Cultural, Morphological, Molecular and Pathogenic Characterization of *Trichoderma* isolates from *Tarai* area of Uttaranchal

Thesis

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By

Deeksha Joshi
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(Deeksha Joshi)

Pantnagar

July 2005

Dr.U.S.Singh
Professor

Department of Plant Pathology
College of Agriculture
G.B. Pant University of
Agriculture and Technology,
Pantnagar- 263145,
Uttaranchal, INDIA

CERTIFICATE

This is to certify that the thesis entitled "Cultural, Morphological, Molecular and Pathogenic Characterization of *Trichoderma* isolates from *Tarai* area of Uttaranchal" submitted in partial fulfillment of the requirements for the degree of **Doctorate of Philosophy** with major in **Plant Pathology** and minor in **Entomology**, of the college of Post-Graduate Studies, G.B. Pant University of Agriculture and Technology, Pantnagar, is a record of bonafide research carried out by **Ms. Deeksha Joshi, Id. No. 24834**, under my supervision and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation and source of literature have been duly acknowledged.

(U.S.Singh)
Chairman, Advisory Committee

Pantnagar
July, 2005

Certificate

We, the undersigned, members of the Advisory Committee of **Ms. Deeksha Joshi, Id. No. 24834**, a candidate for the degree of **Doctor of Philosophy** with major in **Plant Pathology** and minor in **Entomology**, agree that the thesis entitled "**Cultural, Morphological, Molecular and Pathogenic Characterization of *Trichoderma* isolates from *Tarai* area of Uttaranchal**" may be submitted in partial fulfillment of the requirements for the degree.

(U.S. SINGH)
Chairman
Advisory Committee

(G.K. GARG)
Member

(V.S. PUNDHIR)
Member

(S.N. TIWARI)
Member

(R. P. SINGH)
Head & Ex-Officio Member

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INTRODUCTION

Chapter 1

INTRODUCTION

Trichoderma species are widely distributed all over the world and occur in all types of soil and other natural habitats especially those containing high organic matter. This fungus is a secondary colonizer and is frequently isolated from well decomposed organic matter (Papavizas, 1985). They are classified mostly as imperfect fungi. Rapid growth rate in culture and production of numerous spores that are varying shades of green characterize this fungus.

Amongst the various species of *Trichoderma*, *T. viride, T. harzianum* and *T. virens* (previously *Gliocladium virens*) are the most commonly cited ones for biological control. These species alone or in combination with other *Trichoderma* species or chemical fungicides have been used for the control of several diseases like root rots, wilts, damping off, white rot, collar rots etc. in a wide variety of crops (Samuels, 1996). Although *Trichoderma* spp. have been used very widely for the management of soil-borne, seed-borne and foliar diseases, inconsistency in field performance is one of the major problem associated with biocontrol. There could be several factors responsible for inconsistent field performance like inoculum density of pathogen; soil moisture, pH and temperature; rhizosphere competence, soil organic content, atmospheric humidity etc. One factor which has been ignored so far is host and organ specificity of different species and strains of *Trichoderma*. A wide array of mechanisms, employed by *Trichoderma,* contribute towards its biocontrol efficiency. *Trichoderma* spp. are known to exhibit mycoparasitism, antibiosis, enzyme secretion, competition and induction of systemic resistance in plants as a means to inhibit the growth and multiplication of its target fungi. Apart from this, *Trichoderma* spp. indirectly affect pathogen-host plant interaction by solubilizing certain minerals and by promoting plant growth (Howell, 2003). Preliminary work has also shown that different *Trichoderma* spp. exhibit some level of host specificity/selectivity for various pathogens and may rely on different mechanisms for the control of different pathogens. The control of *Rhizoctonia solani* and *Pythium ultimum* by *T. harzianum* is reported to be by direct penetration of host hyphae (Dennis & Webster, 1971b). On the other hand mycoparasitism is not the primary mechanism of biocontrol of *R. solani* and *P. ultimum* by *T. virens* (Howell, 1987).

So far *in vitro* screening for efficient strains of *Trichoderma* is based on its ability to antagonize mycelial growth of fungal pathogens. Many soil-borne fungal pathogens form hard survival structures, sclerotia, which play vital role in its survival and disease cycle. In fungal pathogens like *Sclerotinia* they are sexual structures. So far no information is available on ability of different strains of *Trichoderma* to colonize these hard structures.

Trichoderma spp. are basically soil inhabiting fungi and their growth, multiplication, survival and eventually their biocontrol potential is highly influenced by various soil physical, chemical and biological properties. They are reported to be affected by factors like soil pH, soil temperature and moisture level, water potential and most importantly by the activities of the native antagonistic microflora which is present in the soil where they are applied as biocontrol agents (Papavizas, 1985; Bull, 2002). Therefore, it becomes important that the selected strain should have the ability to compete with the native microflora, establish itself successfully in the crop rhizhoshere/ spermosphere and should have a wide array of mechanisms to inhibit several pathogens. However, the results of many years of research with *Trichoderma* species as biocontrol agents has shown that not all the mechanisms and characteristics needed for an effective biocontrol agent are found in the same organism. Given these considerations, it is expected that the best method for obtaining a potential biocontrol agent might be to isolate *Trichoderma* strains originally from those areas where they are actually expected to function later as a biocontrol agent and where they are already growing under conditions of temperature, moisture etc. similar to those found in nature (Howell, 2003). Soils have tremendous biodiversity and they are home to innumerable microorganisms including several plant pathogens. At any given time the soil may harbour several pathogens causing various diseases at different stages in a number of crop plants. A detailed study of the host selectivity of *Trichoderma* species and the mechanisms employed in these interactions is very important as it may help in the development of effective biopesticides with wide scale application.

At present *Trichoderma* is a readily available source for managing plant diseases but to exploit it for economic gains it is important to understand the biology and systematics, complex mechanisms and interactions of this fungus in relation to other fungi (Samuels, 1996). These aspects emphasize the necessity to explore the indigenous biodiversity of an area and for an in depth study into the various factors which ultimately determine the performance of *Trichoderma* as a biocontrol agent with an aim to come up with effective strains for future use.

Keeping the above factors in view a study was undertaken on 43 *Trichoderma* isolates, isolated from the rhizosphere, with the major objective of defining the biodiversity in this biocontrol agent. Major activities of the study are as follows:

1. Cultural, morphological and molecular characterization of *Trichoderma* isolates collected from various areas in *Tarai* region.
2. Evaluation of the isolates for their antagonistic potential against both hyphae and sclerotia using four major soil borne plant pathogens.
3. Characterization of the isolates with respect to their host selectivity for major pathogens.
4. Development of data base for different isolates.

REVIEW OF LITERATURE

Chapter 2

REVIEW OF LITERATURE

Biological control is a principle of cultural control of plant pathogens that primarily involves alteration of biotic and abiotic environments from one that favours disease/pathogen to one that discourages accumulation of infective or parasitic material and reduces the activity of the pathogen. Broadly speaking, biological control could be defined as- "*any condition under which, or practice whereby, survival and activity of a pathogen is reduced through the agency of any other living organism (except man himself) with the result that there is a reduction in the incidence of disease caused by the pathogen*" (Garret, 1965). Biological control was defined by Baker and Cook (1974) as *the "reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonists, or by mass introduction of one or more antagonists".* Subsequently, they (Cook and Baker, 1983) revised the definition to *'biological control is the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by one or more organisms other than man'.*

Microorganisms as such play an enormously important role in biological control of plant diseases. As naturally occurring resident antagonists, they can be managed or exploited to achieve the desired results (Mathre *et al.*, 1999).

The three most researched and frequently cited microbes for biological control of diseases are *Trichoderma* spp. *Bacillus* spp. and *Pseudomonas* spp. *Trichoderma* has emerged as an extensively studied fungal genera for disease biocontrol and several commercial products based on this fungus have been developed.

2.1 *Trichoderma:* GENERAL CHARACTERISTICS

More than two hundred years ago, when it was first described by Persoon in 1794, mycologists mistook *Trichoderma* Pers.: Fr. for a gasteromycete. The true nature of this fungus was realized only half a century later. Although, *Trichoderma* has not yet given us any "wonder drugs" like penicillin, but the ability of some species to produce enzymes and/or attack or inhibit other fungi causing diseases in plants has attracted major research efforts in several areas specially biological control of plant diseases. At present *Trichoderma* is a readily exploitable source and how this source can be fully utilized for economic gains depends on a clear understanding of the biology, systematics and biocontrol mechanisms of this genus. (Samuels, 1996)

2.1.1 *Trichoderma*: ECOLOGY

Trichoderma species are widely distributed all over the world and occur in all types of soil and other natural habitats especially those containing high organic matter. This fungus is a secondary colonizer and is frequently isolated from well decomposed organic matter. *Trichoderma* species have also been isolated from root surfaces of various plants, from decaying barks and from sclerotia and propagules of other fungi. In general the members of *Trichoderma* exhibit a preference for wet soils. However, there are reports that individual species of *Trichoderma* exhibit different preferences for soil temperature and soil moisture. While species like *T. viride* and *T. polysporum* are generally found in areas with low temperature, *T. harzianum* most commonly occurs in warm climatic regions. *T. hamatum* and *T. koningii* have been reported to occur in diverse climatic conditions. *Trichoderma* spp. may also be sensitive to environmental pollution as indicated by low rate of recovery of *T. viride* from coniferous forests that had been subjected to alkaline dust for a period of 25 years. The presence of carbon dioxide has been reported to favour growth of *Trichoderma*. Apart from these factors, the iron content of the soil, HCO_3^-, salt and organic matter content and presence and absence of other microbes in soil are also important determinants of microsite preference by *Trichoderma* spp. (Papavizas, 1985). *Trichoderma* species have the ability to utilize a wide range of compounds as sole carbon and nitrogen sources and can utilize monosaccharides, dicsaccharides, polysaccharides etc for carbon with ammonia being the most preferred source of nitrogen. The members of *Trichoderma* are generally considered to be aggressive competitors although this trait has also been found to be species dependent.

2.1.1.1 Factors affecting biocontrol potential of *Trichoderma*

Trichoderma being a soil fungus, its growth, multiplication and eventually its biocontrol potential is highly affected by various soil physical, chemical and biological properties. Weindling (1934) noted that pH affected the relative value of *Trichoderma* as an effective antagonist to *R. solani* by affecting the growth and development of each fungus. Backman *et al.* (1975) showed that when fungicides that were toxic to *Trichoderma* were applied for peanut spot control, there was an increase in disease caused by *Sclerotium rolfsii*, a pathogen susceptible to biocontrol by *Trichoderma*. Water potential has also been shown to influence the growth rate, enzyme secretion and *in vitro* enzyme activities of *T. harzianum* (Kredics *et al.,* 2000). Various studies have indicated that soil antagonists to *Trichoderma* are a major factor regulating the potential inoculum level of *Trichoderma* in soil (Papavizas, 1985; Bull, 2002; Green, 1999). This soil antagonism plays a direct role in reducing the efficacy of *Trichoderma* as a biocontrol agent. This was further confirmed in studies done by Naar and Kecskes (1998). They conducted experiments to study the factors influencing the competitive saprophytic ability (CSA) of *Trichoderma* species and compared the growth rate of *Trichoderma* at different temperatures, its cellulolytic activity and sensitivity to *in vitro* inhibitory effect of various test bacteria and fungi. Their studies indicated that tolerance to antagonistic effect of bacteria appears to be the most important factor contributing in CSA of *Trichoderma*.

2.1.2 *Trichoderma:* BIOLOGY

Most species of *Trichoderma* are photosensitive, sporulating readily on many natural and artificial substrates in a concentric pattern in response to diurnal alternation of light and darkness with conidia being produced during the light period. *Trichoderma* cultures exhibit “replacement wall building type” of conidiogenesis. Another interesting property of *Trichoderma* spp. is their ability to produce chlamydospores. These resting structures also have the potential to be exploited for biological control (Papavizas, 1985).

However, the aspect of *Trichoderma* most interesting and fruitful for researchers in the field of agriculture is its physiological activities. This includes the production of cellulase enzymes by *Trichoderma reesei* which cause breakdown of cellulosic materials and bleaching of pulp. This property has been commercially exploited by sugar industry to dispose off sugar by products. Another most important and highly researched property of *Trichoderma* spp. is their potential as biocontrol agents. *Trichoderma harzianum* and *Trichoderma virens* are the most commonly used species for biological control, both alone and in combination with other microbes or chemical adjuvants (Samuels, 1996). Till date *Trichoderma* species are reported to effectively control several plant pathogens like *Rhizoctonia solani, Sclerotium rolfsii, Sclerotinia, Fusarium* spp., *Pythium* etc. which cause several economically important diseases like root and seedling rots, damping off, wilts and blights.

Trichoderma species secrete a number of enzymes, which are reported to play a major role in inhibiting plant pathogenic fungi. They are generally not known to affect human health and are not involved in plant parasitism or in post harvest losses. However, due to their mycoparasitic properties, *T. harzianum* causes serious losses in commercial mushroom production.

Most *Trichoderma* strains are not encountered in nature in association with their perfect stages and are considered to be strictly mitotic, clonal fungi. This apparent lack of sexuality is also a barrier to understanding relationships within and among *Trichoderma* species. Sexual reproduction is known in *Trichoderma* in the sense that the only known teleomorphs of *Trichoderma* are species of *Hypocrea* Fr. and closely related genera (Samuels, 1996).

2.1.3 *Trichoderma* : SYSTEMATICS

About 35 species of *Trichoderma* are currently recognized on the basis of morphological and molecular data.

Trichoderma is a genus of filamentous deuteromycetes with only one known teleomorph, *Hypocrea* Fr. The basic *Trichoderma* morphology has been described as: Colonies having rapid growth, abundant powdery green conidia and ill defined conidiophores. The characteristics of colonies growing on agar media are subtle and it produces submerged mycelium. Reverse of the colonies may be uncoloured or in some species buff, yellow, amber, reddish or yellow green. Conidiation is effuse and chlamydospores are usually present. Conidiophores in most species are repeatedly branched in a regular dendritic manner. However, inspite of the tremendous economic importance of this fungus, the taxonomy of *Trichoderma* is still problematic with no well-defined concept of species. Over the years, speciation concept within the genus *Trichoderma* has altered a lot as a wide range of biochemical and molecular data have come to light.

Rifai (1969) made the first attempt at classification of *Trichoderma* species. Based on his studies, he released a comprehensive taxonomic monograph for this genus. The classification of *Trichoderma* given by Rifai relies primarily on morphological characters. For Rifai, the key to the species of *Trichoderma* lay in the link between *Trichoderma* and the sexually producing genus *Hypocrea* Fr. and many of his aggregate species were based on cultures from ascospores. He classified the different species of *Trichoderma* in 9 species aggregates, which he defined as "aggregations of morphologically very similar and often hardly separable species".

The 9 species aggregates defined by Rifai are:

1. *Trichoderma piluliferum* Webster and Rifai
2. *Trichoderma polysporum* (Link ex Pers.) Rifai
3. *Trichoderma hamatum* (Bon.) Bain.
4. *Trichoderma koningii* Oud.
5. *Trichoderma aureoviride* Rifai
6. *Trichoderma harzianum* Rifai
7. *Trichoderma longibrachiatum* Rifai
8. *Trichoderma pseudokoningii* Rifai
9. *Trichoderma viride* Fers. ex S.F.Gray

The second comprehensive classification of *Trichoderma* species was given by Bisset (1984, 1991a-c, 1992). He too, like Rifai, based his classification on morphological characteristics of *Trichoderma*. He essentially elevated each of Rifai's aggregate species to sectional level and proposed one new section for *Acremonium*-like or *Verticillium*-like anamorphs of *Hypocrea* species. This classification given by Bisset accommodates the full range of morphological variation observed in *Trichoderma* species. Bisset divided the genus *Trichoderma* into five sections:

- Section I- *Trichoderma*

  Type species – *Trichoderma viride, Trichoderma koningii, Trichoderma aureoviride, Trichoderma atroviride.*

  Species in this section have narrow and flexuous conidiophores with branches and phialides uncrowded, frequently paired and seldom in verticils of more than three.

- Section II – *Pachybasium*

  Type species – *Trichoderma harzianum, Trichoderma virens, Trichoderma hamatum, Trichoderma piluliferum, Trichoderma polysporum.*

  Species have highly ramified, broad conidiophores usually arranged in compact pustules or fascicles. Branches and phialides are broad or inflated, relatively short and disposed in crowded verticils. Some species are further characterized by the production of sterile conidiophore extensions and many isolates produce compact conidiogenous pustules with adjacent conidiophores anastomosing.

- Section III – *Longibrachiatum*

  Type species – *Trichoderma longibrachiatum, Trichoderma pseudokoningii*

Member species have sparingly and irregularly branched conidiophores with the phialides irregularly disposed and not usually in whorls or verticils. The species often produce distinctive greenish yellow pigments in the reverse of the cultures.

- Section IV – *Saturnisporum*

  Type species – *Trichoderma saturnisporum, Trichoderma ghanense.*

  The species have a *T.viride* type of branching system with branches and phialides uncrowded and frequently paired, but with inflated phialides and compact conidiogenous pustules as in section *Pachybasium*. They are further differentiated by the bullate or wing like conidial ornamentation.

- Section V – *Hypocreanum*

  This section accomodates the *Hypocrea* anamorphs mostly from section *Hypocrea*. Members are characterized by effuse, usually sparse conidiation, sparingly branched conidiophores and cylindrical to subulate phialides frequently borne in *Verticillium* like divergent verticils.

T. harzianum, T. viride and *T. virens* are the most cited species of *Trichoderma* for biological control of plant diseases. A detailed description of these three species as given by Bisset follows:

Trichoderma harzianum Rifai 1969 (Teleomorph: *Hypocrea albofulva* Berk. and Br. 1873)

Colonies growing rapidly with most isolates 7-9 cm in diameter after 3 days. Aerial mycelium floccose, white to greyish or rarely yellowish. Conidiation predominantly effuse on Potato Dextrose Agar frequently covering the entire surface of the plates, sometimes loosely arranged in flat pustules. Reverse colourless to dull yellowish, buff, pale drab shades or darker. Odour is indistinct or faintly earthy. Hyphae hyaline, smooth walled mostly 2-6 μm in diameter or submerged mycelium occasionally up to 12 μm in diameter. Chlamydospores are fairly abundant and are intercalary or terminal. Conidiophores are hyaline, smooth walled up to 8 μm wide near base and tapering up to 2.5 to 4.5 μm wide for most length. Conidiophores are highly branched with primary branches arising at nearly right angles usually in whorls of 2 or 3 and have a fertile apex. Phialides ampulliform or subglobose or lageniform. Terminal phialides arising in whorls of 2-6. Conidia subglobose to ovoid, apex broadly rounded, base more narrowly rounded and surface is smooth walled.

***Trichoderma virens* (previously *Gliocladium virens*) Miller, Giddens & Foster**

Gliocladium Corda is a genus of hyphomycetes that includes two important fungi used commonly for biological control of plant diseases. These are *G.roseum* Bainier and *G. virens* J.H.Miller, Giddens and Foster. The type species of *Gliocladium* is *G. penicilloides*. A review of literature reveals that there has been confusion surrounding the identification of this taxon. *G. virens* first received attention for its ability to produce antifungal toxins gliotoxin

(Weindling, 1937) and viridin (Brian and McGowan, 1945). von Arx in 1987 transferred *G.virens* to *Trichoderma* without any justification. Bisset followed the same placement in his monograph on *Trichoderma*. This genus is characterized by strictly penicillate conidiophores with branching restricted to the apex. Phialides are narrow and more closely appressed than *Trichoderma*. The conidia are held in drops of liquid on a penicillus of phialides that form at the tips of branches of more or less discrete phialides. These characteristics are similar to the genus *Gliocladium*. However, characters like presence of green conidia, compact phialides, essentially indeterminate conidiophores that branch at right angles and joined in hemispherical tufts, production of chlamydospores in culture and a rapid growth rate are indicative of *Trichoderma*. Therefore, taxonomic conclusions based upon morphological characters alone were not able to allocate *G.virens* to either genus *Trichoderma* or *Gliocladium*.

Trichoderma viride Pers.ex Gray 1821 (= *T. lignorum* Tode ex Harz, 1871) (Teleomorph: *Hypocrea rufa* Fr.)

Conidiophores and their side branches long and slender, without sterile hyphal elongation, phialides not crowded, rather slender, colonies yellowish, bright, dull to dark green, floccose or with compact conidiophore tufts. Conidia roughened, 3.6 to 4.8 x 3.5 to 4.5 μm.

2.1.3.1 Molecular approaches for resolving *Trichoderma* taxonomy

The first efforts at molecular characterization of *Trichoderma* strains and species were undertaken not by professional taxonomists but by frustrated users of the existing taxonomy. Stasz *et al.* (1988) remarked that "...methods are lacking to differentiate among strains for patent purposes or to determine the variability and abundance of strains in natural ecosystems". Taxonomy based on morphological characters alone has not been able to come up with a useful classification for users. Characters derived from nucleic acids and enzymes are becoming more and more attractive for users because they offer more objectivity than traditionally observed and analyzed data. Till date, several workers have attempted to characterize *Trichoderma* species using molecular and enzyme analysis both for solving the taxonomic confusions as well as for accurate identification of the commercially important strains. These studies have revealed that atleast some of the aggregate species are phylogenetically based while at the same time confirming the genetic diversity of the individual aggregates.

Stasz *et al.* (1989) evaluated five aggregate species of *Trichoderma* using enzyme polymorphisms. While they concluded that morphological species are not characterized by specific alleles at multiple loci, they demonstrated what they called, "core groups" of morphological species. Zamir and Chet (1985) divided twenty-three strains of the *T. harzianum* aggregate among five different types according to their isozyme profiles. Schickler *et al.* in 1998 used electrophoretic characterization of chitinase enzymes as a tool to identify *T. harzianum* strains. They observed that each of the three *T. harzianum* strains exhibited a unique pattern of 3-5 different chitinases and concluded that chitinase profiles can be used for identification of *T. harzianum* strains. Isozyme analysis was used by Samuels *et al.* (1994) to confirm that section *Longibrachiatum* is distinct from other sections. More recently Leuchtmann *et al.* (1996) compared 78 representatives of *Trichoderma* sect. *Longibrachiatum* using ten enzyme systems. Their results also supported Bisset's morphologically based taxonomy of section *Longibrachiatum*. Carter and Lynch (1991) had suggested an assay based on the recognition of *Trichoderma* antigens by specific antisera, but observed that the reactivity of the antisera to *Trichoderma harzianum* depends on the type of medium on which fungus has been cultured.

Meyer and Plaskowitz (1989) observed two types of conidial ornamentation among twelve strains referable to *T. viride*, member of sect. *Trichoderma*. Later Meyer (1991) found that mitochondrial DNA restriction fragment data distinguished between those two groups and he suggested that each group, characterized by conidial ornamentation and mtDNA type, could represent a distinct species.

In 1994, Zimand *et al.* used Random Amplified Polymorphic DNA (RAPD) procedure to identify *Trichoderma* strains. RAPD is a simple technique based on PCR amplification of genomic DNA with single primers of arbitrary nucleotide sequences (Williams *et al.,* 1990). Zimand *et al.* (1994) analyzed 47 *Trichoderma* strains originating from various geographical regions and representing three species; *T. harzianum*, *T. viride* and *T. hamatum*. Fujimori and Okuda (1994) also successfully used RAPD's to identify duplicate

Trichoderma strains in microbial screening. RAPD analysis and PCR assay in combination with dilution plating on a semi selective medium have also been used to detect and enumerate propagules of *Trichoderma hamatum* 382, a biocontrol agent utilized in compost mixes (Abbasi *et al.,* 1999). They reported that distinct and reproducible fingerprints were obtained upon amplification of purified genomic DNA of *T. hamatum* with 3 random primers. These three RAPD amplicons were then cloned and sequenced and sequence-characterized amplified region (SCAR) primers were designed. Hermosa *et al.* (2001) also used RAPD markers to estimate the genetic variation among 16 strains of *Trichoderma*, and later used some of the RAPD markers to further design strain specific SCAR markers.

Analysis of ribosomal DNA is one of the most reliable methods for phylogenetic analysis and has been frequently used for mycological investigations. Muthumeenakshi *et al.* (1994); Ospina-Giraldo *et al.* (1999); Hermosa *et al.* (2000) and Kullnig *et al.* (2000) compared internal transcribed spacer (ITS) regions of ribosomal DNA transcriptional unit of *Trichoderma* in an attempt to distinguish various groups within the aggregates and to distinguish between strains. Kuhls *et al.* (1997) used sequence analysis of ITS1 and ITS-2 region of 103 strains of sect. *Longibrachiatum* to reexamine the taxonomy of this section. Muthumeenakshi *et al.* (1994) found three distinct types of ITS-1, and strains characterized by ITS type 2 were aggressive antagonists in commercial mushroom production. Similarly, Ospina-Giraldo *et al.* (1999) characterized 81 isolates of *Trichoderma* comprising of green mold and biocontrol related isolates. They concluded that although the isolates shared a common ancestor, they constitute different phylogenetic groups. Hermosa *et al.* (2000) used ITS sequencing and polymorphism to cluster 17 strains of *Trichoderma* with known biocontrol properties in four groups; *T. harzianum-hamatum, T. longibrachiatum, T. asperellum* and *T. atroviride-koningii* groups. They too concluded that none of the biocontrol strains studied correspond to biotype Th2 or Th4 of *T. harzianum*, which cause mushroom green mold. Kullnig *et al.* (2000) used ITS sequencing to characterize and group strains from Russia, Siberia and Himalayas. Analysis of 28S rDNA helped to resolve the taxonomic confusion related to *Trichoderma virens* (formerly *Gliocladium virens*). The result of rDNA analysis (28S rDNA) and its internal transcribed spacer (ITS) sequences clearly indicated that *T. virens* is phylogenetically distinct from the type species of *Gliocladium*, *G. penicillioides* and is in close proximity to *T. harzianum* thus supporting the taxonomic usage of von Arx and Bissett (Rehner and Samuels, 1994).

Apart from isozyme analysis and molecular marker based techniques; Lieckfeldt *et al.* (2000) used endochitinase gene based phylogenetic analysis of *Trichoderma*.

In recent years, molecular marker based techniques and enzyme analysis have been applied primarily to solve the taxonomic confusions among the various sections/ aggregates within *Trichoderma* and for the identification of commercially important strains. These techniques have also proved useful in classifying/ grouping *Trichoderma* strains based on their biocontrol properties or their ability to cause disease in mushrooms. However, the confusion regarding the taxonomy of various species amongst the group still remains and more efforts are needed for resolving this problem so as to facilitate researchers working on this important fungus.

2.2 *Trichoderma:* As a biocontrol agent

Weindling in 1932, for the first time implicated the role of *Trichoderma lignorum* in the biological control of citrus seedling disease caused by *Rhizoctonia solani*. Since this pioneer work, several reports on successful biocontrol by *Trichoderma* spp. have accumulated. *T. harzianum*, *T .viride* and *T. virens* (earlier *Gliocladium virens*) are the most widely used / cited for biological control. They are reported effective in controlling root rots /wilt complexes and foliar diseases in several crops and are known to inhibit a number of soil borne plant pathogens like *Rhizoctonia solani, Sclerotinia* spp., *Sclerotium rolfsii, Fusarium* spp. etc. and recently root knot nematodes.

2.2.1 Mechanisms of action

One of the most interesting aspect of studies on *Trichoderma* is the varied mechanisms employed by *Trichoderma* species to effect disease control. Members of *Trichoderma* present us with an array of diverse mechanisms. As an antagonist, *Trichoderma* may directly kill the pathogen by mycoparasitism and/or antibiosis. Also, it may adversely affect the growth and development of the pathogen either by antibiosis or by competing for the nutrient, oxygen or space. Indirectly, it may contribute to disease resistance or tolerance in crop plants by induction of systemic resistance in plants (ISR) or by promoting plant growth which manifests itself as increased root and shoot growth, resistance to biotic and abiotic stresses and changes in the nutritional status of the plant (Howell, 2003).

2.2.1.1 Antifungal action

Against fungal pathogens, *Trichoderma* species rely on three major mechanisms viz. Mycoparasitism/ Hyperparasitism, Antibiosis and Competition.

2.2.1.1.1 Mycoparasitism/ Hyperparasitism

One of the most salient characters of the genus *Trichoderma* is its ability to parasitize other fungi. Weindling in 1932 for the first time ascribed the biocontrol of *R. solani* (causing citrus seedling disease) by *Trichoderma lignorum* to mycoparasitism. Mycoparasitism is a complex process involving tropic growth of the biocontrol agent towards the target organism, coiling and finally dissolution of the target organism's cell wall/cell membrane by the activity of enzymes (Weindling, 1932; Chet, 1987; Sharon *et al.,* 2001). Findings within the last few years indicate that hyperparasitism is one of the main mechanisms involved in the antagonism and biocontrol efficacy of *Trichoderma* spp. (Mukhopadhyay, 1994, 1987; Howell, 1987; Sharon *et al.*, 2001).

Studies on the molecular and cellular aspects of the process of mycoparasitism indicate that it is an extremely complex process involving several steps and numerous separate genes and gene products. *Trichoderma* can detect its host from a distance and on detection it starts branching in an atypical way towards the fungus. This process is probably induced by nutrient gradients arising from the host (Chet *et al.,* 1989). Dennis and Webster, 1971a&b conducted experiments using plastic threads similar in diameter to *Pythium ultimum* hyphae and concluded that the coiling of *Trichoderma* is not merely a contact stimulus. *Trichoderma* hyphae were never observed to coil around plastic threads. Later studies done by Elad *et al.* (1983) and Barak *et al.* (1985) indicated the role of lectins in the process of host recognition by *Trichoderma*. Elad *et al.* (1983) isolated a lectin from *R. solani* hyphae and culture filtrate which, they concluded, binds to the galactose residues in cell walls of *Trichoderma*. Extracts from *S .rolfsii* also exhibited agglutinin activity but its properties were different from that of *R. solani*. Inbar and Chet (1994) provided direct evidence for the role of lectins in mycoparasitism. They observed that *Trichoderma harzianum* coiled around nylon fibers, which had been treated with concanavalin A, a lectin, purified from *S. rolfsii*. Attachment of *Trichoderma* to host hyphae is followed by a series of degenerative events and the host cell wall structure is disrupted which promotes osmotic imbalances triggering cell disruption and killing the pathogen.

2.2.1.1.2 Secretion of Enzymes

Most of the pathogenic fungi contain chitin and β-glucans in their cell walls. Dissolution or damage of these structural polymers has adverse effects on the growth of these fungi. Recent research work has implicated a major role of enzymes in biological control by *Trichoderma* species and the secretion of enzymes is reported to be an integral step of the mycoparasitic process of *Trichoderma*. *Trichoderma* species secrete a number of hydrolytic enzymes, which includes chitinases, proteases, cellulases, glucanases and xylanases. Lorito (1998) listed 10 separate chitinolytic enzymes alone. Similar levels of diversity are reported to exist for β-1,3 glucanases (Benftez *et al.*, 1998). Elad *et al.* (1982) tested the secretion of chitinases and β-1,3 glucanases secreted by *T.harzianum* and observed that the enzymes degraded hyphae of *S. rolfsii*. Harman *et al.* (1989) tested the involvement of "chitinase and β-1,3-glucanase" in *Trichoderma* mediated biological control. Geremia *et al.* (1993) purified and biochemically characterized a serine protease enzyme. Elad and Kapat (1999) have suggested the role of proteases in biocontrol of *B. cineria* by *T. harzianum*. For mycoparasitism of Pythiaceous fungi, β-1,4-glucanases may play an important role (Thrane *et al.*, 1997). Recently, Ait-Lahsen (2001) isolated and characterized an exo-α-1,3- glucanase (AGN 13.1) enzyme from *T. harzianum* that degrade α- glycosidic linkages of polysaccharides of cell wall of fungi.

The various enzymes secreted by *Trichoderma harzianum* are reported to act synergistically *in vitro*. These enzymes not only act synergistically amongst themselves but also with chitinolytic enzymes from *T. virens* as well as with antibiotics. Lorito *et al.* (1993) purified two enzymes; endochitinase and cellobiosidase, and observed their effect on spore germination and hyphal elongation of *Botrytis cineria, Fusarium solani, Uncinula necator* and *Ustilago* *avenae*. He observed that combining the two enzymes resulted in a synergistic increase of antifungal activity. The ED_{50} value for a 1:1 mixture of the two enzymes was as low as 10 ug/ml as compared to >30 ug/ml when tested alone. Similar observations on synergistic activity with respect to 1,3- B glucosidase and N-Acetyl-B-Glucosaminidase were made by Lorito *et al.* (1994). Di Pietro *et al.* (1993) reported the synergism between enzymes and the antibiotic gliotoxin.

2.2.1.1.3 Antibiosis

This is the second major mechanism implicated in the biocontrol of pathogens by *Trichoderma*. Two years after reporting the involvement of mycoparasitism in *Trichoderma*- host fungus interactions, Weindling in 1934 reported that a strain of *T. lignorum* produced a "lethal principle" that was excreted into the surrounding medium. He characterized it and demonstrated that it was toxic to both *R.solani* and *Sclerotinia americana* and named it "gliotoxin". Later in 1983, Howell and Stipanovic isolated and described a new antibiotic "gliovirin", from *Gliocladium virens* that was strongly inhibitory to *Pythium ultimum* and *Phytophthora* but was ineffective against *R.solani, Theilaviopsis basicola, Rhizopus arrhizus, Bacillus thuringensis* and *Pseudomonas fluorescens*. Lifshitz *et al.* (1986) attributed the control of *Pythium* species on peas by *T. harzianum* to the production of an antibiotic. Similarly, suppressive activity of *T. virens* to damping off of Zinnias was correlated to production of antibiotic gliotoxin by the bioagent (Lumsden *et al.,* 1992). Mutation studies with *Trichoderma* strains have revealed that mutants deficient for antibiotic production often lack the ability to control Pythium damping off disease (Wilhite *et al.,* 1994). At present

Trichoderma species are reported to produce a number of antibiotics. These include gliotoxin and glioviridin from *T. virens,* viridin, alkyl pyrones, isonitriles, polyketides, peptaibols, diketopiperazines, sesquiterpenes and some steroids from other *Trichoderma* species (Howell, 1998).

2.2.1.1.4 Host Selectivity

Some studies have indicated that *Trichoderma* exhibits some level of host specificity towards the pathogens. Also, the type of mechanism employed by a particular *Trichoderma* species may often be influenced by the pathogen it is attacking. In studies done by Woo *et al.* (1999) it was observed that on disruption of a chitinase gene (*ech*42) activity in *Trichoderma harzianum*, the bioagent showed reduced biocontrol activity against *Botrytis cinerea* on bean leaves. However, the transformant was as effective as the wild type against *P. ultimum* and exhibited enhanced activity against *R. solani* clearly indicating that it relies on different mechanisms to control these three pathogens.

2.2.1.1.5 Sclerotia Colonization

The pathogens *R.solani, S.rolfsii* and *Sclerotinia* spp., which cause several diseases in crop plants, have the ability to survive for long periods in soil and are highly resistant to degradation by microbial attack or fungicides primarily due to the formation of hardy resting structures called sclerotia. These sclerotia may rest in soil for decades and may germinate only in response to root exudates of host plants. They play important role in disease cycle of the pathogen. Very few studies have actually concentrated on the ability of

Trichoderma species to colonize and kill pathogen sclerotia. Jones and Stewart (1997) screened 74 isolates of 7 fungi including 18 isolates of *Trichoderma* species and 39 isolates of *Conithyrium minitans* for their ability to colonize and kill sclerotia of *Sclerotinia sclerotiorum*. Out of 74 isolates only 17 were effective in killing sclerotia. Considerable variation was observed within the 39 isolates of *C. minitans* regarding their ability to kill the sclerotia of *Sclerotinia* spp. Blum and Rodriguez-Kabana (2004) reported higher colonization of sclerotia of *S. rolfsii* by *Trichoderma* in presence of organic amendments like Kudzu and pine bark.

2.2.1.1.6 Competition and Rhizosphere Competence

Competition is considered as a 'classical' mechanism of biological control. It involves competition between antagonist and plant pathogen for space and nutrients (Chet, 1987). The idea of the involvement of this mechanism in biocontrol by *Trichoderma* has gained popularity in recent years. It is assumed that the mechanism of competition is involved in biocontrol, if no evidence for mycoparasitism or antibiosis is found in a particular *Trichoderma*-host fungus interaction (Alexander, 1982; Cook and Baker, 1983). Howell *et al.* (2000) used ultraviolet light irradiation to produce mutants of *T. virens*, deficient for both mycoparsitism and antibiotic production. However, the mutants still retained biocontrol efficacy equal to that of the parent strain against both *P. ultimum* and *R. solani* causing cotton seedling disease. This indicated that neither mycoparsitism nor antibiosis is the principal mechanism involved in the biocontrol of seedling disease in cotton.

The omnipresence of *Trichoderma* in agricultural and natural soils throughout the world proves that it must be an excellent competitor. In studies conducted by Elad and Kapat (1999), they presented information regarding biocontrol of *B. cineria* by *T. harzianum* strain T-39. *B. cineria* conidia require external nutrients for germination and infection. When conidia of T-39 were applied to leaves, germination of conidia of the pathogen was slowed, an effect attributed in part to competition (Elad, 2000). The competitive ability of *Trichoderma* and therefore its biocontrol potential is affected by soil properties.

2.2.1.1.7 Siderophores

Siderophores (Gr. "iron-bearers") are 'low molecular weight, virtually ferric specific ligands, the biosynthesis of which is carefully regulated by iron and the function of which is to supply iron to the cell' (Neilands and Leong, 1986). The structural diversity among the different siderophores is quite considerable and depends on the producing microorganisms. However, a common feature of all siderophores is that they form six coordinate octahedral complexes with ferric iron.

Trichoderma spp. are reported to produce siderophores involved in iron uptake and these are commonly short peptides containing non-protein amino acids. *T. virens* produces three types of hydroxamate siderophores: a monohydroxamate (*cis*- and trans-fusarinines), a dipeptide of *trans*-fusarinine (dimerum acid), and a trimer disdepsipeptide (copragen). Iron is generally present in the microbial environment as the ferric ion (Fe^{+3}), which is virtually insoluble in the presence of O_2 and therefore, is not available for microbial growth. Siderophores chelate Fe (III) and microbial membrane receptor proteins specifically recognize and take-up the siderophore-Fe-complex (Leong, 1986; Hemming, 1986). This results in making iron unavailable to rhizosphere microorganisms, including plant pathogens, which produce less siderophores or different siderophores with lower binding coefficients. The result is less pathogen infection and biological control.

2.2.1.1.8 Metabolism of germination stimulants

Howell (2002) has recently implicated this mechanism in the biological control of pre-emergence damping off of cotton seedlings incited by *P. ultimum* and/or *Rhizopus oryzae*. He found that control by *T. virens* (Strain G6, G6-5) or protoplast fusants of *T. virens/ T. longibrachiatum* was due to metabolism of germination stimulants released by the cotton seed. Germination stimulants are the compounds which normally induce pathogen propagules to germinate. Disease control could be achieved by wild-type strains/ mutant strains deficient for mycoparasitism/ antibiotic production/ induction of terpenoid synthesis. However, he observed that if pathogen propagules were induced to germinate by artificial means none of the above treatments gave effective control.

2.2.1.2 Antinematode action

In recent years *Trichoderma* spp. have been attributed with the ability to control diseases caused by nematodes (Singh *et al.*, 2005a&b). Root dipping in antagonist's suspension not only reduced root knot severity caused by

Meloidogyne but also enhances seedling growth in tomato, brinjal, chili and capsicum (Zaidi *et al.*, 2004). Root dipping of rice seedlings in suspension of *T. harzianum* reduced severity of root lesion nematode and improved seedling growth (U.S. Singh, unpublished information). Culture filtrate of *T. harzianum* and *T. virens* suppressed hatching and release of second stage juveniles of *Meloidogyne*. *Trichoderma harzianum* formed loops and trapped second stage juveniles of *M. incognita*. It penetrated nematode body by forming haustoria like structures and colonized internally replacing all internal organs with fungal mycelia resulting in death of the nematode (U.S. Singh & T. Khan, unpublished information). Egg masses are also penetrated and colonized by *T. harzianum* (Sharon *et al.*, 2001; U.S. Singh and T. Khan, unpublished information). Hyphae of *T. harzianum* were attracted towards nematode body in *Anguina tritici*. This chemotactic response was not recorded against second stage juveniles of *Meloidogyne*. This may be because of rapid motility of juveniles in suspension or on agar medium.

Sharon *et al.* (2001) conducted green house experiments on the potential of *T. harzianum* to control root knot nematode, *M. javanica*. They reported that root galling was reduced and top fresh weight increased in *T. harzianum* pretreated soils. The mycelium of *T. harzianum* coiled around the second stage juveniles of root knot nematode *Meloidogyne javanica* and penetrated them by forming haustoria like structures. Protease production by *T. harzianum* has been associated with the reduction in root galling. However, field experiments are still required to prove the potential of *Trichoderma* as an effective antagonist against nematodes.

2.2.1.3 Plant Growth Promotion

Apart from the direct inhibition of plant pathogens, *Trichoderma* spp. are reported to improve crop health by promotion of plant growth. Plant growth promotion is reported to be one of the indirect mechanisms employed by *Trichoderma* spp. which plays a role in the biocontrol of various plant pathogens and in improvement of plant health (Howell, 2003; Kleifeld and Chet, 1992; Chet 1987). Treatment with *Trichoderma* generally increases root and shoot growth, reduces the activity of deleterious microorganisms in the rhizosphere of plants and improves the nutrient status of the plant. Growth enhancement by *Trichoderma* spp. has been observed even in the absence of any detectable disease and in sterile soil and is not considered to be a side effect of suppression of disease or minor plant pathogens. Secretion of hormone-like metabolites and release of nutrients from soil or organic matter, have been proposed as the mechanisms involved in plant growth promotion (Kleifeld and Chet 1992; Windham, 1986). Altomare *et al.* (1999) investigated the capability of the biocontrol fungus *Trichoderma harzianum* Rifai 1295-22 (T-22) to solubilize *in vitro* some soluble and sparingly soluble minerals and the mechanisms employed in this process. They reported that T-22 was able to solubilize MnO_2, metallic zinc, and rock phosphate (mostly calcium phosphate) in a liquid sucrose-yeast extract medium. They further added that T-22 produced chelating metabolites and used redox activity for solubilizing the minerals. Both of these mechanisms also play a role in biocontrol of plant pathogens, and they may be part of a multiple-component action exerted by T-22 to achieve effective biocontrol under a variety of environmental conditions. Harman in 2000, observed that seed treatment of corn with *T. harzianum* (T-22), planted in low nitrogen soil resulted in plants that were greener and larger in the early part of the growing season. At maturity the treated plants had larger stem diameter and increased yields of grain and silage. He reported a strong interaction between T-22 and the nitrogen fixing bacterium *Bradyrhizobium japonicum*. Yedidia *et al.* (2001) studied the plant growth promoting effects of *T.harzianum* on cucumber plants. He observed that in treated plants there was a large increase in root area and cumulative root length and significant increase in plant dry weight, shoot length and leaf area over that of the untreated control. An increase in the microelement content (*viz*. Cu, P, Fe, Zn, Mn and Na) of plants was also observed.

2.2.1.4 Induced Systemic Resistance (ISR) in Plants

Till recently studies on the mechanism of biological control by *Trichoderma* spp. concentrated on the direct antagonism between the pathogen and the biocontrol agent and direct antagonism through mycoparasitism and/or antibiosis was considered as central mechanism for disease control. The interaction between the bioagent and the host plant was given little attention. However, in recent years this aspect has gained importance. It is becoming increasingly clear that our understanding of the mechanisms of biocontrol has been incomplete. In addition to the ability of *Trichoderma* spp. to attack or inhibit the growth of plant pathogens directly, recent discoveries indicate that they can also induce systemic and localized resistance to a variety of plant pathogens. Magnitude and period of induction is good enough to provide protection to the plants based on this mechanism alone. These new findings are dramatically changing our knowledge of the mechanisms of action and uses of these fungi.

Recent discoveries have shown that in addition to being parasites of other fungi, *Trichoderma* act as opportunistic, avirulent plant symbionts. At least some strains establish robust and long-lasting colonization of root surfaces and penetrate into the epidermis and a few cortical cells below. They produce or release a variety of compounds that induce localized or systemic resistance responses. This restricts further advance of the *Trichoderma* and make the plants resistant to other diseases. These root–microorganism associations cause substantial changes to the plant proteome and metabolism. Plants are protected from numerous classes of plant pathogen by responses that are similar to systemic acquired resistance and rhizobacteria-induced systemic resistance. Root colonization by *Trichoderma* spp. also frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients (Harman *et al.*, 2005).

Several studies revealed that some biocontrol agents including *Trichoderma* spp. are also able to reduce disease through a plant-mediated mechanism that is phenotypically similar to SAR, since the resistance is systemically activated and extends to above-ground plant parts. This type of induced resistance, which is activated by biocontrol agents, is often referred to as induced systemic resistance (ISR) (van Loon *et al.*, 1998). In one of the first comprehensive studies on induction of resistance by *Trichoderma* spp. and the accompanying changes in the host plant, Yedidia *et al.* (1999) demonstrated that inoculating roots of 7 day old cucumber seedlings in an aseptic hydroponic system with *T. harzianum* T-203 spores initiated plant defense responses in both the roots and leaves of treated plants. They observed that *T. harzianum* penetrated the epidermis and outer cortex of the cucumber roots and the treated plants were more developed compared to the untreated plants throughout the experiment. The plant response was marked by an increase in the peroxidase and chitinase activity and by the deposition of callose and cellulose enriched wall appositions on the inner surface of cell walls even in areas beyond the site of fungal penetration. In later studies in 2003 Yedidia *et al.* (2003) presented evidence for the induction of a systemic response in cucumber against angular leaf spot following application of *T. asperellum* to the root system. Hanson (2000) conducted field studies to test the ability of two *T. virens* strains to induce resistance in cotton plants against Verticillium wilt. Cotton seeds treated with dried preparations of *T. virens* were planted in field soil. *Verticillium dahliae* was inoculated in the plants at 6 leaf stage by stem puncture to maintain spatial separation between the two. He observed significant reduction in disease severity with both the strains. *T. harzianum* strain T-39 when inoculated on to roots or leaves of grapes is reported to provided control of disease caused by *B. cineria* on leaves spatially separated from the site of application of the biocontrol agent (De Meyer *et al.*, 1998). Compost mixes amended with *Trichoderma hamatum* have also been observed to significantly reduce severity of Phytophthora root rot and crown root in cucumber in split root bioassays thus indicating a role of induced resistance (Khan *et al.*, 2004).

In a study involving 41 isolates of *T. harzianum* and 5 isolates of *T. virens*, it was observed that when applied through roots, different isolates varied widely in their ability to induce resistance in rice cultivar Pusa Basmati-1 against sheath blight caused by *R. Solani*. Five isolates (PBAT 7, 8, 11, 39 & 43) of *T. harzianum* suppressed sheath blight between 40 to 78 % (Table 1). One of these isolates (PBAT-39) was very effective in suppressing development of zonate leaf spot and anthracnose in sorghum leaves when applied to roots. It also expressed excellent shoot growth promotary effect. However, another isolate PBAT-8, which was equally good growth promotary, was poor inducer of systemic resistance against foliar diseases.

The induction of defense response in plants by *Trichoderma* spp is often associated with accumulation of various antimicrobial compounds like phytoalexins, PR proteins along with the strengthening of cell walls and other barriers in the plant cells. Yedidia *et al.* (2003) reported the accumulation of mRNA of two defense genes (phenylpropanoid pathway gene and lipoxygenase pathway gene) in cucumber plants on treatment with *T. asperellum*. They further reported the accumulation of phenolic secondary metabolites, which may also play a role in the defense response of the plant to various pathogens. Similarly Howell *et al.* (2000) demonstrated that seed treatment of cotton with biocontrol preparations of *T. virens* or application of *T. virens* culture filtrate to cotton seedling radicles induced synthesis of terpenoids desoxyhemigossypol, hemigossypol and gossypol in developing roots in very high concentration and also led to increased peroxidase activity as compared to that of control. These compounds were inhibitory to the cotton seedling pathogen *R. solani* at quite low concentrations.

Various groups of compounds secreted by *Trichoderma* spp. may act as elicitors for the induction of defense responses in plants. Xylanase from *Trichoderma* spp. were reported to induce systemic resistance in cotton, tobacco, grapevine, etc. (Anderson *et al.*, 1993; Bailey and Lumsden, 1998; Elad, 2000; Howell *et al.*, 2000). Elad (2000) reported that cellulase produced by *T. harzianum* acts as elicitor for SAR by triggering peroxidase and chitinase activity. *Fusarium oxysporum* f. sp. *melonis* and powdery mildew infections on green house melon plants were reduced in plants treated with cellulases. Hanson and Howell (2004) characterized elicitors from the culture filtrate of defense inducing *T. virens* strain. They showed that the compound was heat stable, insoluble in chloroform and passed through a 5K molecular weight cut off (MWCO) filter but not a 3K MWCO filter. It was also sensitive to treatment with proteinase indicating that the elicitors are most likely proteins or glycoproteins.

Induced-resistance systems in plants are complex. There are three generally recognized pathways of induced resistance in plants. Two of these pathways involve the direct production of pathogenesis-related (PR) proteins; in one pathway, the production of PR proteins is generally the result of attack by pathogenic microorganisms, and in the other pathway, PR proteins are generally produced as a result of wounding, or necrosis-inducing plant pathogens —for example, herbivory by insects — although both pathways can be induced by other mechanisms. Typically, the pathogen-induced pathway relies on salicylic acid produced by the plant as a signaling molecule, whereas the herbivory-induced pathway relies on jasmonic acid as the signaling molecule (Harman *et al.*, 2005; Singh *et al.*, 2003). These compounds, and their analogues, induce similar responses when they are applied exogenously, and there is considerable crosstalk between the pathways (Harman *et al.*, 2005). The jasmonate-induced pathway is designated as induced systemic resistance. The jasmonate- and salicylate-induced pathways are characterized by the production of a cascade of PR proteins. These include antifungal chitinases, glucanases and thaumatins, and oxidative enzymes, such as peroxidases, polyphenol oxidases and lipoxygenases. Low-molecular-weight compounds with antimicrobial properties (phytoalexins) can also accumulate. The triggering molecules in the *Trichoderma* responses are unknown. The third type of induced resistance has been best-described as being induced by non-pathogenic, root-associated bacteria, and is termed as rhizobacteria-induced systemic resistance (RISR) (Harman *et al.*, 2005). It is phenotypically similar to the jasmonate- and salicylate-induced systems, as it results in systemic resistance to plant diseases. However, it is functionally very different, as the PR proteins and phytoalexins are not induced by root colonization by the rhizobacteria in the absence of attack by plant-pathogenic microorganisms. However, once pathogen attack occurs, the magnitude of the plant response to attack is increased and disease is reduced. Thus, RISR results in a potentiation of plant defense responses in the absence of the cascade of proteins that is typical of the jasmonate- or salicylate-induced systems. Similar observations were recorded by Shoresh *et al.* (2005) who studied the pathway involved in the induction of resistance by *Trichoderma* spp. in cucumber. They reported that treatment with an inhibitor of ethylene action strongly inhibited the protective effect of *Trichoderma* on plants thus indicating that ethylene signal is required for ISR. Moreover, application of jasmonic acid production inhibitor completely abolished the protective effect of *Trichoderma* on plants. These experiments confirmed that like in case of rhizospheric bacteria, induction of resistance by *Trichoderma* also occurs through the jasmonic acid/ ethylene signaling pathway.

The role of a mitogen activated protein kinase TmkA in inducing systemic resistance in cucumber against a bacterial pathogen *Pseudomonas syringae* pv. *lacrymans* was investigated by Viterbo *et al.,* (2005) using *tmkA* loss-of function mutants of *Trichoderma virens*. They observed that the mutants were able to colonize the plant roots as effectively as the wild type strain, but failed to induce a full systemic resistance against the leaf pathogen. Interactions with the plant roots enhanced the level of *tmkA* transcript in *T. virens* and its homologue in *T. asperellum*. At the protein level activation of two forms reacting to the phosphop44/42 MAPK antibody were detected. They further demonstrated that the *tmkA* mutants retained their biocontrol potential in soil against *Rhizoctonia solani,* but were not effective in reducing disease incidence against *Sclerotium rolfsii.* They concluded that unlike in many plant-pathogen interactions, *Trichoderma* TmkA MAPK is not involved in limited root colonization. *Trichoderma*, however, needs MAPK signaling in order to induce full systemic resistance in the plant. Regardless of the exact mechanism responsible, this study demonstrates that a conserved fungal signal transduction pathway is involved in the three-way interaction between biocontrol fungus, plant pathogen, and plant and that different signals control mycoparasitic activity and ability to induce plant systemic resistance.

Therefore it may be concluded that the interactions of *Trichoderma* with its host fungi are very complex often influenced by the particular strain of *Trichoderma* involved, the host fungus in question and maybe several other ecological factors.

More and more studies in recent years are indicating that the mechanisms employed by biocontrol agents for controlling pathogens are many and complex and often vary with the kind of bioagent, pathogen and host plant involved. Mechanisms may also be influenced by the soil type, temperature, pH and other members of the soil microflora. What is perceived by us as biocontrol may actually be the culmination of a number of different mechanisms working synergistically to achieve disease control.

MATERIALS AND METHODS

Chapter 3

MATERIALS AND METHODS

3.1 Isolates of *Trichoderma* Species

Forty three isolates of *Trichoderma* species used in the present study were obtained from Biocontrol laboratory, Dept. of Plant pathology, G. B. Pant Univ. of Agric. & Technol., Pantnagar. These cultures were originally isolated from the rhizosphere soils of various crops growing in the *Tarai* region of Uttaranchal. Three additional cultures of *Trichoderma harzianum*, identified from CABI Biosciences U.K., were also used in the molecular marker based studies. The isolates were coded as PBAT-1 to PBAT-46 for future reference. All the 46 cultures were maintained on potato dextrose agar (PDA, HiMedia Lab. Chemicals) slants at 4^0C for further experiments.

3.2 Purification of *Trichoderma* cultures

Trichoderma isolates were purified by single spore culture. The spores of the isolates were inoculated onto a Petri dish seeded with PDA medium. Sub-culturing was done from the growing front of the single new colony. Small amount of spores were taken on the tip of a sterilized inoculating needle and streaked on PDA poured Petri dishes. This process was repeated by taking inoculum from edge of colonies growing in the freshly streaked Petri plate, and again streaking it in fresh PDA plates. Colony arising from single spore was picked up and inoculated on a fresh plate. This culture was used for further studies.

3.3 Pathogens

The pathogens, *Sclerotium rolfsii* and *Rhizoctonia solani* were isolated from infected okra plants, *Fusarium oxysporum* f.sp. *pisi* was isolated from wilted garden pea plants, while *Sclerotinia sclerotiorum* was isolated from white rot infected French bean plants. The cultures were grown on potato dextrose agar (PDA) at 28 ± 1^0C and maintained at 4^0C.

3.4 Cultural Characterization of *Trichoderma* isolates

3.4.1 Preparation of fresh colonies for inoculum

To study the cultural characteristics and growth of *Trichoderma* isolates, fresh colonies of the isolates were prepared. A block of the fungal mycelium was inoculated onto 90 mm Petri dishes poured with cornmeal dextrose agar (Cornmeal agar + 2% (w/v) dextrose, HiMedia Lab. Chemicals) and the Petri dishes were incubated at 26 ± 2 ^{0}C for 3 days. These fresh cultures were used for further studies.

3.4.2 Study of cultural characteristics and growth rate of *Trichoderma* isolates

The cultural characteristics and growth rates of the 46 *Trichoderma* isolates were determined on Potato dextrose agar (PDA) medium. A 5 mm diameter plug was cut from the actively growing edge of a fresh colony (before the start of conidial production) of the isolates, using a sterile cork borer. The disc was placed in a 90 mm Petri dish, containing 20 ml of PDA medium, approximately 1.5 cm from the edge of the Petri dish with the mycelial surface facing downwards. Three replications were maintained for each isolate. The Petri dishes were incubated in darkness at 25^0C. Another set of Petri dishes were inoculated as above and incubated at 35^0C .The colonies were examined at 24 h intervals and colony radius was measured from the edge of the inoculum plug after 72 h at both 25^0C and 35^0C. The following observations on growth rate and cultural characters of the isolates were recorded:

1. Colony radius on PDA after 72 h at 25^0C
2. Colony radius on PDA after 72 h at 35^0C
3. Time of first appearance of green conidia
4. Pattern of conidiation
5. Color changes in the conidia
6. Presence of any odor or yellow pigmentation in the colony
7. Formation of conidial pustules

3.5 Morphological Characterization of *Trichoderma* isolates

For morphological studies of *Trichoderma*, Cornmeal dextrose agar (CMD) medium was used. This media provides morphological characters more or less approximate to those found in nature. A block of the fungal mycelium was inoculated onto 90 mm Petri dishes seeded with CMD. The cultures were incubated at 20^0C. Microscopic preparations for morphological studies were made from pustules where there were still white conidia, generally within a week of incubation at 20^0C. For preparing slides a very small amount of the material was placed in drop of 3% KOH on a slide. The KOH wets the conidia and allows the conidiophores to spread. Once the mount was prepared, KOH was replaced with lactophenol cotton blue. Before placing the cover slip, the hyphae and conidiophores were separated using needles. After placing the cover slip, the slide was observed under the microscope. Observations were recorded for the following morphological characters:

1. Conidiophores, their branching pattern and angle to main axis.
2. Phialide numbers and their arrangement.
3. Conidial shape and colour.
4. Formation of chlamydospores and their position. (To observe chlamydospore formation cultures were incubated up to 2 weeks at 20^0C).

3.6 Molecular Characterization of *Trichoderma* isolates

PCR amplification of the *Trichoderma* isolates using random primers was performed on a set of 46 isolates. These include the 43 isolates used for all other studies and 3 additional isolates of *T. harzianum* which had been previously identified from CABI Biosciences U.K.

3.6.1 DNA Extraction

The method describe by Mukherjee (1999) was followed for extraction of genomic DNA. The list of buffers and stock solutions is given in Appendix-I

3.6.1.1 Preparation of mycelial mat

The single spore cultures of the isolates were grown on potato dextrose broth (PDB). One ml spore suspension of the isolates was inoculated into 250 ml conical flasks each containing 100 ml of PDB. The flasks were incubated in a shaker (120 rpm) at 25^{0}C. After 48 h the liquid culture was filtered using a vacuum pump and the fungal mycelium was collected on the filter paper in a Buchner funnel. The mycelium was washed with distilled water and frozen and stored at -80^{0}C till used for extraction.

3.6.1.2 Extraction of fungal DNA

The steps in the extraction of fungal DNA are:

1. 2 g of the frozen fungal tissue was ground to a fine powder in liquid nitrogen using a pre cooled mortar and pestle.
2. 300 mg of the powder was transferred to a 2 ml microfuge tube containing 500 µl extraction buffer and mixed thoroughly by vortexing.
3. After thawing, 70 µl of 10% SDS was added to each tube and mixed by inverting the tube.
4. The tube was then incubated at 65°C for 10 min in a water bath.
5. 170 µl of ice cold 5M potassium acetate was then added in each tube and mixed thoroughly.
6. The mixture was incubated on ice for 20 minutes.
7. After 20 minutes 600 µl of chloroform was added to each tube, mixed thoroughly and centrifuged at 11,000 g for 10 min at 4°C.

8. After centrifugation the top aqueous phase (600-700 µl) was transferred to a fresh tube.
9. 500 µl isopropanol was then added to the fresh tube and mixed gently by inverting the tube.
10. The DNA immediately precipitated in the form of a lump.
11. The DNA was pelleted, washed with 70% ethanol & dried for 10 min.
12. Finally DNA was re-dissolved in 100 µl TE buffer (with RNAse) and kept at -20°C.

3.6.2 Quantification of Genomic DNA

The quantification of DNA was done by indirect method by comparing the intensity of bands with marker band (λDNA/Hind III) as well as by direct method by taking absorbance at 260 nm (OD_{260}). Absorbance of DNA samples dissolved in TE buffer was recorded against TE buffer as a blank using UV-VIS spectrophotometer (Spectronic Thermolabsystem). Concentration of DNA was calculated from optical density using following formula:

$$\text{Conc. In } \mu g/\mu l = \frac{OD_{260} \times 50 \times \text{Dilution factor}}{1000}$$

3.6.3 RAPD- PCR of the *Trichoderma* isolates

3.6.3.1 PCR Amplification

PCR amplification was performed in a volume of 25 µl reaction set up as detailed below:

Table 1: Details of the 22 primers used in the study

S. No.	Operon Code	Sequences 5' to 3'	GC Content (%)
1	OPAA 1	AGACGGCTCC	70
2	OPAA 2	GAGACCAGAC	60
3	OPAA 3	TTAGCGCCCC	70
4	OPAA 4	AGGACTGCTC	60
5	OPAA 6	GTGGGTGCCA	70
6	OPAA 7	CTACGCTCAC	60
7	OPAA 12	GGACCTCTTG	60
8	OPAA 14	AACGGGCCAA	60
9	OPAA15	ACGGAAGCCC	70
10	OPAA 16	GGAACCCACA	60
11	OPBB 2	CCCCCGTTAG	70
12	OPBB 3	TCACGTGGCT	60
13	OPBB 5	GGGCCGAACA	70
14	OPBB 6	CTGAAGCTGG	60
15	OPBB 9	AGGCCGGTCA	70
16	OPBB 10	ACTTGCCTGG	60
17	OPBB 11	TGCGGGTTCC	70
18	OPBB 12	TTCGGCCGAC	70
19	OPBB 13	CTTCGGTGTG	60
20	OPBB 14	GTGGGACCTG	70
21	OPB 7	GAAGGCTGGG	70
22	OPB 8	TCGTCGAAGG	60

Component (conc.)	Final conc.	Single tube (μl)
DNA Template(50ng/μl)	50ng/μl	1.0
dNTP mix (10mM each)	800μM	2
Taq Polymerase (3U/μl)	1.5U	0.5
Reaction buffer(10X)	1X	2.5
Primer(20μg/μl)	-	1.0
ddH_2O	18	18
	Total	25

Random primer of 10 nucleotides each, obtained from Operon Technologies Inc. (CA, USA) series OPAA, OPBB and OPB were used. The sequences, operon codes of the primers and GC content are given in Table I.

A master mix (minus template DNA) for 50 samples was prepared to reduce pipetting error. The master mix was then distributed in each tube (24 μl each) and finally 1 μl of different template DNA was added in each tube. The mixture was gently mixed and centrifuged for ten seconds. The PCR amplification was achieved in a MJ Research Thermocycler, programmed as follows.

Cycle	**Denaturation**		**Annealing**		**Polymerization**	
	Temp.	**Time**	**Temp.**	**Time**	**Temp.**	**Time**
First cycle	94°C	5 min	-	-	-	-
45 cycle	94°C	30 sec	35°C	1 min	72°C	2 min
Last cycle	-	-	-	-	72°C	7 min

3.6.4 Agarose Gel Electrophoresis

Submerged gel electrophoresis unit was used for fractionating RAPD markers on agarose gel. Agarose gel (1.2%) was prepared by dissolving appropriate amount of agarose in 1x TAE/0.5x TBE buffer (Sambrook *et al.,* 1989) and adding ethidium bromide stain (1.5 µg/µl). For each well, DNA sample and DNA loading dye (1 x) were mixed in a ratio of 25:2, v/v, and loaded with a micropipette. Electrophoresis was done at 50V for 4 h in 1xTAE/.5x TBE electrophoresis buffer. The gel image was viewed and stored in gel documentation system (Gene Genius Bio Imaging System, Syngene).

3.6.5 Statistical analysis

Each RAPD band was considered an independent character or locus, and bands were scored visually as either present ("1") or absent ("0") for each of the 38 genotypes. Two independent scorings were made on each gel, and only those bands consistently scored were considered for analysis. All the bands were scored just to avoid over/under estimation of genetic similarity (Gherardi *et al.*, 1998). Qualitative differences in band intensity were not considered

Cluster analysis was performed and corresponding dendrogram generated for the 46 isolates using the unweighted pair-group method with arithmetical averages (UPMGA) (Sneath and Sokal, 1973). A cophenetic matrix was computed from the clustering matrix in order to access the goodness of fit of the dendrogram obtained by comparing the cophenetic value matrix with the initial similarity matrix. The significance of the cophenetic correlation was tested by the Mantel correspondence test (Mantel, 1967). All these analysis were done using the NTSYSpc 2.11a (Numerical Taxonomy and Multivariate Analysis System for personal computers) package (Rohlf, 2000).

3.7 Screening of *Trichoderma* isolates for their antagonistic potential

3.7.1 Preparation of pure powder of *Trichoderma* isolates

Pure powder of *Trichoderma* isolates was prepared on barnyard millet (jhangora) grains. The grains were soaked in water for 12 h and then filled in 250 ml Erlenmeyer flasks (@ 50g/ flask). These flasks were autoclaved at 15 lbs psi. for 30 minutes. After cooling to room temperature, the flasks were inoculated with mycelial discs cut from three days old culture of *Trichoderma* isolates, and incubated at 28^0C for 12 days. Barnyard millet grains colonized by *Trichoderma* were air dried under shade and ground with the help of Willy Mill to get a powder. This powder was passed through 50 and 80 mesh size sieves, simultaneously to obtain a very fine powder.

3.7.2 *In vitro* screening for inhibition of hyphal growth using bangle method

All the isolates of *Trichoderma* were tested for their antagonistic potential against the four pathogens by using a modification of the dual culture technique here referred to as bangle method (Zaidi *et al.*, 2004). A 5 mm disc of the pathogen was cut from the edge of a three day old culture using a sterile cork borer and placed in the centre of a Petri plate previously poured with PDA. The mycelial part of the disc was kept in contact with the medium. Spore suspension of *Trichoderma* isolates was prepared by adding pure powder of *Trichoderma* to 0.2% gelatin solution (@2g pure powder/ liter). Glass bangles of 2.2 inch size were flame sterilized and then dipped in the spore suspension. A coating of the spore suspension was formed around the bangles. The bangles were then placed in the Petri dishes previously inoculated with the pathogen. The pathogens, *Rhizoctonia solani* and *Sclerotinia* were inoculated simultaneously with *Trichoderma* while *Sclerotium rolfsii* and *Fusarium oxysorum* f.sp *pisi.* were inoculated 24 and 48 h prior to *Trichoderma*, respectively. A total of 3 replications were maintained for each *Trichoderma* isolate. Control plates were inoculated with bangle dipped in 0.2% gelatin solution. All the plates were incubated at 26±2 ^{0}C. Radial growth of the pathogens in control and treatment plates was recorded when the edge of pathogen colony and *Trichoderma* just came in contact. The formation or absence of inhibition zone, overgrowth of the pathogen by *Trichoderma* and time taken to overgrow the entire plate was also recorded.

3.7.3 Confrontation assay of *Trichoderma* isolates with *Rhizoctonia solani*

All the 43 *Trichoderma* isolates were observed for their mycoparasitic ability against *Rhizoctonia solani* in confrontation assay. Coculture studies were done to study the interaction between the *Trichoderma* isolates and the pathogen. The studies were performed in glass Petri dishes poured with 20 ml PDA. After the medium solidified, a 5 mm disc of the pathogen was cut from the edge of an actively growing culture, using a sterile cork borer, and inoculated in the Petri dish about 1.5 cm from the edge. Another 5 mm disc of a *Trichoderma* isolate was inoculated at the opposite end in the Petri dish. The Petri dishes were incubated at 25^{0}C. After the fungal hyphae of *R. solani* and *Trichoderma* met after 24 to 48 h, a small mat of mycelium was picked up from the zone of interaction between the two and placed on a slide in drop of fluorescent dye. The mycelium was observed under reflected fluorescent microscope (Nikon E1000) and presence or absence of coiling of *Trichoderma* around the host hyphae was recorded.

3.7.4 *In vitro* screening for sclerotial colonization

All the 46 isolates were studied for their ability to parasitize the sclerotia of the pathogens *Rhizoctonia solani*, *Sclerotium rolfsii* and *Sclerotinia sclerotiorum in vitro*. A novel methodology was developed to study the sclerotial colonization by *Trichoderma* species.

A 5 mm disc of the pathogen was cut from the edge of a three day old culture of *Trichoderma* using a sterile cork borer and placed in the centre of a 90 mm Petri plate previously poured with PDA. The mycelial part of the disc was kept in contact with the medium. The inoculated plates were incubated at 26±2^{0}C. After approximately 72 h, when the plates were covered with the growth of *Trichoderma*, they were removed from the incubator. 15 g of sterilized sandy soil (pH 6.8) having 25% moisture content was then spread evenly over the surface growth of *Trichoderma* in the Petri plates. Ten sclerotia of the test pathogen (*R. solani, S. rolfsii* or *S. sclerotiorum*) were then placed at equal distance from each other on the surface of the soil layer. The plates were again incubated at 26±2 ^{0}C for 5 days. Three replications were maintained for each *Trichoderma* isolate. After 5 days, observations were recorded on the number of sclerotia colonized in each replication and per cent colonization was calculated.

3.7.5 Glass house studies

The antagonistic potential of some selected *Trichoderma* isolates was studied further under glass house conditions. Based on the results of the bangle method and sclerotial colonization experiments, 4 of the most promising isolates were selected for glass house studies. The isolates were selected on the criterion that 2 of the selected isolates were highly antagonistic to hyphal growth of all the 4 pathogens (Isolates PBAT-14 and PBAT-43) while the other two selected isolates exhibited good *in vitro* sclerotial colonization (Isolates PBAT-12 and PBAT-16).

The selected four isolates were screened in glass house for their ability to control diseases caused by three sclerotia forming pathogens, *viz.* *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, and one non-sclerotia forming pathogen *viz.* *Fusarium oxysporum* f.sp. *pisi* on three crops. For the glass house experiments, 1 kg plastic pots were filled with steam sterilized soil. The four isolates PBAT-12, PBAT-14, PBAT-16 and PBAT-43 were multiplied on FYM with 30% moisture content (based on air dried FYM), by inoculating 250 g of autoclaved FYM with 10 ml spore suspension of individual *Trichoderma* isolate in 500 ml Erlenmeyer flasks. The inoculated flasks were incubated at 26±2 ^{0}C for 14 days (Zaidi and Singh, 2004). The pathogens were multiplied on autoclaved barnyard millet grains. The grains were soaked in water for 12 h and then filled in 250 ml Erlenmeyer flasks (@ 50g/ flask) and autoclaved at 15 lbs psi for 30 minutes. After cooling to room temperature, the flasks were inoculated with mycelial discs cut from three days old culture of the pathogen and incubated at 28^{0}C for 10 days.

The fully colonized FYM was mixed with the autoclaved soil in plastic pots @ 6 g/ kg soil. After 48 h the test pathogen inoculum (*R. solani, Sclerotinia sclerotiorum* or *S. rolfsii*) was mixed in the soil @1 g of colonized barnyard millet grains/ kg soil. For each of the four *Trichoderma* isolates, 4 sets of pots were prepared. First set contained pots having inoculum of *F. oxysporum pisi*, second set contained inoculum of *S. rolfsii*, third set had inoculum of *R. solani* and the fourth set contained inoculum of *S. sclerotiorum*. Seeds of three crops namely French bean, pea and okra were surface sterilized with 0.05% sodium hypochlorite solution for 10 minutes and then air dried. 10 seeds were planted per pot. Pea seeds were sown in pots having inoculum of *F. oxysporum pisi* and French bean seeds were sown in pots with inoculum of *Sclerotinia sclerotiorum*. Okra seeds were sown in two sets of pots, one having inoculum of *R. solani* and the other set having inoculum of *S. rolfsii*. Pots which did not contain *Trichoderma* colonized FYM served as control. Five replications were maintained for each set including control. All pots were irrigated on alternate days with sterilized water. Soil surface was occasionally stirred with plastic spatula to ensure good soil aeration. Observations were recorded on disease intensity in each set of experiment and per cent disease control was calculated.

3.8Statistical Analysis

The statistical analysis of the bangle method, sclerotial colonization and glass house experiments was done using one factor ANOVA in Microsoft Excel.

RESULTS

Chapter 4

RESULTS

4.1 Cultural Characterization of *Trichoderma* isolates

The cultural characteristics and growth rates of the 43 *Trichoderma* isolates were observed on Potato dextrose agar medium. Observations on growth rate and colony characteristics were recorded.

All the isolates were fast growing reaching a radius of 57 to 62 mm after 72 h at 25^0C and 18 to 21 mm after 72 h at 35^0C. At 35^0C, the isolates produced abnormal compact colonies with irregular margin. Conidiation in the *T. harzianum* isolates was predominantly effuse covering the entire surface of the plates. However, some isolates initially produced flat pustules in concentric rings. The pustules appeared powdery due to dense conidiation. All the *T. virens* isolates showed predominantly effuse conidiation without formation of any pustule.

While most of the isolates produced yellow pigment on Corn Meal Dextrose (CMD) medium, no pigmentation or odour was detected on PDA. Conidial colour change was observed from white to varying shades of green and sometimes from white to yellow green. In most isolates conidia were formed by 48 h and turned green within 72 h. In a few isolates (PBAT-2, 11, 20) green conidia were visible within 48 h (Plates1-43).

4.2 Morphological Characterization of *Trichoderma* isolates

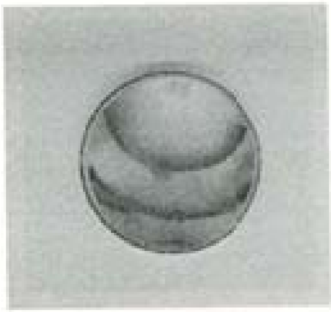
For morphological studies of *Trichoderma*, CMD medium was used. Slides were prepared and observed for various morphological characters under the microscope. Primarily two types of arrangement of conidiophores and phialides was observed among the 43 isolates (Plates1-43). The characters observed were used to classify the isolates into two species using the key provided by Bisset (1991a&b). Out of the 43 isolates, 5 isolates (PBAT-7, 16, 24, 25 and 26) were classified as *T. virens* and 38 isolates were classified as *T. harzianum*.

4.3 Molecular Characterization of *Trichoderma* isolates

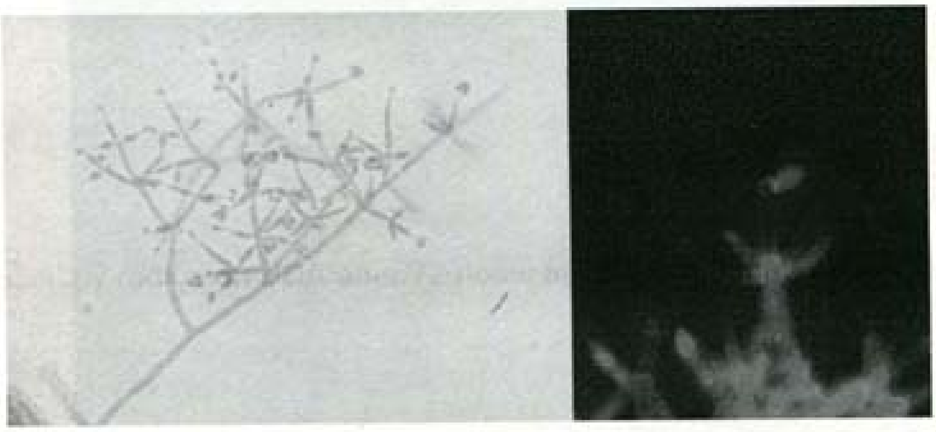
Twenty two random primers obtained from Operon Technologies Inc. (CA, USA) series OPAA, OPBB and OPB (Table 1; Materials & Methods section) were used to assess genetic relatedness among the 43 isolates of *Trichoderma*. These primers generated a total of 158 scorable bands, 157 of which were polymorphic including 26 exclusive bands, which can be used as diagnostic band for specific isolates. Some of the isolates showed more than one exclusive bands with same or different primers (Table 2). The number of bands generated by a primer varied between 3 to 16. The size of bands ranged from 74 to 757 bp. Amplification obtained with individual primers is described as follows:

Plate 1

Trichoderma harzianum PBAT-1



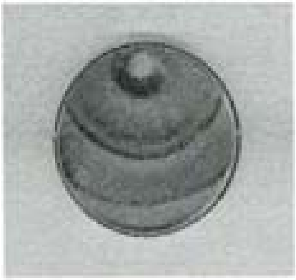
- Colony radius on PDA after 72 hours at 25^0C = 58 mm; at 35^0C = 18 mm
- Pattern of conidiation: Effuse, sometimes as loose flat pustules.
- First green conidia visible after 72 h at 25^0C
- First white conidia formed after 48 h on PDA at 25^0C and conidial colour changes from white to green.
- Yellow pigmentation produced on CMD, no pigment on PDA, odour indistinct.



- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

Plate 2

Trichoderma harzianum PBAT-2

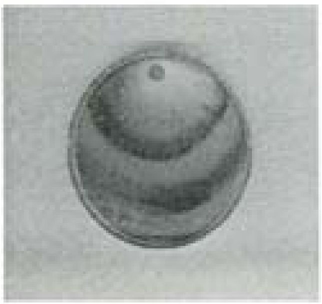


- Colony radius on PDA after 72 hours at 25^{0}C = 57 mm; at 35^{0}C = 21mm
- Pattern of conidiation: Effuse.
- First green conidia visible after 48 h at 25^{0}C
- First white conidia formed after 24 h on PDA at 25^{0}C and conidial colour changes from white to dark green.
- Yellow pigmentation produced on CMD, reverse of colony buff coloured on PDA, odour indistinct.

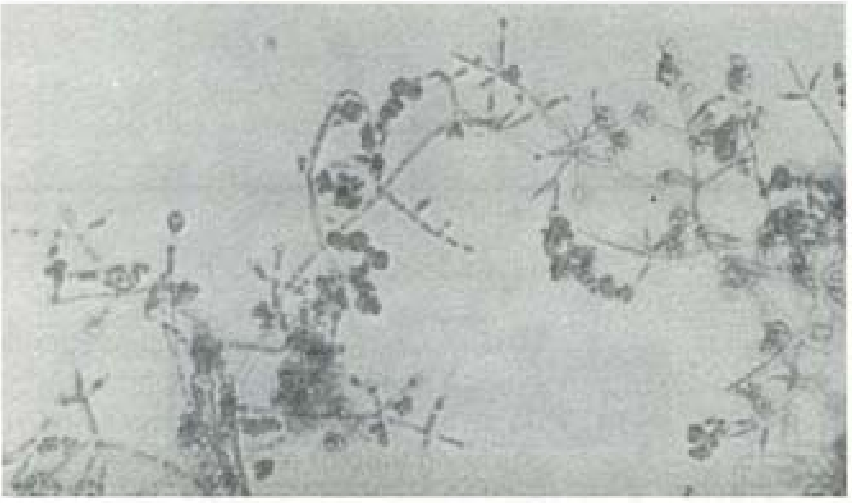
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^{0} angle and often arising in pairs. Fertile apex.
- Phialides lampulliform, arising in crowded and divergent verticils of 2-5.
- Conidia green, ovoid.
- Chlamydospores mostly intercalary.

Plate 3

Trichoderma harzianum PBAT-3



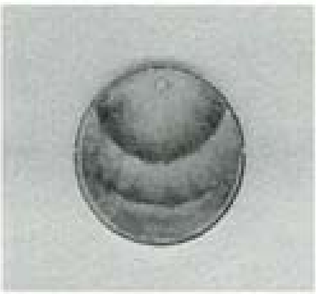
- Colony radius on PDA after 72 hours at 25⁰C = 58 mm; at 35⁰C = 21mm
- Pattern of conidiation: Effuse.
- First green conidia visible between 48 – 72 h at 25⁰C
- First white conidia formed after 48 h on PDA at 25⁰C and conidial colour changes from white to dark green.
- Yellow pigmentation produced on CMD, reverse of colony colourless on PDA, odour indistinct.



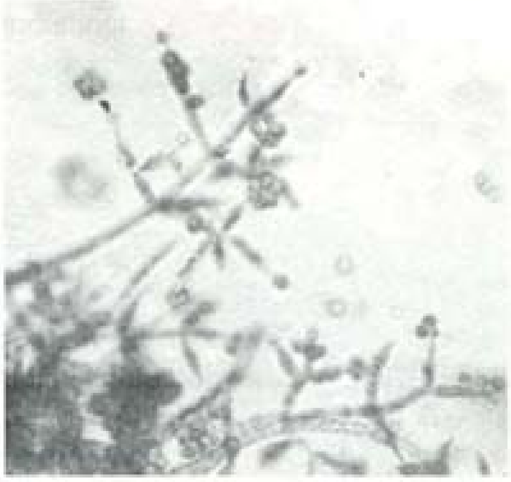
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90⁰ angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, round to ovoid.
- Chlamydospores both terminal and intercalary.

Plate 4

Trichoderma harzianum PBAT- 4



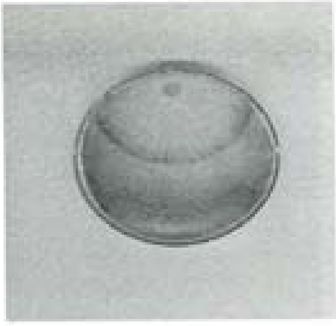
- •Colony radius on PDA after 72 hours at 25⁰C = 57 mm; at 35⁰C = 15 mm
- •Pattern of conidiation: Effuse.
- •First green conidia visible between 48 to 72 h at 25⁰C.
- •First white conidia formed between 48 to 72 h on PDA at 25⁰C and conidial colour changes from white to dark green.
- •Yellow pigmentation produced on CMD, reverse of colony colourless on PDA, odour indistinct.



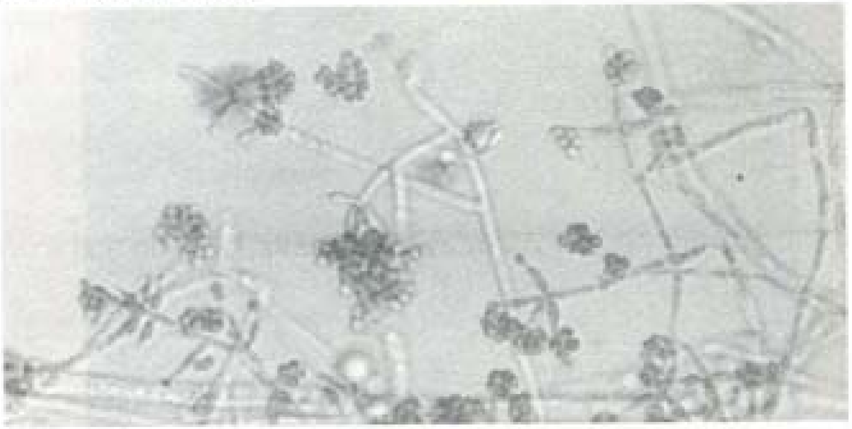
- •Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90⁰ angle and often arising in pairs. Fertile apex.
- •Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- •Conidia green, round to ovoid.
- •Chlamydospores present but not abundant, mostly intercalary.

Plate 5

Trichoderma harzianum PBAT-5



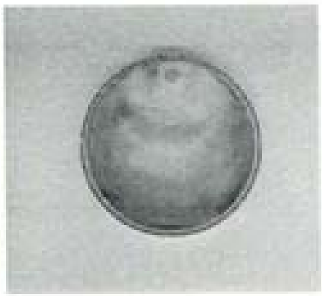
- Colony radius on PDA after 72 hours at 25^{0}C = 56 mm; at 35^{0}C = 20 mm
- Pattern of conidiation: Effuse, sometimes loosely arranged in flat pustules.
- First green conidia visible between 48 to 72 h at 25^{0}C
- First white conidia formed after 48 h on PDA at 25^{0}C and conidial colour changes from white to light green to dark green.
- Yellow pigmentation produced on CMD, reverse of colony colourless on PDA, odour indistinct.



- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores not observed in culture.

Plate 6

Trichoderma harzianum PBAT-6

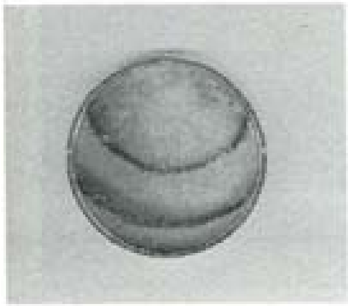


- Colony radius on PDA after 72 hours at 25^0C = 57 mm; at 35^0C = 12 mm
- Pattern of conidiation: Effuse.
- First green conidia visible after 72 hrs at 25^0C
- First white conidia visible between 48 to 72 hrs on PDA at 25^0C and conidial colour changes from white to dark green.
- Yellow pigmentation produced on CMD, reverse of colony colourless on PDA, odour indistinct.

- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but rare, mostly intercalary.

Plate 7

Trichoderma virens PBAT-7

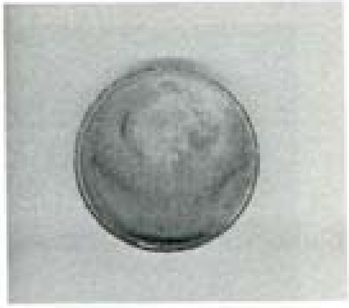


- Colony radius on PDA after 72 hours at 25⁰C = 48 mm; at 35⁰C = 14 mm
- Pattern of conidiation: Predominantly effuse.
- First green conidia visible after 48 h at 25⁰C
- First white conidia visible after 48 h on PDA at 25⁰C and conidial colour changes from white to dark green.
- Yellow pigmentation produced on CMD, reverse of colony colourless on PDA, odour indistinct

- Conidiophores hyaline, sparingly branched at base, branching often divergent. Towards apex it is branched in an irregular manner and each branch terminates in a cluster of 3-5 closely appressed phialides. Fertile apex.
- Phialides ampulliform, arising in convergent, closely appressed clusters of 3-5.
- Conidia green, ovoid.
- Chlamydospores abundant, both intercalary and terminal.

Plate 8

Trichoderma harzianum PBAT-8

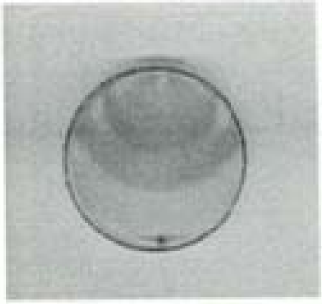


- Colony radius on PDA after 72 hours at 25^{0}C = 58 mm; at 35^{0}C = 12 mm
- Pattern of conidiation: Effuse.
- First green conidia visible after 72 h at 25^{0}C
- First white conidia formed at 72 h on PDA at 25^{0}C. Conidial colour changes from white to dark green.
- Yellow pigmentation produced on CMD, reverse of colony colourless on PDA, odour indistinct.

- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present, both terminal and intercalary.

*Showed a unique band of 231 bp with primer OPAA1
(5'AGACGGCTCC3')

Trichoderma harzianum PBAT 9

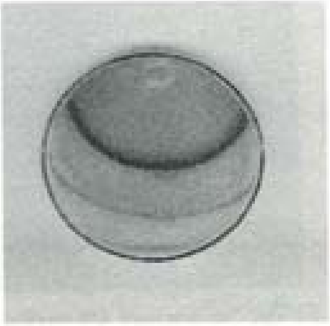


- Colony radius on PDA after 72 hours at 25^0C = 55 mm; at 35^0C = 10 mm
- Pattern of conidiation: Effuse, sometimes loosely arranged in flat pustules.
- First green conidia visible after 48 h at 25^0C
- First white conidia formed between 24 to 48 h on PDA at 25^0C and conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

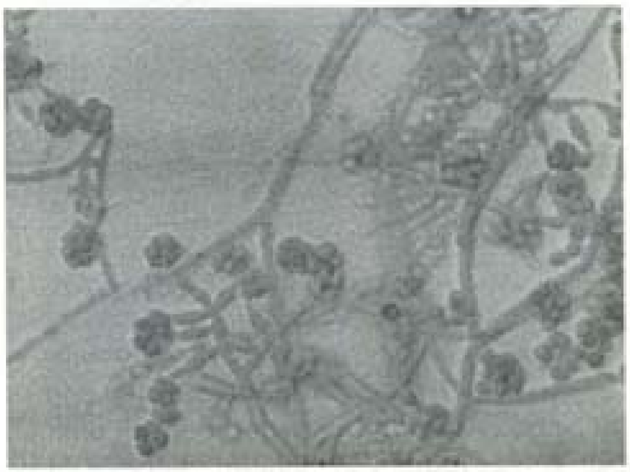
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but rare.

Plate 10

Trichoderma harzianum PBAT 10



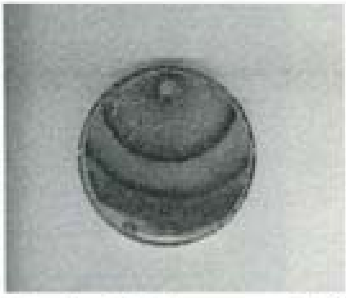
- Colony radius on PDA after 72 hours at 25^0C = 59 mm; at 35^0C = 20 mm
- Pattern of conidiation: Effuse.
- First green conidia visible after 48 h at 25^0C
- First white conidia formed between 24 to 48 h on PDA at 25^0C and conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



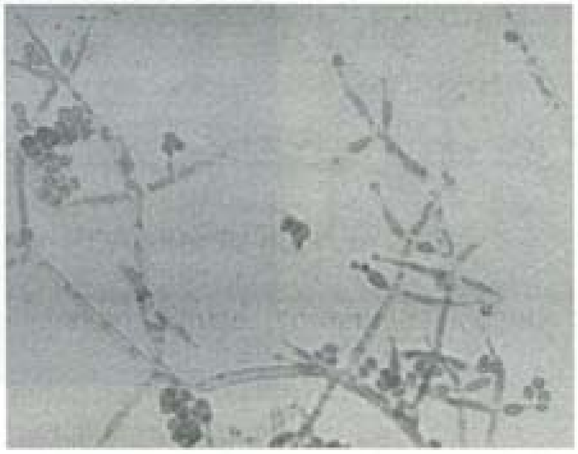
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present, both terminal and intercalary.

Plate 11

Trichoderma harzianum PBAT-11



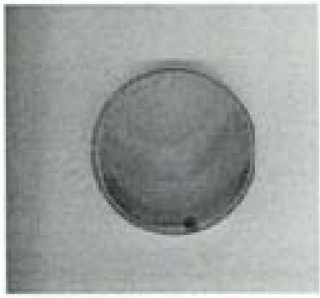
- Colony radius on PDA after 72 hours at 25⁰C = 59 mm; at 35⁰C = 20 mm
- Pattern of conidiation: Effuse, sometimes loosely arranged in flat pustules.
- First green conidia visible after 48 h at 25⁰C
- First white conidia formed between 24 to 48 h on PDA at 25⁰C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



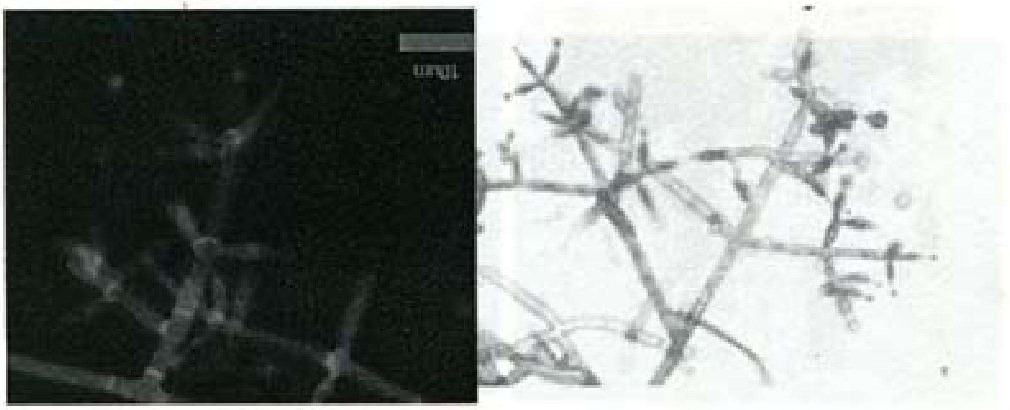
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90⁰ angle and often arising in pairs. Fertile apex.
- Phialides lageniform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but rare, mostly intercalary.

Plate 12

Trichoderma harzianum PBAT-12



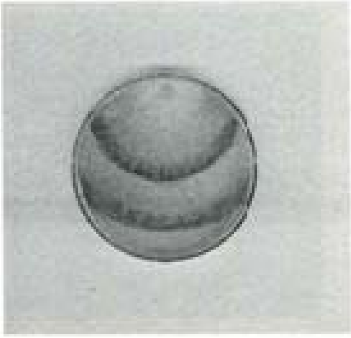
- Colony radius on PDA after 72 hours at 25^{0}C = 50 mm; at 35^{0}C = 18 mm
- Pattern of conidiation: Effuse.
- First green conidia visible between 48 to 72 hrs at 25^{0}C
- First white conidia formed at 48 hrs on PDA at 25^{0}C and conidial colour changes from white to yellow green to green.
- Yellow pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



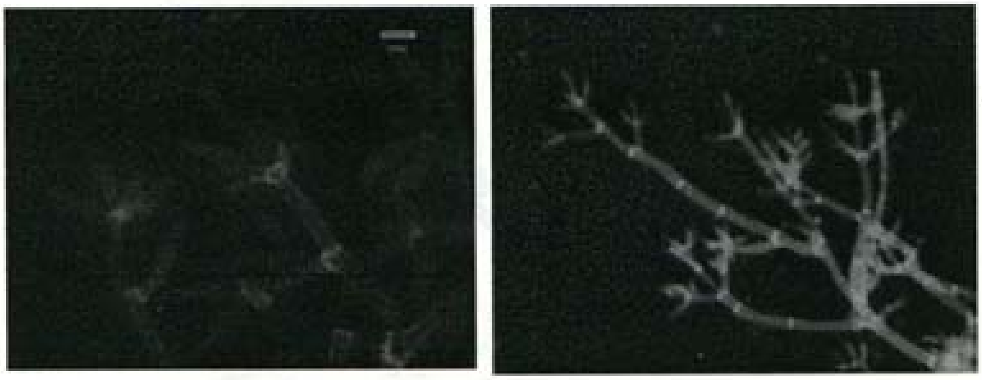
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides lageniform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores abundant, both intercalary and terminal.

Plate 13

Trichoderma harzianum PBAT-13

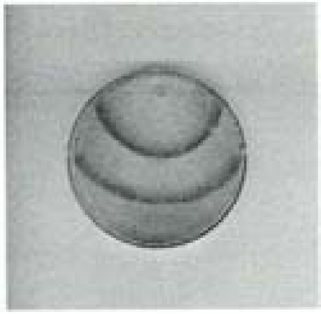


- Colony radius on PDA after 72 hours at 25^0C = 62 mm; at 35^0C = 18 mm
- Pattern of conidiation: Effuse, sometimes loosely arranged in pustules.
- First green conidia visible after 48 h at 25^0C
- White conidia formed between 48 to 72 h on PDA at 25^0C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

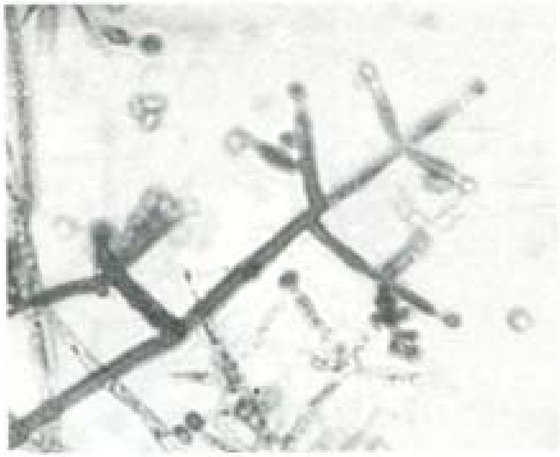


- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides lageniform, arising in crowded and divergent verticils of 3-5.
- Conidia green in colour, ovoid.
- Chlamydospores present but rare, both terminal and intercalary.

Trichoderma harzianum PBAT-14

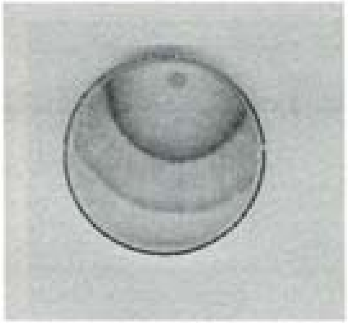


- Colony radius on PDA after 72 hours at 25^{0}C = 58 mm; at 35^{0}C = 20 mm
- Pattern of conidiation: Effuse, sometimes arranged in flat pustules.
- First green conidia visible after 48 h at 25^{0}C
- First white conidia formed at 48 h on PDA at 25^{0}C and conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

Trichoderma harzianum PBAT-15

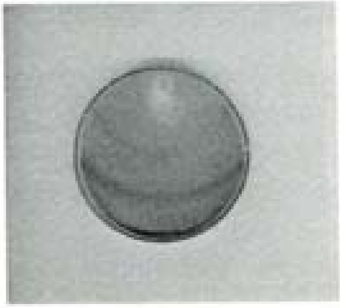


- Colony radius on PDA after 72 hours at 25⁰C = 57 mm; at 35⁰C = 5-9 mm
- Pattern of conidiation: Effuse or sometimes loosely arranged in flat pustules.
- First green conidia visible after 48 hr at 25⁰C
- First white conidia visible between 48 to 72 h on PDA at 25⁰C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

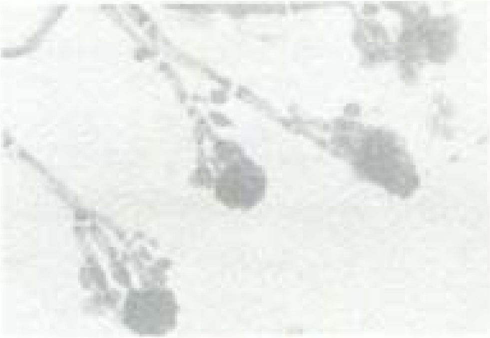
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90⁰ angle and often arising in pairs. Fertile apex.
- Phialides lageniform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores abundant, both intercalary and terminal.

Plate 16

Trichoderma virens PBAT-16



- Colony radius on PDA after 72 hours at 25^0C = 57 mm; at 35^0C = 9 mm
- Pattern of conidiation: Predominantly effuse.
- First green conidia visible after 48 h at 25^0C
- First white conidia formed between 48 to 72 h on PDA at 25^0C and conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

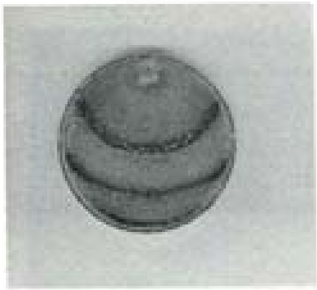


- Conidiophores hyaline, sparingly branched at base, branching often divergent. Towards apex it is branched in an irregular manner and each branch terminates in a cluster of 3-5 closely appressed phialides. Fertile apex.
- Phialides ampulliform, arising in convergent, closely appressed clusters of 3-5.
- Conidia green, ovoid.
- Chlamydospores abundant, both terminal and intercalary.

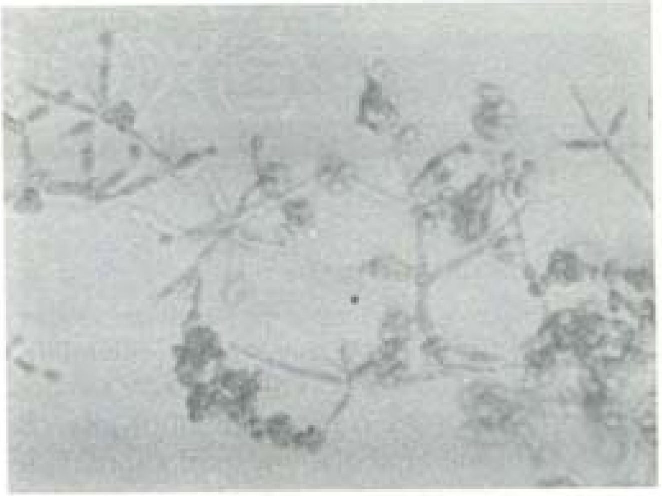
* Shows a unique band of 162 bp with primer OPAA 1 (5'AGACGGCTCC3')

Plate 17

Trichoderma harzianum PBAT 17



- Colony radius on PDA after 72 hours at 25⁰C = 62 mm; at 35⁰C = 22 mm
- Pattern of conidiation: Effuse, sometimes arranged in flat pustules.
- First green conidia visible at 48 h at 25⁰C
- First white conidia formed between 24 to 48 h on PDA at 25⁰C and conidial colour changes from white to light green to dark green.
- Yellow pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



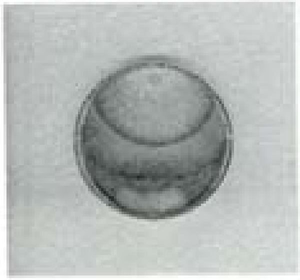
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90⁰ angle and often arising in pairs. Fertile apex.
- Phialides lageniform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

Trichoderma harzianum PBAT 18

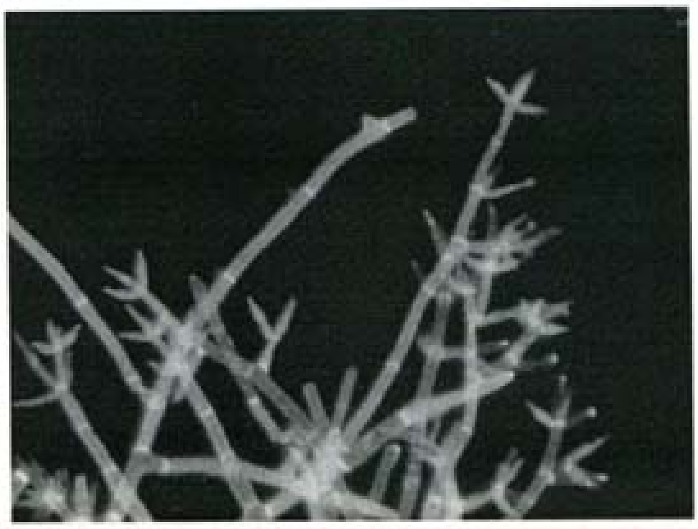
- Colony radius on PDA after 72 hours at 25^{0}C = 57 mm; at 35^{0}C = 21 mm
- Pattern of conidiation: Effuse.
- First green conidia visible after 48 h at 25^{0}C
- First white conidia formed between 24 to 48 h on PDA at 25^{0}C and conidial colour changes from white to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides lageniform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores abundant, both terminal and intercalary.

Trichoderma harzianum PBAT 19



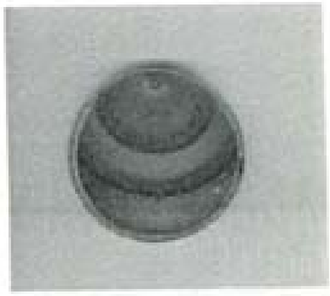
- Colony radius on PDA after 72 hours at 25⁰C = 57 mm; at 35⁰C = 20 mm
- Pattern of conidiation: Effuse, sometimes arranged in flat pustules.
- First green conidia visible after 48 h at 25⁰C
- First white conidia visible formed between 48 to 72 h on PDA at 25⁰C and conidial colour changes from white to yellow green to green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



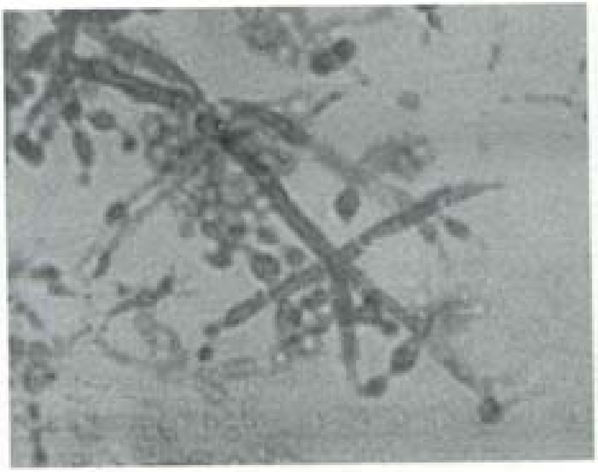
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90⁰ angle and often arising in pairs. Fertile apex.
- Phialides lageniform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present, both terminal and intercalary.

Plate 20

Trichoderma harzianum PBAT 20



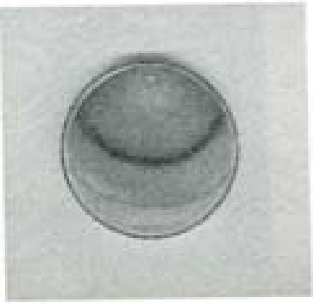
- Colony radius on PDA after 72 hours at 25⁰C = 58 mm; at 35⁰C = 12 mm
- Pattern of conidiation: Effuse.
- First green conidia visible after 48 h at 25⁰C
- First white conidia formed between 24 to 48 h on PDA at 25⁰C and conidial colour changes from white to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



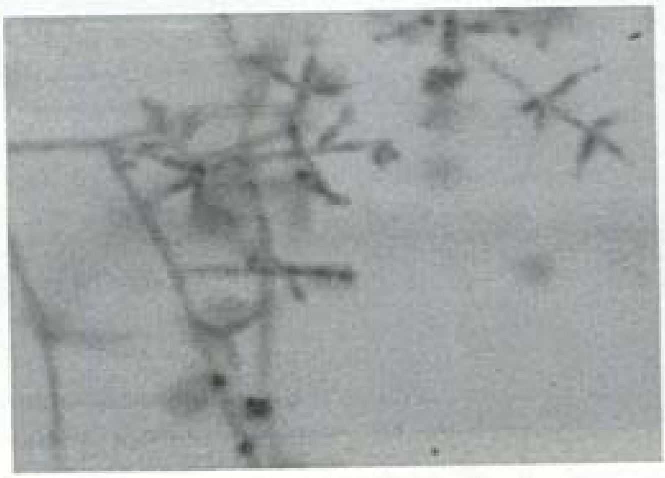
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90⁰ angle and often arising in pairs. Fertile apex.
- Phialides lageniform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

Plate 21

Trichoderma harzianum PBAT 21



- Colony radius on PDA after 72 hours at 25°C = 57 mm; at 35°C = 12 mm
- Pattern of conidiation: Effuse, sometimes arranged in flat pustules.
- First green conidia visible after 48 h at 25°C
- First white conidia formed between 24 to 48 h on PDA at 25°C. Conidial colour changes from white to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

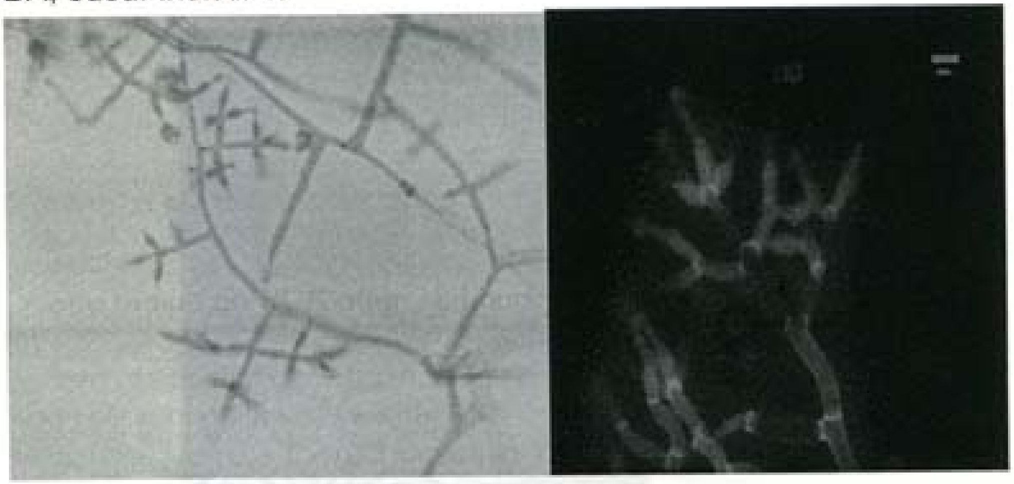


- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90° angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

Trichoderma harzianum PBAT 22

Plate 22

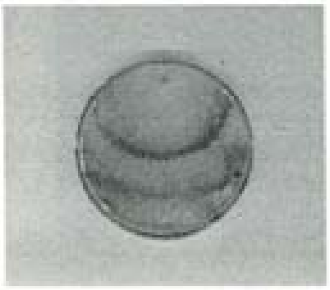
- Colony radius on PDA after 72 hours at 25⁰C = 59 mm; at 35⁰C = 22 mm
- Pattern of conidiation: Effuse.
- First green conidia visible after 48 h at 25⁰C
- First white conidia formed between 24 to 48 h on PDA at 25⁰C and conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90⁰ angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores rare.

Plate 23

Trichoderma harzianum PBAT 23

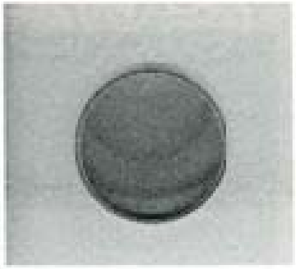


- Colony radius on PDA after 72 hours at 25^0C = 59 mm; at 35^0C =18 mm
- Pattern of conidiation: Effuse, sometimes arranged in flat pustules.
- First green conidia visible after 72 h at 25^0C
- First white conidia formed between 48 to 72 h on PDA at 25^0C and conidial colour changes from white to light green to dark green.
- Yellow pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

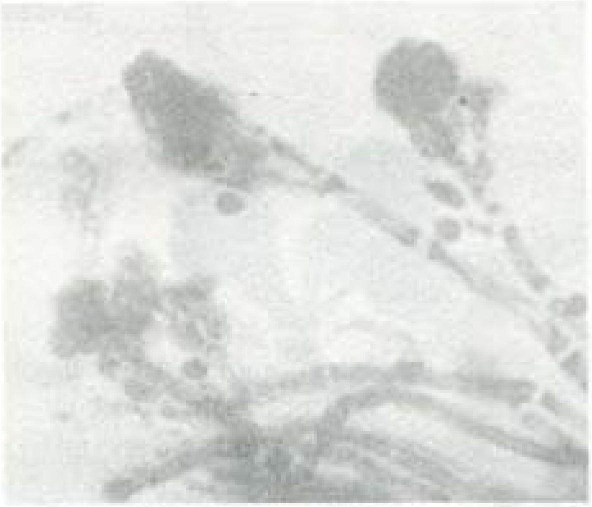
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present, both terminal and intercalary.

Plate 24

Trichoderma virens PBAT 24



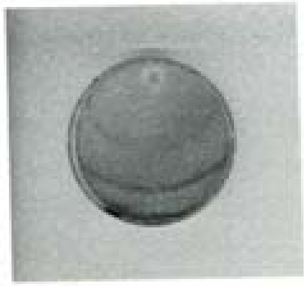
- Colony radius on PDA after 72 hours at 25⁰C = 62 mm; at 35⁰C = 20 mm
- Pattern of conidiation: Predominantly effuse.
- First green conidia visible after 48 h at 25⁰C
- First white conidia visible between 48 to 72 h on PDA at 25⁰C. Conidial colour changes from white to green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



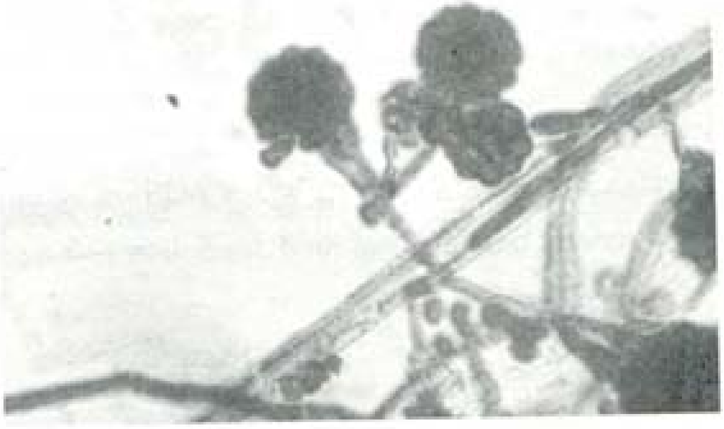
- Conidiophores hyaline, sparingly branched at base, branching often divergent. Towards apex it is branched in an irregular manner and each branch terminates in a cluster of 3-5 closely appressed phialides. Fertile apex.
- Phialides ampulliform, arising in convergent, closely appressed clusters of 3-5.
- Conidia green, ovoid.
- Chlamydospores abundant, both terminal and intercalary.

Plate 25

Trichoderma virens PBAT 25



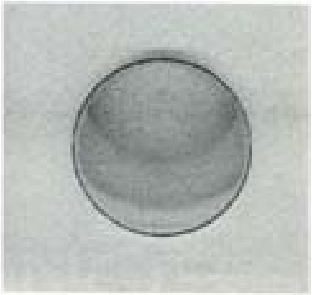
- •Colony radius on PDA after 72 hours at 25⁰C = 62 mm; at 35⁰C = 20 mm
- •Pattern of conidiation: Predominantly effuse.
- •First green conidia visible after 48 h at 25⁰C
- •First white conidia visible between 24 to 48 h on PDA at 25⁰C. Conidial colour changes from white to light green to dark green.
- •No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



- •Conidiophores hyaline, sparingly branched at base, branching often divergent. towards apex it is branched in an irregular manner and each branch terminates in a cluster of 3-5 closely appressed phialides. Fertile apex.
- •Phialides ampulliform, arising in convergent, closely appressed clusters of 3-5.
- •Conidia green, ovoid.
- •Chlamydospores abundant, both terminal and intercalary.

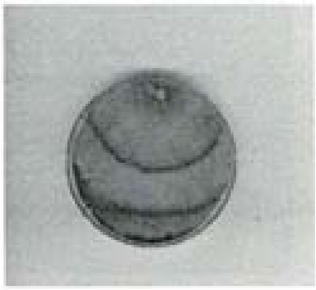
Plate 26

Trichoderma virens PBAT 26

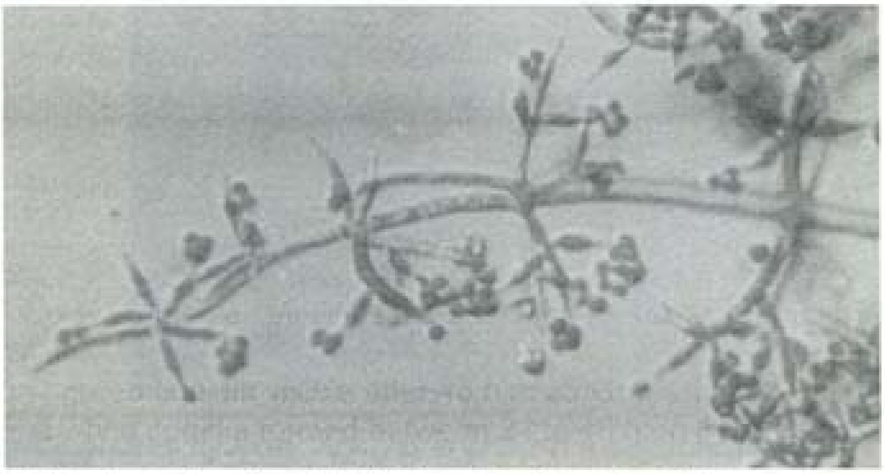


- Colony radius on PDA after 72 hours at 25^{0}C = 61 mm; at 35^{0}C = 20 mm
- Pattern of conidiation: Predominantly effuse.
- First green conidia visible after 48 h at 25^{0}C
- First white conidia formed between 24 to 48 h on PDA at 25^{0}C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.
- Conidiophores hyaline, sparingly branched at base, branching often divergent. Towards apex it is branched in an irregular manner and each branch terminates in a cluster of 3-5 closely appressed phialides. Fertile apex.
- Phialides ampulliform, arising in convergent, closely appressed clusters of 3-5.
- Conidia green, ovoid.
- Chlamydospores abundant, both terminal and intercalary.

Trichoderma harzianum PBAT 27



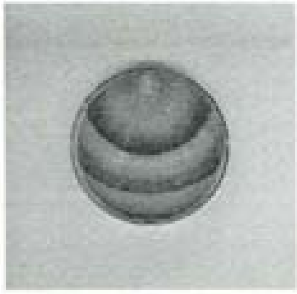
- Colony radius on PDA after 72 hours at 25⁰C = 57 mm; at 35⁰C = 20 mm
- Pattern of conidiation: Effuse, sometimes loosely arranged in flat pustules.
- First green conidia visible after 48 h at 25⁰C
- First white conidia formed between 24 to 48 h on PDA at 25⁰C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



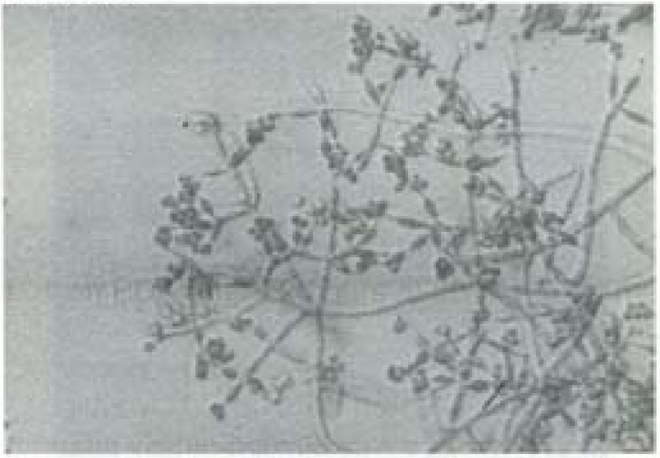
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90⁰ angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

*Showed a unique band of 295 bp with primer OPBB 11 (5'TGCGGGTTCC3')

Trichoderma harzianum PBAT 28



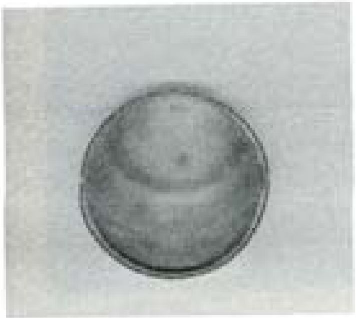
- Colony radius on PDA after 72 hours at 25^{0}C = 57 mm; at 35^{0}C = 20 mm
- Pattern of conidiation: Effuse.
- First green conidia visible after 48 h at 25^{0}C
- First white conidia visible between 24 to 48 h on PDA at 25^{0}C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



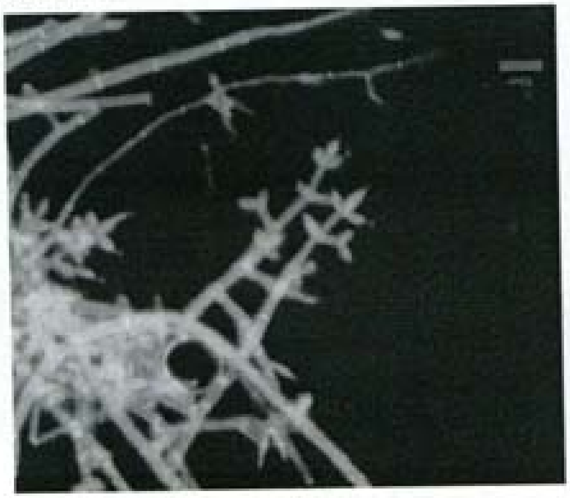
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

Plate 29

Trichoderma harzianum PBAT 29



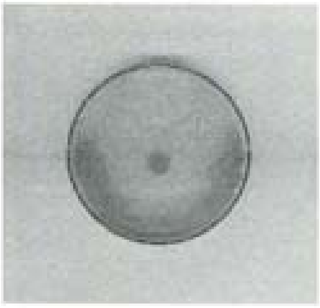
- Colony radius on PDA after 72 hours at 25^{0}C = 57 mm; at 35^{0}C = 20 mm
- Pattern of conidiation: Effuse.
- First green conidia visible after 48 h at 25^{0}C
- First white conidia visible between 24 to 48 h on PDA at 25^{0}C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



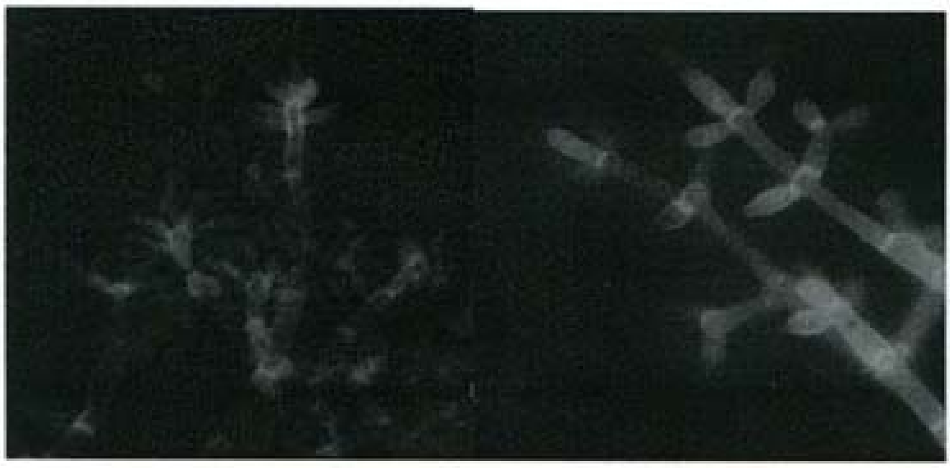
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

Plate 30

Trichoderma harzianum PBAT 30

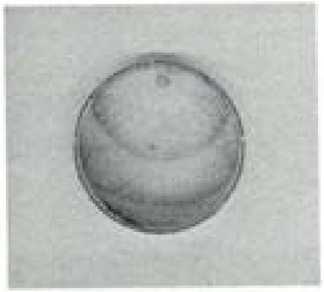


- Colony radius on PDA after 72 hours at 25⁰C = 57 mm; at 35⁰C = 20 mm
- Pattern of conidiation: Effuse
- First green conidia visible after 48 h at 25⁰C
- First white conidia formed between 24 to 48 h on PDA at 25⁰C. Conidial colour changes from white to light green to dark green.
- Yellow pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

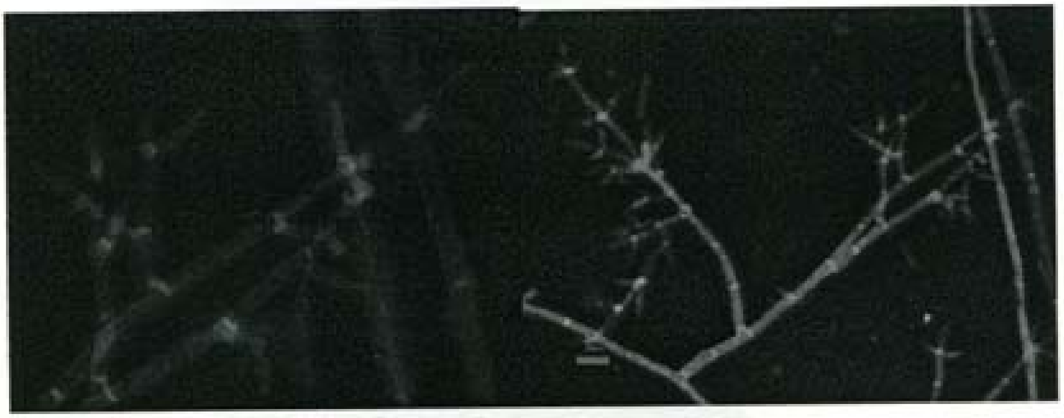


- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90⁰ angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores abundant, terminal and intercalary.

Trichoderma harzianum PBAT 31



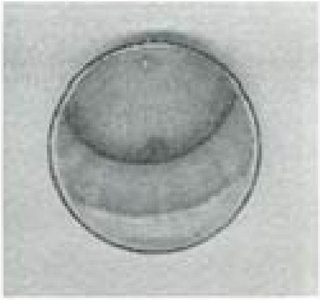
- Colony radius on PDA after 72 hours at 25^{0}C = 57 mm; at 35^{0}C = 20 mm
- Pattern of conidiation: Effuse, sometimes loosely arranged in flat pustules.
- First green conidia visible after 48 h at 25^{0}C
- First white conidia visible between 24 to 48 h on PDA at 25^{0}C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



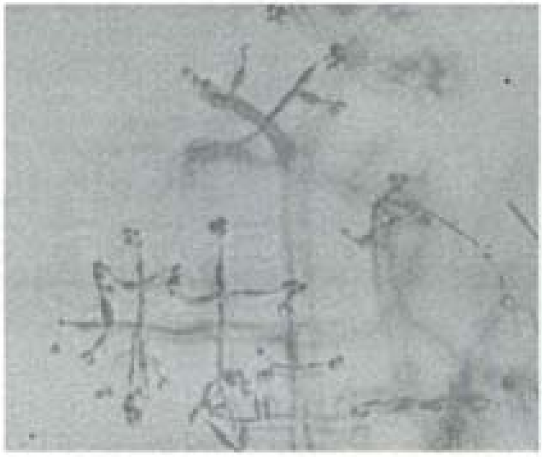
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

Plate 32

Trichoderma harzianum PBAT 32

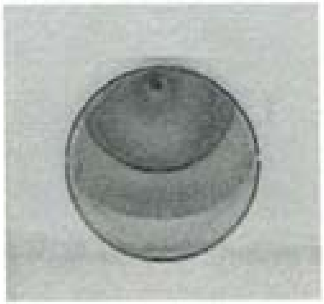


- •Colony radius on PDA after 72 hours at 25^{0}C = 57 mm; at 35^{0}C = 21 mm
- •Pattern of conidiation: Effuse, sometimes loosely arranged in flat pustules.
- •First green conidia visible after 48 h at 25^{0}C
- •First white conidia visible between 24 to 48 h on PDA at 25^{0}C. Conidial colour changes from white to light green to dark green.
- •No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

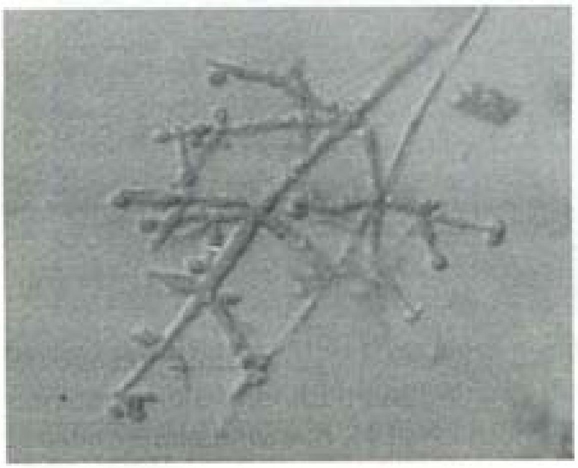


- •Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^{0} angle and often arising in pairs. Fertile apex.
- •Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- •Conidia green, ovoid.
- •Chlamydospores present but not abundant, mostly intercalary.

Trichoderma harzianum PBAT 33



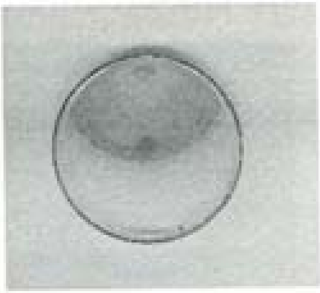
- Colony radius on PDA after 72 hours at 25^{0}C = 58 mm; at 35^{0}C = 18 mm
- Pattern of conidiation: Effuse.
- First green conidia visible after 48 h at 25^{0}C
- First white conidia visible between 24 to 48 h on PDA at 25^{0}C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



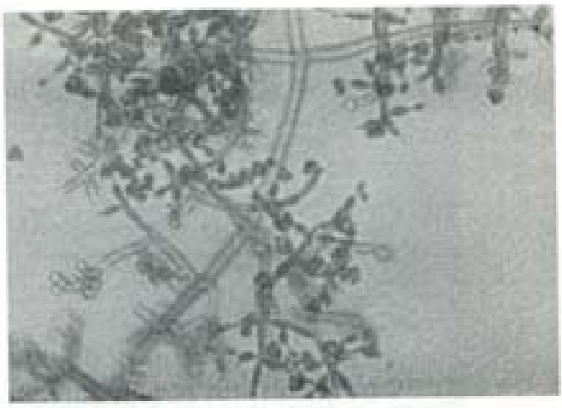
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

Plate 34

Trichoderma harzianum PBAT 34



- Colony radius on PDA after 72 hours at 25^{0}C = 59 mm; at 35^{0}C = 15 mm
- Pattern of conidiation: Effuse
- First green conidia visible after 48 h at 25^{0}C
- First white conidia formed between 24 to 48 h on PDA at 25^{0}C. Conidial colour changes from white to light green to .dark green.
- Yellow pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

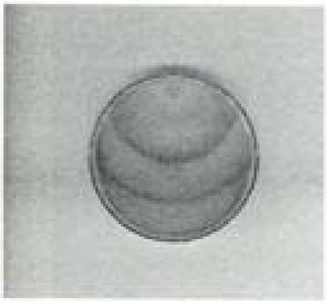


- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^{0} angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

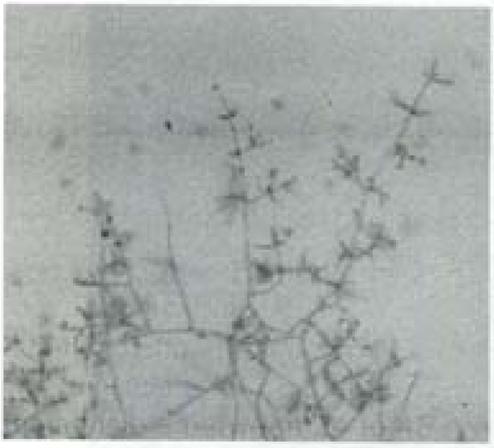
*This isolate showed 19 unique bands with various primers:

OPAA 4 (5' AGGACTGCTC3') 3 bands of 287 bp, 289 bp, 298 bp
OPAA 6 (5' GTGGGTGCCA3') 2 bands of 263 bp, 246 bp
OPAA 7 (5' CTACGCTCAC3') 1 band of 637 bp
OPAA 12 (5' GGACCTCTTG3') 2 bands of 216 bp and 545 bp
OPAA 16 (5' GGAACCCACA3') 2 bands of 329 bp, 508 bp
OPBB 2 (5' CCCCCGTTAG3') 1 band of 580 bp
OPBB 5 (5'GGGCCGAACA3') 2 bands of 325 bp and 423 bp
OPBB 6 (5'CTGAAGCTGG3') 1 band of 151 bp
OPBB 10 (5'ACTTGCCTGG3') 2 bands of 140 bp and 332 bp
OPBB 13 (5'CTTCGGTGTG3') 2 bands of 418 bp and 534 bp
OPB 8 (5'ACCGCGAAGG3') 1 band of 299 bp

Trichoderma harzianum PBAT 35



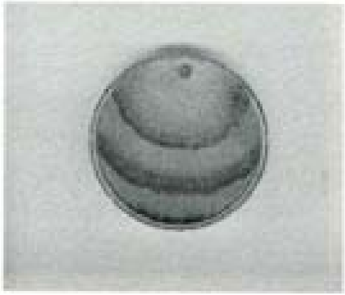
- Colony radius on PDA after 72 hours at 25⁰C = 56 mm; at 35⁰C = 18 mm
- Pattern of conidiation: Effuse, sometimes arranged in flat pustules.
- First green conidia visible after 48 h at 25⁰C
- First white conidia visible between 24 to 48 h on PDA at 25⁰C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



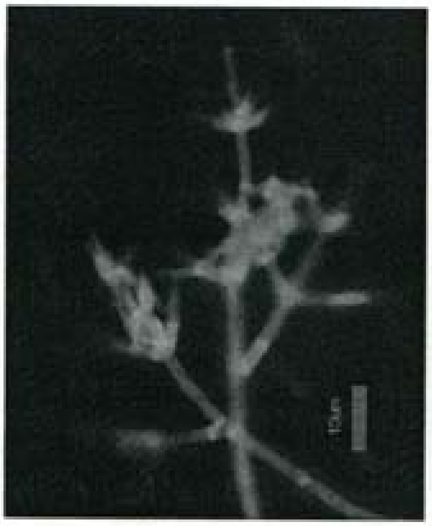
- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90⁰ angle and often arising in pairs. Fertile apex.
- Phialides ampulliform arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

Plate 36

Trichoderma harzianum PBAT 36



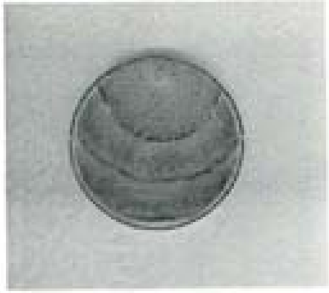
- •Colony radius on PDA after 72 hours at 25^{0}C = 55 mm; at 35^{0}C = 20 mm
- •Pattern of conidiation: Effuse, sometimes arranged in flat pustules.
- •First green conidia visible after 48 h at 25^{0}C
- •First white conidia visible between 24 to 48 h on PDA at 25^{0}C. Conidial colour changes from white to light green to dark green.
- •No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



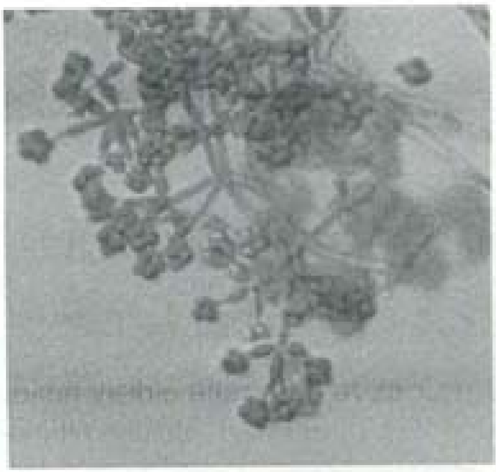
- •Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- •Phialides ampuliform, arising in crowded and divergent verticils of 3-5.
- •Conidia green, ovoid.
- •Chlamydospores abundant, both terminal and intercalary.

Plate 37

Trichoderma harzianum PBAT 37

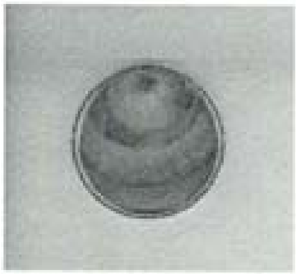


- Colony radius on PDA after 72 hours at 25^0C = 59 mm; at 35^0C = 20 mm
- Pattern of conidiation: Effuse, sometimes loosely arranged in flat pustules.
- First green conidia visible after 48 h at 25^0C
- First white conidia visible between 24 to 48 h on PDA at 25^0C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

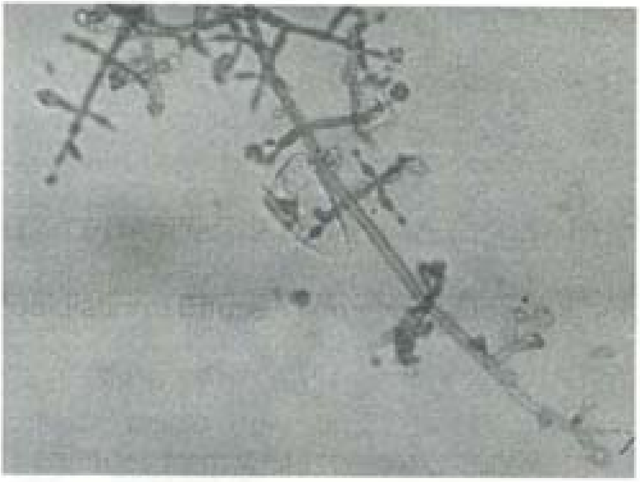


- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialidesampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

Trichoderma harzianum PBAT 38

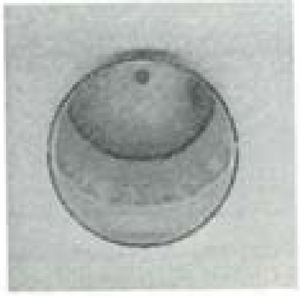


- Colony radius on PDA after 72 hours at 25^0C = 59 mm; at 35^0C = 20 mm
- Pattern of conidiation: Effuse, sometimes loosely arranged in flat pustules.
- First green conidia visible after 48 h at 25^0C
- First white conidia visible between 24 to 48 h on PDA at 25^0C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present.

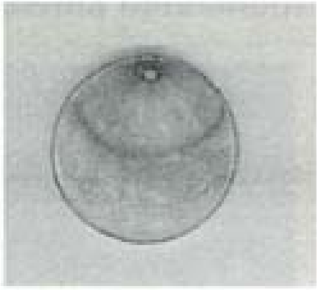
Trichoderma harzianum PBAT 39



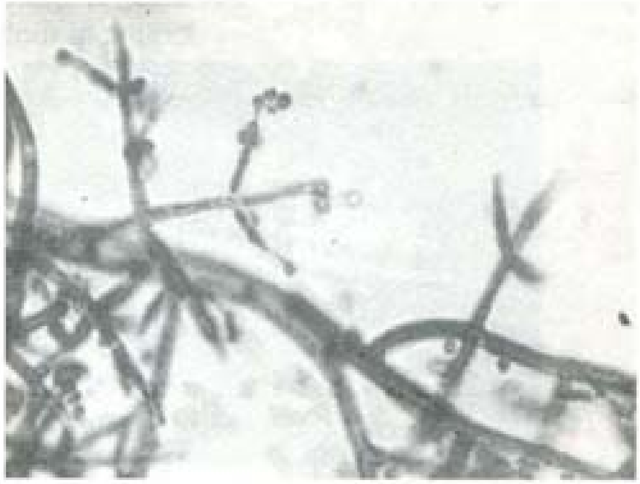
- Colony radius on PDA after 72 hours at 25⁰C = 57 mm; at 35⁰C = 19 mm
- Pattern of conidiation: Effuse.
- First green conidia visible after 48 h at 25⁰C
- First white conidia visible between 24 to 48 h on PDA at 25⁰C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 900 angle and often arising in pairs. Fertile apex.
- Phialides lageniform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores abundant, terminal and intercalary.

Trichoderma harzianum PBAT 40

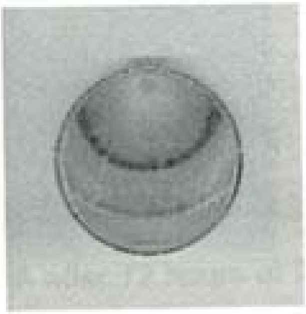


- Colony radius on PDA after 72 hours at 25⁰C = 59 mm; at 35⁰C = 18 mm
- Pattern of conidiation: Effuse, sometimes arranged in flat pustules.
- First green conidia visible after 48 h at 25⁰C
- First white conidia visible between 24 to 48 h on PDA at 25⁰C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

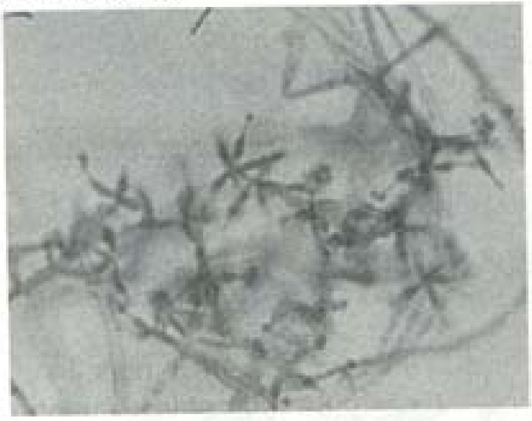


- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 900 angle and often arising in pairs. Fertile apex.
- Phialides flask shaped, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.
- *Showed a unique band of 385 bp with primer OPAA 1 (5'AGACGGCTCC3')
- *Showed a unique band of 340 bp with primer OPBB 2 (5' CCCCCGTTAG 3')

Trichoderma harzianum PBAT 41

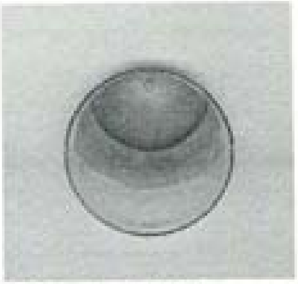


- Colony radius on PDA after 72 hours at 25⁰C = 59 mm; at 35⁰C = 18 mm
- Pattern of conidiation: Effuse, sometimes arranged in flat pustules.
- First green conidia visible after 48 h at 25⁰C
- First white conidia visible between 24 to 48 h on PDA at 25⁰C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 900 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores rare.

Trichoderma harzianum PBAT 42



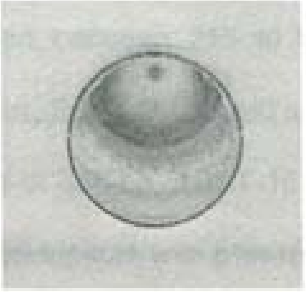
- Colony radius on PDA after 72 hours at 25⁰C = 58 mm; at 35⁰C = 18 mm
- Pattern of conidiation: Effuse, sometimes loosely arranged in flat pustules.
- First green conidia visible after 48 h at 25⁰C
- First white conidia visible between 24 to 48 h on PDA at 25⁰C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.

- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90⁰ angle and often arising in pairs. Fertile apex.
- Phialides ampulliform, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores abundant.

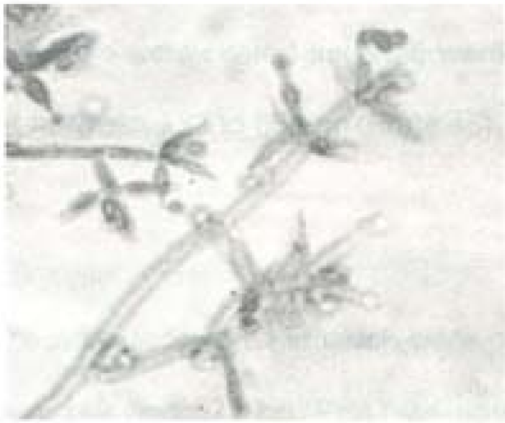
- Showed a unique band of 372 bp with primer OPAA 1 (5'AGACGGCTCC3')
- Showed a unique band of 208 bp with primer OPBB 5 (5'GGGCCGAACA3')

Plate 43

Trichoderma harzianum PBAT 43



- Colony radius on PDA after 72 hours at 25^0C = 55-59 mm; at 35^0C = 15-20 mm
- Pattern of conidiation: Effuse, sometimes loosely arranged in flat pustules.
- First green conidia visible after 48 h at 25^0C
- Pattern of conidiation: Concentric rings with sterile centre
- First white conidia visible between 24 to 48 h on PDA at 25^0C. Conidial colour changes from white to light green to dark green.
- No pigmentation observed on CMD, reverse of colony colourless on PDA, odour indistinct.



- Conidiophores hyaline, highly branched in a dendritic manner, branching mostly at 90^0 angle and often arising in pairs. Fertile apex.
- Phialides ampulliform,, arising in crowded and divergent verticils of 3-5.
- Conidia green, ovoid.
- Chlamydospores present but not abundant, mostly intercalary.

*Showed a unique band of 399 bp with primer OPAA 1 (5'AGACGGCTCC3')

Primer OPAA 1

Total number of 16 RAPD's were scored out of which all 16 were polymorphic. The product size ranged between 215 to 564 bp. Four unique bands were generated with the primer. PBAT-8 showed a unique band of 231 bp, PBAT-40 exhibited a unique band of 385 bp, PBAT-16 showed a unique band of 1622 bp and a unique band of 399 bp size was present in PBAT-43 (Plate 44).

OPAA 2

A total of 3 RAPD's were scored out of which all 3 were polymorphic. The product size ranged between 273 to 591 bp (Plate 44).

OPAA 3

Total number of 6 RAPD's were scored and all 6 were found polymorphic. The product size ranged between 132 to 554 bp (Plate 45).

OPAA 4

A total of 12 RAPD's were scored, all of which were polymorphic. The product size ranged between 148 and 573 bp. PBAT-34 gave three unique bands of size 289, 288 and 298 bp (Plate 45).

OPAA 6

Out of a total 9 RAPD's scored, all 9 were polymorphic. The product size ranged between 114 to 445 bp. Two unique bands of size 263 bp and 406 bp were generated for PBAT-34 (Plate 46).

OPAA 7

Total number of 4 RAPD's were scored out of which all 4 were polymorphic. The product size ranged between 172 and 757 bp. PBAT-34 showed a unique band of size 637 bp (Plate 46).

OPAA 12

A total of 5 polymorphic RAPD's were scored. The product size ranged between 178 to 548 bp. Two unique bands of 216 bp and 545 bp size were generated for PBAT-34 (Plate 47).

OPAA 14

Total number of 6 RAPD's were scored out of all which were polymorphic. The product size ranged between 134 and 595 bp (Plate 47).

OPAA 15

Out of the 5 RAPD's scored, all 5 were observed to be polymorphic. The product size ranged between 103 and 438 bp (Plate 48).

OPAA 16

A total of 9 polymorphic bands were scored. The product size ranged between 132 and 670 bp. PBAT-34 showed two unique bands of 329 bp and 508 bp (Plate 48).

OPBB 2

Total number of 6 RAPD's were scored out of which all 6 were polymorphic. The product size ranged between 163 to 580 bp. Two unique bands of size 580 bp and 340 bp were shown by PBAT-34 and PBAT-40, respectively (Plate 49).

OPBB 3

A total of 7 polymorphic RAPD's were scored. The product size ranged between 143 to 566 bp (Plate 49).

OPBB 5

Total number of 13 RAPD's were scored out of which all 13 were polymorphic. The product size ranged between 120 to 507 bp. Three unique bands were generated. PBAT-34 showed two unique bands of size 325 bp and 433 bp. PBAT-42 showed a unique band of 208bp (Plate 50).

OPBB 6

A total of 9 RAPD's were scored out of which all 9 were polymorphic. The product size ranged between 151 to 424 bp. PBAT-34 showed a unique band of size 151 bp (Plate 50).

OPBB 9

Total number of 10 RAPD's were scored out of which all 10 were polymorphic. The product size ranged between 135 to 451 bp (Plate 51).

OPBB 10

A total of 7 RAPD's were scored and all 7 were observed to be polymorphic. The product size ranged between 140 to 762 bp. Two unique bands of 140 bp and 332 bp were shown by PBAT-34 (Plate 51).

OPBB 11

Total number of 5 RAPD's were scored out of which all 5 were polymorphic. The product size ranged between 155 to 351 bp. One unique band of size 295 bp was shown by PBAT-27 (Plate 52).

OPBB 12

Total number of 5 RAPD's were scored out of which all 5 were polymorphic. The product size ranged between 180 to 635 bp (Plate 52).

OPBB 13

A total of 6 RAPD's were scored out of which all 6 were polymorphic. The product size ranged between 169 to 683 bp. PBAT-34 generated two unique bands of size 418 and 534 bp (Plate 53).

OPBB 14

Total number of 3 RAPD's were scored out of which 2 were polymorphic and 1 was monomorphic. The product size ranged between 123 to 276 bp (Plate 53).

OPB 7

A total of 6 polymorphic RAPD's were scored. The product size ranged between 255 to 452 bp (Plate 54).

OPB 8

Total number of 6 RAPD's were scored out of which all 6 were polymorphic. The product size ranged between 74 to 491 bp. One unique band of size 299 bp was shown by PBAT-34 (Plate 54).

For the 5 isolates of *T. virens* alone, the 22 random primers generated a total of 59 RAPDs out of which only 5 were polymorphic including one unique band and 54 were monomorphic. The unique band was generated with primer OPAA 1 (Size 162 bp) for PBAT-16. Among the 59 scorable bands generated with various *T. virens* isolates, 19 bands were common with various isolates of *T. harzianum*. The remaining 40 bands were generated only in the 5 *T. virens* isolates and in none of the *T. harzianum* isolates. In case of *T. harzianum* isolates alone, the 22 primers generated a total of 118 bands. Out of these, 113 were polymorphic and only 5 were monomorphic for the 41 *T. harzianum* isolates.

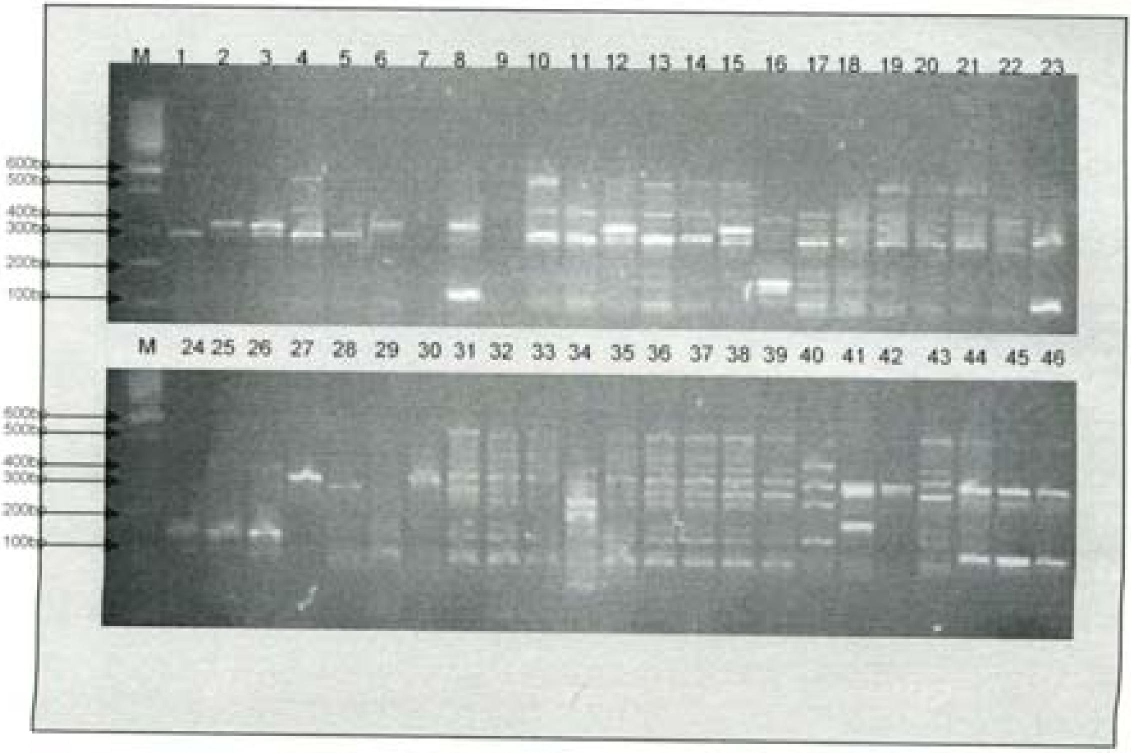
UPGMA cluster analysis was used to produce a dendrogram that separated the 46 isolates into 6 major groups (Figure 1). The first cluster was comprised of all the 5 *T. virens* isolates. This cluster shared only 6% similarity with the remaining 41 isolates identified as *T. harzianum*. The *T. virens* cluster

Table 2: RAPD analysis of forty six isolates of *Trichoderma* species

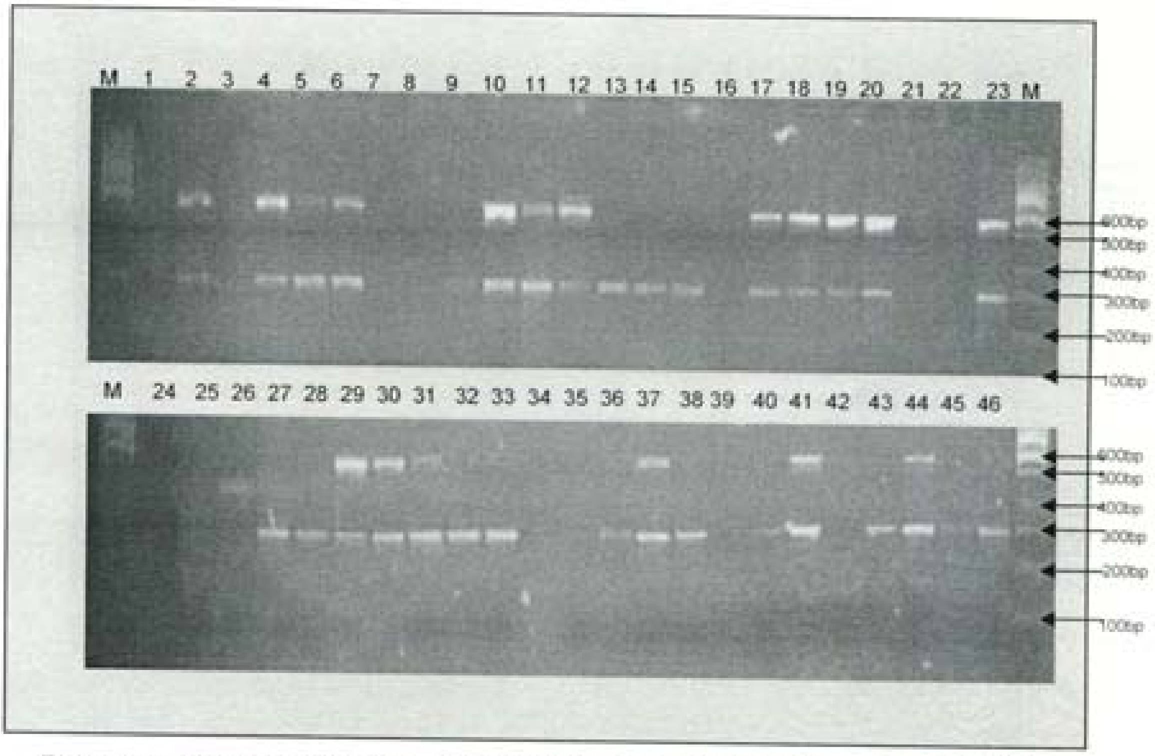
S.No	**Primer code**	**Primer sequences**	**TB**	**M**	**Polymorphism**		**Exclusive Bands**		
					Bands	**%**	**No.**	**Isolate**	**Mol Size (bp)**
1	OPAA1	AGACGGCTCC	16	0	16	100	4	PBAT-8 PBAT 40 PBAT 16 PBAT 43	231 385 162 399
2	OPAA2	GAGACCAGAC	3	0	3	100	0	-	-
3	OPAA3	TTAGCGCCCC	6	0	6	100	0	-	-
4	OPAA4	AGGACTGCTC	12	0	12	100	3	PBAT 34	287 289 298
5	OPAA6	GTGGGTGCCA	9	0	9	100	2	PBAT34	263 406
6	OPAA7	CTACGCTCAC	4	0	4	100	1	PBAT 34	637
7	OPAA12	GGACCTCTTG	5	0	5	100	2	PBAT34	216 545
8	OPAA14	AACGGGCCAA	6	0	6	100	0	-	-
9	OPAA15	ACGGAAGCCC	5	0	5	100	0	-	-
10	OPAA16	GGAACCCACA	9	0	9	100	2	PBAT 34	329 508
11	OPBB2	CCCCCGTTAG	6	0	6	100	2	PBAT 34 PBAT 40	580 340
12	OPBB3	TCACGTGGCT	7	0	7	100	0	-	-
13	OPBB5	GGGCCGAACA	13	0	13	100	3	PBAT 34 PBAT 42	325 433 208
14	OPBB6	CTGAAGCTGG	9	0	9	100	1	PBAT 34	151
15	OPBB9	AGGCCGGTCA	10	0	10	100	0	-	-
16	OPBB10	ACTTGCCTGG	7	0	7	100	2	PBAT 34	140 332
17	OPBB11	TGCGGGTTCC	5	0	5	100	1	PBAT 27	295
18	OPBB12	TTCGGCCGAC	5	0	5	100	0	-	-
19	OPBB13	CTTCGGTGTG	6	0	6	100	2	PBAT 34	418 534
20	OPBB14	GTGGGACCTG	3	1	2	66.6	0	-	-
21	OPB7	GAAGGCTGGG	6	0	6	100	0	-	-
22	OPB8	TCGTCGAAGG	6	0	6	100	1	PBAT 34	299
			158	**1**	**157**	**98.4**	**26**		

TB= Total Bands, M= Monomorphic bands

Plate 44

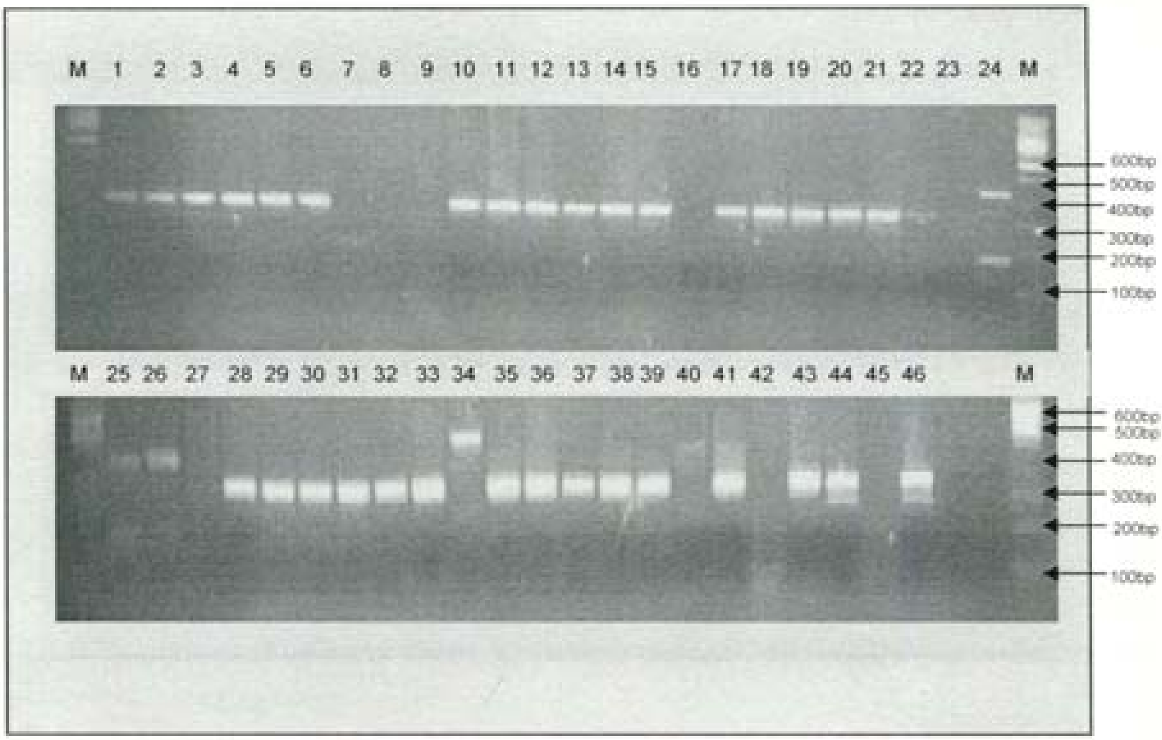


RAPD profile generated in 46 *Trichoderma* isolates with primer OPAA 1

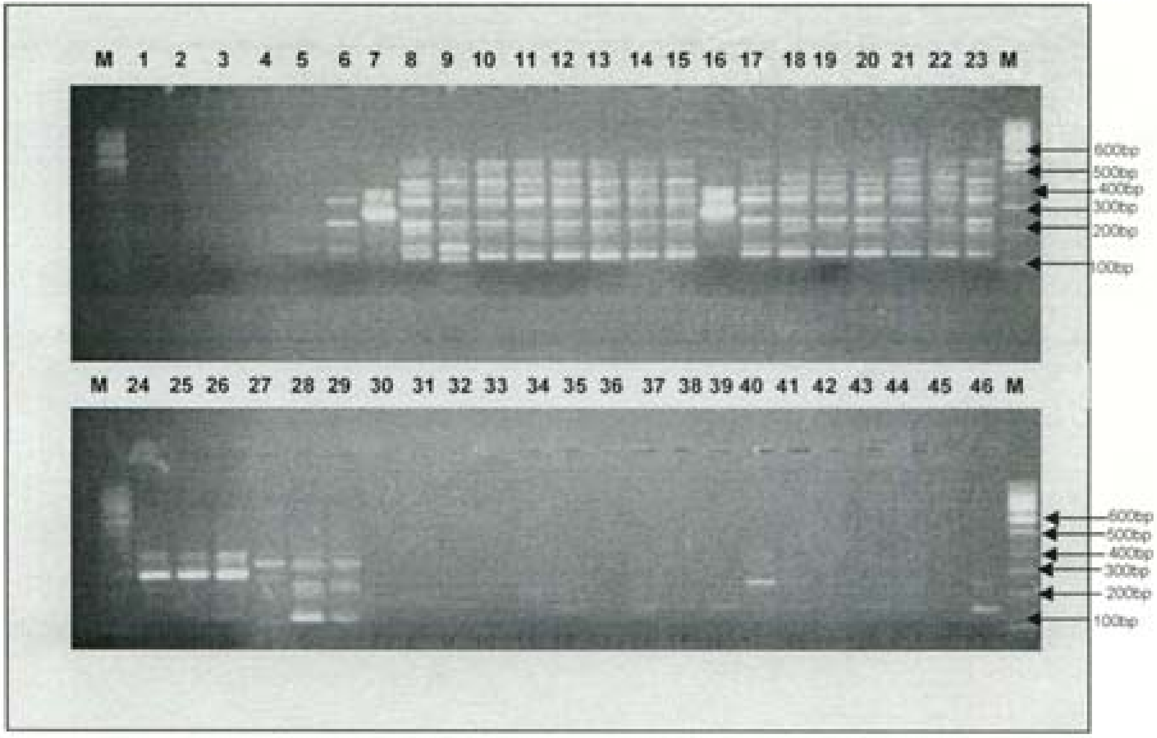


RAPD profile generated in 46 *Trichoderma* isolates with primer OPAA 2

Plate 45

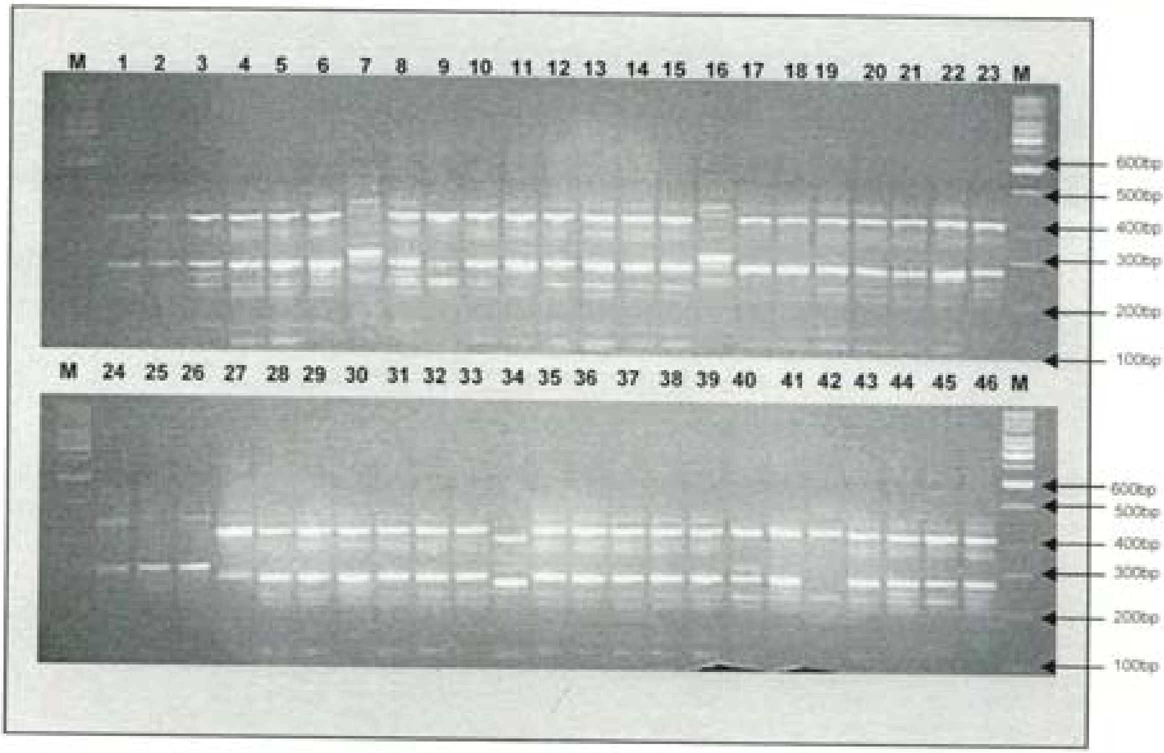


RAPD profile generated in 46 *Trichoderma* isolates with primer OPAA 12

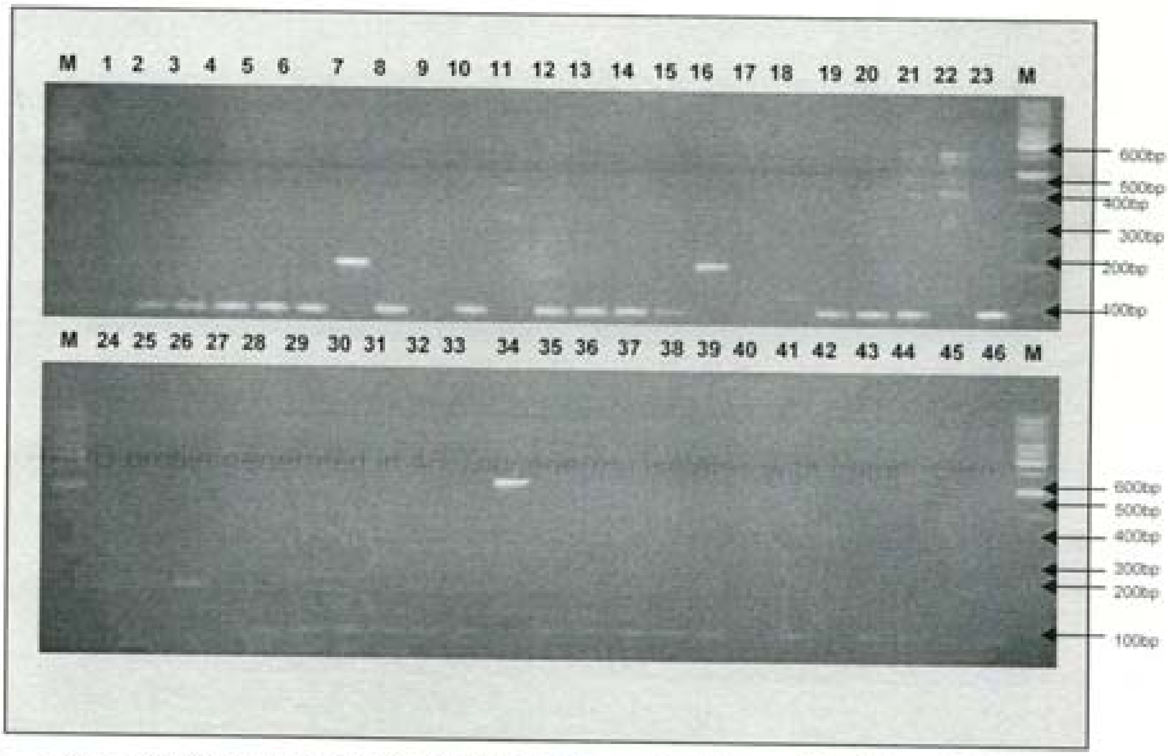


RAPD profile generated in 46 *Trichoderma* isolates with primer OPAA 14

Plate 46

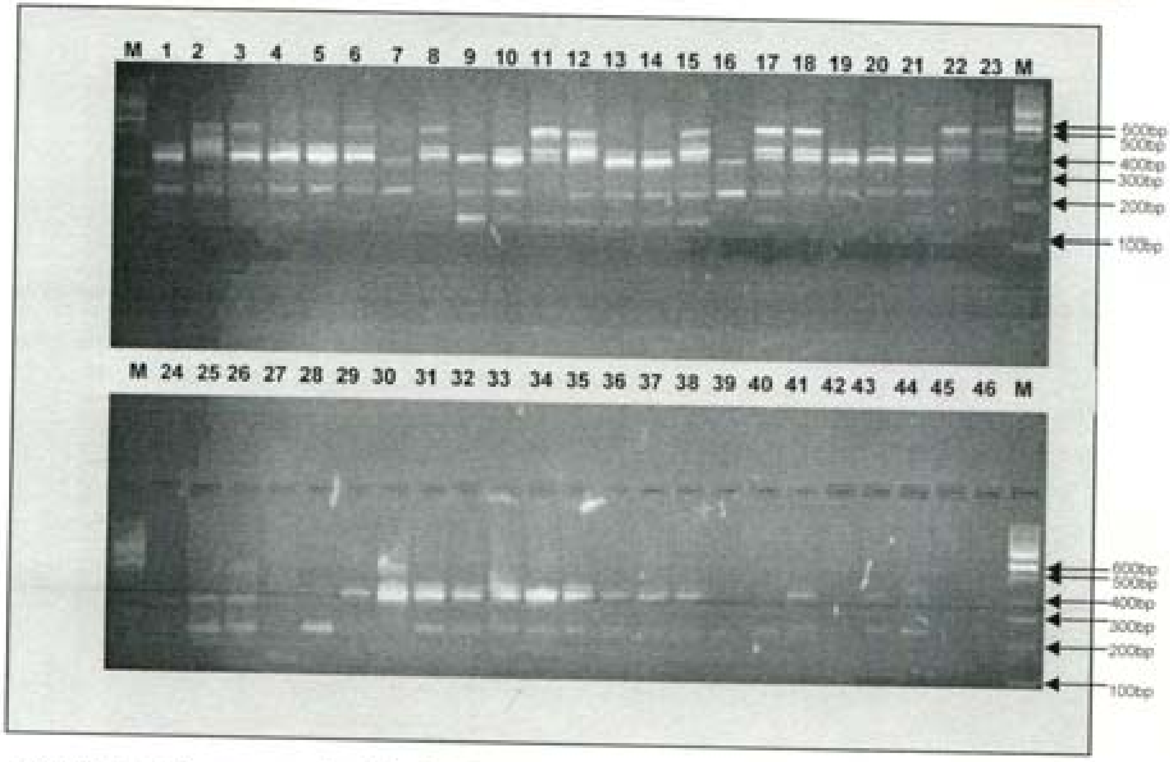


RAPD profile generated in 46 *Trichoderma* isolates with primer OPAA 6

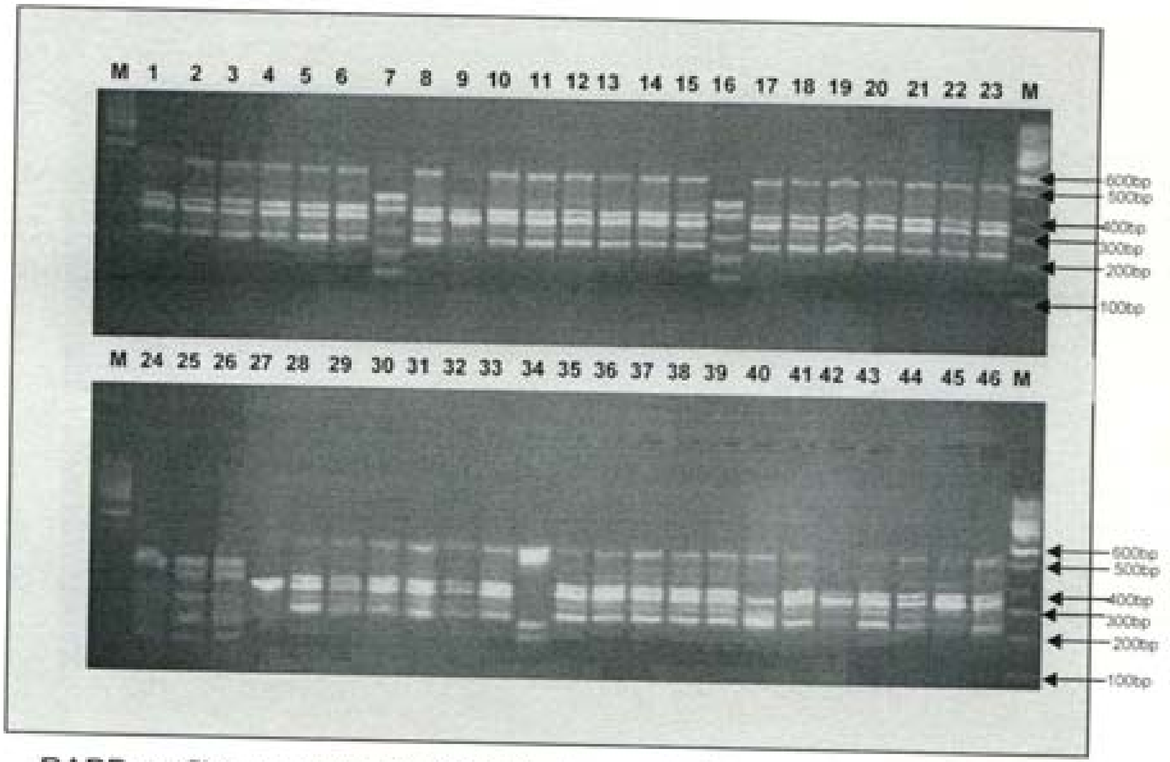


RAPD profile generated in 46 *Trichoderma* isolates with primer OPAA 7

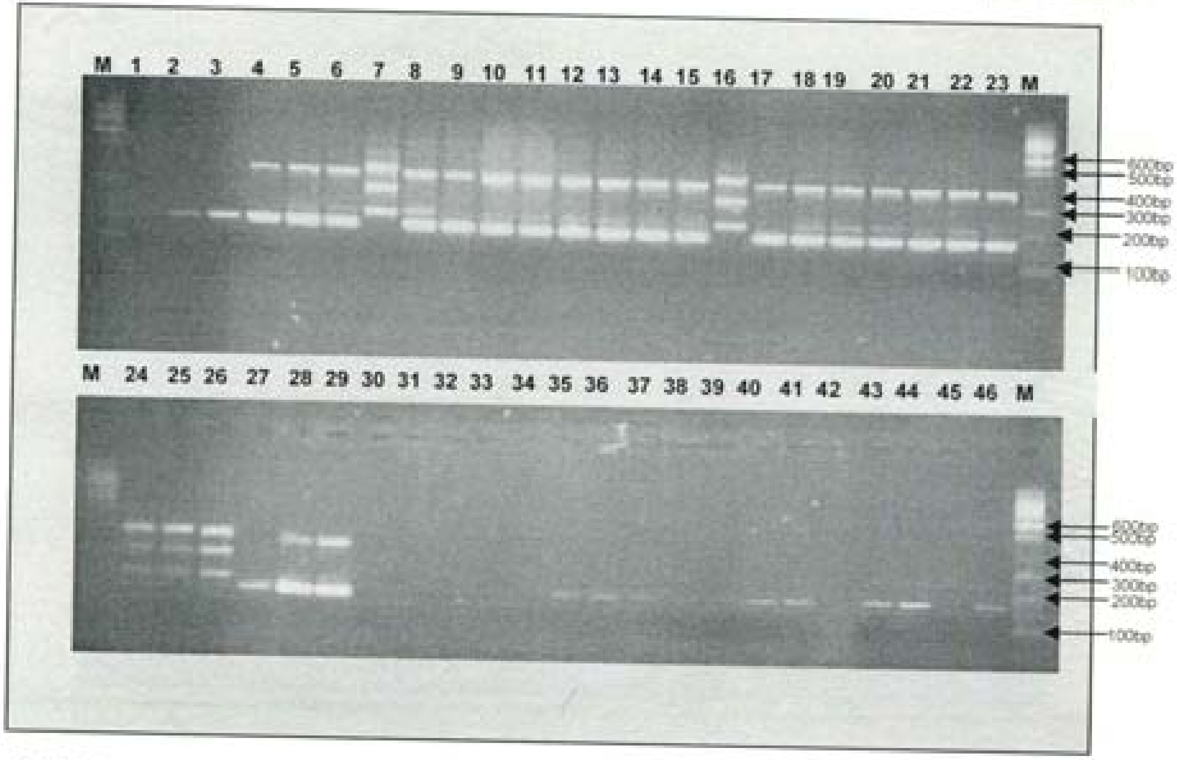
Plate 47



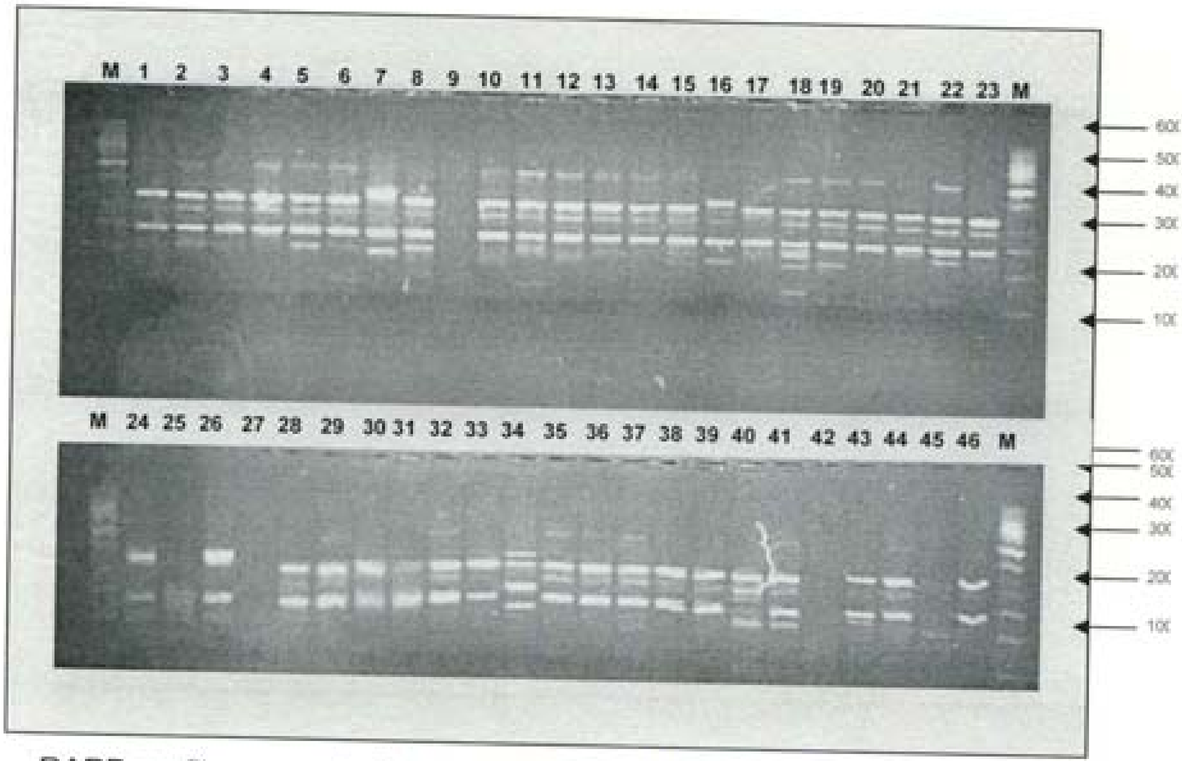
RAPD profile generated in 46 *Trichoderma* isolates with primer OPAA 3



RAPD profile generated in 46 *Trichoderma* isolates with primer OPAA 4

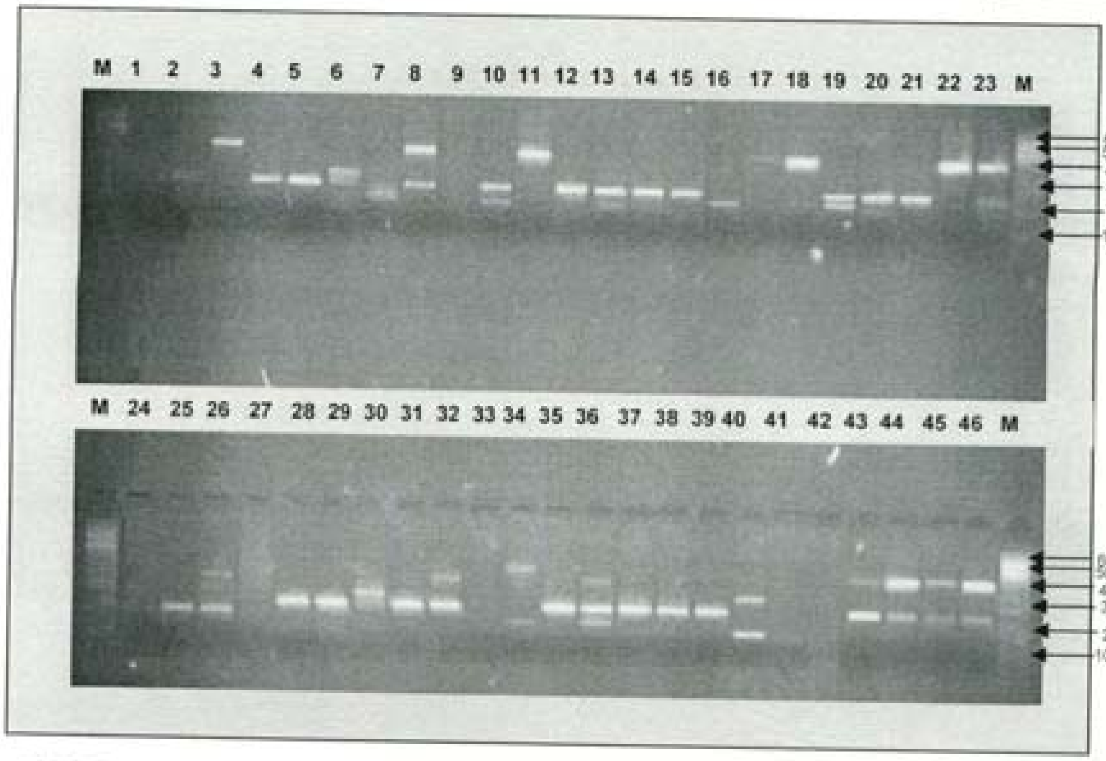


RAPD profile generated in 46 *Trichoderma* isolates with primer OPAA 15

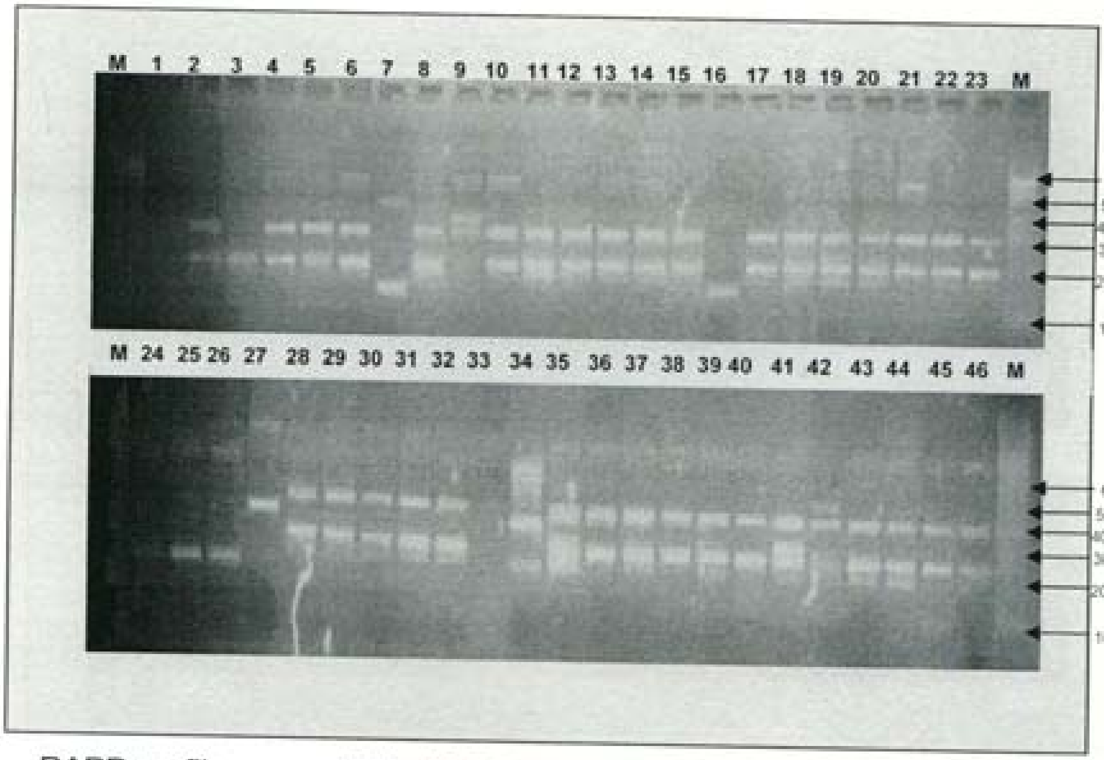


RAPD profile generated in 46 *Trichoderma* isolates with primer OPAA 16

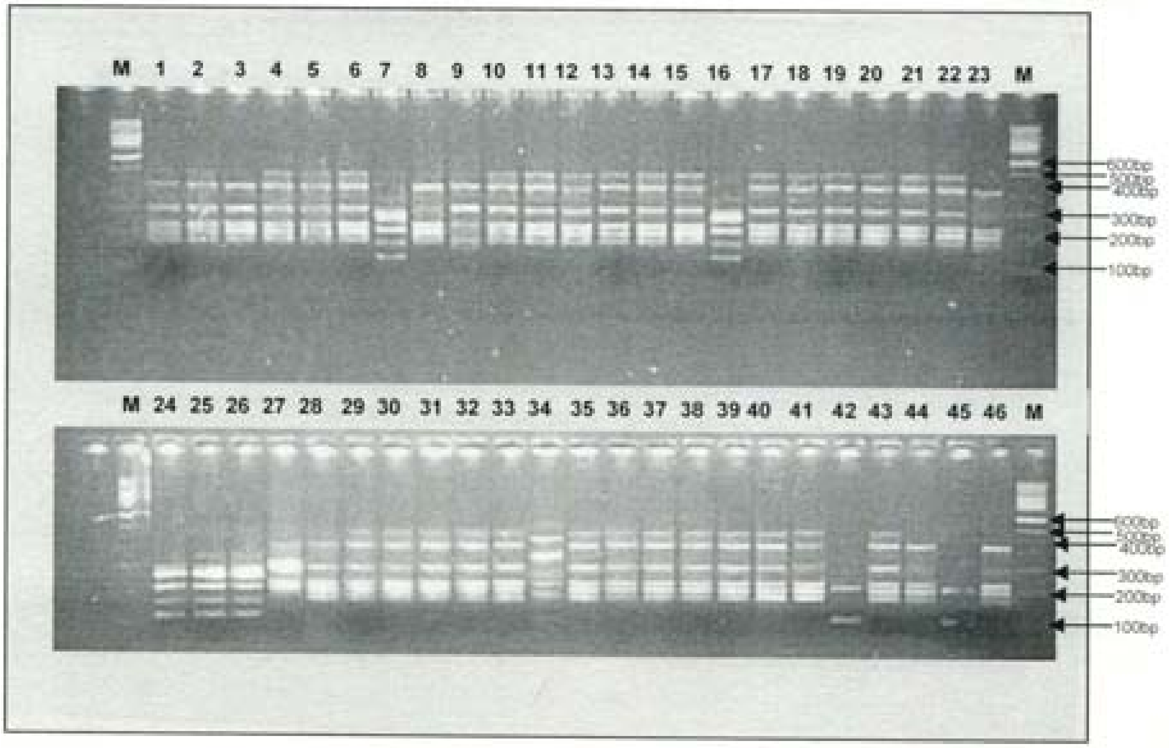
Plate 49



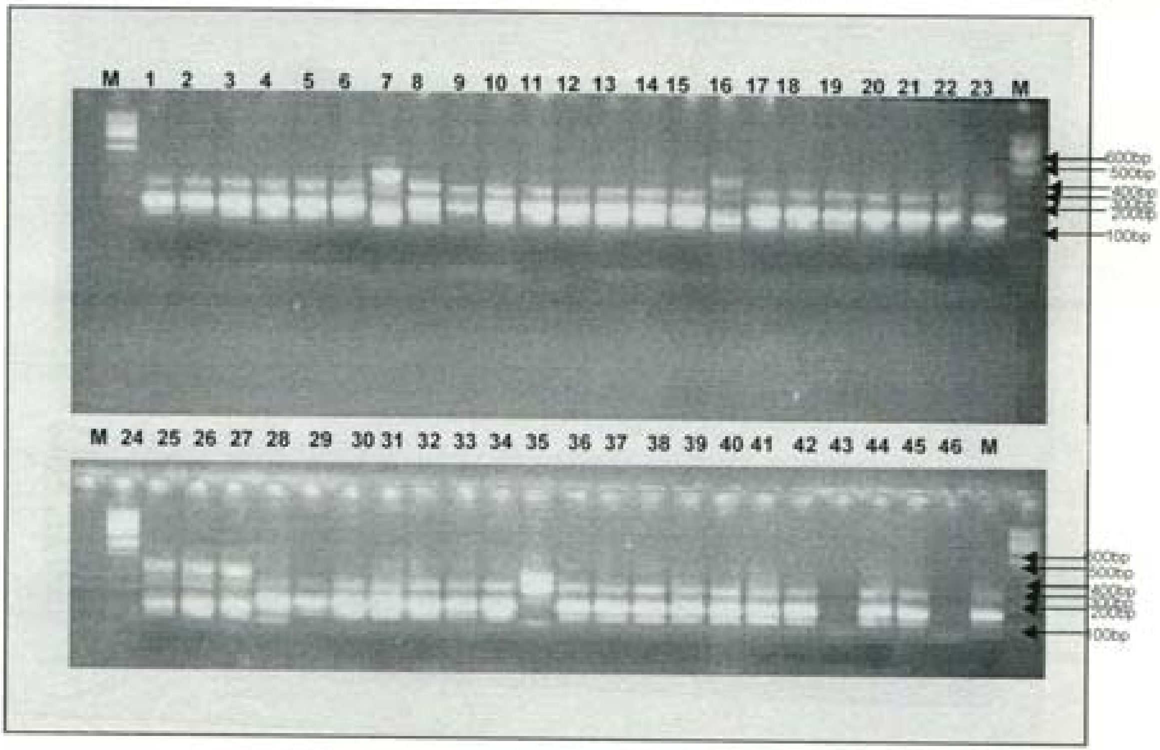
RAPD profile generated in 46 *Trichoderma* isolates with primer OPBB 2



RAPD profile generated in 46 *Trichoderma* isolates with primer OPBB 3

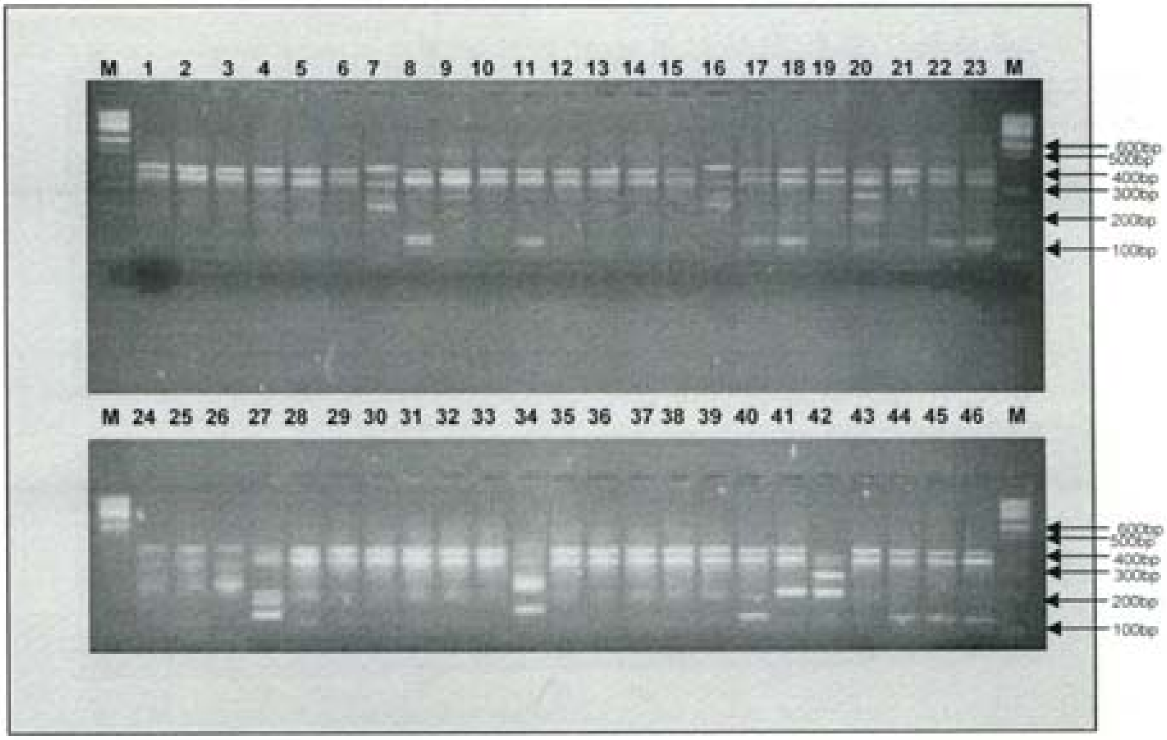


RAPD profile generated in 46 *Trichoderma* isolates with primer OPBB 5

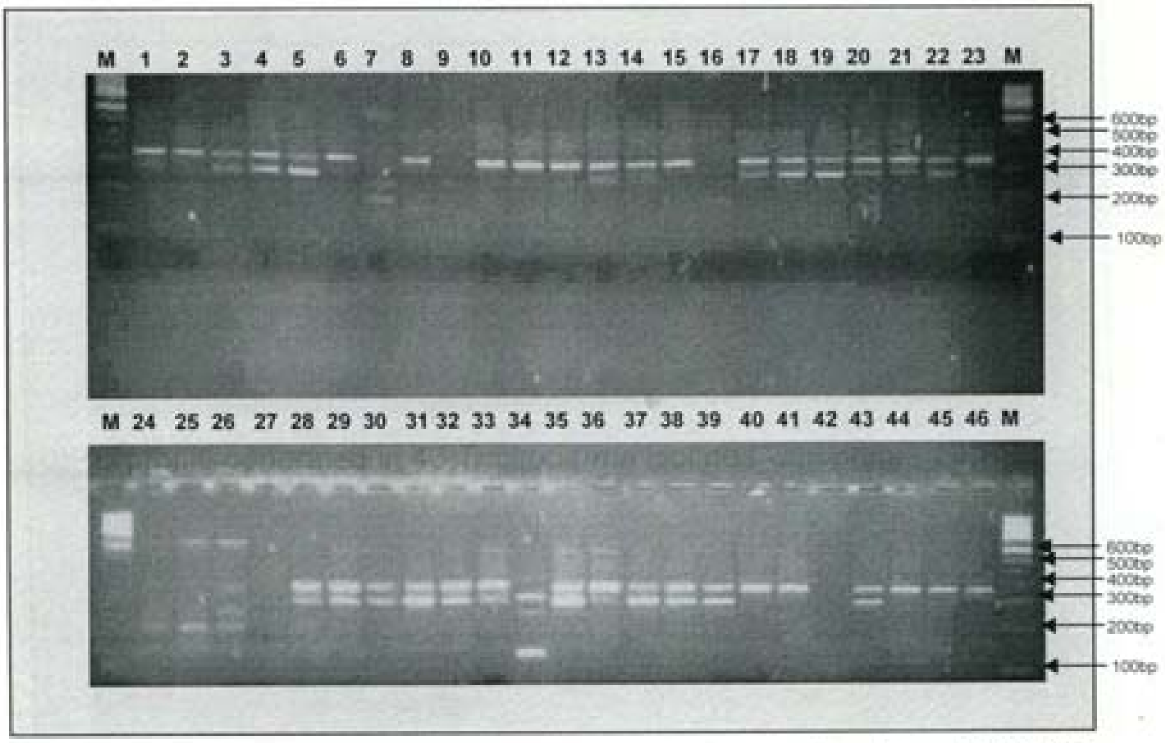


RAPD profile generated in 46 *Trichoderma* isolates with primer OPBB 6

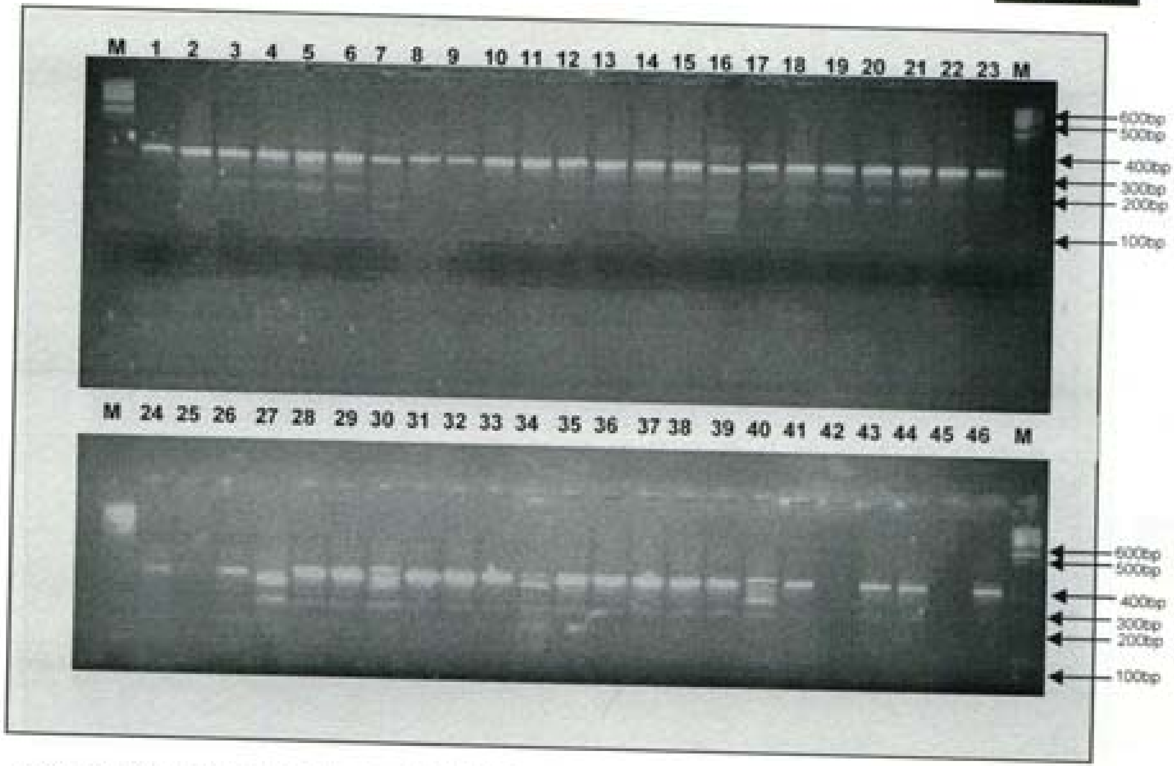
Plate 51



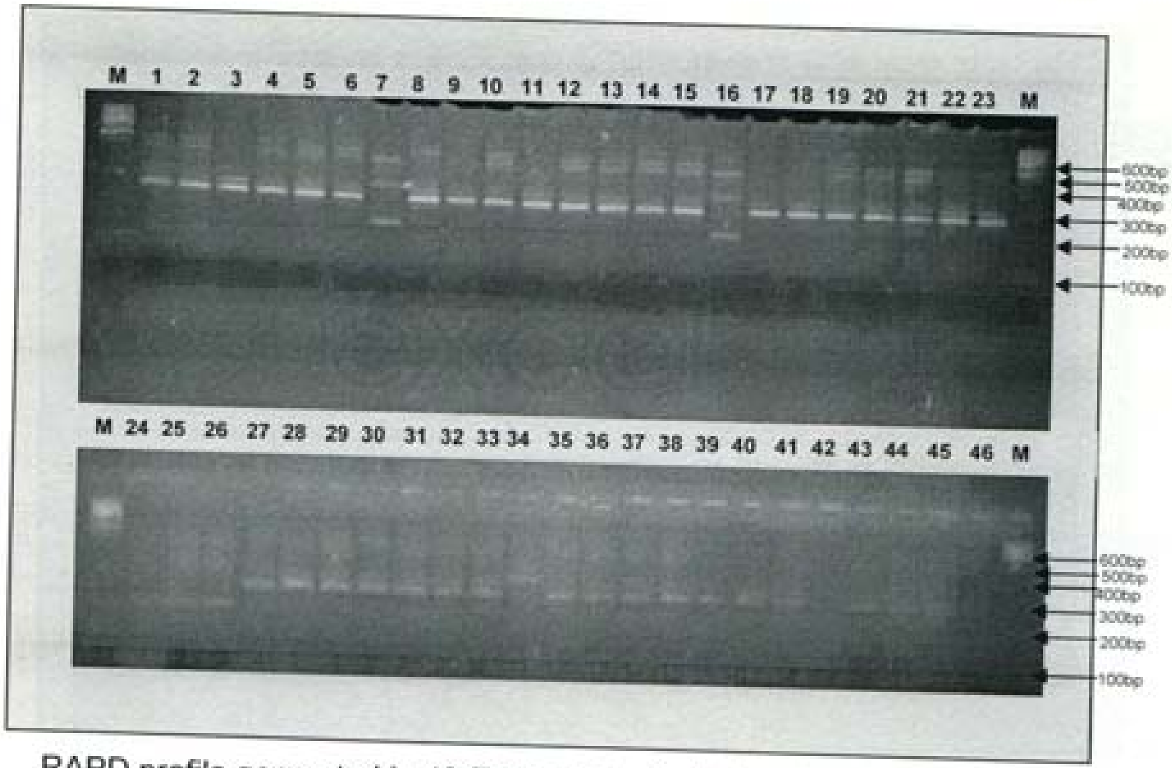
RAPD profile generated in 46 *Trichoderma* isolates with primer OPBB 9



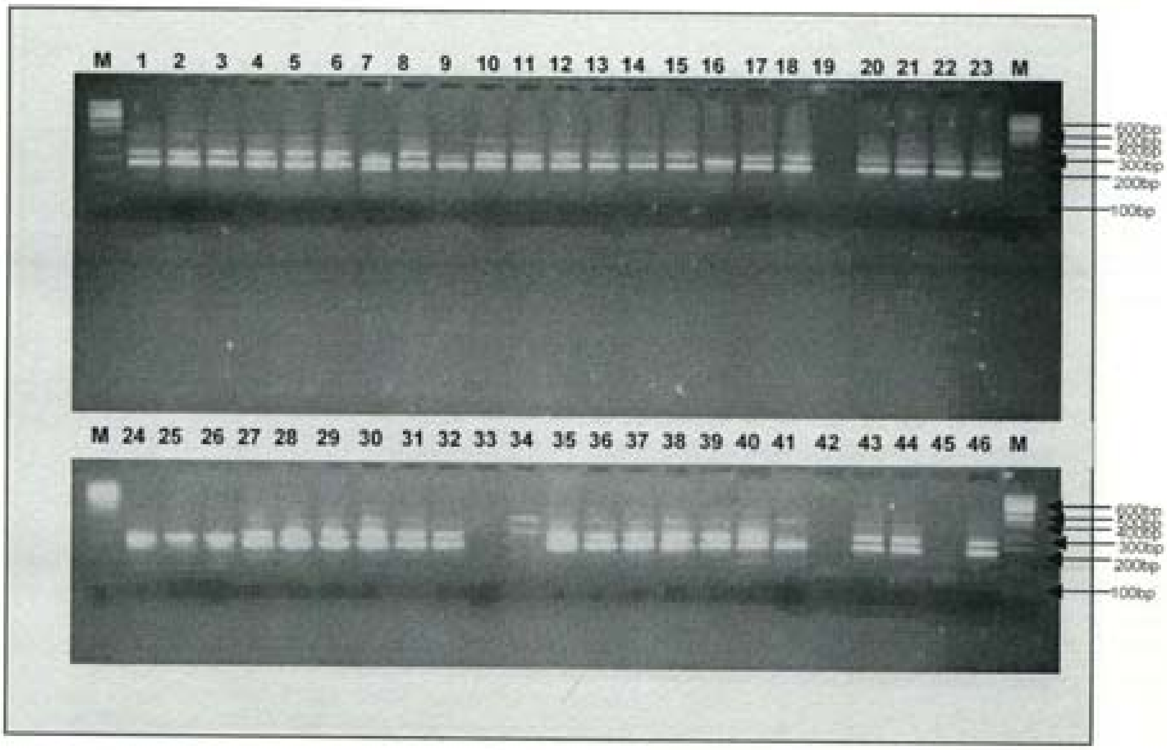
RAPD profile generated in 46 *Trichoderma* isolates with primer OPBB 10



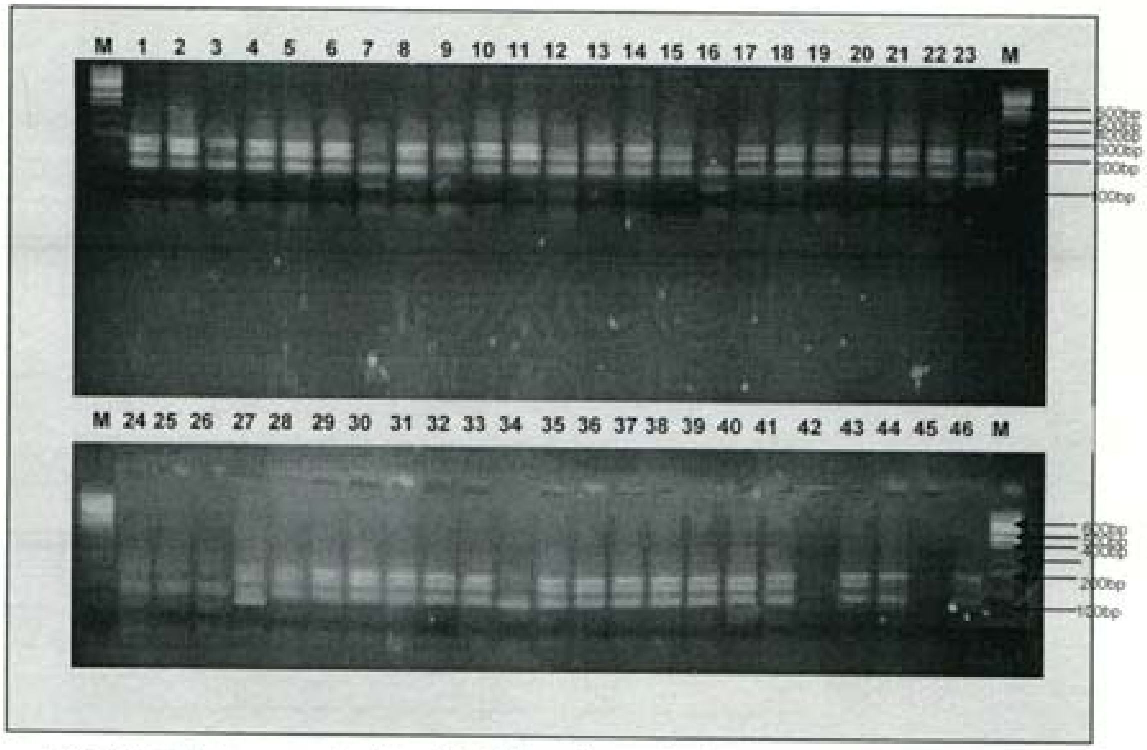
RAPD profile generated in 46 *Trichoderma* isolates with primer OPBB 11



RAPD profile generated in 46 *Trichoderma* isolates with primer OPBB 12

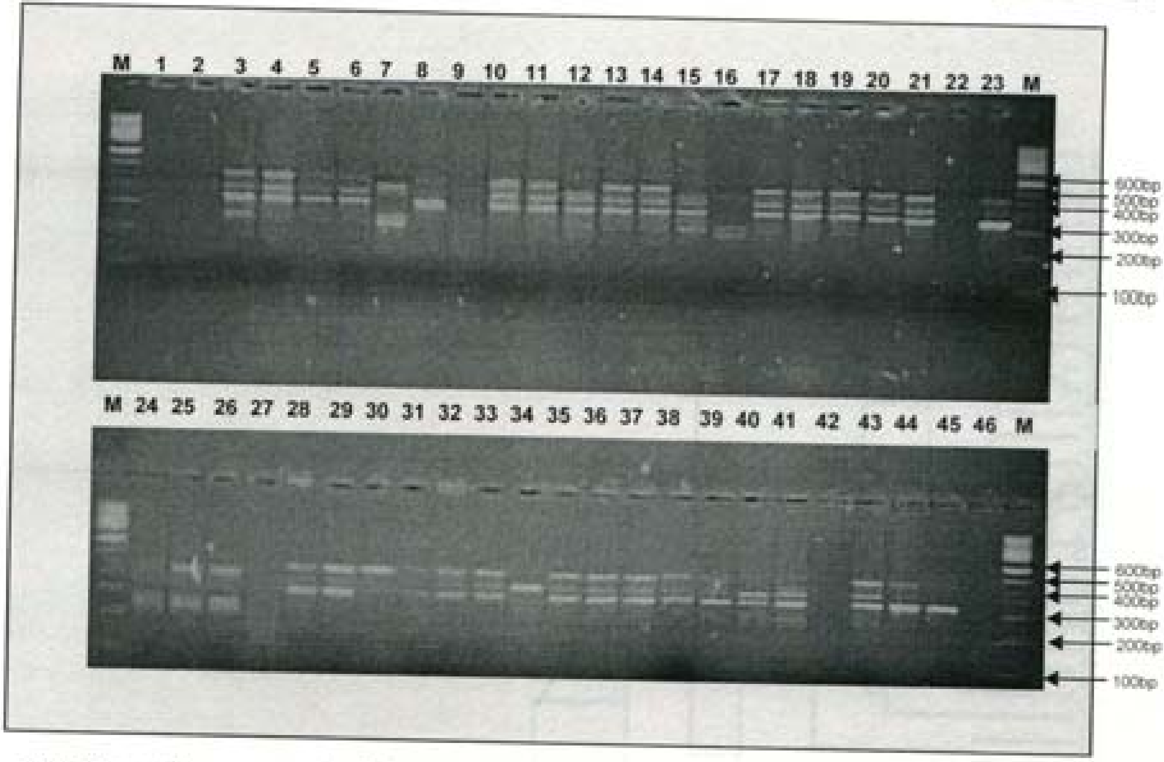


RAPD profile generated in 46 *Trichoderma* isolates with primer OPBB 13

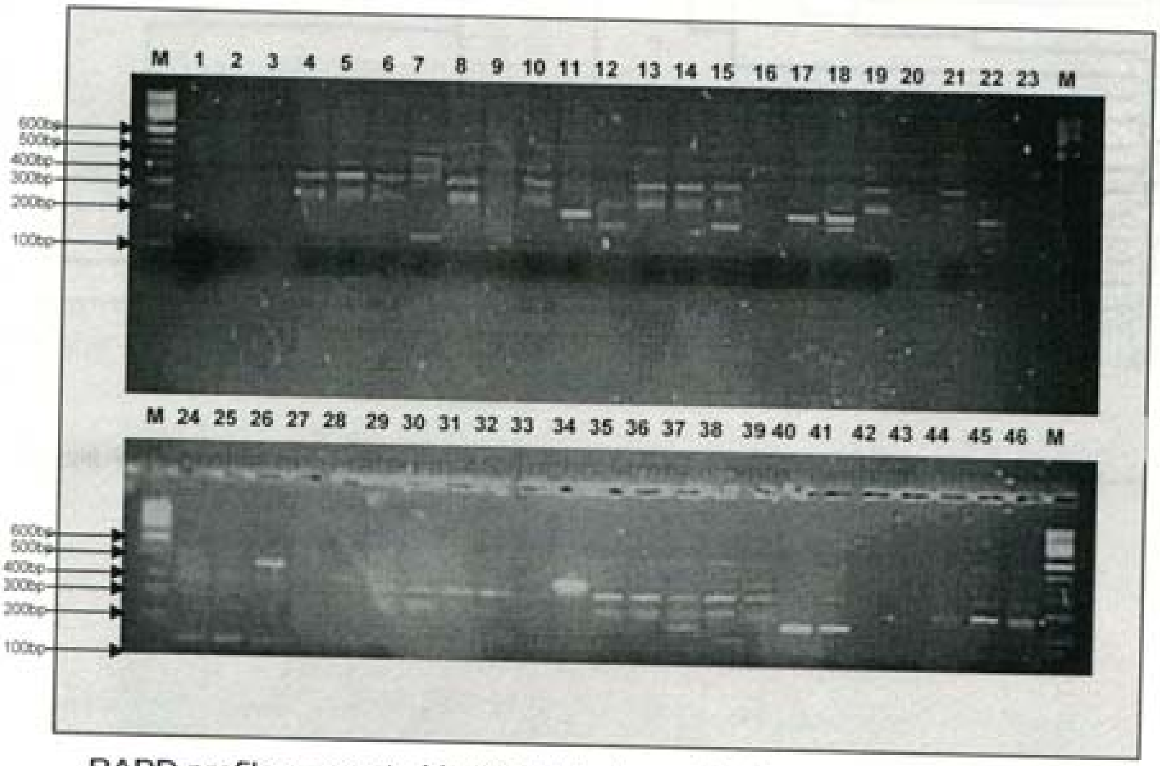


RAPD profile generated in 46 *Trichoderma* isolates with primer OPBB 14

Plate 54



RAPD profile generated in 46 *Trichoderma* isolates with primer OPB 7



RAPD profile generated in 46 *Trichoderma* isolates with primer OPB 8

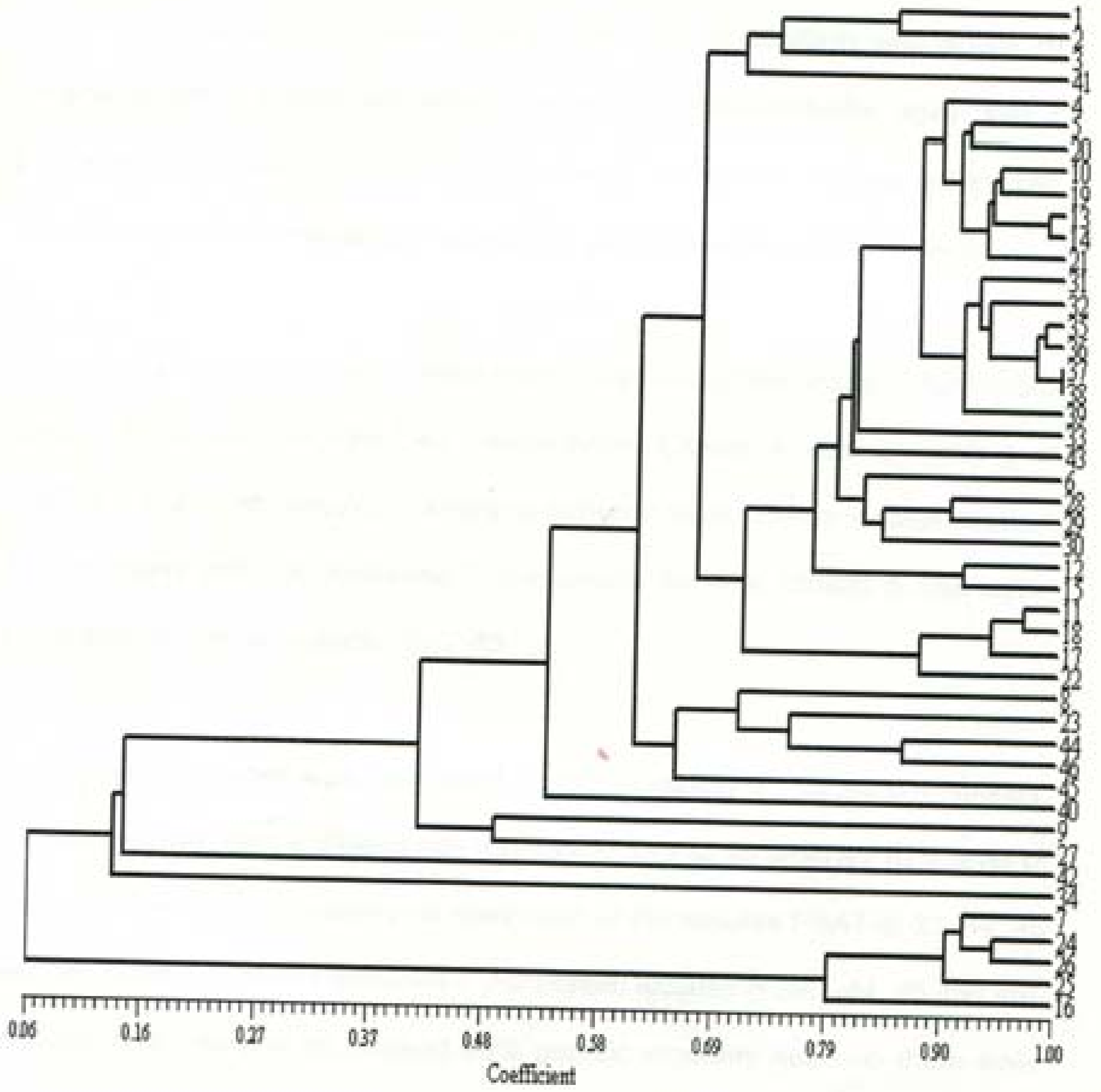


Figure 1 : Dendrogram showing genetic relatedness among different isolates of *Trichoderma*. Isolates 7, 16, 24, 25 and 26 were *T. virens* and others belonged to *T. harzianum*. Cophenetic correlation = 0.99.

was divided into two sublusters at an arbitrary 79% level of similarity. The first subcluster was composed of isolate 16 alone. The second subcluster was further divided into two sub-subcluster at 90% level of similarity with isolate 25 comprising the first sub-subcluster. The second sub-subcluster again into 2 groups at 92% level of similarity. The isolate 7 comprised the first group. The second group was composed of isolates 24 and 26 showing 95% similarity.

The second and third clusters were comprised of the single *T. harzianum* isolates PBAT-34 and PBAT-42, respectively. Cluster 4 was composed of isolates 9 and 27 showing 49% similarity between them. These isolates showed 42% similarity with the remaining *T. harzianum* isolates. Cluster 5 was again composed of a single isolate PBAT-40.

The 6th Cluster was comprised of the remaining *T. harzianum* isolates. This cluster was further divided into two subclusters at an arbitrary 62% level of similarity. The first subcluster was composed of the isolates PBAT-8, 23, 44, 45 and 46. Among the three standard *T. harzianum* isolates (PBAT-44, 45 and 46), isolates PBAT 44 and 46 showed 89% genetic similarity between them while PBAT-45 showed only 65% similarity with all the remaining 4 members of this cluster.

The second subcluster was again clustered into two sub-subcluster at an arbitrary 66% level of similarity. The first sub-subcluster was composed of isolates PBAT-1, 2, 3 and 41. The second sub-subcluster comprised of the remaining isolates showing different levels of genetic similarity among themselves. Isolates PBAT-13 and 14, isolates PBAT- 35 and 36, isolates PBAT-11 and 18 showed >95% genetic similarity. Two isolates PBAT-37 and PBAT-38 exhibited 100% similarity between them.

Clustering of the *Trichoderma* isolates on the basis of dendrogram was supported by a cophenetic correlation coefficient of 0.99, which is considered a very good value. The cophenetic correlation indicates the extent to which the clustering of genotypes demonstrated in the dendogram accurately represents the genetic similarity among the different isolates.

The PCR amplification, performed with RAPD markers to assess the level of polymorphism in 46 isolates of *Trichoderma* (Table 2) showed high degree of genetic diversity particularly among the isolates of *T. harzianum*. Different isolates of *T. virens* exhibited much less genetic diversity.

4.4 Antagonistic potential of different isolates of *Trichoderma*

4.4.1 Against hyphae

The 43 isolates were tested for their ability to inhibit four important soil borne fungal plant pathogens namely *Rhizoctonia solani, Sclerotium rolfsii, Fusarium oxysporum* f. sp. *pisi* and *Sclerotinia sclerotiorum*.

All the 43 *Trichoderma* isolates were observed to inhibit the radial growth of the 4 pathogens *in vitro* (Table 3). In case of *Rhizoctonia solani*, the per cent inhibition of radial growth by various pathogens ranged from19.1% (PBAT-9) to 49.6%. Maximum inhibition was recorded in case of PBAT-34 (49.6%) and ten of the isolates provided more than 45% suppression of hyphal growth of *R. solani*. Among the 5 isolates of *T. virens*, PBAT-7 and PBAT-16 showed 27-30% inhibition. However, PBAT-24, 25 & 26 showed 40-43% inhibition of radial growth of the pathogen. All the 43 isolates were observed to directly overgrow the mycelium of *R. solani* in 4-6 days without formation of any inhibition zone (Table 4, Figure 3). In general the isolates of *T. virens* took more time to overgrow the host hyphae (6 days) than isolates of *T. harzianum* (4 to 5 days).

Against *S. rolfsii* inhibition of hyphal growth was in the range of 30.7% (PBAT-25) to 48.1% (PBAT-18). Among the *T. virens* isolates PBAT-7 & 25 showed ~30% inhibition while the remaining three *T. virens* isolates inhibited the growth of pathogen in range of 39-46% (Table 3, Figure 2). No inhibition zone was observed in the plates at the zone of interaction and the mycelium of *Trichoderma* was observed to directly overgrow the host and cover the entire plate in 5-6 days (Table 4).

The radial growth of *Sclerotinia sclerotiorum* was inhibited in range of 34.2% (PBAT-11) to 52.8% (PBAT-41). Four isolates PBAT-6 (50.2%), PBAT-37 (50.5%), PBAT-41 and PBAT-43 (50.8%) showed > 50% inhibition (Table 3, Figure 4). *T .virens* isolates inhibited the growth of *Sclerotinia* in range of 39 to 43%. An inhibition zone was observed at the site of interaction of *Trichoderma* and host hyphae in all the isolates. The isolates took 7-8 days to overgrow the

Table 3: Antagonistic potential of *Trichoderma* isolates against four plant pathogens

Isolate No.	***Rhizoctonia solani***	***Sclerotium rolfsii***	***Sclerotinia sclerotiorum***	***Fusarium oxysporum***
PBAT-1	38.5(38.8)	42.6(45.9)	45.5(50.9)	36.7(38.8)
PBAT-2	40.9(42.9)	42.9(46.4)	47.5(54.5)	32.9(29.5)
PBAT-3	41.2(43.4)	39.4(40.3)	46.5(52.6)	27.9(21.9)
PBAT-4	46.7(53.0)	43.5(47.4)	47.1(53.7)	18.3(9.8)
PBAT-5	46.9(53.4)	43.4(47.3)	48.0(55.3)	32.9(29.5)
PBAT-6	39.1(39.8)	41.1(43.2)	50.2(59.1)	28.9(23.4)
PBAT-7	30.1(25.2)	31.1(26.7)	39.7(40.8)	35.2(33.3)
PBAT-8	40.3(41.9)	37.3(36.8)	46.0(51.7)	36.6(35.7)
PBAT-9	19.1(10.8)	41.6(44.2)	37.1(36.5)	18.3(9.8)
PBAT-10	36.9(36.1)	43.4(47.3)	37.8(37.6)	21.4(13.6)
PBAT-11	39.5(40.5)	42.6(45.9)	34.2(31.7)	29.3(24.0)
PBAT-12	43.4(47.3)	40.8(42.7)	43.0(46.6)	18.3(9.8)
PBAT-13	40.8(42.7)	46.4(52.5)	46.6(52.9)	19.1(10.8)
PBAT-14	49.3(57.6)	43.1(46.7)	42.8(46.2)	28.5(22.8)
PBAT-15	46.0(51.7)	43.5(47.4)	46.6(52.9)	24.0(16.6)
PBAT-16	27.3(21.2)	39.1(39.8)	43.5(47.4)	15.7(7.4)
PBAT-17	32.2(28.5)	45.5(51.0)	37.6(37.2)	18.3(9.8)
PBAT-18	32.7(29.2)	48.1(55.5)	45.3(50.5)	35.2(33.3)
PBAT-19	35.2(33.3)	43.5(47.4)	43.9(48.2)	12.1(4.7)
PBAT-20	49.2(57.4)	41.5(43.9)	47.5(54.5)	31.4(27.1)
PBAT-21	32.6(29.2)	41.2(43.4)	45.1(50.1)	15.7(7.4)
PBAT-22	43.2(46.9)	48.3(55.7)	47.3(54.1)	22.1(14.1)
PBAT-23	43.5(47.4)	43.7(47.8)	47.1(53.7)	34.4(32.0)
PBAT-24	40.6(42.4)	42.2(45.2)	40.8(42.7)	38.9(39.5)
PBAT-25	43.4(47.3)	30.7(26.2)	38.7(39.2)	36.7(35.8)
PBAT-26	43.8(47.9)	46.6(52.8)	41.0(43.1)	37.4(37.0)
PBAT-27	48.1(55.4)	44.1(48.5)	45.1(50.1)	31.4(27.1)
PBAT-28	44.1(48.5)	45.1(50.2)	48.1(55.3)	18.3(9.8)
PBAT-29	39.4(40.3)	44.8(49.7)	46.2(52.1)	32.0(28.0)
PBAT-30	43.8(47.9)	46.2(52.1)	45.6(51.0)	32.1(28.3)
PBAT-31	40.6(42.5)	45.5(51.0)	47.1(53.7)	17.2(8.8)
PBAT-32	34.6(32.3)	41.2(43.4)	43.9(48.2)	43.9(48.1)
PBAT-33	39.8(41.1)	39.3(40.3)	48.3(55.9)	32.9(29.5)
PBAT-34	49.6(58.0)	39.0(39.7)	39.7(40.8)	38.5(38.8)
PBAT-35	35.8(34.3)	43.1(46.7)	45.9(51.6)	44.9(50.0)
PBAT-36	41.8(44.4)	43.5(47.4)	43.7(47.8)	32.9(29.5)
PBAT-37	44.4(48.9)	42.4(45.6)	50.5(59.6)	31.8(27.7)
PBAT-38	34.6(32.3)	38.7(39.1)	48.0(55.3)	16.5(8.1)
PBAT-39	44.1(48.4)	42.9(46.4)	44.8(49.8)	36.4(35.2)
PBAT-40	46.0(51.7)	44.9(50.0)	43.5(474)	19.8(11.5)
PBAT-41	46.4(52.5)	39.4(40.3)	52.8(63.4)	38.5(22.8)
PBAT-42	33.3(30.3)	42.1(45.0)	45.9(51.6)	34.8(32.6)
PBAT-43	49.3(57.6)	43.1(46.7)	50.8(60.2)	30.9(26.5)
CD 5%	6.6	4.5	3.29	2.9

* Figures in parentheses indicate angular transformed values

Table 4: Observations on the hyphal interaction of *Trichoderma* isolates with four major plant pathogens

Isolate No.	***Rhizoctonia solani***		***Sclerotium rolfsii***	***Sclerotinia sclerotiorum***		***Fusarium oxysporum***	
	IZ	**Time for OG (days)**	**Time for OG**	**IZ**	**Time for OG (days)**	**IZ**	**Time for OG (days)**
PBAT-1	-	4	5	+	7	+	7
PBAT-2	-	4	6	+	7	+	6
PBAT-3	-	4	5	+	7	+	6
PBAT-4	-	4	5	+	7	+	6
PBAT-5	-	4	5	+	7	+	6
PBAT-6	-	5	5	+	8	+	7
PBAT-7	-	6	6	+	8	-	7
PBAT-8	-	4	5	+	8	+	7
PBAT-9	-	5	5	+	8	+	6
PBAT-10	-	5	5	+	8	+	6
PBAT-11	-	4	5	+	-	+	-
PBAT-12	-	4	6	+	7	+	-
PBAT-13	-	4	5	+	7	+	6
PBAT-14	-	4	5	+	7	+	6
PBAT-15	-	4	5	+	7	+	6
PBAT-16	-	6	6	+	8	+	6
PBAT-17	-	4	6	+	-	+	6
PBAT-18	-	4	6	+	8	+	6
PBAT-19	-	4	6	+	-	+	6
PBAT-20	-	4	5	+	7	+	6
PBAT-21	-	5	5	+	7	+	6
PBAT-22	-	4	5	+	7	+	6
PBAT-23	-	4	5	+	8	+	7
PBAT-24	-	6	6	+	8	+	6
PBAT-25	-	6	5	+	-	+	-
PBAT-26	-	6	5	+	8	+	6
PBAT-27	-	4	5	+	8	+	6
PBAT-28	-	4	5	+	7	+	6
PBAT-29	-	4	5	+	7	+	6
PBAT-30	-	4	5	+	7	+	6
PBAT-31	-	4	5	+	7	+	7
PBAT-32	-	4	5	+	7	+	6
PBAT-33	-	4	5	+	7	+	7
PBAT-34	-	4	5	+	7	+	6
PBAT-35	-	4	5	+	7	+	6
PBAT-36	-	4	5	+	7	+	6
PBAT-37	-	4	5	+	7	+	6
PBAT-38	-	4	5	+	7	+	6
PBAT-39	-	4	5	+	7	+	7
PBAT-40	-	4	6	+	7	-	7
PBAT-41	-	4	5	+	8	+	6
PBAT-42	-	4	5	+	8	+	7
PBAT-43	-	4	5	+	7	+	6

IZ= Inhibition Zone, OG= Overgrowth

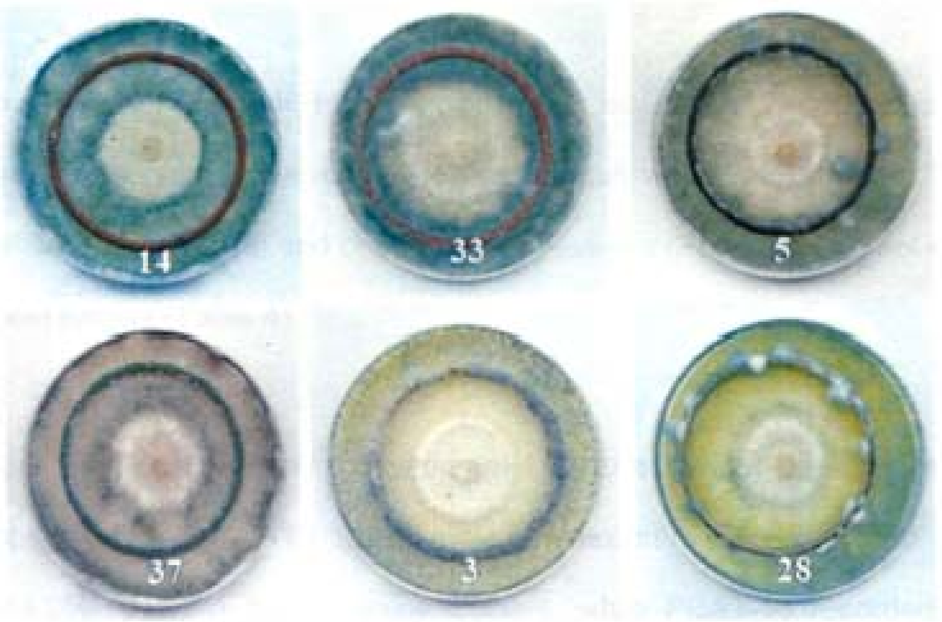


Figure 2: Differential efficacy of different isolates of *Trichoderma harzianum* against mycelial growth of *Sclerotium rolfsii*.

Figure 3: Differential efficacy of different isolates of *Trichoderma harzianum* against mycelial growth of *Rhizoctonia solani*.

host in the Petri dish. However, out of 43 isolates of *Trichoderma*, 39 isolates were observed to overgrow the host fungus while 3 isolates of *T. harzianum* (PBAT-11, 17 & 19) and one isolate of *T. virens* (PBAT-25) did not overgrow the host fungus (Table 4).

Against *Fusarium oxysporum* f.sp. *pisi*, lowest per cent inhibition was observed in PBAT-19 (12.1%) while maximum was recorded in PBAT-35 (44.9%). Among the *T. virens* isolates, while PBAT-16 recorded only 15.7% inhibition in radial growth, the remaining 4 isolates inhibited radial growth by 35-38%. Out of 43 isolates, three isolates of *T. harzianum* (PBAT-11, 12 & 25) did not overgrow the host mycelium. The remaining isolates took 6-7 days for overgrowing the host and covering the entire plate (Table 3 & 4).

Significant difference was observed between the 43 isolates with respect to their antagonistic potential against the four pathogens. This difference was observed among the 38 isolates of *T. harzianum* as well as among the 5 isolates of *T. virens*. The range of inhibition provided by the same set of isolates also varied in different pathogens. It was also observed that individual isolates varied considerably in their antagonistic potential against different pathogens. Isolate PBAT-19 in which the lowest per cent inhibition of radial growth of *Fusarium* was observed (12.1%), exhibited inhibition in range of 35 to 43.9% against the remaining three pathogens. This variability was observed in case of other isolates also. The time taken by the *Trichoderma* isolates to overgrow the pathogens did not vary much between the isolates but their ability to overgrow

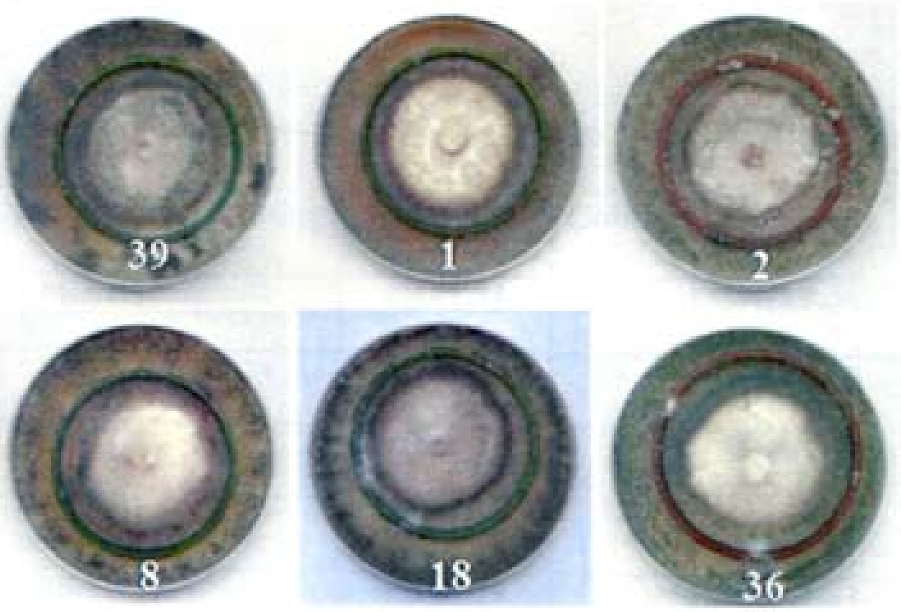


Figure 4 : Differential efficacy of different isolates of *Trichoderma harzianum* against mycelial growth of *Sclerotinia sclerotiorum.*

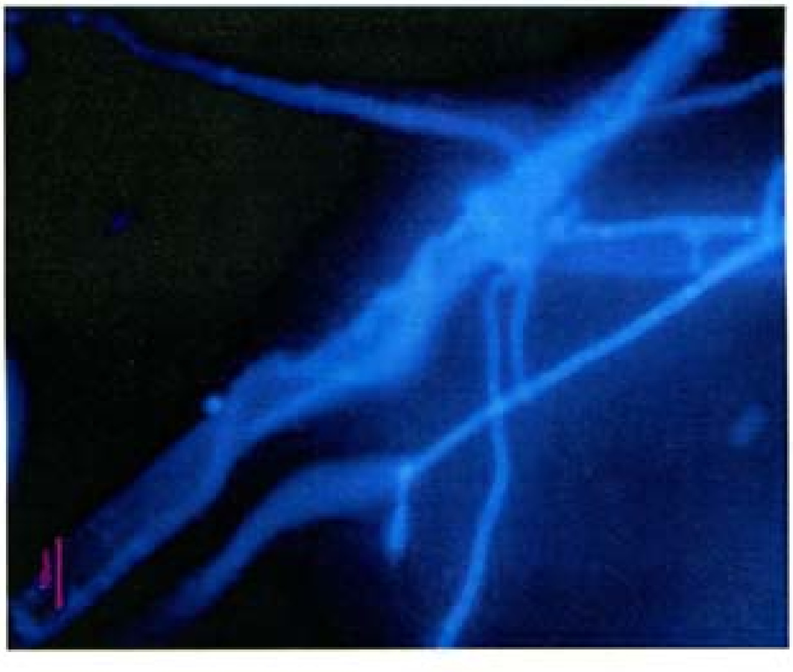


Figure 5: Hyphae of *Trichoderma harzianum* (thin hypahe) coil around hyphae of *Rhizoctonia solani* (thick hyphae)

Table 5: Coiling of *Rhizoctonia solani* hyphae by *Trichoderma* isolates

Isolate no.	Coiling	Isolate no.	Coiling	Isolate no.	Coiling
PBAT-1	-	PBAT-15	+	PBAT-29	+
PBAT-2	+	PBAT-16	-	PBAT-30	+
PBAT-3	+	PBAT-17	+	PBAT-31	+
PBAT-4	+	PBAT-18	+	PBAT-32	+
PBAT-5	-	PBAT-19	+	PBAT-33	+
PBAT-6	+	PBAT-20	-	PBAT-34	-
PBAT-7	-	PBAT-21	+	PBAT-35	+
PBAT-8	+	PBAT-22	+	PBAT-36	+
PBAT-9	+	PBAT-23	+	PBAT-37	+
PBAT-10	+	PBAT-24	-	PBAT-38	+
PBAT-11	+	PBAT-25	-	PBAT-39	-
PBAT-12	+	PBAT-26	-	PBAT-40	+
PBAT-13	+	PBAT-27	+	PBAT-41	+
PBAT-14	+	PBAT-28	+	PBAT-42	-
				PBAT-43	+

the various hosts was different. Three isolates of *T. harzianum* (PBAT-11, 17&19) and one isolate of *T. virens* (PBAT-25) did not overgrow *S. sclerotiorum* while 1 isolate of *T. harzianum* (PBAT-11) and 1 isolate of *T. virens* (PBAT-25) did not overgrow *Fusarium* sp.

4.4.2 Confrontation assay of *Trichoderma* isolates with *Rhizoctonia solani*

The 43 isolates of *Trichoderma* were studied for their mycoparasitic ability against *R. solani*. Hyphal mat from the zone of interaction was observed under microscope for presence or absence of coiling of host hyphae by *Trichoderma*. The results are recorded in Table 5.

Among the 43 isolates of *Trichoderma*, 32 isolates showed mycoparasitic ability by coiling around the hyphae of *R. solani* (Figure 5). No coiling was observed in 6 (PBAT-1, 5, 20, 34, 39 & 42) out of the 38 *T. harzianum* isolates. None of the 5 *T. virens* isolates showed coiling around *R. solani* hyphae. Isolate PBAT-34 which exhibited maximum inhibition of radial growth of *R. solani* also did not show coiling.

4.4.3 Sclerotial parasitization

Fifteen out of 38 isolates of *T. harzianum* and 4 out of 5 isolates of *T. virens* parasitized the sclerotia of at least one pathogen. One isolate of *T. virens* (PBAT-26) did not colonize the sclerotia of any pathogen (Table 6).

Sixteen out of the 43 isolates colonized the sclerotia of *R. solani*, while 11 isolates colonized the sclerotia of *S. rolfsii* and only 5 colonized the sclerotia of *S. sclerotiorum*. Against *R. solani*, maximum colonization of 73% was recorded in PBAT-16 followed by PBAT-22 (63%) and then PBAT-12 (56%). 13 isolates of *T. harzianum* and 3 isolates of *T. virens* could successfully colonize the sclerotia of *R. solani*. The different isolates exhibited colonization in range of 13% (PBAT-32) to 73% (Table 6).

In case of *S. rolfsii* also maximum sclerotial colonization was observed in PBAT-16 (100%) followed by PBAT-25 (76%) and PBAT-24 (73%). Out of the 11 isolates which colonized the sclerotia of *S. rolfsii*, 4 were of *T. virens* and remaining 7 were *T. harzianum*. The colonization was in the range of 10% (PBAT-6) to 100% (PBAT-16). Five isolates colonized the sclerotia of *Sclerotinia sclerotiorum* in range of 36% (PBAT-15) to 96% (PBAT-16) out of which 2 were *T. harzianum* (PBAT-12, 15) and 3 were of *T. virens* (PBAT- 16, 24, 25). Only two isolates of *T. harzianum* (PBAT-15 & PBAT12) and three isolates of *T. virens* (PBAT-16, PBAT-24 and PBAT-25) were observed to colonize the sclerotia of all the three pathogens. PBAT-16 was observed to be the most aggressive colonizer. Individual *Trichoderma* isolates showed considerable difference in their ability to colonize sclerotia of these three pathogens. Isolates PBAT-7 (*T. virens*) and isolates PBAT-19 and PBAT-30 (*T. harzianum*) colonized the sclerotia of only *S. rolfsii*. Similarly eight isolates of *T. harzianum* were observed to colonize the sclerotia of only *R. solani*. Significant

Table 6: Colonization of sclerotia of three major plant pathogens by *Trichoderma* isolates

Isolate No.	***Rhizoctonia solani***	***Sclerotium rolfsii***	***Sclerotinia sclerotiorum***
	Sclerotia colonized (%)	**Sclerotia colonized (%)**	**Sclerotia colonized (%)**
PBAT-1	0	0	0
PBAT-2	0	0	0
PBAT-3	0	0	0
PBAT-4	0	0	0
PBAT-5	0	0	0
PBAT-6	26 (30.9)	10 (14.9)	0
PBAT-7	0	53 (44.9)	0
PBAT-8	0	0	0
PBAT-9	33 (35.2)	50 (47.0)	0
PBAT-10	0	0	0
PBAT-11	0	0	0
PBAT-12	56 (49.2)	60 (50.8)	76 (61.2)
PBAT-13	53 (46.9)	0	0
PBAT-14	0	0	0
PBAT-15	53 (46.9)	43 (41.1)	36 (37.2)
PBAT-16	**73 (59.0)**	**100 (90.0)**	**96 (83.8)**
PBAT-17	0	0	0
PBAT-18	0	0	0
PBAT-19	0	43 (41.1)	0
PBAT-20	53 (46.9)	0	0
PBAT-21	0	0	0
PBAT-22	63 (52.7)	0	0
PBAT-23	0	0	0
PBAT-24	16 19.9)	73 (59.2)	40 (39.1)
PBAT-25	20 (21.9)	76 (61.2)	53 (46.1)
PBAT-26	0	0	0
PBAT-27	0	0	0
PBAT-28	30 (33.0)	0	0
PBAT-29	0	0	0
PBAT-30	0	36 (36.9)	0
PBAT-31	20 (26.0)	40 (39.1)	0
PBAT-32	13 (17.7)	0	0
PBAT-33	0	0	0
PBAT-34	0	0	0
PBAT-35	0	0	0
PBAT-36	0	0	0
PBAT-37	33 (35.2)	0	0
PBAT-38	0	0	0
PBAT-39	0	0	0
PBAT-40	0	0	0
PBAT-41	0	0	0
PBAT-42	36 (36.9)	0	0
PBAT-43	53 (47.0)	0	0
CD 5%	9.5	6.14	3.3

* Figures in parentheses indicate angular transformed values

difference was recorded in the sclerotia colonizing ability of different *Trichoderma* isolates against a specific pathogen (Table 6).

4.4.4 Glass house studies

Four isolates of *Trichoderma* were selected for further studies under glass house conditions. Two isolates viz. PBAT-14 and PBAT-43 exhibited high per cent inhibition of radial growth of all the four pathogens while the other two isolates; PBAT-12 and PBAT-16 were very good sclerotial colonizers.

In pea plants, the lowest per cent disease incidence of wilt (causal organism *Fusarium oxysporum* f.sp. *pisi*) was observed in treatment with PBAT-43 (10%) followed by PBAT-14 (22%). PBAT-12 and PBAT-16 both showed 30% disease incidence. In all the four treatments, there was significantly lower disease incidence as compared to control (38%) (Table 7).

In case of *Rhizoctonia solani* infection on okra, all the four isolates significantly reduced the disease incidence as compared to control. The per cent disease incidence was 30 to 34% in the treated plants as compared to 46% in check. However, between the isolates the differences were not significant at p=0.05.

Against *Sclerotium rolfsii* infection in okra also, all the isolates showed significantly lower disease incidence as compared to check. Lowest disease incidence was observed in PBAT-16 (28%) treatment followed by PBAT-12

Table 7: Studies on host selectivity of four *Trichoderma* isolates against four major plant pathogens in glass house condition

Isolate No.	**French Bean (*S.sclerotiorum*)**	**Okra (*S.rolfsii*)**	**Okra (*R.solani*)**	**Pea (*F.oxysporum pisi*)**
	% incidence	% incidence	% incidence	% incidence
PBAT-12	26 (30.5)	32 (34.4)	32 (34.4)	30 (33.0)
PBAT-14	38 (38.0)	38 (38.0)	34 (35.4)	22 (27.8)
PBAT-16	30 (33.0)	28 (31.8)	30 (33.2)	30 (33.0)
PBAT-43	30 (33.0)	38 (38.0)	32 (34.4)	10 (16.3)
CHECK	56 (48.5)	44 (41.5)	46 (42.6)	38 (37.9)
CD 5%	4.8	3.7	4.4	7.6

*Figures in parentheses indicate angular transformed values

(32%). In treatments PBAT-14 and PBAT-43 the disease incidence was recorded to be 38% in both.

In case of French bean all the four isolates significantly reduced the incidence of *Sclerotinia* infection as compared to control. Treatment with PBAT-12 showed lowest per cent disease incidence (26%) in French bean plants followed by PBAT-16 and PBAT-43 both having 30% incidence. However, the difference between these three isolates was not significant. Plants with PBAT-14 treatment had 38% disease incidence while control plots showed 56% incidence.

DISCUSSION

Chapter 5

DISCUSSION

In recent years there has been an emphasis on the use of indigenous *Trichoderma* strains which are more adapted to the native microflora and therefore stand a better chance of success under natural conditions. In the present study, 43 indigenous isolates of *Trichoderma* (PBAT 1 to 43), earlier isolated from the rhizosphere of various crops growing in the *Tarai* region of Uttaranchal, were characterized based on cultural, morphological and molecular characters and for the presence of host selectivity for specific pathogen.

5.1 Cultural and Morphological Characterization

The variability in the cultural characteristics was not enough with most isolates exhibiting rapid growth, effuse condiation and conidial colour changes from white to varying shades of green, characters typical of most *Trichoderma* species. However, a study of the morphological characters especially the arrangement of conidiophores and phialides revealed two types of arrangement within the 43 isolates. One set of 38 isolates, was characterized by having highly branched conidiophores, with branching in a divergent, dendritic manner, branches often arising at 90^0 angle in pairs or in whorls of 3 with a fertile apex. The phialides were divergent and arranged in whorls of mostly 3-5. The right angled branching of conidiophores with a fertile apex and occurrence of phialides in divergent and crowded whorls of 3-6 are typical characteristics of the species *T. harzianum* sect. *Pachybasium* as described by Bisset (1991a&b). The second set of 5 isolates (PBAT 7, 16, 24, 25 & 26) had hyaline conidiophores, sparingly branched in a divergent manner often at 90^0 angle. Towards the apex the conidiophores branched with each branch terminating in a penicillus of 3-5 closely appressed phialides. The closely appressed phialides (typical of the genus *Gliocladium*) towards the apex and divergent branching often at right angles (typical of the genus *Trichoderma*) are the unique characters exhibited by *T. virens*. These characteristics were initially a major source of confusion for the taxonomists with some workers placing *T. virens* under *Gliocladium* (as *Gliocladium virens*) and other workers placing this species under the genus *Trichoderma*. The taxonomic position of *T. virens* was finally resolved on the basis of analysis of the 28S rDNA and the species was found more closely related to *Trichoderma* (Rehner and Samuels, 1994).The morphological characters observed in combination with the cultural characters like growth rate and chlamydospore production, were used to identify the 43 isolates and classify them under two species *T. harzianum* and *T. virens*.

However, the placement of the 43 isolates on the basis of these characters was not reflected in the genetic relatedness studies especially within 38 isolates classified as *T. harzianum*. The taxonomy of *Trichoderma* has been problematic for long. As is usually the case with other fungal genera, species of *Trichoderma* too were defined originally on the basis of morphology by workers like Rifai (1969) and Bisset (1984, 1991a,b&c). Unfortunately some of the richest characters for species recognition in other fungi are either not variable enough or are difficult to describe in case of *Trichoderma*, which often leads to confusion in accurate identification of species (Samuels, 1996). In recent years several workers have alternatively used molecular marker based studies to resolve the concept of species within the genus *Trichoderma*. The classification of some of the sections erected by Bisset based on morphological characters has been observed to be correct by using molecular marker based studies. However, the sect. *Pachybasium* which contains the species *T. harzianum* and *T. virens* has been a problem. Morphologically the members of this section were found homogenous. However, the morphological similarities contrasted with the molecular analysis done using ITS sequences. Kuhls *et al.* (1997) discussed that the sect. *Pachybasium* should be investigated with large set of strains to resolve this problem. Among the members of sect. *Pachybasium*, the placement of species *T. harzianum* is a source of confusion. Studies on the genetic diversity of strains, previously identified as *T. harzianum* on basis of morphological characters, revealed that these strains occurred in two sections; sect. *Trichoderma* and sect. *Pachybasium*, thus showing the morphological diversity of this species. In the present investigation also, the standard *T. harzianum* isolates used in the molecular analysis showed only 65% genetic similarity within them indicating that *T. harzianum* may represent an aggregate species.

5.2 Host selectivity in *Trichoderma* species

Trichoderma species have a wide host range, but whether they exhibit any selectivity/ specificity for their hosts at strain/ isolate level was studied both with respect to their ability to suppress hyphal growth of various pathogens and their ability to colonize the sclerotia of pathogens. Studies involving the antagonistic activity of different isolates of *Trichoderma* against hyphal growth of four fungal pathogens revealed some interesting observations: (i) in general most of the isolates exhibited medium to high (30-50%) antagonistic potential against *R. solani*, *S. rolfsii* and *Sclerotinia* sp. but the suppression of hyphal growth of *Fusarium* by the isolates was however comparatively less (15-40%); (ii) isolates (PBAT-1, 8, 24, 26, 34, 35, 39, 41) consistently exhibited a high level of antagonistic potential (>35%) against all the 4 pathogens; (iv) some isolates however, varied significantly in their inhibitory activity against the 4 pathogens. PBAT-9 was observed to exhibit high level of antagonistic potential against *S. rolfsii* (41%) and *Sclerotinia* (37%) but showed low inhibitory activity against *R. solani* (19.1%) and *Fusarium* (18.3%). Similarly isolates PBAT-4, 12, 19, 28, 31 and 40 showed high antagonistic activity (>35%) against *R. solani*, *S. rolfsii* and *Sclerotinia* but poor activity against *Fusarium* spp (12 to 18%).

In their ability to colonize the sclerotia of major pathogens also, the isolates showed considerable variability. Although in general the property of colonizing sclerotia is attributed to strains of *T. virens*, in the present investigation some novel observations were made. Out of 43 only 19 isolates were able to colonize sclerotia of at least one pathogen. Out of these 19 sclerotia colonizing isolates, 15 belonged to *T. harzianum* and 4 were *T. virens*. Among the *T harzianum* isolates 13 were observed to colonize the sclerotia of *R. solani*, 7 isolates colonized sclerotia of *S. rolfsii* while two isolates colonized

Sclerotinia sclerotia. Two isolates (PBAT-19 and 30) of *T. harzianum* showed selectivity for sclerotia of *S. rolfsii* while 8 isolates (PBAT-13, 20, 22, 28, 32, 37, 42, 43) were selective for *R. solani*, colonizing the sclerotia of only this pathogen. There was no isolate selective for the sclerotia of *Sclerotinia*. Two *T. harzianum* isolates PBAT-12 and PBAT-15 colonized the sclerotia of all the three pathogens. Among the five *T. virens* isolates three isolates colonized the sclerotia of all the three pathogens(PBAT-16, 24, 25); one isolate (PBAT-7) showed selectivity for *S. rolfsii* and one isolate (PBAT-26) did not colonize any sclerotia. Isolate 16 was observed to be the most virulent sclerotial colonizer. These observations clearly indicate the presence of host selectivity at the isolate/ strain level in *Trichoderma* species. This host selectivity is exercised differentially against hyphal growth of pathogen and its sclerotia. The isolate PBAT-16 which emerged as the most aggressive sclerotial colonizer against the all three pathogens, was not a very efficient suppressor of radial growth against all these pathogens. While it was observed to exhibit 73, 100 and 96% sclerotial colonization against *R. solani, S. rolfsii* and *Sclerotinia* respectively, the inhibition of radial growth of *R. solani* was only 27%. Similarly several isolates of *T. harzianum* (PBAT-1, 8, 26, 34, 35, 39, 41) and one isolate of *T. virens* (PBAT-26) showing high level of suppression of radial growth of different pathogens lacked ability to colonize sclerotia of any pathogen. These observations indicate that antagonism of *Trichoderma* against hyphae and sclerotia of fungal pathogens are not correlated.

The ability of the *Trichoderma* to overgrow the host fungus however seemed to be influenced by both the host and isolate. All the isolates were observed overgrowing the hypha of *R. solani* and *S. rolfsii*. However, some isolates of *Trichoderma* failed to overgrow *Sclerotinia* and *Fusarium*. Mycoparasitism has usually been proposed as the major mechanism employed by *T. harzianum* to inhibit *R. solani*. However, in the current study, 6 isolates of *T. harzianum* and all 5 isolates of *T. virens* did not exhibit coiling of host mycelium in confrontation studies including isolate PBAT-34, which recorded maximum inhibition of hyphal growth of *R. solani* (49.6%) *in vitro*.

To further assess whether the differential activity of *Trichoderma* isolates against hyphal growth and/or sclerotial colonization plays a role in disease control, four isolates of *Trichoderma* were evaluated against pea wilt (caused by *Fusarium oxysporum* f.sp. *pisi*), okra root rot caused by *Sclerotium rofsii* and *Rhizoctonia solani* and white rot of French bean caused by *Sclerotinia sclerotiorum* under glass house conditions. In French bean the lowest disease incidence was observed in PBAT-12 treatment. *Trichoderma harzianum* isolate PBAT-12 is an effective antagonist against both hyphae growth and sclerotia of *S. sclerotiorum*. Against the root rot pathogen *Sclerotium rolfsii*, in okra, treatment with the *T. virens* isolate PBAT-16 resulted in the lowest disease incidence. This isolate too was an effective sclerotial colonizer (100%) and a good hyphal growth inhibitor (39%) against this pathogen. All 4 isolates exhibited good control of *R. solani* and were at par with each other. In case of pea wilt caused by a non sclerotia forming pathogen, *Fusarium*, the plants treated with PBAT-43 recorded the lowest disease incidence followed by PBAT-14 and then PBAT-12 and PBAT-16. *Trichoderma harzianum* isolate PBAT-43 showed good antagonistic potential against this pathogen *in vitro*. The isolates PBAT-12 and PBAT-16 however, were only moderately inhibitory to radial growth of *Fusarium* under *in vitro* condition. The observations made in this experiment indicate that the overall performance of a particular *Trichoderma* isolate against sclerotia forming pathogens is possibly determined by both its antagonistic potential against the hyphal growth of a particular pathogen as well as by its sclerotial parasitizing ability.

No relationship was observed between the *Trichoderma* isolates exhibiting host selectivity for a specific pathogen and their genetic grouping. Isolates PBAT-37 and PBAT-38, which showed 100% genetic similarity to each other, differed significantly in their antagonistic potential against *R. solani* and *Fusarium*. Similar observations were recorded in case of other isolates (PBAT-11 & 18, PBAT-35 & 36) which otherwise showed >95% genetic similarity. In case of PBAT-34, a *T. harzianum* isolate, it was observed that although this isolate exhibited maximum inhibition of hyphal growth of *R. solani in vitro*, it was not observed to coil around the hypha of this pathogen. This isolate was further observed to form a separate cluster related only by 12% with the remaining *T. harzianum* isolates. Similarly PBAT-42, another isolate identified as *T. harzianum*, which formed a separate cluster, did not show coiling of *R. solani* hypha.

The presence of host selectivity in *Trichoderma* at sub-species level may be a major factor affecting its ultimate performance as a biocontrol agent under field conditions. The mechanisms which are employed by *Trichoderma* species to control a pathogen also appear to be variable depending upon the isolate and host pathogen. A study into this complex process of host selectivity needs to be done in order to develop effective biopesticides for future use.

SUMMARY

Chapter 6

SUMMARY

Trichoderma spp. are world wide in occurrence and are easily isolated from soil, decaying wood and other forms of plant organic matter. The potential of *Trichoderma* species as biocontrol agents of plant diseases was first recognized in early 1930's and in subsequent year's control of many diseases has been added to the list. However, even though biological control of plant diseases using *Trichoderma* species has long been an area of fruitful research, commercially it has not yet picked up on a large scale, mostly due to its inconsistent performance under field conditions. Although some work has been done on effect of different environmental factors like soil moisture, temperature, pH etc. field performance little information is available on *Trichoderma* isolate x host fungus interaction, which may be an important factor influencing field performance.

Keeping this in view a study was conducted to explore and characterize 43 isolates of *Trichoderma* species from the *Tarai* region of Uttaranchal. The isolates were characterized on the basis of their cultural, morphological and molecular diversity. The isolates were further studied for their antagonistic potential against four major soil borne plant pathogens with respect to their ability to inhibit hyphal growth as well as to colonize the sclerotia of pathogens and an attempt was made to characterize the isolates for the presence of host selectivity.

Major findings of the work are:-

1. The cultural characteristics of the isolates were not highly variable. Most isolates showed fast growth reaching a radius of 57 to 62 mm after 72 hours at 25^0C and 18 to 21 mm after 72 hours at 35^0C. Conidiation was predominantly effuse with some isolates initially producing flat pustules. Most isolates produced yellow pigment on Corn Meal Dextrose (CMD) medium but no pigmentation or odour was detected on PDA. Conidial colour change was observed from white to varying shades of green and sometimes from white to yellow green. In most isolates conidia were formed by 48 hours and turned green within 72 hours. In a few isolates (PBAT-2, 11, 20) green conidia were visible within 48 hours.

2. The 43 isolates could be divided into two groups based on the arrangement of conidiophores and phialides. First group of 38 isolates was characterized by having highly branched conidiophores, branching in a divergent, dendritic manner with branches often arising at 90^0 angle in pairs or in whorls of 3 with a fertile apex. The phialides were divergent and arranged in whorls of mostly 3-5. The second group of 5 isolates had hyaline conidiophores, sparingly branched in a divergent manner often at 90^0 angle. Towards the apex conidiophores branched in an irregular manner with each branch terminating in a penicillus of 3-5 closely

appressed phialides. Based on the morphological characteristics, the first group was classified as *T. harzianum* and second group as *T. virens* using the key given by Bisset (1991a&b).

3. The PCR amplification, performed with RAPD markers to assess the level of polymorphism in the 46 *Trichoderma* isolates revealed a high degree of genetic diversity. The five *T. virens* isolates formed a separate cluster and showed only 6% similarity with the *T. harzianum* isolates. The diversity was more marked within the *T. harzianum* group. Out of the 41 isolates classified as *T. harzianum*, 5 isolates PBAT-34, PBAT-42, PBAT-27, PBAT-9 and PBAT-40 formed separate clusters and showed only 13% to 54% genetic similarity with the remaining *T. harzianum* isolates. Out of the remaining *T. harzianum* isolates, only about 50% showed 90% or >90% genetic similarity. Clustering of germplasm on the basis of dendrogram was supported by a cophenetic correlation coefficient of 0.99, which is considered a good value.

4. The presence of high level of diversity within the isolates of *T. harzianum* has once again highlighted the fact that the concept of speciation within the genus *Trichoderma* is still highly confusing and further efforts are required to resolve it.

5. Although *Trichoderma* sp. has got a very wide host range, a fairly good degree of host selectivity at the level of strains/ isolates was exhibited by

the 43 isolates studied. It was observed that (i) different isolates of *T. harzianum* or *T. virens* varied widely in their ability to antagonize the same host fungus, (ii) different isolates of *T. harzianum* or *T. virens* exhibited different degree of antagonism against different host fungi. The host selectivity in *Trichoderma* was not observed to be a species related phenomenon and isolates of both *T. harzianum* and *T. virens* showed considerable variation in their antagonistic potential against various pathogens. Isolates PBAT-4, 12, 19, 28, 31 and 40 showed high antagonistic activity (>35%) against *R. solani*, *S. rolfsii* and *Sclerotinia* but poor activity against *Fusarium* spp (12 to 18%).

6. Studies on sclerotia colonization by various *Trichoderma* isolates also showed the presence of host specificity at isolate level in *Trichoderma* species. Isolates of both *T. harzianum* and *T. virens* were observed to colonize the sclerotia of the three pathogens. Nineteen isolates out of 43 colonized the sclerotia of at least one of the pathogens. Only 5 out of the 43 isolates of *Trichoderma* were able to colonize the sclerotia of all three pathogens in which two isolates were of *T. harzianum* (PBAT12 & 15) and 3 isolates were of *T. virens* (PBAT-16, 24 & 25). Three isolates showed specificity for the sclerotia of *S. rolfsii* while 8 isolates colonized the sclerotia of only *R. solani*. Among the five *T. virens* isolates one isolate did not colonize the sclerotia of any pathogen. Significant differences were recorded between the isolates with respect to their sclerotia colonizing potential against a specific pathogen. PBAT-16 was

observed to be the most virulent colonizer against all the three pathogens.

7. Further studies were done under glass house condition with 4 selected isolates to evaluate the role of host selectivity in the performance of *Trichoderma* as a biocontrol agent. Against the non sclerotia forming pathogen *Fusarium* sp., hyphae colonizing isolates PBAT-43 and PBAT-14 showed better disease control. Similarly in case of sclerotia forming pathogens *S. rolfsii* and *Sclerotinia sclerotiorum,* sclerotia colonizing isolates PBAT-16 and PBAT-12 gave better results with higher disease control.

8. Confrontation studies of *T. harzianum* and *R. solani* showed that all *T. harzianum* isolates do not employ mycoparasitism as the major mechanism for inhibition of hyphal growth of *R. solani.* Coiling was absent in 6 isolates of *T. harzianum* all 5 *T. virens* isolates.

9. No relationship was observed between the grouping of the isolates based on genetic relatedness studies and their selectivity for specific pathogens. The isolates showing specificity for inhibition/ colonization of the hyphal growth of specific pathogens/ sclerotia were dispersed over the different clusters. However, isolate PBAT-34, which exhibited maximum inhibition of radial growth of *R. solani in vitro* but did not exhibit any coiling around the host hyphae formed a separate cluster.

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APPENDIX

SOLUTION FOR DNA EXTRACTION

1. 1M Tris Buffer pH 8.0

Dissolve 12.11 g Tris base in 80 ml autoclaved distilled water. Adjust the pH to 8.0 with 1N HCl. Adjust to 100 ml with distilled water. Autoclave the buffer and store at room temperature.

2. 0.5 M EDTA

Dissolve 18.612 g of Na_2 EDTA.$2H_2O$ in 80 ml of distilled water. Adjust the pH to 8.0 with NaOH (by adding 2g of NaOH pellets). Stir vigorously on a magnetic stirrer for several h to ensure that all the solutes have dissolved. Note that Na_2EDTA will not dissolve completely in distilled water in the absence of NaOH. Adjust the volume up to 100 ml.

3. 5M NaCl

NaCl : 29.22 g

Distilled Water : 100 ml

3. DNA Extraction Buffer

	100 ml
1M Tris Buffer	10 ml
0.5M EDTA	10 ml
5M NaCl	10 ml
Distilled Water	70 ml

Adjust the pH to 8.0 with HCl.. Autoclave at 15 lb (psi) for 20 min and store at 4°C.

4. 70 % ethanol	**100 ml**
Absolute Ethyl Alcohol	70 ml
H_2O	30 ml

5. 5 M Potassium Acetate	**100 ml**
Potassium Acetate	49.07 g
Glacial Acetate Acid	11.5 ml

Make up to 100 ml. Autoclave and then store at room temperature.

6. 10 % (w/v) SDS	**100 ml**
SDS	10 g
H_2O	100 ml

Heat to 60° C to dissolve properly. Adjust pH to 7.2 by adding HCl. Make up to 100 ml. Autoclave and store at room temperature.

7. TE Buffer	**100 ml**
1M Tris Buffer	1.0 ml
0.5 EDTA	0.2 ml

Make up to 100 ml by adding water. Autoclave and store at room temperature.

8. Isopropanol

Store at - 20° C in a 100 ml dark colour bottle.

B. SOLUTIONS FOR GEL ELECTROPHORESIS

1. **DNA Loading Buffer (6X)**	**10 ml**
Bromophenol Blue (0.25 %w/v)	0.025 g
Xylene Cyanol FF (0.25 %w/v)	0.025 g
Glycerol in H2O (30%)	3 ml

Dissolve properly. Make up volume to 10 ml. Store at -20° C.

Use sterilized distilled water.

2. **Electrophoresis Buffer (50 x TAE)**	**100ml**
Tris Base	2.4 g
Glacial Acetic Acid	5.7 ml
EDTA (0.5M)	10.0 ml

Dissolve in minimum volume of H_2O adjust pH to 8.0. Make up to 100 ml. Autoclave and store at room temperature.

3. **Electrophoresis Buffer (5 x TBE)**	**1000 ml**
Tris Base	54 g
Tris Boric Acid	27.5 g
0.5M EDTA	20 ml

Dissolve in minimum volume of H_2O adjust pH to 8.0. Make up to 1000 ml. Autoclave and store at room temperature.

4. Ethidium Bromide (10,000 x)	**10 ml**
Ethidium Bromide	100 mg
H_2O	10 ml

Working solution for staining gel is made by dissolving 60 µl Ethidium Bromide stock (10 mg/ml) in 300 ml water. Store at 4^0C. Since Ethidium Bromide is highly carcinogenic. Use gloves while handling.

VITA

Name: Deeksha Joshi
Date of Birth: 14th May
High school: St.Thomas College, Pauri (1992)
Intermediate: LaMartiniere Girls College, Lucknow (1994)
Graduation: B.Sc. (Z,B,C) Lucknow University, Lucknow (1997)
M.Sc.(Ag.) Plant Pathology: G.B.P.U.A&T, Pantnagar (2000)
Ph.D. (Plant Pathology): Joined in year 2000.
*Presently working as Scientist (Plant Pathology) at Vivekanand Institute for Hill Agriculture, Almora 263 601, Uttaranchal

Permanent Address
289
West Canal Road
Majra
Dehradun
Uttaranchal

Name: Deeksha Joshi
Id. No. : 24834
Sem. &Year of admission: 2000-2001, Ist semester
Degree: Ph.D.
Major: Plant Pathology
Minor: Entomology
Thesis Title: "Cultural, Morphological, Molecular and Pathogenic Characterization of *Trichoderma* isolates from *Tarai* area of Uttaranchal"
Advisor: Dr. U.S.Singh

ABSTRACT

Trichoderma species specially *T. harzianum*, *T. virens* and *T. viride* are three most widely used species for biological control of a number of soil borne, seed borne and foliar diseases. Although this fungus has a broad host range and employs a number of mechanisms like mycoparasitism, antibiosis, competition etc. for the control of pathogens the results are still quite inconsistent. Recent studies have indicated that various soil chemical, physical and biological factors may influence the survival of this fungus under natural conditions. Besides some preliminary work has indicated the presence of host selectivity in *Trichoderma* species, a factor which may ultimately influence its biocontrol potential. Keeping this in view, the present investigation was carried out to characterize 43 indigenous *Trichoderma* isolates with respect to their cultural, morphological and molecular characters and in terms of their host selectivity against 4 plant pathogens.

The isolates did not exhibit considerable variation in their cultural characteristics but on basis of morphology they could be classified under two groups. Based on the combined observations of cultural and morphological characters, 38 isolates were classified as *T. harzianum* and 5 isolates as *T. virens*. However, the molecular marker based analysis of the isolates using RAPD technique showed high degree of genetic diversity in the isolates specially within the isolates classified as *T. harzianum*. Experiments were conducted to study the presence of host selectivity in *Trichoderma* isolates both against hyphal growth and sclerotia. Significant differences were observed between the isolates with respect to their antagonistic potential against a specific pathogen. Also individual isolates varied considerable in their activity against different pathogens. The same trend was reflected in their ability to colonize the sclerotia of different pathogens. Only 5 isolates (2 *T. harzianum* and 3 of *T. virens*) were able to colonize the sclerotia of all three pathogens with *T. virens* PBAT-16 being the most virulent colonizer. Individual isolates exhibited differential activity against the radial growth and sclerotia of a specific pathogen. Further experiments conducted under glass house revealed that isolates exhibiting both,a good hyphal inhibition activity and good sclerotia colonizing activity gave highest disease control against sclerotia forming pathogens. Against non sclerotia forming pathogens lower disease incidence was recorded in isolates having higher antagonistic activity against hyphal growth.

No relationship was observed between the host selectivity of the isolates and their genetic groups. However, the presence of host selectivity in *Trichoderma* species emphasizes the need to look into this complex process. Use of strains/ isolates exhibiting a good hyphal as well as sclerotial inhibitory activity is recommended.

(U.S. SINGH)
Advisor

(DEEKSHA JOSHI)
Authoress