition by *Trichoderma* isolates, and biochemical characterization of *Trichoderma* spp. based on production of cellulolytic enzymes: chitinase, glucanase, and protease

Chitinases are defined as glucosyl hydrolases cleaving a bond between the $C_1$and C_4 of two consecutive Glc NAG residues of chitin. The enzymatic degradation of chitin by many microorganisms takes place in two steps: 1. with a chitinase of endotype and 2. an N-acetyl glucosaminidase of exotype. However, in the literature both the enzymes are grouped under the name chitinase. Chitinases are present in a wide range of organisms, including those, which do contain chitin such as fungi, insects and crustaceans and also in organisms that do not contain chitin, such as bacteria, viruses.

Dennis and Webster (1971a) suggested that some *Trichoderma* isolates were found to produce volatile components inhibitory to the growth of other fungi. These isolates possessed a characteristic smell, especially members of the *T. viride* aggregate. The susceptibility of the test fungi varied widely. Dennis and Webster (1971b) studied the production of non-volatile antibiotics among isolates from different species groups of *Trichoderma*. The susceptibility of fungi to these antibiotics varied widely; *Fomes annosus* was the most susceptible and *Fusarium oxysporum*, the most resistant of the test fungi used. *Trichoderma* spp. are known to produce chitinases, β -1, 3-glucanases, proteases and volatile and non-volatile antibiotics (Elad *et al.*, 1982). Prasad and Rangeshwaran (2001) tested nine *Trichoderma* isolates, which included five *T. viride*, three *T. harzianum* and one *T. virens* isolate *in vitro* against *Sclerotium rolfsii*. All the isolates inhibited the mycelial growth of the pathogen and were superior to fungicides. They also showed good plant-growth promoting ability and rhizosphere competence. Cell walls of *Sclerotium rolfsii*, laminarin and chitin when used as a carbon source in the

liquid medium were able to induce chitinases and β -1, 3-glucanase activities by *T. harzianum* with glucose or cell walls of *R. solani* in the liquid medium (Elad *et al.*, 1980, 1983).

Relationship between the mycolytic enzymes and their significance in fungal cell wall lysis and degradation has been established by (Elad *et al.*, 1980, 1983). They found that more protein was induced on cell walls than by glucose suggesting these enzymes may be involved in the degradation of cell walls. *Serratia narcescens* potentially degraded the cell walls of *S. rolfsii* and its components chitin and laminarin produced the enzymes. The enzymes released N-acetyl glucosamine from the cell walls when incubated; indicating the possible degradation of cell walls (Ordentlich *et al.*, 1988). Sivan and Chet (1989a) investigated the effect of two *T. harzianum* strains on *F. oxysporum f. sp. vasinfectum* and *F. oxysporum f. sp. melonis* and found that in spite of not showing any mycoparasitism *in vitro* they were able to induce lytic enzymes in the liquid culture containing cell walls of *F. oxysporum*, *R. solani* and *S. rolfsii*. An isolate of *Trichoderma*, which inhibited the growth of *F. oxysporum f. sp. radicis-lycopersici*, displayed the ability to produce chitinases. The study of cytochemical aspects of chitin breakdown during mycoparasitic action by *Trichoderma* spp. indicated that enzymes play an important role in the antagonistic process (Cherif and Benhamou, 1990). Fourteen isolates of *Trichoderma* were screened to select an effective chitinase producer by Ulhoa and Peberdy (1991) and reported *T. harzianum* to be the best. The chitin concentration 0.5%, temperature 28 C and p^H 6.0 were optimal conditions for maximum enzyme synthesis. *Trichoderma* spp. produced good amount of enzyme when incubated with

chitin, laminarin and cell walls of pathogen as sole carbon source (Ordentlich *et al.*, 1991).

Harman *et al.* (1993) studied the chitinolytic enzymes of *T. harzianum* and purified two enzymes chitibiosidase (40 kDa and 35 kDa) and endochitinase (41 kDa) which were effective in inhibiting a range of chitin containing fungi like *Fusarium, Botrytis, Ustilago* and *Uncinula* etc. They opined that fungal chitinases were better over plant and bacterial chitinases. Ability of two chitinolytic enzymes for their antifungal activity against several fungal species revealed that spore germination and germ tube elongation were inhibited for all chitin containing fungi except *T. harzianum* strain P_1 which was the source for the enzymes (Lorito *et al.*, 1993, 1994). Combination of enzymes resulted in enhanced antifungal activity than their individual enzymes. They further stated that chitinolytic enzymes were more active than enzymes from other sources and more effective against a wide range of fungi and suggested the involvement of enzymes in the biocontrol. Redout *et al.* (1986) induced chitinase and β-1,3-glucanase activities in *T. harzianum* with glucose or cell walls of *R. solani* and found that cell walls induced more proteins than by glucose. *T. viride* produced more lytic enzymes than *T. harzianum*. Haran *et al.* (1996) suggested improved amounts of specific proteins by gene manipulation in biocontrol agents, with developing strains expressing multigene combination like transgenic *T. harzianum* which was transformed to produce increased amounts of specific proteins for better disease control. They also found that type of chitinase production varies from pathogen to pathogen. The differential expression of *T. harzianum* chitinases may influence the overall antagonistic ability of the fungus against a specific host.

Among 35 strains of *T. viride* and *T. harzianum* for their antagonistic ability against *R. solani*, four strains were found to be better. Their chitinase induction was studied with colloidal chitin. Purified fractions of culture filtrate inhibited *R. solani* growth *in vitro* (Krishnamurthy *et al.*, 1999). *T. harzianum* (T-33) and *T. hamatum* (C-1) were able to produce extracellular β -1,3 – glaucanases, and cellulases in culture medium amended with mycelium or fodder yeast and supplemented with avicel cellulose. The addition of fodder yeast (6%) and mycelium (6%) to the basal medium enhanced the production of β -1,3-glucanases than lactose. The medium enriched with mycelium produced more chitinases and cellulases. This enzyme was successfully used for protoplast formation of *Yarrowia lipolylica* (Witkowska *et al.*, 1999). Lima *et al.* (1999) investigated the ability of chitinase (46 kDa endochitinase-CHIT 46) induced from *Trichoderma* isolate (T_6) against cell walls of phytopathogens, *S. rolfsii* and *R. solani in vitro* and amount of enzymes secreted into the medium. A gene encoding for endoglucanase from *T. harzianum* was isolated and transferred to tobacco by Rincon, (2000) with which disease resistance was improved. Cepeda *et al.* (2000) suggested that chitinases may act as inducers of the defense systems of the plant during pathogen attack. Sreelatha *et al.* (2000) studied the dynamics of appearance of intracellular protease in relation to the synthesis of crystal δ-endotoxin to identify the native intracellular protease(s) involved in the proteolytic processing of the crystal δ-endotoxin of *Bacillus thuringiensis* subsp. *tenebrionis in vitro*. Marco and Felix (2002) characterized a protease produced by a *Trichoderma harzianum* isolate, which controls cocoa plant witches' broom disease. The purified enzyme substantially affected the cell wall of the phytopathogen *Crinipellis perniciosa*.

MATERIALS AND METHODS

Antifungal Compounds

Production of volatile antibiotics. Volatile antibiotic study was done following the method of (Dennis and Webster 1971b). Isolates of different species groups of *Trichoderma* were grown on 2% malt extract agar in Petridishes and incubated at 28 C for 48 h. After a given time, the lid of each dish was replaced by a bottom dish containing 2% malt extract agar inoculated with a test fungus. The two dishes were taped together with adhesive tape. The lids of control plates, which had not been inoculated with a *Trichoderma* strain, were also replaced in the same way. Test plates and control plates were set up in triplicate. The assembly was opened after 72 h and colony diameter of Af 11-4 was measured in each plate.

Production of diffusible antibiotics. Diffusible antibiotic study was done following the method of (Dennis and Webster 1971a). Petridishes were prepared containing 15 ml of 2% malt extract agar. The antagonist was grown from an inoculum disc over the surface of a cellophane membrane laid on an agar medium, and the metabolites produced were allowed to diffuse through the cellophane into the agar. Antibiotic activity was then assessed by growing *A. flavus* (Af 11-4) as the test fungus on the medium after removal of the antagonist. The plates were incubated for two days and compared the colony diameter and sporulation in plates with control.

Enzymes

A quantitative assay was performed in the preliminary screening of 48 *Trichoderma* isolates for enzyme production using agar well method. Based on the production of

chitinase enzyme, which was indicated by colour change of the medium seven isolates, were selected for further quantification of enzymes.

Enzyme Assay

1. Protein assay

Reagents

Alkaline sodium carbonate solution

Sodium carbonate solution was prepared by dissolving 2.50 g of sodium carbonate and 500 mg of NaOH in 100 ml of distilled water and the volume was made up to 125 ml with distilled water

Copper sulfate solution

Copper sulfate solution was prepared by dissolving 500 mg of copper sulfate and one gram of sodium potassium tartarate in 100 ml of distilled water.

Copper sulfate reagent

Fifty ml of alkaline sodium carbonate solution was dissolved in one ml of 2% copper sulfate solution.

Folins reagent

Folins reagent and distilled water were dissolved in 1:1 v/v ratio.

Methodology

Protein present in the crude enzyme was estimated using Lowry's method (Lowry *et al.*, 1951). Bovine serum albumin (100 mg100 ml^{-1}) was taken as standard. 0.1, 0.2, 0.3 ml of BSA was taken and volume was made up to one ml with distilled water. BSA was not added in blank. Four ml of alkaline reagent was added in each tube and incubated at room temperature (25 C) for 10 min. 0.4 ml of Folins reagent (1:1) was added and incubated at

room temperature for thirty min and absorbance was read at 550 nm. Protein concentration was expressed as µg of protein present in one ml of the sample.

Chitinase Assay

Preliminary screening:

To select efficient chitin degrading isolates, 48 antagonistic isolates of *Trichoderma* spp. were grown on synthetic medium where carbon source was substituted by colloidal chitin and incubated at room temperature. Controls were maintained without any carbon source.

Preparation of medium:

Synthetic Medium g l^{-1} (SM)

Glucose	--	15.000
$MgSO_4 7H_2O$	--	00.200
KH_2PO_4	--	00.990
KCl	--	00.200
NH_4NO_3	--	01.000
Fe^{2+}	--	00.002
Zn^{2+}	--	00.002
Agar	--	20.000

Chitinases were defined as enzymes cleaving a bond between C_1 and C_4 of two consecutive N-acetyl glucosamine sugars of chitin. Production of lytic enzymes like chitinases by biocontrol fungi plays an important role in controlling fungal pathogens of plants.

Preparation of substrate:

Colloidal chitin.

Colloidal chitin for enzymatic studies was prepared according to the procedure of Berger and Reynolds (1958). To one gram of chitin 2-3 ml of acetone was added to form a paste, the 5-10 ml of conc. HCl was added while grinding slowly in a mortar. The temperature was maintained between 10 and 20 C to arrest hydrolysis. After 15 min, the syrupy liquid was filtered through glass wool and poured into 5% aqueous ethanol with vigorous stirring to precipitate the chitin in a highly dispersed state. The sedimented material was resuspended in water several times to remove excess acid and alcohol and was lyophilized. The recovery of colloidal chitin from chitin was approximately 60%.

Enzyme assay

Reagents

Preparation of buffers:

NAG reagent: 8.84 g of NAG (N - acetyl D-glucosaminidase) was dissolved in 40 ml of water (double distilled) that gives 0.1μM / 0.1M.

Acetate buffer (1M p^H 5)

27.2 g of sodium acetate was added in 200 ml of acetic acid.

Borate buffer (1M p^H 9)

6.1 g of boric acid was taken in 100 ml of potassium hydroxide.

DMAB reagent:

Ten grams of DMAB reagent was dissolved in 100 ml of glacial acetic acid containing 12.5% of 10 N HCl. The reagent could be stored at 2 C for a month without significant

deterioration. Before use it was diluted with 9 volumes of reagent grade glacial acetic acid.

Methodology

Chitinase - β (1-4) - Poly N - acetyl D-glucosaminidase assay was carried out following minor modifications (Reissing *et al.*, 1959).

Sterilized potato dextrose broth flasks were inoculated with 1ml spore suspension of *Trichoderma* isolates and incubated in shaker at 30 C and 120 rpm for 7 days. After 7 days the contents were filtered using Buchner funnel with 2 layers of whatman filter paper under cold conditions (4 C). The filtrate was measured and ammonium sulfate @ (54.1 g/100 ml) was added on a magnetic stirrer under cold conditions. Ammonium sulfate was added slowly with continuous stirring. The filtrate was centrifuged under cold conditions at 12000 rpm for 30 min and the pellet was dissolved in ice-cold distilled water and dialysis was done using dialysis membrane (Fisher Scientific) for 48 h with change of water at every 4 h interval. The water was tested in the beaker with barium chloride for presence of any precipitation. Two to three drops of barium chloride (50 mg/50 ml) was added to 10 ml of water in the beaker, dialysis was stopped, as there was no precipitate in the water. The sample was lyophilized overnight after dialysis to get crude chitinase enzyme. One ml of crude chitinase enzyme (1mg of enzyme was dissolved in 1ml of borate buffer) was added to 2 mg of pure chitin in an eppendorf and incubated for 24 h at 37 C. The solution was centrifuged at 12000 rpm for 10 min under cold conditions. Supernatant was taken in a test tube (166 µl) and 33 µl of borate buffer was added and boiled exactly for 3 min at 100 C and cooled immediately under tap water one ml of DMAB reagent (diluted 10 times in acetic acid) was added and incubated for

20 min at 37 C in a shaking water bath. OD values were taken at 580 nm. Blank was maintained. Specific activity of chitinase was expressed as μ moles of Glc NAc released /mg of protein / h.

200 μl reaction mixture containing 1% chitin, 20 μg of protein (crude enzyme) in 0.1 M acetate buffer (p^H 5.0) is incubated for the desired time in water bath shaker (100 rpm) at 37 C. The reaction is stopped by boiling at 100 C and then centrifuged at 10,000 rpm for 10 min; the supernatant is used for assay.

β- 1,3- Glucanase Assay:

Reagents:

Laminarin 1.0%

Acetate buffer, 0.1M p^H 5.0

DNS reagent (dinitrosalicylic acid):

DNS reagent was prepared by dissolving a mixture of 2 g of dinitrosalicylic acid, 0.4 g phenol, 0.1 g sodium sulfate and 40 g Rochelle salt in 100 ml of 0.1% NaOH and diluted to 200 ml.

Methodology

Glucanase was assayed by estimating the glucose released from laminarin (Miller *et al.*, 1960). The reaction mixture contained 20 μg of lytic enzyme in 400 μg of 0.1 M acetate buffer p^H 5 and 100 μl of 1% laminarin (10 mg / 1ml). Total volume of reaction mixture was 500 μl. The reaction was carried out at 37 C in a shaking water bath for 30 min (100 rpm). After incubation, 3 ml of DNS reagent was added to reaction mixture and boiled for 5 min in a boiling water bath, followed by cooling in running tap water adjusted to ambient temperature. Reading of absorbance was finally made in a spectrophotometer

(Beckman model DV-20) at a wavelength of 640 nm. Specific activity of β-1-3 Glucanase was expressed as (μ. moles) of glucose released/mg/ protein/ h. The amount of Glucanase protein present was determined by running the samples on SDS-PAGE gels using prestained protein Molecular Weight Marker (118kDA, Fermentas).

Protease Assay

Reagents

Borate buffer (1 M p^H 9)

6.1 g of boric acid was taken in 100 ml of potassium hydroxide.

Potassium per chlorate (7% $KClO_4$)

7 ml of $KClO_4$ was dissolved in 100 ml distilled water

10N NaOH

400 g of NaOH pellets were dissolved in 1000 ml distilled water.

Methodology

Protease assay was carried out according to the method of Prestige *et al.*, (1971).

Three mg of Azocaesein was taken in 0.1M Tris HCl p^H 8 and incubated for 1h at 30 C in a total volume of 1ml. The reaction was terminated by adding 2 ml of ice-cold 7% potassium perchlorate. The protein was allowed to precipitate for 15 min at room temperature and centrifuged at 4,000 rpm for 10 min. To the supernatant 0.3 ml of 10 N NaOH was added and intensity of colour was determined at 440 nm. Specific activity of β -1-3 glucanase was expressed as μm of glucose released/mg/protein/h. The amount of protease protein present was determined by running the samples on SDS-PAGE using prestained protein molecular weight marker (118 kDA).

SDS PAGE Electrophoresis

Materials

1. Vertical slab gel electrophoresis unit (Biorad)
2. Power supply
3. Acrylamide
4. Bis acrylamide
5. Ammonium persulphate
6. TEMED.
7. Protein marker (Fermentas)

Stock solutions

Acrylamide/Bis mixture

30 % Acrylamide/Bis mixture	--	30: 0.8 w/w
Acrylamide	--	30.0 g
Bis acrylamide	--	00.8 g
Water	--	100 ml

Resolving gel buffer

Tris – HCl	--	01.0 M (p^H 8.8)
Tris base	--	12.1 g
Water	--	100 ml

The contents were dissolved in about 80 ml water; p^H was adjusted to 8.8 with 1 N HCl and made up to 100 ml with distilled water.

Stacking gel buffer

Tris – HCl	01.0 M (p^H 6.8)
Tris base	12.1 g
Water	100 ml

The contents were dissolved in about 80ml water; p^H was adjusted to 6.8 with 1 N HCl and made up to 100ml with distilled water.

10% SDS

10 g of SDS was dissolved in distilled water with gentle stirring and volume was made up to 100 ml.

0.5% (W/V) bromophenol blue (BPB)

500 mg of BPB was dissolved in 100 ml of distilled water.

Sample buffer

Distilled water	-- 4.57 ml
1 M Tris HCl buffer, p^H 6.8	-- 0.63 ml
40% Glycerol	-- 2.50 ml
10% SDS	-- 2.00 ml
2-mercaptoethanol	-- 0.10 ml
0 5% BPB	-- 0.20 ml

Electrode buffer, p^H 8.3

Tris base	-- 06.0 g (0.05 M)
Glycine	-- 28.8 g (0.384 M)
SDS	-- 01.0 g (0.1%)
Water	-- 1000 ml

Resolving gel preparation (10%)

Distilled water	-- 00.80%
Resolving gel buffer	-- 11.25%
Acrylamide: Bis mixture	-- 10.00 ml
10% SDS	-- 00.30 ml
10% Ammonium persulfate (APS)	-- 300 µl
TEMED	-- 50 µl

Stacking gel preparation (4%)

Distilled water	-- 7.00 ml
Stacking gel buffer	-- 1.25 ml
Acrylamide: Bis mixture	-- 1.50 ml
10% SDS	-- 0.10 ml
10% Ammonium persulfate (APS)	-- 0.20 ml
TEMED	-- 5.00 µl

Gel Staining

Coomassie Brilliant blue Staining

Coomassie Brilliant blue R 250	-- 200 mg
Methanol	-- 100 ml
Acetic acid	-- 14 ml
Water	-- 200 ml

The stain was dissolved and filtered through Whatman N0.1 paper before use.

Destaining solution

Methanol	-- 15 ml

Acetic acid	--	7.0 ml
Water	--	100 ml

Methodology

Preparation of Slab Gel

Preparation of resolving gel

1. Slab gel apparatus was constructed according to the instructions of the manufacturer (Broviga). The vertical slab gel Unit was assembled in the casting mode using 1.5 or 1.0 mm spacers.

2. The resolving gel solution was taken into the sandwich (leave space for pouring stacking gel above the resolving gel).

3. Small quantity of water was added on the side of the slab. The water layers were spread evenly across the entire surface. A very sharp water-gel interface was visible when the gel was polymerized.

4. Casting stand was tilted to pour off the water layer.

5. The surface of the gel was rinsed once with distilled water.

Preparation of stacking gel.

Comb was inserted into the sandwich (1.0 mm or 1.5 mm thickness) and stacking gel solution was poured slowly without trapping any air bubbles below the teeth of the comb, to avoid local distortion in the gel surface at the bottom of the wells resulting in uneven migration of samples.

Preparation of Sample

1. The sample was suspended in an appropriate volume of the sample buffer and kept for heating in boiling water for 3 min.
2. The sample was removed and transferred it on ice until ready for use. The denatured sample was stored at –20 C for future use.

Loading and Electrophoresis

1. The comb was slowly removed from the gel straight up to avoid disturbing the well dividers.
2. The wells were rinsed with tank buffer once and then filled with tank buffer.
3. Samples and molecular weight marker proteins were loaded into separate wells.
4. The lower chamber of the electrophoresis apparatus was filled with tank buffer until the sandwich was immersed in buffer. The trapped bubbles under the ends of the sandwich were coaxed with a long Pasteur pipette.
5. The upper chamber of the electrophoresis apparatus was filled with tank buffer. Buffer was not poured into the sample wells to prevent disturbance of the sample.
6. The unit was connected to the power supply. The cathode was connected to the upper buffer chamber and voltage was set at 50 V initially and once the sample moves out of the well voltage is adjusted to 100 V, current at 40 mA, power at 100 W,
7. When the tracking dye (BPB) reaches nearly the bottom of the gel, the power supply was turned off and the power cables were disconnected.

Staining and Destaining of Gel

Coomasie Brilliant Blue Staining Procedure

1.The sandwich was disassembled and the gel was gently slided into a tray containing the staining solutions.

2.The tray was shaken gently on a shaker for 30 min.

3.The staining solution was removed.

4. Destaining solution was added and gently shaken on shaker. The destaining solution was replaced periodically until the bands were clearly visible.

5.The gels were stored in destaining solution.

RESULTS

Production of volatile antibiotics.

Among 21 isolates of *Trichoderma* species, six *T. harzianum*, five *T. longibrachiatum*, six *T. koningii* and three *T. viride* isolates showed inhibition of *A. flavus* (Af 11-4) colony by producing volatile antibiotics compared to the control. In the control plate Af 11-4 colony diameter was 60 mm where as in other plates Af 11-4 colony diameter was in the range of 10-45 mm. *T. longibrachiatum* (T 102) significantly inhibited the colony growth of Af 11-4 compared to other *Trichoderma* spp. *T. fertile* (T 47), *T. viride* (T 50), and *T. koningii* (T 161) were least effective against Af 11-4 growth (Table 5, Fig 18). Eight isolates belonging to *T. longibrachiatum*, *T. koningii*, and *T. viride* were effective in inhibiting *A. flavus* by producing volatiles.

Production of diffusible antibiotics.

Among 15 *Trichoderma* isolates four *T. harzianum*, three *T. koningii*, three *T. pseudokoningii* and four *T. viride* isolates showed inhibition of *A. flavus* (11-4) colony by producing diffusible antibiotics compared to the control. *T. pseudokoningii* (T 29), *T. harzianum* (T 42), *T. koningii* (T 83) showed significant inhibition compared to other *Trichoderma* spp. *T. viride* (T 62) was the least effective against *A. flavus*. (Table 6, Fig 19).

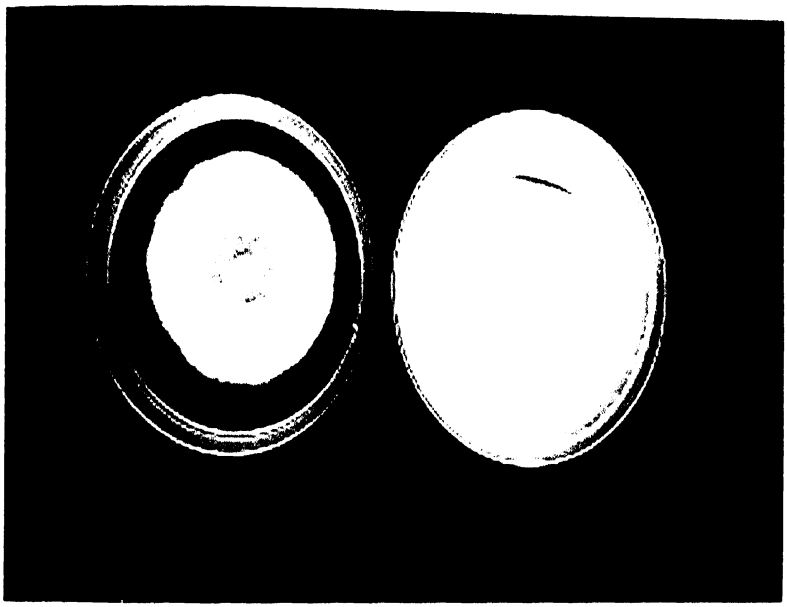


Fig. 18. Effect of volatile antibiotics produced by *T. longibrachiatum* (T 102) over control. Left: Restricted *A. flavus* growth, Right: Control (*T. longibrachiatum)*

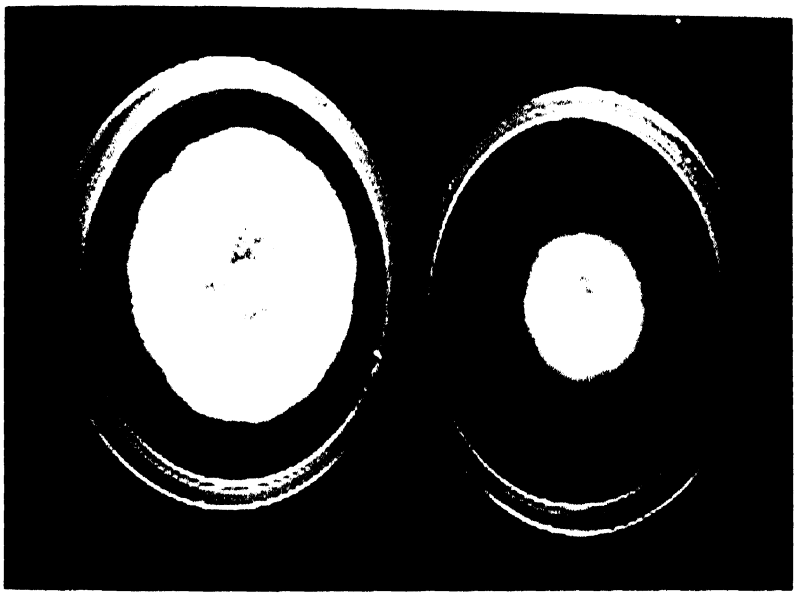


Fig. 19. Effect of diffusible antibiotics produced by *T. koningii* (T 83) over control. Left: Control (*A. flavus*), Right: Restricted *A. flavus* growth

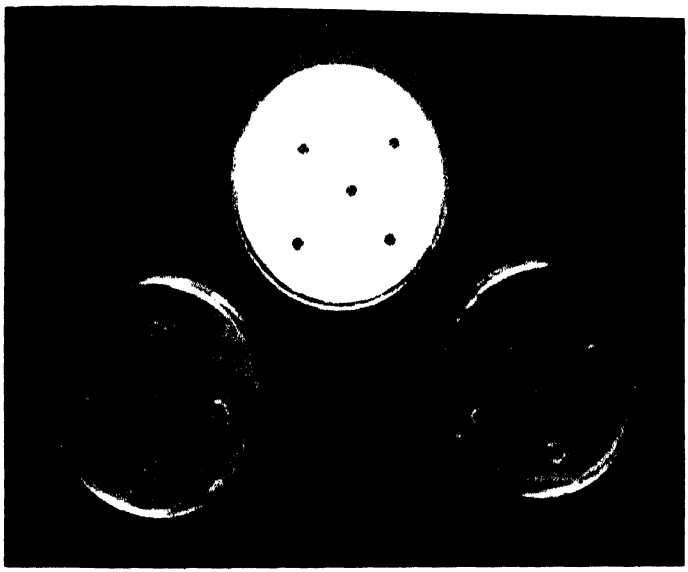


Fig. 20. Preliminary screening of *Trichoderma* isolates for production of Chitinase enzyme. Top: Control (T 35) not producing chitinase enzyme; Lower plates: *Trichoderma* isolates Producing Chitinase enzyme: left (T 83), right (T 102).

Chitinase production by *Trichoderma* spp. in the liquid medium containing colloidal chitin as sole carbon source

Among 48 antagonistic isolates seven were able to degrade colloidal chitin substituted in the medium. Chitin degradation and chitinase enzyme production is indicated by colour change of the medium when compared to control (Fig 20).

Ten different species of *Trichoderma viz., T. harzianum, T. fertile, T. fasiculatum, T. koningii, T. viride, T. atroviride, T. inhamatum, T. aureoviride, T. pseudokoningii* and *T. longibrachiatum* were grown in liquid medium containing colloidal chitin as sole carbon source, and all the species produced chitinases. Isolates *T. koningii* (T 83) and *T. longibrachiatum* (T 102) produced maximum crude protein followed by *T. viride* (T 179). Among the isolates *T. viride* (T 179) produced more crude protein than *T. viride* (T 24). *T. harzianum* (T 20) showed minimum crude protein on par with *T. pseudokoningii* (T 29) among all seven *Trichoderma* isolates (Table 7).

Specific activity of chitinase produced by different *Trichoderma* isolates in liquid medium containing colloidal chitin as a sole carbon source is presented in Table 8. Maximum chitinase activity was associated with the isolate *T. viride* (T 24) more than the other isolate of *T. viride* (T 179). Isolates *T. longibrachiatum* and *T. koningii* also showed appreciable enzyme activity on colloidal chitin when supplied as a sole carbon source.

Glucanase activity was restricted to few species among the seven isolates tested on colloidal chitin as a sole carbon source (Table 9). Only *T. koningii* and *T. longibrachiatum* produced glucanase in the presence of glucose while all others failed to induce any enzyme activity.

On the other hand, all the isolates were capable of producing proteases in liquid medium containing colloidal chitin (Table 10). No enzyme activity was observed in control without any carbon source in the medium. Isolates *T. pseudokoningii* and *T. harzianum* showed maximum protease activity compared to others. Isolate T 179 was the poor producer of protease enzyme.

Trichoderma koningii and *T. longibrachiatum* were good at producing more crude protein, chitinases and glucanases in liquid medium with colloidal chitin as sole carbon source. These two species, on the other hand were poor producers of proteases. *T. harzianum* and *T. psedokoningii* produced maximum proteases and minimum crude protein with low chitinase activity in liquid medium containing colloidal chitin. No glucanase activity was observed by these *Trichoderma* species. *T. viride* isolate T 24 showed high chitinase activity though the amount of crude protein produced is less than the other isolate T 179.

The samples, after running on SDS PAGE, molecular weight of enzyme proteins was compared with 118 kDa β-galactosidase prestained protein marker. Chitinase and protease enzyme production could only be quantified, as the bands were thick and clear. Glucanase production was not quantified as the isolates produced very faint bands. Molecular wt. of chitinase enzyme was 28 kDa and protease was 72 kDa (Fig 21).

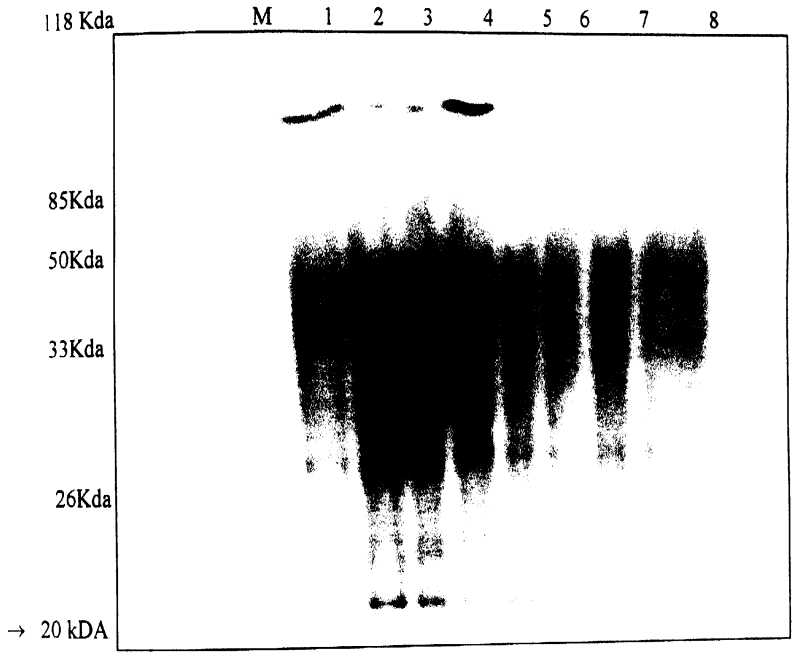


Fig. 21. SDS-PAGE stained with coomasie brilliant blue dye. M indicates the marker. Lane numbers 1-7 indicates seven different *Trichoderma* species and lane number 8 indicates control.

Table 5. Effect of *Trichoderma* volatile antibiotics on the growth of *A. flavus* (Af 11-4).

Isolate no.	*Trichoderma* spp.	*A. flavus* (*Af 11-4*) colony diameter (cm)
T 2	*T. harzianum*	4.0
T 6	*T. longibraciatum*	2.6
T 10	*T. harzianum*	3.8
T 13	*T. koningii*	2.0
T 21	*T. koningii*	3.0
T 24	*T. viride*	2.8
T 34	*T. longibrachiatum*	3.0
T 47	*T. fertile*	4.5
T 49	*T. koningii*	3.3
T 50	*T. viride*	4.5
T 53	*T. harzianum*	4.0
T 56	*T. longibrachiatum*	4.0
T 58	*T. harzianum*	3.2
T 70	*T. koningii*	3.0
T 102	*T. longibrachiatum*	1.0
T 110	*T. longibrachiatum*	4.0
T 129	*T. harzianum*	3.3
T 143	*T. koningii*	3.0
T 161	*T. koningii*	4.5
T 170	*T. harzianum*	3.2
T 179	*T. viride*	2.8
control		6.0
S.E+		0.4757

* The data is the mean of three replications

Table 6. Effect of *Trichoderma diffusible antibiotics on the growth of A. flavus (Af 11-4).*

Isolate no.	*Trichoderma* spp.	*A. flavus* colony diameter (cm)
T 11	*T. harzianum*	1.8
T 12	*T. koningii*	1.4
T 20	*T. harzianum*	2.3
T 29	*T. pseudokoningii*	1.0
T 33	*T. koningii*	2.5
T 37	*T. pseudokoningii*	3.0
T 42	*T. harzianum*	1.0
T 51	*T. viride*	3.0
T 60	*T. viride*	1.5
T 62	*T. viride*	5.0
T 72	*T. harzianum*	2.2
T 83	*T. koningii*	1.0
T 142	*Trichoderma sp.*	3.0
T 205	*T. viride*	2.0
T 206	*T. pseudokoningii*	4.0
Control		5.5
S.E+		0.451

The data is the mean of three replications

Table 7. Amount of protein produced by *Trichoderma* spp. in liquid medium containing colloidal chitin as sole carbon source.

Isolate	**Protein produced in mg / ml of the sample**
T. harzianum (T 20)	06.0
T. pseudokoningii (T 29)	07.0
T. fertile (T 47)	52.0
T. viride (T 24)	29.0
T. koningii (T 83)	75.0
T. longibrachiatum (T102)	66.5
T. viride (T 179)	61.0

Table 8. Specific activity of chitinase produced by *Trichoderma* spp. in liquid medium containing colloidal chitin as sole carbon source.

Isolate	**Specific activity of chitinase / μ M of Glc Nac / mg protein/ h**
T. harzianum (T 20)	166.60
T. pseudokoningii (T 29)	071.40
T. fertile (T 47)	096.15
T. viride (T 24)	551.70
T. koningii (T 83)	346.60
T. longibrachiatum (T102)	360.00
T. viride (T 179)	327.80

Table 9: specific activity of Glucanase produced by *Trichoderma* spp. in liquid medium containing colloidal chitin as sole carbon source.

Isolate	**Specific activity of glucanase / µM of Glc Nac / mg protein/ h**
T. harzianum (T 20)	0.00
T. pseudokoningii (T 29)	0.00
T. fertile (T 47)	0.00
T. viride (T 24)	0.00
T. koningii (T 83)	0.16
T. longibrachiatum (T102)	0.15
T. viride (T 179)	0.00

Table 10. Specific activity of protease produced by *Trichoderma* spp. in liquid medium containing colloidal chitin as sole carbon source.

Isolate	**Specific activity of protease/µM of Glc Nac / mg protein / h**
T. harzianum (T 20)	0.86
T. pseudokoningii (T 29)	0.87
T. fertile (T 47)	0.21
T. viride (T 24)	0.44
T. koningii (T 83)	0.26
T. longibrachiatum (T102)	0.24
T. viride (T 179)	0.19

DISCUSSION

Aflatoxin contamination of groundnut, caused by *Aspergillus flavus* (Af) group of fungi, is a major problem in the rain fed agriculture in the semi-arid tropics. Effective biocontrol of plant pathogens by *Trichoderma* spp. depends upon the ability of enzymatic degradation of the pathogen as well as antibiotic production by the antagonist. Dennis and Webster (1971a,b, c) differentiated the mechanism of biological control among the isolates of *Trichoderma* species into three categories, *viz.*, volatile antibiotics, non-volatile antibiotics and hyphal interaction. In the present study all the three types of antagonistic/enzymatic behavior was observed among the various species of *Trichoderma*. *T. longibrachiatum* (T 102) and *T. viride* (T 24 and T 179) effectively inhibited *A. flavus* by producing volatile antibiotics. On the other hand isolates belonging to *T. koningii* (T 83), *T. viride* (T 62), *T. harzianum* (T 42) and *T. pseudokoningii* (T 29) inhibited the pathogen through diffusible antagonistic substances into the medium. The results suggest that *Trichodermna* species vary in their ability in producing volatile or diffusible (non-volatile) antagonistic substances as a means of inhibition of the pathogen. The results also reveal that isolates of the same species (*T. viride*) produced both volatile and non-volatile substances whereas *T. harzianum* and *T. pseudokoningii* inhibited the growth of the pathogen only through non-volatile antibiotics. Pandey and Upadhyay, (1997) selected *T. viride* as the most potential antagonist based on the fungitoxic effect of its volatile and non-volatile antibiotics against *Fusarium udum*. Volatile and non-volatile compounds produced by *T. viride* were found to be most fungi toxic followed by *Gliocladium virens*.

Prasad and Rangeshwaran (2001) tested nine *Trichoderma* isolates, comprising of *T. viride*, *T. harzianum* and *T. virens* isolates *in vitro* against *Sclerotium rolfsii* and reported the inhibition of the mycelial growth by volatile and non-volatile antibiotics which were superior to fungicides. Non-volatile antibiotics of *T. viride* reduced the biomass of *Colletotrichum capsici* and inhibited the synthesis of DNA, RNA and proteins (Rajathilagam and Kannabiran, 2001). Mycoparasitism involves production of cell wall degrading enzymes to destroy *A. flavus*. Most of the *Trichoderma* spp. were good lytic enzyme producers. In order to select a better biocontrol agent against aflatoxin producing pathogen (*A. flavus*) six *Trichoderma* species were tested for their ability to produce lytic enzymes when provided with direct chitin in the form colloidal chitin. Among the isolates tested, *T. viride* (T24) was a good producer of chitinases followed by *T. longibrachiatum* (T 102). Redout *et al.* (1986) induced chitinase and β-1,3-glucanase activities in *T. harzianum* with glucose or cell walls of *R. solani* and found that cell walls induced more proteins than by glucose. *T. viride* produced more lytic enzymes than *T. harzianum*. Krishnamurthy *et al.* (1990) screened 35 strains of *T. viride* and *T. harzianum* for antagonism against *R. solani* and reported that four strains were better for chitinase induction with colloidal chitin. Chitinase production was enhanced when colloidal chitin was used as a sole source of carbon instead of glucose. A high level of chitinase production with colloidal chitin was observed in the present study. Ulhoa and Peberdy (1991) screened 14 isolates of *Trichoderma* and selected *T. harzianum* as an effective chitinase producer at 0.5% chitin with p^H 6.0 and temperature of 28 C. Harman *et al.* (1993) purified the chitinolytic enzymes of *T. harzianum*, which inhibited a range of chitin containing fungi like *Fusarium*, *Botrytis*, *Ustilago* and *Uncimula*. Lorito *et al.*

(1993) stated that chitinolytic enzymes from *T. harzianum* were biologically more active than enzymes from other sources as they were highly effective against a wide range of fungi.

Glucanases are yet another important group of enzymes that are involved in biological control. Among the *Trichoderma* isolates used in biocontrol of toxigenic *A. flavus*, only *T. koningii* (T 83) and *T. longibrachiatum* (T 102) produced glucanase enzyme. These isolates also produced more crude protein than others. Hadar *et al.* (1979) reported that *T. harzianum* was able to produce β-1,3-glucanase and chitinase when grown on laminarin and chitin as carbon sources and it effectively controlled the damping-off disease caused by *R. solani*. Sivan and Chet (1989a) observed that a strain of *T. harzianum* released β-1,3-glucanase and chitinase into the medium containing laminarin, chitin or fungal cell walls of *F. oxysporum*, *R. solani* and *S. rolfsii* as sole carbon sources. In this study protease production was found to be highest in *T. pseudokoningii* (T 29) and *T. harzianum* (T 20). Marco and Felix (2002) characterized a protease produced by a *T. harzianum* isolate, which controlled witches' broom disease of cocoa. The purified enzyme substantially affected the cell wall of the pathogen *Crinipellis perniciosa*.

T. viride (T 24) showed more chitinase enzyme activity though it did not produce maximum crude protein. This may be due to presence of maximum amount of chitinase produced by *T. viride* (T 24) among the enzymes present in the crude protein. Lytic enzymes undoubtedly contribute to the mechanism of biocontrol and in this case, probably a mixture of enzymes released from *Trichoderma* isolates acted in consortia as a cocktail in suppressing the activity of *A. flavus*. Therefore, the consortia approach to biological control is highly desirable in overcoming the limitations of environment as

well as the intricacies of host pathogen interactions. However, the differential expression of chitinases of *T. harzianum* as suggested by Haran *et al.* (1996) may influence the overall antagonistic or mycoparasitic activity of the fungus against a specific host. The mode of expression depends on the type of pathogen the biocontrol agent attacks and the complex interaction that takes place in the rhizosphere.

Transforming *Trichoderma* by gene manipulation for the production of increased amounts of specific proteins for better disease control has been suggested as a novel tool in exploiting the ability of these biocontrol agents producing lytic enzymes. Incorporating multiple genes may enhance Mycoparasitic ability of biocontrol agents against phytopathogenic fungi.

Recently, yet another novel approach of transforming the host plant itself by incorporating fungal genes to achieve better biological control is gaining momentum. The chitinase genes from *T. harzianum* have been transferred into tobacco resulting in increased hypersensitive response of the plants (Rincon, 2000), which may act as inducers of the plant defense during pathogen attack. Cepeda *et al.* (2000) stated that fungal chitinases may act as inducers of the defense systems of the plant during pathogen attack. Fungal chitinases as revealed in the present study would help in understanding the nature and production of lytic enzymes by *Trichoderma* spp.

This information on *Trichoderma* and identification of efficient, indigenous strains would lead to a better biological control of aflatoxin. This data would also help in the future work on the improvement of *Trichoderma* strains for biocontrol using protoplast fusion technology.

Chapter IV
Evaluation of selected *Trichoderma* isolates for their biological control Potential against *A. flavus*

INTRODUCTION

Contamination of groundnut with aflatoxin is a worldwide problem. The cultivated groundnut (*Arachis hypogaea* L.) is the most important oilseed crop in the rain fed farming system in the arid and semi-arid tropics. Groundnut seed is a valuable source of protein for human and animal nutrition. India and China contribute to nearly 2/3rd of the world production. *Aspergillus flavus* is a widely distributed fungus that survives in soil as spores. These germinate to produce hyphae, which invade the pods directly or, more commonly, through injuries. The number of spores may vary from low to high in a single season and probably depend on such factors as nutrient availability and microbial competition. An important attribute is also the ability of *A. flavus* to survive under extremely dry conditions. Thus, it is able to grow at moisture levels only slightly higher than the 12% used to store pods (Graham, 1982).

Agricultural products are always at risk of contamination with fungi, some of which are able to produce toxic metabolites. These compounds are called mycotoxins and the diseases caused are called mycotoxicoses. Although it has been known for more than 100 years that some kinds of moldy grains, when eaten, may cause illness. Intensive study of mycotoxins and mycotoxicoses only dates from the 1960s, following the occurrence of 'Turkey-x disease' in the United Kingdom the disease that resulted from the presence of aflatoxin B_1 in the feed imported from Brazil (Sargeant *et al.*, 1961). Peanut genotypes with some resistance to invasion by *A. flavus* have been reported (Bartz *et al.*, 1978; Kushalappa *et al.*, 1979; Zambettakis *et al.*, 1977,1981). The *in vitro* screening method has produced inconsistent results when compared to a natural field situation. A more complete understanding of the environmental factors controlling activities of the

aspergilli has allowed development of experimental field conditions under which these fungi thrive and toxin production is favored. Virtually all countries are engaged in international commerce have enacted or have proposed permissible levels of mycotoxin contamination in foods and feeds. Indeed, aflatoxin contamination levels are used in international trade to fix the price of various commodities. Thus, it becomes imperative that methods are available to monitor effectively the mycotoxin content of a range of commodities in both the exporting and importing countries. In addition to groundnut, a number of commodities are currently known to be contaminated with aflatoxins. These include oilseeds, cereals, legumes and spice crops. A wide range of livestock has been found to be affected by aflatoxins to a greater or lesser extent. Among cereal crops, by far the most important contamination occurs in maize. Groundnuts and maize are ingredients in the diet of many poor people and also common ingredients in livestock feeds. Health hazards from ingestion of aflatoxin-contaminated food are much greater in the developing than in the developed world. Most developing countries lie in tropics, where temperatures and relative humidity often favor mold growth, and where no or only limited facilities exist for monitoring groundnut and groundnut products for aflatoxin contamination. The possible presence of aflatoxins in foods and in feeds has had a profound effect on the utilization and trade in groundnuts and its products. Developed countries, which import groundnuts, have set aflatoxin contamination limits for foodstuff ranging from zero to 10 $\mu g\ kg^{-1}$ and this has resulted in import restrictions on aflatoxin-contaminated produce. As a result, many developing countries have been unable to export their groundnuts and groundnut products. For exporting countries to satisfy the regulations, they have to produce groundnuts with no or extremely low aflatoxin

contents. This can only be achieved by following suitable management practices and by storing produce under conditions that minimize the growth of aflatoxin producing fungi. The research, development and monitoring needed to ensure this are dependent upon having simple, specific and cost-effective methods for the detection and estimation of aflatoxins in various agricultural commodities.

Many different methods are available for the estimation of aflatoxins. They include physico-chemical and immuno-analytical methods. Physico-chemical methods are time consuming, require expensive instrumentation, extensive clean up of the samples using solvents; they therefore have serious limitations in cost and labor when large numbers of samples have to be processed. By contrast, immunological methods are simple, rapid, sensitive and specific. Mehan *et al.* (1988) observed an increase in seed infection and aflatoxin contamination from using a labor-intensive method of inoculating developing pegs and pods with an aqueous suspension of *A. flavus*. However, no attempts have been made to assess the effects of large-scale inoculation techniques on soil population levels and seed infection by *A. flavus* group fungi or aflatoxin contamination. Many different methods are available currently for the estimation of mycotoxins. Often these methods are not specific and their sensitivity is generally low compared to that of other methods. As a result they are used only as a tool for screening general toxicity. Physicochemical assay methods such as thin layer chromatography, high performance liquid chromatography, gas chromatography or mass spectrometry are laborious and require expensive instrumentation and clean-up of the samples. Thus these methods are of limited use in routine safety and quality control screening for mycotoxins in agricultural commodities.

Control of plant diseases by biological agents is environmentally safe and compatible with sustainable agriculture. *Trichoderma* spp. are well known biocontrol agents against several plant pathogens (Elad *et al.* 1982). Srilakshmi *et al,* (2001) have isolated several *Trichoderma* isolates from groundnut rhizosphere soil and tested their antagonistic activity against *A. flavus*. They identified 39 antagonistic isolates of *Trichoderma* from their study. In earlier studies (Thakur *et al.*, 2003) several isolates of *Trichoderma* and *Pseudomonas* were characterized for their antagonism against *A. flavus* and for their biocontrol potential and used selected *Trichoderma* spp. both as seed dressing and soil application to determine their effects on population dynamics of aflatoxigenic population of *A. flavus* in the geocarposphere and subsequently on infection of groundnut kernels. They obtained significant reduction in seed infection due to significant reduction in Af population in rhizosphere of the groundnut. The present study was taken up to control aflatoxin contamination in groundnut using *Trichoderma* spp. as biocontrol agents. The objectives of this study were:

1. to evaluate the selected *Trichoderma* spp. under glasshouse and field conditions for their biocontrol activity against *A. flavus* in groundnut, and
2. To estimate aflatoxin content using ELISA.

REVIEW OF LITERATURE

Aflatoxins are known to be hepatotoxic, carcinogenic, and teratogenic. Aflatoxins have been recognized to be more harmful to malnourished than well-nourished animals and humans. Presently 18 different types of aflatoxins have been identified among which aflatoxins B_1, B_2, G_1 and G_2 are produced mostly in groundnut (Healthcote and Hibbert, 1978). The rank order of toxicity, carcinogenicity and mutagenicity is $AFB_1 > AFG_1 > AFB_2 > AFG_2$ indicating that the unsaturated terminal furan of Af B_1 is critical for determining the degree of biological activity of this group of mycotoxins (Heathcote and Hibbert, 1978). Propelled by the discovery of the aflatoxins, much effort has been directed to the investigation of the association between exposure to naturally occurring mycotoxins and long-term adverse health effects in human beings and livestock. Among various mycotoxins, aflatoxins and ochratoxin A have assumed economic importance (Van Egmond., 1991; Bosch and Peers, 1991). They are harmful to human beings, poultry and livestock. Aflatoxins are potent carcinogens and ochratoxin A is a nephrotoxin. Sanders *et al.* (1985) determined the duration of soil moisture and soil temperature stress required for extensive preharvest invasion by *Aspergillus flavus* and contamination of peanuts with aflatoxin. Peanuts subjected to defined temperature and water deficit stress conditions for 30, 40, and 50 days became contaminated, therefore, a threshold stress period for preharvest aflatoxin contamination of peanuts by *A. flavus* was more than 20, and possibly less than 30 days before harvest. Wilson and Stansell, (1983) reported no significant aflatoxin contamination by the aflatoxin producing isolate when irrigation was applied during the last 40 days of the season, in any cultivar. Davidson *et*

al. (1983) compared two runner-type peanut cultivars, "Sunbelt Runner" and "Florunner," under different field conditions for contamination of the seed by *A. flavus* and aflatoxin. Sunbelt Runner had no advantage over Florunner in reducing levels of *A. flavus* and subsequent aflatoxin. Mayura *et al.* (1984) observed that aflatoxin production was more on polished rice than on the unpolished rice and this was attributed to the lipid fraction of the bran layer on unpolished rice. Sanders (1981) had grown in experimental plots with soil moisture and soil temperature modified during the last third of the growing period to produce drought, drought with cooled soil, irrigated and irrigated with heated soil treatments. Drought stress increased the incidence of *A. flavus* and irrigation decreased it, except when soil temperatures were modified.

Hill *et al.* (1983) imposed four soil temperature and moisture treatment regimes on Florunner peanuts 94 days after planting in experimental plots and reported that irrigation caused a higher incidence of *A. niger* than drought which prevented the aflatoxin contamination of undamaged peanuts. Kloepper and Bowen, (1991) examined roots and pods of field-grown peanut sampled at the R_3, R_5 and R_7 developmental stages in comparison to root and pod-free soil for microbial population to assess the geocarposphere and rhizosphere effects. These microorganisms are logical candidates for evaluation as biological control candidates for *A. flavus*. Inoculation of root regions of 1 to 2 week old peanut plants with toxigenic and atoxigenic strains of *A. flavus* resulted in lower levels of aflatoxin B_1 in the peanut kernels at maturity, than those in plants inoculated with the toxigenic strains alone (Chourasia and Sinha, 1994). The results suggest the potential of atoxigenic strains of *A. flavus*, in biological control, against pre-harvest aflatoxin contamination of developing peanuts, subject to practicability of the

approach. Ramakrishna *et al.* (1996) studied colonization of barley grain by *A. flavus* and formation of aflatoxin B_1 in the presence of *Penicillium verrucosum*, *Fusarium sporotrichiodes*, and *Hyphopichia burtonii* and observed five main patterns of aflatoxin B_1 production relative to pure culture but with no consistent relationship with species, a_w, (kernel water activity), temperature or incubation period. Azaizeh *et al.* (1989) tested seven peanut genotypes in greenhouse and micro plot experiments during two consecutive years. However, no significant differences were obtained in the degree of *Aspergillus* infestation of kernels from genotypes of glasshouse experiments. Colonization of peanut pegs was inconsistent among genotypes and between years.

Blankenship *et al.* (1984) determined the threshold geocarposphere temperature range in drought-stressed peanuts for the consistent occurrence of aflatoxin contamination to be between 25.7 C and 27 C. Mixon *et al.* (1984) investigated chemical and soil amendments for effects on seed colonization by *A. flavus* group on aflatoxin contamination of one or more genotypes of peanut. Aflatoxin was not detected in peanuts harvested from gypsum-treated plots, but it was occasionally found in peanuts harvested from the non-gypsum treatments resulting in a highly significant treatment × genotype interaction. Cole *et al.* (1985) reported that undamaged peanuts grown under environmental stress in the form of drought and heat become contaminated with *A. flavus* and aflatoxin in the soil prior to harvest. Increasing the mean temperature up to 29.6 C caused increasing amounts of contamination.

Blankenship *et al.* (1985) subjected four peanut genotypes, selected as resistant to invasion by *A. flavus* in laboratory screening with dehydrated, stored seed to *A. flavus* invasion and aflatoxin contamination. Their study was helpful in selecting germplasm for

resistance to *A. flavus* invasion and assuming resistance to aflatoxin contamination under field conditions. Kisyombe *et al.* (1985) evaluated fourteen peanut genotypes for resistance to *A. parasiticus* infection in 1983 and 1984 in rain-shaded field micro plots where water stress conditions were simulated, and in unshaded micro plots under normal rainfall conditions. Except for J-11, there was no correlation between genotype rankings for resistance to dry seed infection and resistance under field conditions. Hag Elamin *et al.* (1988) reported that groundnut samples, collected soon after harvest, from different districts in the irrigated region were free from aflatoxins. None of the three varieties of groundnuts tested in this work was completely resistant to aflatoxin production. A temperature of 30 C and 86.3% relative humidity are the optimum conditions for both *A. flavus* growth and aflatoxin production in groundnuts.

Mehan *et al.* (1991) found significant interactions when examined preharvest seed infection by *A. flavus* and aflatoxin contamination in selected groundnut genotypes in different soil types at several locations in India in 1985-1990. Irrespective of soil types, *A. flavus*-resistant genotypes showed lower levels of seed infection by *A. flavus* and other fungi than did *A. flavus* susceptible genotypes. The significance of the low preharvest aflatoxin risk in groundnuts grown on Vertisols is highlighted.

In earlier studies (Anjaiah and Thakur, 2000; Desai *et al.*, 2000; and Thakur et *al.*, 2003) several isolates of *Trichoderma* and *Pseudomonas* were characterized for their antagonism against *A. flavus* and for their biocontrol potential. They used selected *Trichoderma* spp. both as seed dressing to determine their effects on population dynamics of aflatoxigenic population of *A. flavus* in the geocarposphere and subsequently on infection of groundnut kernels. Kumar *et al.*, (2002) evaluated the integrated management

package to reduce preharvest seed infection by *A. flavus* in groundnut. Cumulative gain in the levels of *A. flavus* was observed in the plot where no control measures were used. Aflatoxins levels were higher under farmer's storage conditions than under dry conditions.

MATERIALS AND METHODS

Methodology

Biomass of *Trichoderma* isolates

Seven *Trichoderma* spp. tested for antagonism were assessed for their growth on Czapeks - dox broth medium at p^H 4.5, 5.5, 6.5, and 7.5.

Greenhouse evaluation of *Trichoderma* isolates for biocontrol activity againt *A. flavus* in groundnut.

Greenhouse experiment was conducted at ICRISAT Patancheru, during 2001 and 2002.The experiment was established in 64 pots containing a pot mixture of red soil, farmyard manure and sand (2:1:2) in $12_{1/2}$" pots (Fig 22). The temperature in the greenhouse was maintained at 30 ± 2 C throughout the experiment. Seeds of groundnut genotype ICGV 88145 were coated with six *Trichoderma* spp. (10^9 conidia / ml^{-1}) using 0.5% carboxymethyl cellulose (CMC) as an adhesive and used as treatments. Groundnut seeds not coated with *Trichoderma* served as control. Six seeds were planted in each pot and two pots were considered as one replication. Four replications were maintained for each treatment. The experiment was repeated twice.

Biocontrol agents (BCAs)

Six isolates of *Trichoderma* spp. which were highly antagonistic to a highly toxigenic strain of *A. flavus* (Af 11-4) and non pathogenic to groundnut plants (ICGV 88145) were used as potential BCAs. They are:

T. harzianum, T. fertile, T. koningii, T. viride, T. pseudokoningii and *T. longibrachiatum.*

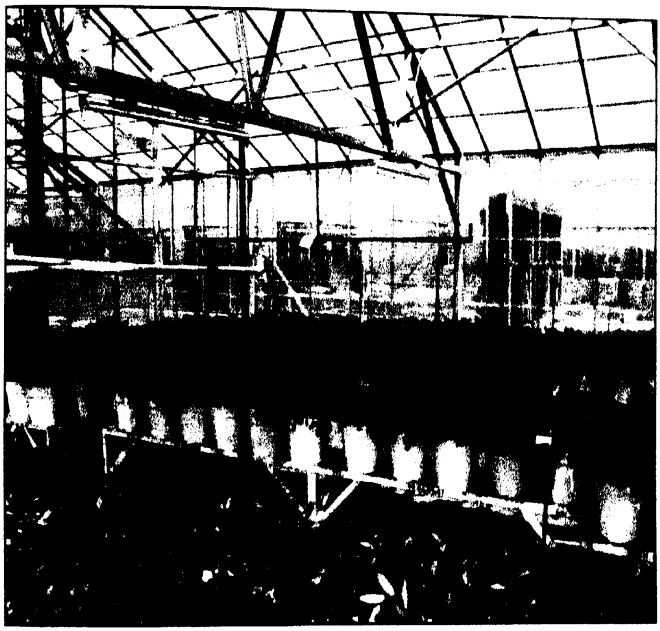


Fig. 22. Biocontrol of *A. flavus* contamination in groundnut using seven different *Trichoderma* species
Design: 8 RBD
Location: ICRISAT
Greenhouse no. 4
Rep: 4
Pots: 64

Preparation of *Trichoderma* Inoculum and seed formulation

Autoclaved pearl millet grains were used as a substrate to multiply *Trichoderma* isolates for 10 days at 28 C in dark. The infested millet grains with *Trichoderma* isolates were mixed with sterile distilled water, stirred for 4-5 min to detach the spores, and filtered through double-layered muslin cloth to obtain spore suspension. Two hundred grams of groundnut seeds were coated with 100 ml of aqueous spore suspension ($1x10^9$ spores/ml) by adding 1 ml of 0.5% carboxy methyl cellulose (CMC) as sticker and 20 g of bentonite powder as filler for seed dressing.

Preparation of *Aspergillus flavus* inoculum:

A. flavus inoculum was multiplied on autoclaved pearl millet grain by incubating for 7 days at 28 C in dark. The inoculum was maintained at mycelial stage only to avoid cross contamination.

Application of *A. flavus* inoculum.

To ensure infection, 10 g of *A. flavus* inoculum/row was applied at the time of flowering (40 days after seedling emergence), 80 days and 120 days adjacent to plants and covered with a layer of soil to avoid cross contamination. Irrigation was given after inoculation with sprinklers.

In case of greenhouse experiment after applying inoculum the pots are covered with a thin layer of soil, and the pots were given measured irrigation for every 2-3 days after 80 days of sowing just for survival of plants, which enhances *A. flavus* infection. Drought was maintained during last 40 days of the experiment giving minimum amount of water.

Soil and pod sampling

Soil and pod samples were sampled at four stages of experiment to monitor the levels of *A. flavus* and *Trichoderma* population. Soil samples were collected in each replication bulking soil from two pots. Initial sampling was done just before sowing, second one prior to application of the pathogen inoculum, and the final sampling at harvest in both experimental plots. Pods from the pots were harvested at 75% maturity level and dried by inverted windrows method for three days.

Soil sampling

Three random samples (10g/sample) from each plot were collected and mixed thoroughly to make a composite sample per plot. Two samplings were done, one at sowing and another at harvesting from geocarposphere.

A. flavus and *Trichoderma* population count in soil.

Soil samples were sieved to fine powder. Aqueous soil suspension (10 g soil + 90 ml sterile distilled water) prepared from each composite sample was mixed and serially diluted in sterile distilled water to 10^{-3} and 10^{-4} concentrations. Diluted soil samples were plated on AFPA (*Aspergillus flavus* and *parasiticus* agar) medium (Pitt *et al.*, 1983) for *A. flavus* population count (0.5 ml). The sample (0.5 ml) was plated on each plate, two plates maintained for each dilution to get total population present per gram of the soil. To get *Trichoderma* population diluted soil sample was plated on TSM (*Trichoderma* selective medium) to get *Trichoderma* population. Plating was similarly done maintaining two plates per dilution.

The plates were incubated for 2-3 days at 28 C in dark. Population density of both *A. flavus* and *Trichoderma* was expressed as colony forming units (cfu) g^{-1} soil.

Aspergillus flavus seed infection

Groundnut pod samples were collected and sub-sample lots are drawn. The sub-samples were shelled and 100 undamaged seeds are randomly collected from each sub-sample. These seeds were used for *A. flavus* seed infection study by blotter plate method. The seed was soaked in sterile distilled water for 3-5 min and surface sterilized with 1% Clorax solution for 1 min followed by three washes with sterile distilled water. This seed was used for plating on Czapeks- dox Agar (CDA) fortified with Rose Bengal (25 mg L-1) and incubated at 28 C for four days in dark. The plates were observed for *A. flavus* seed infection on 5th day.

Aflatoxin analysis

Fifty grams of seeds were soaked in sterile distilled water for 4 h, later dried and incubated at 25 C overnight prior to aflatoxin estimation by using Enzyme linked immunosorbent assay (ELISA), a simple and quick immunoassay protocol for estimation of aflatoxins (Devi *et al.*, 1999).

Data analysis

Analysis of variance (ANOVA) was done using GENSTAT SOFTWARE package for individual experiments to find the treatment means.

Field evaluation of *Trichoderma* isolates for biocontrol activity againt *A. flavus* in groundnut.

Materials and methods

The study was conducted during the 2001 and 2002 growing seasons at ICRISAT, Patancheru. The experiment was designed as a randomly block design with 8 treatments and six replications in each planting (Table 11, 12 and Fig 23). Each replication has a plot with two rows of four meters each. In each row 45 seeds are planted with a gap of 10 cm between the seeds. Thirty cm gap was left between each row. Remaining methodology was similar to greenhouse experiment.

Table 11. Randomization of 8 treatments (BCAs) for the management of *A. flavus* infection under field conditions, Rabi 2001-2002.

		Plot Numbers					
Tr no	**Treatment**	**Rep1**	**Rep2**	**Rep3**	**Rep4**	**Rep5**	**Rep6**
01	T-20	104	207	303	401	501	602
02	T-29	106	202	304	408	507	603
03	T-47	105	201	305	403	508	601
04	T-24	103	206	307	406	505	606
05	T-83	108	205	301	405	502	605
06	T-102	102	203	308	402	503	608
07	T-179	101	204	306	404	506	607
08	Af+No BCA	107	208	302	407	504	604

Plot size: 2 rows x 4 m long/rep.

Table 12. Randomization of 8 treatments (BCAs) for the management of *A. flavus* infection under field conditions, Kharif 2002.

		Plot Numbers					
Tr no	**Treatment**	**Rep1**	**Rep2**	**Rep3**	**Rep4**	**Rep5**	**Rep6**
01	**T-20**	**102**	**201**	**302**	**408**	**506**	**605**
02	**T-29**	**108**	**204**	**307**	**407**	**505**	**606**
03	**T-47**	**104**	**203**	**304**	**406**	**503**	**604**
04	**T-24**	**106**	**207**	**301**	**404**	**508**	**608**
05	**T-83**	**103**	**206**	**303**	**401**	**504**	**601**
06	**T-102**	**101**	**202**	**305**	**403**	**502**	**607**
07	**T-179**	**105**	**205**	**306**	**405**	**507**	**602**
08	**Af+No BCA**	**107**	**208**	**308**	**402**	**501**	**603**

Plot size: 2 rows x 4 m long/rep.

Fig. 23. Biocontrol of *A. flavus* contamination in groundnut using seven different *Trichoderma* species
Design: 8 RBD
Location: ICRISAT
Field: RCE 4B
Season: Rabi 2001/02
Rep: 6

Aflatoxin analysis:

Indirect competitive ELISA:

Materials:

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

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

ELISA Plate Reader (Labsystems Multiskan ^{R} plus).

IPCV-H Polyclonal antibodies.

Mortar and pestle, Muslin cloth, p^{H} meter, incubator, Refrigerator.

Aflatoxin B_1 (Sigma A6636)

Aflatoxin B_1 -BSA conjugate (Sigma A6655)

Bovine Serum Albumin (Sigma A6793)

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

Solutions

Carbonate buffer or coating buffer (p^{H} 9.6)

Na_2CO_3 -- 1.59 g

$NaHCO_3$ -- 2.93 g

Distilled water -- 1000 ml

Phosphate buffer saline (PBS), (p^H 7.4)

Na_2HPO_4	-- 02.38 g
KH_2PO_4	-- 00.40 g
KCL	-- 00.40 g
NaCl	-- 16.00 g
Distilled water	-- 2000 ml.

Phosphate buffered saline Tween (PBS-T)

PBS	-- 1000 ml
Tween-20	-- 0.5 ml

Antibody buffer

PBS-T	--100 ml
Polyvinyl Pyrrolidone (PVP) 40,000 MW	-- 2.0 g
Bovine serum albumin	--0.2 g

Substrate buffer

Substrate buffer for alkaline phosphatase system: p-nitrophenyl phosphate (PNPP) was stored at –20 C. Chemical in tablet form (5,15 or 40 mg tablets are available) was used. Ten percent diethanol amine (v/v) was prepared in distilled water; p^H was adjusted to 9.8 with conc. HCl. This solution can be stored but p^H should be adjusted to 9.8 prior to use. 0.5-mg /ml para nitro phenyl phosphate (PNPP) was prepared in 10% diethanol amine, p^H 9.8 (for each 15 mg tablet 30 ml solution was required). PNPP solution was not allowed

to turn yellow. This may sometimes happen because of alkaline phosphatase (ALP) contamination from skin.

Methodology

Preparation of groundnut seed extracts:

The seed was blend into powder using a blender. The seed powder was titrated in 70% methanol (v/v-70 ml absolute methanol in 30 ml distilled water) containing 0.5% KCl (proportion used in 100 ml for 20 g seed) in a blender, until the seed powder was thoroughly ground. The extract was transferred to a conical flask and shaken for 30 min at 300 rpm. The extract was filtered through Whatman No. 41 filter paper and diluted 1:10 in PBS-Tween (1 ml extract and 9 ml of buffer). To estimate lower levels of AfB_1 (<10 µg/Kg), prior to ELISA a simple liquid-liquid cleanup and concentration (5:1) procedure was adopted. Twenty ml of methanol extract, 10 ml of distilled water and 20 ml chloroform were mixed in a separating funnel and used for clean up. After vigorous shaking for one minute, the lower chloroform layer was collected and evaporated to near dry ness in water bath at 60 C. To the residue 4 ml of PBS-Tween containing 7% methanol was added and used for analysis by ELISA.

AfB_1-BSA conjugate was prepared in carbonate coating buffer at 100 ng/ml concentrations. And 170 µl of the diluted AfB_1-BSA is dispensed to each well of ELISA plate. The plate was incubated in a refrigerator overnight or at 37 C for at least one and half-hour.

The plates were washed in three changes of PBS-Tween, allowing 3 min gap between for each wash (To inhibit non-specific binding of antibodies and thus give false positive reaction).

0.2% BSA prepared in PBS-Tween was added at 170 µl per each well of ELISA plate and incubated at 37 C for 1h.

The plates were washed in three changes of PBS-Tween, allowing 3 min between each wash.

Preparation of Aflatoxin B_1 standards

Healthy groundnut seed extract was prepared as mentioned above.

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

Addition of polyclonal antisera raised each polyclonal antiserum this was predetermined (at ICRISAT) in PBS-Tween against aflatoxin B_1 -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 (100 µl) intended for analysis. The plates were incubated for 30 min at room temperature the mixture containing aflatoxin samples (100 µl) and antiserum (50 µl) to facilitate reaction between the toxins present in the sample with antibody. Entire process was done in ELISA plate and there was no need to pre incubate the toxin and antibody mixture in separate tubes.

The plate was incubated for 1h at 37 C. The plate was washed in three changes of PBS-tween allowing for 3min for each wash. Goat anti-rabbit IgG (1:1000) was prepared and labeled with alkaline phosphatase, in PBS-Tween containing 0.2% BSA. 170 µl was

added to each well and incubate for 1h at 37 C. The plate was washed in three changes of PBS-Tween allowing for 3 min for each wash. Substrate solution (p-nitro phenyl phosphate prepared in 10% diethanolamine buffer, p^H 9.8) was added and incubated for 1h at room temperature.

After satisfactory development of yellow colour in each ELISA plate (colour development takes place in 40 min to 1hour) absorbance is measured at 405 nm in an ELISA reader.

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

$$AfB_1\ (\mu g/Kg) = \frac{AxDxE}{G} \text{ or } \frac{AxE}{CXG}$$

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 (g)

RESULTS

Biomass of *Trichoderma* isolates:

Seven *Trichoderma* spp. tested for antagonism were assessed for their growth on CDA at different p^H and data is presented in (Table 13, Fig 24)

Among the 7 isolates tested, *T. viride* (T 179) produced maximum biomass at all the p^H followed by *T. koningii* (T 83) and *T. longibrachiatum* (T 102). Remaining isolates produced very little biomass and *T. harzianum* (T 20) produced the least biomass. Maximum biomass production was observed at p^H 5.5 for all the isolates followed by p^H 6.5 and 7.5. The increase in biomass was maximum till seventh day, lesser between seventh and tenth day and least thereafter. Isolates of *T. viride* (T 179) and *T. koningii* (T 83) showed better growth even at alkaline p^H of 7.5.

Table.13. Effect of p^H on biomass (g L^{-1}) production of *Trichoderma* isolates in liquid Czapek- dox broth medium at different days and p^H

S no	*Trichoderma* spp.	Isolates	pH											
			4th Day				7th Day				10th Day			
			4.5	5.5	6.5	7.5	4.5	5.5	6.5	7.5	4.5	5.5	6.5	7.5
1	*T. harzianum*	T-20	1.1	0.3	2.2	1.8	0.8	0.5	1.4	1.1	0.8	0.1	1.3	0.4
2	*T. pseudokoningii*	T-29	0.7	1.4	2.7	1.5	1.5	3.9	3.2	2.0	4.0	3.0	2.9	1.1
3	*T. fertile*	T-47	0.6	1.8	2.4	2.0	1.9	1.2	0.4	1.8	1.9	2.0	3.6	3.3
4	*T. viride*	T-24	0.3	2.0	2.2	1.7	1.8	1.1	1.8	1.4	1.7	1.6	1.6	1.8
5	*T. koningii*	T-83	3.7	4.5	4.0	3.8	3.3	5.0	4.8	4.0	4.7	5.8	5.8	5.3
6	*T. longibrachiatum*	T-102	3.0	3.7	3.7	3.3	1.3	4.8	3.6	2.8	1.0	3.3	3.0	2.3
7	*T. viride*	T-179	4.6	6.0	4.8	3.8	5.2	7.7	6.5	5.4	4.4	7.0	6.3	6.0

* The data is the average of three replications

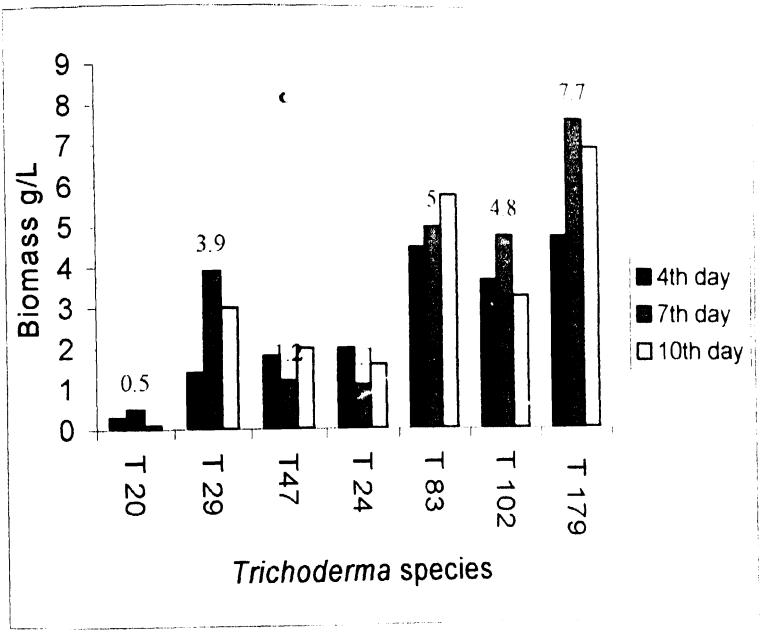


Fig. 24. Biomass production of *Trichoderma* spp. in liquid Czapek-dox medium at p^H 5.5 at different intervals of time.

T 20 (*T. harzianum*)
T 29 (*T. pseudokoningii*)
T 47 (*T. fertile*)
T 24 (*T. viride*)
T 83 (*T. koningii*)
T 102 (*T. longibrachiatum*)
T 179 (*T. viride*)

Management of aflatoxin contamination by *A. flavus* under greenhouse conditions during Experiment I.

Table 14. Effect of *Trichoderma* spp. on pod weight, seed weight, total number of plants, shelling % and biomass of groundnut.

S.no	Isolate	*Trichoderma* spp.	Pod wt (g)	Seed wt (g)	Total Plants	Shelling (%)	Biomass (g)
1	T 20	*T. harzianum*	46.70	25.20	5.000	52.9	097.70
2	T 29	*T. pseudokoningii*	34.60	23.30	4.750	58.6	094.60
3	T 47	*T. fertile*	56.70	37.20	5.000	65.7	104.70
4	T 24	*T. viride*	35.70	28.60	5.500	77.9	091.90
5	T 83	*T. koningii*	48.60	32.90	4.250	68.2	088.80
6	T 102	*T. longibrachiatum*	23.20	15.60	4.750	66.4	087.70
7	T 179	*T. viride*	44.20	30.60	5.750	68.5	095.00
8: Control Af+N0 BCA			39.40	25.70	4.250	65.4	068.40
SE			04.65	03.38	0.368	5.16	004.22
GM			41.10	27.40	4.750	65.5	091.10
CV			11.30	12.30	7.700	07.9	004.60
LSD			22.79	17.01	3.006	16.9	030.14

Note: Design 8 RBD
Location: ICRISAT
Rep: 4
Dilution: 10^{-3}

Different isolates of *Trichoderma* spp. were used for the management of aflatoxins under greenhouse conditions and various growth parameters were estimated. Out of the seven treatments with *Trichoderma* isolates T 47, T 83,T 20 and T 179 increased the pod weight considerably under *A. flavus* inoculated conditions (Table 14). Similarly increase in seed weight was observed with these isolates. Isolates T 24 showed highest shelling (%) followed by T 83, T102 and T 129. Increase in biomass was significant with T 47 treatment while T 20, T 29 and T 179 have also enhanced the biomass.

From the statistical analysis of the data isolates T 47 produced significantly higher biomass than others. However, isolates T 83 and T 179 also performed well under greenhouse conditions.

Table 15. Population dynamics of biocontrol agents and their effect on *A. flavus* under greenhouse conditions.

			Population (cfu / g) of soil					
S.no	**Isolate**	***Trichoderma* spp.**	**Afpop (40d)**	**Afpop (80d)**	**Afpop (120d)**	**Tpop (40d)**	**Tpop (80d)**	**Tpop (120)**
1	T 20	*T. harzianum*	06500.0	03750.0	3000.00	0500.0	0750.0	09250.0
2	T 29	*T. pseudokoningii*	06000.0	14250.0	6000.00	1250.0	0500.0	08250.0
3	T 47	*T. fertile*	23000.0	05000.0	4000.00	1250.0	0750.0	03250.0
4	T 24	*T. viride*	17250.0	05000.0	3500.00	1500.0	0500.0	21250.0
5	T 83	*T. koningii*	22500.0	03250.0	3000.00	1000.0	1000.0	21250.0
6	T 102	*T. longibrachiatum*	16500.0	11750.0	8750.00	0750.0	0500.0	37150.0
7	T 179	*T. viride*	18000.0	05000.0	4500.00	0500.0	0500.0	10250.0
8: Control Af+N0 BCA			34520.0	43000.0	11500.0	0250.0	1000.0	02000.0
SE			12039.4	10658.5	01592.2	0228.2	0297.6	04419.0
GM			18000.0	11375.0	05531.0	0875.0	0562.0	09906.0
CV			00066.9	00093.7	00072.8	0026.1	0052.9	00044.6
LSD			20556.5	32275.5	05924.8	1720.6	1216.6	16084.0

Population dynamics of biocontrol agents and their effect on *A. flavus*

Population of the toxigenic *A. flavus* was estimated at 40, 80 and 120 days from groundnut rhizosphere soils (Fig 25) when treated with different biocontrol agent's *viz*, *T. harzianum* (T 20), *T. pseudokoningii* (T 29), *T fertile* (T 47), *T viride* (T 24), *T koningii* (T 83),*T. longibrachiatum* (T 102) and *T. viride* (T 179) and the results were presented in Table 15.

Biocontrol agents were supplied as a seed treatment and sown in pots and subsequently *A. flavus* was inoculated after 30 days after germination of groundnut seeds The biocontrol agents were allowed to establish till the pathogen is inoculated The *Trichoderma* population established successfully Populations of T 24, T 29, T 47 and T 83 enhanced considerably with cfu over 1000 by 40th day The pathogen populations were drastically reduced in T 29 and T 20 treatments compared to other treatments by 40th day

Isolate T 20 contained *A. flavus* populations throughout the crop period In addition isolates T 47, T 24 and T 179 significantly reduced the pathogen populations by 80th day Al the treatments except T 102 significantly reduced the *A. flavus* population by 120 days

Interestingly population of biocontrol agents, T 24, T 83 and T 102 increased significantly at 120 days under drought conditions An inverse relationship was observed with the increasing populations of BCAs and decreasing population of the pathogen Among the biocontrol agents T 20 and T 24 were highly effective in reducing population of pathogen as revealed from the statistical analysis of the data

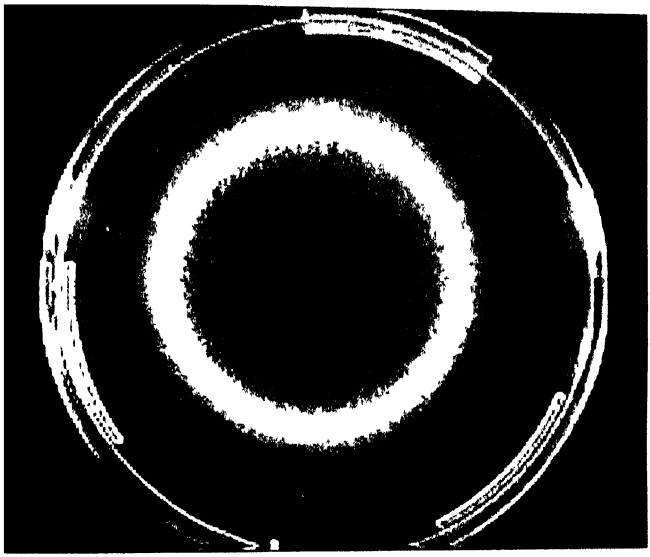


Fig 25 A typical *growth of Aspergillus flavus* colony on *Aspergillus flavus parasiticus* agar medium at 28 C after 5 days

Management of aflatoxin contamination by *A. flavus* under greenhouse conditions during Experimet II.

Table 16. Effect of *Trichoderma* spp. on pod weight, seed weight, total number of plants, shelling (%) and biomass of groundnut.

S.no	Isolate	*Trichoderma* spp.	Pod wt (g)	Seed wt (g)	Total plants	Shelling (%)	Biomass (g)
1	T 20	*T. harzianum*	51.20	36.20	5.250	69.40	104.90
2	T 29	*T. pseudokoningii*	40.40	29.30	5.500	75.00	095.90
3	T 47	*T. fertile*	60.80	46.70	5.250	78.10	106.80
4	T 24	*T. viride*	39.20	30.40	5.500	70.80	101.90
5	T 83	*T. koningii*	49.20	36.80	5.500	71.30	094.90
6	T 102	*T. longibrachiatum*	31.20	26.90	5.500	79.20	092.80
7	T 179	*T. viride*	51.70	36.90	6.000	81.60	096.80
8. Control Af+N0 BCA			42.40	29.00	4.000	72.50	067.80
SE			3.280	04.36	0.217	07.03	004.61
GM			45.80	34.00	5.310	74.40	095.20
CV			07.20	12.80	4.100	09.40	004.80
LSD			21.82	18.64	1.370	13.11	030.27

Out of the seven treatments with *Trichoderma* isolates used for the management of aflatoxins under glasshouse conditions T 47, T 179,T 20 and T 83 increased the pod weight considerably under *A. flavus* inoculated conditions (Table 16) Similarly increase in seed weight was observed with these isolates and also with the isolate T 24 Increase in total number of plants was observed with the isolates T 179, T29, T 24, T 83 and T 102 Isolate T 179 showed highest shelling (%) followed by T102, T 47, and T 29

From the statistical analysis of the data increase in biomass was significant with T 47 treatment while T 20, and T 24 have also enhanced the biomass Isolate T 47 also showed significant increase in seed weight.

Table 17 Population dynamics of biocontrol agents and their effect on *A flavus* under greenhouse conditions.

S.no	Isolate	*Trichoderma* spp.	Population (cfu / g) of soil					
			Afpop (40d)	Afpop (80d)	Afpop (120d)	Tpop (40d)	Tpop (80d)	Tpop (120d)
1	T 20	*T. harzianum*	11750 0	02500 0	5250 0	1500 0	1250 0	03750 0
2	T 29	*T. pseudokoningii*	08250 0	11750 0	7250 0	1250 0	1750 0	04750 0
3	T 47	*T. fertile*	19750 0	05250 0	4500 0	1000 0	1750 0	01250 0
4	T 24	*T. viride*	14250 0	04750 0	3250 0	1500 0	2000 0	18750 0
5	T 83	*T. koningii*	22250 0	02500 0	3500 0	1375 0	1500 0	08250 0
6	T 102	*T. longibrachiatum*	16000 0	09750 0	8250 0	1750 0	2250 0	03000 0
7	T 179	*T. viride*	16000 0	05250 0	4000 0	1250 0	1500 0	10500 0
8 Control Af+N0 BCA			23750 0	17750 0	8000 0	2000 0	2500 0	03250 0
SE			08725 0	05052 3	1439 8	0193 5	0072 2	02072 9
GM			16500 0	07438 0	5750 0	1453 0	1812 0	06688 0
CV			00052 9	00067 9	0025 0	0013 3	0004 0	00031 0
LSD			18581 9	11693 3	6216 0	1773 3	1430 0	12304 7

Population dynamics of biocontrol agents and their effect on *A flavus*

Population of the toxigenic *A. flavus* was estimated at 40, 80 and 120 days from groundnut rhizosphere soils when treated with different biocontrol agents *viz* ,

T. harzianum (T 20), *T. pseudokoningii* (T 29), *T fertile* (T 47), *T viride* (T 24), *T. koningii* (T 83),*T. longibrachiatum* (T 102) and *T. viride* (T 179) and the results were presented in Table 17.

The pathogen populations were drastically reduced in all the treatments considerably by 40th day, 80th day and 120th day except the isolate T 102 which showed increase in *A. flavus* population by 120th day

In addition, statistical analysis of the data revealed that isolate T 29 showed nearly significant reduction in *A. flavus* population by 40th day Isolates T 20, T 83, T 24, T47 and T 179 showed significant reduction in the pathogen populations by 80th day

All the treatments and also the isolate T 29 reduced the *A flavus* population by 120 days

Considerable increase in population of biocontrol agents was seen in all the treatments by 40th day, 80th day and 120th day except the isolates T 47 and T 102 which showed decrease in *Trichoderma* population by 120th day An inverse relationship was observed with the increasing populations of BCAs and decreasing population of the pathogen Among the biocontrol agents T 47 and T 179 were highly effective in reducing population of pathogen as revealed from the statistical analysis of the data

Management of aflatoxin contamination by *A. flavus* under field conditions during rabi 2001/2002.

Table 18 Effect of *Trichoderma* spp on pod weight, seed weight, total number of plants, shelling (%) and biomass of groundnut

S.no	Isolate	*Trichoderma* spp.	Pod wt (g)	Seed wt (g)	Total plants	Shelling (%)	Biomass (g)
1	T 20	*T. harzianum*	314 0	181 4	30 70	57 40	0958 0
2	T 29	*T. pseudokoningii*	307 0	179 6	21 30	59 20	0975 0
3	T 47	*T. fertile*	364 0	182 8	25 70	50 20	1067 0
4	T 24	*T. viride*	286 0	161 5	34 80	55 50	0908 0
5	T 83	*T. koningii*	311 0	186 6	28 20	60 70	1025 0
6	T 102	*T. longibrachiatum*	245 0	145 4	20 00	59 50	0850 0
7	T 179	*T. viride*	212 0	139 8	24 20	65 30	0908 0
8 Control Af+N0 BCA			258 0	149 1	44 30	56 20	0955 0
SE			021 5	18 22	06 55	05 58	0076 7
GM			287 0	165 8	28 60	58 00	0956 0
CV			007 5	011 0	22 90	09 60	0008 0
LSD			102 6	67 69	17 81	10 20	0183 2

Design 8 RBD
Location ICRISAT
Field RCE 4B
Season· Rabi 2001/02
Rep 6
Dilution 10^{-3}

Different isolates of *Trichoderma* spp were used for the management of aflatoxins under field conditions and various growth parameters were estimated Out of the seven treatments with *Trichoderma* isolates T 47, T 20,T 83, T 29 and T 24 showed considerable increase in pod weight when compared to control (Table 18) Increase in seed weight was observed with all these isolates Significant increase in total number of plants was observed with the isolates T 47, T 179, T29 and T 102 All the Isolates except T 47 and T 24 showed increase in shelling (%) over control Increase in biomass was observed with the isolates T 47, T 83, T 29 and T 20 Isolate T 47 performed well under field conditions during rabi season

Table 19 Population dynamics of biocontrol agents and their effect on *A flavus* during rabi season

			Population (cfu / g) of soil					
S.no	**Isolate**	***Trichoderma* spp.**	**Afpop (40d)**	**Afpop (80d)**	**Afpop (120d)**	**Tpop (40d)**	**Tpop (80d)**	**Tpop (120d)**
1	T 20	*T. harzianum*	04000 0	07333 0	02083 0	4167 0	7667 0	2083 0
2	T 29	*T. pseudokoningii*	05167 0	05833 0	07000 0	5833 0	1500 0	2667 0
3	T 47	*T. fertile*	06500 0	15333 0	07333 0	4000 0	4167 0	2167 0
4	T 24	*T.viride*	09000 0	10833 0	13167 0	1083 0	2000 0	2417 0
5	T 83	*T. koningii*	06500 0	08500 0	16500 0	3250 0	5167 0	2000 0
6	T 102	*T. longibrachiatum*	09500 0	09167 0	03833 0	3333 0	5833 0	2667 0
7	T 179	*T. viride*	08833 0	13333 0	13167 0	7667 0	1833 0	2667 0
8 Control Af+N0 BCA			10833 0	18667 0	27000 0	2000 0	1333 0	0917 0
SE			01829 7	15160 0	06592 0	2505 5	1708 0	0603 0
GM			07542 0	17500 0	13354 0	3917 0	3688 0	2198 0
CV			00024 3	00086 6	00049 4	0064 0	0046 3	0027 5
LSD			08688 0	39451 0	19485 0	6049 0	6063 0	2164 6

Population dynamics of biocontrol agents and their effect on *A flavus*

Population of the toxigenic *A. flavus* was estimated at 40, 80 and 120 days from groundnut rhizosphere soils when treated with different biocontrol agents *viz*., *T harzianum* (T 20), *T. pseudokoningii* (T 29), *T fertile* (T 47), *T viride* (T 24), *T koningii* (T 83),*T. longibrachiatum* (T 102) and *T viride* (T 179) and the results were presented in Table 19

Biocontrol agents were supplied as a seed treatment and sown in pots and subsequently *A flavus* was inoculated after 30 days after germination of groundnut seeds The biocontrol agents were allowed to establish till the pathogen is inoculated The *Trichoderma* population established successfully The pathogen populations were drastically reduced in all the treatments by 40th day, 80th day and 120th day Statistical analysis of the data showed that the isolates T 20, T102, T29, and T 47 showed significant decrease in *A. flavus* population when compared to control by 120th day

In addition the biocontrol agent population was more in all the treatments by 40th day, 80th day and 120th day except the isolate T 24, which showed, decreased population by 40th day Notably population of biocontrol agents, increased at 120 days under drought conditions An inverse relationship was observed with the increasing populations of BCAs and decreasing population of the pathogen Among the biocontrol agents T 47 and T 102 were highly effective in reducing population of pathogen as revealed from the statistical analysis of the data

Management of aflatoxin contamination by *A. flavus* under field conditions during kharif 2002.

Table 20 Effect of *Trichoderma* spp on pod weight, seed weight, total number of plants, shelling (%) and biomass of groundnut

S.no	Isolate	*Trichoderma* spp.	Pod wt (g)	Seed wt (g)	Total plants	Shelling (%)	Biomass (g)
1	T 20	*T. harzianum*	245 0	139 60	50 00	58 20	724 0
2	T 29	*T. pseudokoningii*	233 0	134 00	49 50	58 80	696 0
3	T 47	*T. fertile*	285 0	142 00	45 00	49 00	762 0
4	T 24	*T. viride*	228 0	115 10	45 70	50 50	735 0
5	T 83	*T. koningii*	263 0	144 80	47 70	54 00	706 0
6	T 102	*T. longibrachiatum*	208 0	138 10	49 80	66 20	580 0
7	T 179	*T. viride*	156 0	106 20	48 50	67 30	709 0
8 Control Af+N0 BCA			378 0	173 70	47 30	47 80	836 0
SE			023 8	007 99	01 83	05 04	089 7
GM			250 0	136 70	47 90	56 50	718 0
CV			009 5	005 80	03 80	08 90	012 5
LSD			083 1	057 28	08 70	14 55	207 0

Effect of *Trichoderma* spp. on pod weight, seed weight, total number of plants, shelling (%) and biomass of groundnut.

Seven treatments with of *Trichoderma* were used for managing aflatoxin under field conditions. All the *Trichoderma* isolates increased the pod weight considerably under *A flavus* inoculated conditions (Table 20)

Total number of plants increased with the isolates T 20, T 102, T 29, T 179 and T 83 All the isolates showed increase in shelling (%) with all the treatments From the statistical analysis of the data all the isolates showed significant increase in pod weight Isolates T 24 and T 179 produced significant increase in seed weight However, isolates T 102 and T 179 produced significantly high shelling percentage Biomass increased significantly with the isolate T 102. Isolate T 102 performed well under field conditions during kharif season

Table 21. Population dynamics of biocontrol agents and their effect on *A flavus* during kharif season.

S.no	Isolate	*Trichoderma* spp.	Population (cfu / g) of soil					
			Afpop (40d)	**Afpop (80d)**	**Afpop (120d)**	**Tpop (40d)**	**Tpop (80d)**	**Tpop (120d)**
1	T 20	*T. harzianum*	5167	4333	04333	4000	9167	2833
2	T 29	*T. pseudokoningii*	7000	5167	07667	4833	2833	3000
3	T 47	*T. fertile*	3833	2500	05833	4500	3500	3333
4	T 24	*T. viride*	4667	8167	07000	2333	3500	2667
5	T 83	*T. koningii*	4667	4833	09333	2333	4833	3333
6	T 102	*T. longibrachiatum*	2167	2667	08167	3333	6000	3833
7	T 179	*T. viride*	5667	5000	06667	6333	2833	3167
8 Control Af+N0 BCA			7333	8333	12000	2000	1167	1667
SE			1715	3209	03794	1530	1732	0704
GM			5062	5125	07625	3708	4229	2979
CV			0033	5989	00049	0041	0041	0023
LSD			4430	0062	06738	4438	5290	1771

The pathogen populations were drastically reduced in all the treatments by 40th day, 80th day and 120th day (Table 21) Statistical analysis of the data showed that the isolate T 102 showed significant decrease in *A. flavus* population when compared with control by 40th day All the isolates showed significant decrease in *A flavus* population by 80th day Isolate T 20 showed significant decrease in *A. flavus* population by 120th day

In addition the biocontrol agent population was more in all the treatments by 40th day, 80th day and 120th day Interestingly population of biocontrol agent, T 20 increased significantly at 80 days and the isolate T 102 showed increase in biocontrol agent population at 120 days under drought conditions An inverse relationship was observed with the increasing populations of BCAs and decreasing population of the pathogen Among the biocontrol agents T 47, was highly effective in reducing population of pathogen as revealed from the statistical analysis of the data

Effect of *Trichoderma* spp. on seed infection and aflatoxin contamination over control under greenhouse conditions during Experiment II and I.

Table 22 Effect of *Trichoderma* spp on seed infection and aflatoxin contamination in groundnut under greenhouse conditions during Experiment I and II

S.no	Isolate	*Trichoderma* spp.	Exp I		Exp II	
			Seed infection (%)	Aflatoxin (µg/Kg)	Seed infection (%)	Aflatoxin (µg/Kg)
1	T 20	*T. harzianum*	31.50	03 55	60 50	17 50
2	T 29	*T. pseudokoningii*	10 00	03 32	52 50	26 80
3	T 47	*T. fertile*	23 00	02 62	54 50	00 70
4	T 24	*T. viride*	13 50	02 80	51 00	48 50
5	T 83	*T. koningii*	23 00	02 77	45 20	11 30
6	T 102	*T. longibrachiatum*	21 00	02 55	49 00	09 70
7	T 179	*T. viride*	19 20	03 77	41 50	21 80
8 Control Af+N0 BCA			45 50	06 65	76 50	34 50
SE			06 18	01 53	03 67	02 34
GM			23.30	03 51	53 80	21 30
CV			26 50	87 30	06 80	10 90
LSD			22 60	04 49	18 80	44 64

Effect of *Trichoderma* population on seed infection and aflatoxin content

Seven *Trichoderma* isolates were used for studying seed infection and aflatoxin content under glasshouse conditions during Exp I and Exp II During experiment I all the treatments showed less seed infection when studied in laboratory (Fig 26) Similarly all the isolates were producing less aflatoxin compared to control (Table 22) Statistical analysis of the data revealed that isolates T 29, T 24, T 179, and T 102 showed significantly less seed infection (%) Isolates T 47 and T 83 also showed considerable decrease in seed infection (%) Consequently, all the treatments reduced aflatoxin content in groundnut under greenhouse conditions

During experiment II all the isolates showed less seed infection under laboratory conditions compared to control Similarly all the treatments produced less aflatoxin except the isolate T 102. Statistical analysis of the data showed that all treatments except the isolate T 20 showed less seed infection (%) However, all the treatments except the isolate T 24 effectively reduced aflatoxin production during experiment II These isolates also reduced aflatoxin production during experiment I (Fig 27)

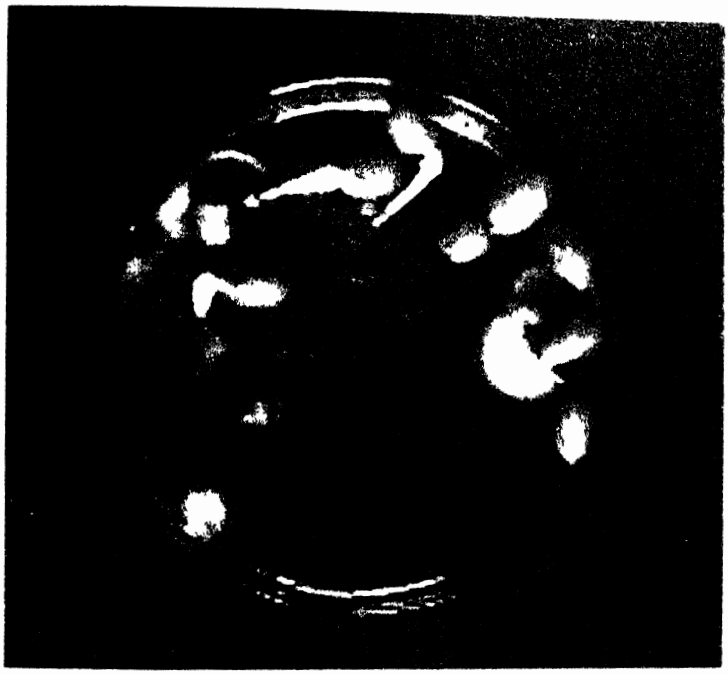


Fig 26 Groundnut seed showing seed infection by *A flavus* after five days at 28 C on *Trichoderma* selective medium

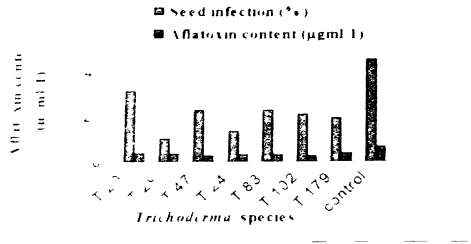
Table 23 Effect of *Trichoderma* spp on seed infection and aflatoxin contamination in groundnut during Rabi 2001/2002 Kharif 2002

S.no	Isolate	*Trichoderma* spp.	Rabi		Kharif	
			Seed infection (%)	Aflatoxin (µg/Kg)	Seed infection (%)	Aflatoxin (µg/Kg)
1	T 20	*T. harzianum*	54 00	05 60	13 2	007 0
2	T 29	*T. pseudokoningii*	64 00	07 80	17 0	005 5
3	T 47	*T. fertile*	62 00	07 90	11 3	007 6
4	T 24	*T. viride*	60 00	05 20	12 8	009 0
5	T 83	*T. koningii*	60 00	04 40	11 3	005 1
6	T 102	*T. longibrachiatum*	56 00	05 20	14 8	011 2
7	T 179	*T. viride*	68 00	06 30	12 3	006 1
8 Control Af+N0 BCA			74 20	09 33	25 7	009 5
SE			08 02	02 67	03 9	012 5
GM			62 50	06 49	14 8	007 6
CV			12 80	41 20	26 7	164 0
LSD			09 60	06 19	09 6	008 0

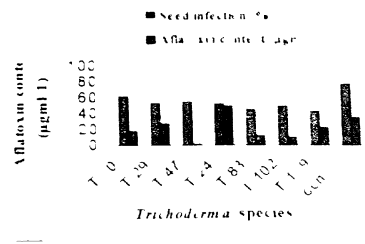
During field experiments conducted in rabi 2001-2002 and kharif 2002 seven *Trichoderma* isolates were used for studying seed infection and aflatoxin contamination During the field experiment (rabi 2001-2002) all the treatments showed reduction in seed infection (Table 23). Similarly all the isolates affected aflatoxin production compared to control. Biocontrol agents *T. koningii* (T 83), *T. longibrachiatum* (T 102) and *T. viride* (T 24) reduced the aflatoxin content considerably. Consequently, all the treatments reduced aflatoxin content in groundnut under field conditions

During the field experiment kharif 2002 all the isolates showed less seed infection under laboratory conditions compared to control. Interestingly even in kharif season all the treatments controlled aflatoxin production except the isolate T 102 Statistical analysis of the data showed that all treatments except the isolate T 29 showed significant reduction in seed infection (%). Similarly the biocontrol agents, *T. koningii* (T 83) and *T. viride* (T 179) also reduced the pathogen populations considerably by the time of harvest

The biocontrol agents T 83 and T 179 effectively reduced the aflatoxin levels during kharif and rabi season (Fig 27).



(a)



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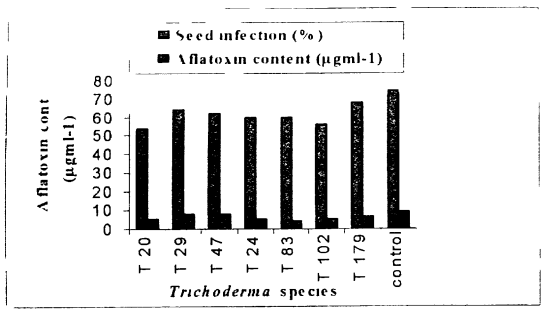
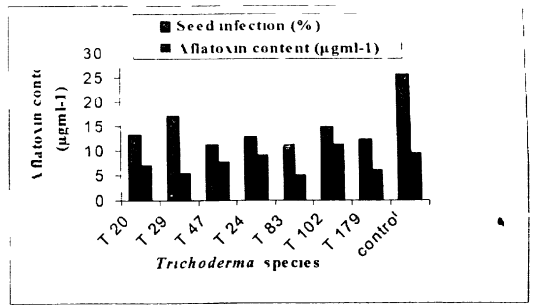


(c)



(d)

Fig. 27. Comparison of seed infection and aflatoxin content (during greenhouse experiments I and II (a &b) and field experiments rabi 2001-2002 and kharif (c &d) using seven different *Trichoderma* species against *A. flavus*

DISCUSSION

Aflatoxin produced by *Aspergillus flavus* in agricultural commodities is a primary concern of the agricultural industry and the consumer (Davidson *et al* 1983) Aflatoxin contamination levels are used in international trade in fixing the prices of the commodities. Soil amendments with chemicals were in vogue to control aflatoxin contamination in groundnut. Aflatoxin was not detected in peanuts harvested from gypsum-treated plots (Mixon *et al.*, 1984) Prevention and control of aflatoxin contamination employing biocontrol agents has attained importance in recent times *Trichoderma* spp. are popular among the biocontrol agents against several plant pathogens including *A. flavus*. Srilakshmi *et al* (2001) reported thirty-nine isolates of *Trichoderma* antagonistic to *A. flavus* isolated from groundnut rhizosphere soil Out of 212 isolates from groundnut rhizosphere soils in this study, in addition to 39 isolates reported earlier nine more were found to be antagonistic to the toxigenic *A. flavus*

Seven *Trichoderma* isolates used for studying the mechanism of antagonism were assessed for their growth on Czapek-dox broth medium at different p^H Among the 7 isolates tested, *T viride* (T 179) produced maximum biomass at all the p^H followed by *T. koningii* (T 83) and *T. longibrachiatum* (T 102). Azaizeh *et al* (1989) tested seven peanut genotypes in greenhouse and micro plot experiments during two consecutive years And, no significant differences were obtained in the degree of *Aspergillus* infestation of kernels from genotypes of glasshouse experiments Colonization of peanut pegs was inconsistent among genotypes and between the years Chourasia and Sinha (1994) conducted greenhouse experiments, in which simultaneous inoculation of root regions of

1 to 2 week old peanut plants with toxigenic and atoxigenic strains of *Aspergillus flavus* and reported lower levels of aflatoxin B_1 in the peanut kernels at maturity, than those in plants inoculated with the toxigenic strains alone We studied the effect of *Trichoderma* spp. on seed infection and aflatoxin contamination under glasshouse conditions Among seven *Trichoderma* isolates used for management of aflatoxin under glasshouse conditions all the treatments except the isolate T 24 effectively reduced aflatoxin production. All the treatments showed considerable effect in decreasing seed infection % *Trichoderma* spp. effectively reduced the seed infection and aflatoxin contamination over control under field conditions. During kharif season all treatments except the isolate T 29 showed significant reduction in seed infection % The biocontrol agents *T. koningii* (T 83) followed by *T. viride* (T 179) effectively reduced the aflatoxin levels during Kharif and rabi seasons. The above results are closely related to (Mixon 1980), who tested six peanut genotypes grown at three locations in laboratory studies for the influence of pod and seed inoculation methods on seed colonization by *A. parasiticus* and/or incidental prior contamination in the field or storage by *A. flavus* or *A. parasiticus.*

In earlier studies (Anjaiah and Thakur., 2000; Desai *et al*, 2000, and Thakur *et al*, 2003) several isolates of *Trichoderma* and *Pseudomonas* were characterized for their antagonism against *A. flavus* and for their biocontrol potential They used selected *Trichoderma* spp. both as seed dressing to determine their effects on population dynamics of aflatoxigenic population of *A. flavus* in the geocarposphere and subsequently on infection of groundnut kernels Kumar *et al* (2002) evaluated the integrated management package to reduce preharvest seed infection by *Aspergillus flavus* in groundnut The beneficial effects of soil treatment with the antagonistic fungus, *Trichoderma* sp was

apparent in improved package plot, despite the addition of *A. flavus* inoculum during flowering stage. The present study was also carried out in a similar manner to determine the potent antagonistic *Trichoderma* isolates in the present study also effectively controlled the aflatoxins in groundnut

Trichoderma spp. are known to be plant growth promoting fungi which were effective in promoting the growth and yield in several crops including wheat, cucumber, tomato and radish (Hyakumachi, 1994). Besides the reduction in aflatoxin contamination and seed infection, in the present study, the *Trichoderma* isolates also improved plant growth in the form of biomass and other agronomic parameters such as pod weight and seed weight.

Among *Trichoderma* isolates used for management of aflatoxin under glasshouse conditions T 47, T 83 and T 179 increased the pod weight and seed weight when compared to control. Isolate T 24 showed increase in shelling % followed by T 179 and T 83 under glasshouse conditions while T 47 and T 20 showed increase in biomass weight Similarly, under field conditions all the isolates showed significant increase in pod weight. Isolates T 24 and T 179 produced significant increase in seed weight However, isolates T 102 and T 179 produced significantly high shelling percentage Biomass increased significantly with the isolate T 102 during rabi season Significant increase in total number of plants was observed with the isolates T 47, T 179, T29 and T 102 during kharif season.

Windham *et al.* (1986) reported increase in the rate of emergence of seedlings, root and shoot dry weights of tomato and tobacco by *T. harzianum* and *T. koningii* They attributed the increase in the growth to a growth-regulating factor Increased growth in

radish was induced by *T. harzianum* when peat and vermiculite were mixed in different ratios and used as a substrate (Paulitz *et al*, 1996) They suggested increased plant growth independent of any detectable root pathogen The biocontrol agents *T harzianum* (T 20) and *T. koningii* (T 83) along with *T fertile* (T 47) enhanced plant growth, pod weight and seed weight in the glasshouse and field experiments in the present study This suggests their growth promotion ability in addition to biocontrol potential The results are in conformity with their above reports on the growth promotion ability of *Trichoderma* spp

Establishment of the biocontrol agent in soil/rhizosphere is one of the important attributes of an effective biocontrol agent Ecologically it has been a difficult proposition of an alleign microorganism to colonize and survive in soil and rhizosphere Antagonists including *Trichoderma* applied to seed failed to establish or proliferate in the rhizosphere (Kommedhal and windels, 1981, Papavizas, 1982) In the present study all the biocontrol agents (*Trichoderma* isolates) established successfully in the rhizosphere of groundnut to combate the toxigenic *A. flavus* Among the biocontrol agents *T harzianum* (T 20) and *T koningii* (T 83) were consistent in reducing the population of *A flavus* under glasshouse and field conditions The biocontrol agents were effective even when they are in less numbers Lewis and Papavizas (1984) stated that the population of *T viride* (T-1-R4) and *T harzianum* (WT-6-24) increased during first three weeks and decreased slowly till 18 weeks and stabilized there after at 10^5 to 10^6/g of soil Achira *et al* (2001) reported that the biocontrol agents, *T. viride* and *Pseudomonas aeruginosa* proliferated during the active growth phase of groundnut resulting in the decline of root pathogens In the present study also the *Trichoderma* spp continued to stay in the rhizosphere of the

groundnut up to harvest in both rabi and kharif seasons The population of *T harzianum* and *T. koningii* were consistant upto the end this may be due to their ability to produce clamydospores which might have helped the biocontrol agents for proliferation and survival in the rhizosphere for a long time The biocontrol agents in general and *T harzianum* (T 20) and *T. koningii* (T 83) in particular are the most effective colonizers of the rhizosphere and reduced the aflatoxin contamination levels in groundnut

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Summary

Biocontrol potential of various isolates of *Trichoderma* spp. was assessed in this study to reduce the preharvest aflatoxin contamination in groundnut. Two hundred and twelve *Trichoderma* isolates were isolated from the groundnut rhizosphere soil collected from four major groundnut-growing districts of Andhra Pradesh (Ananthapur, Chittoor, Cuddapah and Kurnool) and Karnataka (Kolar and Tumkur). About 40% rhizosphere soils harboured *Trichoderma*, with a maximum of 64% *Trichoderma* isolates from Anantapur and a minimum of 11% from Kolar. However, *Trichoderma* population density was maximum in Chittoor soil samples.

Trichoderma isolates were tested for antagonism or mycoparasitism *in vitro* against a toxigenic *A. flavus* (Af 11-4). After preliminary screening of 212 *Trichoderma* isolates, 150 antagonistic isolates were selected which exhibited three types of antagonism *viz.*, contact inhibition (38 isolates), clear inhibition (48 isolates) and mycoparasitism (69 isolates). Isolates (117) that showed inhibition zone and mycoparasitism against *A. flavus* were further screened and 48 isolates were identified using Rifai's key as well as Bisset's key. These isolates belong to ten different species of *Trichoderma viz.*, *T. harzianum*, *T. fertile*, *T. fasiculatum*, *T. koningii*, *T. viride*, *T. atroviride*, *T. inhamatum* *T. aureoviride*, *T. pseudokoningii* and *T. longibrachiatum*.

To estimate the inter-relationships among the 48 isolates of *Trichoderma*, morphological parameters such as growth type, growth rate, colony color, shape of margins, sporulation and sporulation initiation time were used. Clustering of 48 isolates was done using UPGMA based on morphological parameters. Forty-eight isolates were broadly segregated into five clusters representing *Longibrachiatum*, *Pachybasium* and *Trichoderma* sections of Bisset.

Besides morphological identification, AFLP was done to see correlation between morphological features and molecular characters. Thirty-six primer combinations were tested on four isolates preliminarily for selection of primer combinations producing highest number of polymorphic fragments. The generated fingerprints were evaluated for overall clearness of the banding pattern and the numbers of polymorphic bands were recorded. Finally, six E and M primer combinations were chosen for the diversity screening, they are *Eco*RI+AC/*Mse*I+CTG, *EcoRI*+AT/*Mse*I+CTA, *Eco*RI+TG/*Mse*I+CTA, *Eco*RI+AG/*Mse*I+CTG, *Eco*RI+TA/*Mse*I+CAC, *Eco*RI+TA/*Mse*I+CTC. A total of 250 bands were obtained from six primer combinations, which were polymorphic, with an average of 20 polymorphic bands per primer combinations. The genetic similarity coefficients obtained using the Jaccard algorithm was used for generating an UPGMA dendrogram. The isolates were segregated into five major groups Consisting of *Trichoderma* spp. belonging to the sections *Pachybasium*, *Trichoderma* and *Longibrachiatum*. AFLP analysis clearly separated the isolates into a sub group *Pachybasium*. *Trichoderma* section was diversified into four groups among which one group is solely consisted of isolates of *Trichoderma* section while this section dominated in another group. Similarly in MDS analysis of the dataset also segregated the AFLP isolates into five groups with *Trichoderma* section distributed in all except in one group.

Ribosomal DNA of *Trichoderma* isolates was amplified with ITS primers 1&4, which showed amplification of DNA without adding extra $MgCl_2$ to the reaction. Only 25 *Trichoderma* isolates were amplified producing 17 polymorphic bands. Dendrogram clusters were correlated with species designations based on morphological characteristics and geographical collection site. The genetic similarity coefficients obtained using the

Jaccard algorithm were used for generating an UPGMA dendrogram All the Isolates amplified by ITS primers were broadly divided into two major clusters The first group consists of only *T. harzianum* belonging to *Pachybasium* section The second group is subdivided into three sub-groups. The first subgroup was mostly dominated by the species of *Trichoderma* belonging to *Pachybasium* section while the second sub-group is represented mostly by *Trichoderma* section. The third sub-group encompasses the species belonging to both *Trichoderma* and *Longibrachiatum* sections Based on MDS analysis the isolates were divided into four groups. These groups consist of *Trichoderma* spp mostly belonging to the sections *Trichoderma* and *Longibrachiatum*

The antagonistic principles were analyzed from among the 48 isolates in producing volatile and non-volatile antibiotics. Out of 48 isolates 21 produced volatile antibiotics and 15 produced non-volatile antibiotics. Among 21 isolates of *Trichoderma* species six *T. harzianum*, five *T. longibrachiatum*, six *T. koningii* and three *T. viride* isolates showed inhibition of Af 11-4 colony by producing volatile antibiotics. Among 15 *Trichoderma* isolates four *T. harzianum*, three *T. koningii*, three *T. pseudokoningii* and four *T. viride* isolates showed inhibition of Af 11-4 colony by producing diffusible antibiotics

Enzymes being the main weapon of a biocontrol agent, the ability of *Trichoderma* isolates to produce chitinases, glucanases and proteases was been investigated. Ten different species of *Trichoderma viz., T. harzianum, T. fertile, T. fasiculatum, T. koningii, T. viride, T. atroviride, T. inhamatum T. aureoviride, T. pseudokoningii* and *T. longibrachiatum* were grown in liquid medium containing colloidal chitin as sole carbon source and all the species produced chitinases. *T. Koningii* (T 83) and *T. longibrachiatum* (T 102) only induced the enzyme in the presence of glucose. Among all species *T.*

pseudokoningii (T 29) produced more protease. *T. longibrachiatum* (T 102) was the highest producer of chitinases while *T. koningii* (T 83) and *T. pseudokoningii* (T 29) produced more glucanase and protease, respectively.

Based on their ability to produce cellulolytic enzymes and antagonistic behavior, 10 *Trichoderma* isolates were selected for the management of aflatoxins in greenhouse and field experiments. In the present study all the biocontrol agents (*Trichoderma* isolates) established successfully in the rhizosphere of groundnut to combate the toxigenic *A. flavus*. Among the biocontrol agents *T. harzianum* (T 20) and *T. koningii* (T 83) were consistent in reducing the population of *A. flavus* under greenhouse and field conditions. The biocontrol agents were effective even when they were in less numbers. *Trichoderma* spp. continued to stay in the rhizosphere of the groundnut up to harvest in both rabi and kharif seasons. The population of *T. harzianum* and *T. koningii* were consistent up to the end. This may be due to their ability to produce clamydospores, which might have helped the biocontrol agents for proliferation and survival in the rhizosphere for a long time. The biocontrol agents in general and *T. harzianum* (T 20) and *T. koningii* (T 83) in particular are the most effective colonizers of the rhizosphere and reduced the aflatoxin contamination levels in groundnut.

All the treatments, except the isolate T 24 effectively reduced aflatoxin production. All the biocontrol agents considerably decreased the seed infection by *A. flavus*. *Trichoderma* spp. effectively reduced the seed infection and aflatoxin contamination over control under field conditions. During the kharif season all treatments except the isolate *T. pseudokoningii* (T 29) showed significant reduction in seed infection the biocontrol

agents *T. koningii* (T 83) and *T. viride* (T 179) effectively reduced the aflatoxin levels during both kharif and rabi seasons.

Among the biocontrol agents, *T. koningii* (T 83) performed well both in greenhouse and field along with *T. longibrachiatum* (T 102) and *T. viride* (T 179). Further, *T. koningii* (T 83) an isolate from the soils of Anantapur district has effectively reduced the aflatoxin production. It is evident that the isolates with maximum ability to produce cellulolytic enzymes like chitinases have performed well in the greenhouse and field conditions showing a good correlation. Consequently, these biocontrol agents improved the pod and seed weights as well as other growth parameters.

Conclusions

- **Isolation** --- 212 isolates were isolated.
- **Morphological characterization** of 212 isolates and identification of 48 antagonistic *Trichoderma* isolates.
- **Molecular characterization** --- AFLP analysis clearly separated the isolates into sub-groups. ITS primer characterization results were substantiating with morphology data.
- ***In vitro* antagonistic studies** --- Based on antagonistic study 48 isolates were selected.
- **Biochemical studies** --- Twenty-one *Trichoderma* isolates produced volatile antibiotics and 15 isolates produced diffusible antibiotics.
- **Enzymes**
- **Protein** --- *T. koningii* (T 83) produced more protein.
- **Chitinase** --- *T. longibrachiatum* (T 102) produced more chitinase enzyme
- **Glucanase** --- *T. koningii* (T 83) produced more Glucanase enzyme.
- **Protease** --- *T. pseudokoningii* (T 87) produced more Protease enzyme.
- **Evaluation**
- **Greenhouse and Field** --- *T. koningii* (T 83), *T. longibrachiatum* (T 102), *T. viride* (T 179) are statistically on par with each other.
- **Aflatoxin analysis**
- **ELISA** --- *T. koningii* (T 83) isolated from Anantapur district from Nallacheruvu mandalam showed less aflatoxin production

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Publications

Identification of *Trichoderma* Species and their Antagonistic Potential Against *Aspergillus flavus* in Groundnut

P Srilakshmi[1], R P Thakur[2], K Satya Prasad[1], and V P Rao[2] (1 Department of Botany, Osmania University, Hyderabad 500 007, Andhra Pradesh, India, 2 International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, Andhra Pradesh India)

Contamination of groundnut (*Arachis hypogaea*) seed by aflatoxins produced by *Aspergillus flavus* is a major problem affecting quality and trade of groundnut and its products. Among several management options, biological control can play a significant role in reducing pre-harvest

aflatoxin contamination in groundnut Control of plant diseases by biological agents is environmentally safe and compatible with sustainable agriculture. *Trichoderma* spp are well-known biocontrol agents against several plant pathogens (Elad et al. 1982).

Rhizosphere soil samples were collected from major groundnut-growing areas of four districts (Anantapur, Chittoor, Cuddapah, and Kurnool) in Andhra Pradesh, and two districts (Kolar and Tumkur) in Karnataka, India under the National Agricultural Technology Project (NATP) on "Aflatoxin contamination in groundnut mapping and management in Gujarat, Andhra Pradesh, and adjoining areas". *Trichoderma* spp were isolated from the soil samples. The isolates were characterized for morphological traits for speciation, and evaluated for their antagonism against *Aspergillus flavus* to identify highly antagonistic isolates that could be used as potential biocontrol agents for pre-harvest aflatoxin contamination of groundnut

Isolation of *Trichoderma* isolates

Five hundred µl of each soil sample at 10^{-3} and 10^{-4} aqueous dilutions was spread on petri dishes containing *Trichoderma* specific medium (TSM: glucose 3 g, ammonium nitrate 1 g, sodium dihydrogen phosphate 0.9 g, magnesium sulfate 0.2 g, potassium chloride 0.15 g, ferrous sulfate 20 mg, zinc sulfate 20 mg, manganese sulfate 20 mg, rose bengal 30 mg, agar 10 g, and distilled water 1000 ml) After autoclaving at 121°C for 20 min the medium was cooled to 50°C. Then 50 mg streptomycin sulfate, 50 mg chloramphenicol, 10 mg metalaxyl, and 10 mg PCNB (penta-chloro nitro-benzene) were added Two plates were maintained for each dilution The plates were incubated for 4 days in dark at 28°C and typical *Trichoderma* colonies were isolated The colonies were white or whitish green to green, conidiophores long and thick, with or without sterile branches, side branches mostly thick bearing short and plump phialides, phialospores globose or ellipsoidal rough or smooth walled

Of 386 soil samples analyzed, 156 (40.4%) yielded *Trichoderma*, with the maximum (64.1%) *Trichoderma* isolates obtained from soil samples of Anantapur and the minimum (11.1%) from the samples of Kolar (Table 1) A total of 212 isolates of *Trichoderma* spp were obtained (Table 1)

Evaluation for in vitro antagonism

The dual culture method (Denis and Webster 1971c) was used to study the antagonism against a highly aggressive and toxigenic strain of *A. flavus* (Af 11 4) Of the 212 isolates tested, 145 were antagonistic to Af 11-4 Among these, only 39 isolates showed clear inhibition zone against Af 11 4 (Fig 1) These isolates were examined for species identification and further evaluated for antagonism, involving production of volatile and non volatile antibiotics, and hyphal interaction with Af 11 4

Species identification. Thirty nine antagonistic *Trichoderma* isolates were identified according to the identification key (Rifai 1969) based on branching of

Table1. Isolation of *Trichoderma* isolates from soil samples collected from major groundnut-growing districts of Andhra Pradesh and Karnataka, India, rainy season 2000.

District	No. of soil samples analyzed	No. of soil samples with *Trichoderma* isolates	No. of *Trichoderma* isolates obtained	Soil samples with *Trichoderma* (%)
Andhra Pradesh				
Anantapur	53	34	40	64.1
Chittoor	97	48	54	49.5
Cuddapah	23	11	19	47.8
Kurnool	26	15	1	57.6
Karnataka				
Kolar	72		15	11.1
Tumkur	115	40	44	34.8
Total	386	156	212	40.4

conidiophores, shape of the phialides, emergence of phialospores, and shape of phialospores. These isolates were identified into six species, *T. harzianum* (11), *T. hamatum* (1), *T. viride* (9), *T. longibrachiatum* (5), *T. koningii* (9), *T. pseudokoningii* (3), and unknown species (1) (Table 2).

Production of volatile antibiotics. This study was done following the method of Dennis and Webster (1971b). The plates were incubated at 28°C for 72 h. The assembly was opened to measure colony diameter of Af 11-4 in each plate. Twenty-one of the 39 *Trichoderma* isolates showed inhibition of Af 11-4 colony by producing volatile antibiotics compared with the control. In the control plate, the colony diameter of Af 11-4 was 60 mm whereas in other plates it was 10–45 mm. Isolate T 102

Table 2. Species identification of *Trichoderma* isolates antagonistic to *Aspergillus flavus* (Af 11-4).

Trichoderma species	*Trichoderma* isolate number
T. harzianum	T 2, T 10, T 11, T 20, T 42, T 53, T 58, T 72, T 109, T 129, T 170
T. hamatum	T 47
T. viride	T 16, T 24, T 50, T 51, T 60, T 62, T 179, T 188, T 205
T. longibrachiatum	T 6, T 34, T 56, T 102, T 110
T. koningii	T 12, T 13, T 21, T 33, T 49, T 70, T 83, T 143, T 161
T. pseudokoningii	T 29, T 37, T 206
Trichoderma sp (unknown)	T 142

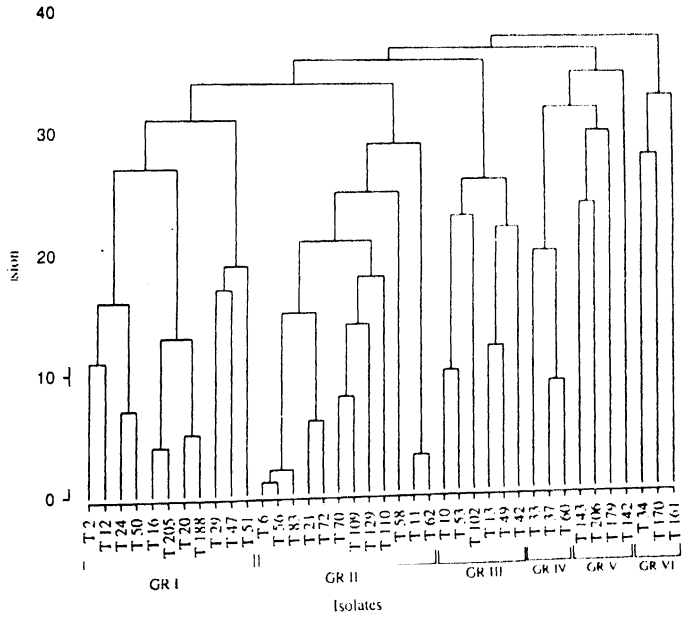

Figure 1. Dendrogram showing 39 *Trichoderma* isolates classified into six groups based on morphological traits and inhibition zone against *Aspergillus flavus* (Af 11-4).

Soil and pod sampling. Soil was sampled at three stages to monitor the levels of *A. flavus* population. Initial sampling was done just before sowing; the second sampling was done prior to application of the pathogen inoculum (at 50% flowering stage), and the final sampling at harvest in both experimental plots. Pods from the improved package plot were harvested at 75% maturity level and dried by inverted windrows method for three days. On the other hand, pods in the control plot (farmers' practice) were harvested at full maturity and dried by leaving them in the field.

Seed infection, aflatoxin content, and *A. flavus* population in soil. Pods were shelled and seeds were surface sterilized before plating them on Czapek Dox agar (CDA) fortified with rose bengal (25 mg L^{-1}) and incubated at 25°C for four days in dark. From each plot, 100 apparently healthy seeds were selected. Number of seeds colonized by typical *A. flavus* was counted and expressed as percent seed infection. Seed samples (50 g) were soaked in sterile distilled water for 4 h, later dried and incubated at 25°C overnight prior to aflatoxin estimation by using enzyme-linked immunosorbent assay (ELISA), a simple and quick immunoassay protocol for estimation of aflatoxins (Devi et al. 1999).

Soil samples (200 g from a composite bulk of 1000 g soil from 5 random spots in a field) were sieved. The fine powder was serially diluted with sterile distilled water to 10^{-3} and 10^{-4} concentrations and plated on AFPA (*Aspergillus flavus* and *parasiticus* agar) medium (Pitt et al. 1983). The plates were then incubated for 2–3 days at 28°C in dark and typical *A. flavus* colonies were counted and population density was expressed as cfu g^{-1} soil.

Other diseases. The crop was given supplemental irrigation to avoid moisture stress throughout the growing season. In both the plots, other important diseases were recorded by selecting ten blocks, each comprising 100 plants row^{-1}. Disease incidence was scored as percentage of infected plants for stem rot (*Sclerotium rolfsii*) and on a 1–9 scale for late leaf spot. Bud necrosis was scored for the presence (+) or absence (–) of infected plants.

Results and Discussion

The indicators for the effectiveness of integrated management of aflatoxin contamination were fungal infection and aflatoxin content in the seed and *A. flavus* population in the soil. Despite the similar initial population levels in both the plots, cumulative gain in the cfu was observed in the plot where farmers' practice was followed indicating unremitting growth in *A. flavus* due to absence of any control measures. On the other hand, the beneficial effects of soil treatment with the antagonistic fungus *Trichoderma* sp was apparent in the plot with improved package, despite the addition of *A. flavus* inoculum during flowering stage, which produced spores at 1.82×10^{7} m^{-1} row (Fig. 1). *Trichoderma* sp being a potential antagonist might have prevented the proliferation of *A. flavus* in the soil. *Trichoderma* sp has the ability to inhibit the growth of *A. flavus* in vitro by production of non-volatile antibiotics (Desai et al. 2000).

Seed infection studies revealed predominance of *A. flavus* infection in plot with farmers' practice (10%) over improved package (2%) (Table 1).

Table 1. Evaluation of an integrated aflatoxin management package and farmers' practice in groundnut (cv ICGS 11) during the rainy season, 2001 at ICRISAT, Patancheru, India

Parameter	Integrated package	Farmers' practice
Seed infection by *Aspergillus flavus* (%)	2	10
Pod yield (kg ha^{-1})	555	544
Late leaf spot damage[1]	6.9 (±0.23)	7.7 (±0.15)
Stem rot incidence (%)	2.5 (±0.62)	31.0 (±4.70)
Bud necrosis incidence[2]	+	+

1. Mean of 10 replications, 1–9 disease rating scale where 9 = susceptible.
2. + = Disease noticed

(*T. longibrachiatum*) showed the maximum inhibition compared with other *Trichoderma* species.

Production of diffusible antibiotics. This study was done following the method of Dennis and Webster (1971a). The plates were incubated for two days and the colony diameter and sporulation of Af 11-4 were compared with the control. Fifteen of the 39 *Trichoderma* isolates showed inhibition of Af 11-4 colony by producing diffusible antibiotics compared with the control. Colony diameter of Af 11-4 in the control plate was 55 mm compared with 10–50 mm in plates with *Trichoderma* isolates. Isolate T 29 (*T. pseudokoningii*), T 42 (*T. harzianum*), and T 83 (*T. koningii*) showed significant inhibition of Af 11-4 growth.

Hyphal interaction. This study was done following the dual culture method (Dennis and Webster 1971c). A block of cellophane (10 mm × 20 mm) was cut from the juncture of the two colonies and mounted in trypan blue-lactophenol, and examined under microscope for hyphal interactions. Isolates T 16 (*T. viride*), T 109 (*T. harzianum*), and T 188 (*T. viride*) showed clear hyphal coiling with Af 11-4 mycelia.

The data of inhibition zone and morphological characters were subjected to average linkage cluster analysis using Euclidian distance as dissimilarity association of GENSTAT Statistical Package (Rothamsted Experiment Station, Herpenden, Herts, UK). The dendrogram prepared from the above classified the 39 *Trichoderma* isolates into six groups (Fig. 1). Further studies are in progress to determine the biological control potential of these isolates against *A. flavus*.

Acknowledgment. The authors thank Mr V Papaiah, Genetic Resources and Enhancement Program, ICRISAT for his help in cluster analysis and making the dendrogram.

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Evaluation of an Integrated Management Package to Reduce Preharvest Seed Infection by *Aspergillus flavus* in Groundnut

K Vijay Krishna Kumar[1], S Desai[2], VP Rao[1], HA Nur[1], P Srilakshmi[1], and RP Thakur[1] (1. International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, Andhra Pradesh, India; 2. National Research Centre for Groundnut (NRCG), PB no. 5, Junagadh 362 001, Gujarat, India)

Aflatoxin contamination in groundnut (*Arachis hypogaea*) is one of the major problems that can occur at preharvest and postharvest stages affecting the quality of the produce and thus trade. Aflatoxins are produced by *Aspergillus flavus* and *A. parasiticus*, which can invade the pods during crop growth, when the conditions are congenial for the pathogen (Hill et al. 1983). An integrated approach through combining chemical, cultural, and biological management options could be a viable option for reducing preharvest contamination of seed in groundnut production systems. The efforts to subdue preharvest aflatoxin problem should be based on the principles of greater ecological sustainability in the long run keeping in view minimal use of pesticides. Through a collaborative project, the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and the Indian Council of Agricultural Research (ICAR) evaluated an integrated package at ICRISAT, Patancheru, Andhra Pradesh, India during the rainy season in 2001 to demonstrate the effectiveness of improved package vis-à-vis farmers' practice.

Methodology

Two treatments, integrated aflatoxin management package (IAMP) and farmers' practice were compared in Alfisol fields at ICRISAT, Patancheru. Each treatment was conducted on a 0.1-ha plot. The IAMP comprised: summer plowing of the field; seed treatment with carbendazim (Bavistin 50 WP) at 4 g kg^{-1} seed; furrow application of *Trichoderma harzianum* at 50 g culture mixed in 50 kg farmyard manure before sowing (to make a final population of 1×10^6 colony forming units (cfu) g^{-1} soil); spray of Nimbucidin (250 ml in 50 L water) for controlling foliar diseases and insects; and a second spray of carbendazim (50 g) + Dithane M-45 (250 g) in 50 L of water, if required; harvesting plants at 75% pod maturity; drying the harvested plants by inverted windrows method for 3 days to avoid contact between the pods and wet soil; and removing insect-damaged and diseased pods. In both practices, hand weeding was done twice, at 20 and 45 days after sowing.

The farmers' practice (as a control) included summer plowing, harvesting pods at full maturity, drying pods by leaving them in the field, and removing damaged pods, but did not include any chemical and biological treatments.

Application of *A. flavus* inoculum. To ensure infection, a highly toxigenic strain of *A. flavus* (Af 11-4) was multiplied on pearl millet (*Pennisetum glaucum*) seeds, mixed with farmyard manure and applied in both the practices when the crop was at 50% flowering stage (the most susceptible stage of the crop).

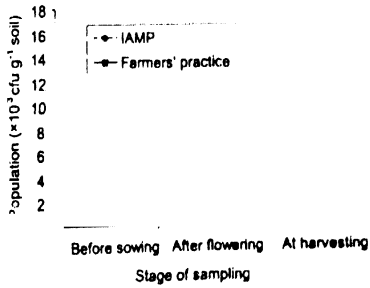

Figure 1. Soil population of *Aspergillus flavus* (Af 11-4) at different crop growth stages in plots with IAMP (integrated aflatoxin management practice) and farmers' practice during the rainy season, 2001 at ICRISAT, Patancheru, India.

Soil and pod sampling. Soil was sampled at three stages to monitor the levels of *A. flavus* population. Initial sampling was done just before sowing; the second sampling was done prior to application of the pathogen inoculum (at 50% flowering stage), and the final sampling at harvest in both experimental plots. Pods from the improved package plot were harvested at 75% maturity level and dried by inverted windrows method for three days. On the other hand, pods in the control plot (farmers' practice) were harvested at full maturity and dried by leaving them in the field.

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Bud necrosis incidence[2]	+	+

1. Mean of 10 replications; 1–9 disease rating scale where 9 = susceptible.
2. + = Disease noticed.

This could be because of inhibition of initial rhizosphere soil population build up of *A. flavus* by seed treatment with systemic fungicide and application of biocontrol agent in the improved package. Although no aflatoxin contamination was recorded in seed samples in both practices, with 10% seed infection levels in farmers' practice it is likely that aflatoxin levels would be higher under farmers' storage conditions than under dry conditions.

The improved package recorded only a marginal increase in pod yield than farmers' practice, reflecting the fact that aflatoxin contamination is more of a qualitative problem in groundnut than quantitative. The concomitant effects of the improved package were evident in scanty incidence of late leaf spot and stem rot diseases over farmers' practice. Further, application of such a package in the long run would improve soil health and might result in improved yields as well.

These results need further confirmation, and relative economics of the two cultivation practices could be compared from on-farm evaluation trials at village level in aflatoxin risk sensitive areas in the target districts of Andhra Pradesh and Karnataka during the rainy season in 2002.

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AFLP and SNP Diagnostics of *Trichoderma* spp. Antagonistic towards Aflatoxin Producing *Aspergillus flavus*

H.K.Buhariwalla[1]*, P. Srilakshmi[1,2], Seetha K[1], R.S. Kanchi[1], S. Chandra[1], K.Satyaprasad[2], F. Waliyar[1], R.P. Thakur[1], and J.H. Crouch[1]

International Crop Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh-502324 India[1], and Department of Botany, Osmania University, Hydrabad 500 007, Andhra Pradesh, India[2]

*Corresponding author. Mailing address: International Crop Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh-502324 India.

Phone: +91 (0) 40 23296161. Fax: +91 (0) 40 23241239. E-mail: H.K.Buhariwalla@cgiar.org

[Abstract]

Trichoderma species offer considerable potential for controlling aflatoxin contamination in groundnut. However, targeted and rapid identification of useful isolates is hampered by an array of practical classification issues. Thus, this study investigates the use of sequence and AFLP analysis for rapid classification of new *Trichoderma* isolates. In addition, we have evaluated the scope for developing rapid and cost effective diagnostics for specific *Trichoderma* species and for identifying isolates most likely to be highly antagonistic to *Aspergillus flavus* species. Unique SNP haplotypes were identified for many but not all species. Sequence-based classification (anchored to GenBank sequences of ex-type strains) was significantly more reliable than morphological characterization but did not provide a comprehensive diagnostic solution. In contrast, AFLP analysis (based on six primer combinations) was able to distinguish all 48 *Trichoderma* isolates use in this study. Yet this level of analysis is still too time consuming and cumbersome for routine diagnostic. Thus, individual AFLP bands have been identified which distinguish closely related species. We have also identified other AFLP bands highly correlated to antagonism to *A. flavus* which represent candidates for the development of a simple PCR-based diagnostic test for rapid y and cost effective identification of *Trichoderma* isolates for the biocontrol of aflatoxin contamination in groundnut.

(204 words) limit 250

been the cause of substantial economic losses in mushroom production (40). However, they have many positive economic benefits which include: the ability to act as biocontrol agents (BCA) (25, 44) induce host-defense responses in plants (15, 57), produce cellulolytic and hydrolytic enzymes (23), antibiotics (18) and degrade organochlorine pesticides (28). Recent investigations have shown that some *Trichoderma* species present in groundnut-cultivated soils are antagonistic to *A. flavus* (47). Moreover, there are several reports of *Trichoderma* spp. inhibiting growth of *A. flavus* and preventing synthesis of aflatoxin B1 (6, 16, 46).

Taxonomic identification of *Trichoderma* species is still largely based on the characterization of a large number of morphological descriptors resulting in the division of the genus into five sections: *Trichoderma*, *Pachybasium*, *Longibrachiatum*, *Saturnisporum* and *Hypocreanum* (6, 7, 8, 38). Unfortunately, identification of *Trichoderma* at the species level is difficult due to a high level of morphological similarity amongst certain species (30). In addition, because of their genotype-by-environment variability morphological markers are often not highly reproducible. Moreover, these conventional methods of identification require a high level of expertise and are time consuming, thus present a bottleneck to the rapid identification of useful isolates (33). As an alternative to conventional classification based on morphological taxonomy, there has been much interest in using molecular taxonomy based on: isozymes (21), RFLP (34, 37), PCR fingerprinting (34), RAPD (1, 56) and sequence polymorphism (30, 31). On this basis, molecular genetic taxonomists have identified four clades: clade A *Trichoderma* sect. including *T. hamatum*, *T. pubescens*, *T. koningii* and *T. atroviride*; clade B containing a large heterogeneous mixture representing the *Pachybrasium* sect. including *T. harzianum* and *T. inhamatum*; clade C the species *Longibrachiatum* and clade D containing *T. aureoviride* (24, 30, 31, 33) have been observed. Many groups have suggested that a multiphasic approach based on: metabolic characteristics, morphological observations and nucleotide sequence information (ITS1 and 2) are more apt in defining species, although morphological and physiological data alone may not accurately reflect phylogeny (21, 24, 31, 33). However, the multiphasic approach does not necessarily offer a reduction in time and expertise required for reliable diagnosis. Accurate and precise classification remains critically important for identifying geographical and phylogenetic hot spots for antagonistic behavior.

Amplified fragment length polymorphism (AFLP) is a genotyping method based on the selective amplification of a subset of DNA fragments generated by restriction enzyme digest (50). This technique has proven to be highly discriminatory and reproducible in comparison to other genotyping assays, and has been extensively used for population structure analysis of other microorganisms (43). These advantages have facilitated the compilation of diversity databases indicating standard reference AFLP banding patterns for harmonizing diagnosis by the community at large (43).

The present study compares AFLP fingerprinting and 28S rDNA (D2) sequence-based diversity analyses with previously reported morphological classification of 48 *Trichoderma* isolates. We have also attempted to identify specific AFLP bands which provide species differentiation or association with antagonistic behavior.

MATERIALS AND METHODS

Collection and conventional characterization of *Trichoderma* isolates. The *Trichoderma* isolates used in this study were collected and identified as described previously (47). In summary rhizosphere soil samples were collected from major groundnut growing areas of four districts in Andhra Pradesh and two districts in Karnataka, (all districts in Southern India) during the 2000 and 2001 rainy seasons (Table 1). A total of 212 *Trichoderma* isolates were obtained from 386 soil samples and single-spore representatives were generated. A subset of 48 isolates showed a clear inhibition zone in dual-culture studies, of which 35 were characterized for production of volatile and diffusible antibiotic, referred to as V and D type antagonism, respectively (data not presented). This morphological and biochemical characterization data were used for comparative and association analyses in the present study.

DNA isolation. The 48 *Trichoderma* isolates were maintained on solid potato dextrose medium (PDA) and incubated at 28°C for 5 days. DNA was extracted from fungal mycelium, ground in liquid nitrogen and lysed with 15 ml of pre-warmed CTAB extraction buffer (2. 5% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 0.03% β-mercaptoethanol). The extraction mixture was incubated in a 65°C water bath for 30 min. and then mixed with an equal volume of chloroform/isoamyl alcohol (24:1). The aqueous layer was separated by centrifugation at 6000 rpm for 10 min., and then further purified with chloroform/isoamyl alcohol. Crude DNA was precipitated with 0.7 (v/v) isopropanol and suspended in TE (50mM Tris-HCl pH 8.0, 10mM EDTA)

containing 7.5 mg RNaseA and incubated overnight at 37°C. Following phenol chloroform:isoamyl extraction and two further chloroform:isoamyl alcohol extractions the DNA was precipitated with two volumes of absolute ethanol containing 1/10th volume of 3M sodium acetate (pH 5.3). The air-dried pellet was then suspended in TE (10 mM Tris, 1 mM EDTA) and the purity of the DNA determined by electrophoretic analysis on 0.8% (w/v) agarose gel containing ethidium bromide. The concentration of the fungal DNA was determined by comparing intensities with that of known concentrations of lambda DNA.

28S-rDNA fungal sequence analysis. The Large Sub-Unit (LSU)-D2 region of rDNA was amplified using primers supplied with the MicroSeq D2 LSU kit (Applied Biosystems Foster City, USA). Fungal DNA was diluted to 1ng/µl and 25µl of diluted DNA was mixed with 25µl of fungal PCR mix supplied by the manufacturer (comprising dNTP, reaction buffer and ampliTaq gold DNA polymerase). Samples were amplified using the following PCR profile: initial hold at 95 °C for 10 mins followed by 40 cycles of 95 °C for 30 sec, 53°C for 30 sec, final hold temperature of 72 °C. PCR products were passed through purification columns (Invitrogen USA) and verified by agarose gel electrophoresis (1.5% w/v). Purified PCR products were used for cycle sequencing with dRhodamine dye terminator chemistry, as per the manufacturer's instructions, using the following PCR profile: 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min. Unincorporated label and sequence primer was removed using a standard ethanol/sodium acetate precipitation procedure. Sequences were analyzed using an Applied Biosystems genetic analyzer model ABI 3700 with a POP 6 gel matrix, filter set E, and DT 3700 pop6 dRhodamine v3 mobility file.

Sequence-based phylogenetic analysis. Forward and reverse sequence data (28S rDNA D2) from 38 isolates was analyzed using Chromas v2.2 (Technelysium Pty Ltd. Australia) software (it was not possible to obtain good quality sequence data from the remaining 10 genotypes in this study). The resultant sequence data were compared with the NCBI database using BLASTn. The nucleotide sequences from the most homologous BLASTn alignments were retrieved from NCBI (Table 2) and used as reference sequences for phylogenetic analysis. It should be noted that the *T. pubescens* in GenBank is listed with the incorrect culture number of DAOM 162.162 whereas the correct accession number is DAOM 166.162. (Pers. Com. C.P. Kubicek). Sequences in Fasta format were aligned with the reference from each of the major *Trichoderma* spp. using the Clustal X (1.81) multiple sequence alignment algorithm using the default options for alignment (48). Nucleotide divergence was estimated using the Kimura's two-parameter (K2P) method (29) and phylogenetic inference was performed by the neighbor-joining (NJ) method (42) implemented by Mega 2.1 software package (32).

Bootstrap analysis was used to resample the dataset (1000 re-samplings) to provide a statistical significance for the particular clusters/clades in the UPGMA and NJ trees (values shown at nodes).

AFLP fingerprinting. AFLP pre-selective and selective reactions were performed according to the manufacturer's instructions (Invitrogen, USA) with some minor modifications. Fungal DNA (400 ng) was cleaved with restriction enzymes *Eco*RI and *Mse*I at 37 °C for 2 hr. Enzyme activity was terminated by heat inactivation (15 mins at 70°C) of cleaved products. Adaptors corresponding to *Eco*RI and *Mse*I restriction sites were ligated to the cleaved fragments using T_4 DNA ligase. Pre-selective amplification of a 10-fold diluted ligated DNA product was carried out using *Eco*RI and *Mse*I primers using the following PCR cycle profile: 94 °C for 30 sec, 56 °C for 60 sec, and 72 °C for 60 sec for a total of 30 cycles. Pre-selective amplification products were then diluted 1:50 prior to selective amplification. The *Eco*RI selective primers were labeled with [γ-^{33}P]-ATP (3000 Ci/mmol). Initially 36 selective primer combinations were pre-screened on four *Trichoderma* isolates for protocol optimization and selection of primer combinations producing the highest number of polymorphic fragments. The following six primer combinations were used to screen all 48 isolates (E-AC/M-CTG, E-AG/ M-CTG, E-AT/ M-CAG, E-TA/ M-CAC, E-TG/M-CTA and E-TA/M-CTC). DNA fragments generated from selective amplifications were separated on denaturing 6% polyacrylamide sequencing gels containing 7.5M urea. Aliquots (3 □l) were mixed with loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured for 5 mins at 95 °C and cooled to 4 °C prior to gel electrophoresis. The dried gels were exposed overnight at room temperature to X-ray films (Kodak X-Omat) with one intensifying screen sandwiched in a film-cassette.

AFLP data collection and diversity analysis. The AFLP autoradiographs were scored for the presence (1) or absence (0) of all polymorphic bands. The resulting 48 × 250 binary data matrix from 6 primer combinations was used to estimate pairwise genetic similarities among the 48 isolates based on Jaccard's coefficient (27). The 48 × 48 similarity matrix was subjected to sequential agglomerative hierarchical nested (SAHN) clustering using UPGMA (unweighted pair-group method analysis) in the NTSYS-pc software version 2.0 (39). Multi-dimensional scaling (MDS) using NTSYS-pc was also applied to the similarity matrix to assess how the isolates relate to one another in a two-dimensional spatial plot using the first two MDS axes. The MDS was used to obtain an independent validation of UPGMA clustering results. Similar clustering analysis using UPGMA was applied to the AFLP data-subset associated with isolates belonging to the clades *T. pubescens* - *T. hamatum* and *T. harzianum* - *T. inhamatum*.

Phylogenetic relationships among isolates obtained from sequence data were used to group the AFLP dataset into four sub-populations to assess the extent to which the sequence-based clustering matched with that of AFLP-based clustering of these sub-populations. For this purpose, allele-frequency based Nei's genetic distance and UPGMA clustering method were employed using TFPGA software (36)

AFLP marker association analysis. The defined AFLP amplification products referred to here can potentially be converted to simple species specific PCR markers, and will therefore be referred to hereafter as markers. The frequency of (0,1) and (1,0) occurrences was computed to identify markers that allowed differentiation between the two pairs of species *T. harzianum* and *T inhamatum* and similarly for *T pubescens* and *T. hamatum*, containing 12 individuals (32 pair-wise combinations) and 20 individuals (99 pair-wise combinations) respectively. The degree to which a particular marker differentiated the two species was then estimated as the ratio D=[{#(0,1)+#(1,0)}/Total #pairs]*100, where #(0,1) denotes the number of pairs in which the first individual has a score of 0, while #(1,0) denotes the number of pairs in which the first individual has a score of 1.

To identify markers that may be significantly associated with D and V type antagonism, the data on each marker were subjected to linear regression analysis using the model

$$y_i = \alpha + \beta x_i + \varepsilon_i$$

where y_i is the antagonism value of individual i, α is intercept, β is the linear regression coefficient, x_i takes value 1 for the presence of a band and 0 otherwise. ε_i is random error assumed to follow a normal distribution with mean 0 and constant variance σ_ε^2. Due to the binary nature of the independent variable x_i, the regression coefficient $\beta=\mu_1-\mu_0$ where μ_1 and μ_0 are means of antagonism values corresponding to the presence and the absence of band respectively for the markers concerned

RESULTS

28S-rDNA phylogenetic analysis. Sequences of 250 nucleotides in length, specific to the D2 LSU region of 28S-rDNA from 38 genotypes was BLASTn analyzed resulting in alignments with 92-100% homology to *Trichoderma* spp. in GenBank (with E values of e^{-146} to e^{-56}). All 38 sequences aligned with one of the following species groups *T. pubescens* - *T. hamatum*, *T. harzianum* - *T.*

inhamatum, *T. atroviride*, *T. koningii* and *T. longibrachiatum*. Sequences showing best alignments were retrieved from GenBank (Table 2), and used as reference sequences for diversity analysis. If 28S-rDNA sequence information was available for ex-type strains this was preferentially used for the analysis, otherwise, strains reported as being typical for the species based on morphology, physiology and molecular genetic characterization (24, 30, 31).

Sequences were first aligned in ClustalX (1.81) and then cross-referenced by eye with the sequence pherograms to check for gaps, base transitions and base transversions. After trimming the ends consisting largely of unaligned regions, the sequence length analyzed was of 218 nucleotides and consisted of no gaps. As some clustering alignment programs are more sensitive to data input order (26), various input orders were compared. When a sequence derived from any isolate used in this study was entered first there were no gaps in the alignment and the phylogenetic trees were consistent. However, this was not the case when the *T. harzianum* GenBank (AF399236) sequence was entered first, so this input order was not used.

Only 14 single nucleotide polymorphisms (SNPs) were observed in the 28S-rDNA region across the 38 genotypes tested, most of which were transversions (Figure 1). There was no intraspecific variation of the 28S rDNA region among isolates of the same *Trichoderma* species, suggesting a high level of sequencing accuracy and sequence conservation within species in this region of the genome. However, certain individual SNPs and groups of SNPs were common across some species. Nevertheless, clear haplotypes were observed for all species apart from *T. pubescens* - *T. hamatum* and *T. harzianum* – *T. inhamatum* groups.

The NJ phylogenetic analysis of 28S-rDNA sequence data from 38 isolates and eight *Trichoderma* spp. reference strains identified four distinct clades (Figure 2). UPGMA analysis generated identical clusters but with higher bootstrap values (figure not shown). Clade 1 included 20 isolates clustered with *T. pubescens* DAOM 166.162 (ex-type strain) and *T. hamatum* DAOM 167057 (ex-neotype strain). All 20 isolates and two reference strains had identical sequences, with their cluster separated from others at a bootstrap value of 42% (UPGMA bootstrap value of 91%). Isolates in this clade did not exhibit any of the SNPs observed in the other species. Clade 2, includes the *T. koningii*, *T. atroviride* and *T. viride* complex, was characterized by seven SNPs. Some polymorphisms

were common to all three species (nucleotide positions 23 and 94) whilst others differentiated one or two of the species (Figure 2). This clade has two subgroups, one with an isolate clustering with *T. koningii* (CBS 979.70) and the other with three isolates clustering with *T. atroviride* (DAOM 165779). The *T. koningii* cluster is differentiated by unique SNPs at nucleotide positions 55 and 62 but there is variation between the isolate and reference strain at nucleotide positions 116 and 150. There are only two SNPs differentiating *T. viride* (ATCC 28020) from *T. atroviride* (DAOM 165779) at nucleotide positions 25 and 116, and, no other isolates in this study possessed those two SNPs. In contrast, there were no unique SNPs for *T. atroviride*. Clade 3 comprises the ex-type reference strain *T. longibrachiatum* (CBS 816.68) and two isolates, characterized by 6 SNPs (of which three are unique SNPs at nucleotide positions 36, 59 and 60). In addition, two other SNPs (at nucleotide positions 113 and 204) were observed in *T. harzianum* - *T. inhamatum*, while a further SNP (at nucleotide position 133) was observed in *T. atroviride* The large number of SNPs in this clade separates it from others at a bootstrap value of 98% (Figure 2) Clade 4 includes *T. inhamatum* CBS 273.78 (ex-type strain) and *T. harzianum* CBS 226.95 (ex-neotype strain) complex and twelve isolates which were characterized by three SNPs. Only one SNP (at nucleotide position 112) was observed to be specific to this clade. This clade was separated from the others at a bootstrap value 75% (UPGMA bootstrap value of 94%).

The G + C content of the D2 LSU sequence for each clade was as follows. 78.6% for clade 3 (*T. longibrachiatum*), 71.4% for clade 2 (*T. koningii*, *T. atroviride* and *T. viride*), 64.3% for clade 1 (*T. pubescens* - *T. hamatum*) and 57% for clade 4 (*T. harzianum* - *T. inhamatum*).

AFLP diversity analysis. A total of 250 intense polymorphic bands were identified by six AFLP primers and no monomorphic bands were observed. The number of polymorphic bands per primer varied as follows: 24 for E-AC/M-CTG; 64 for E-AG/M-CTG; 40 for E-TA/M-CAC; 48 for E-AT/M-CAG; 28 for E-TA/M-CTC and 46 for E-TG/M-CTA (Figure 3). The genetic similarity coefficients obtained using the Jaccard algorithm were used for generating an UPGMA dendrogram (Figure 4a). Pair-wise similarities between isolates (similarity matrix not shown) ranged from 0.11 to 0.97. Indeed, one isolate (T-24) was separated from all others at the 21% similarity level in the UPGMA dendrogram. As a means of validation, a dendrogram was generated based on the AFLP dataset

without missing values, (i.e. if one or several of the primer combinations did not produce bands for a particular accession then that accession was omitted from the analysis) resulting in the removal of 9 accessions. This approach resulted in a dendrogram broadly similar to the one presented in Figure 4a (not shown). Dendrogram clusters were poorly correlated with species designations based on morphological characteristics and geographical collection site Since it was not possible to include reference strains from each of the *Trichoderma* species due to quarantine restrictions, difficulties were incurred in anchoring AFLP analysis to the defined ex-type strains Based on MDS analysis of the AFLP dataset it was possible to separate isolates into 5 different groups (Figure 4b) with three outliers (T-24, T-73 and T-74). However, these groups are poorly correlated with species designations based on morphology or sequence data and poorly correlated with geographical locations

AFLP marker association analysis and clade validations. TFPGA analysis of the AFLP dataset using Nei's genetic distance, with subpopulations defined on the basis of 28S rDNA phylogenetic sequence analysis, confirmed the relationship between clades (Figure 4a compared with Figure 5) However this was not the case when using morphological based species designations This infers that classification based on AFLP diversity analysis is broadly congruent with sequence-based species designations

Further analysis of the AFLP dataset, regarding the two major clades *T pubescens - T hamatum* and *T. harzianum -T inhamatum* suggests that there are two distinct sub-groups within the *T pubescens - T. hamatum* clade separated at the 20% similarity level (Figure 6a) Similarly, there appears to be two distinct clusters within the *T harzianum - T inhamatum* clade separated at the 28% similarity level (Figure 6b) On this basis we attempted identifying candidate AFLP markers able to differentiate amongst these sub-groups (*T pubescens - T hamatum* and *T harzianum - T inhamatum*). For the twelve isolates within the *T harzianum T inhamatum* clade (eight isolates in sub-group 1 and four isolates in sub-group 2) eight markers differentiated the two groups to the extent of D = 100 % (Table 3) Similarly, for the 20 isolates within *T pubescens - T hamatum* clade, (nine isolates in sub-group 1 and eleven in sub-group 2) only one marker differentiated the two groups with D = 100 %.

We also attempted to identify candidate AFLP markers associated with antagonistic characteristics. To this end, volatile (V-type) and diffusible (D-type) antagonism data for 35 *Trichoderma* isolates, was compared with the AFLP dataset of 234 bands. Sixteen AFLP bands were removed from the dataset because of their duplicate binary nature across all 48 isolates. Nine AFLP markers were significantly (P = 0.05) correlated with the D-type antagonism (Table 4a). The two (independent) markers M25 and M40 exhibit the tightest association with D-type antagonism. Eight markers were significantly correlated with V-type antagonism, with markers M21, M155 and M196 being the most tightly associated (Table 4a). Several of these markers are significantly associated with each other based on Fisher exact probability test suggesting these markers may be genetically linked on the same chromosome (Table 4b). These markers associated with antagonism can be considered for conversion to simple PCR based assays.

DISCUSSION

The exact characterization and identification of strains to the species level is the first step in utilizing the full potential of fungi in specific applications (34). However, the current taxonomy of *Trichoderma* is based almost exclusively on species isolated from North America and Western Europe. It has been estimated that there may be more than 200 *Trichoderma* species (44). Thus, the taxonomy of *Trichoderma* is still likely to evolve for some time to come. Meanwhile, the molecular taxonomic classification by Kullnig-Gradinger and co-workers (31) is widely emerging as the benchmark for *Trichoderma* classification. Thus, in this study we have used sequences from ex-type and other key strains as references for phylogenetic analysis with the aim of contributing to the development of a practical solution for a rapid, accurate and cost effective method of classifying newly collected isolates of *Trichoderma*.

Data from the six morphological characteristics (47) was separated into quantitative and qualitative parameters and subjected to UPGMA diversity analysis. The resultant plot failed to differentiate 19 of the 48 isolates which fell into identical clusters of 2, 3 or 4 isolates, while a further 6 clusters were only differentiated at the 99% similarity level. Thus, it is clear that using a sub-set of morphological

characters cannot provide a rapid and accurate diagnostic. However, the evaluation of 40 or more morphological traits (21, 34) presents a major bottleneck for the rapid identification of useful isolates (33).

Sequence-based diagnostic analysis has been useful in establishing coarse-scale phylogeny of Ascomycetes and defining family-level groups (4). In addition, it has been shown that single gene trees are highly correlated with multiple gene trees at the clade level (31). Thus, the commercially available kit MicroSeq has proven successful in the identification of isolates used in this study. Moreover, through using ex-type strain sequences from GenBank as references, it has been possible to classify the 48 isolates used in this study into four phylogenetic clades. This approach appears to be considerably more reliable than the use of morphological characters, which appears to be prone to misidentification (30, 33). In many cases, SNPs unique to a given clade have been validated amongst multiple isolates in this study (clades 1 and 4). These are now good candidates for conversion into multiplexed PCR-based SNP assays for routine rapid and low cost typing. Other potential clade-specific SNPs identified in this study still require validation amongst a larger selection of isolates (clades 2 and 3).

It is widely reported that *T. harzianum* - *T. inhamatum* is the most abundant taxon in virtually all habitats (33). However, of the isolates investigated in this phylogenetic study pre-selected on the basis of antagonism to *Aspergillus*, we found that the predominant taxon was *T. pubescens* - *T. hamatum* (52% of isolates) followed by *T. harzianum* - *T. inhamatum* (32%). Abundance of *A. flavus* antagonistic *Trichoderma* species in groundnut fields could provide an effective biocontrol strategy for reducing the *A. flavus* population and consequently the aflatoxin contamination of groundnut kernels. Further studies would be needed to understand the interaction between specific *Trichoderma* species and *A. flavus* populations in groundnut fields. The global *Trichoderma* biodiversity initiative has reported the identification of a high proportion of new species from collections in Asia and Europe (9, 31, 33,). However, all the isolates studied here cluster with an already well-defined species. Since we have only studied the 48 most antagonistic isolates from the collection of 212 isolates, this may suggest that the best *Trichoderma* biocontrol agents against *A. flavus* are to be found

in already defined species: predominantly *T. harzianum* – *T. inhamatum* and *T. pubescens* - *T. hamatum*.

In this study we have been unable to differentiate the *T. harzianum* - *T inhamatum* and *T pubescens* - *T. hamatum* clades based on 28S-rDNA (31) *T harzianum* and *T inhamatum* have been consistently troublesome to differentiate based on sequence based molecular phylogeny, physiological or morphological characters (14, 24, 33, 45) In contrast, *T pubescens* - *T hamatum* has been differentiated using ITS1 and 2 in other studies. However, AFLP analysis has differentiated virtually all isolates tested in this study (Figure 3a) in contrast to all previous used single assays (31, 34) One of the main advantages of AFLP analysis is that it is able to simultaneously survey many more points on the genome than any other molecular assay. Unfortunately, this very high level of detectable genetic polymorphism across the genome (250 polymorphic bands in this study from just 6 primer combinations) generated highly complex diversity patterns However, AFLP analysis of individual sequence-based clades generates clear sub-groups within *T harzianum* - *T inhamatum* and *T pubescens* - *T. hamatum* (Figure 6a and 6b), expected to relate to the two different species in each pair.

We have investigated the possibility of using *Trichoderma* spp derived from soils in major groundnut production areas in Southern India, as BCA for the control of toxigenic *Aspergillus* spp This endeavor would be significantly enhanced by the development of rapid and precise diagnostics for *Trichoderma* species and diversity groups with high levels of antagonistic behavior against *Aspergillus* species. To this end we have shown that candidate AFLP bands can be identified for conversion to simple PCR based markers which may differentiate between *T harzianum* - *T. inhamatum* and *T. pubescens* - *T. hamatum* the two major groups identified in this study On the other hand Kubicek et al. found species-specific metabolic properties associated with the different *Trichoderma* spp., but they were also not able to differentiate all species (33) Thus, the current study suggests that AFLP may prove to be an appropriate tool for *Trichoderma* species diagnostics Similarly a set of markers has also been associated with the two types of antagonistic behaviour (V and D type) through regression analysis. None of the markers identified were in common between the two antagonistic characteristics assessed.

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TABLE 1. *Trichoderma* isolates used for molecular analysis

Isolate code	*Trichoderma* species [1]	District	*Trichoderma* species
T1	Unknown	Chittoor	*T pubescens*
T2	*T harzianum*	Chittoor	*T pubescens*
T6	*T longibrachiatum*	Chittoor	*T harzianum*
T11	*T harzianum*	Anantapur	*T pubescens*
T12	*T koningii*	Chittoor	*T pubescens*
T 3	*T koningii*	Chittoor	*T pubescens*
T14	Unknown	Chittoor	*T pubescens*
T16	*T viride*	Chittoor	*T harzianum*
T20	*T harzianum*	Chittoor	*T pubescens*
T21	*T koningii*	Chittoor	*T pubescens*
T24	*T viride*	Chittoor	*T pubescens*
T29	*T pseudokoningii*	Chittoor	*T harzianum*
T33	*T koningii*	Chittoor	No data
T34	*T longibrachiatum*	Tumkur	*T harzianum*
T35	Unknown	Chittoor	No data
T37	*T pseudokoningii*	Kolar	No data
T42	*T harzianum*	Chittoor	*T pubescens*
T46	Unknown	Chittoor	No data
T47	*T hamatum*	Tumkur	*T atroviride*
T49	*T koningii*	Chittoor	No data
T50	*T viride*	Chittoor	*T pubescens*
T51	*T viride*	Tumkur	*T pubescens*
T53	*T harzianum*	Tumkur	No data
T56	*T longibrachiatum*	Tumkur	*T pubescens*
T58	*T harzianum*	Chittoor	*T pubescens*
T60	*T viride*	Tumkur	No data
T62	*T viride*	Chittoor	*T atroviride*
T70	*T koningii*	Chittoor	*T harzianum*
T72	*T harzianum* [2]	Anantapur	*T harzianum*
T73	Unknown	Tumkur	No data
T74	Unknown	Tumkur	*T atroviride*
T83	*T koningii*	Anantapur	*T pubescens*
T86	Unknown	Tumkur	*T koningii*
T10	*T harzianum*	Chittoor	*T longibrachiatum*
T102	*T longibrachiatum*	Chittoor	*T harzianum*
T109	*T harzianum*	Chittoor	*T pubescens*
T110	*T longibrachiatum*	Chittoor	*T harzianum*
T117	Unknown	Tumkur	*T pubescens*
T129	*T harzianum*	Tumkur	*T longibrachiatum*
T134	Unknown	Anantapu	No data
T142	Unknown	Chittoor	*T harzianum*
T143	*T koningii*	Chittoor	*T harzianum*
T161	*T koningii*	Tumkur	*T harzianum*
T170	*T harzianum*	Chittoor	*T harzianum*
T179	*T viride*	Chittoor	*T pubescens*
T188	*T viride*	Tumkur	*T pubescens*
T205	*T viride*	Kolar	*T pubescens*
T206	*T pseudokoningii*	Anantapur	No data

[1] Species designation given to [illegible] according to morphological descriptors

[2] Species designation based on sequence analysis

TABLE 2. *Trichoderma* spp representatives used in the phylogeny study including collection number and gene sequence accession numbers ATCC, American Type Culture Collection, Manassas, CBS, Centraalbureau voor Schimmelcultures, Utrecht, DAMO, Department of Agriculture (Mycology), Ottawa, Canada

Species Name	Collection and number	Geographical origin and habitat	GenBank accession number for 28S rDNA sequence
T pubescens (ex-type strain)	DAOM 166162	USA Soil	AF 199248
T hamatum (ex type strain)	DAOM 167057	Canada Soil	AF 199245
T koningii	CBS 979.70	Netherlands Tree	AF 199239
T viride	ATCC 28020	USA Soil	AF 127150
T atroviride	DAOM 165779	USA	AF 199227
T longibrachiatum (ex type strain)	CBS 816.68*	USA	AF 199240
T inhamatum (ex type strain)	CBS 273.78*	Colombia Soil	AF 199237
T harzianum (ex neotype strain)	CBS 226.95	UK	AF 199230

* Reference strains also used in the MicroSeq fungal database v.0050c for fungal identification (http://www.appliedbiosystems.com/techsupp/sw/pps_MSsw.html)

TABLE 3. AFLP markers that differentiate between the group 1 (9 isolates) and group 2 (11 isolates) within clade *T.harzianum* - *T.inhamatum* and similarly group 1 (8 isolates) and group 2 (4 isolates) within clade*T.pubescens* - *T.hamatum* by 100%

T.harzianum - *T.inhamatum* n=20			*T.pubescens* - *T.hamatum* n=12		
Marker	**# pairs**	**D%**	**Marker**	**# pairs**	**D%**
47	32	100.0	74	99	100.0
48	32	100.0			
49	32	100.0			
80	32	100.0			
85	32	100.0			
131	32	100.0			
132	32	100.0			
134	32	100.0			

#pairs = #(0,1)+#(1,0); D=[(#(0,1)+#(1,0))/Total #pairs]*100;

TABLE 4A. AFLP markers significantly associated with antagonism

D-Type	Marker	Intercept	Slope	T-Prob	Adj R2	Duplicate markers	Linked Markers
(n=14)	M199	2.02	1.73	0.049	22.6		
	M44	1.69	1.61	0.008	41.7		M40
	M105	1.78	1.36	0.032	27.5		
	M152	2.79	-1.22	0.050	22.3		M165
	M165	2.93	-1.33	0.028	28.7		M152
	M70	1.87	1.38	0.042	24.3		
	M40	3.36	-1.70	0.004*	47.7	M123	M44
	M25	4.50	-2.61	0.001*	61.7		M27
	M27	3.60	-1.70	0.019	32.6		M25

V-Type	Marker	Intercept	Slope	T-Prob	Adj R2	Duplicate markers	Linked Markers
(n=21)	M21	3.14	1.20	0.026*	20.4		M35, M155, M196
	M155	3.14	1.19	0.022*	20.7	M191	M21, M35, M196
	M196	3.21	1.13	0.026*	20.6		M21, M35, M155
	M187	3.14	0.80	0.045	16.0		
	M35	3.05	0.78	0.048	14.8		M21, M155, M196
	M190	3.67	-0.73	0.051	15.0		
	M239	3.61	-0.72	0.052	14.9		
	M112	3.77	-0.62	0.045	16.2		

Duplicate markers: Markers that have exactly the same 0-1 data pattern across isolates
Linked Markers as inferred from Fisher Exact Probability Test (Table 4b)
* Markers with most significantly association with D and V type antagonism

TABLE 4B. Association between AFLP markers based on Fishers exact test

Type D (n=14)

	M199	M44	M105	M152	M165	M70	M40	M25	M27
M199	*								
M44	0.110	*							
M105	0.604	0.203	*						
M152	0.692	0.657	0.238	*					
M165	0.769	0.500	0.133	***0.002***	*				
M70	0.066	0.095	0.095	0.594	0.720	*			
M40	0.110	***0.023***	0.203	0.238	0.133	0.095	*		
M25	0.275	0.110	0.110	0.308	0.231	0.066	0.110	*	
M27	0.396	0.275	0.275	0.154	0.500	0.176	0.275	***0.033***	*

Type V (n=21)

	M21	M187	M35	M190	M239	M112	M155	M196
M21	*							
M187	0.222	*						
M35	***0.031***	0.664	*					
M190	0.624	0.455	0.392	*				
M239	0.211	0.556	0.314	0.255	*			
M112	0.458	0.259	0.102	0.430	0.414	*		
M155	***0.001***	0.202	***0.026***	0.656	0.237	0.421	*	
M196	***0.001***	0.202	***0.031***	0.656	0.263	0.458	***0.001***	*

Bold italics indicate markers significantly associated to each other

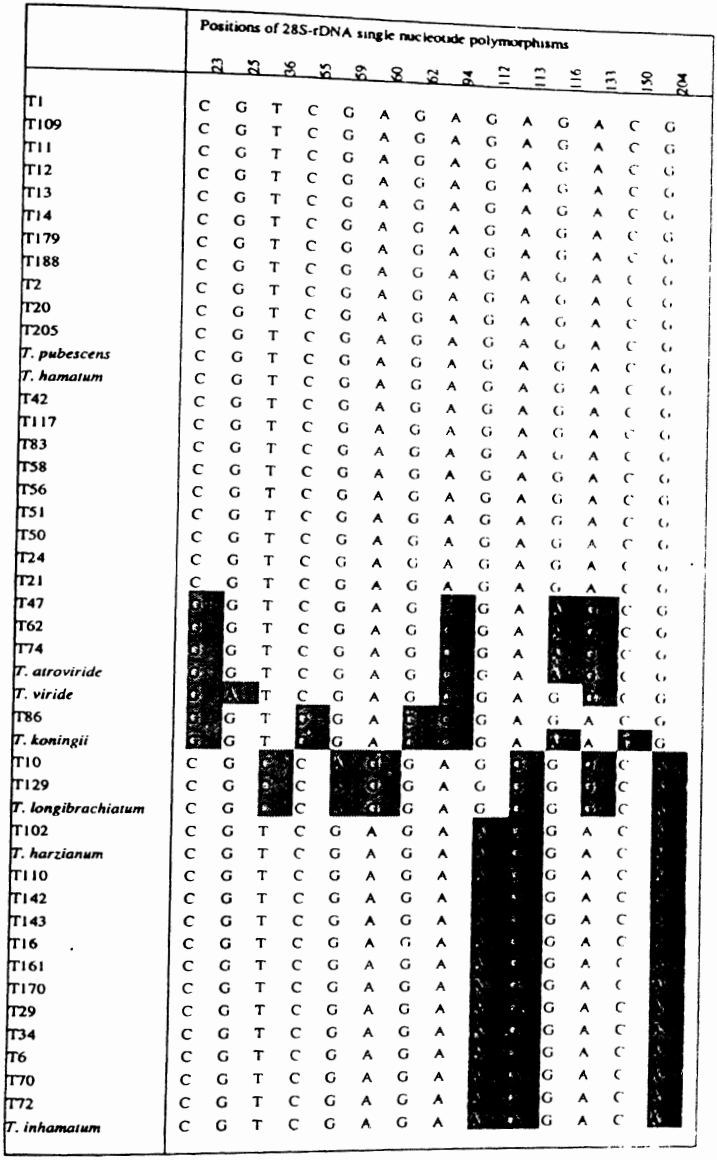

FIGURE 1. Comparative analysis of 28S-rDNA (D2) sequences from 38 *Trichoderma* isolates (identified by collect "T" numbers) and 8 reference isolates (identified by specie name) showing single nucleotide polymorphisms (shaded).

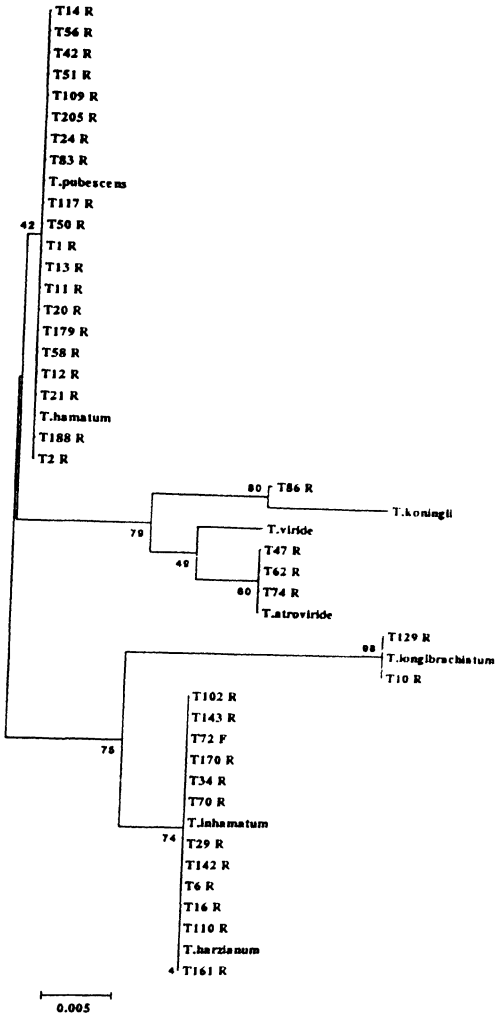

FIGURE 2. Phylogenetic tree of 38 isolates inferred by neighbour-joining analysis of 28S-rDNA sequences. The numbers given over branches indicate the percentage of 1000 bootstrap resampled datasets supporting the clades. Clustering is based on the Kimura two-parameter.

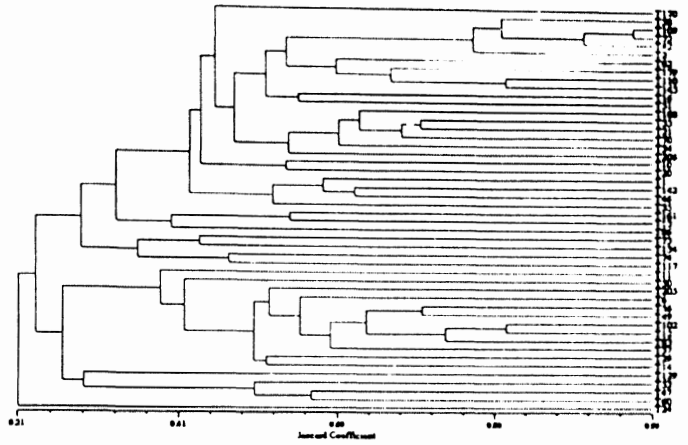

FIGURE 4A. UPGMA dendrogram of 48 *Trichoderma* isolates based on AFLP dataset using Jaccard distance.

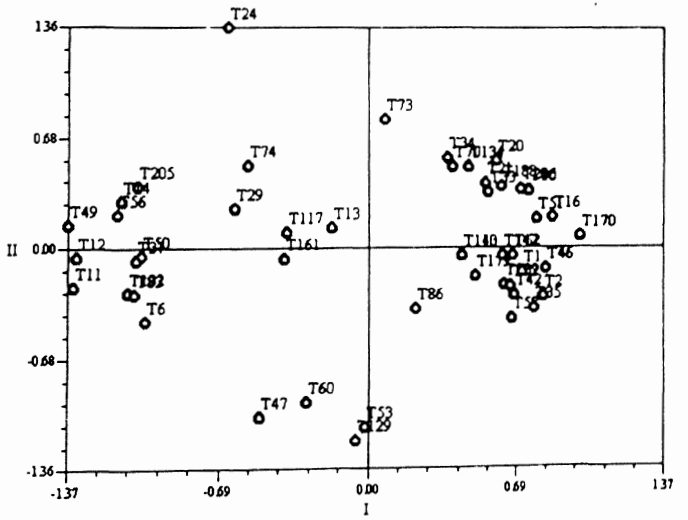

FIGURE 4B. Multi-Dimensional Scale (MDS) plot of 48 *Trichoderma* isolates based on AFLP dataset.

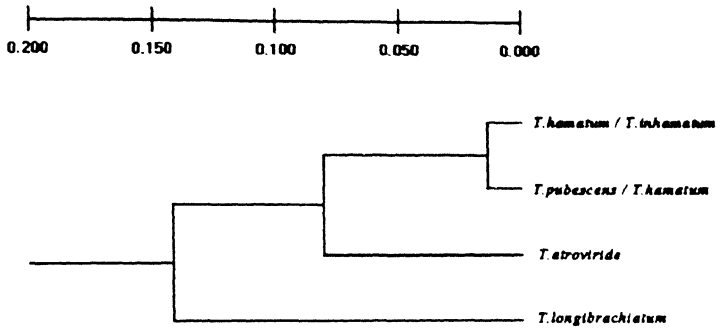

FIGURE 5. UPGMA dendrogram of the AFLP dataset using Nei's genetic distance, with subpopulations defined on the basis of 28S-rDNA phylogenetic sequence analysis.

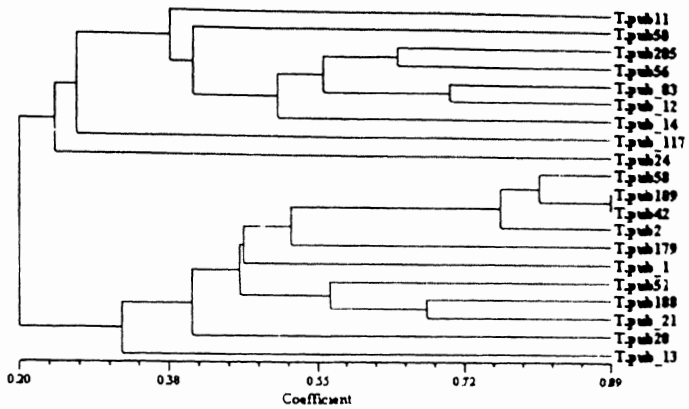

FIGURE 6a. UPGMA dendrogram based on AFLP data from isolates belonging to *T. pubescens - T. hamatum*

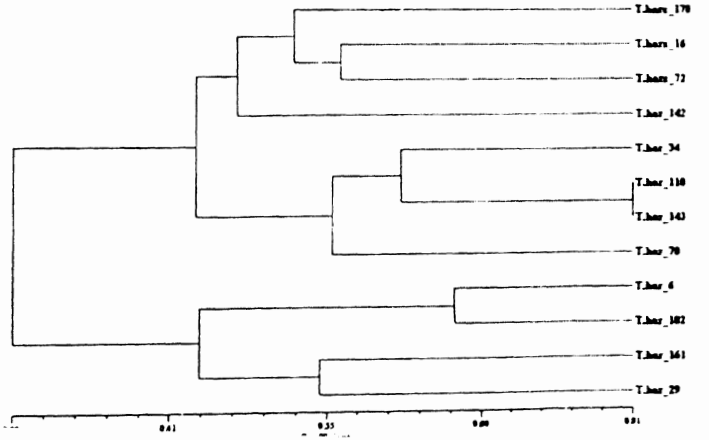

FIGURE 6b. UPGMA dendrogram based on AFLP data from isolates belonging to *T. harzianum - T. inhamatum.*

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