FACTORS AFFECTING THE BIOLOGICAL CONTROL OF *PHYTOPHTHORA CAPSICI* INFECTIONS IN BLACK PEPPER (*PIPER NIGRUM* L.)

Thesis submitted to
Faculty of Science, University of Calicut
In partial fulfilment of requirements for the award of
Doctor of Philosophy in Botany

By
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Under the guidance of
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2004

DECLARATION

I, K. A. Saju, honestly declare that the Ph.D. thesis entitled '**Factors affecting the biological control of *Phytophthora capsici* infections in black pepper (*Piper nigrum* L.)**' is a bonafide record of research work carried out by me at Indian Institute of Spices Research, Calicut under the guidance of Dr Y. R. Sarma, Former Director of IISR. No part of this thesis has been submitted previously to any university for any degree / diploma.

SAJU K. A.

Date: July 2004

Place: Calicut

CERTIFICATE

This is to certify that the Ph.D. thesis entitled '**Factors affecting the biological control of *Phytophthora capsici* infections in black pepper (*Piper nigrum* L.)**' is a record of bonafide research work carried out by Mr. K. A. Saju under my supervision and guidance and that it has not previously formed the basis for award of any other degree / diploma.

Date: July 2004

Place: Calicut

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DEDICATION

This thesis is dedicated to

Sr Josephine
Mr P. S. Sagaran
(Late) Dr K. Kuruvilla
Dr K. V. Paulose
and
Prof. C. M. Mathew
Whose humanitarian and academic inspiration as teachers helped me to take the right path starting from my *harisree* to *post graduation*

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ABBREVIATIONS AND SYMBOLS

^{0}C	degree Celsius
a.i.	active ingredient
ANOVA	Analysis of Variance
BNR	Binucleate *Rhizoctonia*
C / N ratio	Carbon / Nitrogen ratio
CA	Carrot Agar
CDB	Czapek Dox Broth
CF	Culture Filtrate
cfu	colony forming unit
cm / s	centimeter / second
cm	centimeter
CSA	Competitive Saprophytic Ability
cv	cultivar
DMRT	Duncan's Multiple Range Test
EtOAc	ethyl acetate
EtOH	ethyl alcohol
FT	fusant *Trichoderma*
IDM	Integrated Disease Management
IPM	Integrated Pest Management
ISR	Induced Systemic Resistance
KBA	Kings, B Agar
kg	kilogram
LD$_{50}$	Lethal dose for 50% inhibition
mg	milligram
MHC	Moisture Holding Capacity
min	minutes
mm	millimeter
MSL	Mean Sea Level
NARPH	National Repository of *Phytophthora*
NB	Nutrient Broth
NPK	Nitrogen, Phosphorous Potassium
P_{10}VP	Pimaricin, Vancomycin, Pentachloronitrobenzene
PCNB	Pentachloronitrobenzene
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PEG	Poly Ethylene Glycol
PGPR	Plant Growth Promoting Rhizobacteria
ppm	parts per million
PVPH	Pimaricin, Vancomycin, Pentachloronitrobenzene, Hymexazole
RBA	Rose Bengal Agar
RC	Rhizosphere Competence
Rf	retardation factor
RPM	revolution per minute
RT	retention time
SDPT	Serial Dilution Plate Technique
SEM	Scanning Electron Microscopy
TLC	Thin Layer Chromatography
TSM	*Trichoderma* Selective Medium
UV	Ultra Violet
var	variety
w / v	weight / volume
WA	Water Agar
µg	microgram
µl	microlitre

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INTRODUCTION

As environmental concerns over the use of chemicals for plant disease and pest control continues, the search for biological or sustainable alternatives of control has increased. Therefore biological control was suggested as a component of IDM in many crops using antagonistic fungi and bacteria. Biological control is the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organism other than man (Cook and Baker, 1983). This may be accomplished through cultural practices (habitat management) that create an environment favourable to antagonists, host plant resistance or both through plant breeding to improve resistance to the pathogen or suitability of the host plant to the activities of antagonists through the mass introduction of antagonists, non-pathogenic strains or other beneficial organisms or agents. Agrios (1997) defines biological control 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 organism accomplished naturally or through manipulation of the environment, host or by mass introduction of one more antagonists.

1.1 The objective

In black pepper, *Phytophthora capsici* causes foot rot disease and is the most serious disease of the crop in India. Several antagonists like *Trichoderma, Gliocladium, Verticillium* and *Pseudomonas* were isolated and tested for their bioefficacy. Biological control of foot rot of black pepper is of great relevance to the region especially to Kerala and Karnataka since crop loss due to *Phytophthora* infections in black pepper is severe. Although chemical control measures are feasible to some extent the environmental concern over the use of chemicals for disease control continues. *T. harzianum* and *T. virens* have been established as potential biocontrol agents of *P. capsici*. Biological control of *Phytophthora* foot rot of black pepper with *T. harzianum* and *T. virens* has been practiced as a viable technology in recent years (Sarma *et al.*, 1997; Rajan, 1999; Rajan *et al.*, 2002). It offers a powerful means to protect the crop by suppression or destruction of pathogen inoculum or increase the defense systems in plants against pathogens. More information is needed on several factors affecting the biological control in order to realize its full potential in disease suppression. Ecological stability of introduced microorganisms into the environment should be defined and it would help to design more appropriate technologies. Hence the present investigation was undertaken with the following objectives.

1 The mechanism of action of biocontrol agents

2 Their interaction with host plant and pathogen

3 The rhizosphere competence and the ecological fitness of biocontrol agents in the soil ecosystem

4 Microbial consortium approaches for disease management

1.2 Reasons and accomplishments

Understanding modes of action has been useful for refining screening systems to select further useful antagonists in preparing registration packages and for improving activity through molecular manipulation. Improvements in efficacy have also been achieved through studies of physiology, inoculum production, formulation and application process. Liquid and solid substrate fermentations have been refined for *T. harzianum* IISR 1369 and *T. virens* IISR 1370 (Anandaraj and Sarma, 1997; Prakash *et al.*, 1999; Saju *et al.*, 2002c). The greatest impetus to commercial development have been the realization that biocontrol agents must be adapted to the environment where biocontrol is required for long enough for activity to ensure.

Some times concern is raised over the use of biocontrol agents for the control of fungal diseases and insects by the narrow range of environmental factors, particularly of water availability and temperature over which germination, establishment and infection can occur on field crops. Consequently, studies on the ecology of biocontrol fungi against a background knowledge of the pathogen biology, plant growth and the resident microbial community has undoubtedly been a key contributory factor in the increasing reproducibility and success of disease biocontrol. To some extent aspects relating to dispersal and survival can be considered separately from the actual mechanism involved in biological control. It is important to ensure that the biocontrol agent is able to invade and persist in the environment. Terms commonly found useful for fungal plant pathogens such as competitive saprophytic ability (CSA) or rhizosphere competence (RC) may also be applied to biocontrol agents.. Therefore background knowledge is necessary about biocontrol agents with respect to dispersal and survival and ultimately distribution within the complex set of biotic and abiotic environment.

Accordingly, in this study, an attempt has been made to isolate and identify volatile and non-volatile metabolites of *Trichoderma* spp active against *P. capsici*. Metabolites producing isolates with high inhibition of *P. capsici* was identified. The effect of these metabolite-producing isolates on the growth promotion of black pepper and disease suppression was studied *in-vivo*. The influence of abiotic factors such as soil type, soil temperature, pH, pesticides and fertilizers were studied in relation to survival of the antagonist in soil. Rhizosphere competence of biocontrol agents is the ability to grow in the rhizosphere utilizing host exudates so that other invaders cannot enter the zone and attack the root (Ahmad and Baker, 1987). Rhizosphere competence of isolates of *Trichoderma* was determined experimentally. The potential isolate *T. harzianum* IISR 1369 grows well on solid substrates and in liquid cultures with high number of colony forming units (cfu). Non-volatiles of another isolate *T. harzianum* IISR 140 is highly inhibitory to *P. capsici* than that of *T. harzianum* IISR 1369 but it is unable to grow and sporulate well on solid substrate and in liquid culture. An attempt has been made to combine these two characteristics using protoplast fusion technology and developed fusants that are super metabolite producers. Several efficient *Trichoderma* spp and *Pseudomonas fluorescens* isolated from black pepper roots were evaluated in the glass house and field.

2

REVIEW OF LITERATURE

In recent years biological control of plant diseases, insect pests and weeds are gaining momentum all over the world on diverse crops and environments primarily due to the hazardous nature of the chemical pesticides to humans and ecosystems. In that sense integrated pest management (IPM) is an attempt to prevent pathogen, insects and weeds from causing economic crop loss by using a variety of methods that are cost effective and cause least damage to the environment (Agrios, 1997). Consequently, management of pathogen alone took much attention of researchers through eco-friendly means. Thus the concept of integrated disease management (IDM) which is the modification of cultural practices, use of resistant varieties and monitoring of the appearance and development of plant disease epidemics which allow for reduced use of pesticides was developed and put into practice (Agrios, 1997). As stated in the previous chapter, biological control is the reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state by one or more organism accomplished naturally through manipulation of the environment, host or by mass introduction of one or more antagonists (Agrios, 1997). Baker and Cook (1974) and Cook and Baker (1983) also gave a definition similar to above one. Biological control of plant diseases involves one or more natural mechanisms like antibiosis, parasitism, lysis, competition, plant growth promotion, predation and induced systemic resistance. Antibiosis plays an important role in biocontrol by the production of secondary metabolites, which are toxic to the target pathogen(s) and still some other compounds, promote the plant growth directly (Fravel, 1988; Sivasithamparam and Ghisalberti, 1998). These mechanisms are influenced by an array of both abiotic and biotic factors. These factors often limit the interaction between plant pathogen and their antagonists resulting in less than acceptable suppression of disease or reduction in pathogen population (Cook and Baker, 1983; Burpee, 1990). Hence emphasis in this review has been placed on the production of non-volatile and volatile metabolites by *Trichoderma* spp, effect of biocontrol agents on host plants and pathogens, abiotic factors that influence biocontrol agents, their rhizosphere competence and application of protoplast fusion technology for combining desirable characters in biocontrol agents.

2.1 The crop

Black pepper (*Piper nigrum* L.), popularly known as the 'king of spices', is the most important and widely used spice in the world. It is a perennial woody climber believed to be originated in the Western Ghats of India where a number of its wild relatives are documented and studied (Ravindran and Nirmal Babu, 1994; Ravindran *et al.*, 2000). As an export-earning crop, in India, black pepper is grown in an area of 2,57,770 ha (Directorate of Arecanut and Spices Development, Calicut) both as monocrop and mixed crop in arecanut and coconut gardens and coffee and tea plantations. As a climber it is grown on various standards like coconut (*Cocos nucifera*), arecanut (*Areca catechu*), Jack tree (*Artocarpus* sp.), Mango tree (*Mangifera indica*), *Erythrina indica*, *Garuga pinnata*, *Ailanthus* sp., Silver oak (*Grevilea robusta*) and other homestead and forest trees.

Production

Total annual production in India was 79,000 tonnes in 1999-2000. Kerala and Karnataka account for major portion of production of black pepper in the country. During 1999-2000, about 42,806 tonnes of black pepper worth Rs 885 crores were exported to various countries accounting for 46.5% of export earning among spices (Directorate of Arecanut and Spices Development, Calicut). However, in terms of productivity, i.e. production per unit area, India is far behind ~~in~~ countries like Malaysia, Indonesia and Brazil.

Production constraints

In India, there are many constraints that are responsible for the less productivity of black pepper. Diseases and pests, fluctuating price situation, lack of high yielding disease resistant varieties and poor extension system, etc are identified as major factors for low productivity of black pepper. For the purpose of this chapter, constraints due to diseases and pests are detailed.

Diseases and pests

There are various diseases and pests affecting black pepper. ***Phytophthora* foot rot** caused by *P. capsici* is the most destructive of all diseases and occurs mainly during the southwest monsoon season. 'Pollu' disease is common in high altitude areas and is caused by *Colletotrichum gloeosporioides*. This disease appears towards the end of the monsoon. The affected berries show brown sunken patches during early stages and their further development is affected (Anandaraj and Sarma, 1995). Stunted disease is caused by cucumber mosaic virus and in combination with badnavirus (Bhat *et al.*, 2003), it is noticed in parts of Kozhikode, Wayanad and Idukki districts of Kerala and Coorg district of Karnataka. The vines exhibit shortening of internodes to varying degrees. The leaves become small and narrow with varying degrees of deformation and appear leathery, puckered and crinkled. Phylloidy disease is caused by Phytoplasma and is noticed in parts of Wayanad and Kozhikode districts. The affected vines exhibit varying stages of malformation of spikes (Anandaraj, 2000; Sarma *et al.*, 1994). **Slow decline** is a serious disease of black pepper. Foliar yellowing, defoliation and die back are the aerial symptoms of this disease. The root system of diseased vines show varying degrees of necrosis and presence of root galls due to infestation by plant parasitic nematodes such as *Radopholus similis* and *Meloidogyne incognita* leading to rotting of feeder roots (Anandaraj, 2000; Sarma *et al.*, 1994). The damage to feeder roots is caused by these nematodes and *P. capsici* either independently or in combination. Among the insect pests, pollu beetle (*Longitarsus nigripennis*), top shoot borer (*Cydia hemidoxa*), leaf gall thrips (*Liothrips karnyi*) and scale (*Lepidosaphes piperis, Aspidiotus destructor*) and mealy bugs (*Ferrisia virgata*) are the major ones causing severe crop loss (Devasahayam, 2000).

Phytophthora **foot rot**

All parts of the vine are vulnerable to the disease and the expression of symptoms depends upon the site or plant part infected, prevailing environmental conditions and the extent of damage. One or more black spots appear on the leaves, which have a characteristic fimbriate margin (fine fibre like projection at the advancing margins) that rapidly enlarge and cause defoliation. The tender leaves and succulent shoot tips of freshly emerging runner shoots trailing on the soil turn black when infected. The disease spreads to the entire vine from these infected runner shoots and leaves during intermittent showers due to rain splash. If the main stem at the ground level or the collar is damaged, the entire vine wilts followed by shedding of leaves and spikes with or without foliar yellowing. The branches break up at nodes and the entire vine collapses within a fortnight. If the damage is confined to the feeder roots, the expression of symptoms is delayed till the cessation of rain and the vine starts showing declining symptoms such as yellowing, wilting, defoliation and drying up of a part of the vine. This may occur during October-November onwards (Anandaraj, 1997; Anandaraj, 2000; Jahagiridar, 1995; Mehrotra and Aggarwal, 2001; Ramachandran *et al.*, 1986; 1900a; Sarma *et al.*, 1994; Sarma and Anandaraj, 1997).

Integrated management of soil borne diseases of black pepper

P. capsici and nematodes are not spatially separated in a black pepper plantation. Both are soil borne and management of these pathogens would ensure better root system and healthy plant. Hence for the management of soil borne diseases an integrated disease management (IDM) strategy is suggested which include the following precautions and measures (Anandaraj, 2000; Anandaraj and Sarma, 1995; Ramachandran *et al.*, 1990b; Sarma *et al.*, 1994; Sarma *et al.*, 1998; Sarma *et al.*, 2000).

Phytosanitation

Removal and destruction of dead vines along with root system from the gardens is essential as this reduces the build up of pathogen inoculum. Planting material must be collected from disease free gardens and the nursery should be raised perfectly in solarised soil (Anandaraj, 2000; Sarma *et al.*, 1994).

Cultural practices

Adequate drainage should be provided to reduce water stagnation. Injury to the root system due to cultural practices such as digging should be avoided. The freshly emerging runner shoots should not be allowed to trail on the ground. They must either be tied back to the standard or pruned off. The branches of support trees must be pruned at the onset of monsoon. Less humidity and presence of sunlight reduces intensity of leaf infection (Ramachandran *et al.*, 1988; Shea and Broadbent, 1983).

Chemical control

After the receipt of a few monsoon showers all the vines are to be drenched with copper oxychloride (0.2%) @ 5-10 litres / vine or potassium phosphonate (0.3%) @ 5-10 litres / vine or metalaxyl mancozeb (0.125%) @ 5-10 litres / vine (Ramachandran, 1990; Ramachandran and Sarma, 1985a; 1985b). For nematode infested vines apply phorate @ 30 g / vine during May – June and September – October at three places around the base (Ramana, 1990; Ramana and Eapen, 1995).

Biological control

Biological control is the major interest for plant disease problems for last two decades (Agrios, 1997). Interest in the biological control of *Phytophthora* foot rot of black pepper was progressed in the line of isolating several fungal and bacterial isolates from black pepper rhizosphere and testing them for their efficacy (Sarma and Anandaraj, 1996). As a result, several fungi and bacteria like *Trichoderma, Gliocladium, Aspergillus, Penicillium, Bacillus* and *Pseudomonas fluorescens* were obtained and studied. Short-listed isolates were tested in the field for their performance (Rajan, 1999; Rajan *et al.*, 2002; Saju *et al.*, 2003). In recent years biological control of *Phytophthora* infections on other crops using fungal and bacterial agents has been intensified. Non-pathogenic binucleate *Rhizoctonia* fungi (BNR) controlled black shank caused by *Phytophthora parasitica* var. *nicotianae* on green house grown tobacco seedlings in Styrofoam float trays (Cartwright and Spurr Jr, 1998). Most widely used biocontrol agents for the control of Oomycetes plant pathogens are *Trichoderma* spp, *Pseudomonas fluorescens* and *Bacillus subtilis*.

2.2 *Trichoderma* in biological control

The genus *Trichoderma* was introduced into mycological literature by Persoon (1794) and classic studies of Weindling and Fawcett (1936) revealed its biocontrol potential. Later the first authenticative monograph of the genus was written by Rifai (1969) to accommodate nine species. Gams and Bissett (1998) reviewed the taxonomy of *Trichoderma* and listed 33 species. *T. harzianum* and *T. virens* were identified as potential antagonists of *P. capsici* (Rajan, 1999; Rajan *et al.*, 2002). Biocontrol strains have been found that are effective in a variety of habitats and against an assortment of pathogens. *Trichoderma* and *Gliocladium* isolates are being used in many different crops like cotton, grapes, sweet corn, lettuce, onions, peas, plum, apples and carrots against diseases caused by pathogens such as *Pythium, Phytophthora, Rhizoctonia, Sclerotinia, Botritys* and *Fusarium.* Various isolates have been used with success in green house and field applications in soil and in the phyllosphere and as well as in cold storage (Chet, 1987; Papavizas, 1985). Hjeljord and Tronsmo (1998) presented an overview of biocontrol with *Trichoderma* species in which they detailed the criteria for selection, mechanism, formulation etc. Fast growth and multiplication in organic wastes make *Trichoderma* an ideal candidate for biological control of plant pathogens (Prakash *et al.*, 1999; Saju *et al.*, 2000; Saju *et al.*, 2002b). Singh (1994) identified *T. harzianum* and *Chaetomium* sp. as potential biocontrol fungi in management of red rot disease of sugarcane caused by *Colletotrichum falcatum.* Padmodaya and Reddy (1996) found *T. viride* as highly inhibitory to *Fusarium oxysporum* f. sp. *lycopersici* in dual culture followed by *T. harzianum. T. harzianum* and *T. viride* significantly reduced the root rot of sesamum caused by *Macrophomina phaseolina* to 10.1 and 12.8% respectively compared to 60% incidence in the control plots in a field experiment (Sankar and Jeyarajan, 1996). Combining biocontrol agents and chemicals have given better control of disease than either components used alone. Muthumilan and Jeyarajan (1996) reported that integration of *T. harzianum*, *Rhizobium* and carbendazim remarkably reduced the root rot of groundnut caused by *Sclerotium rolfsii.* Mathre *et al.* (1999) reviewed the obstacles in commercializing biocontrol agents. Mass production, carrier media and storage life are important for commercialization of biocontrol agents. McSpadden Gardner and Fravel (2002) listed several products containing microorganisms used for plant diseases control in United States. Fahima and Henis (1990) could multiply *T. hamatum* and *Talaromyces flavus* on roots of diseased and healthy hosts (radish and egg plant) and protected against their pathogens (*R. solani* and *V. dahliae*). Lumsden *et al.* (1990) used a potting medium to demonstrate the biological control potential of *T. harzianum* against damping off of lettuce (*Lactuca sativa*) caused by *Pythium ultimum.* Ramakrishnan *et al.* (1994) developed a formulation of *T. viride* for seed pelleting by mixing talc powder with *Trichoderma* biomass obtained by growing the fungus in molasses-yeast medium for 15 days and drying. Kumar and Marimuthu (1997) reported decomposed coconut coir pith as conducive medium for colonization of *T. viride.* Prakash *et al.* (1999) evaluated neem cake, farmyard manure, coffee husk and tea spent for the mass multiplication of *T. harzianum* and *T. virens* and found that tea spent was the best media. The product could be stored for three months without reduction in the number of propagules. Smith *et al.* (1990) developed a system in the identification of isolates of *Trichoderma* and *Gliocladium* spp with potential for biocontrol of *Phytophthora cactorum. Metarrhizium anisopliae* and *T. viride* are effective in controlling *Atta cephalotes* (an economically important pest in tropical and subtropical agricultural and forestry ecosystems) nests under laboratory and field conditions and superior to the chemical pirimiphos methyl (Lopez and Orduz, 2003). *T. hamatum* and *G. virens* could multiply luxuriantly in coconut water up to 50% dilution with tap water (Anandaraj and Sarma, 1997). The reliability of biocontrol systems can be improved by the use of formulations that provide conducive environments for the bioprotectants and fermentation systems that economically produce propagules of high quality (Harman, 1991). The most appropriate criterion for selection of a biocontrol agent ultimately is considerations of environmental requirements of both the antagonist and of the pathogen, which it is intended to control. Several bacteria especially *Bacillus* spp and *Pseudomonas fluorescens* were identified as biocontrol agents of soil borne plant pathogens. Mixtures of bacterial biocontrol agents also suggested for successful disease control. Mixtures of plant growth promoting rhizobacteria (PGPR) provided greater disease suppression than individual PGPR strains. Mixtures of PGPR can elicit induced systemic resistance (ISR) to fungal, bacterial and viral diseases (Kloepper and Jetiyanon, 2002).

Mechanism of biocontrol with *Trichoderma*

The major mechanisms involved in biological control with *Trichoderma* are lysis, mycoparasitism, antibiosis, competition for nutrients and space, plant growth promotion and rhizosphere competence. Lysis and mycoparasitism requires enzymatic action and lysis reveal susceptibility of host and mycoparasitism shows host resistance. Volatile and non-voaltile metabolites inhibit the growth of the pathogen and the phenomenon is known as antibiosis. Competition for nutrients and space is achieved by fast multiplication and distribution. Both antibiosis and mycoparasitism may be involved in competition for nutrients, indeed production of toxic metabolites is known to be affected by the nutrient status of the growth medium (Fravel, 1988; Ghisalberti and Sivasithamparam, 1991; Howell and Stipanovic, 1995). Recent evidences show that antibiosis and hydrolytic enzymes are not only produced together but act synergistically in mycoparasitic antagonism (Di Pietro *et al.*, 1993; Schirmbock *et al.*, 1994).

2.3 Secondary Metabolites

The basic features of energy production and cell growth in all organisms are essentially similar and the compounds involved in the fundamental reactions of growth and reproduction are the same. These ubiquitous and essential compounds include amino acids, carbohydrates, fatty acids, and nucleic acids and are classified as primary or intermediary metabolites (Sivasithamparam and Ghisalberti, 1998). The concentration of primary metabolites in cells are strictly controlled and kept very low to avoid considerable non-enzymatic side reactions and feed back inhibition. However there are microorganisms those can accumulate considerable quantities of primary metabolites. For example, citric acid an obligatory intermediate in the tri-carboxylic acid (TCA) cycle may accumulate in high quantities in *Aspergillus niger* at low pH and in the absence of metal ions (Sivasithamparam and Ghisalberti, 1998).

In contrast to the ubiquitous nature of primary metabolites there is a much greater number of compounds biosynthesized by organisms that appear not to have a crucial role in the organisms that produce them. These natural products have commonly been referred to as secondary metabolites reflecting the inability to assign a role to them in the internal economy of the organism. In general, they are compounds of limited molecular weight (< 3000 Daltons) and great structural variety. Secondary metabolites are broadly divisible into several characteristic groupings such as polypeptides, terpenes, phenols alkaloids that reflect their origin and biosynthesis. The essential difference between secondary and primary metabolites is that for the former a wide range of compounds can arise from one single intermediate by light variation of the metabolic pathway (Sivasithamparam and Ghisalberti, 1998). Moreover different species of the same family and different isolates of the same species can often produce significantly different compounds leading to the suggestion that secondary metabolites express the individuality of species in chemical terms (Borjesson *et al.*, 1992). On the other hand, widely separate species can produce the same class of secondary metabolites and sometimes even the same secondary metabolites. For example, gibberellic acid, the plant growth hormone present in small amounts in plants is produced in large quantities by the fungus *Gibberella fujikuroi.* Gliotoxin is produced by a number of *Penicillium* sp., *Aspergillus fmigatus* and *Thermoascus crustaceous.*

Secondary metabolites are those compounds that accumulate in the organism and to a large extent are metabolically inactive towards the organism that produces them. Strains able to make these compounds may lose, because of mutations, the capacity to synthesize them without any apparent immediate effect to the organism (Sivasithamparam and Ghisalberti, 1998). Mathur and Bhatnagar (1994) recorded inhibition of *Macrophomina phaseolina* by *T. viride* by volatile and non-volatile metabolites. Similarly *T. harzianum* inhibits *Sclerotium rolfsii* (Upadhyay and

Mukhopadhyay, 1983). Fravel (1988) reviewed antibiosis which is antagonism mediated by specific or nonspecific metabolites of microbial origin by lytic agents, enzymes, volatile compounds or other toxic substances. Fungal metabolites, which are potential antihypercholesterolemics and anticancer agents, were discovered (Haung *et al.*, 1995). Viridiofungins A, B and C are produced by *T. viride* (Huang *et al.*, 1995a; 1995b). Ghisalberti and Sivasithamparam (1991) reviewed the antifungal antibiotics produced by *Trichoderma* spp. In a recent review, Sivasithamparam and Ghisalberti (1998) discussed the secondary metabolites of *Trichoderma* and *Gliocladium* and listed about 140 compounds. Howell (1998) reviewed the phenomenon of antibiosis and inhibition of pathogens in detail.

Non-volatiles

Dennis and Webster (1971a) recorded antibiotic activity of non-volatile compounds in extracts from culture of *Trichoderma* spp. Almassi *et al.* (1991) isolated two compounds from *T. harzianum* strain 71 active against wheat take all fungus *Gaeumannomyces graminis* var. *tritici* which were also reported previously from *T. harzianum* and *T. koningii*. A third compound, an octaketide derived acetal diol, has also been isolated from *T. harzianum* strain 71. The *T. harzianum* strain 73 yielded two new butenolide metabolites containing the 3,4-dialkyl furan-2 (5H), which could antagonize the growth of the take-all fungus. The isolates *T. viride* (H), *T. viride* (A.P.) and *Trichoderma* sp. (D) proved effective in reducing radial growth of *F. oxysporum* f. sp. *lycopersici* in study on production of non-volatile compounds by *Trichoderma* spp (Padmodaya and Reddy, 1996). Ghisalberti and Rowland (1993) later deduced the structures of these new compounds. Cutler *et al.* (1989) isolated a white crystalline compound from *T. koningii* named koningin A that is a ketal that weakly inhibits the growth of etiolated wheat coleoptiles at 10-3M. Ordentlich *et al.* (1992) isolated three substances from growth medium of *T. harzianum* and one was identified as 3-(2-hydroxy propyl)-4-(2-hexadienyl-2 (5H)-furanon. Ghisalberti *et al.* (1990) found that pyrone producing strains of *T. harzianum* 71, *T. koningii* and *T. hamatum* reduced take all of wheat. *T. harzianum* 70 and *T. harzianum* 73 did not produce any pyrones but they reduced the disease to a much lesser extent than isolate 71 with isolate 73 showing distinct host growth promotion effects. They proposed that the success of isolate 71 of *T. harzianum* was related to the pyrones it produces and that the ability of isolates 70 and 73 to reduce take all may be related to mechanisms other than those involving antibiotics. Faull *et al.* (1994) isolated an isonitrile antibiotic homothallin II from UV induced mutant strain of *T. harzianum* with broad antibiotic activity against oomycetes, ascomycetes and basidiomycetes fungi and both gram positive and negative bacteria. Dunlop *et al.* (1989) obtained a new antibiotic as the major metabolite produced in pure culture by *T. koningii* isolated from soil suppressive to the saprophytic growth of the take all fungus *Gaeumannomyces graminis* var. *tritici*. The compound produced in agar culture inhibited the growth of several other soil borne plant pathogens. Ghisalberti *et al.* (1992) isolated harziandione a new class of diterpenes from *T. harzianum*. Howell and Stipanovic (1983) obtained a compound with antibiotic activity from *G. virens* towards *Pythium ultimum* namely Gliovirin. Lewis and Papavizas (1985) reported that mycelial preparations of eight out of 14 isolates of *Trichoderma* spp and *G. virens* reduced survival of *R. solani* at least by 50% in pathogen infested beet seed in soil and in soil infested with sand / cornmeal inoculum of the pathogen. Similarly water extracts of germlings (young, actively growing hyphae on bran) of isolates of *Trichoderma* spp and *Gliocladium virens* affected growth of *R. solani* (R-23) in liquid culture (Lewis and Papavizas, 1987). The chemical composition of the leaked materials includes soluble proteins, carbohydrates, and amino acids and was determined by calorimetry and conductance. Brewer *et al.* (1982) isolated 3 compounds from *T. hamatum* (wild isolates), they are trichoviridin, 3-(3-isocyano-6-oxabicyclo [3,1,0] hex-2-en-5-yl) cyclic acid and 3-(3-isocyanocyclopent-2-enylidene-) propionic acid. At least one of these isocyanides was isolated from all cultures in which the culture broth inhibited the growth of *Micrococcus luteus*. The metabolite gliotoxin was inhibitory to sporangial germination and mycelial growth of *Pythium ultimum* (Roberts and Lumsden, 1990).

Volatile metabolites

Dennis and Webster (1971b) found volatile antibiotics of *Trichoderma* with active coconut odour. Studies on production of volatile compounds by *Trichoderma* spp revealed that *T. viride* (H), *T. viride* (A.P.) and *Trichoderma* sp. (D) are effective in reducing radial growth of *Fusarium oxysporum* f. sp. *lycopersici* (Padmodaya and Reddy, 1996). Cutler *et al.* (1986) obtained 6-pentyl-α-pyrone from *T. harzianum*, which inhibited the growth of etiolated wheat coleoptile and certain selected microorganisms. Scarselletti and Faull (1994) tested 6-pentyl-α-pyrone produced by *T. harzianum* IMI 288012 against *R. solani* and *F. oxysporum* f. sp. *lycopersici* in *in-vitro* plate tests. The compound partially inhibited the growth of *R. solani* (69.6%) and *F. oxysporum* f. sp. *lycopersici* (31.7%) at a concentration of 0.3 mg / ml in agar medium. It completely inhibited the germination of *Fusarium* spores. Maiti *et al.* (1991) reported the production of volatile inhibitors by *T. virens* that particularly suppressed the growth of *Sclerotium rolfsii* in culture. Worasatit *et al.* (1994) reported that pyrone producing strains of *T. koningii* were able to protect wheat root rot. Those not producing pyrone did not reduce the disease when applied to the soil. *Trichoderma* induces oospore formation in *Phytophthora* and is considered as a defensive response to a potential antagonist or competitor (Brasier, 1975a; 1975b). This form of microbial interaction could well be a common feature of plant diseases and in the terminology of Baker and Cook (1974), it would be an antibiosis met with a defensive probiosis.

2.4 Enzymes

Ability of *Trichoderma* spp to degrade polysaccharides and related macromolecules by enzymatic action made them a pioneer organism in enzyme chemistry and industry. The major classes of enzymes are cellulolytic (Koivula *et al.*, 1998), hemicellulolytic (Biely and Tenkanen, 1998), Chitinolytic (Lorito, 1998) and glucanolytic (Benitez *et al.*, 1998). The process of mycoparasitism and lytic enzyme involved in biocontrol are treated later in this chapter.

Montenecourt and Eveleigh (1977) developed a medium and assay procedure to select cellulase-producing strains of *T. viride*. The method employs (i) the use of either rose bengal or oxgall to limit colony size and (ii) phosfon D (tributyl-2, 4-dichlorobenzylphosphonium chloride) to enhance cellulose detection in combination with acid swollen cellulose on agar plates. Evans *et al.* (1992) suggested that an alkaline endoglucanase is an essential part of a *T. reesei* cellulase system capable of efficiently degrading crystalline cellulose. Harman *et al.* (1993) purified chitobiosidase and endochitinase from *T. harzianum*. Kumar and Gupta (1999) obtained strains of *T. viride* antagonistic to root rot pathogen *Macrophomina phaseolina* and tolerant to tebuconazole and showed wide variation in morphological, physiological and biochemical traits especially in enzyme production. Inhibitory effects on spore germination and germ tube elongation of *Botrytis cinerea, Fusarium solani* and *Uncinula necator* were synergistically increased by mixing fungal enzymes and cells of *Enterobacter cloacae* (Lorito *et al.*, 1993a).

2.5 Effect of biocontrol agents on the host plant

There are different mechanisms involved in the process of biocontrol in the presence of host plant. The effect may be indirect in which minor pathogens are suppressed, or direct effect of *Trichoderma* on plant growth promotion and induced systemic resistance (Bailey and Lumsden, 1998; Calvet *et al.*, 1993; Chang *et al.*, 1986; Harman *et al.*, 1989; Inbar *et al.*, 1994; Kleifeld and Chet 1992; Lindsey and Baker, 1967; Shivanna *et al.*, 1994). Three types of reaction on plants could be recorded. They are growth promotive, induced systemic resistance and growth suppressive / pathogenic. Baker *et al.*, (1984) found that *T. hamatum* can stimulate growth of plants including various floricultural and horticultural plants (Chang *et al.*, 1986). Strains of *T. virens* produced the compounds gliotoxin, viridin and gliovirin in soil less growth medium (Lumsden *et al.*, 1992).

Menzies (1993) detected a *T. viride* isolated from a healthy tomato root that was pathogenic to seedlings of cucumber, pepper and tomato in laboratory and glass house experiments. The phytotoxic effect of gliotoxin and viridin are limited compared with their antifungal activities (Lumsden *et al.*, 1992). The 6 pentyl-α- pyrone produced by *T. harzianum* and *T. viride* (Cutler *et al.*, 1986), (-)-harzianopyridone by *T. harzianum* (Cutler and Jacyno, 1991), Koningin A (Cutler *et al.*, 1989) and Koningin B (Cutler *et al.*, 1991) by *T. koningii* inhibited plant growth. *T. koningii* decreased plant dry weight and arbuscular mycorrhizal colonization when inoculated into the rhizosphere of maize and lettuce before or at the same time as *Glomus mosseae* (McAllister *et al.*, 1994).

Addition of *T. harzianum* and *T. koningii* to autoclaved soil increased rate of emergence of tomato and tobacco seedlings over that of the controls. Eight weeks after planting, root and shoot dry weights of tomato and tobacco were increased 213-275 and 259-318% respectively over the controls. When soil fertility was increased the level of increased tomato growth induced by *Trichoderma* spp was enhanced. Radish plants grown in gnotobiotic conditions with *T. harzianum* T-95 were larger than radish plants grown under similar conditions without that agent. The rate of seed germination was increased compared with the controls where *Trichoderma* spp were not present when the seeds were separated from *Trichoderma* spp by a cellophane membrane (Windham *et al.*, 1986).

Sid Ahmed *et al.* (1999) reported that *T. harzianum* reduced *P. capsici* propagules in pepper plants and it is associated with a reduction in root rot between 24 and 76% although plant growth (dry weight) was still reduced by 21.2 to 24.7% compared with the uninoculated control. Lumsden *et al.* (1990) used a potting medium to demonstrate the biological control potential of *T. harzianum* against damping off of lettuce (*Lactuca sativa*) caused by *Pythium ultimum*. They concluded that ATP and chitin measurements are suitable biomass markers for introduced fungal inocula and are inversely associated with colony forming units and esterase activity measurements. Both types of measurements are useful in investigating disease potential and biocontrol activity. Biocontrol activity is primarily linked to a transient increase in *Trichoderma* biomass resulting in a sustained increase in active propagules of the antagonist.

2.6 Effect of biocontrol agents on the pathogen

Mycoparasitism is an exciting phenomenon that plays a role in biocontrol process. *Trichoderma* spp appears to use this mode of action along with competition and antibiosis in the course of their biocontrol activity (Chet *et al.*, 1998). Recognition, attachment, coiling and lytic activity are the various steps involved. Lorito *et al.* (1993b) reported that two chitinolytic enzymes-endochitinase and chitobiosidase-from *T. harzianum* strain P1 were tested for their antifungal activity in bioassays against nine different fungal species. Spore germination (or cell replication) and germ tube elongation were inhibited for all chitin-containing fungi. Harman *et al.* (1993) reported the differential expression of chitinases of varying molecular weights during varying intervals of parasitism and they concluded that differential expression of *T. harzianum* chitinases might influence the overall antagonistic ability of the fungus against a specific host. Elad *et al.* (1983) studied the hyphal interaction between either *T. harzianum* or *T. hamatum* and *Sclerotium rolfsii* or *R. solani* using scanning electron microscopy (SEM). *Trichoderma* spp attached to the host either by hyphal coils, hooks or appressoria. Lysed sites and penetration holes were found in hyphae of the plant pathogenic fungi following removal of parasitic hyphae. High β-1, 3 glucanase and chitinase activities were detected in dual agar cultures when *T. harzianum* parasitized *S. rolfsii*. Interaction sites were stained by fluorescein isothiocyanate-conjugated lectins or calcofluor white. Appearance of fluorescence indicated the presence of localized cell wall lysis at points of interaction between the antagonist and its host. Balakrishnan (1997) and Usman *et al.* (1997) observed lysis and parasitism of hyphae of *Pythium aphanidermatum* infecting ginger by *Trichoderma* spp. Culture fluid of *G. virens* grown on glucose, washed cell walls of *R. solani* (one of its host), olive oil or chitin contained β-glucanase, N-acetylglucosaminidase, lipase and proteinase activities (van Tilburg and Thomas, 1993). Ridout and Lumsden (1993) obtained two polypeptides (18.7 and 33.8 kDa) from mycelial extracts of *G. virens* G 20 and used for the production of antiserum in the eggs of turkey hens. The resulting antisera were used to investigate the association of these polypeptides with gliotoxin production in *G. virens*. Both polypeptides were detected in mycelial extracts from 15 strains that produced gliotoxin but were not detected in extracts from five strains that did not produce gliotoxin or in extracts from three other fungal genera. Di Pietro *et al.*, (1993) studied the synergistic antifungal activity of purified endochitinase and gliotoxin from *G. virens*. Germination of conidia and germ tube elongation of *Botrytis cinerea* was inhibited. *In-vitro* crude culture filtrates of *T. viride* and *Coniothyrium minitans* could lyse walls isolated from hyphal cells or the inner pseudoparenchymatous cells of the sclerotia of *Sclerotinia sclerotiorum* which contained exo-β-(1 → 3) glucanase and endo-β-(1 → 3)- glucanase. Their synergistic action led to almost complete breakdown (Jones *et al.*, 1974). Freitag and Morrell (1992) had grown the white rot fungus *Trametes versicolor* and potential biocontrol organism *T. harzianum* in pure and mixed cultures. Laccase and poly Red-478 peroxidase activities indicate survival of the decay fungus. Most importantly, laccase activities of *Trametes versicolor* and to a smaller extent, cellobiase activities of both fungi were significantly induced in mixed cultures of *T. versicolor* and *T. harzianum*. Katragada and Murugesan (1996) observed extensive colonization of *T. harzianum* over *Fusarium oxysporum* f. sp. *vasinfectum,* vascular wilt pathogen of cotton *in-vitro.* SEM investigation of hyphal interaction showed tightly appressed growth of *T. harzianum* over the host. Once the contact is established between mycoparasite and its host, the mycoparasite produces lytic enzymes that degrade the cell walls of the host.

Trutmann and Keane (1990) recorded parasitism of *T. koningii* on *Sclerotinia sclerotiorum*. Mycoparasitism was necrotrophic and involved disruption of hyphae of *S. sclerotiorum* upon contact. Coiling of *T. koningii* around its host hyphae was common. Extensive growth of *T. koningii* within hyphae of *S. sclerotiorum* was achieved without production of specialized infection structures. At later stages of parasitism *T. koningii* sporulated on remnants of hyphae of *S. sclerotiorum*. Fernandez (1992a) found that inoculation of wheat and black oat strains with *T. harzianum* resulted in a reduction in the incidence of *Cochliobolus sativus* in wheat, *Fusarium graminearum* and other *Fusarium* spp in wheat and black oat. *T. harzianum* was more effective in colonizing the substrate and in reducing the incidence of pathogens in wheat than in black oat but it had no effect on decomposition of either type of residue as determined by early weight loss at the end of the experiment. Inbar *et al.* (1996) studied hyphal interactions between the mycoparasite *T. harzianum* (BAFC cult. No.72) and the soil borne plant pathogenic fungus *Sclerotinia sclerotiorum* in dual culture and in sterile soil by light and scanning electron microscopy. Application of *T. harzianum* to soybean residues resulted in a significant decrease in the incidence of some wheat and soybean pathogens of economic importance in southern and central Brazil. The pathogens were *F. graminearum, Glomerella glycines* and *Macrophomina phaseolina* (Fernandez, 1992a; 1992b). Tu and Vaartaja (1981) studied the parasitic interaction between *G. virens* on *R. solani*. Barak *et al.* (1985) implicated lectins as a basis for specific recognition in the interaction of *Trichoderma* and *S. rolfsii*. Haran *et al.* (1996) observed differential expression of T. *harzianum* chitinases during mycoparasitism. Benhamou and Chet (1993) also studied the ultra structure of mycoparasitic process of *T. harzianum* on *R. solani*. The term mycoparasitism applied strictly to those relationships in which one living fungus acts as a nutrient source for another (Jefries, 1994). Cherif and Benhamou (1990) recorded chitinolytic activity of *Trichoderma* on *F. oxysporum* f. sp. *radicis-lycopersici* and indicated that production of this enzyme may be of great significance in the antagonistic process. Gracia-Garza *et al.* (1997) found that *T. hamatum* TMCS-3 combined with fungus gnats (*Bradysia coprophila*) reduced the viability of sclerotia of *Sclerotinia sclerotiorum* in laboratory pot tests.

2.7 Abiotic factors and biological control

Climate may be a major factor controlling the distribution and survival of *Trichoderma* spp in soil. The effect of these factors, which include soil type, moisture, pH, temperature, nutrient availability and other environmental factors on propagule density in soil and colonization of roots are important (Ousley *et al.*, 1993). However, Bhatnagar (1996) reported that the antagonistic potential of *T. viride*, *T. harzianum* and *T. koningii* was not much altered by changing the environmental conditions. *Trichoderma* spp showed maximum antagonistic potential against *Fusarium udum* at 35±2^{0}C and pH 6.5. The antagonistic potential of *Trichoderma* spp was not much affected by changing the C / N ratio indicating that *Trichoderma* spp can withstand a wide range of variations in C / N ratio without losing their antagonistic vigour. However, *T. koningii* was comparatively more susceptible to pH changes. *Trichoderma* species strains have been isolated from habitats of varying moisture, temperature and nutrient status (Danielson and Davey 1973a; 1973b; 1973c; Papavizas 1985; Roiger *et al.*, 1991). The high level of plasticity in various characteristics and ecological adaptability shown by members of these genera makes them ideal candidates for biocontrol application in a variety of habitats and environments but the sensitivity of different isolates to abiotic environmental factors must be considered. Burpee (1990) presented a review on abiotic factors that influence distribution, survival and functioning of biocontrol agents in various climatic conditions.

The mechanisms employed by biocontrol agents to effect biocontrol of plant diseases are many and complex and their use varies with the kind of biocontrol agent pathogen and host plant involved in the interaction. Mechanisms are also influenced by the soil type, temperature, pH and moisture of the plant and soil environment and by other members of the micro-flora. What we observe and define, as biocontrol may be the colonization of a number of different mechanisms working synergistically to achieve disease control. Our knowledge of the complexity of these systems is currently limited by our ability to perceive them and a great deal of research will have to be undertaken in order to explain exactly what is taking place during the biocontrol process. As with so many other aspects of science, basic knowledge about the mechanisms involved in the biocontrol process will be of immense value to that intent on developing new methods for utilizing biocontrol agents.

Soil type

Studies on the relationship between soil type particularly soil texture and incidence of plant diseases is well documented (Lyda, 1982; Toussoun, 1975). Soil characteristics also influence the survival of biocontrol agents. Montmorillonoid clays increased the competitive effects of *Serratia marcescens* and *Agrobacterium radiobacter* against soil fungi through a pH buffering effect related to high cation exchange capacity (Rozenzweig and Stotsky, 1979). Competitiveness also increased by more rapid utilization of nutrients by the bacteria (Rozenzweig and Stotsky, 1980). In contrast, antagonism of fungi by *Bacillus cereus* (Campbell, 1983; Campbell and Ephgrave, 1983) is not enhanced by clay, possibly because of adsorption of antibiotics on the clay surface (Campbell, 1983). A total of 486 soil samples from various locations throughout the island of Taiwan were tested for ability to suppress sporangial germination of *Phytophthora parasitica, P. palmivora* and *P. capsici.* In general, soils with a pH lower than 5.0 were more suppressive than those with a higher pH although a number of soils with a pH lower than 5 were conducive and some soils with a pH higher than 7 were suppressive (Ann, 1994). Soil type was found to be responsible for failure of biocontrol agents in some conditions. As suggested by Schippers *et al.* (1987) siderophores adsorption by clays may be involved in failures of some PGPR to increase plant growth.

Soil moisture

Moisture influence natural distribution of the various *Trichoderma* spp (Danielson and Davey, 1973a). Initiation of growth from dried mycelial biomass may require addition of an osmoregulant to the formulation (Knudsen *et al.*, 1991). During periods in which humidity and temperature are unsuitable for the antagonists, limited use of chemical fungicides has proven to be an effective strategy for increasing the consistency of biological disease control in soil (Chet, 1987). Knudsen and Bin (1990) quantified radial growth rates and hyphal densities for hyphae originating from alginate pellets containing hyphae of the biocontrol fungus *T. harzianum* using buried nylon mesh technique at varying temperature, soil moisture and food base. Soil moisture levels between -0.03 and – 0.05 Mpa did not significantly affect radial growth rates, however, there was a significant effect of soil matric potential on hyphal density at these times. Addition of wheat bran did not significantly increase radial growth rate however bran did have a highly significant effect on hyphal density within the limits of radial growth. Most diseases incited by soil borne plant pathogens are favoured by wet soils (Cook and Papendik, 1972). Similarly few reports (Adams and Ayers, 1980; Hoch and Abawi, 1979; Hunter *et al.*, 1977) of the effect of the soil moisture on biocontrol agent indicate a general increase in antagonistic activity in wet soils. This apparent anomaly is explained by the fact that the quality and quantity of the antagonistic micro flora is not the same in all soils and the relative competitive advantage of antagonists over pathogen can change with fluctuation in soil moisture (Baker and Cook, 1974; Cook and Baker, 1983; Cook and Papendik, 1970; Schneider, 1982). Eastburn and Butler (1988a; 1988b) found that increasing moisture levels recorded an increase in number of propagules of *T. harzianum*. However their study did not include saturated moisture level.

Soil temperature

Soil temperature influences biocontrol by affecting the disease suppressiveness of soils, predisposing pathogens to antagonism and regulating the activities of specific antagonists (Abd-El Moity *et al.*, 1982; Burpee, 1990). The natural distribution of *Trichoderma* sp. is greatly influenced by habitat temperature and the species groups varying in their temperature optima and tolerances (Domsch *et al.*, 1980). Isolates within a species group also vary in their antagonistic activity at different temperatures (Kohl and Schlosser, 1989; Tronsmo and Dennis, 1978). Soil temperature affected radial growth rates of hyphae from alginate pellets but there was no observed effect of temperature on hyphal density measured during a period of 7-14 days (Knudsen and Bin, 1990). Monaco and Rollan (1999) produced prills by adding *T. koningii* biomass to a sodium alginate suspension and dipping into calcium chloride solutions containing wheat bran. The prills were incubated for 1, 2 months, 1 and two years at 5 and 25^0 C. Wheat bran prills remained biologically active for more than 2 years when kept at 5^0C. Hoitink and Fahy (1986) found that the ability of *Trichoderma harzianum* to induce suppression of *Rhizoctonia* damping off in a container medium prepared with mature composted hardwood tree bark removed from various temperature zones in compost piles were varied significantly. They concluded that some thermophilic fungi *Humicola* spp naturally present in $40\text{-}50^0$ C compost temporarily interfered with the biocontrol activity of *T. hamatum* after compost amended medium was first prepared. *T. hamatum* applied as a seed treatment controlled seed rot of pea or radish in soil infected with *Pythium* spp or *Rhizoctonia solani* respectively, if soil temperatures were between 17 and 34^0C and seeds had been treated with a suspension of conidia with a concentration equal to or greater than 10^6 cfu / ml. The suppressiveness of disease in warm soils has been attributed to a temperature mediated increase in competitive micro flora (Cook and Rovira, 1976). Stressing effect of elevated sub-lethal temperature on *Armillaria mellea* predisposed the pathogen to antagonism by *Trichoderma* spp. This antagonism was related to the relative tolerance of *Trichoderma* to soil temperature $>30^0$C and to reduced growth, reduced production of antibiotics and possibly to increased exudation by *A. mellea* after exposure to high temperature (Munnecke *et al.*, 1981; 1976). Integrated control of diseases caused by *S. rolfsii* in iris (Chet *et al.*, 1982) and *R. solani* in potatoes (Elad *et al.*, 1980) by solar heating of soil and the introduction of *T. harzianum* provide further evidence of the predisposing effect of increases in soil temperature on the biocontrol. Henis and Papavizas (1983) found that necrotrophic parasitism of sclerotia of *S. rolfsii* by *T. harzianum* occurred if the sclerotia were subjected to a localized heat treatment of 90^0C for 15 seconds. They speculated that the susceptibility of the sclerotia was related to a stress induced availability of nutrients for the facultative mycoparasite. In addition to predisposition, temperature can have a direct effect on the interaction between plant pathogens and antagonists in soil. There are several reports of reduced efficacy of biocontrol by *Trichoderma* spp (Dewan and Sivasithamparam, 1988; Harman *et al.*, 1981; Kaiser and Hannan, 1984) and other antagonistic fungi (Adams and Ayers, 1980; Paulitz and Baker, 1987; Turner and Tribe, 1975) at soil temperature $<20^0$C. In fact the lack of acceptable antagonism at low soil temperature would appear to be a major factor limiting the potential success of biocontrol. Since many soil borne pathogens are active at soil temperature <20^0C, selection of low temperature tolerant species or isolates of biocontrol agents would appear to be warranted (Tronsmo and Ystaas, 1980). Addition of chitin or cell walls of *R. solani* to seed coats increased the ability of the mycoparasite *T. hamatum* to protect seeds against *Pythium* spp or *R. solani* and resulted in an increase in population density of *Trichoderma* in the soil (Harman *et al.*, 1981). Parasitism of sclerotia of *S. sclerotiorum* by *T. koningii* was favoured by the presence of exogenous nutrients and was maximal at temperatures between 20 and 35^0 C (Trutmann and Keane, 1990). Tronsmo and Dennis (1978) reported inhibition of growth of *Botrytis cinerea* and *Mucor mucedo* by non-volatile metabolites from *Trichoderma* isolates was most severe at 5^0C where as more isolates produced volatile inhibitors at 20^0C. No effect of temperature was observed on hyphal interaction. *T. viride* and *T. koningii* produced significantly higher population in soil at 25^0 C than at 15 and 30^0 C (Mondal *et al.*, 1996). According to Sanogo *et al.* (2002) *T. stromaticum* produced abundant conidia in cocoa broom at 100% relative humidity and incubation temperature of 20 and 25^0C but none at 30^0C. Sporulation of *T. stromaticum* was not observed at 75% relative humidity at any temperature. At 100% relative humidity and either at 20 or 25^0C, treatment of broom with *T. stromaticum* suppressed *Crinipellis perniciosa* within 7 days. In contrast, at 30^0C treatment with *T. stromaticum* had no effect on the pathogen in broom maintained at either 75 or 100% relative humidity. Mycelium of *C. perniciosa* grew from broom at all temperatures at 100% relative humidity. Conidial germination on broom tissue approximated 80% at temperature from 20 to 30^0C. These results suggest that applying

T. stromaticum under high moisture condition when the air temperature below 30^{0}C may enhance the establishment of this mycoparasite in cocoa plantations.

Soil pH

Soil pH has been reported to influence biocontrol by *Pythium nunn, Trichoderma* spp and *Pseudomonas* spp. Conidia of *T. koningii* germinated and mycelium grew best in acidic conditions (Trutmann and Keane, 1990). Acidic pH (5.0) favoured the growth of *T. viride* and *T. koningii* (Mondal *et al.*, 1996). Low soil pH (<7) favours disease suppression induced by *T. harzianum* (Chet and Baker, 1980; Harman and Taylor, 1988; Liu and Baker, 1980; Marshal, 1982) and *T. hamatum* (Chet and Baker, 1981). Acid conditions may function in enhancing spore germination, mycelial growth, production of conidiophores, antibiotics and the activity of lytic enzymes (Chet and Baker, 1980; Dennis and Webster, 1971a; 1971b; Hadar *et al.*, 1984). In addition low pH reduces the inhibitory effects of soil fungistasis on conidia of *Trichoderma* spp.

Pesticides

The possibility of controlling pathogenic fungi with antagonistic microorganisms introduced as a substitute for or in combination with low sub-lethal doses of fungicides has long been considered and studied (Chet, 1990). The impact of pesticides on biocontrol of soil borne plant pathogens has been reviewed (Griffiths, 1981; Rahe and Utkhede, 1985; Rodrigues-Kabana and Curl, 1980). Increase in diseases caused by *Pythium, Fusarium* or *Phytophthora* resulting from application of PCNB may be attributable to the inhibitory effect of this fungicide on antagonistic fungi and streptomycetes (Bird et al., 1957; Cook and Baker, 1983; Gibson *et al.*, 1961). In contrast low doses of PCNB (4-10 mg a.i. / kg soil) have been used with *T. harzianum* as integrated controls of *S. rolfsii* and *R. solani* on several species of plants (Chet *et al.*, 1979; Chet *et al.*, 1982; Elad *et al.*, 1980; Hadar *et al.*, 1979; Henis *et al.*, 1978). The fungicide may predispose the pathogen to enhanced antagonism by *Trichoderma* similar to the predisposition induced by heat and soil fumigants (Henis and Papavizas, 1983; Munnecke *et al.*, 1976; Ohr *et al.*, 1973). The fumigants carbon disulfide and methyl bromide, which have little adverse effect on *Trichoderma* spp at the dosages applied, reduce the growth and antibiotic production of *Armillaria* leaving the pathogen vulnerable for antagonism (Bliss, 1951; Munnecke *et al.*, 1981; Ohr *et al.*, 1973; Ohr and Munnecke, 1974). Similar predisposing effects have been reported for metham sodium on sclerotia of *S. rolfsii* parasitized by *T. harzianum* (Henis, 1984; Henis and Papavizas, 1983). Methyl bromide plus *T. harzianum* has been used as an integrated control of *S. rolfsii* in field soils planted with tomatoes, peanuts, strawberries or potatoes (Elad *et al.*, 1980; Elad *et al.*, 1981; Elad *et al.*, 1982). Pesticide induced predisposition of soil borne pathogen to microbial antagonism warrants further study as a practical method of integrated disease management in a wide variety of crops (Burpee, 1990). In addition, the efficacy of these types of integrated control may be enhanced by the selection of pesticide resistance in potential biocontrol agent (Baker and Scher, 1987; Papavizas, 1987). Curl *et al.* (1977) found for sterile soil that small doses (2 µg and 10 µg / g of soil) of PCNB together with *Trichoderma* sp. were slightly more efficient against *R. solani* than the biocontrol agent alone. Hadar *et al.* (1979) showed that a combination of a sublethal dose of PCNB and *T. harzianum* reduced the incidence of root rot of eggplant caused by *R. solani* from 40% (non protected check) to 13% while *T. harzianum* alone reduced it to 26%. Ahmad and Baker (1988b) produced a benomyl tolerant mutant of *T. harzianum* which was rhizosphere competent when benomyl was added at 10 μg / g of soil. Transplanting of peanut plants treated with *T. harzianum* into soil fumigated with methyl bromide resulted in significantly less of the diseases caused by *S. rolfsii* and *R. solani* and increased the yield of peanuts (Elad *et al.*, 1982). Combination of a low dose of methyl bromide and *T. harzianum* gave a significantly better control of damping off of carrot seedlings caused by *R. solani* than methyl bromide alone (Strashnov *et al.*, 1985). Disease control with a combination of *Trichoderma* and solar heating of the soil was developed in Israel (Katan *et al.*, 1976). Neither *Trichoderma* nor solar heating alone gave as good control as the combination treatment (Elad *et al.,* 1980). Elad and Chet (1983) found isolates resistant to PCNB or captan and selected. Key and Stewart (1994) studied the sensitivity of *Chaetomium globosum*, *T. harzianum, T. viride* and *Trichoderma* sp. to benomyl, captan, iprodione, mancozeb, procymidone and thiram. The fungi were insensitive to captan, mancozeb and thiram but were sensitive to benomyl ($EC_{50} < 0.3$ μg / ml) and the two dicarboximides iprodione and Procymidone ($EC_{50} < 3.3$ μg / ml). Ultra violet irradiation did not induce resistance to benomyl but a number of biotypes of each fungus were isolated from irradiated cultures that showed resistance to iprodione (EC_{50} values up to 56.4, 177.8, 171.5 and 216.4 μg / ml for *C. globosum*, *T. harzianum*, *T. viride* and *Trichoderma* spp respectively). Fungicide resistant biotypes retained the ability to antagonize the pathogen of onion white rot, *Sclerotium cepivorum*.

Some of the *Trichoderma* species are naturally resistant to pesticides so that no genetic modification is needed for tolerance. Stephen *et al.* (2000) found that *T.*

harzianum used for the control of *Phytophthora* foot rot of black pepper is tolerant to chlorpyriphos and phorate at recommended concentrations for the crop for mealy bug and nematode control respectively.

Fertilizers

Management of soil borne plant pathogens with organic soil amendments is a disease control strategy salvaged from the past. Organic amendments containing high nitrogen such as poultry manure, meet and bone meal and soy meal significantly reduced population of a wide spectrum of soil borne plant pathogens. Pathogen containing was shown to arise from amino acid and (or) nitrous acid generated, the concentration of which are controlled by pH, organic matter content, soil buffereing capacity and nitrification rate. Thus the displacement of pathogen is selective and can persist in fields for several years after single application (Lazarovits, 2001). Research on the effects of organic nutrients on biocontrol in soil can be divided into disease or pathogen suppressive effects of crop residue, specific microbial nutrients and composts (Abd-El Moity *et al.*, 1982; Baker, 1968). Suppressive effects of crop residue on soil borne pathogen and diseases have been reviewed extensively (Cook, 1985; Huber and Watson, 1970; Lewis and Papavizas, 1975). Mechanisms include autolysis (endolysis), heterolysis, fungistasis, the annulment of fungistasis followed by lysis and the production of antibacterial products from residue decomposition (Papavizas and Lumsden, 1980). Chitin, glucose, cellulose, organic acids, bran have been used as soil amendments to enhance the efficacy of antagonists (Baker *et al.*, 1984; Lewis and Papavizas, 1985; 1987; Nelson *et al.*, 1983; Nelson *et al.*, 1988; Nelson and Hoitink, 1983). This emphasized the importance of a nutrient base to counteract fungistasis and enhance the antagonistic activities of fungi as biocontrol agents in soil. This has led to the successful development and experimental use of nutrient based pellet formulation of *Trichoderma* and *Gliocladium* for control of soil borne pathogens (Lewis and Papavizas, 1987; Lumsden and Locke 1989; Papavizas and Lewis, 1989). Conidia of *Trichoderma* spp require exogenous nutrients in order to germinate (Danielson and Davey 1973b). There are few reports on the effects of inorganics on biocontrol or biocontrol agents (Jayaraj, 1995; Jayaraj and Ramabadran, 1988). Incorporation of inorganic sources of nitrogen in soil has been reported to increase or decrease antagonism to plant pathogens (Anas and Reeleder, 1988; Papavizas, 1963). In addition to plant pathogen competition for Fe^{3+} may also suppress the activity of biocontrol agents in soil. Hubbard *et al.* (1983) reported that an isolate of *T. hamatum* that suppressed seed rot of peas in a Colorado soil failed to suppress disease in New York soils with low iron availability. The failure was caused by the chelation of available iron by fluorescent pseudomonads. Harman and Taylor (1988) suggested that improved iron availability was a factor that enhanced biocontrol of seed rot by *T. harzianum* in a solid matrix seed priming system. Jayaraj and Ramabadran (1988) evaluated ammonium nitrate, calcium nitrate, sodium nitrate, potassium nitrate, ammonium sulphate, ammonium chloride and urea *in-vitro* for their effect on the growth and sporulation and on the production of cellulase by antifungal agent *T. harzianum.* The salts were evaluated at 2% (w/v) and 0.5 g N / litre. Ammonium nitrate, ammonium sulphate or sodium nitrate recorded the maximum growth, sporulation and production of cellulase and antifugnal substances respectively. Hoitink and Fahy (1986) provided examples of suppression of pathogen or disease in compost amended soils but specific biocontrol agents or modes of action have not been identified. *T. hamatum* and *T. harzianum* and several species of bacteria are important antagonists of *R. solani* in composted hardwood bark (Hoitink and Fahy, 1986; Kloepper *et al.*, 1980; Kuter *et al.*, 1983, Kwok *et al.*, 1987; Nelson and Hoitink, 1983; Nelson *et al.*, 1983)

2.8 Rhizosphere competence

Rhizopshere competence (RC) is the ability of an organism to establish itself and function in the rhizosphere of plants and is subject to influences of environment and competition with other organism (Ahmad and Baker, 1987b; Harman 1992). Milus and Rothrock (1993) identified bacteria in the genera *Bacillus*, *Pseudomonas* or *Xanthomonas* that will colonize roots well over diverse environments. Ahmad and Baker (1987b) measured the rhizosphere competence of *T. harzianum* for roots of beans, cucumber, maize, radish, and tomato. Mutants tolerant to benomyl were rhizosphere competent in the presence and absence of benomyl (Ahmad and Baker, 1988b). Their experiments indicated that a rhizosphere incompetent biological control agent was induced by mutations to become rhizosphere competent. Rhizophere competence has been achieved by genetic manipulation (Sivan and Harman, 1991; Stasz and Harman, 1990). Ahmad and Baker (1987a) determined competitive saprophytic ability (CSA) of strain of *Trichoderma* spp. CSA was directly correlated with rhizosphere competence. The amount of cellulase produced by these strains was directly correlated with CSA and RC. Rhizosphere competence of the mutants therefore can be at least partially explained by their capacity to utilize cellulose substrates associated with the root. Ahmad and Baker (1988a) cultivated *T harzianum* in czapek dox broth without saccharose with cotton linters, microcrystalline cellulose, wood cellulose or xylan as a sole source of carbon. The rhizosphere competent mutants produced significantly higher biomass than the rhizosphere incompetent wild types. The ability of the mutants to grow more rapidly on complex carbon substrates than their wild type parents and to increase biomass when simple sugars were added along with the cellulose substrate could be of ecological significance and a characteristic of rhizosphere competence. Chao *et al.* (1986) reported that plant roots growing into soil free moist chambers were not colonized by *Trichoderma* spp. When roots were grown in sterile soil *T. harzianum* was detected in the rhizosphere of the upper half of the roots. In untreated soil none of the *Trichoderma* spp could be detected in the rhizosphere more than 3 cm below the planted seed. The *T. harzianum* amdS transformant was stable after at least 6 weeks in non-sterile soil in the green house and after 14 months in sterile soil at 4^0C. Soil inoculation resulted in efective colonization of the roots by the transformants (Pe'er *et al.*, 1991). Although some biocontrol agents may protect seeds from soil borne pathogens they do not proliferate in the rhizoplane and rhizosphere (Chen *et al.*, 1988). Rhizosphere competent biocontrol agents are potentially more effective because they protect not only the seed but also the seedling root. They potentially eliminate the problem of adding large amounts of thalli to induce suppressiveness because substrate provided by the host can support their activity.

2.9 Protoplast fusion technology for strain improvement

The first requirement of successful biocontrol is that a highly effective strain be identified and employed. It is also essential that the production system give rise to highly effective, desiccation tolerant propagules and that delivery systems and formulations permit high activity of the biocontrol agents under conditions of substantial microbial competition, soil and climatic conditions (Burpee, 1990; Harman, 1991). *Trichoderma* and *Gliocladium* spp possess great genetic variability. Some strains have a wide spectrum of activity, other strains may control only specific pathogens while still others may have little or no biocontrol efficacy (Harman, 1991; Lumsden and Locke, 1989; Smith *et al.*, 1990). Similarly some strains may grow poorly under some environmental conditions while others grow well under these same adverse conditions. Therefore several strategies suggests for obtaining strains of *Trichoderma* or *Gliocladium* that are highly effective biocontrol agents. Many very useful strains have been obtained by simply selecting strains from the wild types (Rajan, 1999; Rajan *et al.*, 2002; Saju *et al.*, 1999; 2002a). All cells in *Trichoderma* thalli that Harman and Hayes (1993) have examined are polynucleate and they concluded that wild strains might be heterokaryotic. If so, isolation of homokaryons from mass isolated strain by single spore isolation may give rise to different and more stable strains (Stasz *et al.*, 1988). Chemical mutagenesis has given rise to several effective strains (Ahmad and Baker, 1988b; Papavizas *et al.*, 1982). UV mutation also produced strains with increased biocontrol activity (Kumar and Gupta, 1999). It would also be possible to use sexual or asexual genetic recombination to obtain new strains. If two strains were known to possess good ability to control complementary pathogens a method of combining these desirable traits into single strains would be advantageous.

The results of many years of research with *Trichoderma* species as biocontrol agents has shown that not all the mechanisms and characteristics deemed necessary for optimum biocontrol are found in the same organisms. Very often those strains that have the capacity to produce enzymes and antibiotics that are associated with biocontrol are not the ones that have good storage qualities or function well at temperatures and moisture levels where pathogen flourish. Therefore hybridization of different strains or species will be required in order to combine these beneficial characteristics. The production of hybrids from *Trichoderma* species many of which have no known sexual stage will entail the use of transformation or protoplast fusion in order to obtain strains with optimal sets of characteristics. Once produced and screened, hybrids can yield strains with expanded host, temperature and moisture parameters and they may yield strains with better storage qualities than those exhibited by the parents (Howell, 2002).

The sexual stage of *Trichoderma* and *Gliocladium* is rare and may be entirely lacking for some biocontrol strains and sexual recombinations may not be possible. However parasexuality has been described in this fungus, which results in recombinant progeny strains. Therefore if heterokaryons could be established it might be possible to produce progeny strains of *Trichoderma* that possess desirable attributes of both parents. In the presence of appropriate selection procedures it should be possible to obtain progeny strains that are superior to the parental strains in biocontrol efficacy. Protoplast fusion is a method to effectively induce heterokaryosis and is of current interest because of its application in pure and applied genetics and industrial and agricultural purpose. Strain improvement of biocontrol agents was of great interest for researchers. As in other cases, strain improvement in *Trichoderma* both for industrial and agricultural use was attempted by UV mutation and protoplast regeneration / fusion (Anne, 1992; Davis, 1985; Gracheck and Emert, 1984; Harman and Stasz, 1991; Lalithakumari, 1996;

Lalithakumari *et al.*, 1995). It is applied for developing interspecific, intergeneric superhybrids with higher potentiality than their either parents. Through protoplast fusion technique, improved strains with enhanced antagonistic potential, antibiotics, enzymes, useful mycoproducts, high yielding mushroom etc would effectively be achieved. Protoplast fusion increased the efficacy of the biocontrol strain through enhanced antagonistic potential integrated with acquired tolerance to fungicide against a broad spectrum of phytopathogens. Seed treatments with preparations of *T. virens*, parent, mutant and hybrid strains gave effective biocontrol of pre-emergence damping off. Disease control was attributable to metabolism by the biocontrol agent of pathogen germination stimulants released by the seed (Howell, 2002).

2.10 Biological control in practice

The biological control of plant diseases has long been an area of fruitful study for plant pathologists and there are many publications on this topic (Balakrishnan, 1997; Balakrishnan *et al.*, 1997; Elad *et al.*, 1981; Jayaraj, 1995; Rajan, 1999, Rajan *et al.*, 2002; Usman *et al.*, 1996; Vereet and Wolf, 2002). There are many biocontrol systems use in agriculture that rely on some manipulation of an environment or ecosystem to reduce disease (Harman, 2000).

There are a number of reports on the identification of biocontrol agents for the control of soil borne diseases but few have been found successful in large-scale application and commercialization (Fravel, 1988). An integrated control programme consisting of two to three sprays with a fungicide resistant antagonist and one spray with a benzimidazole or dicarboximide fungicide has been found to provide more constant control than use of the antagonist alone (Gullino *et al.*, 1995). A similar alternation schedule has been recommended for greenhouse cucumbers (Elad *et al.*, 1993). For successful biocontrol a highly efficient strain should be identified which is the primary requirement for the system. Extensive field trials are needed to assess that a particular biocontrol agent is potential antagonist for pathogen. Fravel (1988) who reviewed antibiosis stated that only 5% of the potential biocontrol agents from laboratory or glass house could perform well under field conditions. Unless the initial screening systems mimic conditions those found in the environment a high chance of failure would be the result. However, biocontrol of the soil borne diseases have been successfully practiced and commercialized (Harman, 2000; Sarma *et al.*, 1997). These aspects have been reviewed by earlier workers and cited several examples of biological control in practice (Chet, 1987; Papavizas, 1985; Tronsmo, 1986). As research in this field progresses, knowledge on the biological mechanisms and environmental factors governing the interaction between antagonists and phytopathogenic fungi in disease control also increases. Applied in biologically based formulation an delivery system, appropriately selected *Trichoderma* and *Gliocladium* isolates should provide constant and effective disease control without the dangers of environmental pollution and fungicide resistance so often associated with the use of chemical fertilizers.

2.11 Summary

Secondary metabolites produced by *Trichoderma* spp are greater in number. Identifying such compounds would help understand the mechanism of the species in biocontrol and their chemical identity. Biocontrol agents influence the plant growth by controlling minor pathogens and producing plant growth promoting metabolites and disease suppression. However growth suppressive isolates were also identified. The impact of abiotic environmental factors like soil type, soil moisture, soil temperature, pesticides and fertilizers affect the efficacy of biocontrol agents of soil borne plant pathogenic fungi. Manipulation of abiotic factors to increase performance of biocontrol agents in soil may not necessarily enhance antagonism however a great deal of control can be achieved. Rhizosphere competence is a characteristic that determines the activity of biocontrol agents to propagate and function in the root zone of host plants. Protoplast fusion technology could be successfully employed to produce hybrids of better biocontrol activity and commercial quality.

3

MATERIALS AND METHODS

The materials and methods used in the present investigation are described in detail below. The composition of culture media and working solutions are given in Appendix.

MATERIALS

The crop

Black pepper (*Piper nigrum* L.) cv Karimunda or var. Panniyur I collected from Kozhikode district, Kerala was used for various experiments. The runner shoots collected were used for some experiments. Single nodded rooted cuttings from split-bamboo nursery used in any experiments are indicated.

The pathogen

Phytophthora capsici was obtained from National Repository of *Phytophthora* (NARPH), Indian Institute of Spices Research, Calicut (Table 3.1).

The antagonists

Trichoderma spp and *Pseudomonas fluorescens* were obtained from repository of biocontrol agents, Indian Institute of Spices Research, Calicut (Table 3.1). Twenty-eight isolates of *Trichoderma*, which showed high percentage of inhibition of *P. capsici in-vitro* in dual plate technique, were selected (Anon., 2000; 2002; Saju *et al.*, 1999; 2002). These twenty-eight isolates representing various species groups of *Trichoderma* and *T. harzianum* IISR 1369 and *T. virens* IISR 1370, two promising isolates currently used in the biocontrol of *P. capsici* (Rajan, 1999; Rajan *et al.*, 2002) were taken for the study of volatile and non-volatile metabolites.

Table 3.1 Details of *Phytophthora capsici, Trichoderma* spp and *Pseudomonas fluorescens* isolates used in various experiments

Species	Repository No.*	Old No.	Crop	Area
Phytophthora capsici	99-101	----	Black pepper	Kerala
P. capsici	02-52	----	Black pepper	Kerala
T. harzianum	IISR 1369	P 26	Black pepper	Kerala
T. virens	IISR 1370	P 12	Black pepper	Kerala
T. aureoviride	IISR 126	TAV 7	Ginger	Kerala
T. aureoviride	IISR 130	TAV 12	Ginger	Kerala
T. aureoviride	IISR 140	TAV 22	Ginger	Kerala
T. aureoviride	IISR 143	TAV 25	Ginger	Kerala
T. aureoviride	IISR 148	TAV 30	Ginger	Kerala
T. hamatum	IISR 150	THAM 6B	Ginger	Kerala
T. hamatum	IISR 151	THAM 7A	Ginger	Kerala
T. hamatum	IISR 160	THAM 18B	Ginger	Kerala
T. harzianum	IISR 165	TH 5	Ginger	Kerala
T. harzianum	IISR 167	TH 9	Ginger	Kerala
T. harzianum	IISR 169	TH 12	Ginger	Kerala
T. harzianum	IISR 173	TH 25	Ginger	Kerala

Continued

Table continued

Species	Repository No.*	Old No.	Crop	Area
T. harzianum	IISR 177	TH 32	Ginger	Kerala
T. pseudokoningii	IISR 186	TPK 3	Ginger	Kerala
T. pseudokoningii	IISR 187	TPK 4	Ginger	Kerala
T. polysporum	IISR 1101	TP 9	Ginger	Kerala
T. longibrachiatum	IISR 1103	TLB 1	Ginger	Kerala
Trichoderma sp.	IISR 1107	VAL 114	Black pepper	TN
Trichoderma sp.	IISR 1112	VAL 129	Black pepper	TN
T. koningii	IISR 1119	TK	not known	UM
Trichoderma sp.	IISR 1124	----	Black pepper	TN
Trichoderma sp.	IISR 1136	----	Black pepper	Karnataka
Trichoderma sp.	IISR 1141	----	Black pepper	Karnataka
Trichoderma sp.	IISR 1152	----	Black pepper	Karnataka
Trichoderma sp.	IISR 1240	----	Black pepper	TN
Trichoderma sp.	IISR 1267	----	Black pepper	WB
Trichoderma sp.	IISR 1270	----	Black pepper	WB
Trichoderma sp.	IISR 1275	----	Black pepper	Kerala
Pseudomonas fluorescens	IISR 6	IISR GB 12	Black pepper	Kerala
P. fluorescens	IISR 11	AP 13 K	Black pepper	AP
P. fluorescens	IISR 13	AP 19	Black pepper	AP
P. fluorescens	IISR 41	BX	Black pepper	Kerala

TN Tamil Nadu; WB West Bengal; UM University of Madras, Chennai; AP Andhra Pradesh

Trichoderma species were identified based on Rifai (1969)

*Accessions to culture collection, Indian Institute of Spices Research, Calicut

METHODS

Mass production of *Trichoderma* spp

Trichoderma spp multiplied on broken sorghum grain was used as source inoculum. Approximately 400 ml of water was added to 1 kg of broken sorghum to obtain 40% moisture. About 250 g of this material was taken in polypropylene bags of size 10 X 12 inches and tied with rubber bands. It was autoclaved for 1 hour at 121^0C (15 lb) and allowed to cool.

Spore suspension was prepared from a 4 days old culture of *Trichoderma* spp, grown on PDA (50 ml) in 250 ml conical flask, using 300 ml sterile distilled water. It was filtered through sterile muslin cloth. Each 1 ml of this suspension approximately contained 1 X 10^7 colony forming units. Suspension was taken in a sterile syringe and 5 ml was injected into each sterilized bag containing sorghum and mixed thoroughly. The bags were incubated at room temperature (28-30^0C) for 15 days (Prakash *et al.*, 1999). The 15 days old cultures were used as inoculum for various experiments. The number of colony forming units (cfu) per gram of sorghum grain was determined by serial dilution plate technique (Johnson and Curl, 1972; Dhingra and Sinclair, 1985) using *Trichoderma* Selective Medium (TSM) (Elad and Chet, 1983). The number of cfu present in sorghum used is mentioned in each experiment.

Mass production of *Pseudomonas fluorescens*

Pseudomonas fluorescens was grown in nutrient broth (NB) in conical flasks. Broth (100 ml) was taken in 250 ml flasks and sterilized. The broth was inoculated with a loopful of culture from two days old nutrient agar plates. The flasks were incubated at room temperature on orbital shaker for 48 hours. The cultures were diluted with water before application. The quantity applied is mentioned in each experiment. The number of colony forming units (cfu) per ml of broth was determined by serial dilution plate technique (Johnson and Curl, 1972; Dhingra and Sinclair, 1985) using Kings' B Agar medium. The number of cfu present in broth after dilution with water is mentioned in each experiment.

Estimation of colony forming units of *Trichoderma* spp, *Pseudomonas fluorescens* and other fungi

The number of colony forming units of *Trichoderma* present per gram of soil / carrier media was determined by serial dilution plate technique (Johnson and Curl, 1972; Dhingra and Sinclair, 1985) using *Trichoderma* Selective Medium (TSM) (Elad and Chet, 1983). *Pseudomonas fluorescens* was enumerated on Kings' B Agar (KBA) and other fungi on Rose Bengal Agar (RBA). Suspension was prepared by adding 5 g of soil / carrier media to 50 ml of sterile distilled water in 100 ml conical flasks. The suspensions were shaken vigorously. Serial dilutions were prepared from the suspension in order to get a desired level. Aliquots (1 ml) of serial dilutions were mixed homogenously with selective agar medium in the culture plates. After incubation for 5 days at 28-30^{0}C the developed colonies of the applied species of fungi or bacteria were enumerated by visual observation. The number of bacteria was enumerated after 48 hours.

Preparation of *Phytophthora capsici* inoculum for challenge inoculation

Phytophthora capsici from NARPH was sub-cultured on carrot agar and grown for 4 days at room temperature (28-30^{0}C). Discs of 1 cm diameter were cut from the plates and put in another plate having 15 ml of sterile distilled water. The plates were incubated under continuous fluorescent light for 48 hours. The discs were observed under the microscope for presence of mature sporangia. Sample plates were given a cold shock for 10 minutes in a refrigerator and again observed under the microscope for the release of zoospores (Erwin and Ribeiro, 1996; Ribeiro, 1978). Discs with sporangia were used for challenge inoculation of plants and number of discs used was mentioned in concerned experiment. The *P. capsici* isolate 99-101 was used for challenge inoculation of plants and 02-52 was used for *in-vitro* testing of metabolites of *Trichoderma.*

Statistical design and analysis

Experiments were performed by following appropriate design (Gomez and Gomez, 1984). Statistical analysis was performed on a computer using MSTATC software (Version 1.41, Russel D. Freed, MSTAT Director and Scott P. Eisensmith, Deputy Director, Crop and Soil Science Department, Michigan State University). The details and type of test performed was given under each experiment.

Thirty isolates of *Trichoderma* spp were screened for their volatile and non-volatile effect on the growth of *P. capsici* (see section 3.5 and 3.7). Nine isolates were identified as superior over the others in the inhibition of *P. capsici* by volatiles and eight were found as superior in the inhibition of *P. capsici* by non-volatiles. Out of the above seventeen short-listed isolates three were both volatile and non-volatile producers. Therefore, the resulting fourteen isolates were taken for *in-vitro* and *in-vivo* studies.

IN-VITRO STUDIES

3.1 *In-vitro* interactions between *Trichoderma* spp and *Phytophthora capsici*

The mycoparasitism of fourteen *Trichoderma* isolates on *P. capsici* was studied on Water Agar (WA) at pH ranges from 4.5 to 8.5 with one-unit increments (Sid Ahmed *et al.*, 1999). A 4 mm diameter disc of *P. capsici* was inoculated at one place of water agar and a similar sized disc of *Trichoderma* species was kept 5 cm away from it. After 48 hours the colony was observed under an inverted microscope (Nikon, Japan) for any mycelial interaction between the two fungi and it continued until 6 days. Any coiling and penetration of hyphae of *P. capsici* by *Trichoderma* spp was recorded and photographed. Also noted was the parasitism of sporangium by *Trichoderma* spp, evacuation of hyphae of *P. capsici* etc. *P. capsici* alone served as the control.

IN-VIVO STUDIES

3.2 *In-vivo* effect of *Trichoderma* spp on black pepper plants

Fourteen isolates of *Trichoderma* were identified as highly inhibitory to *P. capsici* by their volatiles and non-volatiles. *In-vivo* effect of these isolates on black pepper and suppression of *P. capsici* were tested as described by Rajan (1999) and Sid Ahmed *et al.* (1999).

Single nodded rooted cuttings of black pepper cv Karimunda from split bamboo nursery were planted in polythene bags (3 X 6 inch) with about 500 g nursery mixture (soil : sand : farm yard manure 1:1:1). Test isolates of *Trichoderma* were grown on sorghum as described earlier (Prakash *et al.*, 1999) and 20 g was applied to each bag. The cultures were 15 days old. The number of colony forming units of *Trichoderma* isolates present in sorghum grain was given (Table 3.2).

After two months, one set of plants was challenge inoculated with *P. capsici* isolate 99-101. Five carrot agar discs of *P. capsici* having 1 cm diameter were added to each bag at two places after removing the topsoil and replacing the same. The plants were kept in microprocessor controlled green house (Cassia Siamia Technologies, Hyderabad) having temperature of 20^0C and relative humidity 90%. The number of plant death, disease severity and root rot were recorded after 15 days. Disease symptoms in black pepper after infection with *P. capsici* are light yellowing and wilting of the leaves followed by general necrosis and plant death. Root rot is also common without many symptoms on the aerial parts. Results of number of plant death / disease severity / root rot were converted to percentages for presentation. Growth parameters viz. plant height, shoot and root dry weights were determined for another set of plants. The data were analysed by ANOVA followed by range test for mean comparison.

Table 3.2 Colony forming units (cfu) of *Trichoderma* isolates present in sorghum grains

Sl No.	*Trichoderma*	No. of cfu / g
1	*T. harzianum* IISR 1369	12×10^{11}
2	*T. virens* IISR 1370	7×10^{11}
3	*T. aureoviride* IISR 126	24×10^{10}
4	*T. aureoviride* IISR 140	11×10^{10}
5	*T. aureoviride* IISR 143	18×10^{10}
6	*T. aureoviride* IISR 148	25×10^{10}
7	*T. hamatum* IISR 150	7×10^{9}
8	*T. hamatum* IISR 151	19×10^{9}
9	*T. hamatum* IISR 160	14×10^{9}
10	*T. harzianum* IISR 165	29×10^{11}
11	*T. harzianum* IISR 173	23×10^{11}
12	*T. pseudokoningii* IISR 186	3×10^{10}
13	*Trichoderma* sp. IISR 1107	9×10^{11}
14	*Trichoderma* sp. IISR 1141	7×10^{10}

3.3 Rhizosphere competence of *Trichoderma* spp

Rhizosphere competence of 14 *Trichoderma* isolates which were able to suppress *P. capsici* by volatile and non-volatile metabolites were studied by the method of Ahmed and Baker (1987b) with modification. About 500 g of soil was taken in polythene bags. Single nodded rooted cuttings of black pepper cv Karimunda from split bamboo nursery were planted one in each bag and simultaneously applied 1 g *Trichoderma* multiplied on sorghum grains. The number of colony forming units of *Trichoderma* isolates present in sorghum grain was given (Table 3.2).

One set of cuttings from split bamboo nursery was washed in tap water to remove the adhering soil and debris. It was then planted in polythene bags. After 30 days, 1 g of *Trichoderma* grown on sorghum grains was applied around the plant and covered with soil. The following were the experimental details.

Design: split-plot

Main plot: Time of application (2)

1 Application during planting

2 Application after 30 days of planting

Sub plot: *Trichoderma* isolates (14)

1 Control

2 *T. harzianum* IISR 1369

3 *T. virens* IISR 1370

4 *T. aureoviride* IISR 126

5 *T. aureoviride* IISR 140

6 *T. aureoviride* IISR 143

7 *T. aureoviride* IISR 148

8 *T. hamatum* IISR 150

9 *T. hamatum* IISR 151

10 *T. hamatum* IISR 160

11 *T. harzianum* IISR 165

12 *T. harzianum* IISR 173

13 *T. pseudokoningii* IISR 186

14 *Trichoderma* sp. IISR 1107

15 *Trichoderma* sp. IISR 1141

Replications 3

Plot size: one plant / bag; three bags / replication

After one month of *Trichoderma* application the polythene bags were gently cut open and the plant with root system and adhering soil was taken. The roots were removed and gently taped to remove excess of adhering soil. The root along with adhering rhizosphere soil was taken in pre-weighed filter paper and dried at room temperature (26-28^{0}C) for 24 hours. It was weighed and added to 50 ml sterile distilled water taken in wide-mouthed bottle. It was shaken vigorously and also stirred with a forceps to suspend all the soil from the root to the water. The roots were taken out and blotted with filter paper. It was weighed and the weight of the rhizosphere soil calculated. The soil suspension was serially diluted and aliquots (1 ml) from a suitable dilution were plated on Actinomycetes Isolation Agar, Rose Bengal Agar, Nutrient Agar and *Trichoderma* selective medium.

The number of colonies developed on NA was counted after 48 hours. The number of cfu on other media was taken after 5 days. The number of cfu was transformed to log values prior to statistical analysis. The data was analysed by FACTOR.

3.4 Effect of abiotic factors in relation to survival of *Trichoderma* spp

To test the hypothesis that abiotic factors are responsible for controlling the distribution and survival of *Trichoderma* spp in soil the effect of soil type, moisture, temperature, pH, pesticides and fertilizers in relation to the survival of biocontrol agents was determined experimentally. Five *Trichoderma* isolates viz., *T. harzianum*

IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 with high rhizosphere competence, fast growth and sporulation were taken.

3.4.1 Effect of soil type on the survival of *Trichoderma* spp

Effect of soil type on the abundance of *Trichoderma* spp was studied as described by Rozenweig and Stotsky (1979) with modification. Soil was collected from black pepper plots at Calicut (clayey), Peruvannamuzhi (sandy clay) and Thamarassery (sandy loam). The properties of the soil such as texture and moisture holding capacity (MHC) were determined. It was dried evaporated to dryness at room temperature (26-28^{0}C). About 500 g was taken in plastic pots and planted with single nodded rooted cuttings of black pepper cv Karimunda cut from split bamboo nursery. About 1 g each of *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 multiplied on sorghum grain was added to each pot and the pot were watered regularly. The number of cfu of *Trichoderma* spp present in sorghum grain was mentioned (Table 3.3). The bags were kept in a glass house. There were six replications per treatment.

Table 3.3 Colony forming units (cfu) of *Trichoderma* isolates present in sorghum grains

Sl No.	*Trichoderma*	No. of cfu / g
1	*T. harzianum* IISR 1369	$31 X 10^{11}$
2	*T. virens* IISR 1370	$14 X 10^{11}$
3	*T. aureoviride* IISR 126	$41 X 10^{10}$
4	*T. harzianum* IISR 165	$35 X 10^{11}$
5	*T. harzianum* IISR 173	$13 X 10^{11}$

Soil samples were taken from the pots at 0 hour, after 15, 30, 45, 60, 75 and 90 days of treatment. The soil samples were air-dried and the number of colony forming units (cfu) per gram was determined by serial dilution plate technique. The number of cfu was transformed to log values prior to statistical analysis. The effect of soil type on propagule density in soil at every time interval was tested for each *Trichoderma* spp individually using analysis of variance.

3.4.2 Soil moisture effects on the survival of *Trichoderma* spp

Soil moisture effects on the survival of *Trichoderma* spp was studied following the method of earlier workers (Adams, 1987; Knudsen and Bin Li, 1990; Stark and Firestone, 1995) with modification. The soil used in the experiment was collected from Kozhikode, Kerala. This soil had a moisture holding capacity (MHC) of 64.87%. Soil was air-dried and sieved (<2 mm size) and about 100 g soil was taken in plastic pots of size 500 ml and water was added to get 20, 30, 40, 50, 60, 70 and 80% levels of MHC. Sealing the holes of the pots prevented the drainage of water and kept in a glass house. About 1 g of *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 multiplied on sorghum grain was added to each bag and mixed thoroughly. The number of cfu of *Trichoderma* spp present in sorghum grain was mentioned (Table 3.3). The pots were then weighed and water was added every 10 days to return soil water content that evaporated. The pots were covered with polythene sheets and tied with rubber bands. There were three replications per treatment.

Samples of soil were removed at 0 hour, after 15, 30, 45, 60, 75 and 90 days of treatment. The samples were air-dried and number of cfu per gram of soil was determined by serial dilution plate technique. The number of cfu was transformed to log values prior to statistical analysis. The effect of soil moisture on propagule density at every time interval was tested for each *Trichoderma* spp individually using analysis of variance.

3.4.3 Soil temperature effect on the relative abundance of *Trichoderma* spp

Temperature effect was studied in a model plant soil ecosystem as described by Saremi *et al.* (1999). Forest soil was collected from Peruvannamuzhi, Kozhikode district, Kerala. Soil was air-dried and sieved to remove stones and large particles then mixed thoroughly. Polythene bags of 3 X 6 inch size were used. The bags were filled with 500 g soil.

About 1 g of *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 multiplied on sorghum was applied to each bag and mixed thoroughly. The number of cfu of *Trichoderma* spp present in sorghum grain was mentioned (Table 3.3). Each bag was planted with single nodded rooted cuttings of black pepper cv Karimunda from split bamboo nursery. The bags were kept under natural light at fluctuating temperature regimes of <20, 20-26, 26-32°C in a microprocessor controlled green house (Cassia Siamia Technologies, Hyderabad). Bags were watered at two days interval. Six bags were used to monitor changes in fungal propagule densities in soil through time as measured by soil dilution.

Six bags were sampled from each temperature regime for each fungus. Samples were collected 0, 15, 30, 45, 60, 75 and 90 days after application. Care was taken to avoid resampling the same sites in the pots. Samples were removed the day after watering the bags. Sample collected from each bag were air dried, crushed, mixed thoroughly. Number of cfu per gram of soil was determined by serial dilution plate technique. Aliquot (1 ml) was plated on TSM. Dilution plates were incubated for 5 days and colony count were taken. The number of colony forming units transformed to log values prior to statistical analysis. The effect of temperature on propagule density in soil at every time interval was tested for each *Trichoderma* species individually using analysis of variance.

3.4.4 Effect of pH on *Trichoderma* spp and *Phytophthora capsici* in culture

The pH of PDA was adjusted with 1M HCl / NaOH in 1.0 pH units increments from 4.5 to 8.5. It was sterilized and the pH was checked after autoclaving using sample flask. The media was poured into petri-plates and inoculated at the center with mycelial discs of 2 days old *P. capsici, T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173. There were four replications per treatment. The mycelial growth was recorded after 24 and 48 hours. The colony diameter of each species after 48 hours was subjected to analysis of variance individually.

3.4.5 *In-vitro* toxicity of pesticides to *Phytophthora capsici* and *Trichoderma* spp

The sensitivity of *P. capsici, T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 to eight pesticides were evaluated by poisoned foot technique (Nene and Tapliyal, 1993). The pesticides were metalaxyl, mancozeb, potassium phosphonate, copper oxychloride, phorate, carbendazim, chlorpyriphos and quinalphos. Pesticides were added to molten Potato Dextrose Agar (PDA) medium after sterilization and poured into 9 cm diameter petri dishes. Each pesticide was evaluated at 10, 100, 300 and 500 ppm concentrations based on active ingredient. Unamended potato dextrose agar served as inoculated

control. After the media had solidified the plates were amended with 4 mm diameter agar plugs cut from the margin of 2 days old culture of each fungus. Three replicates of each combination of fungus / fungicide concentration were made.

After 48 hours of incubation at 28-30^{0}C the diameter of the colony was measured for each plate and percent inhibition of growth relative to the control was calculated using the formula (C-T) / C X 100 where C is the diameter of control colony and T is the diameter of colony in the treated plate. The LD_{50} value of each pesticide was calculated for each species. The pesticide was considered to be toxic to *Trichoderma* at a particular concentration when 50% or more growth of the fungus was inhibited. The percent inhibition was transformed to corresponding angular values prior to statistical analysis. The data was analysed by ANOVA.

3.4.6 Effect of simultaneous application of pesticides on the survival of *T. harzianum* IISR 1369 in soil

Toxicity of pesticides to *T. harzianum* IISR 1369 ~~is~~ was tested in soil as suggested by Stephen *et al.* (2000). About 200 g soil was taken in cups and planted with single nodded rooted cuttings of black pepper cv Karimunda. About 1 g of *T. harzianum* IISR 1369 multiplied on sorghum grains was added to each bag and mixed thoroughly. The sorghum grains approximately contained 20 X 10^{11} cfu / g. Required quantities of pesticides were added to each cup in order to get 10, 100, 300 and 500 ppm concentration based on their active ingredient. There were four replications per concentration. Soil samples were drawn at 0 hour, 7, 15, 30, 45, 60 and 90 days. The cfu was determined by SDPT. The number of cfu was transformed to log values prior to statistical analysis. The effect of pesticide on propagule density in soil at every time interval was tested by analysis of variance.

3.4.7 Effect of organic and inorganic fertilizers on the survival of *T. harzianum* IISR 1369 applied to black pepper rhizosphere

Inorganic sources of nitrogen, phosphorus, potassium and organic amendments on the survival of *T. harzianum* IISR 1369 applied to black pepper rhizosphere were studied by the method of Paulitz and Baker (1987) and Jayaraj (1999) with modification.

About 4 kg of soil was taken in polythene bags. Single nodded rooted cuttings of black pepper cv Karimunda from split bamboo nursery were planted two, in each bag. About 50 g of *T. harzianum* IISR 1369 multiplied on sorghum grain was added to each bag. The sorghum grains approximately contained 20 X 10^{11} cfu / g. The plants were watered regularly. Soil sample was drawn and the number of cfu of *Trichoderma* was determined. Required quantity of fertilizers for 4 kg soil (Table 3.2) based on the recommendation for bush pepper was added (Sadanandan, 1994; 2000; Sadanandan and Hamza, 1996). After 10 days soil sample was taken and determined the cfu. Fertilizer application and determination of propagules of *T. harzianum* (before and after application) were followed at bimonthly interval for a year. The following were the experimental details.

Design: CRD

Treatments (9)

1 Control

2 Urea

3 Rock phosphate

4 Muriate of potash

5 NPK

6 Farm yard manure

7 Dried leaves

8 Coir pith compost

9 Neem cake

Replications 3

Plot size : 2 bags / replication

For determination of cfu, samples from two replications (4 bags) were mixed and analysed. The number of colony forming units was determined by serial dilution plate technique on *Trichoderma* selective medium for each replicate. The number of cfu was transformed to log values prior to statistical analysis. The effect of fertilizers on propagule density in soil at every time interval was tested using analysis of variance.

Table 3.4 Details of fertilizer dose for bush pepper

Chemical / organic Fertilizer	Nutrient content and percentage		Quantity needed (g) for 10 kg soil at bimonthly interval	Quantity needed (g) for 4 kg soil at bimonthly interval
Urea	N	46%	2.1	0.84
Rock phosphate	P	18%	2.77	1.1
Muriate of Potash	K	60%	1.66	0.66
U + RP + MoP	NPK		2.1, 2.77, 1.66	0.84, 1.1, 0.66
Farm yard manure	NPK		200	80
Leaf litter	NPK		100	40
Coir pith compost	NPK		50	20
Neem cake	NPK		30	12

All are general recommendations for bush pepper. NPK=1g: 0.5g: 0.2g at bimonthly interval for 10 kg soil

STUDIES ON VOLATILES AND NON-VOLATILES

3.5 Screening of *Trichoderma* spp for the effect of their volatile metabolites on the growth of *P. capsici*

Thirty isolates of *Trichoderma* spp were screened for the effect of their volatile metabolites on the growth of *P. capsici* by following the method of Dennis and Webster (1971b). Carrot agar (CA) medium was poured into 90 mm diameter petri plates and inoculated with two days old 4 mm diameter mycelial disc of *Trichoderma* species. A similar sized disc of *P. capsici* isolate was cut from a two days old culture and inoculated onto carrot agar in another plate. The lid of the plate inoculated with *Trichoderma* was removed and is covered with bottom of another carrot agar plate inoculated with *P. capsici*. The plates were sealed around the edges with cellophane tape and incubated at room temperature (28-30^{0}C). There was no *Trichoderma* in the case of control and both the plates were inoculated with same *P. capsici* isolate. There were three replications per treatment.

The colony diameter of *P. capsici* was measured after 72 hours. The percent inhibition of *P. capsici* by volatiles of *Trichoderma* spp was calculated using the formula (C-T) / C X 100 where, C is the diameter of the control colony of *P. capsici* and T is the diameter of *P. capsici* colony in the treatment. The percent inhibition was transformed to corresponding angular values and subjected to analysis of variance followed by range test for mean comparison. After 72 hours *P. capsici* colony was sub-cultured onto fresh carrot agar medium and observed for any changes to colony characters in comparison with control.

3.6 Analysis of volatiles of *Trichoderma* spp by gas chromatography

The present work is the chemical study on the complex odor of *Trichoderma* spp from India used for biocontrol of foot rot of black pepper. *T. harzianum* IISR 1369 and *T. virens* IISR 1370 were investigated for volatile components using gas chromatography as suggested by Geiger (1993) and Rapior *et al.* (2000) with little modification. About five ml of Czapek Dox Broth (CDB) was taken in 15 ml capacity head space vials and sterilized. The headspace vials were specially made for gas chromatographic analysis. It was inoculated with spores of *T. harzianum* IISR 1369 and *T. virens* IISR 1370 and sealed with Aluminium lined rubber cork specially made for headspace vials. The vials were incubated at room temperature (28-30^0 C) for 10 days. The analysis of grown up cultures was carried out with a gas chromatograph (Perkin Elmer Autosystem GC) and a detector (Flow Ionization Detector) coupled with headspace sampler (Perkin Elmer Headspace Sampler). The carrier gas was Nitrogen with a linear gas velocity of 28 cm/s. The injector and detector temperatures were 200 and 270^{0}C respectively. The column was temperature programmed as follows: 50^{0}C (3 min) to 200^{0}C (3^{0}C / min). CDB without *Trichoderma* served as control.

3.7 Screening of *Trichoderma* spp for the effect of their non-volatile metabolites on the biomass of *P. capsici*

Above thirty isolates of *Trichoderma* were also screened for their effect of non-volatile metabolites on the mycelial dry weight of *P. capsici* as described by Singh *et al.* (1992) with little modification. About 100 ml each Czapek Dox Broth (CDB) having pH 6.5 was taken in 250 ml conical flasks and sterilized. The broth was inoculated with a 4 mm diameter disc of *Trichoderma* cut from 4 days old culture and incubated at room temperature (28-30^{0}C) for 21 days. Three flasks were inoculated for each *Trichoderma* species. The contents were filtered through whatman No.1 filter paper to remove mycelia and some amount of spores. It was then filtered through G5 filter (sintered glass funnel, with 0.22 μm pore size, Borosil) under vacuum to make it cell free. The filter sterilized culture filtrate was added to carrot broth in 100 ml conical flask in order to get 10, 25, 50 and 75 percent concentration. The total content in the flask was 20 ml. The flasks were inoculated with 4 mm diameter disc of *P. capsici* cut from two days old culture and incubated at room temperature (28-30^0 C) for 14 days. The flask containing carrot broth without culture filtrate and with required amount of CDB served as control. There were three replications per concentration.

After fourteen days, contents were filtered through a pre-weighed filter paper to remove the broth. The mycelia were washed thrice with distilled water. The mycelia on the filter paper were dried at 50^0 C for 3 days in a hot air oven. The filter paper together with the dry mycelia was weighed and dry weight of mycelia was calculated. The percent inhibition of *P. capsici* by non-volatile metabolites of *Trichoderma* spp was calculated using the formula (C-T) / C X 100 where, C is the dry weight of the control colony of *P. capsici* and T is the dry weight of the *P. capsici* colony in the treatment. The percent inhibition at each concentration was transformed to corresponding angular values and subjected to analysis of variance followed by range test for mean comparison.

3.8 Isolation and identification of non-volatile metabolites

Non-volatiles were isolated as described by Dunlop *et al.* (1989) with modification. Czapek dox broth with pH 6.0 was taken in conical flasks and sterilized. The broth was inoculated with a loop-full of spores from the surface of 4 days old culture of *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148. These isolates were selected based on high inhibition of *P. capsici* by their culture filtrate and high rhizosphere competence ability.

About one litre of the broth was inoculated with (100 ml in 250 ml conical flask) each fungus. Uninoculated CDB served as control. The flasks were incubated at room temperature (28-30^{0}C) for 21 days. The contents of the flasks were passed through four layered sterile muslin cloth and then through sterile whatman No. 1 filter paper to remove the mycelial mat and spores. The pH of the culture filtrates was checked using a pH meter (ELICO).

3.8.1 Extraction of active compounds and testing for inhibition of *P. capsici*

About 100 ml of cell free culture filtrate was extracted twice with equal volume of ethyl acetate (EtOAc) (Dunlop *et al.*, 1989). The solvent fractions were combined and dried over sodium sulfate (Na_2SO_4) spread over cotton pad in a funnel. The solvent phase was concentrated to about 1 ml under vacuum using flash evaporator (Heidolf, Germany) at 35^{0}C with 250 rpm. Then 2 ml of pure solvent was added to the concentrate in the rotary flask and taken out with a micropipette. The extract was kept open in a laminar flow and the volume reduced to 1 ml.

Concentrate from ethyl acetate was tested for its antifungal activity as described by Dunlop *et al.* (1989). Carrot agar plates were inoculated with 5 mm diameter discs of *P. capsici* isolate 02-52 cut from two days old culture. Different quantities of EtOAc fractions were dispensed directly on the top of *P. capsici* inoculum plug. The quantities applied were 10, 20, 40, 50, 100 and 200 μl. Required quantity of pure solvent served as control for each concentration. Also maintained was an absolute control without any application. There were three replications per treatment / concentration. After dispensing, the plates were kept open in the laminar airflow allowing the solvent to evaporate completely. Colony diameter was measured after 24, 48 and 72 hours and analysed by ANOVA. Percent inhibition in growth was calculated using the formula (C-T) / C X 100 where, C is the diameter of control colony of *P. capsici* and T is the diameter of colony in the treated plate.

3.8.2 Thermo stability of ethyl acetate extract of culture filtrate of *Trichoderma* spp

The thermo stability of the EtOAc fraction was also tested following the method of Yoon *et al.* (1989). About 100 µl was taken in ependorf tubes and autoclaved at 121^{0}C for 15 minutes. Since all the solvent evaporated during autoclaving, pure solvent was added to dissolve the fraction. It was then tested for its antifungal activity as described above. Colony diameter was measured after 24, 48 and 72 hours and analysed by ANOVA. Percent inhibition in growth was calculated using the formula (C-T) / C X 100 where, C is the diameter of control colony of *P. capsici* and T is the diameter of colony in the treated plate.

3.8.3 Effect of ethyl acetate extract of culture filtrate of *Trichoderma* spp on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

Mycelial discs (5 mm dia) cut from the peripheral growth of a 4 day old culture of *P. capsici* were transferred to petri-dishes (90 mm dia) containing 20 ml of 10, 100, 300, 400, 500, 1000, 2000 and 4000 ppm of ethyl acetate extract of culture filtrate of *Trichoderma* spp. For preparing above concentrations of extract, I ml of EtOAc extract obtained from 100 ml of culture filtrate was used. Each petriplate contained 20 ml of the solution. EtOAc was allowed to evaporate before adding water to prepare different concentrations. Plates containing sterile distilled water without extract served as controls. Plates were incubated at room temperature (28-30^{o}C) under continuous fluorescent light. The number of sporangia formed on the peripheral mycelial growth of the discs was counted after 48 hours under the microscope (X 40). There were three replicate plates and each one contained 10-12 mycelial discs. Two counts were taken from a disc and ten discs were used to take the count in each plate. Percent reduction in the number of sporangia formed was calculated using the formula,

$$\left(\frac{\text{No. of sporangia in the control} - \text{No. of sporangia in the treatment}}{\text{No. of sporangia in the control}} \right) \text{X } 100$$

Percent reduction in the number of sporangia was analysed by ANOVA after transforming the percent values to corresponding angular values.

The cultures were given a cold shock for 10 minutes in a refrigerator and taken out and kept at room temperature (28-30^{0}C) for half an hour. The discs were observed under the microscope (X 40) for [illegible] ~~number~~ of zoosporangia ~~released~~ and zoosporangia without release of zoospores. The number was counted from random areas of the fungal growth from each mycelial disc. There were three replicate plates and each one contained 10-12 mycelial discs. Two counts were taken from a disc and ten discs were used to take the count in each plate. Percentage of sporangia released their zoospores were calculated using the formula,

$$\left[\frac{\text{No. of sporangia opened}}{(\text{No. of sporangia opened} + \text{No. of sporangia not opened})}\right] \text{X } 100$$

Zoospores were collected separately from fresh cultures released within two hours and was added (50 μl) to cavity slides each containing 50 μl of different concentrations (10, 100, 300, 400, 500, 1000, 2000 and 4000 ppm) of ethyl acetate fraction. The slides were kept in petri-dishes with moist-lined filter paper and incubated at room 23-25^{0}C. The number of germinated and ungerminated zoospores was counted after 12 hours. Percentage of zoospores germinated was calculated using the formula,

$$\left[\frac{\text{No. of zoospores germinated}}{(\text{No. of zoospores germinated} + \text{No. of zoospores not germinated})}\right] \text{X } 100$$

There were three replicate wells per concentration and from each replication ten fields were counted (X 40).

The percent reduction in number of sporangia formed in each concentration of the extract was subjected to analysis of variance. Percentage of zoosporangia released and percentage of zoospores germinated were transferred to arcsine values and subjected to analysis of variance separately.

3.8.4 Thin layer chromatography

Individual compounds in EtOAc fraction was separated by thin layer chromatography (Dunlop *et al.*, 1989; Ghisalbeti and Rowland, 1993; Sadasivam and Manickam, 1996). Silica gel G and water was taken in 1:2 proportions and shaken well in a 250 ml round bottom flask. Then it was applied to dry, pre-heated glass plate using an applicator (MERCK) at a thickness of 0.25 mm. It was dried at room temperature for 24 hours and stored for further work. The plates were heated to 110^{0}C for 30 minutes and then allowed to cool prior to applying samples. Samples of EtOAc concentrate were applied as spots using capillary tubes. From 100 ml of culture filtrate, 1 ml of EtOAc extract was obtained and it was applied on the TLC plate as single spot.

Ethyl acetate and hexane was used as solvent system (80 / 20) (Dunlop *et al.*, 1989; Ghisalberti and Rowland, 1993). Solvents (100 ml) were taken in the TLC chamber half an hour before to equilibrate with the vapour. The plates with samples were dried under fan and kept in TLC chamber with solvent system (100 ml) for developing the chromatogram. The plates were removed just before the solvent system reached the top of the plate. It was dried under room temperature and visualized the spots. Sample plate was sprayed with 10% H_2SO_4 in methanol and heated at 110^{0}C for 15 minutes and observed for any additional spots. The distance traveled by the each spot~~s~~ were measured and the Rf (Retardation factor) value for each compound was calculated using the formula,

$$\left(\frac{\text{Distance traveled by the solute front}}{\text{Distance traveled by the solvent front}}\right)$$

Each spot / compound was scraped out and taken in 15 ml glass vial. Ethyl alcohol (EtOH) was added to it in the ratio 1:2 (v / v) and shaken well. It was kept in the refrigerator allowing settling down the silica gel. After 24 hours the EtOH was collected with a micropipette. The compounds were soluble in EtOH as observed by the colour. The EtOH fractions were kept open in a laminar airflow allowing it to evaporate and reduced to 1 ml.

Antifungal activity of each compound was tested as described by Dunlop *et al.* (1989). Carrot agar plates were inoculated with 5 mm diameter discs of *P. capsici* cut from two days old culture. About 200 µl was dispensed over the disc of *P. capsici.* Required quantity of pure EtOH served as control. Also maintained was an absolute control without any application. There were three replications per treatment per compound. Colony diameter was measured after 24, 48 and 72 hours and analysed by ANOVA. Percent inhibition in growth was calculated using the formula (C-T) / C X 100 where, C is the diameter of control colony of *P. capsici* and T is the diameter of colony in the treated plate.

STRAIN IMPROVEMENT

3.9 Strain improvement of *Trichoderma* using protoplast fusion technology

The potential isolate *T. harzianum* IISR 1369 grows well on solid substrates and in liquid cultures with high number of colony forming units (cfu). Culture filtrate (CF) of another isolate *T. aureoviride* IISR 140 is highly inhibitory to *P. capsici* than that of *T. harzianum* IISR 1369 but it is unable to grow well and sporulate on solid substrate and in liquid culture. An attempt has been made to combine these two characteristics using protoplast fusion technology.

About 100 ml potato dextrose broth adjusted to pH 6.0 was taken in 250 ml conical flasks and sterilized. The broth (100 ml each) was inoculated with a loop-full of spores from the surface of 4 days old culture of *T. harzianum* IISR 1369 and *T. aureoviride* IISR 140 in separate flasks. This was incubated at room temperature (28-30^{0}C) on an orbital shaker with 120 rpm. After 24 hours the contents of the flasks were filtered through whatman No.1 filter paper and washed three times each with 100 ml sterile distilled water.

About 25 mg each of cellulase onozuka (Yakult Honsha Co. Ltd), Hemicellulase and Driselase (Sigma) were dissolved in 5 ml of 0.6 M NaCl in 30 ml capacity vials. The enzyme-osmoticant solution was sterilized using a sterile millipore membrane (pore size 0.22 μm) syringe filter. To this sterile enzyme-osmoticant solution was added about 100 mg of mycelia using a sterile spatula. The vials were incubated on an orbital shaker with 120 rpm at room temperature (28-30^{0}C). The protoplast formation was checked at 1, 2, 3, and 4 hours. After 4 hours the protoplasts were harvested by filtration through a sterile double-layered cheesecloth. The protoplasts were then counted with a haemocytometer and used immediately. The regeneration of protoplasts was checked by spreading 100 μl of protoplast-enzyme-osmoticant solution on PDA amended with osmoticant (Annamalai and Lalithakumari, 1991). The protoplasts in the enzyme-osmoticant solution were centrifuged at 1000 rpm for 10 minutes and the supernatant was poured off. The protoplasts were suspended in 2 ml 0.6 M NaCl.

Protoplast fusions were performed as suggested earlier (Anne, 1992; Hanson and Howell, 2002) using approximately 100 μl of osmoticant with protoplast of each species taken in sterile test tube (Figure 3.1). For controls, 200 μl of *T. harzianum* IISR 1369 and *T. aureoviride* IISR 140 was taken separately. To this was slowly added 200 μl of 30% polyethylene glycol (w / v) (PEG, MW 4000) containing 10 mM $CaCl_2$. PEG was also used in 40% and 50% concentration. But for controls only 40% PEG was used. It was gently shaken and then added 100 μl of 0.6 M NaCl stepwise to reach a total volume of 1 ml. The fusion frequency was checked after 30 minutes by taking the counts of hetero / homokaryons and nonfusant protoplasts.

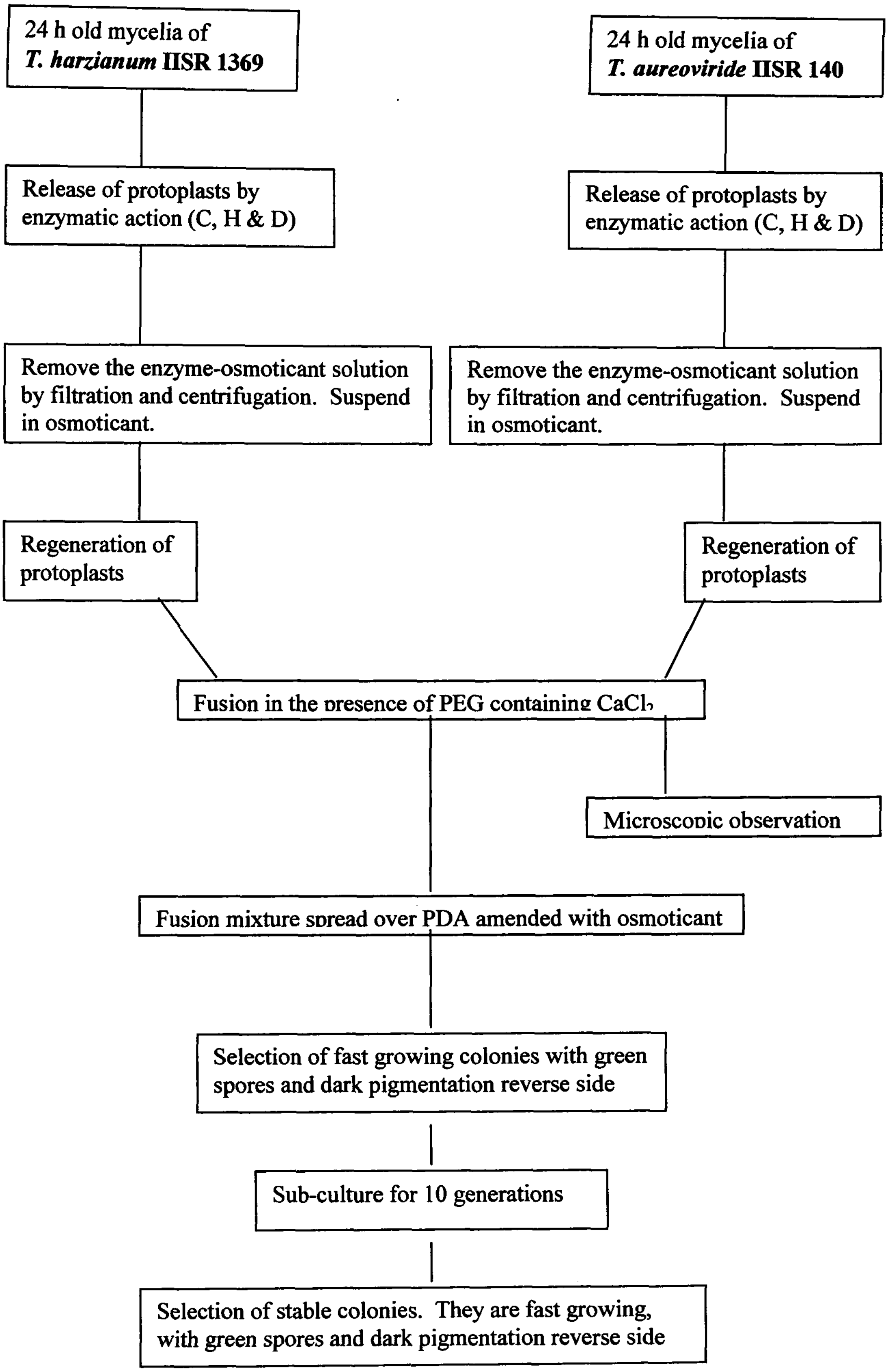

Figure 3.1 Chart showing the various steps involved in protoplast fusion between *T. harzianum* IISR 1369 and *T. aureoviride* IISR 140. C cellulase, H hemicellulase and D driselase

The fusion mixture (100 µl) was spread over PDA, pH 6.0, amended with 0.6M NaCl. Then protoplast / fusion product regeneration was checked everyday. Fast growing colonies with dark reverse side were selected and sub cultured. Its colony morphology and spore colour was compared with the parents. The fusants were sub cultured at 5 days interval for 10 generations. Stable intermediates in colony morphology and spore colour was retained and others were discarded. Their morphological and cultural characteristics were recorded by growing on PDA. These were tested for their antagonism by inhibition of *P. capsici* by adopting dual plate technique. The interactions were studied in petri dishes (90 mm diameter) containing carrot agar medium. A disc (5 mm diameter) from the edge of an actively growing 48 hours old *P. capsici* colony was transferred to each dish and similar sized disc of fusant *Trichoderma* cut in the same manner was placed at a distance of 6 cm on the opposite end of the plate. The dishes were incubated at room temperature (26 ± 2^0C). Plates inoculated with *P. capsici* without fusant *Trichoderma* served as control. The interactions were observed daily and calculated the percentage of inhibition according to the formula, (C-T / C) X 100 where, C is the radius of control colony of *P. capsici*, T is the distance in mm travelled by the *P. capsici* colony in the paired plate. The radii were measured after 72 hours of growth in all the cases. Percent inhibition was analysed by ANOVA after transforming the percent values to corresponding angular values.

3.9.1 Secondary metabolites production by fusant *Trichoderma*

The production of non-volatiles was examined in liquid media by culturing the isolates for 21 days in CDB, pH 6.0. Cultures were filtered through four-layered sterile muslin cloth followed by whatman No. 1 filter paper. About 100 ml of the filtrate was extracted twice with an equal volume of ethyl acetate EtOAc. The EtOAc fractions were combined and evaporated to dryness with a flash evaporator (Heidolf, Germany) and the residue was again dissolved in 3 ml of EtOAc. It was kept open in a laminar flow and the volume reduced to 1 ml. Inhibitory activity of the extracts was assayed against *P. capsici* isolate 02-52. Carrot agar plates were inoculated at the center with 5 mm diameter disc of *P. capsici* taken from two days old culture. Aliquots of EtOAc fraction (10, 20, 40, 50, 100, 200 µl) were directly dispensed over the disc and the plates were kept open in a laminar airflow allowing evaporation of the solvent completely (Dunlop *et al.*, 1989). Equal amount of pure solvent served as control. Also maintained was an absolute control without any application. Colony diameter was measured after 24, 48 and 72 hours and analysed by ANOVA. Percent inhibition in growth was calculated using the formula (C-T) / C X 100 where, C is the diameter of control colony of *P. capsici* and T is the diameter of colony in the treated plate.

3.9.2 Thermo stability of ethyl acetate extract of culture filtrate of fusant *Trichoderma*

Thermo stability of the EtOAc fractions was also tested as described in the section 3.8.3 following the method of Yoon *et al.* (1989). Colony diameter was measured after 24, 48 and 72 hours and analysed by ANOVA. Percent inhibition in growth was calculated using the formula (C-T) / C X 100 where C is the diameter of control colony of *P. capsici* and T is the diameter of colony in the treated plate.

3.9.3 Effect of ethyl acetate extract of culture filtrate of fusant *Trichoderma* on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

Ethyl acetate extract of culture filtrate of fusant *Trichoderma* (FT 9, FT 11, FT 14, FT 15, FT 16, FT 17 and FT 21) were tested against zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici* as described in section 3.8.3.

DISEASE MANAGEMENT

3.10 Evaluation of *Trichoderma* spp and *Pseudomonas fluorescens* for suppression of *P. capsici*

Field trial was conducted at Thamarassery, Kozhikode district, Kerala to study the disease suppressive effect of *Trichoderma* spp and *Pseudomonas fluorescens* and some of their combinations in comparison with existing potent ones. Black pepper was planted in July 2000 using 7 years old Areca nut as standard.

Experimental details:

Design:	RBD
Treatments	17
Replications	3

No. of plants per replications 20

Pits were taken 30 cm away from the base of the trunk in the northeastern side. The plants consisted of Karimunda, Panniyur 1, Panniyur 2, Panniyur 4, Panniyur 5, which were first raised in poly bags. The plants were cut into uniform length of 30 cm before planting. *Trichoderma* spp was multiplied on sorghum grains and *P. fluorescens* in nutrient broth. About 50 g of *Trichoderma* was added to each pit during planting of cuttings. About 1 litre of nutrient broth, 48 h old shake culture, was diluted to 10 L with water and 50 ml was added to each pit. In treatments where combinations were used 25 g of *Trichoderma* and 25 ml of *P. fluorescens* were added. The application was repeated in September 2000. There were two applications in 2001. In the year 2002, only one application was done, in June. The number of colony forming units of *Trichoderma* and *P. fluorescens* isolates was mentioned (Table 3.5).

Table 3.5 Colony forming units (cfu) of *Trichoderma* and *P. fluorescens* isolates

Sl No.	*Trichoderma / P. fluorescens*	No. of cfu / g
1	*T. virens* IISR 112	$23 X 10^{11}$
2	*T. virens* IISR 18	$12 X 10^{11}$
3	*T. harzianum* IISR 1369	$14 X 10^{11}$
4	*T. virens* IISR 1370	$10 X 10^{11}$
5	*T. aureoviride* IISR 143	$30 X 10^{11}$
6	*T. pseudokoningii* IISR 187	$19 X 10^{11}$
7	*T. harzianum* IISR 167	$20 X 10^{11}$
8	*T. harzianum* IISR 178	$72 X 10^{11}$
9	*P. fluorescens* IISR 11	$44 X 10^{9}$
10	*P. fluorescens* IISR 13	$27 X 10^{9}$
11	*P. fluorescens* IISR 41	$19 X 10^{9}$
12	*P. fluorescens* IISR 6	$26 X 10^{9}$

Colony forming units of *P. fluorescens* mentioned is per ml of solution after diluting with water

Mean of five applications done in 2000, 2001 and 2002

Soil samples were collected before planting. Plants were monitored for growth and incidence of disease. Periodically soil samples were collected from the base along with small root pieces from 5 plants randomly in each replication. The samples were pooled and 100 g was used to detect the presence of *Phytophthora* by baiting using *Albizzia* leaves. About 150 ml of water was added to it and stirred well and 10 leaves of *Albizzia* was added and after 48 hours the leaves were observed under the microscope for the presence of sporangia (Anandaraj and Sarma, 1990).

Remaining soil was air dried for 24 hours and number of colony forming units of *Trichoderma, P. fluorescens* an other fungi were determined by serial dilution plate technique in *Trichoderma* Selective Medium, Kings' B agar and Rose Bengal Agar respectively. *Trichoderma* species were distinguished based on colony morphology. Since markers were not available *P. fluorescens* was not enumerated specifically. Number of cfu on KB was considered as *P. fluorescens* even though other species also grow on this media.

Final plant height was measured in June 2002. Increase in plant height was analyzed by analysis of variance followed by range test for mean comparison using MSTATC software. The number of cfu of *Trichoderma, P. fluorescens* and other fungi were converted to log cfu / g and pooled over the years. The pooled data was analyzed by analysis of variance.

There was no natural infection of *Phytophthora* in the field planted black pepper and since it was a private field destructive sampling was not undertaken to score root rot. Hence the same set of treatments was carried out in glass house by taking large quantity of soil (4 kg / pot). Cuttings of black pepper var. Panniyur were planted in polythene bags. *Trichoderma* spp multiplied on sorghum grains were added (50 g / plant). Bacteria were grown in nutrient broth (NB). One litre of nutrient broth was diluted to 10 L using water and 50 ml was added to each plant. In treatments where combinations are involved half of the cultures were added. There were 10 plants per treatments. After six months the plants in each treatment was challenge inoculated with *P. capsici* isolate 99-101 and number of plant death / root rot was recorded. Results of plant death / root rot were converted to percentages for presentation.

4

RESULTS

Thirty isolates of *Trichoderma* spp were screened for their volatile and non-volatile effect on the growth of *P. capsici.* Nine isolates were identified as superior over the others in the inhibition of *P. capsici* by volatiles and eight were found as superior in the inhibition of *P. capsici* by non-volatiles. Out of the above seventeen short-listed isolates three were both volatile and non-volatile producers. Therefore, the resulting fourteen isolates were taken for *in-vitro* and *in-vivo* studies.

IN-VITRO STUDIES

4.1 *In-vitro* interactions between *Trichoderma* spp and *Phytophthora capsici*

Out of fourteen isolates of *Trichoderma* which were high volatile and non-volatile producers six of them showed antagonistic interactions with *P. capsici* on water agar *in-vitro*. *T. aureoviride* IISR 126, *T. aureoviride* IISR 140, *T. aureoviride* IISR 143, *T. aureoviride* IISR 148 and *T. harzianum* IISR 165 showed coiling, parallel growth and penetration of hyphae of *P. capsici*. *T. aureoviride* IISR 126 caused sporangial proliferation. *T. aureoviride* IISR 143 showed sporangial parasitism. *T. harzianum* IISR 165 caused shortening of hyphal tips of *P. capsici*. *Trichoderma* sp. IISR 1107 induced deformation of sporangia (Table 4.1). These interactions were observed in acidic pH ranging from 4.5 to 6.5 (Plate 4.1 a - x). However, some of the isolates of *Trichoderma* were not able to make any visible interactions *in-vitro*.

Table 4.1 *In-vitro* interactions between *Trichoderma* spp and *Phytophthora capsici*

Trichoderma spp	Nature of interactions with *P. capsici*
T. harzianum IISR 1369	No visible interaction
T. virens IISR 1370	No visible interaction
T. aureoviride IISR 126	Parallel growth, sporangial proliferation
T. aureoviride IISR 140	Hyphal coiling, penetration
T. aureoviride IISR 143	Coiling, penetration, sporangial parasitism, hyphal evacuation
T. aureoviride IISR 148	Parasitism
T. hamatum IISR 150	No visible interaction
T. hamatum IISR 151	No visible interaction
T. hamatum IISR 160	No visible interaction
T. harzianum IISR 165	Shortening of hyphal tips, parasitism
T. harzianum IISR 173	No visible interaction
T. pseudokoningii IISR 186	No visible interaction
Trichoderma sp. IISR 1107	Sporangial deformation
Trichoderma sp. IISR 1141	No visible interaction

IN-VIVO STUDIES

4.2 *In-vivo* effect of *Trichoderma* spp on black pepper plants

Fourteen isolates of *Trichoderma* spp were tested *in-vivo* for their effect on the host plant. All the isolates were found to be growth promoting. The percent increase in plant height over the control varied from 68 to 128%. *T. harzianum* IISR 1369, *T. hamatum* IISR 151, *T. hamatum* IISR 160 and *T. pseudokoningii* IISR 186 showed highest plant growth. *Trichoderma* isolates also caused increase in the biomass of the plants (Plate 4.2 a - n). *T. harzianum* IISR 1369 recorded highest shoot biomass (Table 4.2). However root biomass was uniform among various treatments.

When challenge inoculated with *P. capsici*, five of the *Trichoderma* isolates viz. *T. aureoviride* IISR 126, *T. aureoviride* IISR 140, *T. aureoviride* 148, *T. harzianum* IISR 165, *T. harzianum* IISR 173 were able to suppress the pathogen. Where the above *Trichoderma* isolates were applied, only 20% of the plants were showed death / yellowing compared to 100% in the control (Table 4.2). This was followed by *T. harzianum* IISR 1369, *T. virens* IISR 1370 and few other isolates where 40% of the plants were showed mortality. These isolates recorded average performance in the growth promotion. Thus the characteristics of growth promotion and disease suppression among *Trichoderma* spp are varied among different isolates.

Table 4.2 Growth promotion of black pepper and suppression of *Phytophthora capsici* by *Trichoderma* spp

Treatment	Increase in plant height (cm)	% death	Shoot dry weight (g)	Root dry weight (g)
Control	30.0_{b}	100	2.49_{b}	0.57_{a}
T. harzianum IISR 1369	65.0_{a}	40	4.48_{a}	0.75_{a}
T. virens IISR 1370	58.4_{ab}	40	2.96_{ab}	0.81_{a}
T. aureoviride IISR 126	55.2_{ab}	20	3.82_{ab}	0.71_{a}
T. aureoviride IISR 140	62.2_{ab}	20	3.42_{ab}	0.77_{a}
T. aureoviride IISR 143	50.4_{ab}	40	3.17_{ab}	0.76_{a}
T. aureoviride IISR 148	51.2_{ab}	20	2.64_{b}	0.59_{a}

continued

Table continued

Treatment	Increase in plant height (cm)	% death	Shoot dry weight (g)	Root dry weight (g)
T. hamatum IISR 150	59.2 $_{ab}$	60	2.52 $_{b}$	0.64 $_{a}$
T. hamatum IISR 151	65.2 $_{a}$	60	4.01 $_{ab}$	0.69 $_{a}$
T. hamatum IISR 160	68.4 $_{a}$	60	3.88 $_{ab}$	0.63 $_{a}$
T. harzianum IISR 165	51.6 $_{ab}$	20	2.98 $_{ab}$	0.64 $_{a}$
T. harzianum IISR 173	53.8 $_{ab}$	20	3.11 $_{ab}$	0.63 $_{a}$
T. pseudokoningii IISR 186	68.0 $_{a}$	40	3.38 $_{ab}$	0.60 $_{a}$
Trichoderma sp. IISR 1107	52.4 $_{ab}$	40	2.58 $_{b}$	0.58 $_{a}$
Trichoderma sp. IISR 1141	58.8 $_{ab}$	40	2.69 $_{b}$	0.61 $_{a}$

Values followed by same letter(s) in a column do not differ significantly according to Duncan's Multiple Range Test at 5% level

4.3 Rhizosphere competence of *Trichoderma* spp

Ability to colonize the roots of black pepper by fourteen *Trichoderma* isolates (Plate 4.11 c-h) was studied. These isolates were able to suppress *P. capsici* by their volatile and non-volatile metabolites.

Rhizosphere competence of *Trichoderma* isolates differed considerably when applied during planting and after planting. *T. harzianum* IISR 1369, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140, *T. aureoviride* IISR 148, *T. aureoviride* IISR 165 and *T. harzianum* IISR 173 colonised the roots in higher numbers when applied during planting. *T. hamatum* IISR 150, *T. hamatum* IISR 151 and *T. hamatum* IISR 160 colonized the roots significantly in higher numbers when applied after planting. Colonisation of *T. virens* IISR 1370, *T. aureoviride* IISR 143, *T. pseudokoningii* IISR 186 and *Trichoderma* sp. IISR 1141 was almost same at both the conditions of applications.

Plate 4.1 a	Coiling around the *P. capsici* hyphae by *T. aureoviride* IISR 140 (X 100)
Plate 4.1 b	*T. aureoviride* IISR 143 parasitizing the sporangia of *P. capsici* (X 100)
Plate 4.1 c	Sporangial proliferation of *P. capsici* induced by *T. aureoviride* IISR 126 (X 100)
Plate 4.1 d	Parallel growth of hyphae of *P. capsici* and *T. aureoviride* IISR 126 and penetration of the former by the latter (X 100 Phase Contrast)
Plate 4.1 e-f	Sporangial proliferation of *P. capsici* induced by *T. aureoviride* IISR 126 (X 100)
Plate 4.1 g	Shortening of hyphal tips of *P. capsici* induced by *T. aureoviride* IISR 165 (X 100)
Plate 4.1 h-k	Penetration of hyphae of *P. capsici* by *T. aureoviride* IISR 140 (**h** X 100 Phase Contract, **i** X 100 Phase Contrast, **j** X 200 Phase Contrast, **k** X 200)
Plate 4.1 l	Parasitism of *P. capsici* hyphae by *T. aureoviride* IISR 143 (X 100 Phase Contrast)
Plate 4.1 m	*P. capsici* hyphae, healthy (X 100 Phase Contrast)
Plate 4.1 n	Evacuation of *P. capsici* hyphae induced by *T. aureoviride* IISR 143 (X 40)
Plate 4.1 o	Coiling around the *P. capsici* hyphae by *T. aureoviride* IISR 143 (X 100)
Plate 4.1 p	*T. aureoviride* IISR 143 parasitising the sporangia of *P. capsici* (X 100)
Plate 4.1 q-x	Coiling around the *P. capsici* hyphae by *T. aureoviride* IISR 143 (**q** X 100, **r** X 100, **s** X 200, **t** X 100, **u** X 100, **v** X 100, **x** X 100)

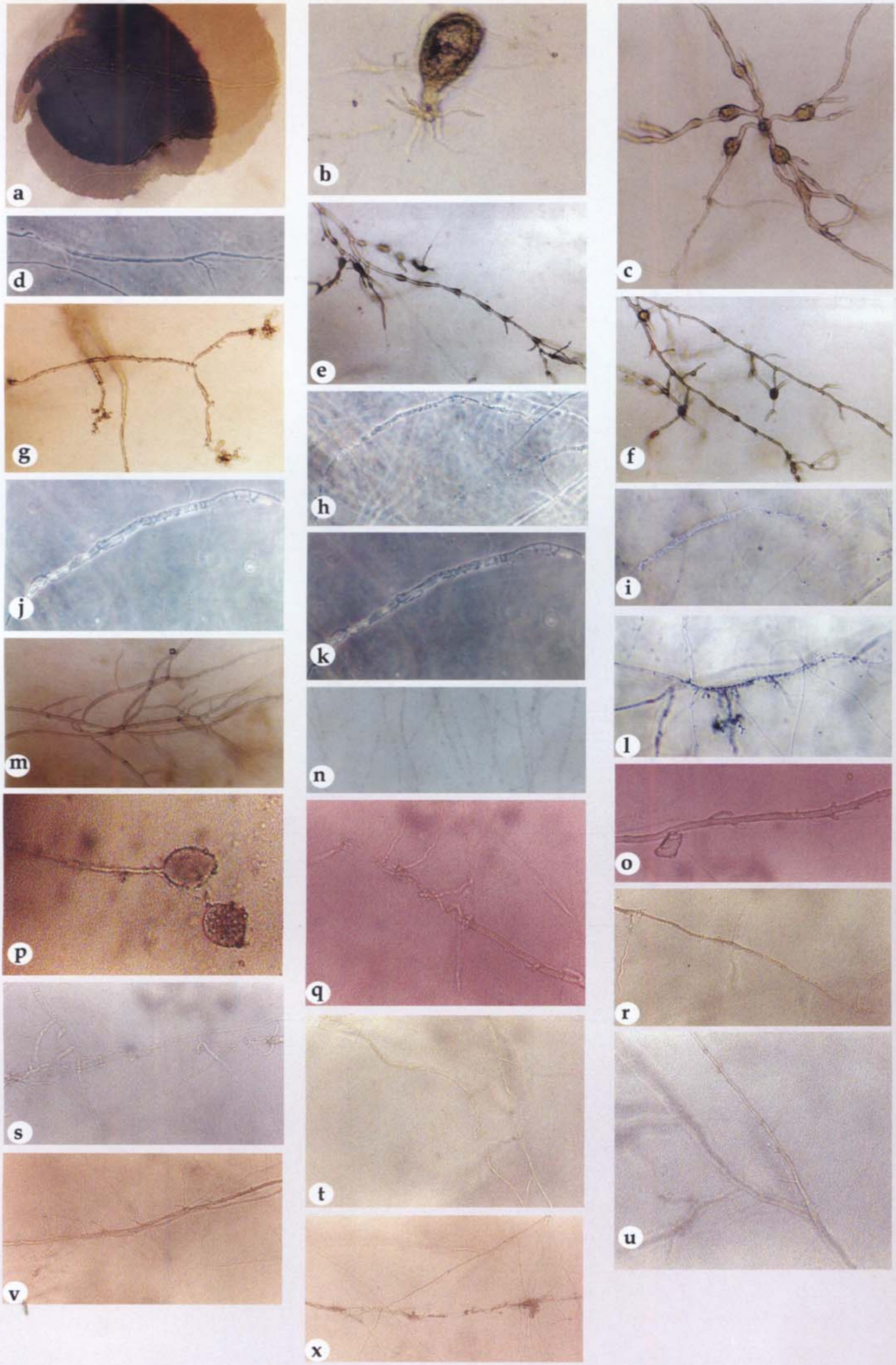
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Plate 4.2 a	Growth promotion of black pepper by *T. pseudokoningii* IISR 186 and *T. hamatum* IISR 151
Plate 4.2 b	Growth promotion of black pepper by *T. aureoviride* IISR 126 and *T. hamatum* IISR 150
Plate 4.2 c	Growth promotion of black pepper by *T. harzianum* IISR 173 and *T. virens* IISR 1370
Plate 4.2 d	Growth promotion of black pepper by *T. aureoviride* IISR 140 and *T. hamatum* IISR 160
Plate 4.2 e	Growth promotion of black pepper by *Trichoderma* sp. IISR 1141 and *T. aureoviride* IISR 148
Plate 4.2 f	Growth promotion of black pepper by *T. harzianum* IISR 1369 and *Trichoderma* sp. IISR 1107
Plate 4.2 g	Growth promotion of black pepper by *T. harzianum* IISR 165 and *T. aureoviride* IISR 143 in comparison with control
Plate 4.2 h	Increase in root biomass of black pepper by *T. aureoviride* IISR 143 and *T. harzianum* IISR 165 in comparison with control
Plate 4.2 i	Increase in root biomass of black pepper by *T. hamatum* IISR 151 and *T. pseudokoningii* IISR 186
Plate 4.2 j	Increase in root biomass of black pepper by *T. virens* IISR 1370 and *T. harzianum* IISR 173
Plate 4.2 k	Increase in root biomass of black pepper by *T. hamatum* IISR 160 and *T. aureoviride* IISR 140
Plate 4.2 l	Increase in root biomass of black pepper by *T. aureoviride* IISR 126 and *T. hamatum* IISR 150
Plate 4.2 m	Increase in root biomass of black pepper by *T. harzianum* IISR 1369 and *Trichoderma* sp. IISR 1107
Plate 4. 2 n	Increase in root biomass of black pepper by *Trichoderma* sp. IISR 1141 and *T. aureoviride* IISR 148


a


b


c


d


e


f


g


h


i


j


k


l


m


n

In general, the propagules of *T. virens* IISR 1370 *T. aureoviride* IISR 126, *T. aureoviride* IISR 140, *T. aureoviride* IISR 148, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 colonised the roots significantly in higher numbers (log cfu / g = >5). Rhizosphere competence ability of *T. harzianum* IISR 1369 is varied when applied during planting (log cfu / g = 4.6) and after one month of planting (log cfu / g = 3.4). *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165, and *T. harzianum* IISR 173 were taken for the study of abiotic factors (Figure 4.1). *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148 were not taken for abiotic studies since they are slow growing and less sporulating. In all the treatments the population of bacteria, actinomycetes and other fungi were also determined (Figures 4.2, 4.3 and 4.4). Data shown that population of native organisms ~~was~~ were not affected the colonization of the *Trichoderma* and vice versa.

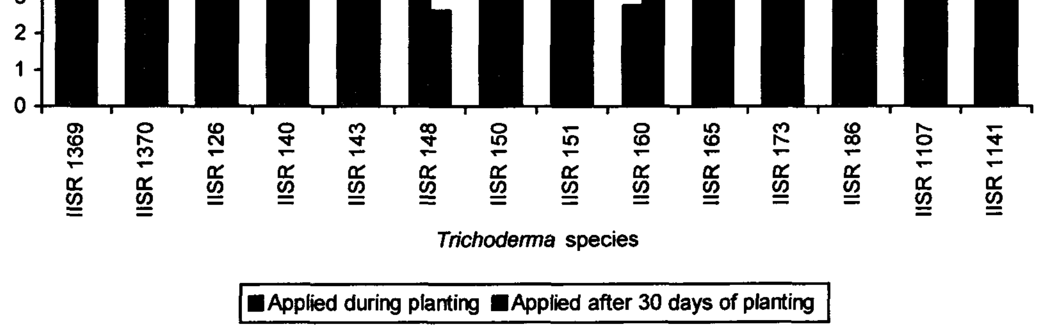


Figure 4.1 Population of *Trichoderma* isolates in the rhizosphere soil of black pepper.

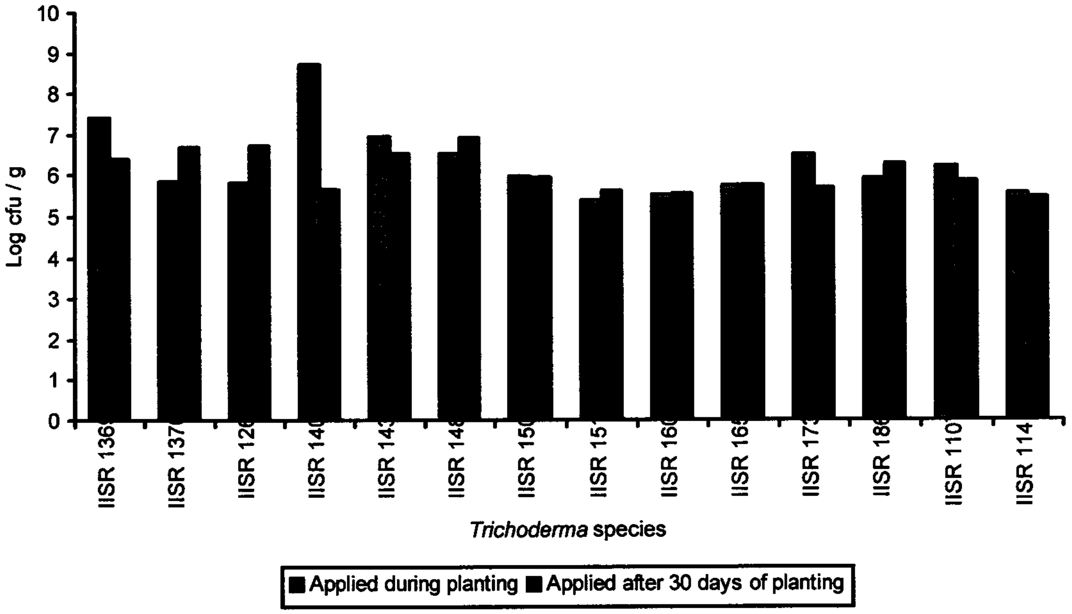


Figure 4.2 Population of bacteria in the rhizosphere soil of black pepper.

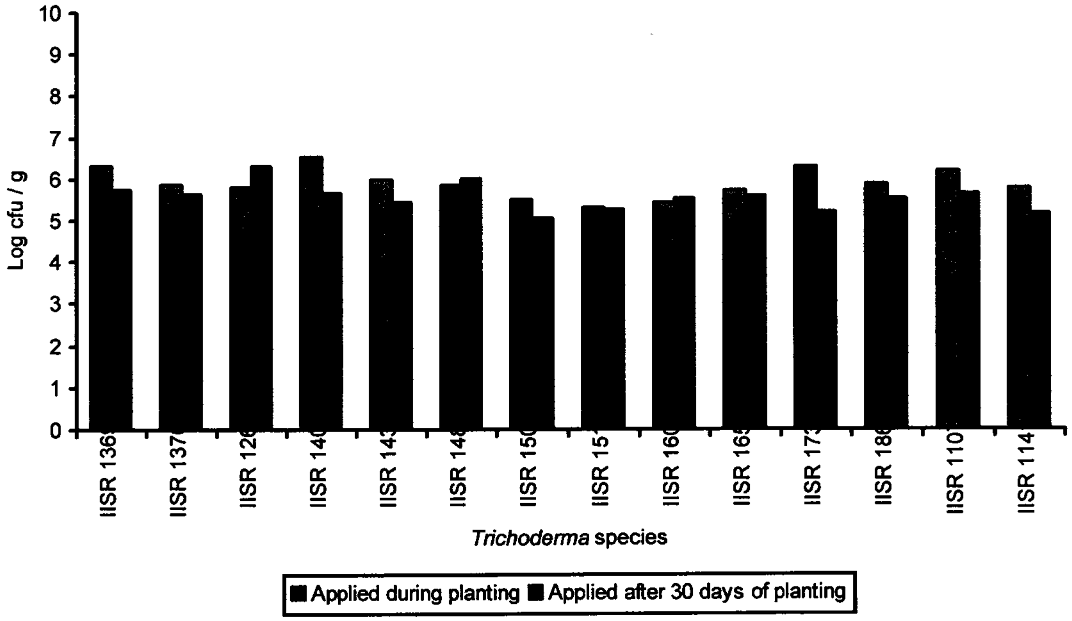


Figure 4.3 Population of actinomycetes in the rhizosphere soil of black pepper.

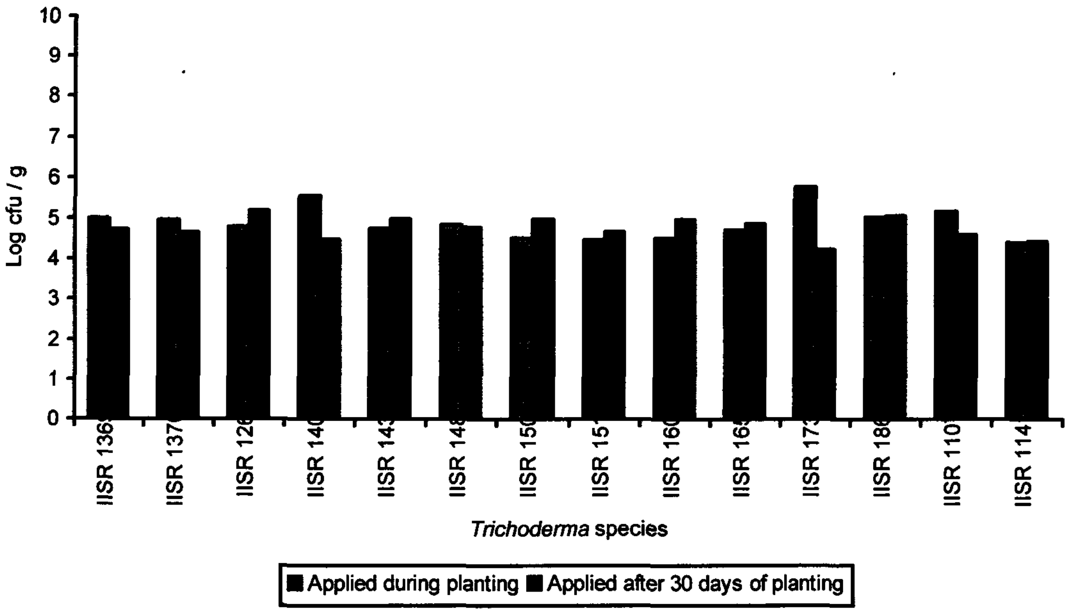


Figure 4.4 Population of other fungi in the rhizosphere soil of black pepper.

4.4 Effect of abiotic factors in relation to survival of *Trichoderma* spp

Effect of soil type, moisture and temperature on the survival of *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 was determined experimentally. These isolates were selected based on their high rhizosphere competence, fast growth and sporulation. Changes in the pH of agar culture and pesticides on the growth of *P. capsici* and above-mentioned *Trichoderma* spp were determined *in-vitro*. Simultaneous application of pesticides and *T. harzianum* IISR 1369 in soil and its effects on the population of latter was determined. Effect of organic and inorganic fertilizers in response to the survival of *T. harzianum* IISR 1369 in soil was also studied.

4.4.1 Effect of soil type on the survival of *Trichoderma* spp

Influence of soil types on the survival of *Trichoderma* spp was studied. Soil from Calicut is tentatively classified as clayey, soil from Peruvannamuzhi as sandy clay and Thamarassery as sandy loam based on texture. The soil possessed a moisture holding capacity of 71.83, 70.11 and 68.69% respectively. The population of *T. harzianum* IISR 1369 showed reduction by 30 days and thereafter the population remained more or less unchanged (Figure 4.5). A similar trend was observed with *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 also (Figure 4.6, 4.7, 4.8 and 4.9). In general, there was no significant (P=0.05) difference in the population of *Trichoderma* spp in three different soils at all time interval. Therefore species of *Trichoderma* possess great survival capability in different soils and adapted for use in areas irrespective of texture of soil prevailing.

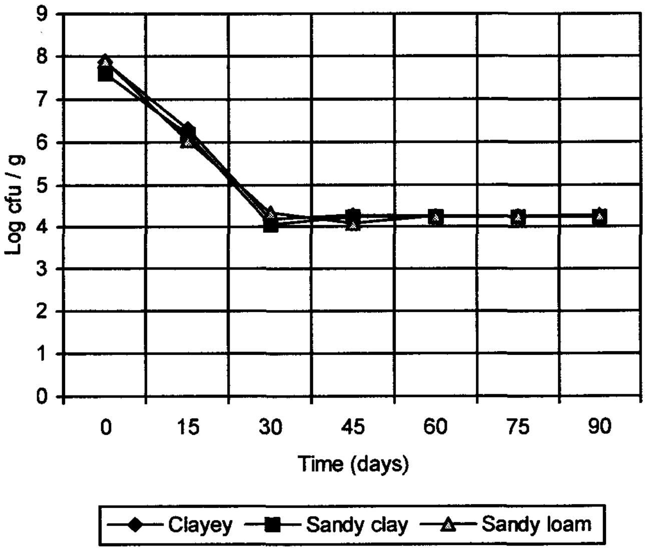


Figure 4.5 Propagule densities of *T. harzianum* IISR 1369 over time in three different soils

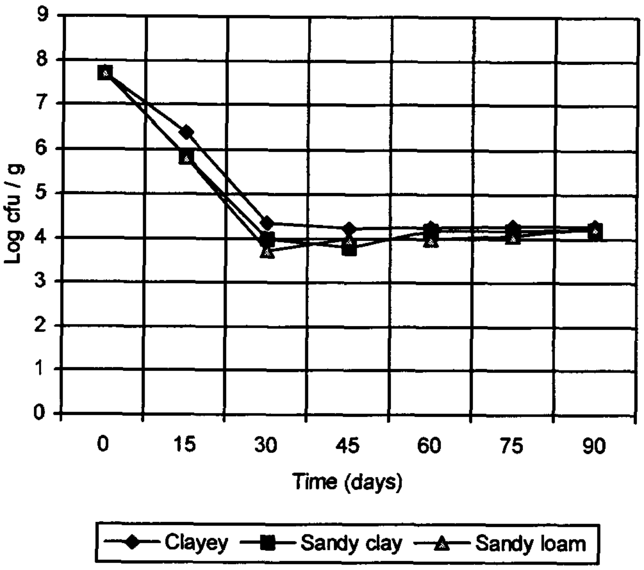


Figure 4.6 Propagule densities of *T. virens* IISR 1370 over time in three different soils

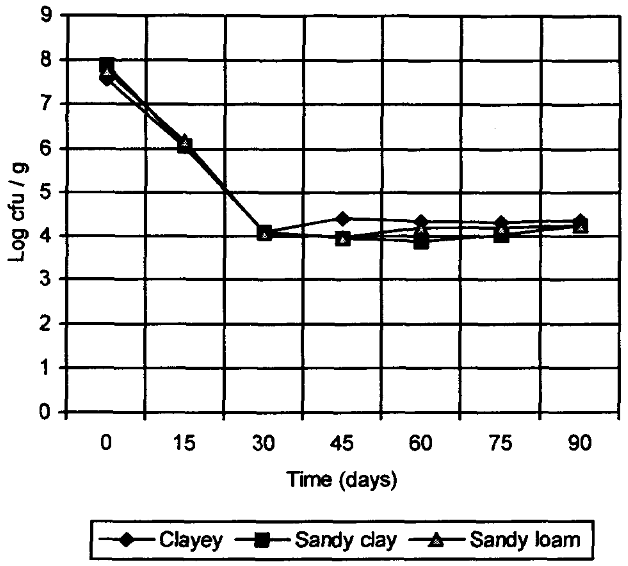


Figure 4.7 Propagule densities of *T. aureoviride* IISR 126 over time in three different soils

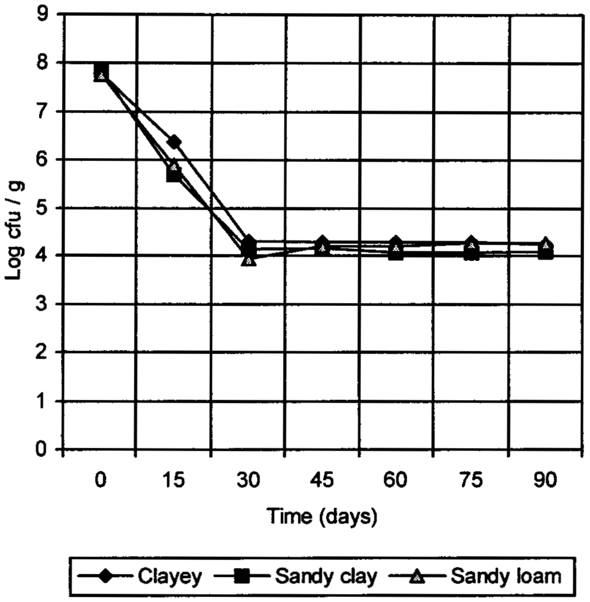


Figure 4.8 Propagule densities of *T. harzianum* IISR 165 over time in three different soils

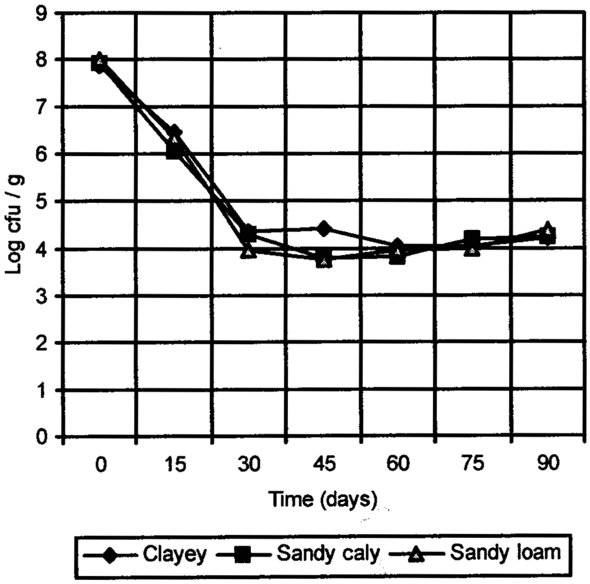


Figure 4.9 Propagule densities of *T. harzianum* IISR 173 over time in three different soils

4.4.2 Soil moisture effects on the survival of *Trichoderma* spp

Effect of soil moisture ranging from 20-80% of the moisture holding capacity on the survival of *Trichoderma* spp was studied. Population of *T. harzianum* IISR 1369 showed a decline within 15 days at all moisture levels. After 30 days the population showed fluctuation with respects to different moisture levels. Population at 80, 70 and 20% moisture levels declined gradually over a period of 90 days to the level of 10^2 cfu / g of soil (Figure 4.10). There was significant (P=0.05) reduction in the number of cfu of *T. virens* IISR 1370 at 70 and 80% moisture levels. Other moisture levels did not affect the population significantly even though it declined from the initial level (Figure 4.11). Population of *T. aureoviride* IISR 126 also declined sharply at moisture levels of 70 and 80% (Figure 4.12). *T. harzianum* IISR 165 also showed a similar trend as that of *T. virens* IISR 1370 and *T. aureoviride* IISR 126 (Figure 4.13). For *T. harzianum* IISR 173 decline in population was noticed at 20, 70 and 80% moisture levels as in the case of *T. harzianum* IISR 1369 (Figure 4.14).

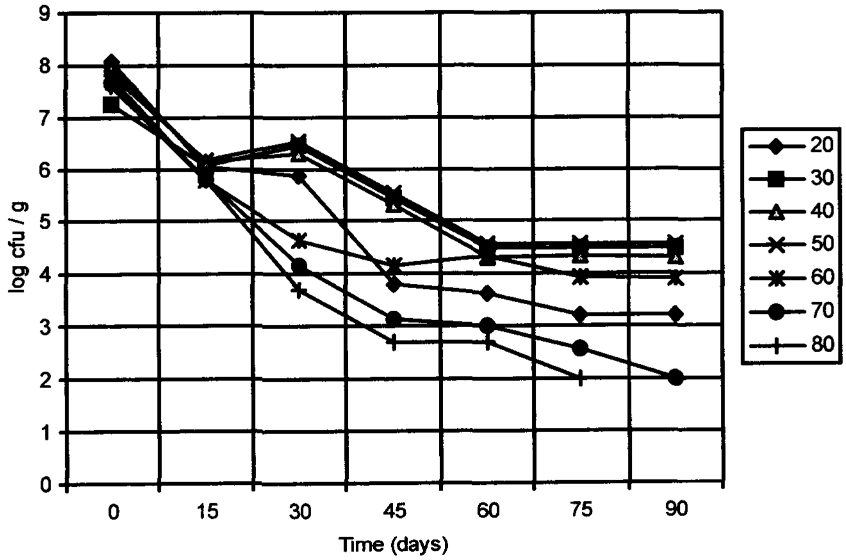


Figure 4.10 Changes in propagule densities of *T. harzianum* IISR 1369 over time at different soil moisture levels ranging from 20-80%

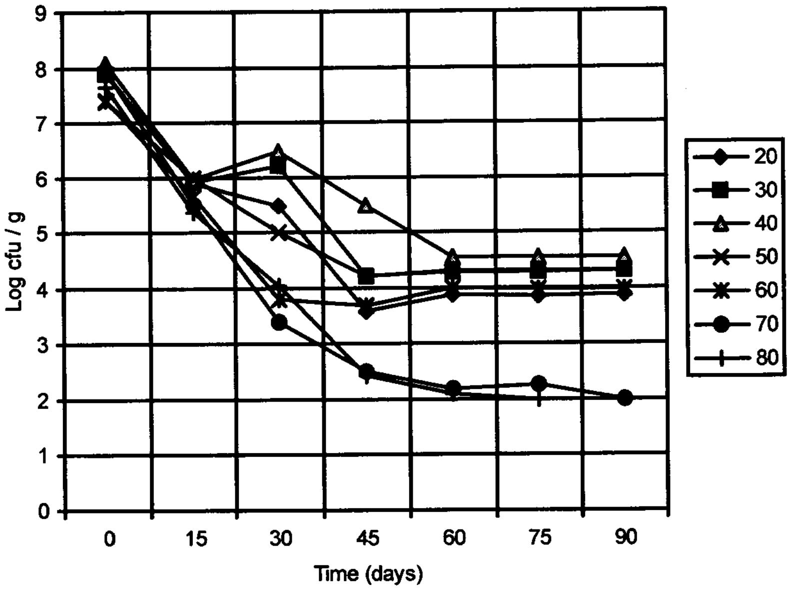


Figure 4.11 Changes in propagule densities of *T. virens* IISR 1370 over time at different soil moisture levels ranging from 20-80%

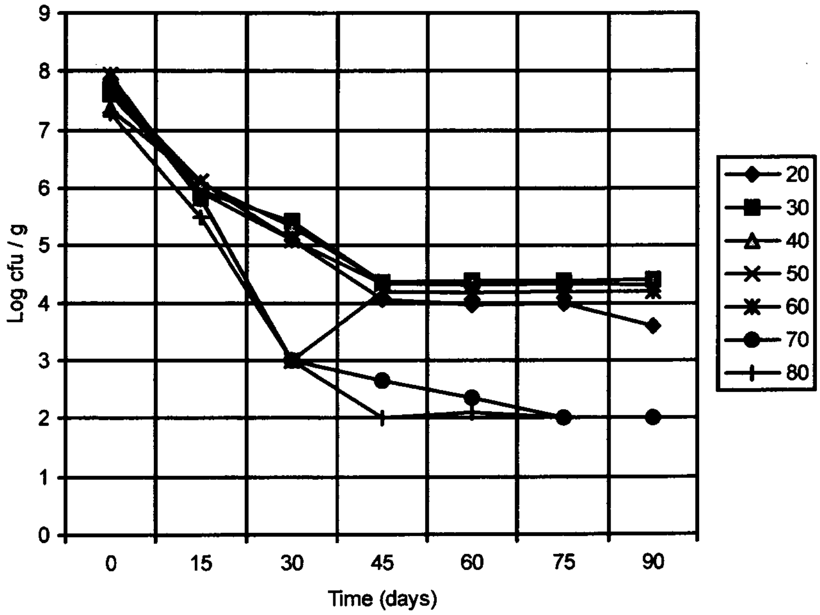


Figure 4.12 Changes in propagule densities of *T. aureoviride* IISR 126 over time at different soil moisture levels ranging from 20-80%

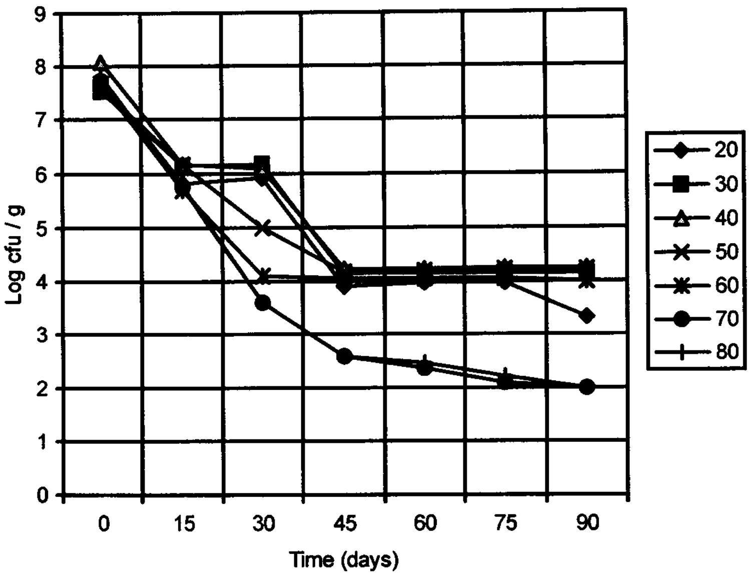


Figure 4.13 Changes in propagule densities of *T. harzianum* IISR 165 over time at different soil moisture levels ranging from 20-80%

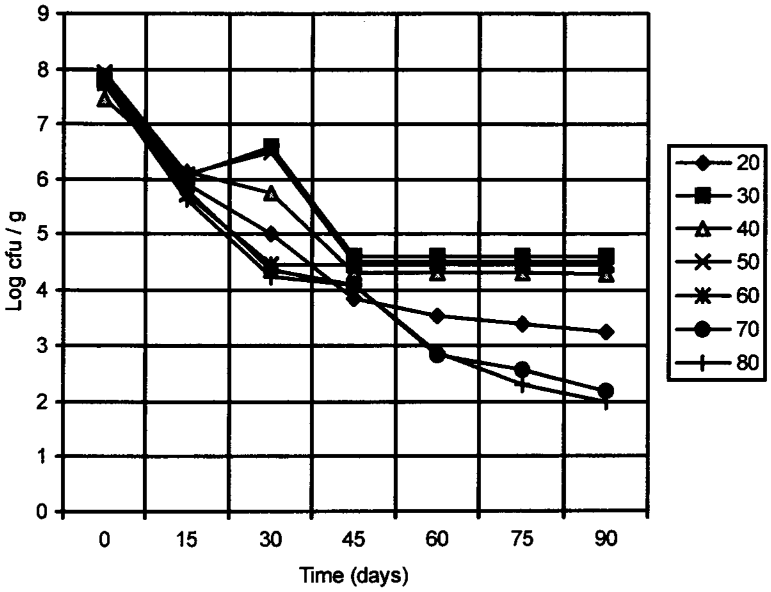


Figure 4.14 Changes in propagule densities of *T. harzianum* IISR 173 over time at different soil moisture levels ranging from 20-80%

4.4.3 Soil temperature effects on the relative abundance of *Trichoderma* spp

Effect of soil temperature on the abundance of *Trichoderma* spp was studied at three temperature ranges (<20^0C, 20-26^0C and 26-32^0C) over a period of 90 days. Population of *T. harzianum* IISR 1369 showed decline at 15 days followed by fluctuation at 30 days at all temperature ranges and thereafter population levels were steady (Figure 4.15). *T. virens* IISR 1370 showed similar reduction at 15 days and an increase at 30 days. Afterwards population at temperature ranges <20^0C and 20-26^0C was not affected. However, the population at 26-32^0C was significantly below than the other two temperature ranges (Figure 4.16). Population of *T. aureoviride* IISR 126 showed reduction at 15 days and thereafter did not vary significantly at all the three temperature ranges (Figure 4.17). Population of *T. harzianum* IISR 165 recorded decrease at 15 days and increase at 30 days and thereafter the population levels at all the temperature ranges were steady (Figure 4.18). Population of *T. harzianum* IISR 173 showed decline at 15 days followed by an increase at 30 days. It again declined at 45 days and thereafter continued without much change at other time intervals. However the population in the lower temperature range was significantly below than the other two ranges (Figure 4.19).

In general, propagule density of each fungus in soil declined during the first 15 days then it has changed more drastically at 30 days. Then the change was less and it has maintained the population up to 90 days. Temperature had a significant ($P=0.05$) effect on the propagule density at three temperature ranges of *T. virens* IISR 1370 and *T. harzianum* IISR 173. At the same time *T. harzianum* IISR 1369, *T. aureoviride* IISR 126 and *T. harzianum* IISR 165 were able to tolerate a wide range of temperature levels. It shows their high plasticity and adaptability at varying temperature ranges. However, *T. virens* IISR 1370 recorded significant reduction in population at high temperature range. In the case of *T. harzianum* IISR 173, population at low temperature range was significantly less. Here, different isolates of the same species (viz. *T. harzianum* IISR 1369, *T. harzianum* IISR 165 and *T. harzianum* IISR 173) behaved in different manner with respect to temperature range indicating variability existing in isolates for varying climatic conditions.

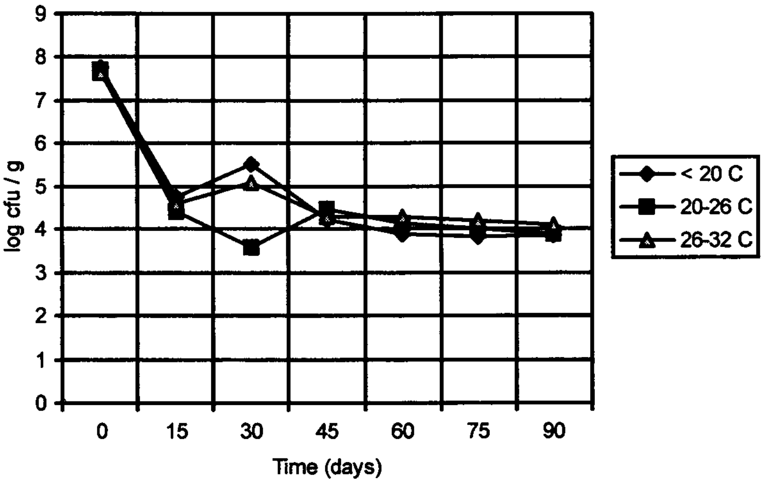


Figure 4.15 Proplagule densities of *T. harzianum* IISR 1369 in soil over time at three temperature ranges

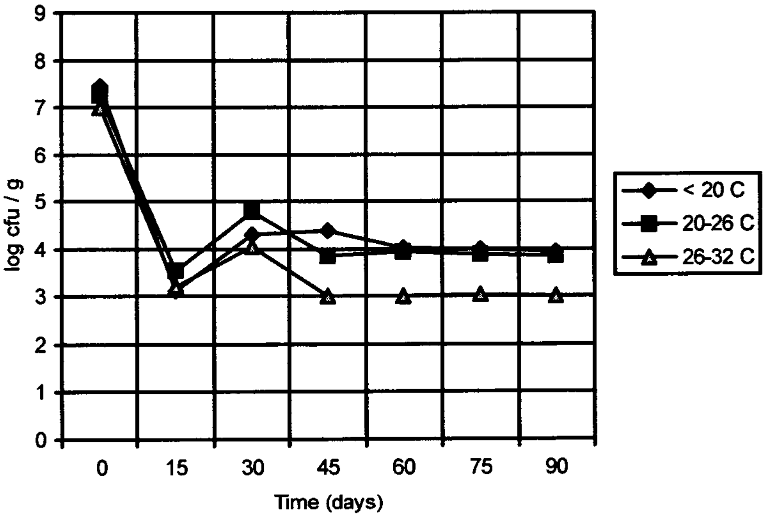


Figure 4.16 Propagule densities of *T. virens* IISR 1370 in soil over time at three temperature ranges

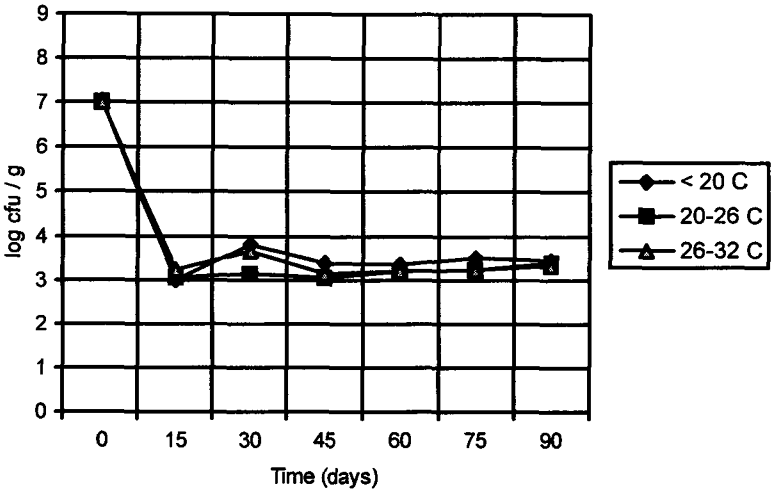


Figure 4.17 Propagule densities of *T. aureoviride* IISR 126 in soil over time at three temperature ranges

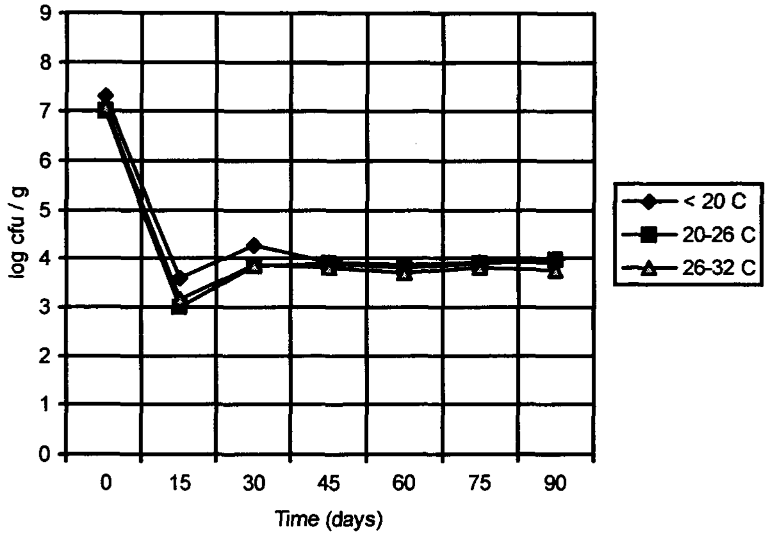


Figure 4.18 Propagule densities of *T. harzianum* IISR 165 in soil over time at three temperature ranges

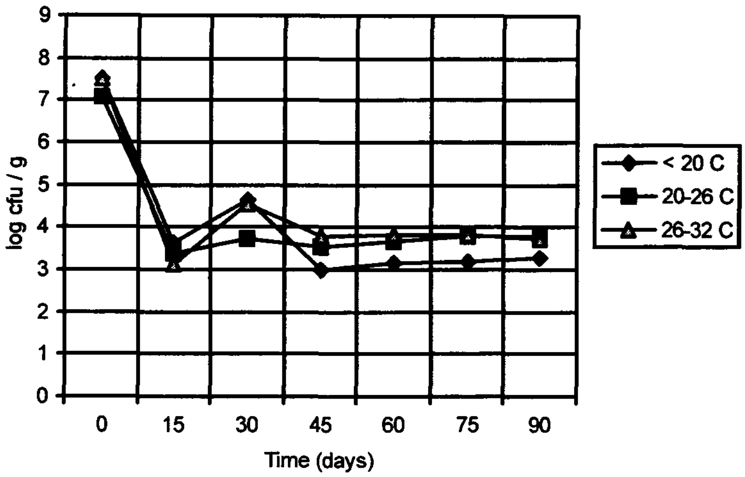


Figure 4.19 Propagule densities of *T. harzianum* IISR 173 in soil over time at three temperature ranges

4.4.4 Effect of pH on *Trichoderma* spp and *Phytophthora capsici* in culture

Growth of *P. capsici*, *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 was studied at pH levels from 4.5 to 8.5 with increments of one unit. *P. capsici* was able to grow well in acidic pH. Its colony diameter was reduced at pH 7.5 and above (Figure 4.20). *T. harzianum* IISR 1369 showed a linear growth over the wide range of pH studied. Acidic or basic nature of the medium did not affect the linear growth rate of this fungus (Figure 4.21). *T. virens* IISR 1370 was able to grow well at pH 4.5. Between pH 5.5-8.5 the growth rate was found gradually decreasing (Figure 4.22). *T. aureoviride* IISR 126 required a pH 6.5 for well growth. It showed small colony growth both at highly acidic and basic pH (Figure 4.23). Acidic nature of the medium did not affect the growth of *T. harzianum* IISR 165 (Figure 4.24). The growth of *T. harzianum* IISR 173 was not affected by pH range 4.5-7.5. At pH 8.5 the colony diameter showed a sharp decline (Figure 4.25).

Thus for *T. harzianum* IISR 1369 and *T. harzianum* IISR 165, pH of the growth medium was not a limiting factor. But for other isolates of *Trichoderma* spp studied, pH was a deciding factor of its growth. In general, other *Trichoderma* spp and *P. capsici* favoured acidic pH for growth.

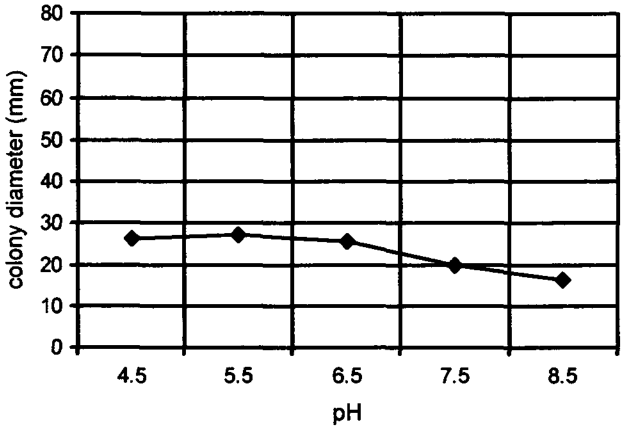


Figure 4.20 Effect of pH on the linear growth of *Phytophthora capsici* on potato dextrose agar after 48 hours

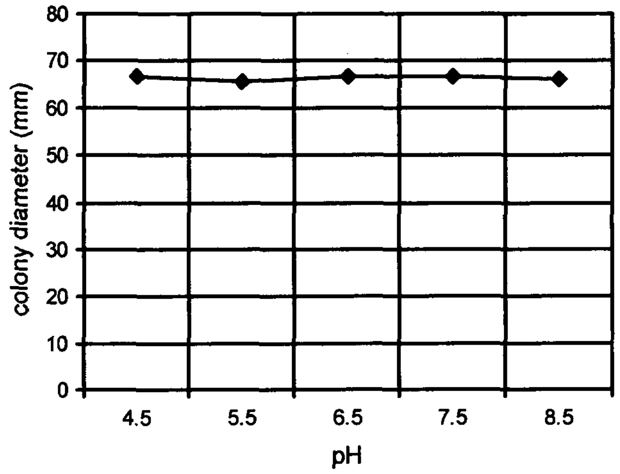


Figure 4.21 Effect of pH on the linear growth of *T. harzianum* IISR 1369 on potato dextrose agar after 48 hours

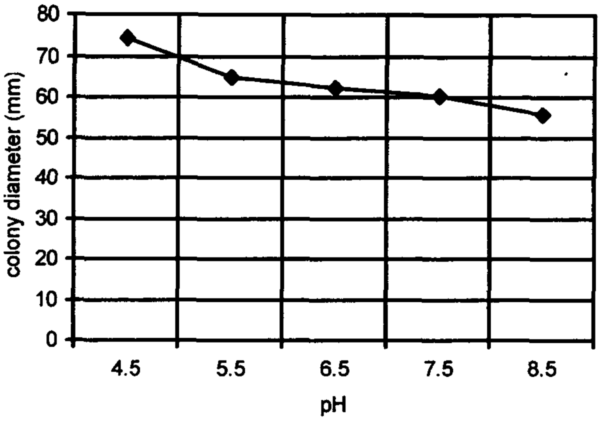


Figure 4.22 Effect of pH on the linear growth of *T. virens* IISR 1370 on potato dextrose agar after 48 hours

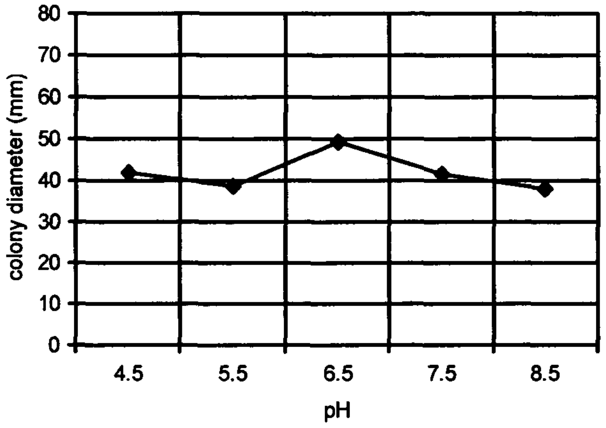


Figure 4.23 Effect of pH on the linear growth of *T. aureoviride* IISR 126 on potato dextrose agar after 48 hours

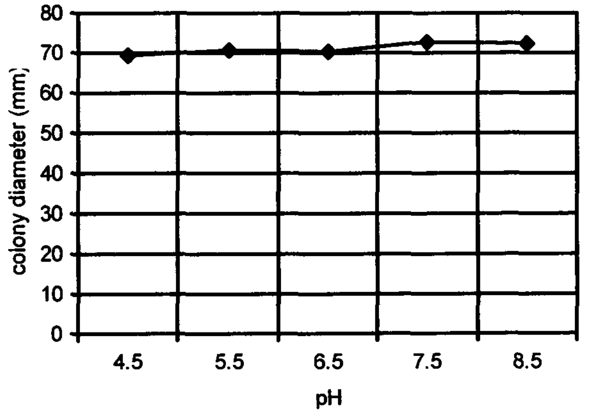


Figure 4.24 Effect of pH on the linear growth of *T. harzianum* IISR 165 on potato dextrose agar after 48 hours

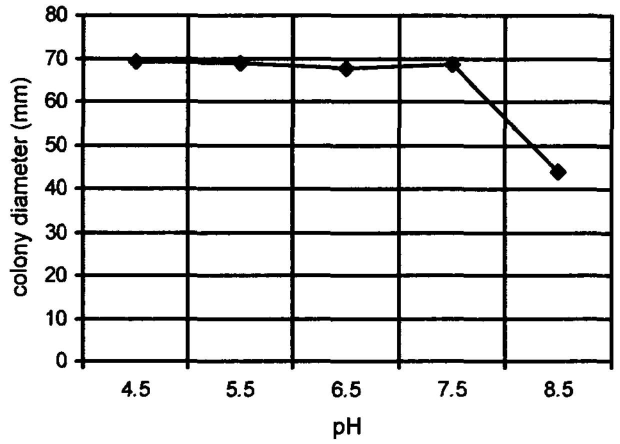


Figure 4.25 Effect of pH on the linear growth of *T. harzianum* IISR 173 on potato dextrose agar after 48 hours

4.4.5 *In-vitro* toxicity of pesticides to *Phytophthora capsici* and *Trichoderma* spp

P. capsici, T. harzianum IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 were tested *in-vitro* for their sensitivity to pesticides (Plate 4.3 a).

P. capsici was highly sensitive to metalaxyl, mancozeb and copper (10 ppm) (Plate 4.3 d - e). It was sensitive to potassium phosphonate at 100 ppm. Phorate increased the growth of *P. capsici* at 10 and 100 ppm concentrations and the LD_{50} value was very high. *P. capsici* was sensitive to chlorpyriphos at 300 ppm and to quinalphos at 500 ppm (Table 4.3). Since phorate, chlorpyriphos and quinalphos are used for the control of nematode, mealy bug and thrips infecting black pepper its response to the fungus *P. capsici* was investigated.

Lower concentrations of metalaxyl and copper were also highly sensitive to *P. capsici* (Table 4.4). At 1 ppm concentration metalaxyl inhibited *P. capsici* by 81.1% reduction in colony growth and at 5 ppm by 87%. At 10 ppm concentration of copper, *P. capsici* was inhibited by 40.87% and the LD_{50} value is 12.23. Thus *P. capsici* is highly sensitive to metalaxyl than copper (Plate 4.3 b - c).

Table 4.3 Sensitivity of *P. capsici* to pesticides *in-vitro*

Pesticide	% reduction in growth at (ppm) concentration				LD_{50} value
	10	100	300	500	
Metalaxyl	100	100	100	100	------
Mancozeb	45.2	77.39	100	100	11.06
Potassium Phosphonate	18.26	65.22	73.91	63.48	76.66
Copper	40.87	77.39	100	100	12.23
Phorate	-16.5	-13.0	7.82	12.17	2054.2
Chlorpyriphos	26.09	39.13	55.65	75.65	269.54
Quinalphos	11.3	13.91	32.17	46.96	532.37
CD at 5%				2.59	

Table 4.4 Sensitivity of *P. capsici* to lower concentrations of metalaxyl and copper *in-vitro*

Fungicide	% reduction in growth at (ppm) concentration			LD_{50} value
	1	5	10	
Metalaxyl	81.1	87.0	100	0.62
Copper	4.3	35.0	40.0	12.23
CD at 5%			2.084	

T. harzianum IISR 1369 was sufficiently tolerant to metalaxyl, mancozeb, potassium phosphonate, copper and phorate (Table 4.5). But it was highly sensitive to carbendazim. It was moderately sensitive to quinalphos (LD_{50} value 86.54) (Plate 4.3 f, g & h).

Table 4.5 Sensitivity of *T. harzianum* IISR 1369 to pesticides *in-vitro*

Pesticide	% reduction in growth at (ppm) concentration				LD_{50} value
	10	100	300	500	
Metalaxyl	2.50	14.17	21.67	36.39	687.00
Mancozeb	0	21.11	37.22	43.33	576.97
Potassium Phosphonate	0	0	0	7.77	3217.5
Copper	0	0	37.7	42.5	588.24
Phorate	0	13.06	38.33	36.11	692.33
Carbendazim	100	100	100	100	-------
Quinalphos	36.94	57.78	79.17	83.33	86.54
CD at 5%				1.02	

T. virens IISR 1370 was sufficiently tolerant to metalaxyl, mancozeb, potassium phosphonate and phorate. It was moderately tolerant to copper and less tolerant to chlorpyriphos. It was highly sensitive to carbendazim and quinalphos (Table 4.6) (Plate 4.3 i, j & k)

T. aureoviride IISR 126 was sufficiently tolerant to metalaxyl, mancozeb, potassium phosphonate and phorate. It was less tolerant to copper, chlorpyriphos and quinalphos. It was highly sensitive to carbendazim (Table 4.7) (Figure 4.35 l, m & n).

T. harzianum IISR 165 was sufficiently tolerant to metalaxyl, mancozeb, potassium phosphonate, copper and phorate. It was less tolerant to chlorpyriphos and quinalphos. It was highly sensitive to carbendazim (Table 4.8) (Plate 4.4 a, b, c & d).

T. harzianum IISR 173 was sufficiently tolerant to metalaxyl, mancozeb, potassium phosphonate, copper and phorate. It was less tolerant to chlorpyriphos. It was highly sensitive to carbendazim and quinalphos (Table 4.9) (Plate 4.4 e, f & g).

Table 4.6 Sensitivity of *T. virens* IISR 1370 to pesticides *in-vitro*

Pesticide	% reduction in growth at (ppm) concentration				LD_{50} value
	10	100	300	500	
Metalaxyl	14.4	32.5	34.44	37.78	661.73
Mancozeb	0	35.0	46.67	47.22	529.44
Potassium Phosphonate	0	2.77	15.00	13.33	1879.7
Copper	0	21.39	60.0	62.5	250.0
Phorate	0	0	15.0	30.28	825.63
Carbendazim	100	100	100	100	-----
Chlorpyriphos	32.22	50.83	74.44	85.28	100
Quinalphos	52.22	76.67	81.67	87.22	9.57
CD at 5%				0.581	

Table 4.7 Sensitivity of *T. aureoviride* IISR 126 to pesticides *in-vitro*

Pesticide	% reduction in growth at (ppm) concentration				LD_{50} value
	10	100	300	500	
Metalaxyl	9.44	29.17	37.78	42.50	588.24
Mancozeb	0	25.28	44.72	45.0	555.56
Potassium Phosphonate	0	0	10.56	17.5	1428.5
Copper	0	11.67	57.78	62.78	86.54
Phorate	0	6.38	41.39	45.28	552.12
Carbendazim	100	100	100	100	------
Chlorpyriphos	21.94	51.94	85.56	90.28	96.26
Quinalphos	26.94	60.83	80.56	85.83	82.20
CD at 5%				1.85	

Table 4.8 Sensitivity of *T. harzianum* IISR 165 to pesticides *in-vitro*

Pesticide	% reduction in growth at (ppm) concentration				LD_{50} value
	10	100	300	500	
Metalaxyl	14.53	6.55	25.92	26.49	943.75
Mancozeb	-2.56	8.26	19.94	21.08	1185.9
Potassium Phosphonate	-2.56	-2.56	-2.56	0.85	29411
Copper	-2.56	0.28	22.50	27.06	923.87
Phorate	-2.56	-2.56	25.07	35.04	713.27
Carbendazim	96.58	96.58	100	100	------
Chlorpyriphos	29.34	35.89	58.68	84.33	85.21
Quinalphos	74.35	84.33	82.33	88.60	6.72
CD at 5%				5.48	

Table 4.9 Sensitivity of *T. harzianum* IISR 173 to pesticides *in-vitro*

Pesticide	% reduction in growth at (ppm) concentration				LD_{50} value
	10	100	300	500	
Metalaxyl	0	11.67	21.39	24.44	1022.9
Mancozeb	0	20.56	24.17	30.83	810.9
Potassium Phosphonate	0	0	0	5.83	4288.1
Copper	0	0	32.5	40.83	612.29
Phorate	0	9.44	33.06	36.67	681.76
Carbendazim	100	100	100	100	-------
Chlorpyriphos	33.61	53.61	80.28	86.67	93.27
Quinalphos	69.17	80	86.11	87.78	7.23
CD at 5%				0.57	

Plate 4.3 a *Trichoderma* spp tested *in-vitro* for their sensitivity to pesticides

Plate 4.3 b Sensitivity of *P. capsici* to lower concentrations of copper

Plate 4.3 c Sensitivity of *P. capsici* to lower concentrations of metalaxyl

Plate 4.3 d Sensitivity of *P. capsici* to copper

Plate 4.3 e Sensitivity of *P. capsici* to metalaxyl

Plate 4.3 f Sensitivity of *T. harzianum* IISR 1369 to metalaxyl and mancozeb

Plate 4.3 g Sensitivity of *T. harzianum* IISR 1369 to quinalphos

Plate 4.3 h Sensitivity of *T. harzianum* IISR 1369 to chlorpyriphos

Plate 4.3 i Sensitivity of *T. virens* IISR 1370 to quinalphos

Plate 4.3 j Sensitivity of *T. virens* IISR 1370 to metalaxyl and mancozeb

Plate 4.3 k Sensitivity of *T. virens* IISR 1370 to chlorpyriphos

Plate 4.3 l Sensitivity of *T. aureoviride* IISR 126 to metalaxyl and mancozeb

Plate 4.3 m Sensitivity of *T. aureoviride* IISR 126 to chlorpyriphos

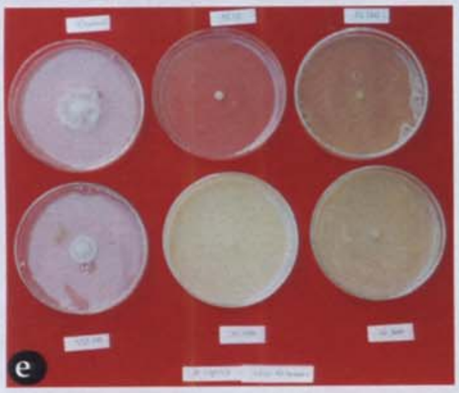
Plate 4.3 n Sensitivity of *T. aureoviride* IISR 126 to quinalphos


a
T. harzianum HSR 1369
T. virens HSR 1370
T. aureoviride HSR 126
T. harzianum HSR 165
T. harzianum HSR 173


b


c


d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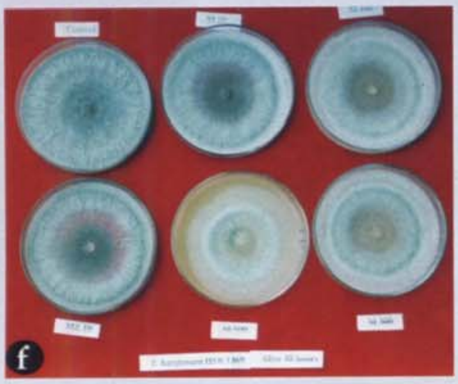
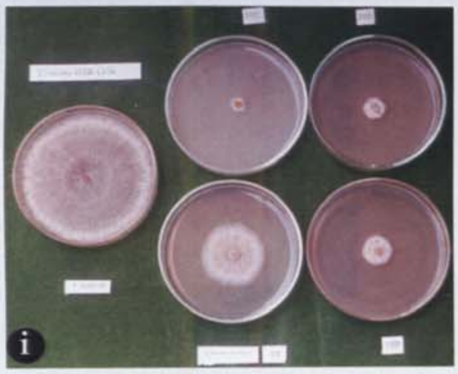

f


g


h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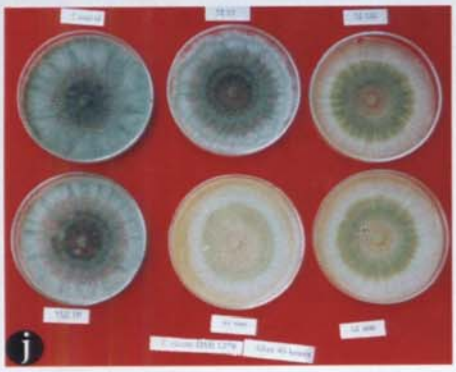
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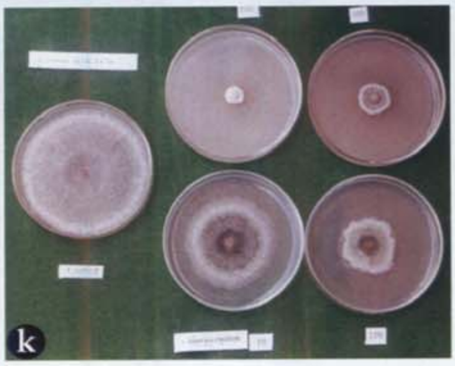

k


l


m

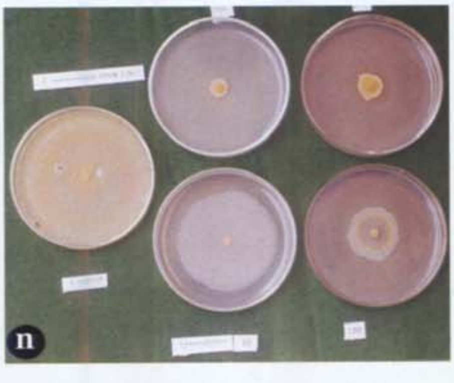

n

Plate 4.4 a Sensitivity of *T. harzianum* IISR 165 to chlorpyriphos

Plate 4.4 b Sensitivity of *T. harzianum* IISR 165 to quinalphos

Plate 4.4 c Sensitivity of *T. harzianum* IISR 165 to carbendazim

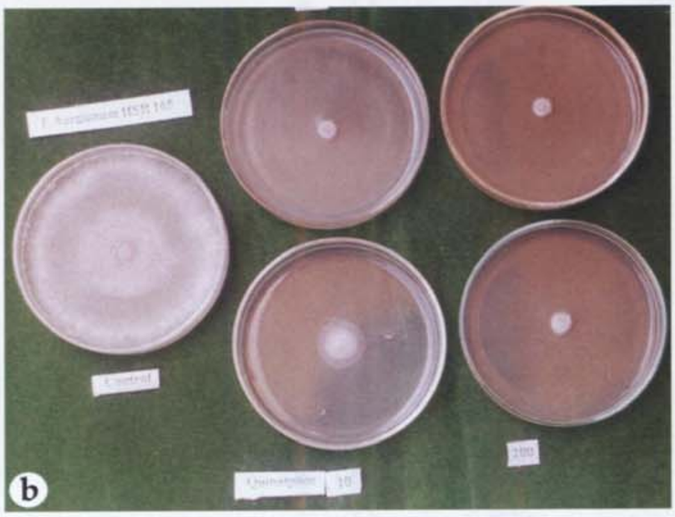
Plate 4.4 d Sensitivity of *T. harzianum* IISR 165 to metalaxyl and mancozeb

Plate 4.4 e Sensitivity of *T. harzianum* IISR 173 to quinalphos

Plate 4.4 f Sensitivity of *T. harzianum* IISR 173 to chlorpyriphos

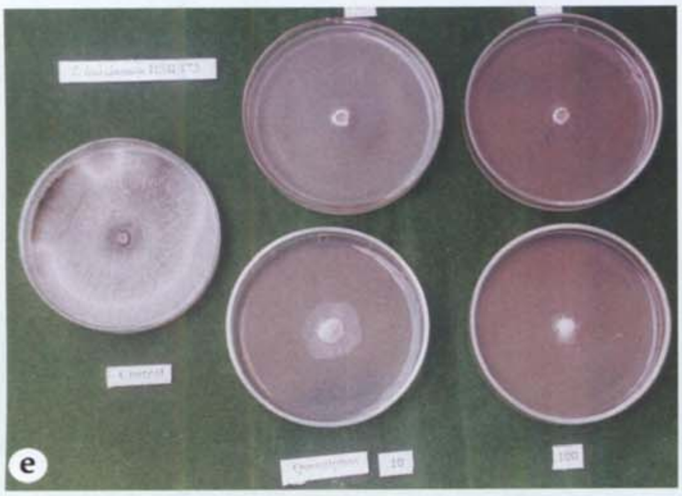
Plate 4.4 g Sensitivity of *T. harzianum* IISR 173 to metalaxyl and mancozeb

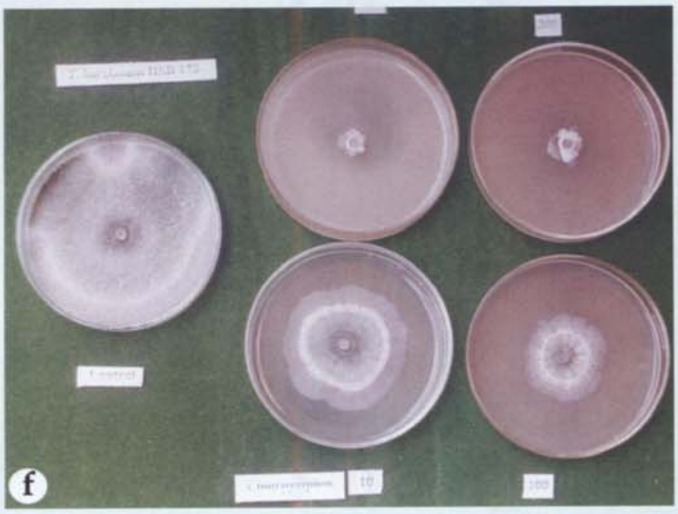

a
Control
10
100


b
Control
10
200


c
Control
10
100


d
Control
MZ 10
After 48 hours


e
Control
10
100


f
Control
10
100


g
Control
MZ 10
After 48 hours

4.4.6 Effect of simultaneous application of pesticides on the survival of *T. harzianum* IISR 1369 in soil

Different concentrations of pesticides and *T. harzianum* IISR 1369 were applied simultaneously into soil and the population was monitored for a period of 90 days. There was significant (P=0.05) reduction in population at higher concentration of pesticides. Metalaxyl caused a sharp decline in population of *T. harzianum* IISR 1369 within 15 days at 300ppm and above (Figure 4.26). Mancozeb caused a sharp decline in population at 500ppm concentration within 15 days (Figure 4.27). At 500 ppm concentration of copper oxychloride the population of *T. harzianum* caused a reduction within 15 days. Afterwards the population was not affected much (Figure 4.28). Quinalphos also caused a reduction in population within 15 days at 500ppm concentration (Figure 4.29). However, in general, simultaneous application of pesticides was not affected the population of *T. harzianum* IISR 1369 upto 300ppm concentration.

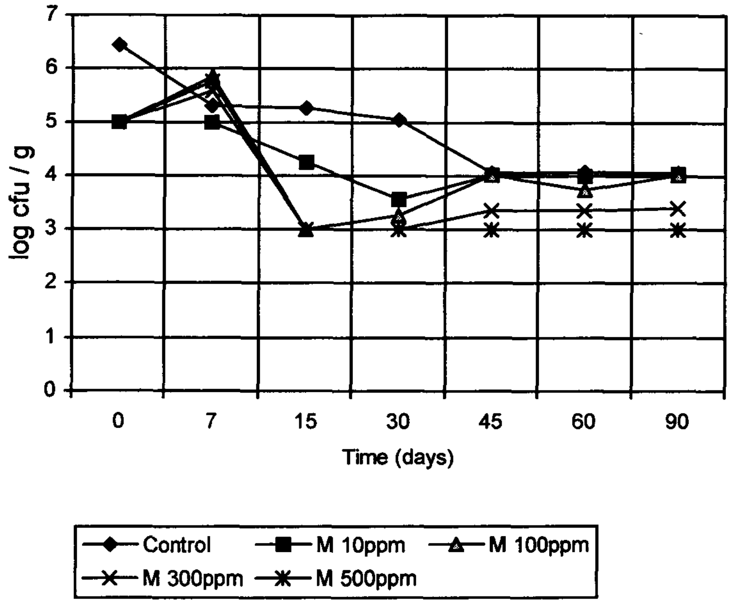


Figure 4.26 Population of *T. harzianum* IISR 1369 in metalaxyl applied soil

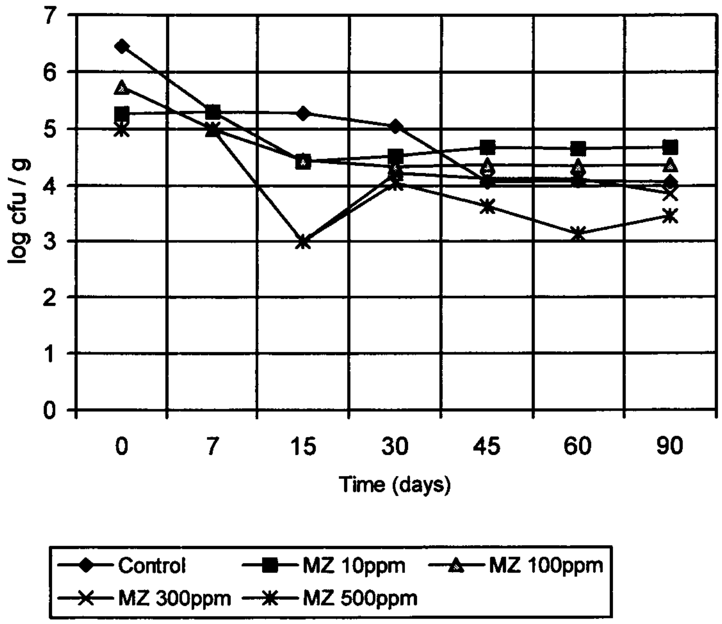


Figure 4.27 Population of *T. harzianum* IISR 1369 in mancozeb applied soil

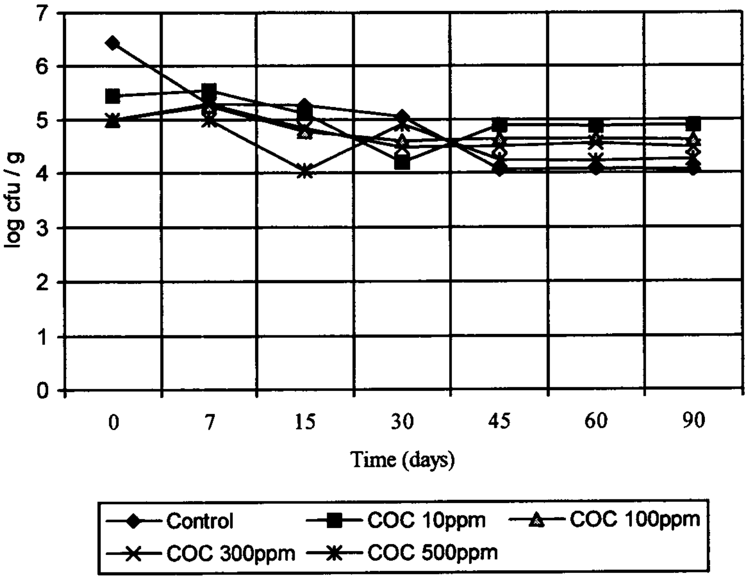


Figure 4.28 Population of *T. harzianum* IISR 1369 in copper oxychloride applied soil

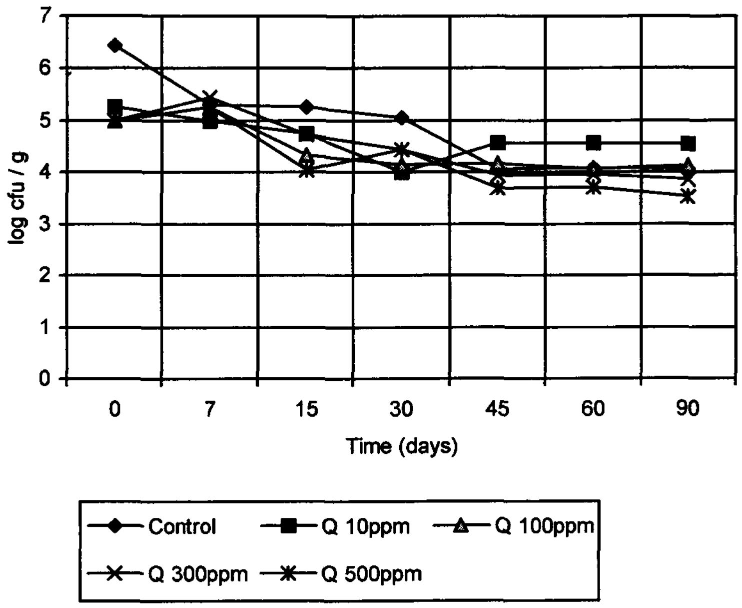


Figure 4.29 Population of *T. harzianum* IISR 1369 in quinalphos applied soil

4.4.7 Effect of organic and inorganic fertilizers on the survival of *T. harzianum* IISR 1369 applied to black pepper rhizosphere

Application of urea (N), rock phosphate (P), muriate of potash (K) and NPK together did not affect significantly (P=0.05) the survival of *T. harzianum* IISR 1369. It showed higher population than the control (Figure 4.30, 4.31, 4.32 and 4.33). Application of organics like farmyard manure, coir pith compost and neem cake also supported the survival of *T. harzianum* (Figure 4.34, 4.35 and 4.36). Survival of *T. harzianum* in dried leaves amended soil was found to be on par with control (Figure 4.37). Therefore application of inorganic and organic fertilizers was not affected the survival of *T. harzianum*. In general, these fertilizers contributed to the increase in population of *T. harzianum* IISR 1369 than the control.

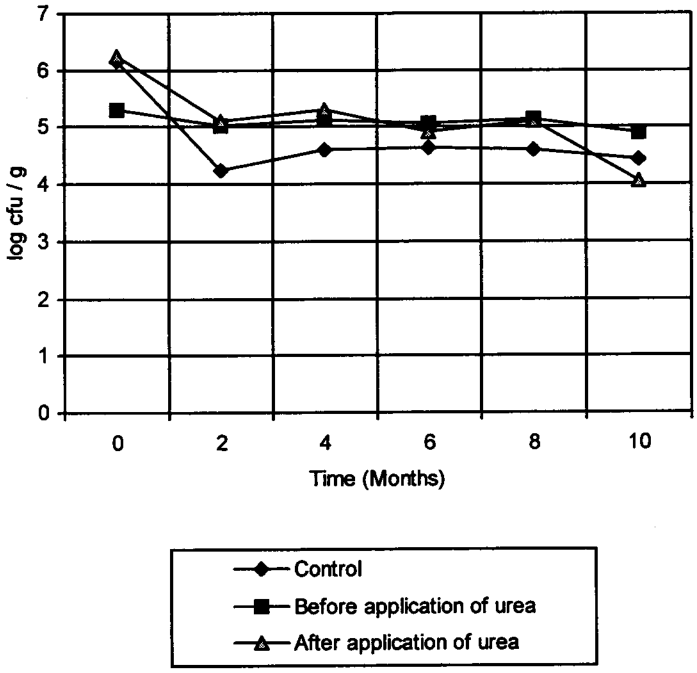


Figure 4.30 Population of *T. harzianum* IISR 1369 in urea (N) applied soil

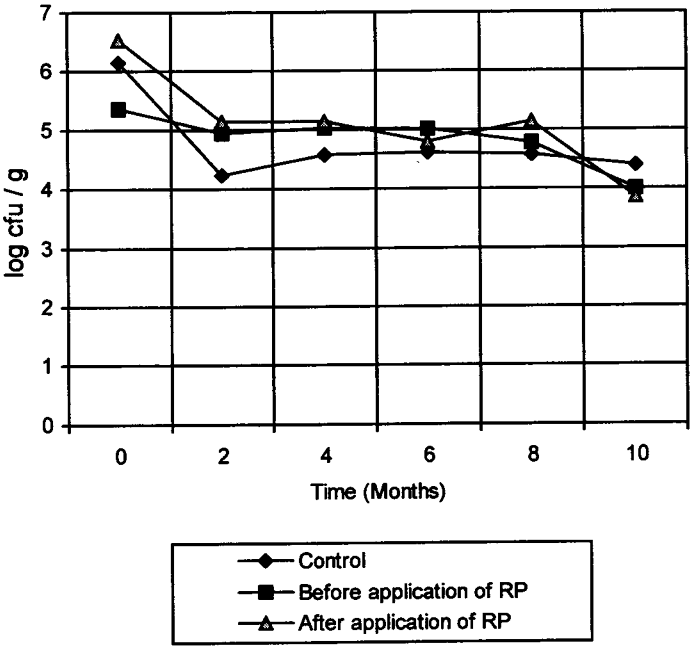


Figure 4.31 Population of *T. harzianum* IISR 1369 in Rock Phosphate (P) applied soil

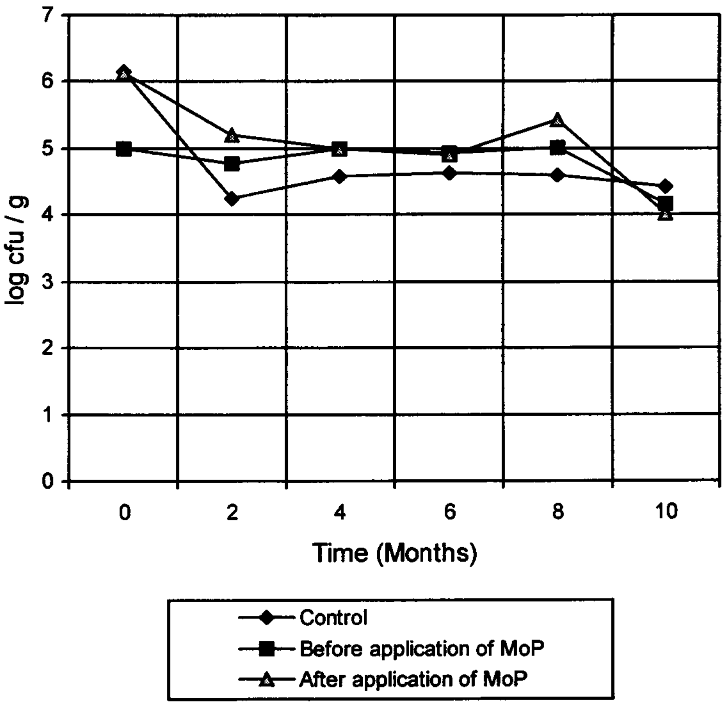


Figure 4.32 Population of *T. harzianum* IISR 1369 in Muriate of Potash (K) applied soil

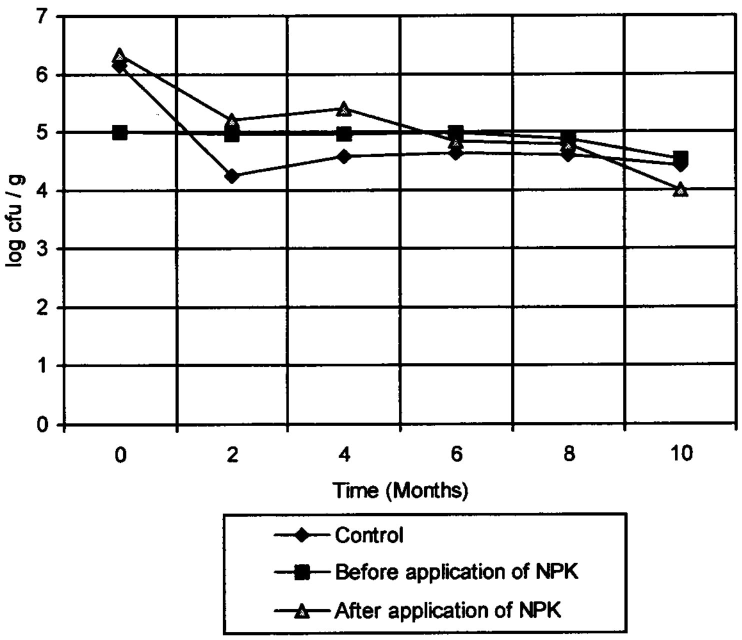


Figure 4.33 Population of *T. harzianum* IISR 1369 in NPK applied soil

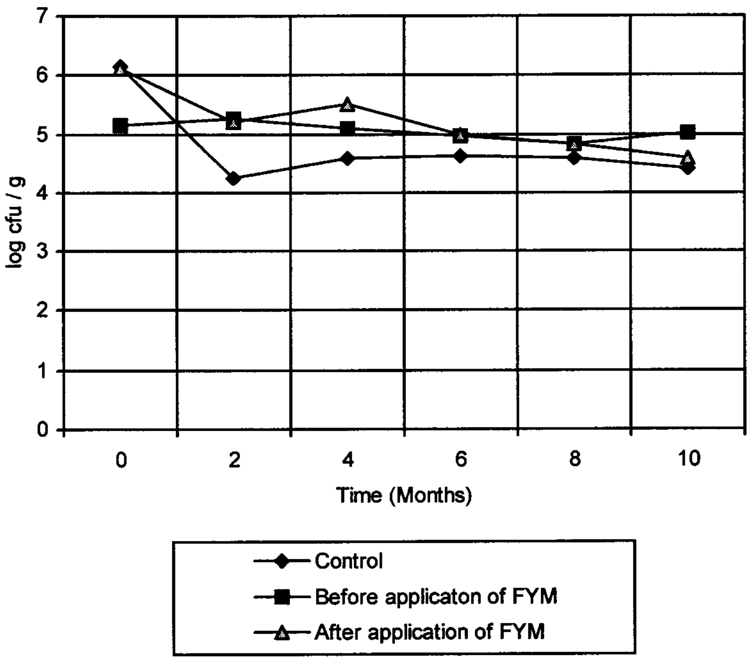


Figure 4.34 Population of *T. harzianum* IISR 1369 in farmyard manure (FYM) applied soil

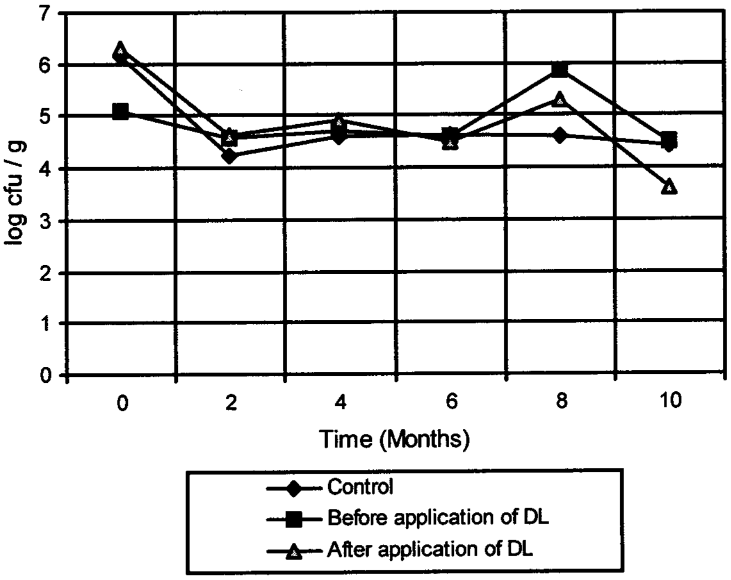


Figure 4.35 Population of *T. harzianum* IISR 1369 in dried leaves (DL) applied soil

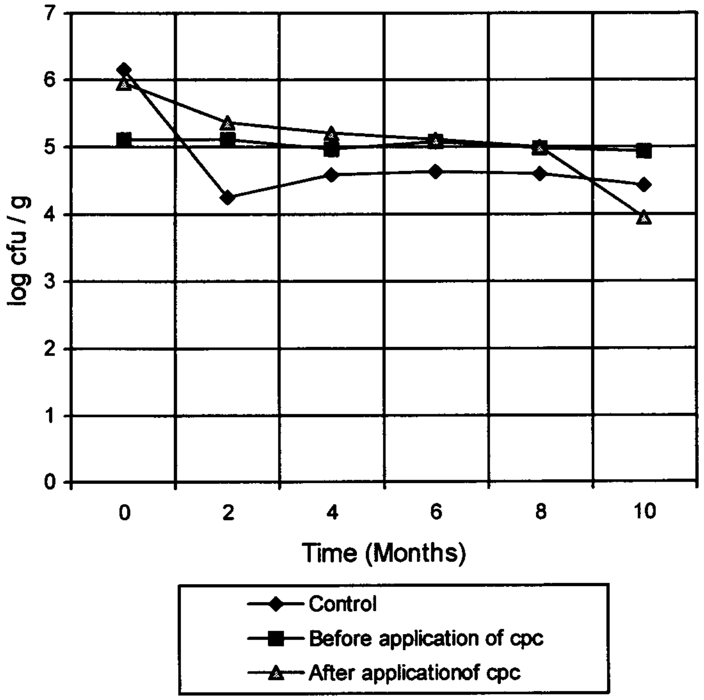


Figure 4.36 Population of *T. harzianum* IISR 1369 in Coirpith compost (CPC) applied soil

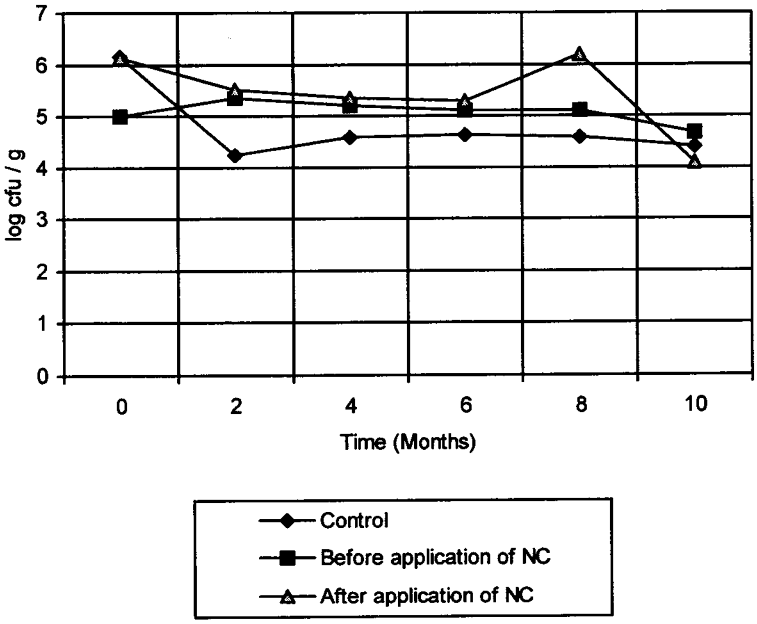


Figure 4.37 Population of *T. harzianum* IISR 1369 in Neem Cake (NC) applied soil

STUDIES ON VOLATILES AND NON-VOLATILES

4.5 Screening of *Trichoderma* spp for the effect of their volatile metabolites on the growth of *P. capsici*

Thirty isolates of *Trichoderma* spp were screened for their volatile effect on the growth of *P. capsici*. Severe inhibition of mycelial growth was induced by many isolates. Percent inhibition of growth of *P. capsici* by volatiles of *Trichoderma* spp varied from 2.54 to 29.21% (Table 4.10). The inhibited colonies were often usually compact and dense. When such colonies were sub-cultured on to fresh carrot agar medium, apparently normal growth of the test fungi occurred. Inhibition in colony diameter was often accompanied by aerial growth. Nine isolates were identified as superior over the others in the inhibition of *P. capsici* by volatiles. *T. hamatum* IISR 160 recorded highest percentage of inhibition of *P. capsici* (29.21%) followed by *T. aureoviride* IISR 148 (28.13%), *T. aureoviride* IISR 140 (25.22%), *T. virens* IISR 1370 (21.85%), *T. harzianum* IISR 165 (21.39%), *T. harzianum* IISR 173 (20.93%), *T. hamatum* IISR 150 (19.25%), *Trichoderma* sp. IISR 1141 (18.33%) and *T. hamatum* IISR 151 (16.33%) (Table 4.10).

Table 4.10 Inhibition of growth of *Phytophthora capsici* by volatiles of *Trichoderma* spp

Sl No.	*Trichoderma* spp	Percent inhibition of *P. capsici*
1	*T. harzianum* IISR 1369	12.35_{cfghi}
2	*T. virens* IISR 1370	21.85_{abcd}
3	*T. aureoviride* IISR 126	14.50_{defg}
4	*T. aureoviride* IISR 130	3.31_{nop}
5	*T. aureoviride* IISR 140	25.22_{abc}

continued

Table continued

Sl No.	*Trichoderma* spp	Percent inhibition of *P. capsici*
6	*T. aureoviride* IISR 143	6.83_{jklmn}
7	*T. aureoviride* IISR 148	28.13_{ab}
8	*T. hamatum* IISR 150	19.25_{cde}
9	*T. hamatum* IISR 151	16.33_{def}
10	*T. hamatum* IISR 160	29.21_{a}
11	*T. harzianum* IISR 165	21.39_{bcd}
12	*T. harzianum* IISR 167	5.00_{lmnop}
13	*T. harzianum* IISR 169	2.54_{op}
14	*T. harzianum* IISR 173	20.93_{bcd}
15	*T. harzianum* IISR 177	5.92_{klmno}
16	*T. pseudokoningii* IISR 186	2.54_{op}
17	*T. pseudokoningii* IISR 187	6.83_{ijklmn}
18	*T. polysporum* IISR 1101	3.92_{mnop}
19	*T. longibrachiatum* IISR 1103	11.13_{ghijkl}
20	*Trichoderma* sp. IISR 1107	15.11_{efgh}
21	*Trichoderma* sp. IISR 1112	7.14_{ijklmn}
22	*T. koningii* IISR 1119	8.21_{hijklm}
23	*Trichoderma* sp. IISR 1124	6.38_{jklmn}
24	*Trichoderma* sp. IISRT 1136	10.51_{fghijk}
25	*Trichoderma* sp. IISR 1141	18.33_{cde}
26	*Trichoderma* sp. IISR 1152	6.99_{ijklmn}
27	*Trichoderma* sp. IISR 1240	3.31_{nop}
28	*Trichoderma* sp. IISR 1267	5.92_{klmno}
29	*Trichoderma* sp. IISR 1270	4.54_{mnop}
30	*Trichoderma* sp. IISR 1275	11.28_{fghij}

Values followed by same letter(s) in the column do not differ significantly according to Duncan's Multiple Range Test (DMRT) at 5% level

4.6 Analysis of volatiles of *Trichoderma* spp by gas chromatography

The gas chromatographic spectrum of *T. harzianum* IISR 1369 showed 35 volatile components and that of *T. virens* IISR 1370 showed 44 components (Table 4.11). Total amount of volatile metabolites produced was deviated for the two *Trichoderma* spp. Otherwise the volatile profiles of the examined fungal species were highly variable. However one peak (RT 11.98) of *T. virens* IISR 1370 was common with control CDB. Two compounds (RT 0.748 and 28.78) were produced by both *T. harzianum* IISR 1369 and *T. virens* IISR 1370 (Plates 4.5, 4.6 and 4.7).

Table 4.11 Volatile constituents of *T. harzianum* IISR 1369 and *T. virens* IISR 1370

RT	*T. harzianum*	*T. virens*	CDB
0.518	-	+	-
0.748	+	+	-
0.997	+	-	-
1.007	-	-	+
1.157	-	+	-
1.868	-	+	-
1.912	+	-	-
2.595	-	-	+
2.7	+	-	-
3.265	+	-	-
3.837	+	-	-
4.103	+	-	-
4.505	+	-	-
4.965	+	-	-
5.767	-	-	+
5.775	-	+	-
6.217	+	-	-
6.695	-	-	+

continued

Table continued

RT	*T. harzianum*	*T. virens*	CDB
6.735	+	-	-
7.318	-	+	-
8.522	-	-	+
9.093	-	+	-
9.402	-	+	-
9.67	-	+	-
9.817	-	-	+
10.255	+	-	-
10.605	-	-	+
10.928	-	+	-
11.445	+	-	-
11.988	-	+	+
12.017	-	-	+
12.125	+	-	-
12.705	-	+	-
12.813	-	-	+
13.535	+	-	-
13.572	-	+	-
13.8	-	-	+
14.248	-	-	+
14.32	+	-	-
14.615	-	+	-
14.628	+	-	-
14.892	-	+	-
15.282	-	-	+
15.46	-	+	-
15.567	+	-	-
16.1	-	+	-
16.17	+	-	-
16.622	-	-	+
17.148	-	+	-

continued

Table continued

RT	*T. harzianum*	*T. virens*	CDB
17.198	-	-	+
17.793	-	-	+
17.995	-	+	-
18.18	-	-	+
18.27	-	+	-
18.912	-	-	+
18.968	-	+	-
19.192	-	+	-
19.198	+	-	-
19.313	-	-	+
19.590	-	-	+
19.842	-	-	+
20.048	-	+	-
20.958	-	-	+
21.688	+	-	-
21.827	-	+	-
21.932	-	-	+
22.145	-	-	+
22.152	-	+	-
22.238	+	-	-
22.433	+	-	-
22.578	-	-	+
22.855	-	+	-
23.173	-	-	+
23.182	-	+	-
23.270	+	-	-
23.352	-	-	+
24.023	+	-	-
25.070	-	-	-
25.197	-	-	+
25.413	-	+	-

continued

Table continued

RT	*T. harzianum*	*T. virens*	CDB
25.555	-	-	+
25.808	-	+	-
26.933	-	-	+
27.052	-	+	-
27.4	-	-	+
27.838	-	+	-
28.733	-	-	+
28.785	+	+	-
29.150	-	-	+
29.175	-	+	-
29.535	-	+	-
29.867	-	+	-
30.027	-	-	+
30.080	+	-	-
30.362	-	+	-
30.715	-	+	-
30.822	-	-	+
30.833	+	-	-
31.157	+	-	-
31.250	+	-	-
31.435	-	+	-
31.437	+	-	-
31.445	-	-	+
32.375	-	-	+
32.593	-	-	+
33.168	-	-	+
33.355	-	+	-
33.363	+	-	-
33.480	+	-	-
33.675	-	-	+
33.910	-	-	+

continued

Table continued

RT	*T. harzianum*	*T. virens*	CDB
34.587	-	+	-
34.722	+	-	-
35.098	+	-	-
35.107	-	+	-
35.573	-	+	-
37.462	-	+	-
38.212	-	+	-

RT retention time of compounds in minutes

4.7 Screening of *Trichoderma* spp for the effect of their non-volatile metabolites on the mycelial dry weight of *P. capsici*

Above-mentioned thirty isolates of *Trichoderma* spp were also screened for their effect on the mycelial dry weight of *P. capsici*. Non-volatiles of almost all isolates studied were inhibitory to *P. capsici*. Percent inhibition of mycelial dry weight of *P. capsici* by non-volatiles of *Trichoderma* spp varied (23.82-79.8%) with the concentration (75%) tested.

Lower concentration tested caused little reduction in growth. Eight isolates were found to be highly efficient in production of non-volatile metabolites that inhibited the mycelial dry weight of *P. capsici*. The following isolates with higher percentage of inhibition of *P. capsici* by their culture filtrate were selected. They are *T. aureoviride* IISR 126 (79.08%), *T. aureoviride* IISR 140 (77.31%), *T. aureoviride* IISR 143 (73.34%), *T. virens* IISR 1370 (71.75%), *T. aureoviride* IISR 148 (71.60%), *Trichoderma* sp. IISR 1107 (71.33%), *T. pseudokoningii* IISR 186 (70.25%), and *T. harzianum* IISR 1369 (57.79%) (Table 4.12).

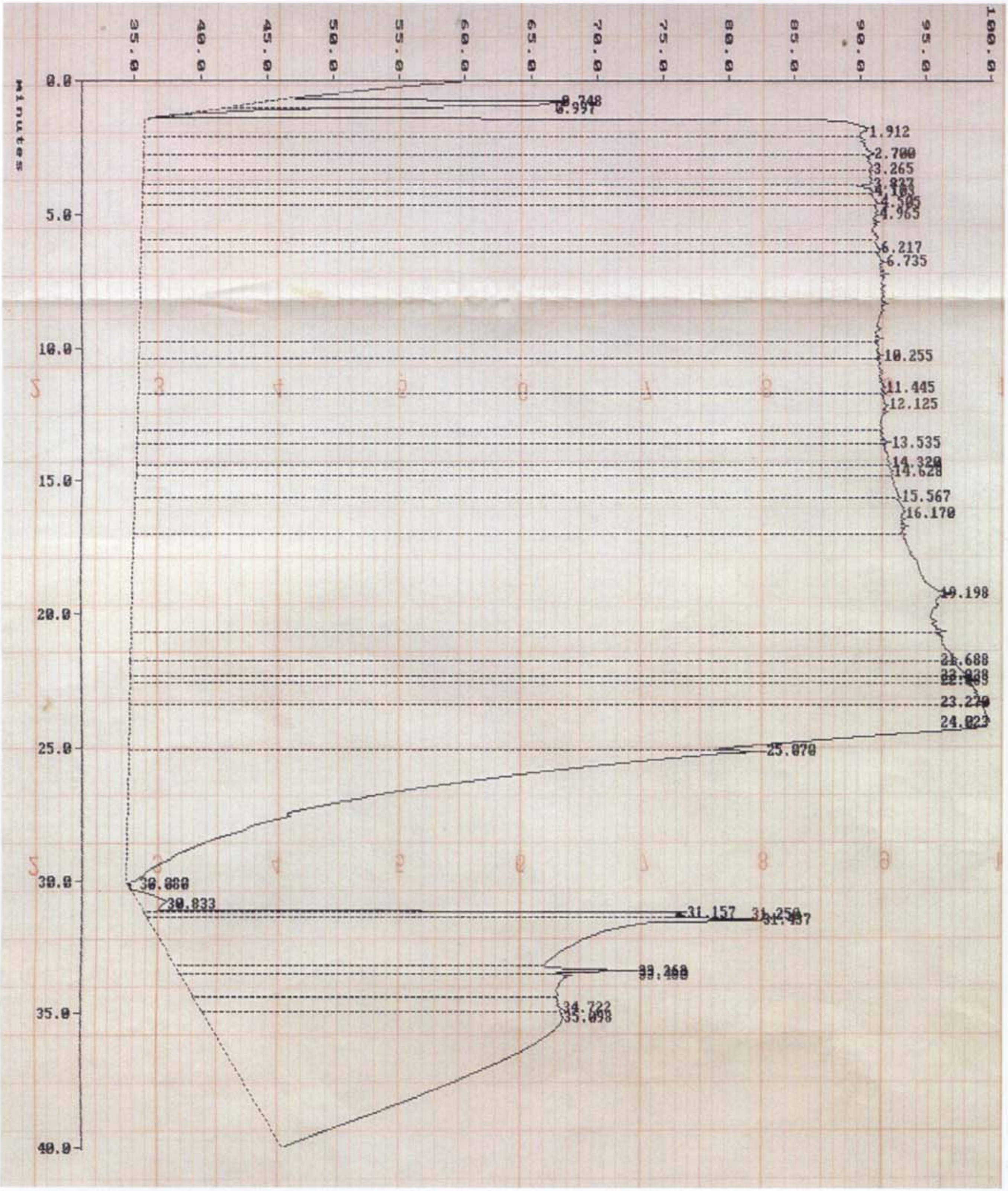


Plate 4.5 Chromatogram of volatiles produced by *T. harzianum* IISR 1369 on CDB

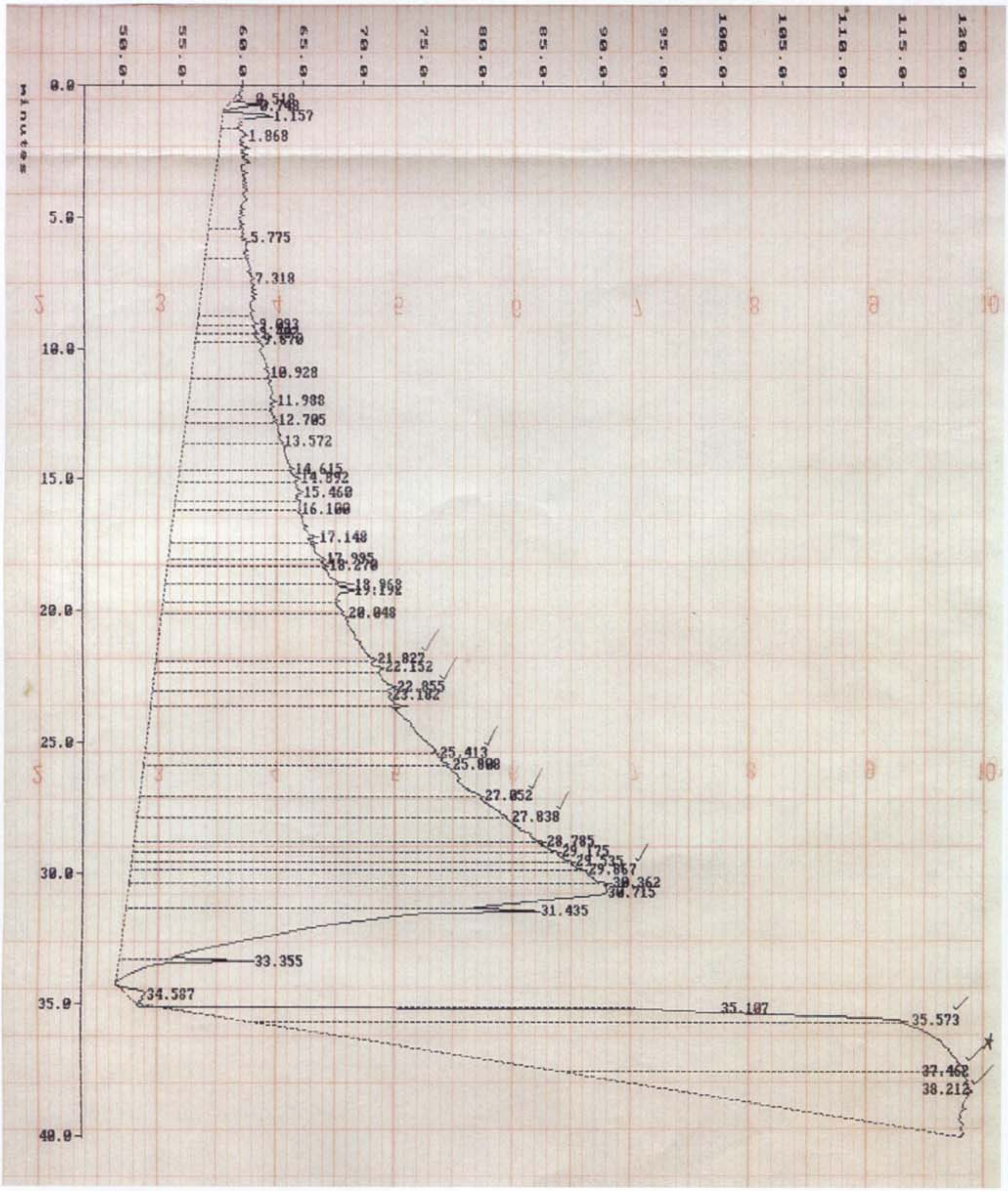


Plate 4.2 Chromatogram of volatiles produced by *T. virens* IISR 1370 on CDB

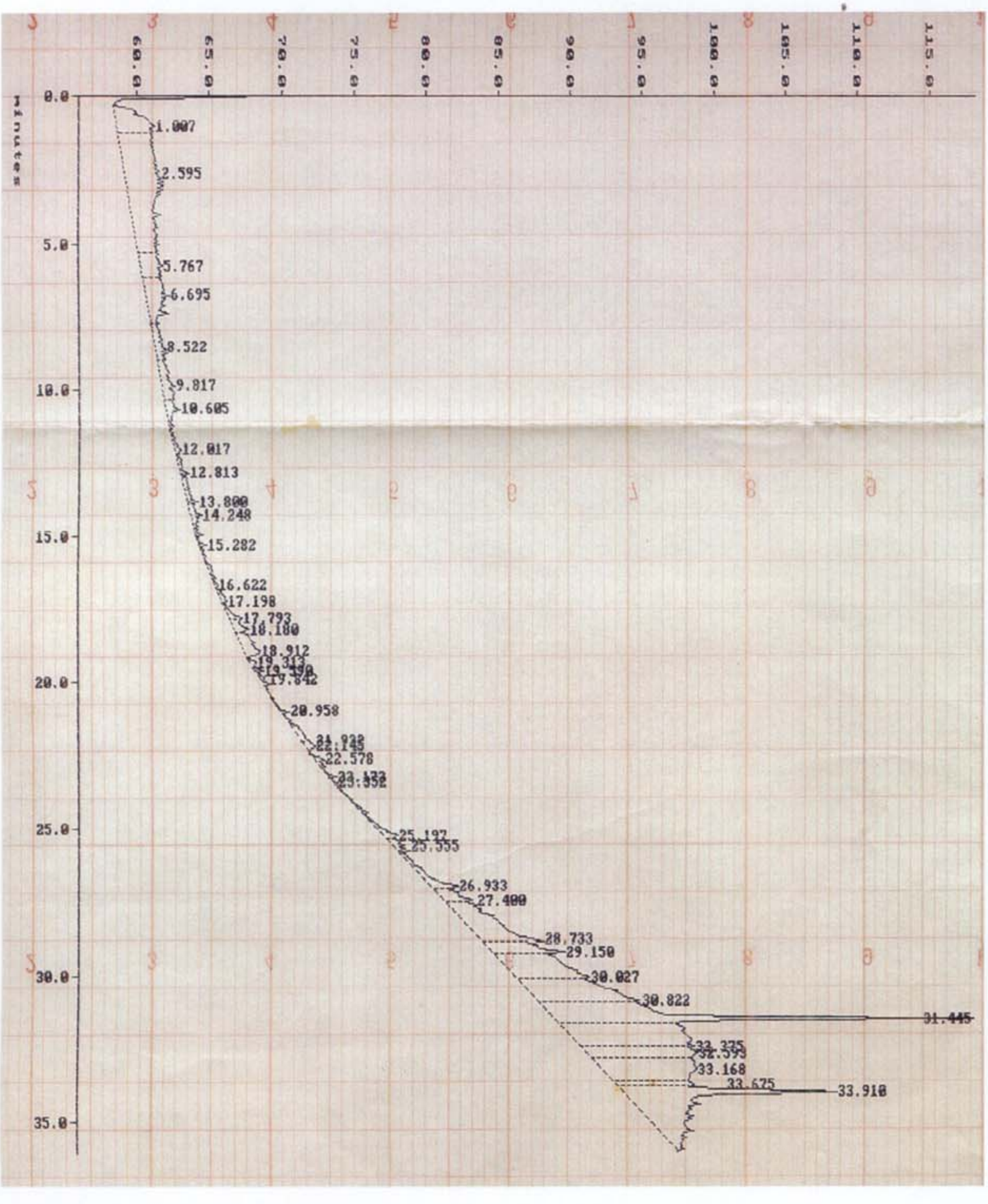


Plate 4.7 Chromatogram of volatiles from CDB (control)

Table 4.12 Inhibition of mycelial dry weight of *P. capsici* by non-volatile metabolites of *Trichoderma* spp

Sl No.	*Trichoderma* sp.	Percent inhibition at different concentration			
		10	25	50	75
1	*T. harzianum* IISR 1369	21.83_{fghijk}	30.25_{fghijk}	37.40_{hijklm}	57.79_{ef}
2	*T. virens* IISR 1370	36.41_{cd}	40.31_{def}	56.13_{defg}	71.75_{abcd}
3	*T. aureoviride* IISR 126	15.50_{jkl}	37.46_{efg}	77.66_{a}	79.08_{a}
4	*T. aureoviride* IISR 130	27.89_{ef}	38.53_{efg}	39.23_{hijkl}	42.72_{ghi}
5	*T. aureoviride* IISR 140	21.93_{fghij}	46.17_{cde}	63.58_{bcde}	77.31_{ab}
6	*T. aureoviride* IISR 143	52.57_{b}	67.11_{a}	69.01_{abcd}	73.34_{abc}
7	*T. aureoviride* IISR 148	25.63_{efg}	55.52_{bc}	58.46_{cdef}	71.60_{abcd}
8	*T. hamatum* IISR 150	23.77_{efghi}	$27.31_{ghijklm}$	40.66_{hijk}	43.84_{ghi}
9	*T. hamatum* IISR 151	17.49_{ijkl}	$29.38_{fghijkl}$	32.07_{jklmno}	40.89_{hi}
10	*T. hamatum* IISR 160	20.55_{ghijkl}	20.66_{klm}	23.00_{no}	23.82_{k}
11	*T. harzianum* IISR 165	19.87_{ghijkl}	25.43_{hijklm}	24.50_{mno}	50.62_{fg}
12	*T. harzianum* IISR 167	14.68_{kl}	24.39_{ijklm}	24.91_{mno}	28.60_{k}
13	*T. harzianum* IISR 169	23.77_{efghi}	$27.31_{ghijklm}$	40.66_{hijk}	43.84_{ghi}
14	*T. harzianum* IISR 173	16.38_{jkl}	18.41_{lm}	20.67_{o}	24.02_{k}
15	*T. harzianum* IISR 177	15.20_{jkl}	17.78_{m}	36.52_{ijklmn}	38.46_{ij}
16	*T. pseudokoningii* IISR 186	59.70_{ab}	62.40_{ab}	71.80_{abc}	70.25_{abcd}
17	*T. pseudokoningii* IISR 187	29.76_{de}	38.30_{efg}	41.13_{hijk}	45.63_{ghi}
18	*T. polysporum* IISR 1101	17.49_{ijkl}	$29.38_{fghijkl}$	32.07_{jklmno}	40.89_{hi}
19	*T. longibrachiatum* IISR 1103	17.64_{hijkl}	$27.52_{ghijklm}$	40.39_{hijk}	44.70_{ghi}
20	*Trichoderma* sp. IISR 1107	15.86_{jkl}	68.59_{a}	74.99_{ab}	71.33_{abcd}
21	*Trichoderma* sp. IISR 1112	13.81_{l}	20.72_{jklm}	24.32_{mno}	26.27_{k}
22	*T. koningii* IISR 1119	62.20_{a}	57.33_{abc}	72.00_{abc}	73.46_{abc}
23	*Trichoderma* sp. IISR 1124	26.72_{efg}	38.48_{efg}	28.82_{klmno}	45.90_{ghi}
24	*Trichoderma* sp. IISR 1136	19.98_{ghijkl}	35.27_{efghi}	37.12_{hijklm}	41.35_{ghi}
25	*Trichoderma* sp. IISR 1141	17.49_{ijkl}	$29.38_{fghijkl}$	32.07_{jklmno}	40.89_{hi}
26	*Trichoderma* sp. IISR 1152	29.76_{de}	38.30_{efg}	41.13_{hijk}	45.63_{ghi}
27	*Trichoderma* sp. IISR 1240	24.78_{efgh}	25.41_{hijklm}	25.92_{lmno}	30.75_{jk}
28	*Trichoderma* sp. IISR 1267	15.20_{jkl}	17.78_{m}	36.52_{ijklmn}	38.46_{ij}
29	*Trichoderma* sp. IISR 1270	25.59_{efg}	$28.39_{ghijklm}$	42.56_{ghij}	45.10_{ghi}
30	*Trichoderma* sp. IISR 1275	14.72_{jkl}	24.05_{ijklm}	43.05_{ghij}	48.33_{gh}

Values followed by same letter(s) in a column do not differ significantly according to DMRT at 5% level

Thus nine isolates which were highly efficient in volatile production and eight isolates with non-volatile production were selected for *in-vitro* interaction and *in-vivo* studies. Out of the above 17 isolates, three of them were found to be both volatile and non-volatile producers. They are *T. aureoviride* IISR 148, *T. aureoviride* IISR 140 and *T. virens* IISR 1370. Among the eight non-volatile producers, five were found as highly rhizosphere competent. They are *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140, *T. aureoviride* IISR 148 and *T. harzianum* IISR 1369. They were taken for extraction of non-volatiles.

4.8 Isolation and identification of non-volatile metabolites

Non-volatile compounds were isolated from *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148 (Plate 4.8 a). The culture filtrate of *T. harzianum* IISR 1369 was colourless and that of *T. virens* IISR 1370 was pale red. The culture filtrate of three isolates of *T. aureoviride* was highly dark red in colour. The pH of the culture filtrate of above five species was 4.8, 5.8, 6.2, 6.0 and 5.9 respectively.

4.8.1 Extraction of active compounds and testing for inhibition of *P. capsici*

On extraction with Ethyl acetate (EtOAc), culture filtrate of *T. harzianum* IISR 1369 gave colourless liquid and *T. virens* IISR 1370 gave a reddish oily liquid. *T. aureoviride* IISR 126, *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148 gave a yellow oily liquid of varying intensity. Concentrated EtOAc fraction inhibited *P. capsici* in culture (Table 4.13). *T. harzianum* IISR 1369, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148 inhibited *P. capsici* completely (100%) at 50 µl concentration. In the case of *T. virens* IISR 1370, ethyl acetate extract inhibited *P. capsici* completely (100%) at very low concentration (20 µl) (Plate 4.8 b, c, d, e & f).

Table 4.13 Effect of ethyl acetate extract of culture filtrate of *Trichoderma* spp on the growth of *P. capsici* in agar culture, measured as colony diameter (mm)

Ethyl acetate extract	Quantity (µl)	Growth period (days at 26^0C) 1	2	3	% inhibition
T. harzianum IISR 1369	10	9	17.00	35.00	15.66
	20	10	17.17	25.83	19.70
	40	8	12.33	21.83	36.72
	50	0	0	0	100
T. virens IISR 1370	10	4	24.17	31.5	24.09
	20	0	0	0	100
	40	0	0	0	100
	50	0	0	0	100
T. aureoviride IISR 126	10	14.16	24.83	39.67	4.40
	20	13.3	23.67	27.00	16.07
	40	10.6	25.67	28.00	18.84
	50	0	0	0	100
T. aureoviride IISR 140	10	8	16	34.00	18.07
	20	11	14	23.17	27.97
	40	7	12	21.17	38.63
	50	0	0	0	100
T. aureoviride IISR 1148	10	4.5	17	30.00	27.71
	20	8.5	15.67	20.83	35.25
	40	3	6.67	9.17	73.42
	50	0	0	0	100
Ethyl acetate control	10	16.17	31.83	41.5	
	20	13	24.67	32.17	
	40	12.17	26	34.5	
	50	12.5	26	34.5	
Absolute control		17.67	36.33	45.17	
CD at 5%		0.981	0.927	1.462	

4.8.2 Thermo stability of ethyl acetate extract of culture filtrate of *Trichoderma* spp

The concentrated and steam heated EtOAc fractions were able to inhibit *P. capsici* completely up to 48 hours even though it was not lethal to *P. capsici*. It was tested at 100 µl concentration. However growth was noticed after 72 hours (Table 4.14) (Plate 4.8 g).

Table 4.14 Effect of steam heated ethyl acetate extract of culture filtrate of *Trichoderma* spp on the growth of *P. capsici* in agar culture, measured as colony diameter (mm)

Species	Quantity (µl)	Growth period (days at 26^{0}C) 1	2	3	% inhibition
T. harzianum IISR 1369	100	0	0	14.5	60.27
T. virens IISR 1370	100	0	0	10	72.60
T. aureoviride IISR 126	100	0	0	14	61.64
T. aureoviride IISR 1140	100	0	0	9	75.34
T. aureoviride IISR 148	100	0	0	12	67.12
Ethyl acetate	100	13	26.5	36.5	
Absolute control		17.83	36.83	44.67	
CD at 5%		0.648	0.582	0.127	

4.8.3 Effect of ethyl acetate extract of culture filtrate of *Trichoderma* spp on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

Ethyl acetate extract of culture filtrate of *Trichoderma* spp was tested for the effect on sporangia formation, zoospore liberation and zoospore germination of *P. capsici*.

Plate 4.8 a *Trichoderma harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148 taken for extraction of non-volatiles – colony morphology and pigmentation on reverse side of the plate.

Plate 4.8 b Inhibition of *P. capsici* by concentrated ethyl acetate extract of culture filtrate of *T. harzianum* IISR 1369.

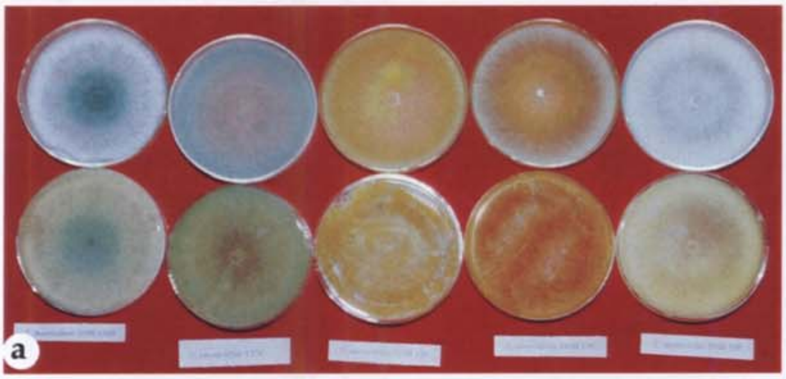
Plate 4.8 c Inhibition of *P. capsici* by concentrated ethyl acetate extract of culture filtrate of *T. virens* IISR 1370.

Plate 4.8 d Inhibition of *P. capsici* by concentrated ethyl acetate extract of culture filtrate of *T. aureoviride* IISR 126.

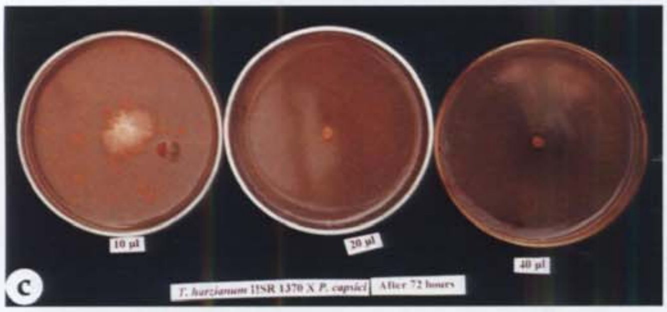
Plate 4.8 e Inhibition of *P. capsici* by concentrated ethyl acetate extract of culture filtrate of *T. aureoviride* IISR 140.

Plate 4.8 f Inhibition of *P. capsici* by concentrated ethyl acetate extract of culture filtrate of *T. aureoviride* IISR 148.

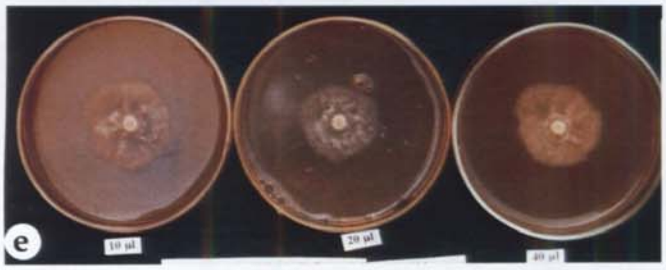
Plate 4.8 g Inhibition of *P. capsici* by steam heated ethyl acetate extract of broth culture of *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148.


a


b
Blank 10 µl
Blank 20 µl
Blank 40 µl
Abs. Control
10 µl
20 µl
40 µl
T. harzianum IISR 1369 X P. capsici
After 72 hours


c
10 µl
20 µl
40 µl
T. harzianum IISR 1370 X P. capsici
After 72 hours


d
10 µl
20 µl
40 µl
T. aureoviride IISR 140 X P. capsici
After 72 hours


e
10 µl
20 µl
40 µl


f
10 µl
20 µl
40 µl


g
Abs. Control
Blank 100 µl
IISR 1369
IISR 1370
IISR 126
IISR 140
IISR 148
Steam sterilized

From 100 ml of culture filtrate, about 1 ml of EtOAc extract was obtained and this extract diluted to get 10, 100, 300, 400, 500, 1000, 2000 and 4000 ppm concentration of solutions in water. Increasing concentration of ethyl acetate extract of culture filtrate of *T. harzianum* IISR 1369 caused a decline in the number of sporangia formed. Percent reduction in the number of sporangia formed per microscopic field (X 40) was varied from 4.86% at 10 ppm to 81.68% at 4000 ppm. Peripheral growth of mycelia from the disc was also reduced. Liberation of zoospores was 90.97% at 10 ppm and 35.32% at 4000 ppm as against 86.06% in the control. Percent germination of zoospores was 76.67% at 10 ppm, which reduced to zero at 400 ppm and above as against 97.22% germination in the control (Figure 4.38). Similar results were obtained with the ethyl acetate extract of culture filtrate of *T. virens* IISR 1370 where percent reduction in the number of sporangia varied from 5.31% at 10 ppm to 85.60% at 4000 ppm. Percent liberation of zoospores was 91.77% and 28.23% at 10 and 4000 ppm respectively. Percent germination of zoospores was 74.24% at 10 ppm, which was reduced to zero at 400 ppm and above (Figure 4.39). In the case of *T. aureoviride* IISR 126 percent reduction in the number of sporangia varied from 3.82% at 10 ppm to 87.62% at 4000 ppm. Percent liberation of zoospores was 89.94% and 37.87% at 10 and 4000 ppm respectively. Percent germination of zoospores was 83.33% at 10 ppm, which was reduced to zero at 400 ppm and above (Figure 4.40). When the extract of *T. aureoviride* IISR 140 was tested, percent reduction in the number of sporangia varied from 5.66% at 10 ppm to 88.63% at 4000 ppm. Percent liberation of zoospores was 92.51% and 24.4% at 10 and 4000 ppm respectively. Percent germination of zoospores was 83.33% at 10 ppm, which was reduced to zero at 400 ppm and above (Figure 4.41). Similarly for *T. aureoviride* IISR 148, percent reduction in the number of sporangia varied from 3.98% at 10 ppm to 87.05% at 4000 ppm. Percent liberation of zoospores was 86.81% and 18.46% at 10 and 4000 ppm respectively. Percent germination of zoospores was 79.15% at 10 ppm, which was reduced to zero at 400 ppm and above (Figure 4.42).

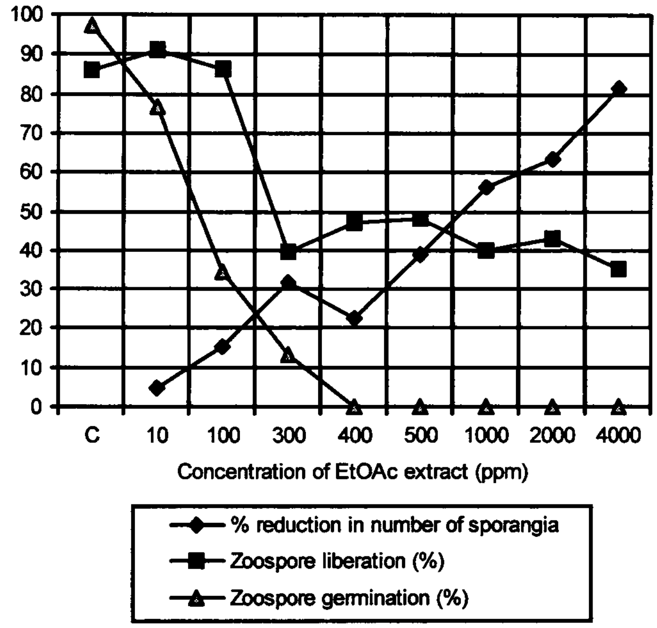


Figure 4.38 Effect of EtOAc extract of culture filtrate of *T. harzianum* IISR 1369 on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

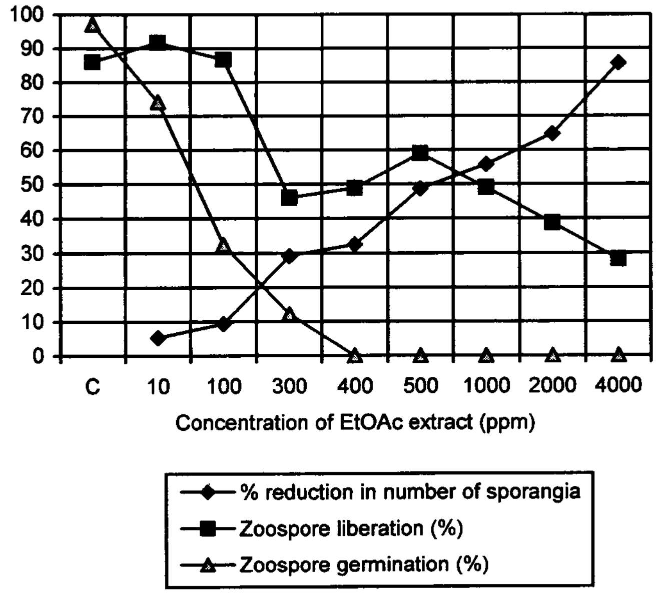


Figure 4.39 Effect of EtOAc extract of culture filtrate of *T. virens* IISR 1370 on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

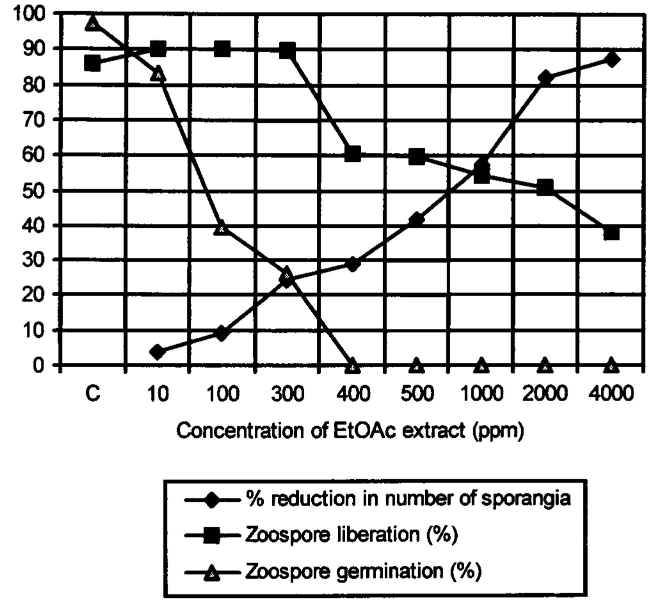


Figure 4.40 Effect of EtOAc extract of culture filtrate of *T. aureoviride* IISR 126 on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

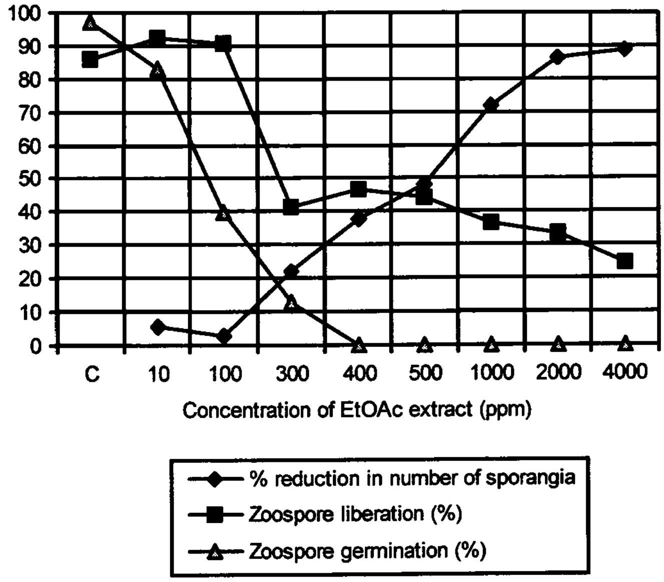


Figure 4.41 Effect of EtOAc extract of culture filtrate of *T. aureoviride* IISR 140 on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

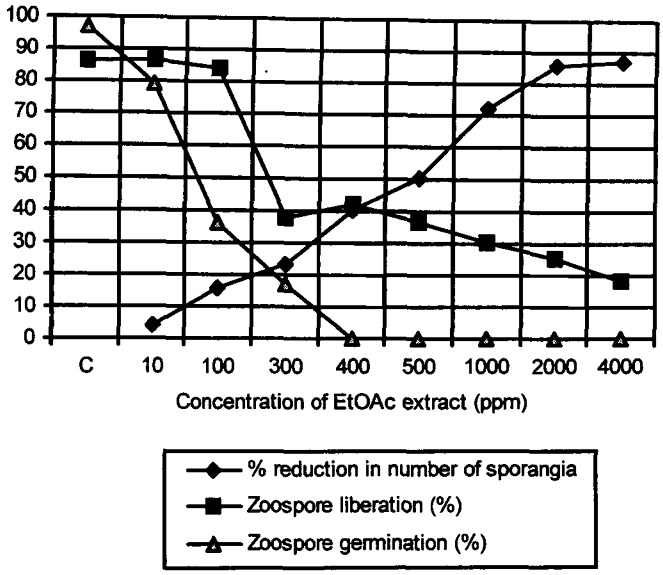


Figure 4.42 Effect of EtOAc extract of culture filtrate of *T. aureoviride* IISR 148 on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

4.8.4 Thin layer chromatography

No compounds were detected from ethyl acetate extract of CDB (control) before and after spraying with 10% H_2SO_4 in methanol (Plate 4.9 a & b). Ethyl acetate extract of culture filtrate of *T. harzianum* IISR 1369 showed one visible compound (Rf 0.06) on TLC plate. The basal spot (Rf 0.0) also was visible. Four additional compounds were detected after spraying with 10% H_2SO_4 in methanol (Plate 4.9 c & d and Figure 4.43). Compound 2 (Rf 0.6) partially inhibited (30.09%) *P. capsici* (Table 4.15 and Plate 4.10 a).

Ethyl acetate extract of culture filtrate of *T. virens* IISR 1370 showed six visible compounds on TLC plate. No additional compounds were detected after spraying with 10% H_2SO_4 in methanol (Plate 4.9 e & f and Figure 4.44). Compound 1 (Rf 0.65) and 3 (Rf 0.38) inhibited *P. capsicci* and showed 60.67 and 56.80 %

inhibition respectively. Compound 2 (Rf 0.53) partially inhibited (41.74%) *P. capsici* (Table 4.16 and Plate 4.10 b, c & d).

Table 4.15 Inhibition of *P. capsici* by purified non-volatile compounds of *T. harzianum* IISR 1369 measured as colony diameter (mm)

Compound	Rf value	Growth period (days at 26^{0}C)			%
		1	2	3	inhibition
1	0.74	11.17	22.17	32.83	4.36
2	0.6	8.33	14.17	24	30.09
3	0.4	10.17	17.33	27.17	20.85
4	0.23	11.17	18.33	29.83	13.10
5	0.06	11	20	30.5	11.15
6	0.0	11.17	15.5	23.33	32.04
Ethyl alcohol		13	26.17	34.33	
Absolute control		17.83	36.50	39.5	
CD at 5%		0.88	0.851	3.798	

Table 4.16 Inhibition of *P. capsici* by purified non-volatile compounds of *T. virens* IISR 1370 measured as colony diameter (mm)

Compound	Rf value	Growth period (days at 26^{0}C)			%
		1	2	3	inhibition
1	0.65	0	9.67	13.5	60.67
2	0.53	8.83	12.33	20	41.74
3	0.38	0	8.5	14.83	56.80
4	0.26	11.33	21.17	31.17	9.20
5	0.12	11.17	18.17	28.83	16.02
6	0.0	11	18.17	28.83	16.02
Ethyl alcohol		13	26.17	34.33	
Absolute control		17.83	36.50	39.5	
CD at 5%		0.779	0.891	3.776	

Ethyl acetate extract of culture filtrate of *T. aureoviride* IISR 126 showed five visible compounds on TLC plate. No additional compounds were detected after spraying with 10% H_2SO_4 in methanol (Plate 4.9 g & h and Figure 4.45). Compound 2 (Rf 0.69) partially inhibited (33.49%) *P. capsici* (Table 4.17 and Plate 4.10 e & f).

Table 4.17 Inhibition of *P. capsici* by purified non-volatile compounds of *T. aureoviride* IISR 126 measured as colony diameter (mm)

Compound	Rf value	Growth period (days at 26^{0}C)			%
		1	2	3	inhibition
1	0.8	11.33	24	34.0	0.96
2	0.69	10.17	16	22.83	33.49
3	0.51	11.17	22.67	33.83	1.45
4	0.34	10.83	21.17	32.3	5.91
5	0.09	11	21.17	32.3	5.91
Ethyl alcohol		13	26.17	34.33	
Absolute control		17.83	36.50	39.5	
CD at 5%		0.944	0.848	4.302	

Ethyl acetate extract of culture filtrate of *T. aureoviride* IISR 140 showed 12 visible compounds on TLC plate. No additional compounds were detected after spraying with 10% H_2SO_4 in methanol (Plate 4.9 i, j & m and Figure 4.46). Compounds 5 (Rf 0.51) and 9 (Rf 0.29) partially inhibited *P. capsici* and percent inhibition were 49.98 and 47.56% respectively. Compounds 1, 2, 4 and 8 (Rf 0.74, 0.69, 0.60 and 0.37) partially inhibited *P. capsici* and showed 41.24, 31.05, 30.09 and 29.59% inhibition respectively (Table 4.18 and Plate 4.10 g & h).

Table 4.18 Inhibition of *P. capsici* by purified non-volatile compounds of *T. aureoviride* IISR 140

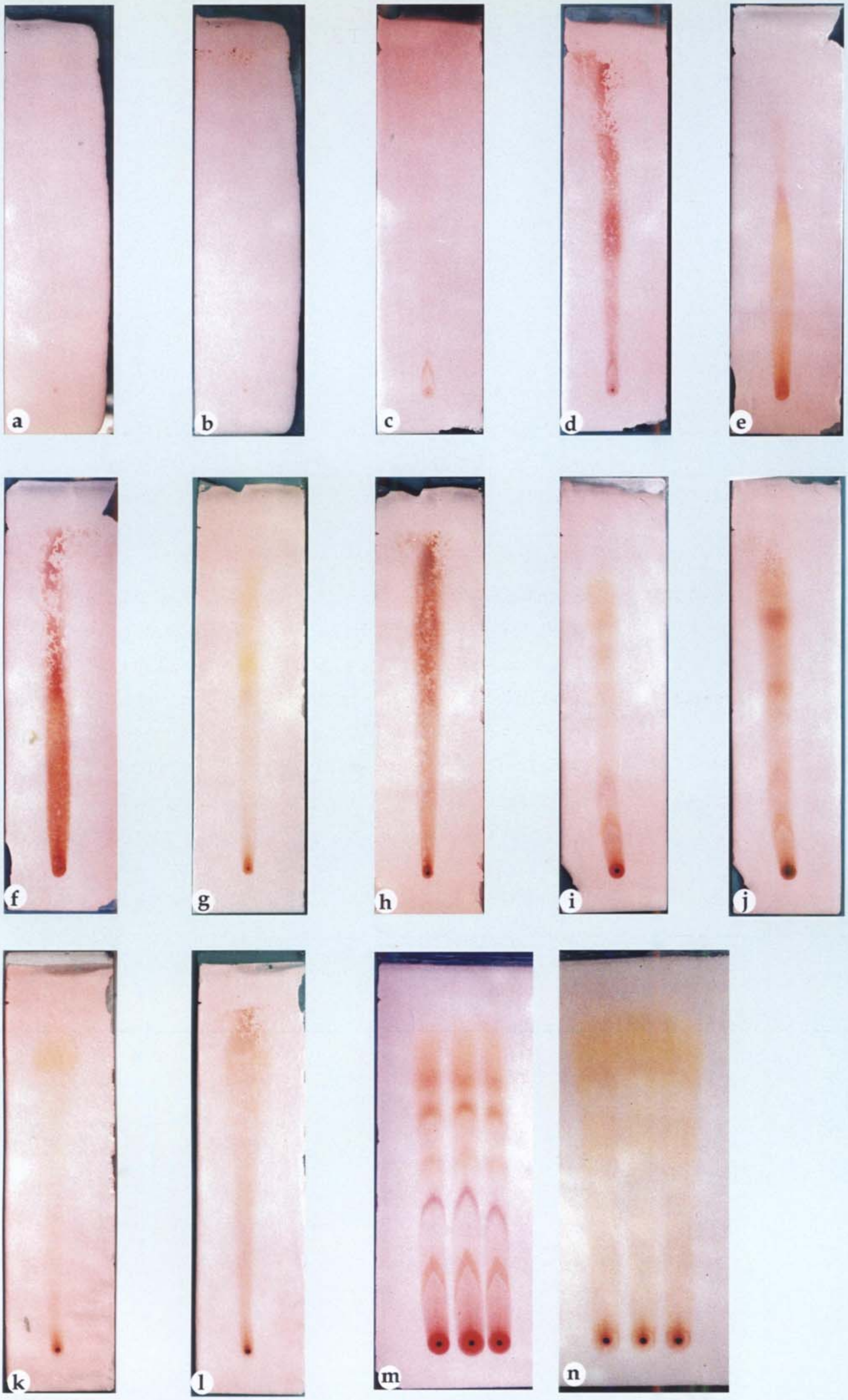
Compound	Rf value	Growth period (days at 26^{0}C) 1	2	3	% inhibition
1	0.74	11.67	14.17	20.17	41.24
2	0.69	11.17	14.67	23.67	31.05
3	0.63	11.67	17.67	25.83	24.75
4	0.60	11	15.17	24	30.09
5	0.51	0	11	17.17	49.98
6	0.46	11	14	25.33	26.21
7	0.40	11.83	17.17	27	21.35
8	0.37	11.67	14.17	24.17	29.59
9	0.29	0	10.33	18	47.56
10	0.18	11.33	15.17	25.67	25.22
11	0.09	11.17	14.5	26.17	23.76
12	0.0	11.17	15.33	25.67	25.22
Ethyl alcohol		13	26.17	34.33	
Absolute control		17.83	36.50	39.5	
CD at 5%		0.467	0.571	2.176	

Ethyl acetate extract of culture filtrate of *T. aureoviride* IISR 148 showed nine visible compounds on TLC plate. No additional compounds were detected after spraying with 10% H_2SO_4 in methanol (Plate 4.9 k, l & n and Figure 4.47). Compounds 2, 4 and 5 (Rf 0.63, 0.54 and 0.46) partially inhibited *P. capsici* and showed 34.45, 30.09 and 30.09% inhibition respectively (Table 4.19 and Plate 4.11 a & b).

Table 4.19 Inhibition of *P. capsici* by purified non-volatile compounds of *T. aureoviride* IISR 148 measured as colony diameter (mm)

Compound	Rf value	Growth period (days at 26^{0}C)			%
		1	2	3	inhibition
1	0.74	11.17	15.83	26.17	23.76
2	0.63	10.5	13.67	22.5	34.45
3	0.60	11.17	14.33	25.17	26.68
4	0.54	11.17	18.83	24	30.09
5	0.46	11.33	14.67	24	30.09
6	0.37	11.33	14.83	26.5	22.80
7	0.26	11	15.67	26.67	22.31
8	0.11	11.17	15.83	28	18.43
9	0.03	10.67	16.83	24.83	27.67
Ethyl alcohol		13	26.17	34.33	
Absolute control		17.83	36.50	39.5	
CD at 5%		0.595	0.569	2.812	

Plate 4.9 a-b	TLC of ethyl acetate extract of CDB (control) before (**a**) and after (**b**) spraying with 10% H_2SO_4 in methanol
Plate 4.9 c-d	Distribution of non-volatile compounds from *T. harzianum* IISR 1369, applied to TLC plates and developed in EtOAc : Hexane (8:2), (**c**) before and (**d**) after spraying with 10% H_2SO_4 in methanol
Plate 4.9 e-f	Distribution of non-volatile compounds from *T. virens* IISR 1370, applied to TLC plates and developed in EtOAc : Hexane (8:2). (**e**) before and (**f**) after spraying with 10% H_2SO_4 in methanol
Plate 4.9 g-h	Distribution of non-volatile compounds from *T. aureoviride* IISR 126, applied to TLC plates and developed in EtOAc : Hexane (8:2). (**g**) before and (**h**) after spraying with 10% H_2SO_4 in methanol
Plate 4.9 i-j & m	Distribution of non-volatile compounds from *T. aureoviride* IISR 140, applied to TLC plates and developed in EtOAc : Hexane (8:2). (**i & m**) before and (**j**) after spraying with 10% H_2SO_4 in methanol
Plate 4.9 k-l & n	Distribution of non-volatile compounds from *T. aureoviride* IISR 148, applied to TLC plates and developed in EtOAc : Hexane (8:2). (**k & n**) before and (**l**) after spraying with 10% H_2SO_4 in methanol


a
b
c
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i
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k
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m
n

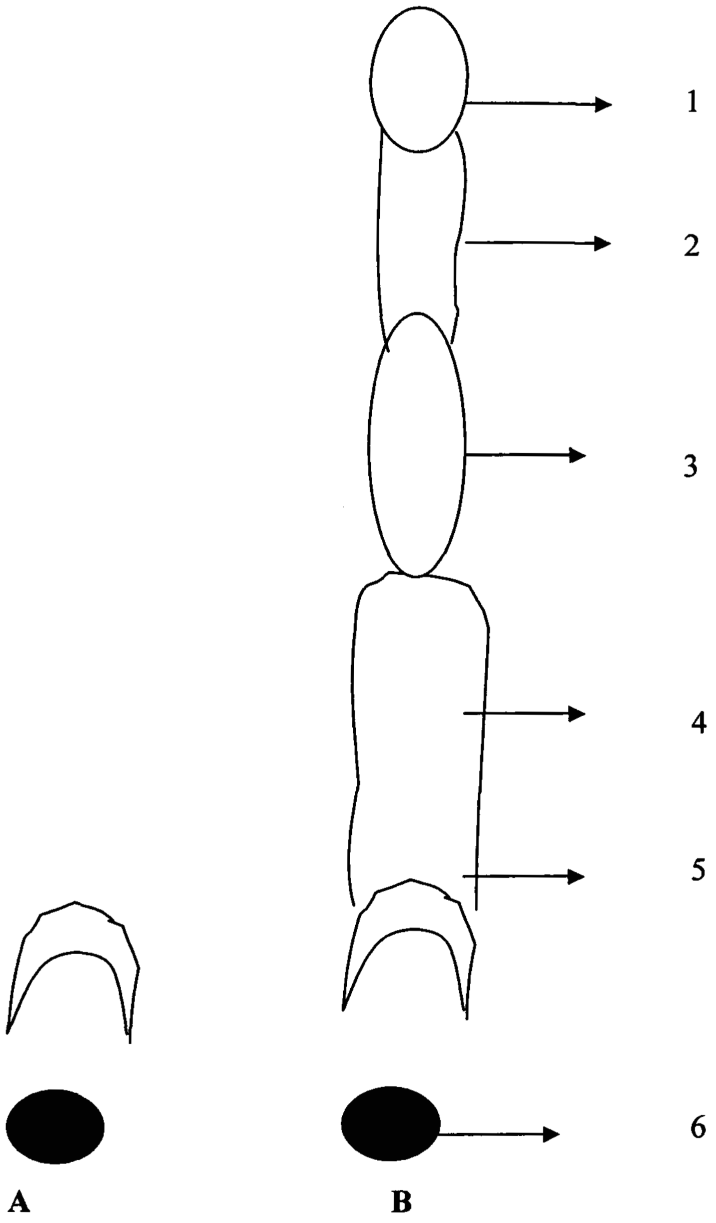


Figure 4.43 Diagrammatic sketch of distribution of non-volatile compounds of *T. harzianum* IISR 1369 applied to TLC plates and developed in ethyl acetate : hexane (8:2), (**A**) before and (**B**) after spraying with 10% H_2SO_4 in methanol. Compounds are numbered 1 to 6.

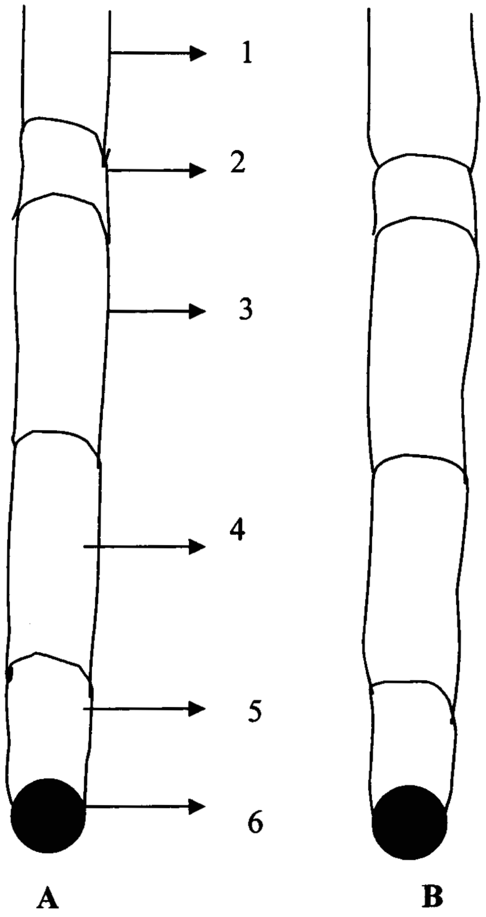


Figure 4.44 Diagrammatic sketch of distribution of non-volatile compounds of *T. virens* IISR 1370 applied to TLC plates and developed in ethyl acetate : hexane (8:2), (**A**) before and (**B**) after spraying with 10% H_2SO_4 in methanol. Compounds are numbered 1 to 6.

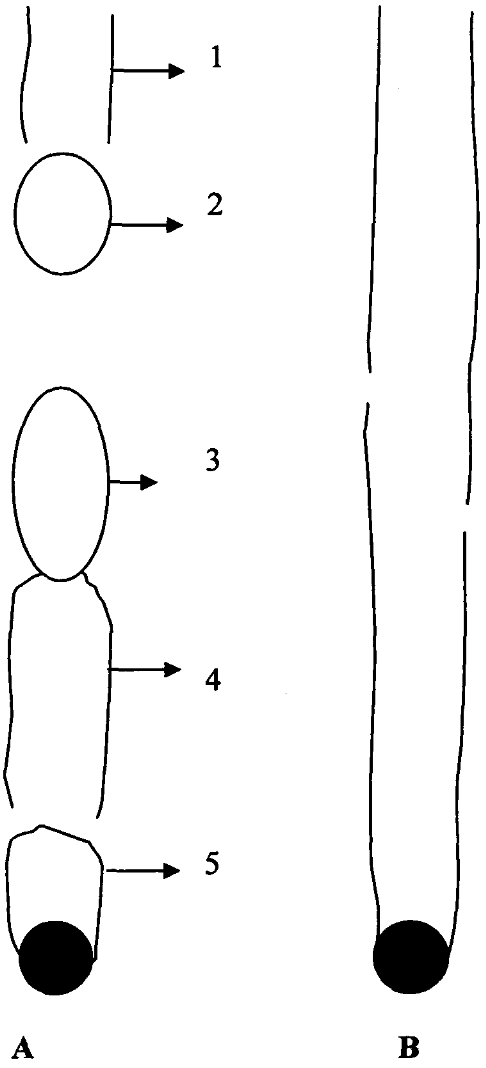


Figure 4.45 Diagrammatic sketch of distribution of non-volatile compounds of *T. aureoviride* IISR 126 applied to TLC plates and developed in ethyl acetate : hexane (8:2), (**A**) before and (**B**) after spraying with 10% H_2SO_4 in methanol. Compounds are numbered 1 to 5.

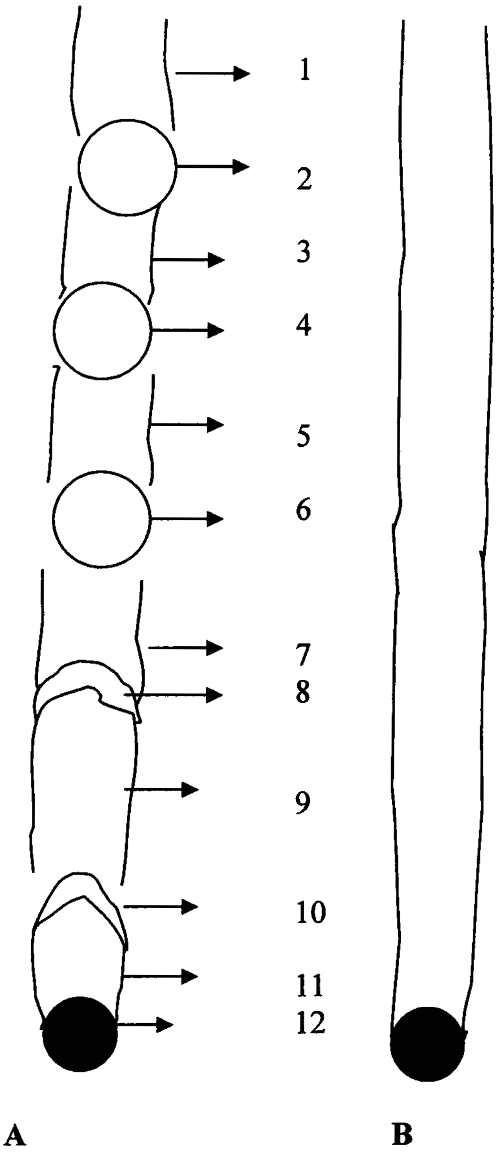


Figure 4.46 Diagrammatic sketch of distribution of non-volatile compounds of *T. aureoviride* IISR 140 applied to TLC plates and developed in ethyl acetate : hexane (8:2), **(A)** before and **(B)** after spraying with 10% H_2SO_4 in methanol. Compounds are numbered 1 to 12.

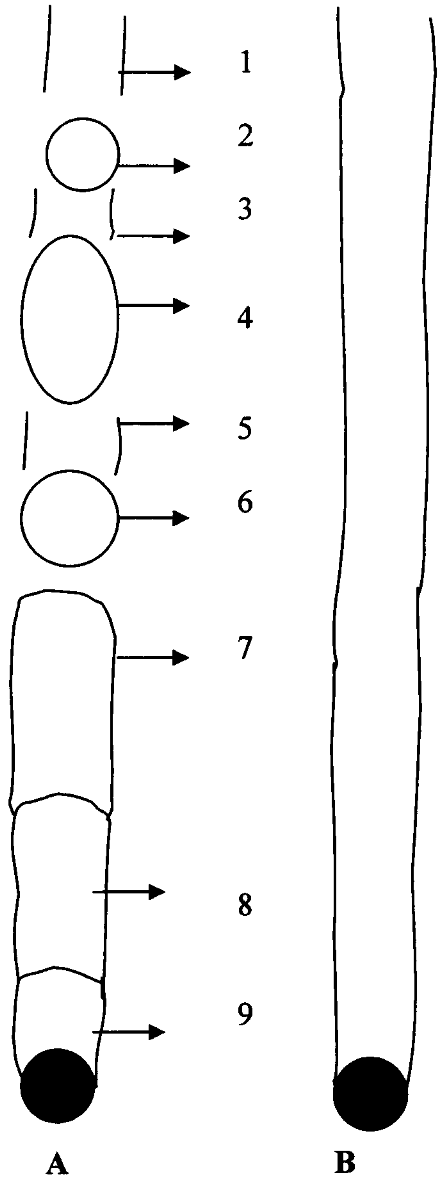
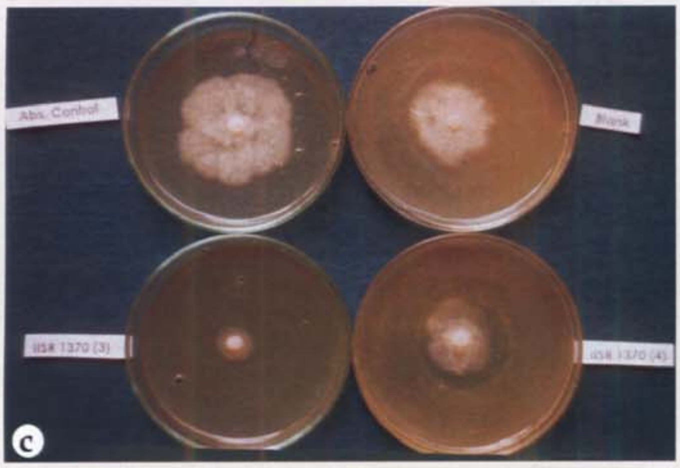


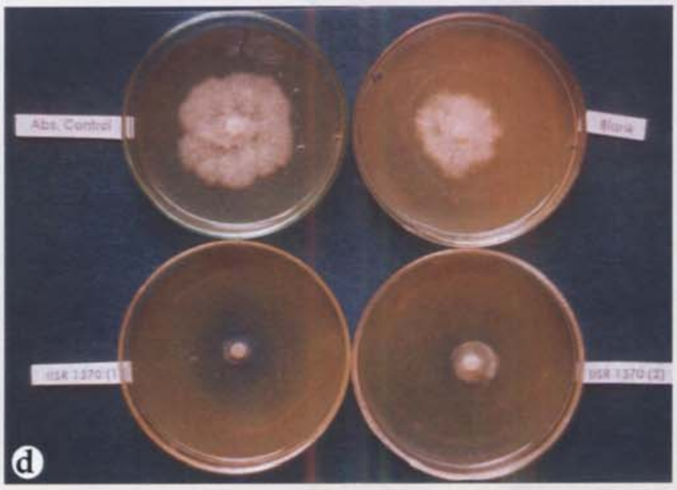
Figure 4.47 Diagrammatic sketch of distribution of non-volatile compounds of *T. aureoviride* IISR 148 applied to TLC plates and developed in ethyl acetate : hexane (8:2), **(A)** before and **(B)** after spraying with 10% H_2SO_4 in methanol. Compounds are numbered 1 to 9.

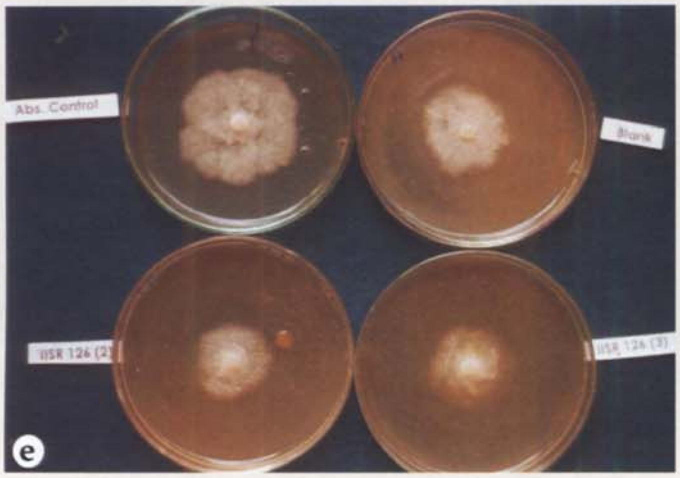
Plate 4.10 a	Inhibition of mycelial growth of *P. capsici* by purified non-volatile compounds of *T. harzianum* IISR 1369
Plate 4.10 b-d	Inhibition of mycelial growth of *P. capsici* by purified non-volatile compounds of *T. virens* IISR 1370
Plate 4.10 e-f	Inhibition of mycelial growth of *P. capsici* by purified non-volatile compounds of *T. aureoviride* IISR 126
Plate 4.10 g-h	Inhibition of mycelial growth of *P. capsici* by purified non-volatile compounds of *T. aureoviride* IISR 140

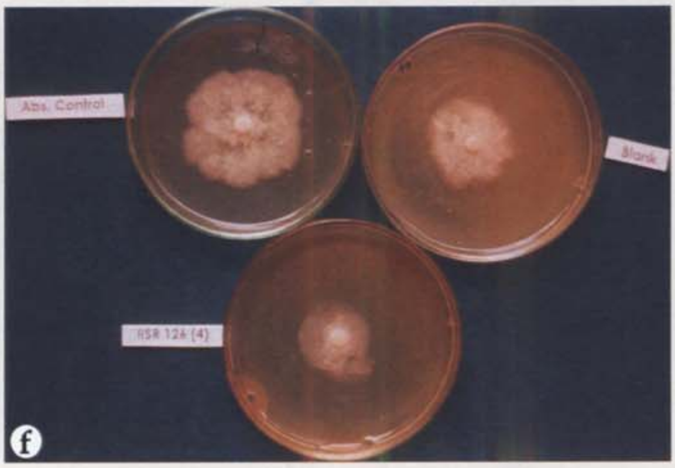

Abs. Control
Blank
(1)
(2)
(3)
(4)
IISR 1367
(5)
(6)
a

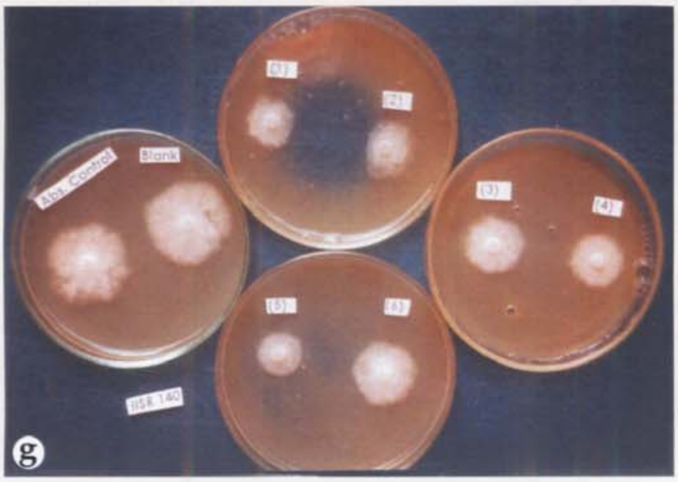

Abs. Control
Blank
IISR 1370 (5)
b


Abs. Control
Blank
IISR 1370 (3)
IISR 1370 (4)
c


Abs. Control
Blank
IISR 1370 (1)
IISR 1370 (2)
d

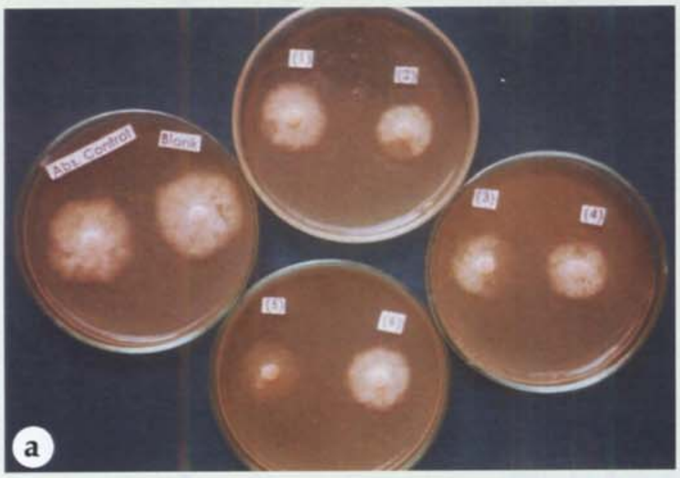

Abs. Control
Blank
IISR 126 (2)
IISR 126 (3)
e


Abs. Control
Blank
IISR 126 (4)
f


(1)
(2)
Abs. Control
Blank
(3)
(4)
(5)
(6)
IISR 140
g

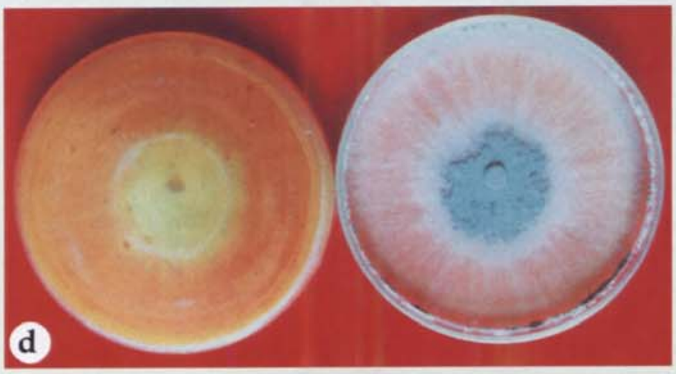

(7)
(8)
Abs. Control
Blank
(9)
(10)
(11)
(12)
IISR 140
h

Plate 4.11 a-b	Inhibition of mycelial growth of *P. capsici* by purified non-volatile compounds of *T. aureoviride* IISR 148
Plate 4.11 c	*In-vitro* inhibition of *P. capsici* by *Trichoderma* spp in dual culture, *T. pseudokoningii* IISR 186 (top left), *Trichoderma* sp. IISR 1141 (bottom left) and *T. hamatum* IISR 160 (bottom right)
Plate 4.11 d	Colony morphology and culture characteristics of *T. pseudokoningii* IISR 186
Plate 4.11 e	Colony morphology and culture characteristics of *T. harzianum* IISR 1369 and *T. virens* IISR 1370
Plate 4.11 f	Colony morphology and culture characteristics of *T. aureoviride* IISR 126, *T. aureoviride* IISR 143 *and T. aureoviride* IISR 148
Plate 4.11 g	Colony morphology and culture characteristics of *T. harzianum* IISR 173, *Trichoderma sp.* IISR 1141 and *T. harzianum* IISR 165
Plate 4.11 h	Colony morphology and culture characteristics of *T. hamatum* IISR 160, *Trichoderma sp.* IISR 1107 *and T. hamatum* IISR 150


Abs. Control
Blank
(1)
(2)
(3)
(4)
(5)
(6)
a


Abs. Control
Blank
(7)
(8)
(9)
blank
b


P. capsici X T.
IISR 186
c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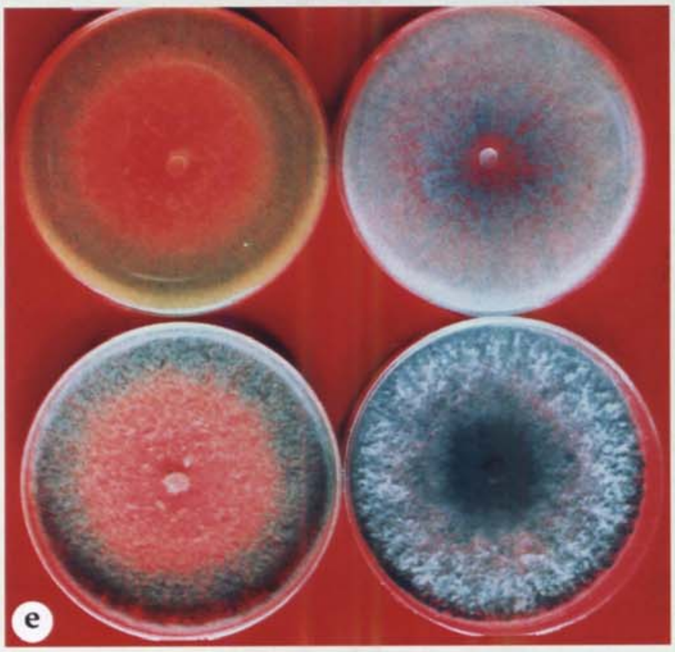
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STRAIN IMPROVEMENT

4.9 Strain improvement of *Trichoderma* using protoplast fusion technology

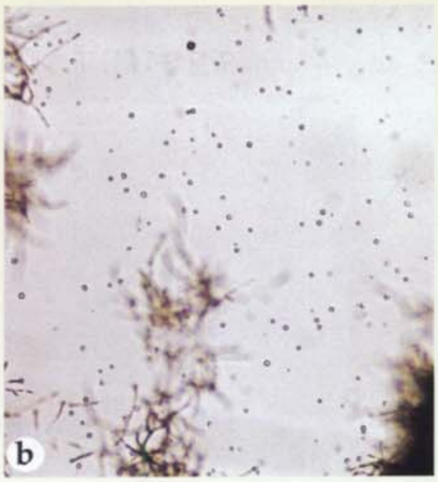
The potential isolate *T. harzianum* IISR 1369 grows well on solid substrates and in liquid cultures with high number of colony forming units. Culture filtrate of another isolate *T. aureoviride* IISR 140 is highly inhibitory to *P. capsici* than that of *T. harzianum* IISR 1369 but it is unable to grow well and sporulate on solid substrates and in liquid cultures. An attempt has been made to combine these two characteristics using protoplast fusion technology.

T. harzianum IISR 1369 released a maximum of 115 X 10^4 protoplasts per ml of the enzyme-osmoticant solution at room temperature (26-28^{0}C) and *T. aureoviride* IISR 140 released 95 X 10^4 protoplasts / ml (Plate 4.12 a - d). When it was incubated at 21-23^{0}C, protoplasts were not released.

After incubation for half an hour in fusion mixture, heterokaryons / homokaryons were observed under the microscope (Plate 4.12 e-n). Fusants were isolated from 30, 40 and 50% PEG used. Several colonies were found on PDA plates containing NaCl as osmoticant from the fusant treatments. Also colonies were found on PDA from either the mixed parental types without PEG / $CaCl_2$ or from the parental types incubated alone in the fusion buffer. Plates were completely covered with fungal colonies in all treatment, with colonies of both parental type visible on plates from mixed cultures either with or without PEG treatment indicating that protoplasts of both species were viable. Protoplasts started germination after 12 hours. It has divided continuously and formed into a mass (Plate 4.12 o). After 48 hours hyphal tips were noticed around dividing protoplast mass (Plate 4.13 a-c). After 72 hours colonies could be seen on the plates and were whitish in colour. Later, colonies with yellow pigment underside were marked and sub cultured.

Plate 4.12 a	*T. harzianum* IISR 1369 (left) and *T. aureoviride* IISR 140 (right) taken for protoplast fusion
Plate 4.12 b	Release of protoplasts from *T. harzianum* IISR 1369 (X 40)
Plate 4.12 c-d	Release of protoplasts from *T. aureoviride* IISR 140 (X 40)
Plate 4.12 e	Protoplast fusion - homofusants / heterofusants (X 200)
Plate 4.12 f	Protoplast fusion - homofusants / heterofusants (X 100)
Plate 4.12 g	Protoplast fusion (multiple) - homofusants / heterofusants (X 200)
Plate 4.12 h	Protoplast fusion (Triple) - homofusants / heterofusants (X 200 Phase Contrast)
Plate 4.12 i	Protoplast fusion (Double) - homofusants / heterofusants (X 100 Phase Contrast)
Plate 4.12 j	Protoplast fusion in progress (Double) - homofusants / heterofusants (X 100 Phase Contrast)
Plate 4.12 k	Protoplast fusion (Double) - homofusants / heterofusants (X 200 Phase Contrast)
Plate 4.12 l	Protoplast fusion (Double) - homofusants / heterofusants (X 200 Phase Contrast)
Plate 4.12 m	Protoplast fusion (Double) - homofusants / heterofusants (X 100)
Plate 4.12 n	Protoplast fusion (Double) - homofusants / heterofusants (X 100 Phase Contrast)
Plate 4.12 o	Regeneration of protoplast – dividing mass of protoplast.

a



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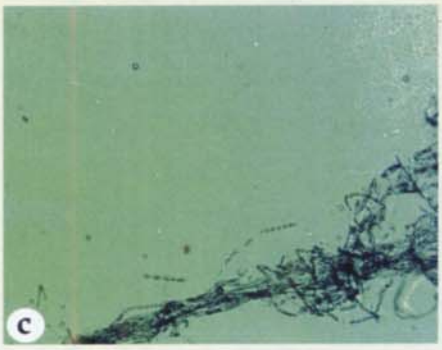

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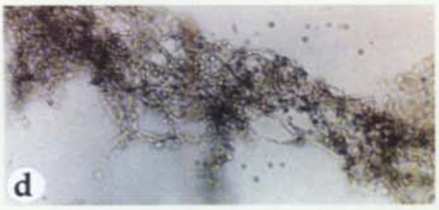

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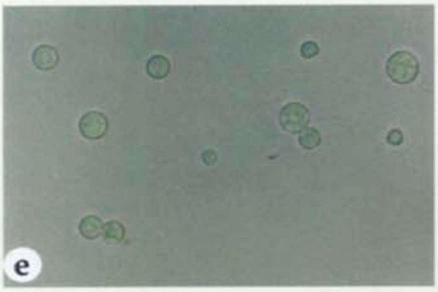

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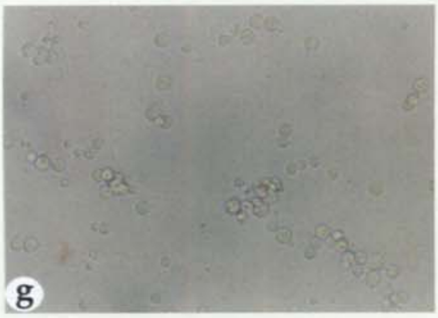

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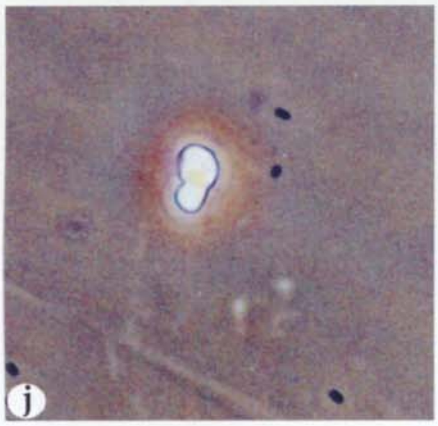

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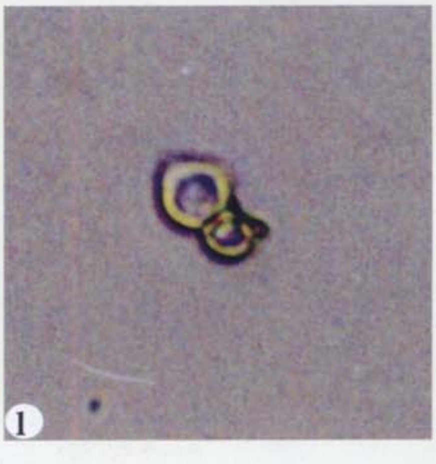

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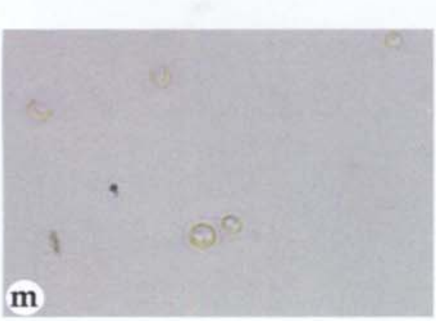

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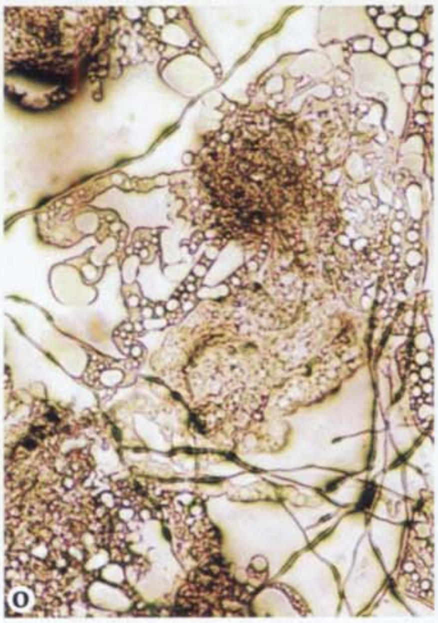


o

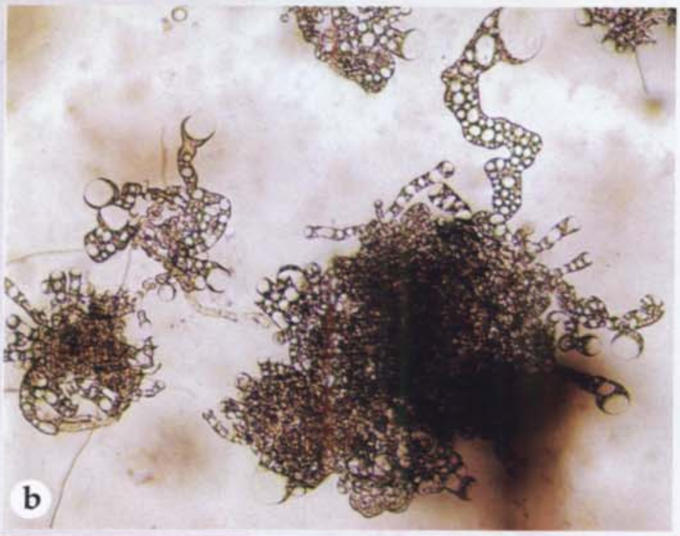
They were relatively smaller than the wider white colonies. The white colonies were turned to green later indicating that they were either protoplast regenerated colonies or homofusants of *T. harzianum* IISR 1369. There were 32 colonies selected with yellow pigmentation underside. They were designated as FT1......FT32 (fusant *Trichoderma*).

During sub-culturing for 10 generation continuously some of them looked same as that of *T. harzianum* IISR 140 or some others that is intermediate in colony morphology (Plate 4.13 d-e) reverted to their parental types. Out of 32, seven colonies were retained their intermediate colony morphology with varying degrees of dark pigmentation (secondary metabolites) on reverse side of the culture plate. They were FT 9, FT 11, FT 14, FT 15, FT 16, FT 17 and FT 21 (Plate 4.13 f-h). Their colony colours were varied from greenish yellow to hyaline, which turn green later with dark reverse side as compared to parents (Plate 4.13 d-e). Their growth, colony, spore characters were recorded by growing them on PDA over a period of 15 days (Table 4.20).

Dual plate experiments showed that percent inhibition of *P. capsici* by fusant *Trichoderma* varied from 32.14 to 39.64% (Table 4.21). FT 15 recorded highest percentage of inhibition of *P. capsici* followed by FT 11, FT 17 and FT 21(Plate 4.14 a).

Plate 4.13 a-c	Hyphal tip formation from dividing protoplast mass
Plate 4.13 d	Colony morphology of *T. harzianum* IISR 1369 and *T. aureoviride* IISR 140
Plate 4.13 e	Pigmentation reverse side of the colony of *T. harzianum* IISR 1369 and *T. aureoviride* IISR 140
Plate 4.13 f-h	Colony morphology and pigmentation of protoplast fusants between *T. harzianum* IISR 1369 and *T. aureoviride* IISR 140 (**f** FT 9 (left), FT 11 (middle), FT 14 (right); **g** FT 15 (left), FT 16 (right); **h** FT 17 (left), FT 21 (right)


a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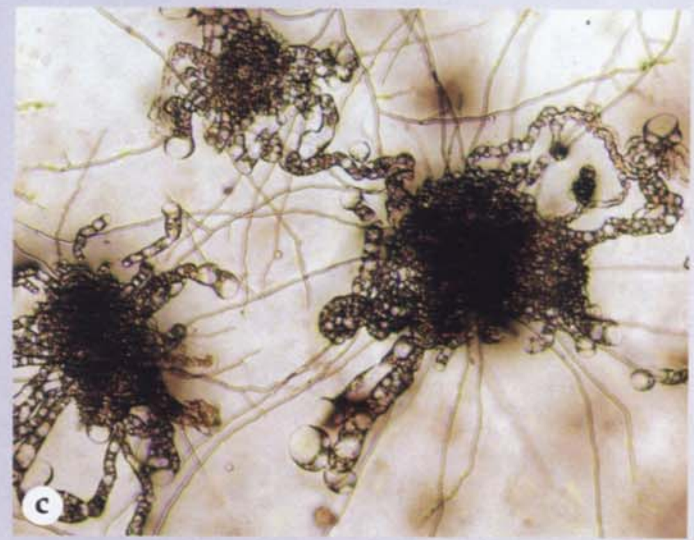
f
FT 9
FT 11
FT 14


c


FT 15
F 16
g


d


FT 17
FT 21
h

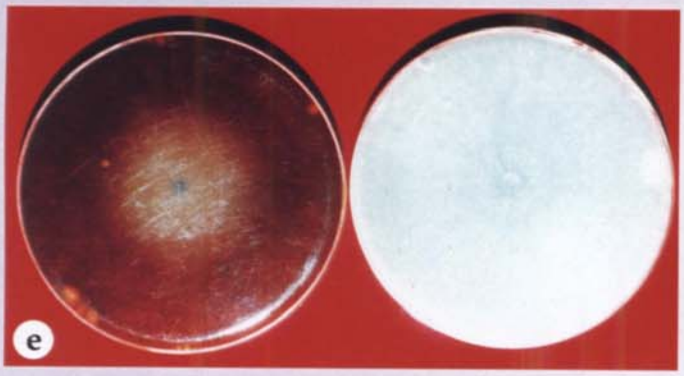

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Table 4.20 Morphological characteristics of protoplast fusion progeny of *T. harzianum* IISR 1369 and *T. aureoviride* IISR 140

Parent/Fusant	Characteristics
Parents	
T. harzianum IISR 1369	Colony whitish green initially gradually turns to green and then to dark green with tuft of abundant green spores, reverse side whitish
T. aureoviride IISR 140	Colony yellowish, adhering towards the agar surface, spores, yellow less in number, reverse side dark red
Fusants	
FT 9	Colony greenish, initially more sporulation towards the margin than the center which becomes uniform later, spores green, pale yellow reverse side which turn dark
FT 11	White colony gradually turn green, hyaline mycelia still visible, thick mycelial mat, heavy sporulation, mature spores green, pale yellow reverse side
FT 14	Light green colony gradually turn dark green, spores hyaline which turn dark green later, pale underside reverse side turning dark
FT 15	Yellowish white colony, hyaline / yellow spores which turn green, thick mycelial mat, dark reverse side which turn darker
FT 16	Colony greenish, dark green spores, heavy sporulation, pale yellow reverse side
FT 17	Colony hyaline, tuft mycelial mat, sporulation less, light green spores dark yellow reverse side which turn darker
FT 21	Colony greenish, green spores, heavy sporulation, pale yellow reverse side

Table 4.21 *In-vitro* screening of protoplast fusion progeny for inhibition of *P. capsici*

Fusant *Trichoderma*	Percent inhibition of *P. capsici*
T. harzianum IISR 1369	35.62
T. aureoviride IISR 140	30.65
FT 9	38.81
FT 11	41.54
FT 14	29.65
FT 15	43.54
FT 16	35.62
FT 17	41.54
FT 21	37.62
CD at 5%	1.257

4.9.1 Secondary metabolites production by fusant *Trichoderma*

Non-volatiles of fusant *Trichoderma* was obtained by growing them in czapek dox broth. Ethyl acetate extract of culture filtrate was tested for inhibition of *P. capsici in-vitro*. The concentrated ethyl acetate extract of broth culture of FT 9, FT 14, FT 15, FT 16, and FT 17 inhibited (100%) *P. capsici* at 50 µl concentration and that of FT 11 and FT 21 inhibited (100%) at 10 µl concentration (Table 4.22) (Plate 4.14 b-h).

4.9.2 Thermo stability of ethyl acetate extract of culture filtrate of fusant *Trichoderma*

The steam heated EtOAc fractions were able to inhibit *P. capsici* completely up to 48 hours even though it was not lethal to *P. capsici*. It has found started growing at 72 hours (Plate 4.15 a). However, secondary metabolites of FT 11 inhibited the growth of *P. capsici* completely showing that it is thermo stable (Table 4.23).

Table 4.22 Effect of ethyl acetate extract of culture filtrate of fusant *Trichoderma* on the growth of *P. capsici* in agar culture, measured as colony diameter (mm)

Fusant	Quantity (μl)	Growth period (days at 26^{0}C) 1	2	3	% inhibition
FT 9	10	0	12.83	30.0	32.06
	20	0	16.5	27.33	33.87
	40	0	16.66	22.83	38.56
	50	0	0	0	100
FT 11	10	0	0	0	100
	20	0	0	0	100
	40	0	0	0	100
	50	0	0	0	100
FT 14	10	19.67	32.33	37.5	15.08
	20	16.17	24.83	32.16	22.18
	40	16.67	29.33	27.0	27.34
	50	0	0	0	100
FT 15	10	15.83	27.83	39.5	10.55
	20	12.17	26	33.5	18.94
	40	10.67	22	30.0	19.26
	50	0	0	0	100
FT 16	10	18.83	35	43.16	2.26
	20	13	29.33	39.66	4.04
	40	9.67	23.66	32.5	12.54
	50	0	0	0	100
FT 17	10	18	36.66	43.66	1.13
	20	11.83	26.16	35.33	14.51
	40	10	26	30.0	19.26
	50	0	0	0	100
FT 21	10	0	0	0	100

continued

Table continued

Fusant	Quantity (µl)	Growth period (days at 26^{0}C) 1	2	3	% inhibition
	20	0	0	0	100
	40	0	0	0	100
	50	0	0	0	100
Ethyl acetate	10	20.17	34.33	44.16	
	20	18.17	32	41.33	
	40	16.17	27.16	37.16	
	50	16.67	27.16	37.16	
Absolute control		22.5	36.0	50.5	
CD at 5%		0.44	0.865	0.976	

Table 4.23 Effect of steam heated ethyl acetate extract of culture filtrate of fusant *Trichoderma* on the growth of *P. capsici* in agar culture, measured as colony diameter

Fusant	Quantity (µl)	Growth period (days at 26^{0}C) 1	2	3	% inhibition
FT 9	100	0	9	11.83	67.58
FT 11	100	0	0	0	100
FT 14	100	0	10	11.83	67.58
FT 15	100	0	9	11	69.86
FT 16	100	0	8	10	72.60
FT 17	100	0	8	10	72.60
FT 21	100	0	8	10	72.60
Ethyl acetate	100	18	27.16	36.5	
Absolute control		22.5	36	50.5	
CD at 5%		0.167	0.394	0.326	

Plate 4.14 a *In-vitro* screening of protoplast fusants between *T. harzianum* IISR 1369 and *T. aureoviride* IISR 140

Plate 4.14 b Inhibition of *P. capsici* by concentrated ethyl acetate extract of culture filtrate of FT 9

Plate 4.14 c Inhibition of *P. capsici* by concentrated ethyl acetate extract of culture filtrate of FT 11

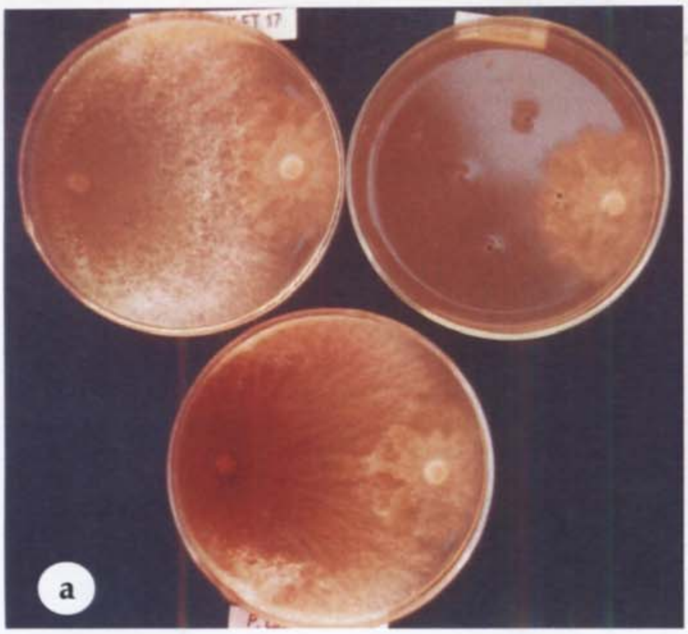
Plate 4.14 d Inhibition of *P. capsici* by concentrated ethyl acetate extract of culture filtrate of FT 14

Plate 4.14 e Inhibition of *P. capsici* by concentrated ethyl acetate extract of culture filtrate of FT 15

Plate 4.14 f Inhibition of *P. capsici* by concentrated ethyl acetate extract of culture filtrate of FT 16

Plate 4.14 g Inhibition of *P. capsici* by concentrated ethyl acetate extract of culture filtrate of FT 17

Plate 4.14 h Inhibition of *P. capsici* by concentrated ethyl acetate extract of culture filtrate of FT 21


FT 17
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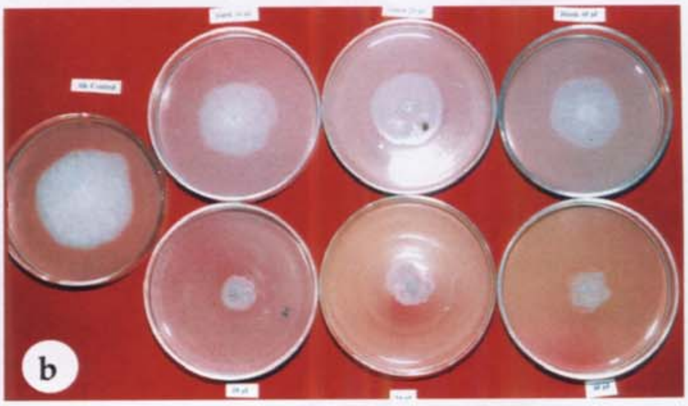
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c
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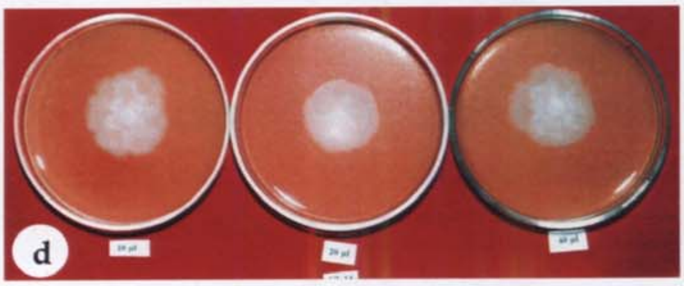
40 µl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10 µl
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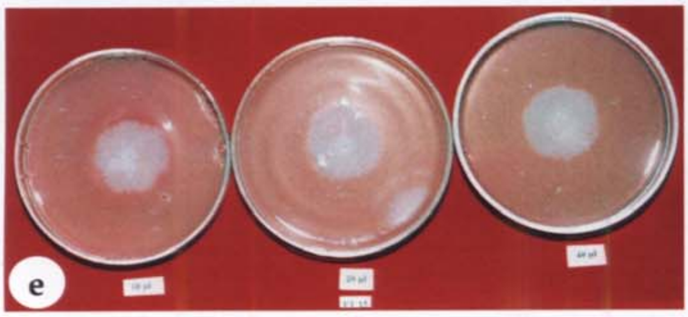
40 µl


e
10 µl
20 µl
40 µl


f
10 µl
20 µl
40 µl


g
10 µl
20 µl
40 µl


h
10 µl
20 µl
40 µl

4.9.3 Effect of ethyl acetate extract of culture filtrate of fusant *Trichoderma* on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

Ethyl acetate extract of culture filtrate of fusant *Trichoderma* was tested for the effect on sporangia formation, zoospore liberation and zoospore germination of *P. capsici*. From 100 ml of culture filtrate, about 1 ml of EtOAc extract was obtained and this extract diluted to get 10, 100, 300, 400, 500, 1000, 2000 and 4000 ppm concentration of solutions in water. Increasing concentration of ethyl acetate extract of culture filtrate of FT 9 caused a decline in the number of sporangia formed. Percent reduction in the number of sporangia formed per microscopic field (X40) was varied from 7.54% at 10 ppm to 91.44% at 4000 ppm. Peripheral growth of mycelia from the disc was also reduced. Liberation of zoospores was 87.24% at 10 ppm and 43.97% at 4000 ppm as against 91.37% in the control. Percent germination of zoospores was 52.78% at 10 ppm, which reduced to zero at 300 ppm and above as against 97.22% germination in the control (Figure 4.48). Similar results were obtained with the ethyl acetate extract of culture filtrate of FT 11 where percent reduction in the number of sporangia varied from 10.37% at 10 ppm to 92.83% at 4000 ppm. Percent liberation of zoospores was 86.97% and 40.08% at 10 and 4000 ppm respectively. Percent germination of zoospores was 48.33% at 10 ppm, which was reduced to zero at 300 ppm and above (Figure 4.49). In the case of FT 14 percent reduction in the number of sporangia varied from 5.37% at 10 ppm to 93.63% at 4000 ppm. Percent liberation of zoospores was 88.06% and 42.06% at 10 and 4000 ppm respectively. Percent germination of zoospores was 66.67% at 10 ppm, which was reduced to zero at 300 ppm and above (Figure 4.50). When the extract of FT 15 was tested, percent reduction in the number of sporangia varied from 5.61% at 10 ppm to

89.87% at 4000 ppm. Percent liberation of zoospores was 88.95% and 33.92% at 10 and 4000 ppm respectively. Percent germination of zoospores was 80.83% at 10 ppm, which was reduced to zero at 300 ppm and above (Figure 4.51). Similarly for FT 16, percent reduction in the number of sporangia varied from 0% at 10 ppm to 92.96% at 4000 ppm. Percent liberation of zoospores was 83.50% and 38.37% at 10 and 4000 ppm respectively. Percent germination of zoospores was 73.89% at 10 ppm, which was reduced to zero at 300 ppm and above (Figure 4.52). In the case of FT 17 percent reduction in the number of sporangia varied from 12.41% at 10 ppm to 93.01% at 4000 ppm. Percent liberation of zoospores was 87.50% and 39.52% at 10 and 4000 ppm respectively. Percent germination of zoospores was 80.83% at 10 ppm, which was reduced to zero at 300 ppm and above (Figure 4.53). When the extract of FT 21 was tested, percent reduction in the number of sporangia varied from 4.91% at 10 ppm to 91.04% at 4000 ppm. Percent liberation of zoospores was 83.50% and 38.37% at 10 and 4000 ppm respectively. Percent germination of zoospores was 73.89% at 10 ppm, which was reduced to zero at 300 ppm and above (Figure 4.54).

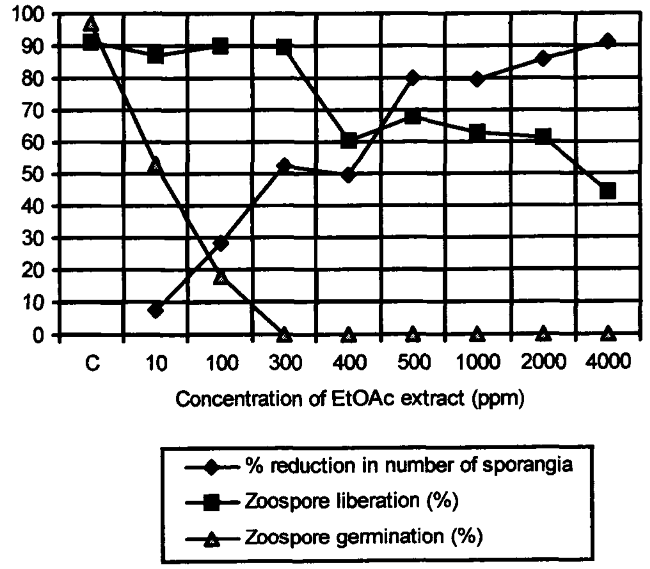


Figure 4.48 Effect of EtOAc extract of culture filtrate of FT 9 on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

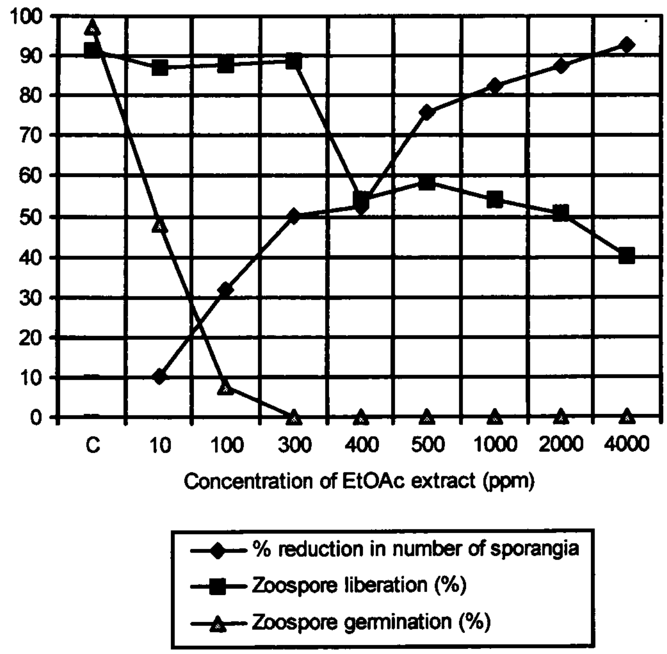


Figure 4.49 Effect of EtOAc extract of culture filtrate of FT 11 on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

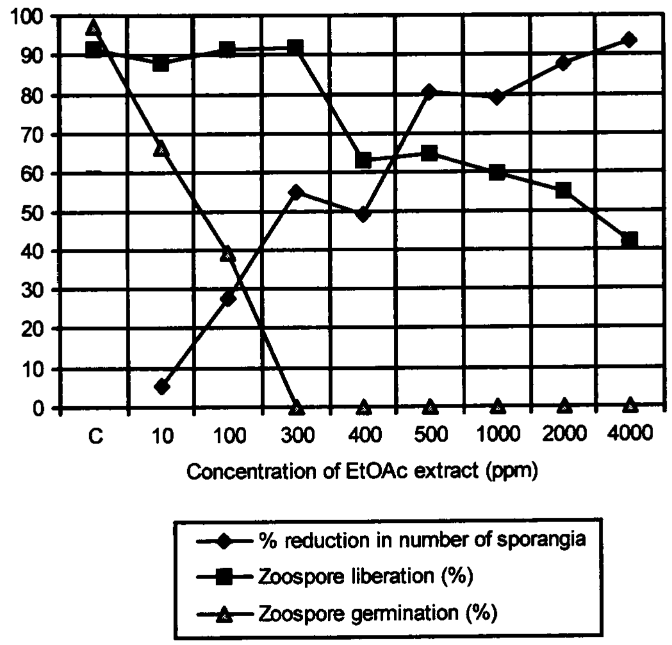


Figure 4.50 Effect of EtOAc extract of culture filtrate of FT 14 on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

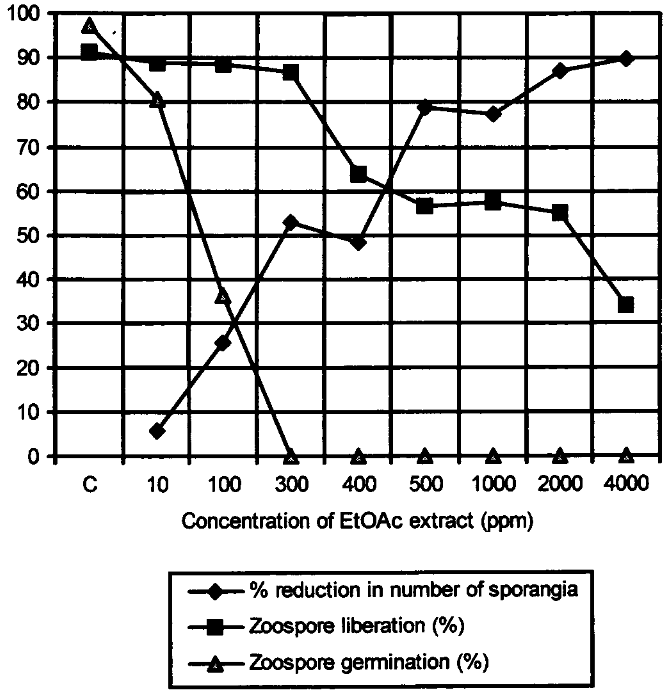


Figure 4.51 Effect of EtOAc extract of culture filtrate of FT 15 on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

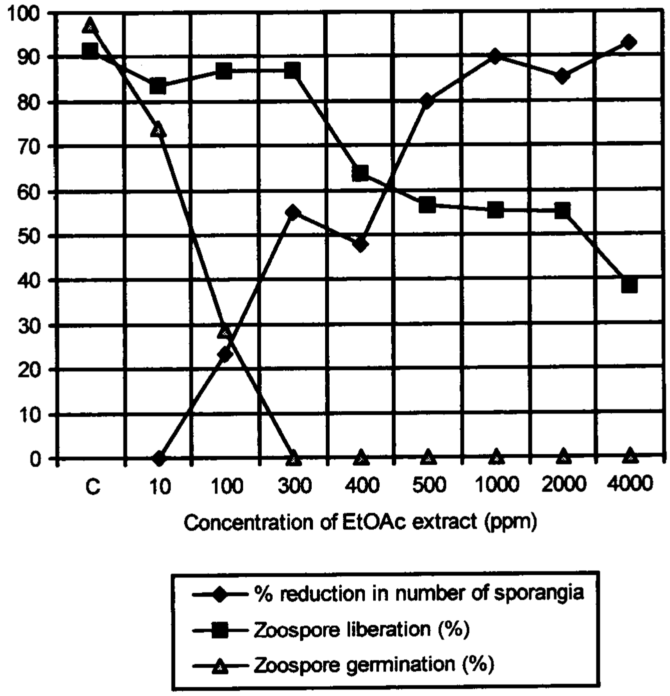


Figure 4.52 Effect of EtOAc extract of culture filtrate of FT 16 on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

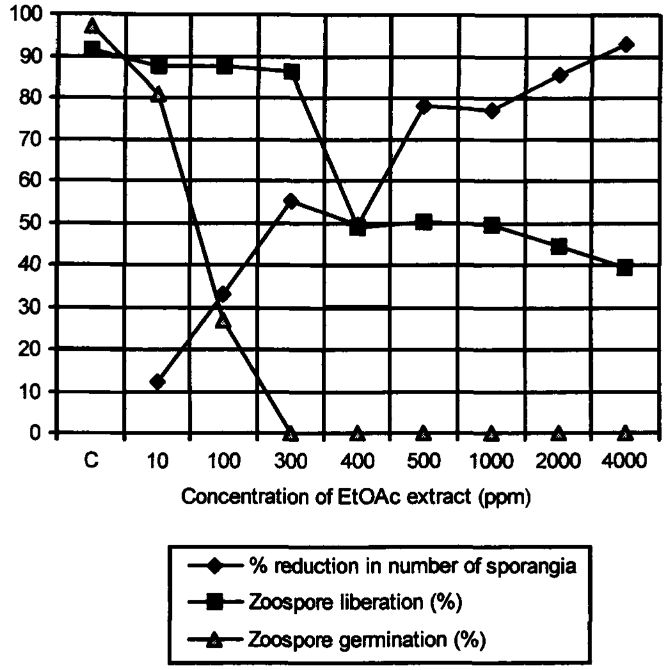


Figure 4.53 Effect of EtOAc extract of culture filtrate of FT 17 on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

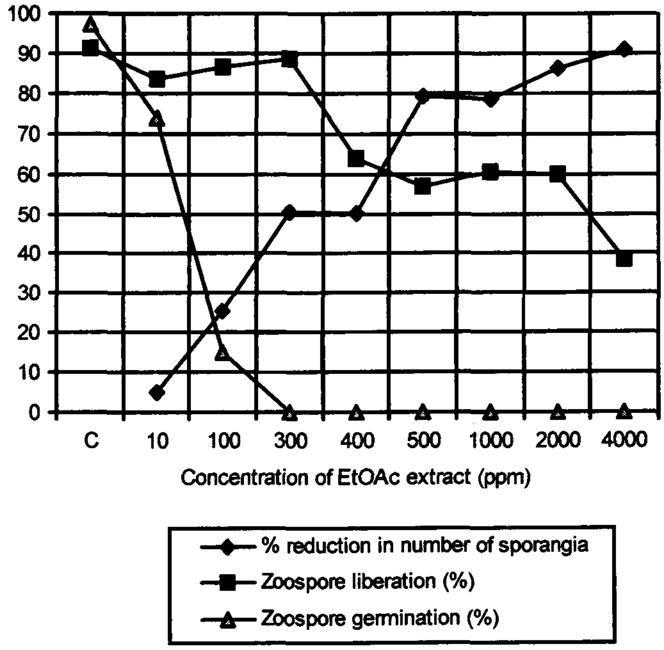


Figure 4.54 Effect of EtOAc extract of culture filtrate of FT 21 on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

DISEASE MANAGEMENT

4.10 Evaluation of of *Trichoderma* spp and *Pseudomonas fluorescens* for suppression of *P. capsici*

Field experiment was conducted to study the disease suppressive effect of *Trichoderma* spp and *Pseudomonas fluorescens* and some of their combinations in comparison with existing potent ones. The treatment with *T. harzianum* IISR 1369 + *P. fluorescens* IISR 41 recorded maximum plant height of 204.9 cm followed by *T. harzianum* IISR 1369 + *P. fluorescens* IISR 6 (199.0 cm) and *T. virens* IISR 1370 + *P. fluorescens* IISR 11 (192.6 cm) (Table 4.24). Single application of fungi like *T. harzianum* IISR 1369, *T. aureoviride* IISR 143, *T. pseudokoningii* IISR 187, *T. harzianum* IISR 167 were on par with *P. fluorescens* IISR 6 and combination of *T. virens* IISR 1370 + *P. fluorescens* IISR 13 (Table 4.24). In general, treatments applied with *Trichoderma* spp and *P. fluorescens* were recorded increase in plant height than the control.

Disease incidence was not noticed in any of the treatments including control hence the exact role of these biocontrol agents in disease suppression could not be studied at field level. However, *Phytophthora* was detected in all the various treatments at periods of interval by baiting. Population of *Trichoderma* in various treatments varied form 10^3…10^6 cfu / g of soil in dry and wet months. A decline in population in dry months and an increase in wet months was recorded. However, the pooled analysis of population over the years showed that *Trichoderma* was able to survive maintaining a population level of 10^4 cfu / g (Figure 4.55). A similar trend was observed in the case of *P. fluorescens* also (Figure 4.56). The population of *P. fluorescens* was higher than *Trichoderma*. Pooled analysis of population of other fungi also showed variation over the period (Figure 4.57).

In green house test, when challenge inoculated with *P. capsici,* treatments applied with *P, fluoreoscens* IISR 13 and *P. fluorescens* IISR 41 were highly disease suppressive showing only 10% disease incidence as compared to 90% in the control. Combination of *T. harzianum* IISR 1369 + *P. fluorescens* IISR 41, *T. harzianum* IISR 1369 + *P. fluorescens* IISR 6 and *T. virens* IISR 1370 + *P. fluorescens* IISR 13 were also on par with the above treatments (Table 4.24). Hence it is inferred that *P. fluorescens* IISR 41 and *P. fluorescens* IISR 13 can be utilized for the suppression of *P. capsici.* Combination of *T. harzianum* IISR 1369 + *P. fluorescens* IISR 41, *T. harzianum* IISR 1369 + *P. fluorescens* IISR 6 and *T. virens* IISR 1370 + *P. fluorescens* IISR 13 are disease suppressive and promote growth of black pepper (Plate 4.15 b-d). Since the combination of *T. harzianum* IISR 1369 + *P. fluorescens* IISR 41 has given maximum plant growth and less disease, it can be recommended as a strategy for disease management of foot rot of black pepper.

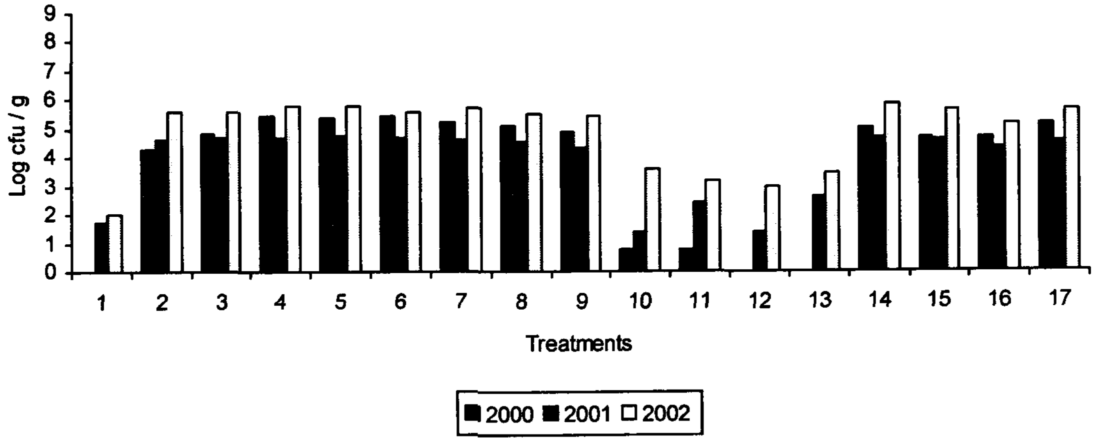


Figure 4.55 Population of *Trichoderma* applied to black pepper in the field.

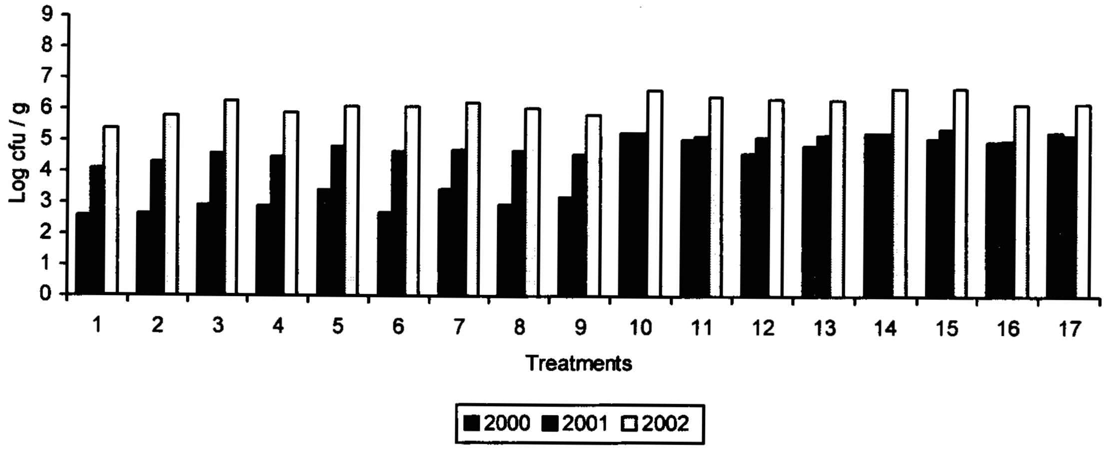


Figure 4.56 Population of *P. fluorescens* applied to black pepper in the field

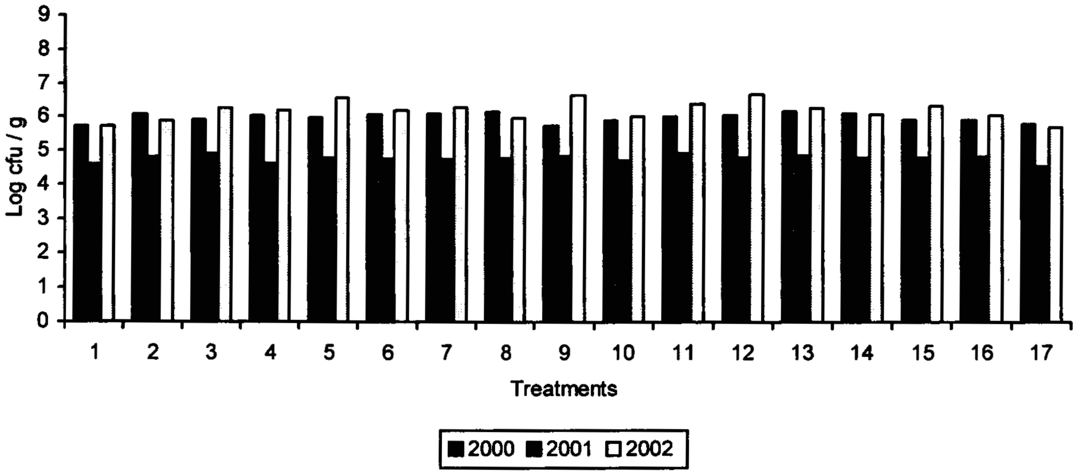


Figure 4.57 Population of other fungi in black pepper field applied with *Trichoderma* and *P. fluorescens*

Table 4.24 Effect of *Trichoderma* spp and *P. fluorescens* on the growth of black pepper and suppression of *P. capsici*

Sl No.	Treatment	Increase in plant height (cm)*	% incidence of of root rot**
1	Control	149.0 $_{fg}$	90
2	*T. virens* IISR 112	157.8 $_{ef}$	30
3	*T. virens* IISR 18	144.8 $_{g}$	40
4	*T. harzianum* IISR 1369	175.1 $_{c}$	30
5	*T. virens* IISR 1370	171.7 $_{cd}$	30
6	*T. aureoviride* IISR 143	180.7 $_{c}$	20
7	*T. pseudokoningii* IISR 187	175.8 $_{c}$	50
8	*T. harzianum* IISR 167	175.9 $_{c}$	40
9	*T. harzianum* IISR 178	157.3 $_{ef}$	40
10	*P. fluorescens* IISR 11	150.5 $_{fg}$	20
11	*P. fluorescens* IISR 13	164.3 $_{de}$	10
12	*P. fluorescens* IISR 41	165.4 $_{de}$	10
13	*P. fluorescens* IISR 6	176.1 $_{c}$	30
14	*T. harzianum* IISR 1369 + *P. fluorescens* IISR 41	204.9 $_{a}$	10
15	*T. harzianum* IISR 1369 + *P. fluorescens* IISR 6	199.0 $_{ab}$	10
16	*T. virens* IISR 1370 + *P. fluorescens* IISR 13	179.3 $_{c}$	10
17	*T. virens* IISR 1370 + *P. fluorescens* IISR 11	192.6 $_{b}$	20

Values followed by same letter(s) in a column do not differ significantly according to Duncan's multiple range test at 5% level

* Data from field experiment

** Data from challenge inoculation of black pepper with *P. capsici* in the glass house (Number of plants showing root rot)

Plate 4.15 a Inhibition of *P. capsici* by steam heated ethyl acetate extract of culture filtrate of fusant *Trichoderma* in agar culture

Plate 4.15 b Growth of black pepper in the glass house after application of *T. harzianum* IISR 1369 + *P. fluorescens* IISR 41in comparison with control

Plate 4.15 c Growth of black pepper in the glass house after application of *T. harzianum* IISR 1369 + *P. fluorescens* IISR 6 in comparison with control

Plate 4.15 d Growth of black pepper in the glass house after application of *T. virens* IISR 1370 + *P. fluorescens* IISR 11 in comparison with control


Abs. Control
Blank 100 µl
FT 9
FT 11
FT 14
FT 15
FT 16
FT 17
Steam sterilized
FT 21
a


b


c


d

5

DISCUSSION

The present investigation was aimed to understand the mode of action of *Trichoderma* especially of volatiles and non-volatiles and the ecological factors responsible for their long-term survival and activity in soil for sustainable disease management of *Phytophthora* foot rot / root rot in black pepper. Studies on secondary metabolites partly explain the biocontrol mechanism of *Trichoderma* spp through antibiosis, plant growth promotion etc. Data on abiotic factors that influence the inundative biocontrol organisms is a prerequisite for large-scale field demonstration and adoption. Black pepper is cultivated in soils, which are acidic in nature (5.0-6.5) with adequate organic matter content. It is grown as a pure crop as well as mixed crop both in the plains as well as in high altitudes up to 3000-3500 ft MSL. The edaphic factors vary in different agro climatic regions, which will have profound impact on the soil microflora. Ecological fitness of the biocontrol agents with a background knowledge on the pathogen biology and crop cultivation would help in standardization of strategies for greater adoption and functioning of biocontrol agents for disease management in spice crops.

Thirty isolates of *Trichoderma* spp were screened for their effect of volatiles and non-volatiles on the growth of *P. capsici*. Nine isolates were identified as superior over the others in the inhibition of *P. capsici* by volatiles and eight were found as superior in the inhibition of *P. capsici* by non-volatiles. Out of the above seventeen short-listed isolates three were both volatile and non-volatile producers. Therefore, the resulting fourteen isolates were taken for *in-vitro* and *in-vivo* studies.

IN-VITRO STUDIES

In-vitro* interactions between *Trichoderma* spp and *Phytophthora capsici

Out of the several antagonistic mechanisms described for *Trichoderma* in the biocontrol of plant pathogens, mycoparasitism is usually studied in *in-vitro* conditions. Among the fourteen isolates of *Trichoderma* studied *in-vitro*, six of them showed antagonistic interactions with *P. capsici*. *In-vitro* interactions observed involve parallel growth of hyphae of *Trichoderma* and *Phytophthora*, penetration of hyphae of *P. capsici* and coiling of hyphae of *P. capsici* by *Trichoderma* spp. Apart from this, hyphal evacuation, sporangial parasitism, sporangial deformation and shortening of hyphal tips were observed with *P. capsici* after pairing with *Trichoderma*. However, little evidence exists for these types of interactions in soil. Parasitism occurs on account of lytic enzymes and parasitic isolates may be good in enzyme production, which can degrade the pathogen cell walls. Isolates with sporangial parasitism are important as they prevent the most explosive phase, the indirect germination of sporangia. Few reports are available on the parasitism of *Phytophthora capsici* by *Trichoderma* spp (Sid Ahmed *et al.*, 1999). However, *in-*

vitro interactions between *Trichoderma* spp and *Pythium aphanidermatum* were studied. These include coiling, penetration, hyphal evacuation of the pathogen etc (Balakrishnan, 1997).

IN-VIVO STUDIES

In-vivo effect of *Trichoderma* spp on black pepper plants

Fourteen isolates of *Trichoderma* spp were tested *in-vivo* for their effects on the host plant. *In-vivo* effect of *Trichoderma* spp on black pepper recorded growth promotion, which varied from 68 to 128%. Plant height was maximum when *T. harzianum IISR* 1369, *T. hamatum* IISR 151, *T. hamatum* IISR 160 and *T. pseudokoningii* IISR 186 were applied. Shoot biomass increased when *Trichoderma* was applied compared to root biomass, which was uniform among various treatments. Two mechanisms have been put forward most frequently to explain the nature of the increased growth response induced by certain members of the soil micro-flora. The first is the enhanced growth of plants induced by biocontrol agents is considered predominantly through suppression of minor pathogens (Kloepper and Schroth, 1979; Salt, 1978; Sivan *et al.*, 1984). The second hypothesis is that a microbial agent produces growth-regulating metabolites. Sivasithamparam and Ghisalberti (1998) listed several such metabolites from *Trichoderma.* When challenge inoculated with *P. capsici* five of the *Trichoderma* isolates viz., *T. aureoviride* IISR 126, *T. aureoviride* IISR 140, *T. aureoviride* IISR 148, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 were able to suppress the disease where 20% of the plants showed mortality as against 100% in the control. Disease suppression is more important rather than growth promotion of host plant because this can be easily achieved by routine agronomic inputs. However biocontrol agents also provide induced systemic resistance (ISR) protecting the crop from pathogen attack. Hence isolates with high suppression of *P. capsici* are promising. Here two *T. harzianum* and three *T. aureoviride* isolates were identified to suppress *P. capsici*. But no single isolate possess characteristics responsible for disease suppression and growth promotion. It implies that characteristics of disease suppression and growth promotion are distributed in different isolates. However disease suppressive isolates identified here also possess average ability (70.66 – 107.33% increase in plant height over control) for growth promotion. The success of biocontrol with these agents needs field evaluation. Fravel (1988) estimated that only 5% of the potential biocontrol agents chosen from screening tests actually worked in field trials. Even when using tests plants unless the culture conditions mimic natural conditions, the potential of the antagonist can be over or under estimated. Since non-sterile soil was used in this experiment the results are indication of natural performance of *Trichoderma* spp.

Rhizosphere competence of *Trichoderma* spp

The ability to colonise rhizosphere is crucial for bacteria or fungi to function as biocontrol agents for soil borne plant pathogens (Milus and Rothrock, 1993). As described earlier, rhizosphere competence is the ability of a microorganism to grow and function in the developing rhizosphere (Ahmad and Baker, 1987). The present rhizosphere competence modified assay method using black pepper is developed to improve measurement in time and space of the activity of potential rhizosphere inhabitants with ease.

If *Trichoderma* spp is applied during the planting time it is thought that they would be able to colonize the roots in large numbers. This is true in the case of many species of *Trichoderma* isolates as shown in Figure 4.1. *T. harzianum* IISR 1369, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140, *T. aureoviride* IISR 148, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 colonised the roots in higher numbers when applied during planting. *T. hamatum* IISR 150, *T. hamatum* IISR 151 and *T. hamatum* IISR 160 colonized the roots significantly in higher numbers when applied after planting. Colonisation of *T. virens* IISR 1370, *T. aureoviride* IISR 143 and *T. pseudokoningii* IISR 186 and *Trichoderma* sp. IISR 1141 was almost same at both the conditions of applications.

The propagules of *T. aureoviride* IISR 126, *T. aureoviride* IISR 140, *T. aureoviride* IISR 148, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 colonised the roots significantly in higher numbers (log cfu / g= >5). Rhizosphere competence ability of *T. harzianum* IISR 1369 is varied when applied during planting (log cfu / g = 4.6) and after one month of planting (log cfu / g = 3.4). However, colonization when applied during planting and after planting do not differ much in the case of native bacteria, actinomycetes and other fungi.

Since non-sterilized soil was used the system allowed rhizosphere competence to be measured on the basis of population density of *Trichoderma* spp as a function of rhizosphere soil. Rhizosphere competence is directly correlated with the ability to utilize host exudates and proliferate in the rhizosphere. Rhizosphere competent biocontrol agents are potentially more effective because they protect not only the root but also the root rot (Milus and Rothrock, 1993). They partially eliminate the problem of adding large amounts of thalli to induce suppressiveness because substrates provided by the host can support their activity. Organic matter also found to increase the population of *T. harzianum* IISR 1369 (Saju *et al.*, 1999; 2002b).

Therefore applying an organic base can stimulate the proliferation of rhizosphere microflora.

Effect of abiotic factors in relation to survival of *Trichoderma* spp

Understanding environmental parameters that control the survival and activity of introduced biocontrol agents are often less studied and understood. These ecological factors are basis for predicting the performance and modifying the soil environments to some extent for better activity of biocontrol agents to obtain an acceptable level of disease biocontrol. Very often farmers ask these questions rather before scientists look into the problems. Hence background knowledge on ecological factors that influence biocontrol agents are essential.

Effect of soil type on the survival of *Trichoderma* spp

Different soil types viz., clayey (Calicut), sandy clay (Peruvannamuzhi) and sandy loam (Thamarassery) did not influence significantly the survival of any of the *Trichoderma* isolate studied. Instead, after a decline during the first 30 days, the population remained relatively unchanged during the course of experiment. The soil possessed a moisture holding capacity of 71.83, 70.11 and 68.69% respectively. This indicated that the texture of the soil alone during the course of routine glass house raising of cuttings would not affect the survival of *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173. However soil organic matter content was found to be a crucial factor for increasing the population of *Trichoderma* spp in soil (Knudsen and Bin Li, 1990; Paulitz and Baker, 1987; Saju *et al*., 2000; 2002b). Paulitz and Baker (1987) recorded increase in population of *Pythium nunn,* which reduced the damping-off of cucumbers induced by *Pythium ultimum.*

Soil moisture effects on the survival of *Trichoderma* spp

Propagules of *T. harzianum* IISR 1369 and *T. harzianum* IISR 173 survived best in moisture levels of 30-60% and that of *T. virens* IISR 1370, *T. aureoviride* IISR 126 and *T. harzianum* IISR 165 at 20-60%. However none of the moisture levels tested caused a complete disappearance of *Trichoderma* propagules. Higher percentage of moisture caused decrease in population but it has maintained a population of 10^2-10^3 cfu / g during the experimental time.

In field experiments highest population of *Trichoderma* was recorded during wet season (Eastburn and Butler, 1991; Rajan 1999; Rajan *et al.*, 2002). This is because soil moisture during wet season varies and do not remain uniform always. But in pot experiments the moisture levels are maintained for a longer period. This also explains that *Trichoderma* is able to survive even after heavy rain and higher matric potential. *T. harzianum* IISR 1369 and *T. harzianum* IISR 173 recorded population decline at 20% moisture level. However in this study, 60% and above moisture levels were found as saturated and therefore the decline in population occurred. At 20% moisture level, population decline occurred possibly because of desiccation of propagules and acute shortage of moisture for long time for activity.

Low water availability can inhibit microbial activity by lowering intracellular water potential and thus reducing hydration and activity of enzyme (Stark and Firestone, 1995). Eastburn and Butler (1991) made similar observation with *T. harzianum* and most active at higher matric potential. In a study, which did not include saturated condition (Eastburn and Butler, 1988a; 1988b), the population densities of *T. harzianum* positively correlated with increase in soil moisture at micro site level.

Danielson and Davey (1973a) found that population of *Trichoderma* were highest in wet hard wood forest soils in the southeastern United States and in Washington State. They also indicated that population of *Trichoderma* was more in a lake shore with saturated moisture level than away from the shore where the moisture content was less.

Soil temperature effects on the relative abundance of *Trichoderma* spp

The cfu of *Trichoderma* spp in soil as measured by serial dilution plate technique consisted of both residual propagules from the original inoculum and new propagules formed during the experiment. The effects of temperature on propagule density should therefore be interpreted as the sum of effects on the survival and activity phases of the life cycle of these fungi. The findings indicated that temperature was not a major factor affecting propagule density of *T. harzianum* IISR 1369, *T. aureoviride* IISR 126 and *T. harzianum* IISR 165 in soil. However *T. virens* IISR 1370 and *T. harzianum* IISR 173 were affected by temperature. Here different isolates of same species, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 behaved differently in response to temperature. *T. harzianum* IISR 1369, *T. aureoviride* IISR 126 and *T. harzianum* IISR 165 favoured by all the temperature ranges. Lower temperature ranges (<20^{0}C and 20-26^{0}C) favoured *T. virens* IISR 1370 in the present experiment with propagule densities in soil showing significant reduction at higher temperature (26-32^{0}C). In contrast higher temperature ranges (20-26^{0}C and 26-32^{0}C) favoured *T. harzianum* IISR 173 with significant reduction in propagule density at low temperature range (<20^{0}C). Effects of those temperatures on the *Trichoderma* spp were consistent with the putative effect of climate on the survival of these species. Temperature also has a profound effect on the production and activities of enzymes and antibiotics involved in biocontrol by *Trichoderma* species (Burpee, 1990). What occurs in a petri dish may not occur at all in the soil around a germinating seed (Howell, 2003). *T. stromaticum* produced abundant conidia on cocoa broom at 100% relative humidity and incubation temperature of 20 and 25^0C but none at 30^0C (Sanogo *et al.*, 2002). Their results suggested that applying *T. stromaticum* under high-moisture conditions where the air temperature is below 30^0C would enhance the establishment of this mycoparasite in cocoa plantations. The present study used a soil-plant system with modification form Saremi *et al.* (1999) rather than soil incubation studies (Adams, 1975; 1987; Masangkay *et al.*, 1999). Since the system mimicked natural conditions, temperature responses observed could be a case in field conditions.

Effect of pH on *Trichoderma* spp and *Phytophthora capsici* in culture

P. capsici was able to grow well in acidic pH. Its colony diameter was reduced at pH 7.5 and above. *T. harzianum* IISR 1369 and *T. harzianum* IISR 165 were rather insensitive to pH changes. *T. virens* IISR 1370 and *T. harzianum* IISR 173 favoured acidic pH *T. aureoviride* IISR 126 showed maximum growth at pH 6.5. In general, *Trichoderma* spp have wide plasticity and adaptability at different pH levels. However the pH changes in soil and survival of population of *Trichoderma* was not studied. Acidic pH favoured the growth and survival of *Trichoderma* (Chet and Baker, 1980; Mondal *et al.*, 1996; Trutmann and Keane, 1990). Incidentally acid conditions also favoured pathogen multiplication in the present study. However, fast growth, sporulation and establishment of *Trichoderma* spp may take over the momentum in soil when competition occurs between *P. capsici* and *Trichoderma* spp.

***In-vitro* toxicity of pesticides to *Phytophthora capsici* and *Trichoderma* spp**

P. capsici was sufficiently sensitive to metalaxyl, mancozeb, copper (10 ppm) and potassium phosphonate (100 ppm). Phorate increased the growth of *P. capsici* at 10 and 100 ppm concentrations. *P. capsisi* was sensitive to chlorpyriphos at 300 ppm and to quinalphos at 500 ppm. Metalaxyl was found as highly efficieint systemic fungicide for controlling *P. capsici* infecting black pepper (Ramachandran, 1990; Ramachandran and Sarma, 1985a; 1985b; 1989). However, fungitoxic effect of endosulfan and quinalphos on *Phytophthora palmivora* was observed (Anonymous, 1985; Ramachandran and Sarma, 1988).

The four fungal antagonists, *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 were sufficiently insensitive to metalaxyl, mancozeb, potassium phospohonate and copper. The only exception is *T. aureoviride* IISR 126 which has very low copper tolerance but insensitive to metalaxyl, mancozeb and potassium phosphonate. The *in-vitro* studies suggested that they could be successfully integrated with these pesticides in field situation without detrimental effect on the biocontrol agent. Working with the same isolate of *T. harzianum* IISR 1369 Stephen *et al.* (1999; 2000) found that the isolate was resistant to chlorpyriphos. Rajan and Sarma (1997) also reported compatibility of potassium phosphonate with different species of *Trichoderma* and *Gliocladium.* Carbendazim was toxic to *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 but insensitive to phorate. Quinalphos was toxic to *T. virens* IISR 1370, *T. harzianum* IISR 165 and *T.*

harzianum IISR 173 but less toxic to *T. harzianum* IISR 1369 and *T. aureoviride* IISR 126 (moderately tolerant). It is likely that growth of the biocontrol agent would be inhibited if they were exposed to field concentrations of these pesticides. *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 were moderately tolerant to chlorpyriphos.

Effect of simultaneous application of pesticides on the survival of *T. harzianum* IISR 1369 in soil

Since metalaxyl, mancozeb, copper and quinalphos up to 300 ppm did not affect the population of *T. harzianum* IISR 1369 in soil, the organism is relatively tolerant to field concentrations of above pesticides. Therefore these pesticides can be used for disease / pest control while using *T. harzianum* IISR 1369 for suppression of *P. capsici*. Compatibility of biocontrol agents with pesticides is of great advantage for disease control where a chemical spray is a must for some pathogen / insect control. Sawant and Mukhopadhyay (1991) integrated metalaxyl with *T. harzianum* for controlling *Pythium* damping off in sugar beet. Very often low concentration of chemicals makes the pathogen vulnerable for attack by biocontrol agents (Baker and Cook, 1974; Cook and Baker, 1983; Kay and Stewart, 1994). Therefore biocontrol agents with tolerance to pesticides would be of immense use in integrated disease management. The present lead needs to be exploited in IDM for black pepper.

Effect of organic and inorganic fertilizers on the survival of *T. harzianum* IISR 1369 applied to black pepper rhizosphere

Urea, Rock phosphate and Muriate of potash in recommended concentration for bush pepper did not harm the antagonist *T. harzianum* IISR 1369 in the rhizosphere of black pepper. Further, the population of *Trichoderma* in those treatments showed significant increase over control. *In-vitro* experiments of Jayaraj and Ramabadran (1988) showed that *T. harzianum* could utilize ammonium nitrate, ammonium sulphate and sodium nitrate evaluated at 2% concentration. Therefore soil application of *T. harzianum* IISR 1369 along with fertilizers can be recommended. Similarly, Jayaraj (1995) reported utilization inorganic sources of nitrogen by *T. harzianum* in *in-vitro* and *in-vivo* studies.

Organic fertilizers are often used and found enhancing the soil micro flora and micro fauna. They also enhanced the population of *Trichoderma.* However in the present study, dried leaves of *Glyricidia* did not cause significant increase in population. Saju *et al.* (2000; 2002b) observed increase of *T. harzianum* IISR 1369 in soil amended with organics but without the presence of host plant. This would help to maintain a stable population of biocontrol agents around the host plants throughout the season. The enzymes and antibiotics produced by *Trichoderma* species involved in biocontrol process are strongly influenced by the substrate on which the fungus is grown (Howell, 2003).

Application of *T. harzianum* to the soil as a wheat bran culture significantly reduced viability of *Armillaria* in woody blocks of inoculum (Otieno *et al.*, 2003). Soil amendment with coffee pulp also reduced the inoculum viability *Armillaria* but did not affect the incidence of *Trichoderma* in the blocks of inoculum. Thus direct application of wheat bran formulation of *T. harzianum* into soil surrounding woody *Armillaria* inoculum sources can suppress the pathogen. Organics such as farm yard manure, coir pith, coir pith compost and neem cake were found to be enhancing the population of *T. harzianum* IISR 1369 (Saju *et al.*, 2000: 2002b)

Studies on the abiotic factors that influence the biocontrol fungi have necessarily been dependant on indirect estimates of activity and number. This is primarily because direct observations of biocontrol fungi in soil are often impossible to make. Significant amount of variations were seen between essentially identical experiments and such variations most likely result from working with natural soil (Eastburn and Butler, 1991). In the present investigation, effect of soil type, temperature, pesticides and fertilizers were studied using natural soil. Even though environmental variation was held to a minimum, soil characteristics such as pH, organic matter content and microbial population were not uniform and subject to change between trials. However, if the goal is to obtain a realistic understanding of how organisms operate in natural settings then non-sterile, non-uniform system must be used in these studies are adequate.

STUDIES ON VOLATILES AND NON-VOLATILES

Screening of *Trichoderma* spp for the effect of their volatile metabolites on the growth of *P. capsici*

The percent inhibition of colony growth of *P. capsici* by volatiles of *Trichoderma* sppp varied (2.54 to 29.21%). Volatile affects little reduction in growth as compared to the percentage of inhibition recorded by dual plating (Anon., 2000; 2002; Saju *et al.*, 1999; 2002a). This is probably because of the micro quantities of volatile metabolites produced and their possible escape in petriplates especially in dual cultures. However even at lower quantities they are strong inhibitors of mycelial growth, and also influence the virulence of the *P. capsici* as measured by the decreased lesion size on black pepper leaves after exposure to volatiles (Anon., 2000; 2002; Saju *et al.*, 2001). When exposed colonies were sub-cultured onto fresh CA medium, normal growth was restored indicating their fungistatic nature. Volatiles of all the isolates of *Trichoderma* inhibited *P. capsici* to a varying degree. Out of the thirty isolates screened, nine isolates were identified as superior over the others in the inhibition of *P. capsici* by volatiles and the percent inhibition varied from 16.33 to 29.21. *T. hamatum* IISR 160 recorded highest percentage of inhibition of *P. capsici* (29.21%) followed by *T. aureoviride* IISR 148 (28.13%), *T. aureoviride* IISR 140 (25.22%), *T. virens* IISR 1370 (21.85%), *T. harzianum* IISR 165 (21.39%), *T. harzianum* IISR 173 (20.93%), *T. hamatum* IISR 150 (19.25%), *Trichoderma* sp. IISR 1141 (18.33%) and *T. hamatum* IISR 151 (16.33%) (Table 4.10).

Analysis of volatiles of *Trichoderma* spp by gas chromatography

T. harzianum IISR 1369 and *T. virens* IISR 1370 produced a variety of volatile organic compounds in culture. About 35 volatile components have been obtained from *T. harzianum* IISR 1369 and *T. virens* IISR 1370 produced 44 compounds. However one peak of *T. virens* IISR 1370 was common with control CDB. Only two compounds (RT 0.748, 28.785) were common for both *T. harzianum* IISR 1369 and *T. virens* IISR 1370. This indicated diversity of metabolite production among the *Trichoderma* spp. This supports the concept of chemical identity of species, which can help in species identification (Sivasithamparam and Ghisalberti, 1998). The identity of compounds needs further investigation. The production and type of volatiles produced appeared independent of the isolate used.

The major volatile compound isolated from *Trichoderma* with inhibitory property of pant pathogen is 6-pentyl-α-pyrone (6pp) (Cutler *et al.*, 1986; Scarselletti and Faull, 1994). Since in these experiments the isolated compound was not purified, the exact inhibitory principle in the volatiles still remains unidentified. However the

GC profiles showed a variety of volatile compounds produced by *Trichoderma* spp. Marked differences in the production of volatile metabolites have been reported between closely related species and even between strains of the same fungus. Volatile production is influenced by species, substrate and duration of growth (Borjesson *et al.*, 1992). A closer look at the range of metabolites produced by the most common biocontrol fungi is essential to ascertain their antimicrobial properties and the chemical identity of the isolates, which produced the volatiles. Volatile metabolites of *T. koningii,* induced the production of oospores in *Phytophthora cinnamomi* of the A2 mating type (Pratt *et al.*, 1972). Homothallin I (*T. koningii*) and homothallin II (*T. harzianum* and *T. koningii*) have been recorded to cause induction of oospores (Brasier, 1971; Edenborough and Hebert, 1988; Faull *et al.*, 1994; Pratt *et al.*, 1972).

Screening of *Trichoderma* spp for the effect of their non-volatile metabolites on the biomass of *P. capsici*

The culture filtrate of *Trichoderma* spp with non-voaltile metabolites inhibited mycelial biomass of *P. capsici* at 75% concentration and percent inhibition varied from 23.82 to 79.08. The conditions like culture medium, pH and incubation temperature adopted were found to be optimum for production of non-volatiles by *Trichoderma* spp were adopted (Dunlop *et al.*, 1989; Ghisalberti *et al.*, 1990; 1992). Eight isolates were selected based on inhibition of *P. capsici.* They are *T. aureoviride* IISR 126 (79.08%), *T. aureoviride* IISR 140 (77.31%), *T. aureoviride* IISR 143 (73.34%), *T. virens* IISR 1370 (71.75%), *T. aureoviride* IISR 148 (71.60%), *Trichoderma* sp. IISR 1107 (71.33%), *T. pseudokoningii* IISR 186 (70.25%), and *T. harzianum* IISR 1369 (57.79%) (Table 4.12).

Thus nine isolates which were highly efficient in volatile production and eight isolates with non-volatile production were selected for *in-vitro* interaction and *in-vivo* studies. Out of the above 17 isolates, three of them were found to be both volatile and non-volatile producers. They are *T. aureoviride* IISR 148, *T. aureoviride* IISR 140 and *T. virens* IISR 1370.

Among the eight non-volatile producers, five were found as highly rhizosphere competent. They are *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140, *T. aureoviride* IISR 148 and *T. harzianum* IISR 1369. These isolates were tested for the extraction of non-volatiles.

Isolation and identification of non-volatile metabolites

A number of compounds from *Trichoderma* spp responsible for inhibition of plant pathogens have been obtained. However, very few have been isolated and studied against oomycetous pathogens. Hence non-volatiles active against *P. capsici* were isolated and studied. *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148 were tested for the isolation of non-volatiles.

Extraction of active compounds and testing for inhibition of *P. capsici*

Non-volatiles from *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148 were extracted using ethyl acetate, since it gave maximum extraction (Dunlop *et al.*, 1989; Faull *et al.*, 1994). Concentrated ethyl acetate fraction of culture filtrate of *T. virens* IISR 1370 was highly inhibitory (100%) to *P.capsici* when 20 µl of crude fraction was tested as compared to other isolates where 50 µl was required for 100% inhibition, thereby indicating that concentrated ethyl acetate extract contained major principles from culture filtrate. Similar tests were carried out earlier (Dunlop *et al.*, 1989).

Thermo stability of ethyl acetate extract of culture filtrate of *Trichoderma* spp

Heating the EtOAc extracts partly affected the stability of the compounds. It needed 100 µl of the autoclaved extract to cause inhibition as against 20-50 µl in the case of extract without autoclaving. And after 48 hours, mycelium started growing out from the inoculum disc indicating that the extract was not able to cause complete inhibition.

Effect of ethyl acetate extract of culture filtrate of *Trichoderma* spp on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

Ethyl acetate extract of culture filtrate of *Trichoderma* spp was tested for the effect on sporangia formation, zoospore liberation and zoospore germination of *P. capsici.* The results showed that ethyl acetate extract of culture filtrate of *Trichoderma* spp was more effective against zoospore germination compared to sporangia formation and zoospore liberation. Even though higher concentration of the extract affected the three phases of the pathogen, production of such high concentration in soil needs detailed investigation. The concentration effective against zoospore germination is almost the same (400 ppm) for all the five *Trichoderma* spp, whereas sporangial formation and zoospore liberation required significantly higher concentration for inhibition. This clearly indicated that the non-volatiles of *Trichoderma* would induce disease suppression under field conditions by checking the germination of zoospores of *P. capsici.*

Thin layer chromatography

Out of the six compounds obtained from *T. harzianum* IISR 1369, only compound 2 (Rf 0.6) was found partially inhibitory to *P. capsici* showing 30.09% inhibition. This showed that *T. harzianum* IISR 1369 is weak in antibiotic production. And also individual compounds may be weak in causing inhibition. However, ethyl acetate extract of culture filtrate inhibited *P. capsici* at 50 µl concentration. The possibility of other compounds not dissolvable in EtOAc can be ruled out as weak extraction was found with solvents like petroleum ether, n-butanol, toluene, hexane and chloroform. Hence it can be inferred that no single compound from *T. harzianum* IISR 1369 inhibited *P. capsici* completely but their combination (EtOAc extract) cause high inhibition.

In *T. virens* IISR 1370, out of six compounds, compound 1 and 3 (Rf 0.65 and 0.38) showed greater inhibition (60.67 and 56.80% inhibition respectively) of *P. capsici* and compound 2 partially inhibited (41.74%) *P. capsici*. But the combined EtOAc fraction inhibited *P. capsici* completely (Table 4.13). This suggests the combined action of various compounds would be more effective in suppressing the plant pathogens. Therefore in terms of non-volatile production *T. virens* IISR 1370 is superior to *T. harzianum* IISR 1369. Higher inhibition of *P. capsici* by non-volatile compounds of *T. viride* IISR 1370 showed that one of the major mechanism involved in biocontrol may be the production of non-volatile antibiotics (Rajan, 1999 and Rajan *et al.*, 2002). Extensive field trials involving *T. harzianum* IISR 1369 and *T. virens* IISR 1370 showed reduction of disease in treatments with *T. virens* IISR 1370. However the survival of cfu of *T. virens* IISR 1370 was significantly below compared to that of *T. harzianum* IISR 1369. *Trichoderma* technology has been commercialized for black pepper disease control for large-scale adoption (Sarma and Anandaraj, 1997). Since *T. harzianum* IISR 1369 only partially inhibit *P. capsici* by compound 2 and complete inhibition by all 6 compounds (Ethyl acetate extract), it is hypothesized that apart from combined action of non-volatile antibiotics, its high competitive saprophytic ability with competition for nutrients are the major modes of action of *T. harzianum* IISR 1369 in suppression of *Phytophthora* caused foot / root rot in black pepper.

In *T. aureoviride* IISR 126, out of five compounds, only compound 2 (Rf 0.69) partially inhibited (33.49%) *P. capsici.* However the EtOAc extract completely inhibited *P. capsici* at 50 µl concentrations. This is explained based on the fact that all individual compounds present would act in a combined manner and individual compounds are less effective in causing reasonable amount of inhibition.

T. aureoviride IISR 140 produced 12 compounds. Compound 5 and 9 (Rf 0.51 and 0.29) showed inhibition of *P. capsici* and percent inhibition was 49.48 and 47.56% respectively. Compounds 1, 2, 4 and 8 (Rf 0.74, 0.69, 0.60 and 0.37) partially inhibited *P. capsici* and showed 41.24, 31.05, 30.09 and 29.59% inhibition respectively. Other compounds also caused little inhibition of *P. capsici.* Here also the combined action of individual compounds was evident.

T. aureoviride IISR 148 produced 9 compounds. Compounds 2, 4 and 5 (Rf 0.63, 0.54 and 0.46) partially inhibited *P. capsici* and showed 34.45, 30.09 and 30.09% inhibition respectively. Other compounds showed little inhibition. However from the testing of the EtOAc fraction it was evident that the individual compounds act in combination to cause greater inhibition.

The present study showed that a diversity of metabolite production in *Trichoderma* spp with varying degree of biological activity towards *P. capsici* and many of the compounds act in combination. However, further detailed studies are warranted on the chemical configuration of these metabolites. The role of these secondary metabolites in biocontrol of *Phytophthora* is indicative. Few reports are available on the action of secondary metabolites on oomycetes especially *Phytophthora* and *Pythium*. Homothallin I and homothallin II and their corresponding amine viz. N-formyl derivative (amine from homothallin II, formamide from homothallin II) have been identified from *T. koningii* from the culture broth (Edenborough and Hebert, 1988). Corresponding N, N-dimethyl anlogue (N, N-dimethylamine from homothallin II) has been isolated form *T. koningii* after treatment of an unstable precursor, probably the diketone, with demethylamine. The present study revealed an array of non-volatiles responsible for the inhibition of *P. capsici*. However their production and concentration in soil need to be studied in order to elucidate their role in the bicontrol process. Mechanisms of fungal antagonism and defense often include the production of biologically active metabolites by one species that excerts effects on potential competitors and (or) predators. To obtain evidence for the occurrence of this phenomenon in the field needs careful and definitive ecological studies. Nevertheless the results summarized here demonstrate the broad array of possible benefits that can arise from interdisciplinary studies in the ecology, chemistry and molecular biology of fungal antagonism and defense. Results of this investigation of the mechanism involved in the fungal antagonism and defense using volatile and non-volatile metabolites and have potential implications in many areas of study including fungal ecology, secondary metabolites and chemotaxonomy. Apparently many questions remain unanswered on the nature of the antifungal metabolites produced by *Trichoderma* species and their role in biocontrol. However the efforts to characterize metabolites of these isolates and their ecological significance need to be studied in greater detail.

STRAIN IMPROVEMENT

Strain improvement of *Trichoderma* using protoplast fusion technology

The potential isolate *T. harzianum* IISR 1369 grows well on solid substrates and in liquid cultures with high number of colony forming units (cfu). Culture filtrate of another isolate *T. aureoviride* IISR 140 is highly inhibitory to *P. capsici* than that of *T. harzianum* IISR 1369 but it is unable to grow well and sporulate on solid substrates and in liquid cultures. An attempt has been made to combine these two characteristics using protoplast fusion technology.

A large number of protoplasts without exposure to fusion conditions were tested for growth on PDA. Since these cultures exactly resembled either parent in spore colour and colony morphology they were considered as protoplast regenerated colonies of single parent or homofusants. They were not studied further. Fusion frequency was 0.7 in 40% PEG. Colonies form the fusion mixture also grown on PDA. The fusants selected were intermediate in colony morphology and whatever reverted to their parental types was discarded. Mrinalini (1993) also produced fusants intermediate in colony morphology in fusion between *T. harzianum* and *T. longibrachiatum*. *T. harzianum* IISR 1369 produce green colonies with luxuriant sporulation. *T. aureoviride* IISR 140 is yellow with little sporulation. In *T. harzianum* no pigmentation is seen reverse side of the culture plate but *T. aureoviride* produce dark red pigmentation reverse side. The fusants were generally of the type that they produce greenish to greenish yellow colony with dark red reverse side having varying intensity. Therefore none resembled exactly the parental type. However in fusion reported between *T. virens* and *T. harzianum*, 17 out of 24 stable strains formed colonies similar to those of *T. virens* (Shin and Cho, 1993). Similarly in an intergeneric fusion between *T. longibrachiatum* and *Phanerochaete* *chrysosporium,* the fusant obtained was phenotypically similar to the *T. longibrachiatum* parent and quite dissimilar to the *Phanerochaete chrysosporium*. parent. The fusant was reported to differ from the *T. longibrachiatum* parent in pigment production, sporulation, growth rate and enzymatic activity (Nutsubidze *et al*., 1991). In the present study, the fusants were phenotypically intermediate in characters such as sporulation and colony morphology. However progeny from other protoplast fusions have been reported to different from the parental strains in characteristics such as pigmentation (Shin and Cho, 1993; Kumari and Panda, 1994) secondary metabolite production (Kumari and Panda, 1994) and nutritional status (Kumari and Panda, 1994; Stasz *et al*., 1989).

Variability in secondary metabolite production has been reported in the progeny from other protoplast fusion (Kumari and Panda, 1994; Nutsubidze *et al*., 1991). In the seven fusants produced, variation was observed in secondary metabolite production. This variability may be important in the biocontrol of diseases. Pe'er and Chet (1990) also observed variability in biocontrol activity in protoplast fusants. However, it is important to identify the stable ones in that the biocontrol activity would be stable.

The maintenance of activity in fusants indicated that it was possible to successfully combine some biocontrol activity (secondary metabolite production) from *T. aureoviride* with growth qualities of *T. harzianum* (fast growth and sporulation). The fusant *Trichoderma* inhibited *P. capsici* in dual culture experiment and percent inhibition varied form 32.14 to 39.70 compared to the parents, which showed 35.56 and 30.68% inhibition respectively. Fusants exhibited good growth and sporulation and high amount of secondary metabolite production. However its *in-vivo* performance in growth promotion and disease suppression is warranted.

Secondary metabolites production by fusant *Trichoderma*

Out of seven fusants secondary metabolites of FT 11 and FT 21 are highly inhibitory (100%) to *P. capsici* at 10 µl concentration. Other fusants also showed inhibition but at higher concentration (which required 50 µl of EtOAc extract). However FT 11 and FT 21 inhibited *P. capsici* at 10 µl concentration of EtOAc extract. Secondary metabolites of either parent were not able to inhibit *P. capsici* at such a low concentration (10 µl). This shows superiority of fusants over parents in secondary metabolites production. Similar enhancement in secondary metabolite production has been reported (Hanson and Howell, 2002; Mrinalini, 1993). Further purification and identification of non-volatiles compounds are to be carried out.

Thermo stability of ethyl acetate extract of culture filtrate of fusant *Trichoderma*

Even though the steam heated EtOAc extract of fusants inhibited *P. capsici* initially, the colonies started growing after 48 hours. However the growth rate was highly reduced. Hence secondary metabolites are partially thermostable. However in FT 11 secondary metabolites is thermostable as there was no growth even after 72 hours in the culture plate after inoculation. Similarly Yoon *et al.* (1989) isolated active substance form broth culture of Streptomyces A-2 antagonistic to *P. capsici*, *P. nicotiana* var. *parasitica* and *Rhizoctonia solani*, which retained their properties following autoclaving.

Effect of ethyl acetate extract of culture filtrate of fusant *Trichoderma* on zoosporangia formation, zoospore liberation and zoospore germination of *P. capsici*

The results showed that ethyl acetate extract of culture filtrate of fusant *Trichoderma* was more effective against zoospore germination than sporangia formation and zoospore liberation. Ethyl acetate extract of culture filtrate of parents also showed same trend in the inhibition of three life phases of *P. capsici.* Even though at higher concentration of the extract, it has affected the three phases of the pathogen, production of such high concentration in soil needs investigation. The concentration effective against zoospore germination is almost the same (300 ppm) for all the seven fusant *Trichoderma* (100% inhibition). Therefore the non-volatiles of fusant *Trichoderma* were more active against *P. capsici* than their parents, whereas sporangium formation and zoospore liberation required higher concentration for inhibition. This clearly indicated that the non-volatiles of fusant *Trichoderma* might be effective to control the disease under field conditions by checking the germination of zoospores of *P. capsici*, thereby checking infection.

DISEASE MANAGEMENT

Evaluation of *Trichoderma* spp and *Pseudomonas fluorescens* for suppression of *P. capsici*

Field experiment was conducted to study the disease suppressive effect of *Trichoderma* spp and *P. fluorescens* and some of their combinations in comparison with existing potent biocontrol agents (*T. harzianum* IISR 1369 and *T. virens* IISR 1370).

The potential of *T. harzianum* IISR 1369 and *T. virens* IISR 1370 as biocontrol agents in foot rot management of black pepper has been established under field conditions (Rajan, 1999; Rajan *et al.*, 2002). *T. virens* IISR 112 and *T. virens* IISR 18 were found as suppressing foot rot in glass house tests. *T. aureoviride* IISR

143 showed hyphal coiling and penetration of *P. capsici* in *in-vitro* tests. *T. pseudokoningii* IISR 187, *T.. harzianum* IISR 167 and *T. harzianum* IISR 178 inhibited *P. capsici* in dual plate tests (Anon., 2000; 2002; Saju *et al.*, 1999; 2002a). *P. fluorescens* IISR 11, *P. fluorescens* IISR 13, *P. fluorescens* IISR 41 and *P. fluorescens* IISR 6 were found to suppress root rot of black pepper in glass house experiments (Paul *et al.*, 2000). Combinations of *T. harzianum* IISR 1369 with *P. fluorescens* IISR 41 and *P. flurescens* IISR 6 and *T. virens* IISR 1370 with *P. fluorescnes* IISR 13 and *P. fluorescens* IISR 11 were not mutually antagonistic in petriplate studies and hence compatible. For inundative biological control, data on filed performance of biocontrol agents is necessary. Data showed that combination of *Trichoderma* and *P. fluorescens* were superior over others in growth promotion. Single application of fungi was superior to single application of bacteria (Table 4.24). In preliminary experiments *T. harzianum* IISR 1369 was compatible with *P. fluoresocens* IISR 41 and *P. fluorescens* IISR 6. Similarly *T. virens* IISR 1370 was not inhibiting *P. fluorescens* IISR 13 and *P. fluorescens* IISR 11.

The population of *Trichoderma* in soil also showed that they are able to survive throughout the year. An increase in population of *Trichoderma* was observed during wet months and a decline during dry months. This is common with many of the *Trichoderma* spp studied (Eastburn and Butler, 1991).

The population of *P. fluorescens* could be estimated, only based on an increase in number of bacteria in treated plants compared to control. Randomly taken colonies from dilution plates inhibited *P. capsici* in *in-vitro* tests. Population of other fungi was on par in various treatments. The presence of other members of soil micro-flora also may influence biocontrol activity by inhibiting the growth and development of the biocontrol agents or by metabolising its enzymatic and / or antibiotic products.

This may not entirely eliminate the biocontrol activities of *Trichoderma* species, but it might well limit their efficacy in terms of the length of time that they are effective (Howell, 2003). Even though *Phytophthora* was detected in soil through baiting, no infection of vines was noticed. The presence of *Phytophthora* in various treatments was negligible in January, February and April 2001. However they have been detected during rainy season.

In green house test, when challenge inoculated with *P. capsici,* treatments applied with *P. fluoreoscens* IISR 13 and *P. fluorescens* IISR 41 were highly disease suppressive showing only 10% disease incidence as compared to 90% in the control. Combination of *T. harzianum* IISR 1369 + *P. fluorescens* IISR 41, *T. harzianum* IISR 1369 + *P. fluorescens* IISR 6 and *T. virens* IISR 1370 + *P. fluorescens* IISR 13 were also on par with the above treatments (Table 4.24). Hence it is inferred that *P. fluorescens* IISR 41 and *P. fluorescens* IISR 13 can be utilized for the suppression of *P. capsici.* Combination of *T. harzianum* IISR 1369 + *P. fluorescens* IISR 41, *T. harzianum* IISR 1369 + *P. fluorescens* IISR 6 and *T. virens* IISR 1370 + *P. fluorescens* IISR 13 are disease suppressive and promote growth of black pepper (Plate 4.15 b-d). Since the combination of *T. harzianum* IISR 139 + *P. fluorescens* IISR 41 has given maximum plant growth and less disease, it can be recommended as a strategy for disease management of foot rot of black pepper.

The present investigation established the potential of *Trichoderma* spp and *P. fluorescens* individually inducing growth promotion and disease suppression. However the combinations of both indicated increased growth and root rot suppression thereby indicating the growth-mediated defense in black pepper-*P. capsici* pathosystem. The leads obtained in the present investigation need to be exploited in disease management programmes in field. However, the combination of

T. harzianum IISR 1369 + *P. fluorescens* IISR 41 need further field evaluation / demonstration before advocating the consortium to farmers.

6

SUMMARY AND CONCLUSIONS

Identifying secondary metabolites responsible for biocontrol of plant pathogens has been useful for refining screening systems to select further useful antagonists. Commercial development of biocontrol formulation should be with realization that biocontrol agents must be adapted to the environment where biological control is practiced to ensure long lasting effects. Therefore abiotic factors that influence biocontrol agents need be studied in depth. With this view the results obtained are encouraging to use *Trichoderma* spp over a wide range of climatic and soil conditions. The investigations were carried out with specific reference to *Phytophthora*-black pepper pathosystem.

IN-VITRO STUDIES

Among the fourteen isolates of *Trichoderma* studied *in-vitro*, six of them showed antagonistic interactions with *P. capsici*. *In-vitro* interactions observed involve parallel growth of hyphae of *Trichoderma* and *Phytophthora capsici*, penetration of hyphae of *P. capsici* and coiling of hyphae of *P. capsici* by *Trichoderma* spp etc. Apart from this, hyphal evacuation, sporangial parasitism, sporangial deformation and shortening of hyphal tips were observed with *P. capsici* after pairing with *Trichoderma.*

IN-VIVO STUDIES

Fourteen isolates of *Trichoderma* spp were tested *in-vivo* for their effect on the host plant. In *in-vivo* tests *Trichoderma* spp induced growth promotion of black pepper and disease suppression. The percent increase in plant height over the control varied from 68-128%. Plant height was maximum when *T. harzianum* IISR 1369, *T. hamatum* IISR 151, *T. hamatum* IISR 160 and *T. pseudokoningii* IISR 186 were applied. Shoot biomass increased when *T. harzianum* IISR 1369 was applied. However, root biomass was uniform among various treatments. When challenge inoculated with *P. capsici,* five of the *Trichoderma* isolates viz. *T. aureoviride* IISR 126, *T. aureoviride* IISR 140, *T. aureoviride* IISR 148, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 were able to suppress the disease where 20% of the plants showed mortality as against 100% in the control.

In a modified method to study rhizosphere competence modified experiment it was found that out of the fourteen isolates studied *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140, *T. aureoviride* IISR 148, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 were identified as highly rhizosphere competitive (log cfu / g = >4.5) compared to other isolates (log cfu / g=<4.5).

The effect of soil type, moisture and temperature on propagule density in soil was determined experimentally for five *Trichoderma* spp viz. *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173. *Trichoderma* spp were introduced into soil in polybags planted with black pepper. Different types of soils viz. clayey (Calicut), sandy clay (Peruvannamuzhi) and sandy loam (Thamarassery) did not influence the survival of *Trichoderma* spp as determined by soil dilution plating over a period of 90 days.

Trichoderma spp were inoculated into soil with different moisture level ranging from 20-80%. Population of *T. harzianum* IISR 1369 and *T. harzianum* IISR 173 at 80, 70 and 20% moisture levels declined gradually over a period of 90 days. The number of cfu of *T. virens* IISR 1370, *T. aureoviride* IISR 126 and *T. harzianum* IISR 165 significantly reduced at 80 and 70% moisture levels. However the population never reduced to zero.

Trichoderma spp were inoculated into soil in polybags planted with black pepper. Changes in the density of propagules in soil were monitored over three months at three temperature ranges ($<20^0$C, 20-26^0C and 26-32^0C). There was no significant difference in propagule density of *T. harzianum* IISR 1369, *T. aureoviride* IISR 126 and *T. harzianum* IISR 165 at three temperature ranges studied. *T. virens* IISR 1370 recorded greatest propagule density in soil at lower temperature ranges ($<20^0$C and 20-26^0C). The propagule density of *T. harzianum* IISR 173 remained higher at higher temperature ranges (20-26^0C and 26-32^0C).

P. capsici was able to grow well in acidic pH. *T. harzianum* IISR 1369 showed a linear growth over the wide range of pH studied. *T. virens* IISR 1370 was able to grow well at pH 4.5 whereas *T. aureoviride* IISR 126 required a pH 6.5 for good growth. Acidic or basic nature of the medium does not affect the growth of *T. harzianum* IISR 165. The growth of *T. harzianum* IISR 173 was not affected by pH range 4.5 –7.5. At pH 8.5 the colony diameter showed a sharp decline. The adaptability of *Trichoderma* in wide range of soil pH is an advantage since *P. capsici* also thrives under acidic conditions.

P. capsici was sufficiently sensitive to metalaxyl, mancozeb, copper and potassium phosphonate even at lower doses. At the same time phorate increased the growth of *P. capsici* at 10 and 100 ppm concentration. *P. capsici* was sensitive to cholrpyriphos at 300 ppm and to quinalphos at 500 ppm. The four fungal antagonists, *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 were sufficiently insensitive to metalaxyl, mancozeb, potassium phospohonate and copper. The only exception was *T. aureoviride* IISR 126 which has showed copper tolerance but insensitive to metalaxyl, mancozeb and potassium phosphonate. Carbendazim was toxic to *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148 but they were insensitive to phorate. Quinalphos was toxic to *T. virens* IISR 1370, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 but less toxic to *T. harzianum* IISR 1369 and *T. aureoviride* IISR 126 (moderately tolerant). *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. harzianum* IISR 165 and *T. harzianum* IISR 173 were moderately tolerant to chlorpyriphos.

Since metalaxyl, mancozeb, copper, and quinalphos up to 300 ppm did not affect the population of *T. harzianum* IISR 1369 in soil, the organism would be relatively tolerant to field concentrations of above pesticides. Therefore these pesticides can be integrated along with *T. harzianum* IISR 1369. However growth additive effect of phorate on *P. capsici* would also indicate to discourage using the same in IDM.

Application of urea, rock phosphate, muriate of potash and NPK together did not affect the survival of population of *T. harzianum* IISR 1369. It showed higher population over control. Application of FYM, coirpith compost and neem cake also supported increase in population of *T. harzianum*. However, dried leaves of *Glyricidia* did not cause significant increase in population. Therefore application of fertilizers in recommended concentration will not affect *T. harzianum* IISR 1369 in soil.

STUDIES ON VOLATILES AND NON-VOLATILES

Thirty isolates of *Trichoderma* spp were screened for their volatile effects on the growth of *P. capsici*. Percent inhibition of growth of *P. capsici* by volatiles of *Trichoderma* spp varied from 2.54 to 29.21%. Nine isolates were identified as superior over the others in the inhibition of *P. capsici* and the percent inhibition varied from 16.33 to 29.21.

Volatile metabolites from *T. harzianum* IISR 1369 and *T. virens* IISR 1370 cultured on CDB in headspace vials were injected into GC for analysis. Volatiles of *T. harzianum* IISR 1369 consisted of 35 compounds and that of *T. virens* 44 compounds. Thus volatile profiles were highly variable. However, one peak of *T. virens* IISR 1370 was common with control CDB. Only two compounds (RT 0.748 and 28.785) were produced by both *T. harzianum* IISR 139 and *T. virens* IISR 1370. However, individual compounds could not be identified in the present study.

Thirty (30) isolates were also screened for their non-volatile effect on the mycelial dry weight of *P. capsici*. Percent inhibition of mycelial biomass of *P. capsici* by non-volatiles of *Trichoderma* spp varied from 23.82 to 79.08 with respect to the concentration of culture filtrate (75%) tried. Eight isolates were found to be highly efficient in production of non-volatile metabolites that inhibited the growth of *P. capsici* and percent inhibition varied from 57.79 to 79.08.

T. harzianum IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148 were taken for the isolation of non-volatiles. Concentrated ethyl acetate extract of culture filtrate of *T. virens* IISR 1370 was highly inhibitory (100%) to *P. capsici* when 20 µl of crude fraction was tested as compared to other isolates (*T. harzianum* IISR 1369, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148) where 50 µl was required for 100% inhibition of *P. capsici*. Heating the EtOAc extracts partly affected the stability of the compounds. It needed 100 µl of the autoclaved extract to cause inhibition as against 20-50 µl in the case of extract without autoclaving. However, after 48 hours mycelium started growing out from the disc. This indicated partial thermostable nature of metabolites produced by the *Trichoderma* spp. EtOAc extract of culture filtrate of *Trichoderma* spp was more effective against zoospore germination (100% inhibition at 400 ppm concentration) whereas sporangia formation and indirect germination of zoosporangia of *P. capsici* required higher concentration of EtOAc extract.

The non-volatiles of *T. harzianum* IISR 1369 consists of six compounds out of which compound 2 (Rf 0.6) partially inhibited (30.09%) *P. capsici*. *T. virens* IISR 1370 produced six non-volatile compounds. Compounds 1 and 3 (Rf 0.65 and 0.38) inhibited *P. capsici* and showed 60.67 and 56.80 % inhibition respectively and compound 2 (Rf 0.53) inhibited partially (41.74%). *T. aureoviride* IISR 126 produced 5 compounds and compound 2 (Rf 0.69) showed partial inhibition (33.49%) of *P. capsici*. *T. aureoviride* IISR 140 produced 12 compounds. Compounds 5 and 9 (Rf 0.51 and 0.29) inhibited *P. capcisi* and percent inhibition were 49.98 and 47.56% respectively. Compounds 1, 2, 4 and 8 (Rf 0.74, 0.69, 0.60 and 0.37) partially inhibited *P. capsici* and showed 41.24, 31.05, 30.09 and 29.59% inhibition respectively. *T. aureoviride* IISR 148 produced 9 compounds. Compounds 2, 4 and 5 (Rf 0.63, 0.54 and 0.46) partially inhibited *P. capsici* and showed 34.45, 30.09 and 30.09% inhibition respectively.

STRAIN IMPROVEMENT

The potential isolate *T. harzianum* IISR 1369 grows well on solid substrates and in liquid media with high cfu count. Culture filtrate of another isolate *T. aureoviride* IISR 140 is highly inhibitory to *P. capsici* than that of *T. harzianum* IISR 1369, but its growth and sporulation both in solid and liquid cultures is poor. An attempt has been made to combine these two characteristics of *T. harzianum* and *T. aureoviride* using protoplast fusion technology. All the seven fusants produced were morphologically intermediate between two parental strains. They exhibited biocontrol activity (secondary metabolite production) from *T. aureoviride* IISR 140, with fast growth and sporulation qualities of *T. harzianum* IISR 1369. The fusant *Trichoderma* inhibited *P. capsici* in dual culture assay and percent inhibition varied form 32.14 to 39.70. Fusants differed in the production of secondary metabolites. Concentrated ethyl acetate fraction of culture filtrate of fusants, FT 11 and FT 21 was highly inhibitory (100%) to mycelial growth of *P. capsici* when 10 µl of crude fractions was tested as compared to other fusants (FT 9, FT 14, FT 15, FT 16 and FT 17) where 50 µl was required for 100% inhibition of *P. capsici.* Heating the EtOAc extracts partly affected the stability of the compounds. It needed 100 µl of the autoclaved extract to cause inhibition as against 10-50 µl in the case of extract without autoclaving. Partially the compounds are thermostable. And after 48 hours the mycelium started growing out from the disc. However EtOAc extract of FT 11 retained the inhibitory property even after autoclaving. EtOAc extract of culture filtrate of fusant *Trichoderma* was more effective against zoospore germination (100% inhibition at 300 ppm concentration) whereas the inhibition of sporangia formation and indirect germination of zoospores of *P. capsici* required higher concentration for inhibition.

DISEASE MANAGEMENT

Field testing with eight *Trichoderma* isolates and four *P. fluorescens* strains and combination of *Trichoderma* and *P. fluorescens* showed that combination of *T. harzianum* IISR 1369 + *P. fluorescens* IISR 41 (204.9 cm) was good in growth promotion followed by *T. harzianum* IISR 1369 + *P. fluorescens* IISR 6 (199 cm) and *T. virens* IISR 1370 + *P. fluorescens* IISR 11 (192.6 cm). This was followed by *T. virens* IISR 1370 + *P. fluorescens* IISR 13 (179.3 cm). Single application of *P. fluorescens* IISR 6, *T. harzianum* IISR 167, *T. pseudokoningii* IISR 187, *T. aureoviride* IISR 143 and *T. harzianum* IISR 1369 were on par. When challenge inoculated in green house, *T. harzianum* IISR 1369 + *P. fluorescens* 41, *T. harzianum* IISR 1369+ *P. fluorescens* 6, *T. virens* IISR 1370 + *P. fluorescens* IISR 13 and single application of *P. fluorescens* IISR 41 and *P. fluorescens* IISR 13 showed only 10% disease incidence as against 90% in the control. Hence the combination of *T. harzianum* IISR 1369 + *P. fluorescens* IISR 41 can be recommended since it gives both growth promotion and disease suppression as a strategy for disease management of foot rot of black pepper.

CONCLUSIONS

Trichoderma spp were hyperparasitic on the mycelia and sporangia of *Phytophthora capsici* infecting black pepper *in-vitro.* They exhibited growth promotion of black pepper and reduced root rot caused by *Phytophthora capsici in-vivo.* More rhizosphere competent isolates of *Trichoderma* were identified. *Trichoderma* spp over a wide range of climatic and soil conditions were highly adoptable. Many non-volatile compounds inhibitory to mycelial growth of *P. capsici* were isolated from *T. harzianum* IISR 1369, *T. virens* IISR 1370, *T. aureoviride* IISR 126, *T. aureoviride* IISR 140 and *T. aureoviride* IISR 148. They also inhibited sporangia formation, indirect germination of sporangia and germination of zoospores. Seven new strains capable of fast growth and sporulation and production of higher levels of non-volatiles were obtained by protoplast fusion between *T. harzianum* IISR 1369 X *T. aureoviride* IISR 140. Non-volatiles of fusants inhibited sporangial formation, indirect germination of sporangia and germination of zoospores at low concentration. Based on the comparative growth promotion of black pepper vines in the field by *Trichoderma* and *P. fluorescens* isolates and glass house experiments with challenge inoculation with *P. capsici*, combination of *T. harzianum* IISR 1369 + *P. fluorescens* IISR 41 can be recommended as a strategy for disease management of foot rot of black pepper. Protection of black pepper from *P. capsici* in the present study indicated growth mediated defense coupled with suppression of *P. capsici* propagules by *Trichoderma* through its various mechanisms as outlined earlier.

Chemical characterisation of volatiles and non-volatiles that inhibit *P. capsici* would be useful for refining screening systems to select further useful antagonists. The leads obtained in the area of strain improvement need be exploited for the development of highly efficient biocontrol agents to develop new formulation for biocontrol. Combination of *T. harzianum* IISR 1369 + *P. fluorescens* IISR 41 need further large-scale filed evaluation / demonstration before advocating the consortium to farmers.

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APPENDIX

Composition of culture media

Composition and preparation of the culture media / solutions used in this study are given below.

Carrot agar

Add 2% agar to carrot broth

Carrot broth

Carrot	200 g
Water	1000 ml

Cut the carrot into small pieces. Homogenize in a mixer with little amount of water. Pass through double-layered muslin cloth and take the juice. Add water to the homogenized tissue to get 1 liter of juice.

Czapek dox broth

NaNO	2 g
K_2HPO_4	1 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4$	0.01 g
Sucrose	30 g
Distilled water	1000 ml

pH 6.0

If glass distilled water is used, add 1 ml each of 1% $ZnSO_4$ and 0.5% $CuSO_4$. Heat the full chemicals without sucrose in a water bath for 15 minutes. After cooling add sucrose and agar. Do not filter.

Kings' B Agar

Proteose peptone	20 g
K2HPO4	2.5 g
Glycerol	15 ml
MgSO4	6 g
Agar	15 g
Distilled Water	1000 ml

pH 7.2

*Cycloheximide	100 ppm (0.1g)
*Ampicillin	50 ppm (0.05g)
*Chloremphenicol	12.5 ppm (0.0125g)

*Do not autoclave. Add just before pouring.

Nutrient agar

Beef extract	3 g
Peptone	5 g
Sodium chloride	5 g
Agar	15 g
Distilled water	1000 ml

Potato dextrose agar

Add 2% agar to potato dextrose broth

Potato dextrose broth

Potato	200 g
Dextrose	20 g

Peel the potato and cut into small pieces. Boil for half an hour in 500 ml water and take the extract. Add the dextrose.

Potato carrot agar

Potato	20 g
Carrot	20 g
Agar	20 g
Distilled water	100 ml

Boil the potato and carrot for half an hour in 500 ml water and take the extract.

PVPH medium (Tsao and Occano, 1969)

Pimaricin	10 ppm
Vancomycin	200 ppm
PCNB	100 ppm
Hymexazole	50 ppm

Add the chemicals to 100ml sterile distilled water in coloured bottles or wrap with black polythene cover and keep in refrigerator. To 100 ml of sterile molten carrot agar add 10ml of PVPH solution, shake well and dispense into petriplates.

$P_{10}VP$

Pimaricin	10 ppm
Vancomycin	200 ppm
PCNB	100 ppm

Prepare the stock as in the case of PVPH and keep in refrigerator. To 100 ml of sterile molten potato dextrose agar add 10 ml of PVPH solution, shake well and dispense into petriplates.

***Trichoderma* selective medium** (Elad and Chet, 1983)

$MgSO_4$	0.2 g
K_2HPO_4	0.9 g
KCl	0.15 g
NH_4NO_3	1 g
Glucose	3 g
Fenaminosulf or ridomil	0.3 g
Rose Bengal	0.15 g
Agar	20 g
Distilled water	1000 ml
*Quintozene or PCNB	0.2 g
*Chloramphenicol	0.25 g

*Do not autoclave. Add and shake well just before pouring.