ENHANCING BIO - EFFICACY OF *Trichoderma* spp. FOR THE MANAGEMENT OF SOIL BORNE FUNGAL PATHOGENS

By

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(2011-21-113)

THESIS

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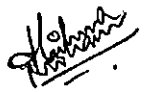
DECLARATION

I hereby declare that the thesis entitled **"Enhancing bio-efficacy of *Trichoderma* spp. for the management of soil borne fungal pathogens"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.



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CERTIFICATE

Certified that this thesis entitled **"Enhancing bio-efficacy of *Trichoderma* spp. for the management of soil borne fungal pathogens"** is a bonafide record of the research work done independently by **Mrs. Hima V. M.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Introduction

INTRODUCTION

The anamorphic fungal genus, *Trichoderma* coming under the Phylum *Ascomycota* and the Order Hypocreales is a cosmopolitan fungus commonly seen in soil and root ecosystems. It includes more than 200 species and is occurring all over the world in different geographical regions and climatic zones. They are one of the most exploited fungal biocontrol agents in the field of agriculture for the management of crop diseases caused by a wide range of fungal phytopathogens. In 1987, Chet reported that *Trichoderma* spp. are active mycoparasites against a range of economically important aerial and soil borne plant pathogens, and can be successfully used as a biocide in greenhouse and field applications. The antagonistic mechanism of *Trichoderma* spp. is a complex process involving competition, mycoparasitism, antibiosis and the production of extra cellular enzymes such as chitinase, glucanase, cellulase, proteinase *etc.* and induced systemic resistance (ISR) (Dinakaran *et al.,* 2003).

Soil borne diseases mainly caused by fungal pathogens are the major constraints in the agriculture sector, all the time. The fungi *viz., Pythium* spp., *Phytophthora* spp., *Ganoderma* spp., *Sclerotium* spp., *Rhizoctonia* spp., *Fusarium* spp., *Macrophomina* spp. *etc.* thriving in the soil ecosystem are the major phytopathogens which are posing serious threat for the cultivation of crops. The diseases like pre - and post - emergence damping off, collar and root rot, wilt *etc.* caused by them are economically very important and severe enough to cause extensive and devastating damage to the crops. Most of them are generalistic and unspecific in their host range and hence are much difficult to control by chemicals. It has been experienced that excessive use of chemical fertilizers and plant protection chemicals for maximizing crop yield has resulted in deterioration of physical, chemical and biological health of the arable land. This situation eliminates the ecologically beneficial

microbes from soil as well as the group of microorganisms which otherwise improve crop health. Biocontrol offers a chance to improve the crop production within the existing resources, and growing problems of resistance of chemical pesticides to the target pathogen population. Hence, the current concern about the environment indicates a need to limit the application of chemicals for plant disease control. With the increasing interest in developing alternatives to chemical fungicides, the development of *Trichoderma* sp. as bio protectant has become the focal point for research and development.

In this context, several researches have been published, indicated the role of *Trichoderma* spp. as a successful control measure against soil borne diseases *viz.*, *T. harzianum* for the control of *S. rolfsii* and *Rhizoctonia solani* (Elad *et al.,* 1980); *Trichoderma* spp. against *Pythium* damping off in tomato (Patil *et al.,* 2012); *T. harzianum* against *Phytophthora infestans* (Fatima *et al.,* 2015) and *Trichoderma* spp. against *Fusarium* wilt in pomegranate (Sonawane *et al.,* 2015).

The value of the global biopesticide market is expected to reach $4,556.37 Million by 2019, at a compound annual growth rate of 15.30% from 2014 to 2019 (source: Marketsandmarkets.com, 2014; last access 31/03/2015). At present, *Trichoderma* based biocontrol products share 60% of all fungal based products in the world market with a regular increase in the number (Waghunde *et al.,* 2016). However, the efficacy of *Trichoderma* spp. as biocontrol agent in natural soils may be limited by soil fungistasis, competition by other soil microbes, poor plant root colonization or unfavourable environmental conditions (Bae and Knudsen, 2000). Hence, genetic manipulation of *Trichoderma* spp. for strain improvement has come in importance. The perusal of literature revealed that mutagenesis and the protoplast fusion technique are the quickest and the easiest methods which are commonly used for the strain improvement of fungus. It has been reported that

these biotechnological tools are able to combine the advantageous properties of distinct promising strains of various bioagents to improve the antifungal production and the antagonistic potential against a broad spectrum of phytopathogens. It was successfully applied in the breeding of *Trichoderma* spp. (Mohamed and Haggag, 2010) and was revealed that most of the selected mutants and fusants showed superiority in the growth rate and antagonistic activity against soil borne fungal pathogens like *Fusarium oxysporum, Pythium ultimum, Sclerotium rolfsii* and *Sclerotinia sclerotiorum.*

Harman (2000) reported that *Trichoderma* spp. could colonize the roots of plants and moreover, promote plant growth. It was already known that induced systemic resistance mediated through the production of defense related enzymes namely peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, cellulases and β 1, 3 glucanases, is also the one indirect tool by which the biocontrol agents afforded resistance against pathogens.

Considering all these aspects, the present investigation was taken up to study the biocontrol efficiency of native isolates and induced strains of *Trichoderma* spp. against important soil borne fungal pathogens. In this study, *Pythium aphanidermatum* causing rhizome rot of ginger has been taken as test pathogen.

The research programme entitled 'Enhancing bio-efficacy of *Trichoderma* spp. for the management of soil borne fungal pathogens' envisaged the following aspects.

1) Isolation of native *Trichoderma* spp. and *in vitro* evaluation of their antagonistic potential
2) Pot culture experiment for the evaluation of biocontrol efficiency of the selected native isolates of *Trichoderma* spp.

3) Evaluation on biocontrol efficiency of the selected native isolates of *Trichoderma* spp. under field condition
4) Identification of promising isolates of *Trichoderma* spp.
5) Study on the strain variation of *Trichoderma* spp.
6) *In vitro* screening of mutants and protoplast fusants against the pathogens
7) Characterization of the selected mutants and fusants
8) Pot culture experiment for the evaluation of biocontrol efficiency of selected mutants and fusants
9) Studies on Induced systemic resistance
10) Evaluation of biocontrol efficiency of selected mutants and fusants under field experiment

Review of Literature

2. REVIEW OF LITERATURE

The ultimate aim of the agriculture sector is the feeding of an expanding world population. In agriculture, parasites and the pathogens are the major threats for the crop production. An estimate showed that 37 per cent of crop loss is due to pests, of which 12 per cent is due to pathogens (Radheshyam *et al.*, 2012). Among this, soil borne diseases are one of the major constraints faced by the farmers. The diseases caused by the pathogens survived in the soil matrix and in residues on the soil surface are called as soil borne diseases (Rani and Sudini, 2013). They also reported that the fungi *viz.*, *Pythium* spp., *Phytophthora* spp., *Sclerotium* spp., *Rhizoctonia* spp., *Fusarium* spp. *etc.* thriving in the soil ecosystem are the major phytopathogens which are posing serious threat to the farmers for the cultivation of crops. Fungi are the most aggressive microorganisms that threaten the food security (Benitez *et al.*, 2004). The diseases like pre - and post - emergence damping off, collar and root rot, wilt *etc.* caused by them are economically very important and severe enough to cause extensive and devastating damage to the crops. They often substantially reduce the quality and quantity of agricultural commodities (Seitz *et al.*, 1982; Alderman *et al.*, 1996). The infestation of micro organisms affects the health of humans and livestock especially if the contaminating organism produces toxic residues in or on consumable products (Diekman and Green, 1992; Nelson *et al.*, 1993 and Cheeke, 1995). In the opinion of Brimner and Boland (2003) plant diseases should get significant concern since the plant health is intimately related to the welfare of people, animals and the environment.

Rhizome rot, commonly known as soft rot, is one of the most destructive diseases of ginger (Dohroo, 2005) which causes a loss of 50 – 90 per cent, occurring in major production areas like tropical regions of India

(Stirling *et al.*, 2009). They indicated that ginger crops in Kerala and Tamil Nadu are sometimes almost totally destroyed by rhizome rot.

The perusal of literature revealed several strategies for the control of plant diseases. This included improvement of cultural practices, breeding of resistant varieties, storage conditions unfavourable for pathogen attack and survival, application of chemical fungicides and biological control (Radheshyam *et al.*, 2012). In many countries, chemical fungicides are widely used to control plant pathogens. Fungicides are often applied in greater quantities than herbicides and insecticides in agricultural production and hence have drastic effect on the environment and consumer (Vinale *et al.*, 2008). Moreover, since they pollute the atmosphere, damage the environment, leave residues which leads to the development of resistant strains among the target organism with repeated use, they are not found economical for the long run (Naseby *et al.*, 2000). Cigdem and Merih (2003) reported that non-degradable components of their compounds can accumulate over the years and can enter the food chain which can cause higher toxicity in animals. And it was also revealed that modern methods of disease management though they are effective in the control of plant pathogens, may have negative impact on non – target or beneficial organisms (Baker and Dunn, 1990; Gray Jr. *et al.*, 1994 and Ekelund *et al.*, 2000). Thus the excessive use of chemical fertilizers and plant protection chemicals for maximizing crop yield has resulted in the deterioration of physical, chemical and biological health of the arable land. Hence, the current concern about the environment indicates a need to limit the application of chemicals for plant disease control.

In 1999, Lorito and Scala suggested that the achievement of not only a satisfactory but also an environment friendly control measure against the plant diseases was one of the major challenges faced by modern agriculture.

2.1. BIOCONTROL OF PLANT DISEASES

The term biocontrol was initially used to refer the biological control of insects as the suppression of insect populations by the actions of their native or introduced enemies. However, this term was used later to indicate the biological control of pathogens also. It was defined as a population-leveling process, in which the population of one species lowers the number of another species by mechanisms such as competition, parasitism and antibiosis (Cook and Baker 1983; Vasudevan *et al.*, 2002).

Pal and Gardener (2006) suggested that in plant pathology, the term biological control was applied for the use of microbial agents to mitigate diseases as well as suppress weed populations so as to improve crop yield and to preserve stored products. The negative effects of the chemical fungicides compelled to look for alternative disease management practices which included the use of biocontrol agents and pathogen resistant crop cultivars (Radheshyam *et al.*, 2012). According to them, biological control of plant disease was the use of beneficial microorganisms, such as specialized fungi, bacteria and actinomycetes to attack and control plant pathogens and the diseases they caused.

Fungal based biocontrol agents are getting wide acceptance next to bacteria because of their broader spectrum in terms of disease control and production of yield (Copping and Menn, 2000). Hence, currently it is well established and in several cases, it is complimentary or even replacing the chemical counterparts (Whipps and Lumsden, 2001). In 2001, Avis and co workers reported that biological control can be considered as more natural and environmentally acceptable alternative to the existing chemical treatment methods. Mathivanan *et al.*, 2006 considered biological control as one of the best viable alternative to chemical fungicides.

2.2. *Trichoderma* spp. AS BIOCONTROL AGENT

Fungal biocontrol agents are the most successful tools in the combat against plant pathogenic fungi (Burges, 1998; Butt *et al.*, 1999 and Butt *et al.*, 2001). Among them, *Trichoderma* spp. are one of the most exploited fungal biocontrol agents in the field of agriculture for the management of crop diseases caused by a wide range of fungal phytopathogens (Elad, 2000; Mathivanan *et al.*, 2000). Verma *et al.* (2007) reported that *Trichoderma* based fungicides were the most commonly used ones.

The antagonistic effect of *Trichoderma* spp. was first reported by Weindling in 1932 and he observed secretion of some substances from the hyphae of *T. lignorum.* Later it was identified as gliotoxin and was found lethal to both *Rhizoctonia solani* and *Sclerotium americana.* In 1974, Coley-Smith and co workers showed by means of microtome sections that medulla of infected sclerotia of *S. delphinii* were completely replaced by hyphae and chlamydospores of *Trichoderma hamatum* on agar plates. That was the first report on mycoparasitism of *Trichoderma* spp. Later, Henis *et al.* (1982) also observed the abundant production of chlamydospores by *Trichoderma* spp. within the infected sclerotia of *S. rolfsii.* One of the first characterized secondary metabolites of *Trichoderma* spp. was the peptide antibiotic paracelsin from *T. reesei* (Bruckner and Graf, 1983; Bruckner *et al.*, 1984).

In 2004, Benitez and co workers reported that the most important *Trichoderma* spp. having antagonistic properties were *T. atroviride, T. harzianum, T. virens* and *T. asperellum.* According to them, *Trichoderma* spp. were able to control ascomycetes, basidiomycetes and oomycetes. The antagonistic effect of *Trichoderma* spp. on nematodes was also reported by several workers (Dababat *et al.*, 2006; Kyalo *et al.*, 2007 and Goswami *et al.*, 2008).

Radheshyam *et al.* (2012) reported that *Trichoderma* spp. had successfully used for the management of diseases caused by *Rhizoctonia solani* in bean, tomato, peanut, rice, lettuce; by *Sclerotium rolfsii* in lupine, tomato, peanut, sugarbeet; by *Pythium* spp. in pea, tomato, brinjal, tobacco and sugarbeet and by *Macrophomina phaseolina* in sesame and okra. According to Sriram *et al.* (2013), *Trichoderma* spp. could be used in different crops like rice, wheat, pulses, vegetables, coconut, black pepper, cardamom, ginger, banana, sugarcane, sunflower, ground nut, soybean, cotton, castor and tobacco against a wide range of plant pathogens. In 2015, Srivastava *et al.* reported that *Trichoderma* spp. was highly effective on root rot, foot rot, collar rot, stem rot, damping off, wilt, blight and leaf spot of crops like pulses, oil seeds, cucurbitaceous crops (cucumber, bottle gourds, ridge gourd), solanaceous crops like tomato, brinjal, chilli, capsicum *etc.* and were also found effective against sheath rot, sheath blight and bacterial leaf blight of rice. In short, nowadays, *Trichoderma* based bio control products share 60 per cent of all fungal based products in the world market with a regular increase in the number (Waghunde *et al.,* 2016).

2.2.1. History of *Trichoderma* spp.

The first description of the fungus, *Trichoderma* dates back to 1794 (Persoon, 1794). He first proposed the genus name *Trichoderma* on the basis of macroscopic similarity. The four species categorized in this genus were *T. viride, T. nigriscens, T. aureum* and *T. roseum,* collected in Germany. But later these four species were found to be unrelated to each other. The name *Trichoderma* is now applied to the most frequently encountered green forms typified by the original *T. viride* species described by Persoon, 1794. In 1865, a link to the sexual state, *Hypocrea* sp. was suggested by Tulasne and Tulasne. However, the different species assigned to the genus *Trichoderma / Hypocrea* were difficult to distinguish morphologically and hence, the taxonomy and

species identification were vague until around 1969. In 1969, based on the colony growth rate and microscopic characters, the first concept for genus identification was developed by Rifai. Some new species were subsequently described and keyed out (Domsch *et al.*, 1980). Thereafter numerous new species of *Trichoderma / Hypocrea* were discovered, and by 2006, the phylogenetic classification has rapidly reached 100 (Druzhinina *et al.*, 2006).

In 2010, Schuster and Schmoll reported that The Index Fungorum database (http://www.indexfungorum.org/Names/Names.asp) listed 471 different names for *Hypocrea* species and 165 records for *Trichoderma.* Currently, the International Subcommission on *Trichoderma / Hypocrea* listed 104 species (http://www.isth.info/biodiversity/index.php) which have been characterized at the molecular level (Waghunde *et al.*, 2016).

2.2.2. Mechanism of action of *Trichoderma* spp.

Isolates of the genus *Trichoderma* have been shown to antagonise a wide range of soil-borne phytopathogenic organisms through the mechanisms *viz.*, competition for space and nutrients (Verma *et al.*, 2007), parasitism, and antibiosis (Mukherjee *et al.*, 2008). It has been proved that *Trichoderma* spp. can colonise the root system of plants and were able to enter into the roots as endophytes and provided protection to host plants from pathogen infection (Harman, 2011). Singh *et al.* (2011) reported that these fungi could bring many benefits *viz.*, improved photosynthetic efficiency, increased nutrient and water up-take, and tolerance to abiotic stresses to their host plants.

According to Harman *et al.* (2004), disease suppression by *Trichoderma* spp. was mainly based on its hyper parasitism, antibiosis, induced resistance in the host plant and competition for nutrients and space. It was showed that direct mechanisms of disease control by *Trichoderma* spp.

included mycoparasitism mediated by the synthesis of lytic enzymes (Benitez *et al.*, 2004; McIntyre *et al.*, 2004; Howell and Puckhaber, 2005). At the same time Harman *et al.* (2004) suggested that indirect mechanisms of disease control included competition for space and nutrients, induction of defense responses in plants, biofertilization and antibiosis.

Trichoderma spp. were found to be aggressive competitors because of their fast growth and rapid colonization of substrates to exclude pathogens such as *Fusarium* spp. (Papavizas, 1985). He also reported that the most common reason for the death of many microbes in the presence of *Trichoderma* spp. was the starvation and scarcity of limiting nutrients. They mostly found in rhizosphere of plants (Harman, 2000). Since they were good colonizers of cellulosic materials, they could often be found wherever decaying plant material was available (Kubicek *et al.,* 2008; Jaklitsch, 2009). Carbon and iron are the two essential elements required by most of the filamentous fungi for viability. Competition for carbon was effective mode of biocontrol not only in *Trichoderma* but also some other fungi such as strains of *Fusarium oxysporum* (Sarrocco *et al.*, 2009; Alabouvette *et al.,* 2009). Radheshyam *et al.* (2012) reported that competition was a proposed mechanism of action in *Trichoderma* spp. although it was not proven to be the main activity.

The complex process of mycoparasitism included chemotropic growth of hyphae of *Trichoderma*, recognition of the host by the mycoparasites, secretion of extra cellular enzymes, penetrations of the hyphae and lysis of the host (Zeilinger *et al.,* 1999). In 2002, De Marco and Felix proposed that in *Trichoderma* species, central mechanism responsible for the antagonistic property was mycoparasitism. According to Punja and Utkhede (2003), *Trichoderma* spp. were the most widely studied mycoparasitic fungi. Harman *et al.* (2004) suggested that the remote sensing activity of hyphae of

Trichoderma spp. was partially because of their ability to produce pathogenesis related proteins, mostly glucanase, protease and chitinase. In 2012, the cell wall degrading enzymes of *Trichoderma* spp. such as β -1, 3-glucanases and different chitinolytic enzymes were suggested to be the key enzymes in mycoparasitism by Radheshyam *et al.* Gajera *et al.* (2013) reported that constitutive secretion of exochitinases from *Trichoderma* spp. at low level could degrade fungal cell wall. It resulted in the release of oligomers which played a central role in growth inhibition of pathogenic fungal strains.

In 1994, Schirmbock *et al.* defined antibiosis as the process of secretion of anti – microbial compounds by antagonist fungi to suppress and / or kill pathogenic fungi in the vicinity of its growth area. It was revealed that small sized diffusible compounds or antibiotics produced by *Trichoderma* spp. could inhibit the growth of other microorganisms (Benitez *et al.*, 2004). Raaijmakers *et al.* (2009) reported that the typical coconut smell of *T. viride* indicated the presence of volatile compounds including harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-pentyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid *etc.* which were found to be inhibitory to pathogen growth. In 2014, different pathways for producing secondary metabolites *viz.*, pyrone, polyketide, peptaibol, flocculosin terpenoid/steroid, gliotoxin and gliovirin were illustrated and summarized by Daguerre *et al.*

2.2.3. Isolation of *Trichoderma* spp.

According to Harman *et al.* (2004), *Trichoderma* spp. were the most frequently isolated soil fungi. However, there is still considerable interest in finding of *Trichoderma* spp. with more biocontrol effectiveness.

A total of 20 isolates of *Trichoderma* spp. were isolated from the roots of cotton plants and were cultured on potato-dextrose agar (PDA) and rose bengal agar at 28°C for five days. (Asran-Amal *et al.*, 2005). In 2009, Gil and co workers isolated biocontrol agents *viz., Trichoderma* spp., *Gliocladium* spp. and actinomycetes for the quantitative estimation of their population in soil using dilution plate technique. In their experiment, they used 10^{-1} to 10^{-6} soil dilutions and potato dextrose agar (PDA), modified potato dextrose agar (PDA amended with rose bengal, chloramphenicol, and strepto- mycin sulfate - PDAm), Martin medium cellulolytic fungi (CF) medium, medium E for *Trichoderma* spp. (TME) and selective medium for *Trichoderma* spp. (TSM) as the culture media for the isolation of *Trichoderma* spp. and *Gliocladium* spp.. Among the various media used, they observed PDAm as the most effective medium for the isolation of *Trichoderma* spp. and *Gliocladium* spp.

Kumar *et al.* (2012) collected soil samples from different ecological habitat of spice crops of South Andaman, Andaman and Nicobar Islands for the isolation of *Trichoderma* spp. They prepared five-fold serial dilutions of each soil samples in sterilized distilled water and 0.5 ml diluted sample was poured on the surface of Trichoderma Specific Medium (TSM) to isolate *Trichoderma* spp.. Terna *et al.* (2016) isolated seven isolates of *Trichoderma* spp. from farm and refuse dump soils in Ibadan, Nigeria using serial dilution plate technique at concentration of 10^{-6}. They used potato dextrose agar as the culture medium. Kamaruzzaman *et al.* (2016a) isolated ten isolates of *Trichoderma* spp. in the similar way by collecting soil samples from different locations of Bangladesh.

2.2.4. *In vitro* antagonism of *Trichoderma* spp.

Several researchers have showed the antagonistic potential of *Trichoderma* spp. against soil borne as well as foliar fungal pathogens

(Mohamed and Haggag, 2006; Etebarian, 2009; Mishra, 2010; Eshgi *et al.*, 2015).

Gomathi and Ambikapathy, 2011 reported that in dual culture experiment, the percentage of inhibition of growth on the pathogen, *Pythium debaryanum* by *Trichoderma harzianum, T. koningii* and *T. viride* were 66.6, 62.5 and 60.4 percentage respectively. Muthukumar *et al.* (2011) tested eight isolates of *Trichoderma* spp. for the growth inhibition of *Pythium aphanidermatum*, of which isolate from chilli rhizosphere (TVC3) exhibited maximum growth inhibition of the pathogen (88.0%) compared with the control. This was followed by THC1 (83.9%) and TVC5 (80.0%). The least inhibition percentage expressed was 52.6 by isolate TVC2. In 2012, Patil *et al.* evaluated the antagonistic potential of three different species of *Trichoderma viz., T. harzianum, T. flavofuscum* and *T. viride* against four isolates of *Pythium* spp. *viz., P. aphanidermatum, P. vexans, P. ultimum* and *P. viniferum.* They reported that the result of dual culture experiment with all the *Trichoderma* spp. showed the inhibition percentage in a range of 27.78 (*T. flavofuscum* against *P. ultimum*) to 69.23 (*T. viride* against *P. vexans*) and the result of volatile metabolites showed the per cent of inhibition in a range 38.46 (*T. viride* against *P. vexans*) to 87.78 (*T. harzianum* against *P. aphanidermatum*).

The antagonistic potential of *T. harzianum* was tested against 38 isolates of *Phytophthora infestans* and the results showed a range of 56.94 to 86.75 per cent of inhibition on the growth of the pathogens (Fatima *et al.*, 2015). Ambuse and Bhale, 2015 described the antagonistic efficacy of *Trichoderma* spp. against *Phytophthora colocasiae* by dual culture method under *in vitro* conditions. *Trichoderma viride, T. harzianum, T. virens, T. koningii* and *T. pseudokoningii* species were used for the antagonistic study. Their results indicated that all tested *Trichoderma* spp. were found > 50 per

cent antagonistic to the pathogen. Among them, highest inhibition percentage of 77.77 per cent was exhibited by *T. viride* and *T. harzianum* followed by *T. pseudokoningii* showed 61.11 per cent of inhibition on the growth of pathogen.

Trichoderma reesei was the most effective mycoparasite in interactions with *Ganoderma* isolates, followed by *T. koningii* and *T. harzianum* (Widyastuti *et al.*, 2003). The range of inhibition percentage in that case was 56.4 – 100. Susanto *et al.* (2005) documented that *T. harzianum* could exhibit the highest inhibition capacity of 97.8 per cent on the growth of *G. boninense* in dual culture analysis. A total of 48 native isolates of *T. harzianum* were taken by Siddiquee *et al.* (2009) for screening of their antagonistic properties against *G. boninense* (strain PER 71), causal organism of basal stem rot of oil palm, using dual culture techniques. The results showed that all the isolates inhibited the growth of PER 71 with the growth inhibition percentage values ranged from 47.86 to 72.06 per cent.

Kumar *et al.* (2012) tested antagonistic ability of 12 isolates of *Trichoderma* spp. against soil borne pathogen *S. rolfsii* and foliar borne pathogens *viz., Colletotrichum capsici* and *C. gloeosporioides*. The results showed that all the isolates recorded > 50 per cent inhibition over the pathogen *S. rolfsii* and > 27 and > 36 per cent of inhibitions on the foliar pathogens *C. gloeosporioides* and *C. capsici* respectively. The antagonistic potential of six isolates of *Trichoderma* spp. was evaluated by Parmar *et al.*, 2015 against a soil borne pathogen of ground nut, *S. rolfsii*. Their results indicated that *T. viride* could inhibit 61 per cent of growth of the pathogen followed by *T. harzianum* which showed 55 per cent reduction in growth of the same pathogen. The least inhibition percentage was recorded by *T. pseudokoningii* (44%).

In the dual culture experiment, the direct use of *Trichoderma* spp. could exhibit high inhibition per cent against *Fusarium oxysporum* f. sp. *psidii* (61-69%) (Kumar *et al.*, 2007) and *F. solani* (58-68%) (Gupta and Mishra, 2009). In 2012, the antagonistic efficacy of *T. harzianum* against three fungal pathogens *viz., Cladosporium spherospermum, Aspergillus niger* and *Fusarium oxysporum* was evaluated by Lone *et al.* Their results revealed that *T. harzianum* caused the maximum growth inhibition in *A. niger* (75%) followed by *C. spherospermum* (72.2%) and *F. oxysporum* (25%). The dual culture experiment conducted by Sonawane *et al.* (2015) using six isolates of *Trichoderma* spp., showed a range of 92.4 to 100 per cent of inhibition on the growth of *Fusarium oxysporum.*

Kamaruzzaman *et al.* (2016 b) evaluated 10 isolates of *Trichoderma* spp. isolated from soil, for their antagonistic efficacy against seven phytopathogens *viz., Fusarium oxysporum, Sclerotium rolfsii, Rhizoctonia solani, Aspergillus flavus, Penicillium* sp., *Colletotrichum gloeosporioides* and *Phomopsis vexans* using dual culture technique. Six days after inoculation, the highest inhibition per cent of 99.58, 99.70, 99.85, 100, 99.58, 91.87 and 100 were exhibited by isolates of *Trichoderma* spp. against the pathogens *F. oxysporum, S. rolfsii, R. solani, A. flavus, Penicillium* sp., *C. gloeosporioides* and *P. vexans* respectively.

In 2010, Guigon-Lopez and co workers evaluated the antagonistic potential of six *Trichoderma* strains, among which five of them belonged to *T. asperellum* and one of them belonged to *T. longibrachiatum.* They reported that *T. asperellum* strains, TC 74, T 341 and T 359, developed the highest *in vitro* growth rate and also showed the highest antagonistic activity against the plant pathogens *B. cinerea, R. solani, M. phaseolina* and *P. omnivora, Fusarium* sp. with the inhibition per cent of growth ranged from 60 - 75; 51 - 59; 44 - 64; 28 -37 and 5 – 14 per cent respectively. Naglot *et al.* (2015)

observed that the per cent inhibition of a tea pathogen, *F. solani* by 21 native isolates of *Trichoderma* spp. isolated from tea gardens of north-eastern region of India, ranged from 42 – 76 per cent.

In 2016, the *in vitro* antagonistic efficacy of four isolates of *Trichoderma* spp. *viz.*, *T. asperellum* (T1), *T. longibrachiatum* (T2) and two isolates of *T. harzianum* (T3 and T4) was evaluated by Al-qaysi and Alwan, against *R. solani* and the results showed the highest inhibition percentage of 50 by *T. harzianum* (T3) followed by T2 recorded 47.5 per cent inhibition. The range of inhibition percentage was found to be 42.2 to 50.

In 2003, Howell reported that the mycoparasitic activity of *Trichoderma* sp. against various phytopathogens and oomycetes are due to the lytic activity of cell wall-degrading enzymes. Later, Guevara and Zambrano (2006) tested the cellulase activity of nine bacterial strains on Carboxy Methyl Cellulose agar plates flooded with congo red. They observed the cellulase activity of bacterial strains in a range of 1.5 to 3.0. Kamala and Indira, 2011 conducted an experiment to evaluate the cellulase activity of 20 indigenous isolates of *Trichoderma* spp. from Manipur using Czapek - mineral salt agar medium and hex-adecyltrimethyl ammonium bromide. From their results, it was evident that the highest cellulase activity was observed in case of T 73 with an inhibition zone of 62.33 mm, which was followed by T 105 (50.67 mm) and T 68 (44.33). The least cellulase activity was produced by T 11 (11.67 mm).

2.2.5. Disease management of *Trichoderma* spp. under *in vivo* condition

A plenty of researches have been conducted regarding the effective use of *Trichoderma* spp. for managing various kinds of diseases. Wijesinghe *et al.* (2010) reported that several *Trichoderma* species were used as biocontrol

agents against plant pathogenic fungi such as *Sclerotinia* spp., *Verticillium* spp., *Rhizoctonia solani, Fusarium oxysporum, Colletotrichum gloeosporioides* and *Thielaviopsis paradoxa* under both green house and field conditions. Soil treatments with *T. harzianum* spores suppressed infestations of *F. oxysporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *melonis* (Radheshyam *et al.*, 2012).

It was demonstrated that *T. hamatum* inoculation of potting mix with a high microbial capacity, significantly reduced the severity of *Rhizoctonia* damping off of radish and *Rhizoctonia* crown and root rot of poinsettia (Krause *et al.*, 2001). The same species of *Trichoderma* induced systemic resistance in cucumber and reduced the severity of *Phytophthora* leaf blight when they were inoculated into the compost amended potting mix (Khan *et al.*, 2004). In 2005, Chakraborty proved the efficacy of *T. harzianum* (Rifai), a potent biocontrol agent in inhibiting the growth of *F. solani*, causal organism of wilt of brinjal, both under *in vitro* and field condition. In 2008, Coskuntuna and Ozer reported that seed treatment with *T. harzianum* decreased the incidence of basal stem rot in onion comparable to the imidazole fungicide, prochloraz. Dubey *et al.* (2007) reported that the integration of *T. harzianum* (10^5 spores / ml/ g seed) and carboxin (2 g /Kg seed) as seed treatment resulted in enhanced seed germination (12.0 -14.0%) and grain yields (42.6 – 72.9%) and reduced wilt incidence (44.1 – 60.3%) in chickpea.

Morsy *et al.* (2009) conducted an *in vivo* experiment using *T. viride* and *Bacillus subtilis* with tomato plants under pot culture and field condition. They reported that an integrated application of *T. viride* and *B. subtilis* recorded a survival rate of 80 per cent and 86.7 – 90 per cent from the wilt in pot and field condition respectively. They also reported that this combination treatment could promote the growth characters *viz.*, number of branches /plant, height, number of fruits /plant and yield of tomato.

A study to evaluate the biocontrol potential of three indigenous *Trichoderma* isolates (T 73, T 80 and T 105) against damping-off disease in common beans caused by *P. aphanidermatum* was conducted under pot culture experiment (Kamala and Indira, 2011). The result of the experiment revealed that in greenhouse condition, isolate T 105 significantly reduced the pre- and post-emergence damping-off disease incidence and showed highest disease control percentage of 82.86 and 90.72 per cent respectively. Surekha *et al.*, 2013, showed that seeds of black gram coated with spores of *T. viride* were found effective in reducing disease incidence and severity of *Fusarium* wilt and *Alternaria* blight under greenhouse conditions. They also reported that *T. viride* could act as the best antagonist against *A. alternata* than *F. oxysporum* and it alone reduced the disease incidence by 59 and 62 per cent by 7^{th} day and 14^{th} days respectively.

Harman *et al.* (2004) reported that some strains of *Trichoderma* spp. promoted plant growth, increased nutrient availability, improved crop production and enhanced disease resistance in plants. Many biocontrol agents were not only able to control the pathogens but were able to promote plant growth and development (Vinale *et al.*, 2008).

In 2004, the ability of *T. harzianum* T 22 and *T. atroviride* P 1 to improve the growth of lettuce, tomato and pepper plants was investigated under green house and filed conditions by Vinale *et. al.* In their study, in all the cases, the crop productivity was found to be increased up to 300 per cent compared to the control plants. The effect of *T. harzianum* on the growth response of tomato plants was shown by Tanwar *et al.* (2010). They reported that among the different treatments, a combined application of *Glomus mosseae, Acaulospora laevis* and *T. harzianum* was most effective in increasing the shoot length, root biomass, flowering and fruiting in plants over

control. They also observed high fresh and dry shoot weight, root length and leaf area in plants treated with *T. harzianum* alone.

Kamaruzzaman *et al.* (2016 a) evaluated four *Trichoderma* isolates *viz.*, *T. harzianum* (ST 5), *T. viride* (ST 6), *T. virens* (ST 7) and *T. atroviride* (ST 9) for their plant growth promotion characters in peanut. When the treatments were given as seed treatment, *T. harzianum* (ST 5) gave maximum length and weight of shoot, weight of roots with pods, weight of pods and number of nodules per plant. *T. viride* (ST 6) showed higher plant growth, nodulation and yield compared to *T. virens* (ST 7) and *T. atroviride* (ST 9). In the control treatment, minimum growth, yield and nodulation were observed. Daryaei *et al.* (2016) reported that the conidial treatment of *T. atroviride* LU 132 @ 2×10^6 conidia/g of potting mix was able to colonise ryegrass root systems, and it increased plant dry matter and also protected the plants from the attack of a soil-borne pathogen, *R. solani.*

2.2.6. Compatibility of *Trichoderma spp.* with fungicides and insecticides

In an integrated approach of disease management, it is necessary to check the compatibility of antagonists with pesticides. Stephen *et al.* (2000) studied the compatibility of *T. harzianum* with phorate (6 - 36 ppm a.i.) and chlorpyriphos (10 - 40 ppm a. i.) under *in vitro* condition and in soil. They reported that phorate and chlorpyriphos could be safely applied with *T. harzianum* for the management of *Phytophthora* foot rot, nematodes and mealy bugs on black pepper. Experiments were also conducted to evaluate the compatibility of different combinations of carbendazim, mancozeb and phorate with biocontrol agents such as *T. viride, T. harzianum, Gliocladium virens* and *Enterobacter aerogenes* (Gupta and Sharma, 2004). Their findings indicated that, of the various chemicals tested under *in vitro* condition, carbendazim was found inhibitory to all the fungal antagonists whereas,

mancozeb and phorate were least inhibitory at 200 ppm. Vinale *et al.* (2004) reported a high level of tolerance of *T. harzianum* and *T. atroviride* to concentrations of copper oxychloride varying from 0.1 up to 5mM.

Saju (2005) reported that the pesticides, metalaxyl, mancozeb, copper oxychloride and quinalphos up to 300 ppm could not affect the population of *T. harzianum* (IISR 1369) which indicated its high compatibility with the field concentrations of these chemicals. Hanada *et al.* (2009) tested the compatibility of conidial germination of *T. martiale* strain ALF 247 with two commonly used contact and systemic fungicides, copper hydroxide (1.5% a. i.) and fosetyl-Al (0.4% a. i.). The results showed that under the concentrations and incubation periods *i. e.* for 30, 60, 90, 120 and 180 minutes used in the study, the average conidial germination varied from 91.3 - 94.7 per cent, indicated the high compatibility of conidia of *T. martiale* strain ALF 247 with these fungicides.

In 2010, Bhai and Thomas tested the compatibility of *T. harzianum* with commonly used three fungicides *viz.,* Bordeaux mixture, copper oxychloride and mancozeb and six insecticides *viz.,* phorate, chlorpyriphos, quinalphos, monocrotophos and phosalone, in cardamom at their recommended dosages *viz.,* 1%, 0.25%, 0.25%, 20g/plant, 0.07%, 0.05%, 0.075% and 0.07% respectively. The results showed that among the fungicides, Bordeaux mixture was found highly inhibitory (100%) to *T. harzianum* and among the insecticides, quinalphos recorded maximum inhibition per cent of 55.84. Copper oxychloride, phorate and phosalone were found highly compatible with *T. harzianum* without any inhibition on growth. They observed the same results in pot and field experiments. Gangwar and Sharma (2013) reported that *Trichoderma* could be easily applied to seeds treated with metalaxyl and thiram but not with mercurials.

In 2004, Vijayaraghavan and Abraham reported that mancozeb was compatible with *Trichoderma* sp.. Bagwan (2010) reported that thiram, copper oxychloride and mancozeb at 0.2 % were compatible with *T. harzianum* and *T. viride*. He also reported that *Trichoderma* spp. was most sensitive to captan, tebuconazole, vitavax, propiconazole and chlorothalonil. His findings indicated that none of the insecticides tested *viz.*, monocrotophos (0.05%), imidacloprid (0.008%), chlorpyriphos (0.2%), profenofos (0.05%) and carbosulfan 25% EC (0.05%), inhibited the growth of *Trichoderma* spp. above 10 per cent. Sarkar *et al.* (2010) also proved the compatibility of *T. harzianum* with copper oxychloride and copper hydroxide. Deepthi (2013) reported that an isolate of *Trichoderma* spp., GRHF 4 was more compatible with mancozeb followed by copper oxychloride.

Gowdar *et al.* (2006) reported complete inhibition (100%) of *Trichoderma* sp. with carbendazim at 0.1 and 0.2 per cent concentrations followed by 96.88 and 88.44 per cent inhibition with thiophanate methyl at 0.1 and 0.2 per cent concentrations respectively. In 2014, Thoudam and Dutta conducted a study to evaluate the compatibility of *T. atroviride* against the fungicides, tebuconazole, tetraconazole, sulphur + copper, carbendazim + mancozeb, mancozeb, captan, copper oxychloride and hexaconazole with different concentrations of 1, 10, 50 and 100 ppm, under *in vitro* condition. In their experiment, it was found that at 1 ppm concentration, the fungicide, carbendazim was highly compatible with the antagonist, whereas it showed cent per cent inhibition in all other concentrations used. Except, carbendazim all other fungicides were found to be compatible with *T. atroviride*.

Tapwal *et al.* (2012) reported the compatibility of *Trichoderma* sp. with fungicides, mancozeb, carbendazim and metalaxyl + mancozeb at concentrations of 50, 100, 200 and 300 ppm and insensitivity of it to blue copper and captaf. In 2016, Singh *et al.* observed very less mycelial growth of

T. harzianum in the medium amended with four different concentrations *viz.*, 500, 1000, 1500 and 2000 ppm of the fungicide, carbendazim. An *in vitro* experiment was conducted by Samuelian in 2016, to investigate the inhibitory effect of mancozeb at 2.5 l/ha and 5 l/ha on *Trichoderma* isolates, *T. virens* (BRIP60169), and *T. harzianum* (BRIP60170 and BRIP60384). He reported that in the two concentrations tested, mancozeb showed complete inhibition on the mycelial growth of all *Trichoderma* isolates analysed.

2.4. MORPHOLOGICAL CHARACTERIZATION OF *Trichoderma* spp.

Morphological characterization was a potential method for the identification of *Trichoderma* species (Gams and Bissett 2002; Samuels *et al.*, 2002 and Anees *et al.*, 2010). Samuels *et al.* (2002) considered cultural characteristics such as growth rate, colony colour and colony appearance as taxonomically useful characteristics for *Trichoderma* spp. In 2010, Bhagat and Pan reported that cultural and anamorphic characteristics were the major determinants for morphological identification of *Trichoderma* spp. at species level.

Bhagat and Pan (2010) studied cultural characteristics of 38 isolates of *Trichoderma* spp. in three media, potato dextrose agar (PDA), oat meal agar (OMA) and malt extract agar (MEA). The results indicated that there were clear differences in growth pattern and other cultural characters of antagonists in three media. In general, the mycelial growth was found denser in PDA than in MEA and OMA. The sporulation was first noticed in PDA followed by MEA. The spores were appeared in green to dark green and sometimes yellowish green to dark green with white tinge. They had also reported that *T.viride* and its related species were able to secrete α-pyrone, responsible for a sweet coconut like aroma.

In 2014, Sharma and Singh determined cultural characteristics and growth rates of 30 isolates of *Trichoderma* spp. on Potato dextrose agar medium. They reported that all the isolates were found fast growing reached a radius of 42.5 to 56.5 mm after 72 h at 25°C and 20 to 37.8 mm after 72 h at 35°C. The colour of conidia was changed from white to varying shades of green. In most isolates, conidia were formed by 48 h and turned green within 72 h.

Herath *et al.* (2015) studied the morphological characters of an isolate of *Trichoderma* spp. by culturing it on Czapeck dox agar (CDA) medium. He reported that isolate grew rapidly on the media forming a cottony green colony with concentric rings. The conidiophores were found erect and arose from short side branches. The conidia were globose, pale green with smooth walls and were occurred in clusters with a size of 2.5 µm ± 0.1.

Kamaruzzaman *et al.* (2016b) conducted morphological characterization of 10 isolates of *Trichoderma* spp. and reported that mycelial growth rate of the isolates tested varied considerably up to 72 hours. All the isolates exhibited regular type of growth. In their study, the colour of spores varied as light green, whitish green, dark green and yellowish green, indicated the differences in spore production.

2.5. MOLECULAR CHARACTERIZATION OF *Trichoderma* spp.

Plaza *et al.*, 2004 reported that nucleic acid detection methods such as PCR became a common tool for the identification and characterization of microbial communities. Joo *et al.* (2005) conducted the molecular identification of the isolated strain of *Trichoderma* spp. using ITS 1 and ITS 4 as primers. Nielsen *et al.* (2005) amplified the ITS 1 and ITS 2 regions of the ribosomal DNA of *Trichoderma* spp. using the primers ITS 5 and ITS 4 and

the length of the fragments was determined on 1 per cent agarose gel. The isolate was identified as *T. brevicompactum.*

Molecular identification of 21 isolates of *Trichoderma* spp. was carried out by Lopes *et al.*, 2012 at species level using the primers ITS 1 and ITS 2. They identified nine isolates as *T. asperellum*, seven as *T. harzianum*, three as *T. tomentosum*, one each as *T. koningiopsis* and *T. erinaceum.*

The amplification of rRNA gene region of 20 isolates of *Trichoderma* spp. was carried out by Nikolajeva *et al.*, 2012 using the primers, ITS 1 and ITS 4. Out of 20, six strains were proved to be *T. asperellum* and five belonged to *T. viride*, two strains belonged to *T. koningii* and two belonged to *T. viridescens* but five further species (*T. citrinoviride, T. hamatum, T. harzianum, T. longibrachiatum* and *T. rossium*) were represented by single isolates.

In 2015, an isolate of *Trichoderma* spp. was molecularly identified as *T. erinaceum* using ITS primers, ITS - 1 and ITS - 4 by Herath *et al.* They used those primers to amplify a ~ 600 bp fragment of the rDNA. They reported that the homology search against the GenBank data revealed 100% similarity to the ITS region of *T. erinaceum.*

Various molecular techniques have been used in *Trichoderma* research including random amplified polymorphic DNA (RAPD) analysis (Hermosa *et al.*, 2001), restriction fragment length polymorphism (RFLP) analysis (Dodd *et al.*, 2004), amplified fragment length polymorphism (AFLP) analysis (Buhariwalla *et al.*, 2005) *etc.* In 2015, Skoneczny and co workers suggested that among those methods, RAPD was the simplest and cheapest technique which was characterized by low repeatability and transferability between different laboratories.

Genetic variability among the isolates of *Trichoderma* spp. was studied by Venkateswarlu *et al.* (2008) by using molecular techniques like RAPD and ITS - PCR. In their study, five random primes *viz.*, OPA 8, OPA 9, OPA 10, OPA 12 and OPA 13 generated reproducible polymorphism among the isolates of *Trichoderma* spp. In ITS - PCR, ITS region of rDNA was amplified using genus specific ITS – 1 and ITS - 4 universal primers. They observed the amplified products in the size ranged from 504 to 566 bp.

In 2010, Gupta *et al.* conducted DNA RAPD - PCR analysis of seven isolates of *Trichoderma* spp. and found that out of 10 RAPD oligonucleotides (OPA 1 – OPA 10) tested, seven markers OPA 1, 3, 5, 7, 8, 9 and 10 efficiently differentiated the isolates of *Trichoderma* spp. by showing reproductive banding patterns. A total of 248 bands were obtained from those markers along with a 61.84 per cent similarity among the seven isolates of *Trichoderma* spp.

2.6. STRAIN IMPROVEMENT IN *Trichoderma spp.*

2.6.1. Mutation

Mutation is one of the successful tools to enhance the antagonistic effects of *Trichoderma* spp. against fungal plant pathogens (Mohamed and Haggag, 2010).

Rey *et al.* (2001) developed a mutant of *T. harzianum* strain 2413. They observed increased activity of extracellular enzymes and α- pyrone in the mutant. The mutant also showed increase in resistance against *R. solani* and *B. cinerea* than the wild type under different controlled environmental conditions.

Zaldivar *et al.* (2001) practiced mutation in a wild type strain of *T. aureoviride* which proved its ability to grow on cellulose as carbon source,

using a chemical mutagent, N-methyl-N-nitro-N-nitrosoguanidine (NG, Sigma). Surviving conidia after the chemical treatment were plated on potato dextrose agar with the addition of 0.1 per cent Triton X-100 and 4 g/l L - sorbose as colony restrictors. They reported that the mutant strain 7-121 secreted three times more β -1, 4- endoglucanase and four times more β - glucosidase than the wild type. In addition, the mutant strain showed enhanced production of fungal cell wall degrading enzymes, chitinases, β -1, 3- glucanases and proteases.

In 2006, Mohamed and Haggag developed mutants by exposing culture of *T. harzianum* to two dosages of γ irradiation (200 Gy and 500 Gy). From the surviving colonies, they selected and identified four isolates (Th20M53, Th50M6, Th50M11 and Th50M46) as stable mutants. They reported that the mutant strains, Th50M6 and Th50M11 showed increased antagonistic activity against, *F. oxysporum* under *in vitro* experiments. In the pot culture and field experiments, the mutants were found significantly reduced incidence of wilt disease and improved yield and mineral contents of tomato plants than the wild type.

Mohamed and Haggag (2010) developed seven mutants by taking *T. koningii* (3mutants) and *T. reesei* (4 mutants) as parents, using a combination of UV - light and nitrate treatment ($NaNO_2$ - 500µg/ml). They found that most of the selected mutants were showing superiority in the growth rate and their antagonistic activity against selected soil borne fungal pathogens like *Fusarium oxysporum, Pythium ultimum, Sclerotium rolfsii* and *Sclerotinia sclerotiorum*. The mutants recorded higher reduction rate ranged between 92.6 - 98.3, 98.2 - 99.8, 89 - 98.3 and 73.3 - 99.2 per cent against *Fusarium* spp., *Pythium* spp., *Sclerotium* spp. and *Sclerotinia* spp. respectively than their parents.

In 2012, Radha *et al.* employed chemical and physical methods to develop mutant strains of *Aspergillus niger* on casein – agar medium. They developed 11 chemical mutants by the treatment of ethyl methane sulfonate (EMS @ 6.0 mg for one hour) and nine physical mutants by UV irradiation for a period of 60 minutes. They observed increased acid protease activity in mutant strains than the wild type. They reported that the physical mutant, UV 9 showed enhanced protease activity by 2.01 fold than wild type.

2.6.2. Isolation of protoplasts and protoplast fusion

During 1970s the protoplast isolation was a time consuming process. In 1976, Peberdy *et al.* reported that optimization of protoplast isolation depended on many factors including choice of osmotic stabilisers, pH and age of mycelium. Literature showed that the first source of cell wall lysing enzymes used for the isolation of protoplasts was the snail *Helix pomatia.* Later various micro organisms like *Cytophaga* sp., *Streptomyces venezulae* and *Trichoderma harzianum* were used for the same purpose (Peberdy, 1979). In 1980, Peberdy introduced a new enzyme Novozyme 234 isolated from the cell wall of *Trichoderma* spp. in Nottingham. That was a breakthrough in the field of protoplast isolation. Hamlyn *et al.* (1981) used this enzyme for the isolation of protoplasts from *Aspergillus nidulans* and they reported that millions of protoplasts were produced with just one hour's of incubation in the enzyme solution. In the same year, in Canada, Stephen and Nasim discovered the same enzyme, Novozyme 234 under the name of Mutanase and published their method of protoplast isolation in various yeast species.

It was Anne and Peberdy (1976) in Nottingham, used polyethylene glycol (PEG) first time to induce protoplast fusion in fungi. In 2002, Hanson and Howell reported that intra-specific fusants of *Trichoderma koningii* could show better biocontrol activity to *Rhizoctonia solani.*

Prabavathy *et al.* (2006 a) isolated protoplast from *Trichoderma reesei* strain *PTr 2* using Lysing enzymes (Sigma Chemicals Co., USA) with 0.6 M KCl as osmotic stabilizer. They conducted intra-strain protoplast fusion using 40 per cent polyethylene glycol with STC (sorbitol, Tris–HCl, $CaCl_2$) buffer. Fused protoplasts were regenerated on carboxymethyl cellulose agar (CMCA) selective medium and from them 15 fusants were selected. They reported that most of the fusants exhibited fast mycelial growth and abundant sporulation on PDA as compared to non-fusant and parent strains. Furthermore, they observed increased carboxymethyl cellulase activity in fusants especially in two fusants, *SFTr 2* and *SFTr 3* which recorded more than two-fold increase in enzyme activity than the non-fusants and parent strain *PTr 2*. In another study, they reported that the intra-fusants of *Trichoderma harzianum* increased chitinase and biocontrol activity than their parents (Prabavathy *et al.,* 2006 b).

El- Bondkly and Talkhan (2007) demonstrated the significance of the protoplast fusion approach as a technique to develop superior hybrid strains of filamentous fungi lacking inherent sexual reproduction. They isolated protoplasts from *T. harzianum* using Novozyme 234 as enzyme and 0.7M KCl as osmotic stabilizer. They carried out intra-strain protoplast fusion using PEG with STC (Sorbitol, Tris-HCl, $CaCl_2$) buffer and produced 18 self-fusant strains. Among them, four produced maximum chitinase with a two fold increase compared to the parent strain and most self-fusant strains exhibited increased antagonistic activity against *Cephalosporium acremonium*, *Aspergillus niger* and *Rhizoctonia solani* than the parent strain.

Srinivasan *et al.* (2009) isolated protoplasts from 16 hour old cultures of *T. harzianum* and *T. reesei* using lysing enzyme and the fusion of protoplasts was achieved by using PEG as fusing agent. Their studies revealed that the developed 20 fusant strains were growing 60 – 70 per cent faster than the parents, up to 3rd generation. The fusant strains displayed 40 - 50 per cent increased cellulase production and 10 - 20 per cent increase in chitinase

production than the parental strains and hence 7 – 8 per cent higher antagonistic activity than the parents.

Mohamed and Haggag (2010) practised the inter-specific protoplast fusion between *T. koningii* and *T. reesei* which yielded fusion frequency up to 1.25 per cent. They isolated 13 fusants. In dual culture, the fusants showed highest reduction rate ranged from 96.4 - 98.6, 90.4 - 94.6, 90.2 - 98.2 and 92.8 - 99.3 per cent against *Fusarium oxysporum, Pythium ultimum, Sclerotium rolfsii* and *Sclerotinia sclerotiorum* respectively compared to their parents.

In 2010, Hayat and Christias developed four fusants F1, F2, F3 and F4 by using protoplasts of A and R strains of *Sclerotium rolfsii.* From their study, it was revealed that the use of 1M sucrose as osmotic stabilizer and lysing enzyme @ 15mg/ml were found optimum for the isolation of protoplasts from strains A and R. They also reported that PEG 4000 could be effectively used for the fusion of protoplasts.

In 2014, Patil *et al.* developed fusants using filamentous chitinolytic fungal strains *A. oryzae* NCIM 1272 and *T. harzianum* NCIM 1185 as parents. They used cocktail containing 5 mg ml^{-1} lysing enzymes from *T. harzianum*, 0.06 mg ml^{-1} β- glucuronidase from *Helix pomatia* and 1 mg ml^{-1} purified *Penicillium ochrochloron* chitinase as enzyme and 0.8 mol l^{-1} sorbitol as an osmotic stabilizer. Intergeneric protoplast fusion was carried out using 60 per cent polyethylene glycol as fusogen. They observed enhanced chitinase activity in the fusants which ultimately provided potential strain for degradation of shellfish waste.

2.7. SYSTEMIC RESISTANCE INDUCED BY *Trichoderma* spp.

Induced systemic resistance (ISR) is one of the two major pathways involved in the defense mechanism of plants. It was initially described in

plants which were colonized by non pathogenic rhizobacteria. ISR is jasmonic acid or ethylene dependent pathway through which defense gene expressions are enhanced in plants. In 2005, Zhu- Salzman *et al.* stated ISR as a state of enhanced defensive capacity developed by a plant when stimulated by microorganism or environmental stress.

All plants are having endogenous defense mechanisms that can be induced in response to attack by insects and pathogens (Bostock *et al.,* 2001). Girish and Umesha (2005) reported that plants had a wide array of active defense responses which contribute to resistance against a variety of pathogens. They also reported that plants responded to bacterial pathogens by activating defense responses which included changes in cell metabolism and enzyme activities of cells. The changes in enzyme activities consisted of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), lipoxygenase, superoxide dismutase, and β -1, 3 - glucanase. Selvaraj and Ambalavanan (2013) suggested that application of biocontrol agents could elevate the production of defense related enzymes *viz.*, peroxidase (PO), polyphenoloxidase (PPO) and phenylalanine ammonia lyase (PAL) and accumulation of phenol in plants. Prasad *et al.* (2013) stated that fungi that colonize and penetrate plant root tissues could induce systemic resistance in plants. This was triggered by a series of morphological and biochemical changes resulted in the synthesis of defense chemicals against pathogens.

Peroxidase is an enzyme involved in the synthesis of lignin which is associated with the defense mechanism of plants. It is involved in substrate oxidation, cell wall lignification, photosynthesis, respiration and growth regulation and plays key roles in plant-pathogen interactions. In 1999, Yedidia *et al.* reported that seedlings of cucumber inoculated with *T. harzianum* showed increased peroxidase and chitinase activities in the roots and the leaves of treated plants, within 48 and 72 h, respectively which ensured induction of systemic resistance. Significant increase in the peroxidase activity

due to the seed treatment of *T. asperellum* strain T 34 in cucumber plants was reported by Segarra *et al.* in 2007. They reported that peroxidase activity in the control plants was between 0.20 and 0.34 µmol phenol red gram fresh weight^{-1} min^{-1} over the assay period.

PPO is a copper containing enzyme that oxidizes phenolics to highly toxic quinines which is responsible for the terminal oxidation of diseased plant tissue and there by attributed to the resistance. It is induced in plants *via* octadecanoid pathway. Quinones developed could also play a significant role in the lignin biosynthesis (Umesha, 2006).

PAL is the first enzyme of phenyl propanoid metabolism in higher plants. It has a significant role in the regulation of accumulation of phenolics, phytoalexins and lignins which are found to be key factors responsible for the disease resistance. Phenolics were a group of chemicals composed of one or more aromatic benzene rings with one or more hydroxyl groups (Armstrong, 2003). Phenolics are fungi toxic in nature and can increase the physical and mechanical strength of the host cell wall.

A pot culture experiment was conducted by Uppala (2007) to evaluate the efficiency of endophytes in inducing systemic resistance in amaranth. In this study, plants were artificially inoculated with *Rhizoctonia solani.* The results revealed that the isolates, EB – 20 and EB – 22 recorded maximum PO and PPO activity whereas standard cultures of bioagents *viz., T. harzianum* (IISR), *Pseudomonas fluorescens* (KAU) and *P. fluorescens* (TNAU) showed high PAL activity. It was also reported that the uninoculated healthy plants recorded higher PO activity but lower PPO and PAL activity than control.

In 2007, the induction of systemic resistance in ginger by the application of rhizobacteria was studied by Vijayaraghavan. In this study, appreaciable increase in PO, PPO and PAL activity over time was reported in

all the rhizobacterial treated plants after challenge inoculation of pathogen, *Ralstonia solanacearum*. Native PAGE analysis revealed six isoforms of PO and four isoforms of PPO in plants treated with rhizobacteria RB – 144, RB - 22, RB – 82, RB – 66, RB - 11 and *Pseudomonas fluorescens* whereas in each case, only three isoforms were noted in control.

Parmar and Subramanian (2012) conducted an experiment in tomato plants by challenge inoculating them with the pathogen, *Fusarium oxysporum* f. sp. *lycopersici*. They observed the increased production of defense related enzymes *viz.*, PO, PPO and PAL and total phenol in plants inoculated with the pathogen than the healthy plants. Similarly, in 2015, a study on induction of systemic resistance in tomato plants by the challenge inoculation of wilt pathogen, *Ralstonia solanacearum* was conducted by James. This study indicated that the plants treated with microbial consortium containing *Trichoderma viride* - 1, *T. viride* - 2, *T. harzianum* - 1, *Bacillus subtilis*, and *Streptomyces thermodiastaticus* showed higher activity of defense related compounds such as phenols, oxidative enzymes and PR proteins compared to healthy plants.

Murthy *et al.* (2013) revealed that treatment with *T. asperellum* (T 4 and T 8) could increase the production of defense related enzymes, peroxidase (PO), polyphenol oxidase (PPO) and phenyl alanine ammonia lyase (PAL) and the phenol in tomato plants challenge inoculated with *Ralstonia solanacearum*. They observed the highest activity of PO on 9th day after challenge inoculation in all the treatments which was then found decreased. One day after challenge inoculation, significant increase in the activity of PPO was observed and reached the highest level on 9th day. The activity of PAL was found highest on 7th day after challenge inoculation and was then declined. The treatment of T 4 and T 8 showed the maximum activity of PAL as 81 and 78 per cent respectively, relative to control plants. Phenol content in

plants were found increased from 1st day after challenge inoculation and reached the maximum level on 7th day by recording 620 µg g-1 catechol compared to the control (265 µg g-1 catechol).

Selvaraj and Ambalavanan, 2013 conducted a pot culture experiment in anthurium to study the induction of various defense enzymes and phenols by the two isolates of three biocontrol agents *viz., Pseudomonas fluorescens, Bacillus subtilis* and *Trichoderma viride.* Their study revealed that the application of biocontrol agents triggered the activity of these enzymes and phenols upon inoculation of the pathogen. They observed the peak activity of these compounds at six days after the inoculation of the pathogen. Native PAGE analysis of the enzymes showed the presence of additional three isoforms (PO 1 to PO 3) of PO and four isoforms (PPO 1 to PPO 4) of PPO which were absent in healthy plants.

Materials & Methods

3. MATERIALS AND METHODS

The present investigation on 'Enhancing bioefficacy of *Trichoderma* spp. for the management of soil borne fungal pathogens' was conducted in the Department of Plant Pathology, College of Horticulture, Vellanikkara, Thrissur during the period 2011 – 2016. The details of the materials used and the techniques adopted for the investigation are described below.

3.1. SURVEY AND COLLECTION OF SOIL SAMPLES

Purposive sample surveys were conducted in different locations of northern, central and southern districts of Kerala. Soil samples were collected from different locations of each districts of Kerala for the isolation of native *Trichoderma* spp.

3.2. ISOLATION OF NATIVE *Trichoderma* spp.

The rhizosphere soil collected from different types of lands *viz.*, garden, plantation, vegetable field, ginger field and forest of northern, central and southern regions of Kerala, were pooled separately, shade dried and the total microbial population of *Trichoderma* spp. were quantitatively estimated by serial dilution plate technique (Johnson and Curl, 1972). Potato Dextrose Agar medium (PDA), Rose Bengal Agar medium and *Trichoderma* Selective medium (Appendix 1) were used for estimating the microbial count of *Trichoderma* spp. at dilutions of 10^{-3} and 10^{-4}.

Dried soil of 10 g was transferred to 90 ml of sterilized water in 250 ml conical flask and shake well for 10 min in a shaker to get 10^{-1} dilution. The serial dilutions up to 10^{-4} were prepared from this dilution. The fungal

microorganisms were isolated on PDA, Rose Bengal Agar medium and *Trichoderma* Selective Medium by pour plate method. Transferred one milli litre from 10^{-3} and 10^{-4} dilutions separately to sterilized Petri dishes, poured the media and spread uniformly with a gentle swirl. Petri dishes were incubated at room temperature and next day onwards, the observations on total number of microbial colonies of *Trichoderma* spp. developed on the three media in each dilutions were recorded. The colonies showing the cultural characters of *Trichoderma* spp. were transferred to PDA slants. These isolates were purified, sub cultured and maintained as pure culture for further studies. The isolates of *Trichoderma* spp. obtained from the soil samples were hereafter mentioned as native isolates. The native isolates were numbered representing *Trichoderma* spp. shortly 'Tr' in the serial order.

3.3. ESTIMATION OF P^H OF SOIL SAMPLES

To investigate the relationship between the isolates of *Trichoderma* spp. and the chemical nature of soil, P^H of the soil samples were estimated by potentiometric method using a P^H meter (Jackson, 1958).

Soil samples were shade dried separately and sieved through a mesh size of two milli meter. 10 g of the dried soil samples were taken in separate glass beakers and added 25 ml distilled water to make a soil water suspension of 1: 2.5. This suspension was stirred continuously for 15 – 30 min and P^H was recorded using a P^H meter.

3.4. ISOLATION OF SOIL BORNE FUNGAL PATHOGENS

The important soil borne fungal pathogens *viz., Pythium aphanidermatum, Phytophthora capsici, Ganoderma lucidum, Rhizoctonia solani, Sclerotium rolfsii* and *Fusarium oxysporum* f. sp. *cubense* associated

with the diseased rhizome, stem and leaf of the selected crop plants *viz.*, ginger, pepper, coconut, rice, pepper and banana respectively were isolated on PDA medium.

3.4.1. Isolation of *P. aphanidermatum* from rhizome of ginger

The infected plants uprooted from different locations were brought to the laboratory and washed the rhizomes under tap water to remove soil particles. Small bits of infected portions along with some healthy portions were surface sterilized with one per cent sodium hypochlorite solution, washed in three changes of sterile water and then transferred to sterile Petri dishes containing solidified PDA medium. The dishes were incubated at room temperature (28 ± 2°C) and observed for the growth of pathogen from next day onwards. The isolates were purified by hyphal tip method and the pure cultures were maintained on PDA slants by periodical sub culturing.

3.4.2. Isolation of *P. capsici* from collar region of black pepper

The pathogen associated with the collar region of infected vine of black pepper was isolated and maintained the pure cultures on PDA slants by using the same procedure as described in 3.4.1.

3.4.3. Isolation of *G. lucidum* from basidiocarps

Basidiocarps collected from infected stem of coconut palms were brought to the laboratory and washed well under tap water to remove dirt particles. Tissues from inner part of basidiocarp *ie.* at the junction where pileus attach to the stipe portion were used for the isolation of pathogen using the procedure as described in 3.4.1.

3.4.4. Isolation of *R. solani* from the sheath of rice

The rice plants showing symptoms of sheath blight were collected and brought to the laboratory. The pathogen associated with the infected portions was isolated and maintained the pure cultures on PDA slants by following the same procedure as described in 3.4.1.

3.4.5. Isolation of *S. rolfsii* from the infected vine of black pepper

The infected vine of black pepper was collected and the pathogen, *S. rolfsii* was isolated from the collar region and maintained the pure culture on PDA slants as per the procedure explained in 3.4.1.

3.4.6. Isolation of *F. oxysporum* f. sp. *cubense* from banana

The infected rhizome of banana collected from the field were brought to the laboratory and washed under tap water to remove soil particles. Portions of the rhizome showing brown streaks were cut into small pieces of about one to 1.5 cm along with some healthy portions. The pathogen associated with the diseased specimens was isolated and maintained the pure cultures on PDA slants by following the same procedure as described in 3.4.1.

3.5. PRELIMINARY SCREENING OF NATIVE ISOLATES OF *Trichoderma* spp.

The native isolates of *Trichoderma* spp. isolated from soil samples collected from northern, central and southern districts of Kerala were preliminary screened for their antagonistic efficiency against two important soil borne fungal pathogens, *viz.*, a fast growing pathogen, *S. rolfsii* and a slow

growing pathogen, *F. oxysporum* f. sp. *cubense* by dual culture method outlined by Skidmore and Dickinson (1976) under *in vitro* condition on PDA medium. With the help of a cork borer, 8mm diameter of mycelial discs from actively grown culture of pathogens was transferred separately to the centre of PDA mediated Petri dishes. Mycelial disc of 8mm diameter of each isolates of *Trichoderma* spp. was transferred to the same plate and was placed at two cm away from the pathogen. In each Petri dish, disc of two isolates of *Trichoderma* spp. were placed on either sides of the pathogen. Three replications were maintained for each treatment. The pathogen grown as monoculture in the centre of the Petri dish served as control. All the plates were incubated at room temperature and were examined for the antagonistic activity. The measurements on the radial growth of pathogen and the antagonists were taken daily till the control plate showed full growth. The native isolates of *Trichoderma* spp. showing antagonistic properties were selected for further studies.

3.6. *IN VITRO* EVALUATION OF NATIVE ISOLATES OF *Trichoderma* spp. AGAINST SOIL BORNE FUNGAL PATHOGENS

The native isolates of *Trichoderma* spp. which showed antagonistic property during preliminary screening were used for further evaluation under *in vitro* condition against six soil borne fungal pathogens *viz., P. aphanidermatum, P. capsici, G. lucidum, R. solani, S. rolfsii* and *F. oxysporum* f. sp. *cubense* by dual culture technique (Dennis and Webster, 1971). The antagonistic property of these isolates was compared with the reference cultures of biocontrol agents *viz., T. viride* and *T. harzianum*. Mycelial disc of eight milli meter diameter was cut from actively growing cultures of each pathogen and placed separately at the centre of one half of the Petri dish. Similarly eight milli meter disc of the selected antagonist, *Trichoderma* spp. was transferred and placed at the centre of the other half of the same Petri dish. Three replications were maintained for each treatment and the pathogen

grown as monoculture served as control. The plates were examined for the antagonistic activity and the measurements on the radial growth of pathogen and the antagonist were taken daily till the control plates showed complete growth.

Per cent inhibition on the growth of pathogen over control was calculated by the formula suggested by Vincent (1927).

PI = [C-T/C] ×100

PI = Per cent inhibition

C = Radial growth of pathogen (mm) in control

T = Radial growth of pathogen (mm) in treatment

The mode of reaction of the antagonist on the growth of pathogen was studied by following the method given by Purkayastha and Bhattacharya (1982).

Types of reaction:-

Homogenous (H) : Free intermingling of hyphae

Overgrowth (O) : Pathogen overgrown by antagonists

Cessation of growth (C) : Cessation of growth at line of contact

Aversion (A) : Development of clear zone of inhibition

3.7. *IN VITRO* EVALUATION OF NATIVE *Trichoderma* spp. FOR THE CELLULASE ACTIVITY

The isolates of *Trichoderma* spp. which showed antagonistic activity against all the six pathogens were selected for the evaluation of their cellulase activity under *in vitro* condition by following the method suggested by Kasana *et al.* (2008). Spore suspension of the selected isolates of *Trichoderma* spp. was prepared with eight milli meter disc of the cultures in one milli litre sterile

water. 50µl of this suspension was poured into agar wells made with eight milli metre cork borer at the centre of carboxy methyl cellulose agar (CMC) (Appendix 1) mediated plates (Guevara and Zambrano, 2006). Inoculated plates were incubated at 28 ± 2°C in triplicates and flooded with Gram's iodine on two and three days after inoculation (DAI). Observations on the diameter of diffusion zones and the growth of isolates of *Trichoderma* spp. were recorded and the hydrolyzing capacity was estimated using the formula, given below.

$$\text{Hydrolysing capacity} = \frac{\text{Diameter of the diffusion zone}}{\text{Diameter of the growth of } \textit{Trichoderma} \text{ spp.}}$$

3.8. POT CULTURE EVALUATION ON ANTAGONISTIC EFFICACY OF SELECTED ISOLATES OF *Trichoderma* spp.

A pot culture experiment was laid out to study the antagonistic efficiency of the selected native isolates of *Trichoderma* spp. by taking ginger as test crop and *Pythium aphanidermatum* as test pathogen. The selected isolates of *Trichoderma* spp. from the cellulase test and the dual culture experiment along with two standard biocontrol agents of *Trichoderma* spp. and a standard bacterial biocontrol agent were used for the pot culture experiment. The details of the experiment are furnished below.

Design	: CRD
Treatments	: 18
Replications	: 3
No. of plants/ replication	: 12
Crop	: Ginger
Variety	: Rio – de - Janeiro

Treatment details :

T1 – T4 : Selected isolates of *Trichoderma* spp. from northern zone

T5 – T8 : Selected isolates of *Trichoderma* spp. from central zone

T9 – T12 : Selected isolates of *Trichoderma* spp. from southern zone

T13 : Reference culture of *Trichoderma viride*

T14 : Reference culture of *Trichoderma harzianum*

T15 : Reference culture of *Pseudomonas fluorescens*

T16 : Copper hydroxide (0.2%) at 7DAI

T17 : Control (with pathogen)

T18 : Absolute control

Concentrations of fungal and bacterial isolates used for the experiment were 10^6 spores ml^{-1} and 10^8 cfu ml^{-1} respectively and were given as soil application at the time of planting and 40 days after planting (DAP). The challenge inoculation of pathogen was done at 45 DAP.

3.8.1. Preparation of potting mixture and sterilization

The potting mixture was prepared by mixing soil, sand and cow dung in the ratio of 1:1:1. It was then sterilized chemically by drenching with formalin five per cent and was covered with polythene sheet for 10 days. The potting mixture was kept open for seven days before filling into the earthen pots of size 12" × 10", which were used for planting.

3.8.2. Preparation of inoculum of *Trichoderma* spp.

The selected isolates of *Trichoderma* spp. and the two standard cultures of *Trichoderma* spp. *viz., T. viride* and *T. harzianum* were grown

separately in potato dextrose broth (Appendix 1) for 10 days. The fungal mat along with spores was blended uniformly with the medium and was further diluted with sterile water to get a concentration of 10^6 spores ml^{-1}. This was used as inoculum and was applied as soil drenching @ 50 ml/plant.

3.8.3. Preparation of inoculum of *Pseudomonas fluorescens*

The standard bacterial biocontrol agent of KAU, *Pseudomonas fluorescens* was grown in nutrient broth (Appendix 1) for 48 h. The bacterial growth was blended uniformly with the medium and then the concentration was adjusted to 10^8 cfu ml^{-1} with sterile water. This inoculum was applied to soil @ 50 ml/plant.

3.8.4. Preparation of fungicide solution

The fungicide, copper hydroxide was used at a concentration of 0.2 per cent for the soil application and was prepared by dissolving two grams of Cu $(OH)_2$ in one litre of water and was applied to soil seven days after the challenge inoculation with the pathogen.

3.8.5. Challenge inoculation of pathogen

Five days old culture of *Pythium aphanidermatum* was used for artificial inoculation on ginger crop. All the treatments except absolute control were inoculated with the pathogen at 45 days after planting. For the preparation of inoculum, five days old culture of the pathogen was mixed with sterile sand @ culture in three Petri dishes / kg sand and was applied to soil @ 100 g/pot.

3.8.6. Observations recorded

Observation on the germination percentage, pre-emergence rot, plant biometric characters and disease incidence at different intervals were recorded.

3.8.6.1. Germination percentage and pre-emergence rot

The number of rhizomes germinated, earliness of germination and pre-emergence rot of rhizome were recorded one month after planting and calculated the per cent germination and per cent pre-emergence rot.

3.8.6.2. Biometric observations

The biometric observations *viz.,* number of tillers /plant, height of the plant and the number of leaves /tiller were taken at 40DAP, 70DAP and 100DAP.

3.8.6.2.a. Number of tillers

The total number of tillers in each plant was counted once in thirty days up to 100DAP.

3.8.6.2.b. Height of the plant

The height of each tiller of each plant from collar region to the growing tip of the plant was measured at 30 days interval up to 100 DAP.

3.8.6.2.c. Number of leaves

The total number of fully opened leaves in each tiller of each plant was counted once in thirty days up to 100DAP.

3.8.6.3. Assessment of disease incidence

To assess the disease incidence, number of infected plants and total number of plants in each treatment were recorded and per cent disease incidence was calculated using the formula.

$$\text{Per cent disease incidence (PDI)} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

The observations on disease incidence were taken at seven days after the artificial inoculation of the pathogen, *P. aphanidermatum*.

3.9. *IN VITRO* EVALUATION OF COMPATIBILITY OF SELECTED ISOLATES OF *Trichoderma* spp. WITH THE SELECTED PESTICIDES

The most efficient isolates of *Trichoderma* spp. selected from the pot culture experiment were screened for the tolerance against selected fungicides and insecticides by poisoned food technique (Zentmeyer, 1955).

3.9.1. *In vitro* evaluation of *Trichoderma* spp. against selected fungicides

An *in vitro* evaluation of selected isolates of *Trichoderma* spp. was carried out for the fungicide tolerance against four different fungicides on PDA medium by poisoned food technique (Zentmeyer, 1955).

The details of fungicides used for the evaluation are given in Table 1

Table. 1. Fungicides used for *in vitro* evaluation of *Trichoderma* spp.

Sl. No.	Chemical Name	Trade Name	Concentration (%)
1	Carbendazim	Bavistin 50% WP	0.1
2	Copper hydroxide	Kocide 77% WP	0.15
3	Mancozeb	Dithane M – 45 75% WP	0.3
4	Bordeaux mixture	-	1

Hundred milli litre of PDA medium was sterilized in 250 ml conical flask. To get the required concentration, the corresponding quantity of each fungicide was mixed separately with the sterilized medium in conical flask, shaked well and poured into sterilized Petri plates @ 20 ml per plate. Mycelial discs of 10 mm diameter were cut from the actively growing culture of different isolates of *Trichoderma* spp. and placed at the centre of each Petri dish containing the poisoned medium. Three replications were maintained for each fungicide and medium without fungicide served as control. All the dishes were incubated at room temperature at 28 ± 2°C. The radial growth of each isolates of *Trichoderma* spp. was recorded daily till the control plate was fully covered with the growth of the isolate. The per cent of inhibition on the growth of isolates was calculated by using the formula.

PI = [C-T/C] ×100

PI– Per cent inhibition

C – Radial growth of isolate (cm) in control

T – Radial growth of isolate (cm) in treatment

3.9.2. *In vitro* evaluation of *Trichoderma* spp. against insecticides

An *in vitro* experiment with selected isolates of *Trichoderma* spp. was carried out for the evaluation of insecticide tolerance against five different insecticides on PDA medium by poisoned food technique as described in 3.9.1.

The details of insecticides used for the evaluation are given below

Table. 2. Insecticides evaluated against *Trichoderma* spp.

Sl. No.	Chemical name	Trade name	Concentration (%)
1	Malathion	Fytanon	0.1
2	Dimethoate	Rogor 30 EC	0.05
3	Quinalphos	Ekalux 25 EC	0.05
4	Chlorpyriphos	Mr. Bon	0.25
5	Flubendiamide	Fame 480 SC	0.01

The per cent of inhibition on the growth of isolates of *Trichoderma* spp. was calculated by the formula mentioned in 3.9.1.

3.10. EVALUATION OF ANTAGONISTIC POTENTIAL OF SELECTED NATIVE ISOLATES OF *Trichoderma* spp. UNDER FIELD CONDITION

For the evaluation of antagonistic potential of selected native isolates of *Trichoderma* spp., a field experiment was laid out by taking ginger as test crop and *Pythium aphanidermatum* as test pathogen. The selected five native isolates of *Trichoderma* spp. from the pot culture experiment, reference cultures of *Trichoderma* spp. and *Pseudomonas fluorescens* along with one fungicide were used for this experiment. The details of the experiment are as follows

Design : RBD
Treatments : 10
Replications : 3
No. of plants/replication : 32
Crop : Ginger
Variety : Rio – de- Janeiro

Treatments : -

T1 – T5 : Promising native isolates of *Trichoderma* spp. selected from the pot experiment

T6 : Reference culture of *Trichoderma viride*

T7 : Reference culture of *T. harzianum*

T8 : Reference culture of *Pseudomonas fluorescens*

T9 : Copper hydroxide (0.2%) at 7 DAI

T10 : Control

Concentration of fungal and bacterial isolates used for the experiment was 10^6 spores ml^{-1} and 10^8 cfu. ml^{-1} respectively and were given as soil application @ 150 g/bed at the time of planting and 40 days after planting (DAP).

3.10.1. Preparation of the field

The field was ploughed well and the stubbles were removed. For the planting of ginger rhizome, beds of size 2 × 1 m^2 were prepared at a spacing of 50 cm.

3.10.2. Preparation of inoculum of selected isolates of *Trichoderma* spp.

Talc based formulation of five selected isolates of *Trichoderma* spp. was prepared and used for the field experiment. The isolates of *Trichoderma* spp. were grown separately in potato dextrose broth for 10 days. The fungal mat along with spores was blended uniformly with the medium and was further mixed with sterile talc @ 400 ml culture solution /kg of talc. This was used as inoculum for this experiment.

3.10.3. Preparation of inoculum of *Pseudomonas fluorescens*

The bacterial reference culture, *P. fluorescens* was grown in nutrient broth for 48h. The bacterial cells were blended uniformly with the medium and further mixed with sterilized talc @ 400 ml bacterial suspension /kg of talc. This inoculum was used for the soil application.

3.10.4. Preparation of fungicide solution

The fungicidal solution of copper hydroxide was prepared as described in 3.8.4.

3.10.5. Observations recorded

The observations on germination percentage of rhizome, pre emergence rot, the plant biometric observations *viz.*, number of tillers / plant, height of the plant and the number of leaves / tiller and disease incidence were recorded.

3.10.5.1. Observations on germination percentage and pre emergence rot

The per cent germination of rhizome and the per cent of pre emergence rot were estimated in the similar way as described in 3.8.6.1.

3.10.5.2. Biometric observations

The biometric observations on number of tillers / plant, height of the plant and the number of leaves / tiller were recorded as in 3.8.6.2.

3.10.5.2.a. Number of tillers

The observation on number of tillers in each plant was recorded as described in 3.8.6.2.a.

3.10.5.2.b. Height of the plant

The observations on the height of the plant were taken similarly as in 3.8.6.2.b.

3.10.5.2.c. Number of leaves

Total number of leaves in each tiller of the plant was taken similar to that in 3.8.6.2.c.

3.10.5.3. Assessment of disease incidence

The incidence of disease in each treatment was assessed and the per cent disease incidence was calculated as described in 3.8.6.3.

3.11. IDENTIFICATION OF PROMISING ISOLATES OF *Trichoderma* spp.

The promising native isolates of *Trichoderma* spp. selected from the field experiment were identified based on the cultural, morphological and molecular characteristics.

3.11.1. Cultural characterization of selected *Trichoderma* spp.

The observations on the cultural characters *viz.*, growth rate, colour, shape, texture and sporulation of colony of the selected isolates of *Trichoderma* spp. were recorded.

3.11.2. Morphological characterization of selected *Trichoderma* spp.

Permanent aqueous mounts were prepared from the pure cultures of selected isolates of *Trichoderma* spp. and observed for different structures under compound microscope. Microphotographs and observations on

morphological characters *viz.,* micrometry of hypha, phialides and spores were recorded.

Based on the cultural and morphological characters, the isolates of *Trichoderma* spp. were tentatively identified and for further confirmation and species level identification, cultures were sent to Indian Type Culture Collection (ITCC), IARI, New Delhi.

3.11.3. Molecular characterization of *Trichoderma* spp.

The molecular variability among the promising isolates of *Trichoderma* spp. selected from the field experiment was studied by Inter Transcribed Sequence – Polymerised Chain Reaction (ITS-PCR) and Random Amplified Polymorphic DNA (RAPD) assay.

3.11.3.1. Preparation of cultures of *Trichoderma* spp.

The selected isolates of *Trichoderma* spp. were grown on potato dextrose broth at 28± 2°C for three days before DNA extraction.

3.11.3.2. Genomic DNA extraction from *Trichoderma* spp.

The DNA of the promising isolates of *Trichoderma* spp. was extracted by using the protocol given by Chakraborty *et al.* (2010) with necessary modifications.

For the extraction of genomic DNA, the cultures of selected isolates were prepared as mentioned above in 3.11.3.1. The mycelia of isolates were incubated with lysis buffer containing 250 mM Tris – HCl (p^H 8.0), 50 mM EDTA (p^H 8.0), 100mM NaCl and 2% SDS, for 1 hr at 60°C followed by centrifugation at 12,000 rpm for 15 min. The supernatant was then extracted

with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min. The aqueous phase separated out was further extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 12,000 rpm for 15 min. The aqueous phase was then transferred in a fresh tube and mixed with equal volume of chilled isopropanol. The vial was then incubated at - 20°C for 30 min. for the precipitation of genomic DNA. DNA was pelleted by centrifuging at 12,000 rpm for 15 min. and washed in 70 per cent ethanol by centrifugation. The pellets were air dried, suspended in distilled water and stored at - 20°C for further use.

3.11.3.3. Qualitative and quantitative estimation of DNA

The quality and quantity of the isolated DNA was estimated by Agarose gel electrophoresis and nanodrop.

3.11.3.3.1. Agarose gel electrophoresis of the extracted DNA

Buffers used for electrophoresis:

1) Composition of 50 X TAE buffer (Stock)

2M Tris base	: 24.20 g
1M Glacial Acetic Acid	: 5.71 ml
0.5 M EDTA	: 18.06 g
Distilled water	: 100 ml
p^H	: 8.0

Preparation:

Each chemical was dissolved in separate beakers using distilled water and all were mixed finally. The p^H was adjusted to 8.0 by using 0.1 N HCI or NaOH and volume was made up to 100 ml and sterilized by autoclaving at 15 lbs for 15 min.

Preparation of working solution:

To prepare 100 ml of working solution of 1X TAE buffer, two milli litre stock solution of TAE buffer was taken and made up to 100 ml with sterile distilled water.

2) Composition of loading dye (6X)

Glycerol	: 30 ml
Bromophenol blue (saturated)	: 0.25 g
Xylene cyanol	: 0.25 g
Double distilled water	: 100 ml

Preparation:

Contents were mixed well and divided in to one milli litre aliquot, sterilized and stored at ⁻20°C for further use.

Preparation of gel:

Gel plates were washed thoroughly with cleaning solution followed by distilled water and dried. The two open sides of the plates were sealed with cellophane tape. Gel solution was prepared by mixing 0.8 g of agarose in 100 ml of 1X TAE buffer (0.8% gel) in a conical flask and boiled in a microwave oven until a clear solution was obtained and two micro litre of ethidium bromide (10 µg/ml) was added. The solution was poured on to the sealed plate after inserting a suitable comb and allowed to polymerize.

Loading and running of gels:

The inserted comb was gently removed from the gel after polymerization. The gel plate was placed in horizontal apparatus filled with 1X TAE buffer with the well side directed towards the cathode. 10 µl of the samples were mixed with loading dye and loaded in the wells with the help of

micropipettes. After loading, the electrophoresis unit was connected to power pack with a regulated electric power supply of 70 V. The power was turned off when the loading dye reached at about three centimeter from the anode end.

Gel documentation:

At the end of the run, the gel was carefully taken out from the electrophoresis unit and was viewed under U. V. light of 320 nm in a transilluminator. The DNA intercalated with ethidium bromide dye emitted orange fluorescence under U.V. light. The image was documented and stored using the Gel documentation and analysis system, Alpha Imager TM- 1200 (Alpha Innotech, USA).

3.11.3.3.2. Estimation of quantity of DNA by nanodrop

The DNA isolated was quantified by reading absorbance at 260 nm and 280 nm against distilled water blank using nanodrop. The purity of DNA was assessed from the ratio of OD value at 260 to OD value at 280. The quantity of DNA in the sample was calculated using the formula:

$OD_{260} = 1$ is equivalent to 50 µg double stranded DNA ml^{-1}

Hence, quantity of DNA present in the sample = $OD_{260} \times 50 \times$ dilution factor (µg/ml)

3.11.3.4. Amplification of genomic DNA using ITS - PCR

The amplification of ITS region of genomic DNA of isolates was carried out by adopting ITS- PCR technique in which ITS - 1 and ITS - 4 were used as forward and reverse primers.

Table 3. Sequences of primers used in ITS – PCR

Sl. No.	Primers	Sequence
1	ITS – 1	TCCGTAGGTGGACCTGCGG
2	ITS – 4	TCCTCCGCTTATTGATATGC

Standardization of ITS – PCR:

The ITS – PCR has been standardized and the following conditions were used for the amplification of DNA from the native isolates of *Trichoderma* spp..

Reaction mixture for ITS – PCR:

10X Taq buffer	: 2.5 µl
dNTPS	: 1.0 µl
3 U Taq DNA polymerase	: 0.2 µl
Forward primer	: 0.1 µl
Reverse primer	: 0.1 µl
Genomic DNA	: 1.0 µl
Sterile double distilled water	: 20 µl
Total volume	: 25 µl

Conditions used for ITS – PCR:

Stage – I : Initial denaturation at 94°C for 5 min

Stage – II : Denaturation at 94°C for 30s

Annealing at 56°C for 45s and

Extension at 70°C for 2 min

Number of cycles : 35

Stage – III : Final extension at 72°C for 7 min

Hold at 4°C for 10 min

Amplicons were subjected to one per cent agarose gel electrophoresis with 1.0 X TAE as running buffer. The banding pattern was visualized under U.V. trans – illuminator and the DNA banding profiles were documented in the gel documentation system (Alpha Innotech, USA) and compared with 1 Kb DNA ladder.

Sequencing of amplicon:

After amplification, the amplicons of the isolates of *Trichoderma* spp. were sent to SciGenom, Cochin for sequencing.

3.11.3.5. RAPD profiles through Polymerized Chain Reaction (PCR)

Six different random primers belong to operon series *viz.*, OPA – 1, 3, 4, 5, 8 and 9 (Operon technologies Inc.,) were used to detect polymorphism among the isolates under the study. The primer sequences used in RAPD technique are given below.

Table 4. Sequences of oligonucleotide primers used in RAPD

Sl. No.	Primers	Sequence
1	OPA – 1	CAGGCCCTTC
2	OPA – 3	AGTCAGCCAC
3	OPA – 4	AATCGGGCTG
4	OPA – 5	AGGGGTCTTG
5	OPA – 8	GTGACGTAGG
6	OPA – 9	GGGTAACGCC

Standardization of RAPD technique:

The RAPD technique has been standardized and the following conditions were used for the amplification of DNA from the native isolates of *Trichoderma* spp..

Reaction mixture for RAPD:

10X Taq buffer : 2.0 µl

50mM $MgCl_2$: 1.5 µl

dNTPS : 1.0 µl

Taq DNA polymerase : 0.4 µl

Primer (1: 9) : 2.0 µl

Genomic DNA (60 ng/ µl) : 2.0 µl

Sterile double distilled water : 11.1 µl

Total volume : 20 µl

Conditions used for RAPD amplification:

Stage – I : Initial denaturation at 94°C for 4 min

Stage – II : Denaturation at 94°C for 45s

Annealing at 35°C for 1 min and

Extension at 72°C for 2 min

Number of cycles : 35

Stage – III : Final extension at 72 °C for 5 min

Amplicons were subjected to 1.5 % agarose gel electrophoresis with 1.0 X TAE as running buffer. The banding pattern was visualized under U.V. trans – illuminator. The DNA banding profiles were documented in the gel documentation system (Alpha Innotech) and compared with 1Kb plus DNA ladder.

3.12. STUDY ON THE STRAIN VARIATION OF *Trichoderma* spp.

An attempt was carried out to induce strain variation in two selected native isolates of *Trichoderma* spp. selected from the field experiment. Mutation and protoplast fusion techniques suggested by Mohamed and Haggag, 2010, were adopted for inducing strain variation. The isolates of *Trichoderma* spp. selected for this study showed variations in the cultural, morphological and antagonistic characters. These two isolates of *Trichoderma* spp. were designated as parental isolates 1 and 2.

3.12.1. Induction of strain variation by mutation

The parental isolates 1 and 2 were inoculated separately on PDA medium and incubated at room temperature for sporulation. Conidial suspensions of each isolate were prepared by dislodging the conidia taken from the medium in a sterile eppendorf tube containing sterilized physiological saline (0.85% NaCl) and Tween-80. After dispersing the spore clumps, the concentration of the conidial suspensions was adjusted to 10^6 spores /ml. Then the conidial suspension was divided into two equal halves and were transferred to two sterilized Petri plates. One of the plates with conidial suspension was treated as control. The conidial suspension in the other plate was treated with sodium nitrate ($NaNO_2$) @ 500µg /ml and was irradiated under ultraviolet lamp under aseptic condition for different periods of 20, 40, 60 and 80 minutes. The distance between the conidial suspension and the U.V. lamp was adjusted to 30 cm. After irradiation, the plates were incubated at room temperature (28 ± 2°C) for one hour in dark. Later, 100 µl of the conidial suspensions were taken from the U.V. treated as well as control plate and were separately spread plated on Czapek – Dox Agar medium supplemented with 0.1% Triton X-100. The plates were incubated at room temperature until the fungal colonies were observed.

3.12.1.1. Preliminary screening of mutant isolates of *Trichoderma* spp.

All the isolates obtained after the U.V. exposure of the selected parental isolates were preliminary screened for their antagonistic efficiency against a virulent soil borne pathogen, *Sclerotium rolfsii* by dual culture method under *in vitro* condition as explained in 3.5.

3.12.1.2. *In vitro* evaluation of selected mutants against soil borne fungal pathogens

The mutants selected from the preliminary screening based on their antagonistic efficiency were further evaluated under *in vitro* condition against six soil borne fungal pathogens *viz., P. aphanidermatum, P. capsici, R. solani, S. rolfsii* and *F. oxysporum* f. sp. *cubense* by dual culture technique and the per cent inhibition on growth of pathogen over control was calculated as per the procedure described in 3.6.

The antagonistic potential of the mutants was compared with their parental isolates of *Trichoderma* spp. as well as the reference cultures of *Trichoderma* spp. *viz., T. viride* and *T. harzianum* by adopting the same method.

3.12.2. Induction of strain variation by protoplast fusion

The parental isolates of *Trichoderma* spp. were grown separately on PDA medium at 28 ± 2°C for sporulation. The spore suspension prepared from the seven day old culture was used for the isolation of protoplast.

3.12.2.1. Preparation of protoplasting medium (PM) or Regeneration medium (RM)

The medium suggested by Kirimura *et al.,* 1986, was used for pre growing fungal strains for the isolation of protoplast.

Composition of medium:

Glucose	: 80 g
NH_4NO_3	: 2g/l
KH_2PO_4	: 10g/l
$MgSO_4.7H_2O$	: 0.25g/l
$FeCl_3.6H_2O$	: 0.02g/l
$MnSO_4$	: 0.014 g/l
P^H	: 4.5

3.12.2.2. Protoplasting buffer (PB)

The parental isolates were inoculated in PB buffer consists of phosphate buffer (0.1M, P^H 5.8-6.0) containing 0.7 M NaCl, 0.2M $CaCl_2$ and Glucanex (20 mg/ml) and incubated at 30°C with gentle shaking for up to 4 h.

3.12.2.3. Fusion buffer (FB)

The buffer of FB consists of phosphate buffer (0.05M, P^H 7.5) containing 30% (W/V) poly ethylene glycol (PEG) 6000, 50 mM $CaCl_2$ and 0.7M NaCl.

3.12.2.4. Isolation of protoplast

Spore suspension (100 µl) of each parent, was inoculated in 50 ml PM and incubated for 24 h at 30°C on rotary shaker (180 rpm). The mycelium formed was recovered by centrifugation @ 12,000 rpm for 15min at 4°C. The recovered mycelial pellet was washed twice with sterilized physiological saline (0.85% NaCl) and re-suspended in 1.5 ml PB. The mixture was investigated by phase contrast microscope.

3.12.2.5. Purification of protoplast

After the release of maximum number of mature protoplasts, the crude protoplast suspension was filtered through syringe filter (5 µm pore size) for purifying the released protoplasts.

3.12.2.6. Viability check of protoplasts

To check the viability, the purified protoplast suspension was stained with one per cent Evans Blue dye, which stained the dead cells and did not stain the viable cells.

3.12.2.7. Protoplast fusion

Equal volumes of purified protoplast suspension from the two parents were mixed well and centrifuged @ 12,000 rpm for 15 min at 4°C. The pellet formed was re - suspended in 2 ml FB and incubated for 20 min at 30°C. The mixture was investigated under phase contrast micro scope. After incubation, the suspension was plated on to the surface of malt extract agar containing 0.7 M NaCl and incubated for seven days at 28 ± 2°C. Colonies growing on the surface of the plates were isolated on PDA.

3.12.2.8. Preliminary screening of fusants of *Trichoderma* spp.

All the fusants obtained after PEG mediated fusion of protoplasts of the selected parental isolates of *Trichoderma* spp. were preliminary screened for their antagonistic efficiency against the soil borne pathogen, *S. rolfsii* as described in 3.5.

3.12.2.9. *In vitro* evaluation of selected fusants against soil borne fungal pathogens

Antagonistically potential fusants selected after preliminary screening were further evaluated under *in vitro* condition against soil borne fungal pathogens *viz., P. aphanidermatum, P. capsici, R. solani, S. rolfsii* and *F. oxysporum* f. sp. *cubense* by dual culture technique and the per cent inhibition of growth of pathogen over control was calculated as per the procedure described in 3.6.

The antagonistic potential of the fusants was compared with their parental isolates of *Trichoderma* spp. as well as the reference cultures of *Trichoderma* spp. *viz., T. viride* and *T. harzianum* by the same method.

3.13. CHARACTERIZATION OF PROMISING MUTANTS AND FUSANTS

The promising mutants and fusants selected for the pot culture and field experiments were identified based on their cultural, morphological and molecular characteristics.

3.13.1. Cultural characterization of mutants and fusants

The cultural characters *viz.,* growth rate, colour, shape, texture and sporulation of colony were studied on PDA media.

3.13.2. Morphological characterization of mutants and fusants

Permanent aqueous mounts were prepared from the pure cultures of selected mutants and fusants of *Trichoderma* spp. and were observed for different structures under compound microscope. Microphotographs and

observations on morphological characters *viz.,* micrometry of hypha, length and width of hyphal cells, size of phialides and spores were taken.

3.13.3. Molecular characterization of mutants and fusants

The molecular variability among the selected mutants and fusants was studied by Inter Transcribed Sequence – Polymerised Chain Reaction (ITS – PCR) and Random Amplified Polymorphic DNA (RAPD) as described in 3.11.3.

3.14. POT CULTURE EXPERIMENT ON EVALUATION OF BIO CONTROL EFFICIENCY OF MUTANTS AND FUSANTS

A pot culture experiment was laid out to study the biocontrol efficiency of selected mutants and fusants by taking ginger as test crop and *Pythium aphanidermatum* as test pathogen. A total of two mutants and two fusants, selected from the dual culture experiment were taken for this experiment. The bio control efficiency of these isolates were compared with their parental isolates, reference cultures of *T. viride* and *T. harzianum*, reference culture of *P. fluorescens* and a fungicide, copper hydroxide (0.2%). The details of the experiment are furnished below.

Design	: CRD
Treatments	: 12
Replications	: 3
No. of plants/ replication	: 12
Crop	: ginger
Variety	: Rio – de- Janeiro

Treatments :

T1 – T2 : Parental isolates 1 and 2

T3 – T4 : Two selected mutants

T5 – T6 : Two selected fusants

T7 : Reference culture of *Trichoderma viride*

T8 : Reference culture of *Trichoderma harzianum*

T9 : Reference culture of *Pseudomonas fluorescens*

T 10 : Copper hydroxide (0.2%) at 7 DAI

T 11 : Control

T 12 : Absolute control

Concentration of fungal and bacterial isolates used for the experiment was 10^6 spores ml^{-1} and 10^8 c.f.u. ml^{-1} respectively and were given as soil application at the time of planting and 40 days after planting (DAP).

3.14.1. Preparation of potting mixture and sterilization

The potting mixture for the experiment was prepared and sterilized as per the procedure described in 3.8.1.

3.14.2. Preparation of inoculum of *Trichoderma* spp. for applications

A total of eight isolates of *Trichoderma* spp. *viz.*, two parental isolates, two selected mutants, two selected fusants and two standard cultures of *Trichoderma* spp. were used for this experiment. Their inoculums were prepared on potato dextrose broth as per the procedure mentioned in 3.8.2.

3.14.3. Preparation of inoculum of *Pseudomonas fluorescens*

The inoculum of standard bacterial bio control agent of KAU, *P. fluorescens* was prepared as per the procedure in 3.8.3.

3.14.4. Preparation of fungicide solution

Solution of fungicide, copper hydroxide was prepared by following the method described in 3.8.4.

3.14.5. Challenge inoculation of pathogen

The inoculum of the pathogen, *P. aphanidermatum* was prepared and was artificially inoculated in ginger as per the method explained in 3.8.5.

3.14.6. Observations recorded

Observations on the germination percentage, pre-emergence rot, plant biometric characters and disease incidence were recorded at regular intervals as mentioned in 3.8.6.

3.15. ASSAY OF DEFENSE RELATED ENZYMES

The pot culture experiment given in 3.14 which was laid out by selecting four promising genetically improved isolates, their parental isolates and reference cultures, was also used to assess the effect of these isolates in the induction of systemic resistance in plants.

The plants were challenge inoculated with the fungal pathogen, *P. aphanidermatum* at 45 DAP as described in 3.8.5. The estimation on the activity of defense related enzymes such as peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and total phenol was carried out by spectroscopy with the leaf samples collected on the day of inoculation as well as 1, 3 and 5 days after inoculation (DAI) of the pathogen.

3.15.1. Assay of defense related enzymes in ginger

500mg of leaf samples from each treatment was weighed and homogenized in five milli litre, 10 mM sodium phosphate buffer using a pre-cooled pestle and mortar and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was taken in two milli litre eppendorf tubes and stored at ⁻20°C and this forms the extract for estimation of PO, PPO and PAL.

3.15.1.1. Assay of peroxidase (PO) activity

The assay was carried out as per the protocol of Rathmell and Sequeira (1974) with slight modifications. The crude enzyme (100 µl) was added and mixed with 1.5 ml 0.05M pyrogallol and 0.5 ml of one per cent hydrogen peroxide. Initial rate of increase in absorbance was measured for five minutes at one minute interval at 436 nm. Peroxidase activity was expressed as units of PO min^{-1} g^{-1} fresh tissue.

3.15.1.2. Assay of poly phenol oxidase (PPO) activity

As per the protocol of Mayer *et al.* (1965), 700 µl of sodium phosphate buffer (P^H 6.0) was added to 20 µl of the crude enzyme. To this 100 µl of 0.2 M catechol was added and the initial rate of increase in absorbance was measured to five minutes at 420 nm. The enzyme activity was expressed as units of PPO min^{-1} g^{-1} fresh tissue.

3.15.1.3. Assay of phenylalanine ammonia lyase (PAL) activity

Phenylalanine ammonia lyase was estimated as per the protocol of Brueske (1980). To 500 µl of enzyme extract, 500 µl of 0.5M Tris HCl buffer (P^H 8.5) was added. To this 500 µl of 0.15 M L-phenylalanine was added and incubated at 37°C for 60 min. The reaction was stopped by adding 500 µl of one Molar trichloro acetic acid (TCA) and incubated at 40°C for five minutes.

It was centrifuged to remove any particles and the absorbance was read at 270 nm in a U.V. visible spectrophotometer (Spectronic R Genesys – 5). The control tube contained L-phenylalanine added after TCA. The rate of the reaction was expressed as µmol of trans cinnamic acid formed g^{-1} fresh tissue. The standard was prepared with different concentrations of trans cinnamic acid.

3.15.1.4. Assay of total phenol

Total phenol in ginger plants was estimated as per the protocol of Malick and Singh (1980). One gram of leaf sample was weighed and ground using a pestle and mortar in 10 times the volume of 80 per cent of methanol. The homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was saved and the residue was re – extracted with five times the volume of 80 per cent methanol, centrifuged and the supernatants were pooled together and this was used for quantitative estimation of phenols by spectroscopy. Five milli litre of the supernatant was taken, evaporated to dryness and the residue was dissolved in five milli litre of distilled water. From the above residue dissolved, one milli litre was pipetted out into a test tube and the volume was made up to three milli litre with distilled water. Folin – Ciocalteau reagent (0.5 ml) was added to the test tube. After three minutes, two milli litre of 20 per cent Na_2CO_3 solution was added to each tube and mixed thoroughly. The tubes were placed in a boiling water bath for exactly one minute, cooled and the absorbance was measured at 650 nm (Spectronic – 20 D+) against a reagent blank. The rate of reaction was expressed as µg of phenol formed g^{-1} of fresh tissue. A standard curve was prepared with different concentrations of catechol.

3.16. EVALUATION OF ANTAGONISTIC POTENTIAL OF MUTANTS AND FUSANTS IN FIELD CONDITION

The mutants and fusants used for the pot culture experiment were further evaluated for their antagonistic potential under field condition by taking ginger as test crop and *Pythium aphanidermatum* as test pathogen. The bio control efficiency of mutants and fusants was compared with their parental isolates of *Trichoderma* spp., two reference cultures of *Trichoderma* spp., bacterial reference culture, *Pseudomonas fluorescens* and a fungicide, copper hydroxide (0.2%). The details of the experiment are as follows.

Design	: RBD
Treatments	: 11
Replications	: 3
No. of plants/ replication	: 32
Crop	: ginger
Variety	: Rio – de- Janeiro

Treatments :

T1 – T2	: Parental isolates 1 and 2
T3 – T4	: Two selected mutants
T5 – T6	: Two selected fusants
T7	: Reference culture of *T. viride*
T8	: Reference culture of *T. harzianum*
T9	: Reference culture of *P. fluorescens*
T 10	: Copper hydroxide (0.2%) at 7 DAI
T 11	: Control

Concentration of fungal and bacterial isolates used for the experiment was 10^6 spores ml^{-1} and 10^8 c.f.u. ml^{-1} respectively and were given as soil

application @ 150g talc based formulation /bed at the time of planting and 40 days after planting (DAP).

3.16.1. Preparation of the field

The field for the evaluation of bio control efficiency of mutants and fusants was prepared as described in 3.10.1.

3.16.2. Preparation of inoculum of *Trichoderma* spp.

The talc based formulations of mutants and fusants selected for the field experiment, their parental isolates and two reference cultures of *Trichoderma* spp. were prepared and applied in the field by following the procedure described in 3.10.2.

3.16.3. Preparation of inoculum of *Pseudomonas fluorescens*

The talc based formulation of the bacterial reference culture, *P. fluorescens* was prepared and applied in the soil as per the method explained in 3.10.3.

3.16.4. Preparation of fungicide solution

The fungicidal solution of copper hydroxide was prepared and used in the field experiment as per the procedure in 3.10.4.

3.16.5. Observations

The observations on germination percentage, pre-emergence rot, first appearance of symptom, disease incidence and the biometric observations were recorded in the same manner as in 3.10.5.

3.16.5.1. Biometric observations

Observations on number of tillers / plant, height of the plant and the number of leaves / tiller were recorded at regular intervals as given in 3.10.5.2.

3.16.5.2. Assessment of disease incidence

The incidence of disease in each treatment of the field experiment was estimated in the similar way as explained as in 3.10.5.3.

3.17. STATISTICAL ANALYSIS

Analysis of variance was done on the data collected using statistical package WASP 2. Multiple comparisons among the treatments were done by using DMRT (Duncan's Multiple Range Test).

Results

4. RESULTS

The present investigation was carried out to develop an antagonistically potential *Trichoderma* spp. for the management of important soil borne fungal diseases of crop plants. For this, soil samples were collected from different locations of northern, central and southern zones of Kerala and isolated *Trichoderma* spp. by serial dilution method. *In vitro* and *in vivo* evaluations of the native isolates of *Trichoderma* spp. were conducted to find out their efficacy in antagonism, reducing the disease incidence, improving plant biometric characters and inducing systemic resistance in crop plants. To improve these characters, two parental isolates were selected and mutation and protoplasmic fusion of these isolates were carried out. Performance evaluations of the improved strains with their parents were conducted under *in vitro* and *in vivo* conditions. The results of experiments are presented below.

4.1. SURVEY AND COLLECTION OF SOIL SAMPLES

Purposive sample surveys were conducted in 51 different locations of northern, central and southern districts of Kerala for the collection of soil samples. The soil samples were collected from the rhizosphere of healthy plants grown in these areas. The details of locations surveyed are given in Table 5.

4.2. ENUMERATION AND ISOLATION OF NATIVE *Trichoderma* spp.

The isolation of *Trichoderma* spp. was carried out by plating 10^{-3} and 10^{-4} dilutions of soil samples on solidified Potato dextrose agar (PDA), Rose bengal agar (RBA) and *Trichoderma* selective medium (TSM). The initiation of growth of the fungus was observed two days after inoculation (DAI) and number of colonies of *Trichoderma* spp. was recorded from next day onwards. The data on enumeration of total number of isolates of *Trichoderma* spp. are furnished in Table 6.

Table 5. Locations surveyed for the isolation of *Trichoderma* spp.

Sl. No.	Soil sample	District	Location	Type of land	pH of soil	No. of isolates
Northern Region of Kerala						
1	SS8-MALA	Malappuram	Thirurangadi	Plantation	5.8	2
2	SS10-MANAN	Wayanad	Mananthavadi	Garden	6.8	1
3	SS11-EDVK	Wayanad	Edavaka	Rice	5.7	1
4	SS12-WAYA	Wayanad	Thondernad	Rice	5.6	0
5	SS13-THRLERI-A	Wayanad	Thrissileri	Rice	6.0	1
6	SS14-THRLERI-B	Wayanad	Thrissileri	Rice	6.4	3
7	SS23-ADR	Kannur	Adoor	Plantation	4.3	3
8	SS24-KOORA	Kozhikode	Koorachund	Banana	5.0	3
9	SS27-ECAVE	Wayanad	Edakkal cave	Forest	5.75	5
10	SS28-EDAK	Wayanad	Edakkal	Coffee	5.0	4
11	SS29-GAMB	Wayanad	Ambalavayal	Ginger	4.0	1
12	SS30-CAMB	Wayanad	Ambalavayal	Coffee	4.5	4
13	SS31-CHRCH	Wayanad	Pulpally	Pepper	5.85	1
14	SS32-ADI	Wayanad	Adikolly	Pepper	5.6	3
15	SS33-CHERU	Wayanad	Cheruthottil	Pepper	5.8	1
16	SS40-MTHANGA	Wayanad	Muthanga	Forest	5.85	3
17	SS44-STHNGLI	Kasaragod	Seethangoli	Cashew nut	7.1	2
Central region of Kerala						
18	SS1-DNY	Palakkad	Dhony	Forest	6.3	5
19	SS2-PKD	Palakkad	Palakkad	Ginger field	6.0	0
20	SS3- PKD	Palakkad	Palakkad	Plantation	6.3	4
21	SS4-KMLA	Palakkad	Karimala	Plantation	5.2	2
22	SS5-KSLA	Palakkad	Kariyanshola	Plantation	6.1	6
23	SS6-PKLM	Palakkad	Parambikulam	Plantation	6.0	0
24	SS7-PKLM	Palakkad	Parambikulam	Forest	6.1	3
25	SS9-PKD	Palakkad	Thathamangalam	Rice	5.0	1
26	SS22-KLYR	Idukki	Kaliyar	pineapple	6.5	5

27	SS25-KNRA1	Thrissur	Kannara	Banana	5.55	2
28	SS26-KNRA2	Thrissur	Kannara	Nutmeg	4.85	3
29	SS34-KVK	Thrissur	Vellanikkara	Uncultivated land	6.2	3
30	SS35-NARA	Ernakulam	Narakkal	Pokkali rice field	3.6	2
31	SS36-KUZHU	Ernakulam	Kuzhuppilly	Pokkali rice field	3.4	4
32	SS38-MKPARA	Thrissur	Malakkapara	Forest	4.25	1
33	SS39-ATHRPILLI	Thrissur	Athirapilli	Forest	6.15	2
34	SS48-VELLANI	Thrissur	Vellanikkara	Ginger	5.35	3
Southern region of Kerala						
35	SS15-ASHTA	Kollam	Ashtamudi	Banana	7.3	2
36	SS16-KVLM1	Alappuzha	Kavalam	Coconut	6.7	1
37	SS17-KAYAM	Alappuzha	Kayamkulam	Banana	5.2	1
38	SS18-KVLM2	Alappuzha	Kavalam	Banana	5.6	3
39	SS19-VELBa	Trivandrum	Vellayani	Banana	6.4	2
40	SS20-VELCa	Trivandrum	Vellayani	Cassava	6.1	1
41	SS21-VELAm	Trivandrum	Vellayani	Amaranth	5.7	3
42	SS37-PANACHI	Kottayam	Panachikkad	Garden	5.4	4
43	SS41-KAMBLM	Trivandrum	Kallambalam	Banana	6.6	3
44	SS42-THRVLLA	Pathanamthitta	Thiruvalla	Plantation	7.1	3
45	SS43-KRVATTA	Alappuzha	Karuvatta	Plantation	5.8	2
46	SS45-NYR	Trivandrum	Neyyar	Forest	5.3	2
47	SS46-CHNGSRI	Kottayam	Changanasseri	Nutmeg	5.8	4
48	SS47-VNKULAM	Pathanamthitta	Vennikulam	Rubber	4.8	3
49	SS49-KLPUZHA	Kollam	Kulathurpuzha	Forest	5.1	4
50	SS50-THAZHVA	Kollam	Thazhava	Ginger	7.1	3
51	SS51-PULILLA	Kollam	Puliella	Ginger	5.95	3
Total						**128**

Table 6. Enumeration of colonies of *Trichoderma* spp.

Sl. No.	Soil sample	Colony count of *Trichoderma* spp. (4DAI)						No.of isolates
		PDA		RBA		TSM		
		10^{-3}	10^{-4}	10^{-3}	10^{-4}	10^{-3}	10^{-4}	
Soil samples from northern region of Kerala								
1	SS8-MALA	1	0	0	0	1	0	2
2	SS10-MANAN	0	0	0	1	0	0	1
3	SS11-EDVK	0	0	0	0	1	0	1
4	SS12-WAYA	0	0	0	0	0	0	0
5	SS13-THRLERI-A	0	0	0	1	0	0	1
6	SS14-THRLERI-B	0	0	1	1	1	0	3
7	SS23-ADR	0	0	0	2	1	0	3
8	SS24-KOORA	0	3	0	0	0	0	3
9	SS27-ECAVE	0	1	2	0	2	0	5
10	SS28-EDAK	1	0	2	0	1	0	4
11	SS29-GAMB	0	0	0	1	0	0	1
12	SS30-CAMB	0	1	0	1	2	0	4
13	SS31-CHRCH	0	0	0	0	1	0	1
14	SS32-ADI	1	0	1	0	1	0	3
15	SS33-CHERU	1	0	0	0	0	0	1
16	SS40-MTHANGA	1	0	1	0	1	0	3
17	SS44-STHNGLI	0	0	0	1	0	1	2
Total								**38**
Soil samples from central region of Kerala								
18	SS1-DNY	1	1	0	1	2	0	5
19	SS2-PKD	0	0	0	0	0	0	0
20	SS3- PKD	0	1	2	0	1	0	4
21	SS4-KMLA	0	0	1	0	0	1	2
22	SS5-KSLA	1	1	3	0	1	0	6
23	SS6-PKLM	0	0	0	0	0	0	0
24	SS7-PKLM	0	0	0	0	3	0	3

25	SS9-PKD	0	0	0	0	0	1	1
26	SS22-KLYR	0	0	1	2	2	0	5
27	SS25-KNRA1	1	0	1	0	0	0	2
28	SS26-KNRA2	0	0	2	1	0	0	3
29	SS34-KVK	0	0	2	1	0	0	3
30	SS35-NARA	0	1	0	0	0	1	2
31	SS36-KUZHU	1	0	1	1	0	1	4
32	SS38-MKPARA	0	0	0	0	1	0	1
33	SS39-ATHRPILLI	0	0	0	2	0	0	2
34	SS48-VELLANI	0	1	0	1	1	0	3
Total								**46**
Soil samples from southern region of Kerala								
35	SS15-ASHTA	0	1	0	0	0	0	1
36	SS16-KVLM1	0	0	1	1	0	0	2
37	SS17-KAYAM	0	0	1	0	0	0	1
38	SS18-KVLM2	0	1	1	0	1	0	3
39	SS19-VELBa	1	0	1	0	0	0	2
40	SS20-VELCa	1	0	0	0	0	0	1
41	SS21-VELAm	1	0	0	1	0	1	3
42	SS37-PANACHI	1	0	2	0	1	0	4
43	SS41-KAMBLM	0	0	2	1	0	0	3
44	SS42-THRVLLA	1	0	1	1	0	0	3
45	SS43-KRVATTA	1	0	0	1	0	0	2
46	SS45-NYR	0	0	0	0	2	0	2
47	SS46-CHNGSRI	0	1	1	1	1	0	4
48	SS47-VNKULAM	0	0	0	1	2	0	3
49	SS49-KLPUZHA	1	0	2	0	1	0	4
50	SS50-THAZHVA	1	0	1	0	1	0	3
51	SS51-PULILLA	0	1	0	1	1	0	3
Total								**44**

A total of 128 isolates of *Trichoderma* spp. including 38 from northern, 46 from central and 44 from southern regions of Kerala were isolated. All the isolates were purified and sub cultured on PDA medium and were named as Tr 1 to Tr 128.

4.3. ESTIMATION OF pH OF SOIL SAMPLES

The pH of soil samples collected from different locations was estimated to find out the effect of pH on the occurrence of *Trichoderma* spp. The results are presented in Table 5 and 7. From the table, it was observed that majority of the isolates of *Trichoderma* spp. (50.78%) were obtained from soil samples having a pH range of 5.6 - 6.5. The soil samples in pH range of 4.6 - 5.5 yielded 34 isolates of *Trichoderma* spp. and 6.6 – 7.5 yielded 18 isolates. It was also evident that even in the pH range of 2.5 - 3.5, *Trichoderma* spp. found surviving (3.13%) and four isolates were obtained from this pH range.

4.4. ISOLATION OF SOIL BORNE FUNGAL PATHOGENS

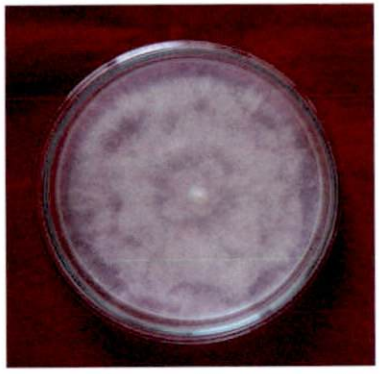
The major soil borne fungal pathogens *viz., Pythium aphanidermatum, Phytophthora capsici, Ganoderma lucidum, Rhizoctonia solani, Sclerotium rolfsii, Fusarium oxysporum* f. sp. *cubense* associated with the diseased specimens of ginger, pepper, coconut, rice, pepper and banana respectively were isolated on PDA medium. The details of pathogens are furnished in Table 8. All the six pathogens were purified and maintained as pure cultures by sub-culturing at frequent intervals (Table 8 and Plate 1).

Table 7. Effect of pH on the occurrence of *Trichoderma* spp.

Sl. No.	Range of pH	Soil samples	No. of isolates of *Trichoderma* spp.	Per cent
1	2.5 – 3.5	SS36 - KUZHU	4	3.13
2	3.6 – 4.5	SS23 – ADR, SS29 – GAMB, SS35 – NARA, SS38 – MKPARA	7	5.47
3	4.6 – 5.5	SS4 – KMLA, SS9 – PKD, SS17 – KAYAM, SS24 – KOORA, SS26 – KNRA2, SS28 – EDAK, SS30 – CAMB,SS37 – PANACHI,SS45 – NYR, SS47 – VNKULAM, SS48 – VELLANI, SS49 – KLPUZHA	34	26.56
4	5.6 – 6.5	SS1 – DNY, SS2 – PKD, SS3 – PKD, SS5 – KSLA, SS6 – PKLM, SS7 – PKLM,SS8 – MALA, SS11 – EDVK, SS12 – WAYA, SS13 – THRLERI- A, SS14 – THRLERI- B, SS16 – KVLM, SS18 – KVLM 2, SS19 – VEL Ba, SS20 – VEL Ca, SS21 – VEL Am, SS25 – KNRA 1, SS27 – ECAVE, SS31 – CHRCH, SS32 – ADI, SS33 – CHERU, SS34 – KVK, SS39 – ATHRPILLI, SS40 – MTHANGA,SS43 – KRVATTA, SS46 – CHNGSRI,SS51 – PULILLA	65	50.78
5	6.6 – 7.5	SS10 – MANAN, SS15 – ASHTA, SS22 – KLYR, SS41 – KAMBLM, SS42 – THRVLLA,SS44 – STHNGLI, SS50 – THAZHVA	18	14.06
	TOTAL		**128**	

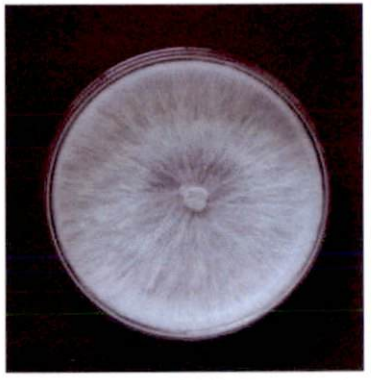
Plate 1. Soil borne fungal pathogens used for the *in vitro* evaluation of antagonism

Pythium aphanidermatum **(Ginger)**



Phytophthora capsici **(Pepper)**

Ganoderma lucidum **(Coconut)**



Sclerotium rolfsii **(Pepper)**

Fusarium oxysporum **f. sp.** ***cubense*** **(Banana)**

Rhizoctonia solani **(Rice)**

Table 8. Soil borne fungal pathogens used for *in vitro* study

Sl. No.	Pathogens	Host
1	*Pythium aphanidermatum*	Ginger
2	*Phytophthora capsici*	Pepper
3	*Ganoderma lucidum*	Coconut
4	*Rhizoctonia solani*	Rice
5	*Sclerotium rolfsii*	Pepper
6	*Fusarium oxysporum* f. sp. *cubense*	Banana

4.5. PRELIMINARY SCREENING OF NATIVE ISOLATES OF *Trichoderma* spp.

Out of 6 soil borne fungal pathogens, the most virulent and fast growing pathogen, *S. rolfsii* and a very slow growing pathogen, *F. oxysporum* f. sp. *cubense* were selected for the preliminary screening of the native isolates of *Trichoderma* spp. From the preliminary screening, 41 native isolates of *Trichoderma* spp. were selected which included 11 isolates from northern region, 11 from central and 19 from southern region of Kerala. The isolates selected after preliminary screening are presented in Table 9.

Table 9. Native isolates of *Trichoderma* spp. selected after preliminary screening

Sl. No.	Region of Kerala	No. of isolates selected	Name of isolates of *Trichoderma* spp.
1	North	11	Tr 9, Tr 10, Tr 25, Tr 28, Tr 48, Tr 52, Tr 59, Tr 61, Tr 62, Tr 72, Tr 76
2	Centre	11	Tr 2, Tr 14, Tr 20, Tr 23, Tr 43, Tr 57, Tr 58, Tr 78, Tr 81, Tr 83, Tr 121
3	South	19	Tr 31, Tr 32, Tr 34, Tr 36, Tr 41, Tr 42, Tr 90, Tr 97, Tr 101, Tr 104, Tr 107, Tr 109, Tr 111, Tr 112, Tr 114, Tr 118, Tr 119, Tr 126, Tr 127
Total		**41**	

4.6. *IN VITRO* EVALUATION ON THE ANTAGONISTIC EFFICIENCY OF NATIVE ISOLATES OF *Trichoderma* spp.

The evaluation on antagonistic efficiency of 41 selected native isolates against six soil borne fungal pathogens *viz., P. aphanidermatum, P. capsici, G. lucidum, R. solani, S. rolfsii, F. oxysporum* f. sp. *cubense* was carried out under *in vitro* condition. The antagonistic efficiency of these isolates was compared with the reference cultures of *Trichodema* spp. *viz., T. viride* and *T. harzianum*. The results are presented in the Table 10 to 15.

4.6.1. Evaluation of antagonistic efficiency of native isolates of *Trichoderma* spp. from northern region of Kerala

The results of antagonistic efficiency of selected 11 isolates of *Trichoderma* spp. are presented in Table 10 and 11.

4.6.1.1. *Pythium aphanidermatum*

The results of the evaluation of native isolates of *Trichoderma* spp. against *Pythium aphanidermatum* are given in (Table 10). Among the 11 isolates tested, four isolates of *Trichoderma* spp. *viz.*, Tr 9, Tr 10, Tr 48 and Tr 52 were recorded cent per cent inhibition on the growth of pathogen followed by Tr 28 and Tr 76 which showed 94.44 per cent. All the isolates were found antagonistically effective against *P. aphanidermatum* by showing > 74 per cent inhibition, which was higher than that of reference culture, *T. viride* (72.56%). Except five *viz.*, Tr 25, Tr 59, Tr 61, Tr 62 and Tr 72 all isolates recorded higher per cent of inhibition than *T. harzianum* (81.89).

All isolates overgrew the pathogen and recorded overgrowth as mechanism of antagonism (Plate 2a and 9a).

4.6.1.2. *Phytophthora capsici*

The result of dual culture experiment against the pathogen, *P. capsici* revealed that three isolates of *Trichoderma* spp. *viz.*, Tr 9, Tr 10 and Tr 52 were highly effective against this pathogen by exhibiting cent per cent of inhibition (Table 10). This was followed by Tr 48 (96.67%) and Tr 59 (90%). All isolates, except Tr 62 and Tr 25 recorded > 80 per cent inhibition over the growth of pathogen. The reference cultures *viz., T. viride* and *T. harzianum* showed 100 per cent inhibition on the growth of *P. capsici.*

All isolates recorded over growth as mechanism of antagonism (Plate 2b and 9b).

4.6.1.3. *Ganoderma lucidum*

Among the isolates of *Trichoderma* spp. tested, the isolate, Tr 10 alone showed cent per cent inhibition on the growth of *G. lucidum*. This is followed by Tr 52, recorded 93.94 per cent inhibition and Tr 9 showed 70.30 per cent inhibition (Table 10). All other isolates recorded inhibition on the growth of pathogen in the range of 44.85 to 62.42 per cent.

The mechanism of antagonism was found as over growth in case of all isolates of *Trichoderma* spp. (Plate 2c and 9f).

4.6.1.4. *Fusarium oxysporum* f. sp. *cubense*

Among 11 isolates, seven isolates of *Trichoderma* spp. *viz.*, Tr 9, Tr 10, Tr 28, Tr 48, Tr 52, Tr 72 and Tr 76 recorded cent per cent inhibition on the growth of pathogen, *F. oxysporum* f. sp. *cubense* (Table 11).The isolates *viz.,* Tr 25, Tr 59, Tr 61, Tr 62 and *T. harzianum* showed antagonistic property in the range of 31.78 to 44.44 which was less than that of the reference culture, *T. viride* (61.56).

All the native isolates of *Trichoderma* spp. except Tr 59 exhibited cessation type of mechanism of antagonism by ceasing the growth of the pathogen at line of contact. The reference culture *T. viride* showed over growth as mechanism of antagonism against the pathogen (Plate 3a and 9c).

4.6.1.5. *Sclerotium rolfsii*

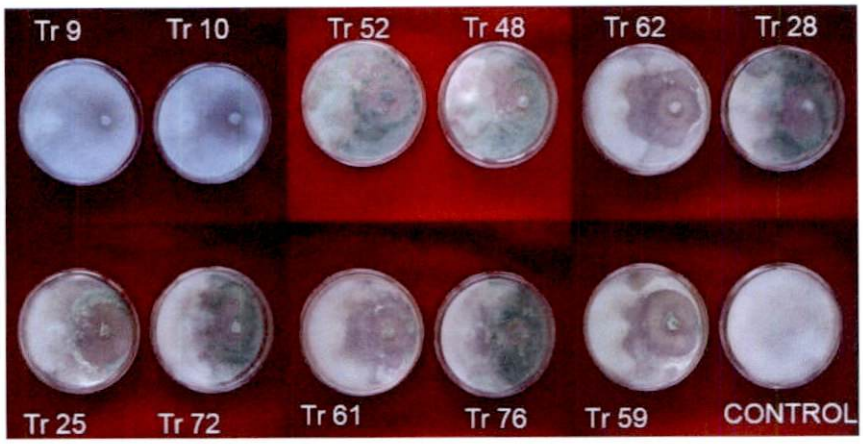
All the isolates exhibited per cent of inhibition in a range of 55.56 to 65.56 against the pathogen, *S. rolfsii.* The isolate, Tr 9 showed maximum efficiency of 65.56 per cent followed by Tr 10 (64.44%), Tr 28 (63.33%), Tr

Table 10. Evaluation of antagonistic efficiency of isolates of *Trichoderma* spp. from northern zone against *Pythium aphanidermatum*, *Phytophthora capsici* and *Ganoderma lucidum*

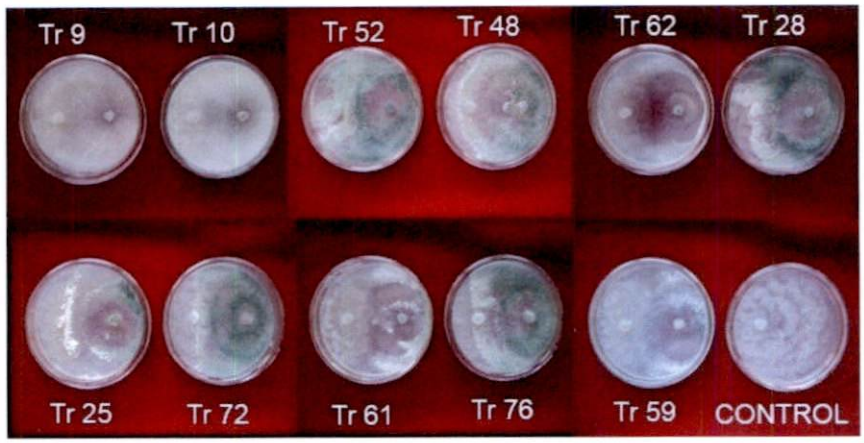
Sl. No.	Isolate of *Trichoderma* spp.	*Pythium aphanidermatum*				*Phytophthora capsici*				*Ganoderma lucidum*			
		Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action
		P	T			P	T			P	T		
1	Tr 9	0.00	9.00	100.00	O	0.00	9.00	100.00	O	1.63	7.37	70.30	O
2	Tr 10	0.00	9.00	100.00	O	0.00	9.00	100.00	O	0.00	9.00	100.00	O
3	Tr 25	2.00	7.00	77.78	O	2.00	7.00	77.78	O	2.17	6.83	60.61	O
4	Tr 28	0.50	8.50	94.44	O	1.05	7.95	88.33	O	3.03	5.97	44.85	O
5	Tr 48	0.00	9.00	100.00	O	0.30	8.70	96.67	O	2.10	6.90	61.82	O
6	Tr 52	0.00	9.00	100.00	O	0.00	9.00	100.00	O	0.33	8.67	93.94	O
7	Tr 59	2.05	6.95	77.22	O	0.90	8.10	90.00	O	2.87	6.13	47.88	O
8	Tr 61	2.05	6.95	77.22	O	1.40	7.60	84.44	O	2.07	6.93	62.42	O
9	Tr 62	2.30	6.70	74.44	O	2.40	6.60	73.33	O	2.27	6.73	58.79	O
10	Tr 72	2.00	7.00	77.78	O	0.95	8.05	89.44	O	2.33	6.67	57.58	O
11	Tr 76	0.50	8.50	94.44	O	1.00	8.00	88.89	O	2.33	6.67	57.58	O
12	*T. harzianum*	1.63	7.37	81.89	O	0.00	9.00	100.00	O	2.63	6.37	52.12	O
13	*T. viride*	2.47	6.53	72.56	O	0.00	9.00	100.00	O	2.20	6.80	60.00	O
14	CONTROL	9.00				9.00				5.50			

*Mean of three replications P : Pathogen T : Antagonist
PIOC : Per cent inhibition over control O : Overgrowth of antagonist over pathogen

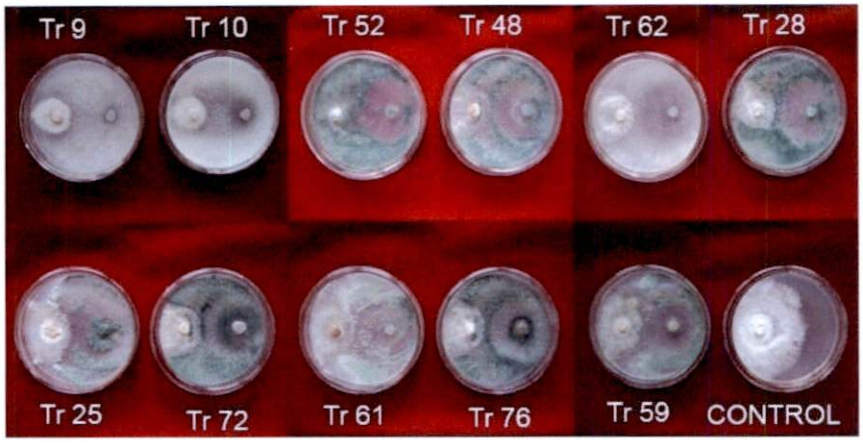
Plate 2. *In vitro* evaluation of antagonism of selected native isolates of *Trichoderma* spp. from Northern zone of Kerala – I



a. **Antagonism against *P. aphanidermatum***



b. **Antagonism against *P. capsici***



c. **Antagonism against *G. lucidum***

76 (62.56%), Tr 48 and Tr 52 (61.11%). The minimum antagonistic efficiency of 55.56 per cent was recorded by Tr 59 (Table 11). All native isolates of *Trichoderma* spp. tested exhibited higher antagonistic property than reference cultures against the pathogen.

Except Tr 9 and Tr 48, all the isolates exhibited aversion type of mechanism of antagonism against *S. rolfsii* by developing a clear zone of inhibition in between the mycelial growth of pathogen and antagonist. The isolates Tr 9, Tr 48 and the reference cultures of *Trichoderma* spp. ceased the growth of pathogen at the line of contact (Plate 3b and 9d).

4.6.1.6. *Rhizoctonia solani*

The result of dual culture experiment with the pathogen *R. solani* revealed that the isolate, Tr 10 showed the maximum efficiency by recording 50.33 percentage of inhibition followed by Tr 9 (49.22%) and Tr 52 (45.22%) (Table 11). All the isolates including the reference cultures exhibited > 40 per cent of inhibition over the growth of pathogen. Among them Tr 61 recorded the minimum inhibition of 40.00 per cent against the pathogen.

All the 11 isolates of *Trichoderma* spp. and the reference cultures exhibited homogenous type of reaction and the hyphae of pathogen and antagonist freely intermingled with each other (Plate 3c and 9e).

4.6.2. Evaluation of antagonistic efficiency of isolates of *Trichoderma* spp. from central region of Kerala.

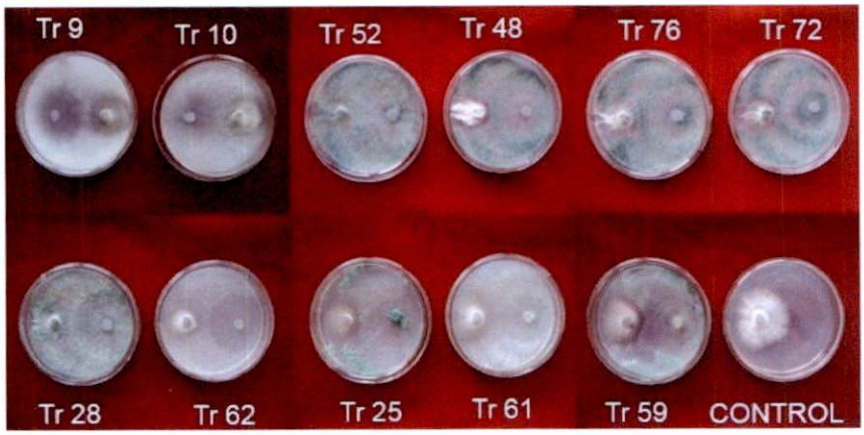
A total of 11 isolates of *Trichoderma* spp. obtained from central region of Kerala were selected from the preliminary screening and were further

Table 11. Evaluation of antagonistic efficiency of isolates of *Trichoderma* spp. from northern zone against *Fusarium oxysporum* f. sp. *cubense*, *Sclerotium rolfsii* and *Rhizoctonia solani*

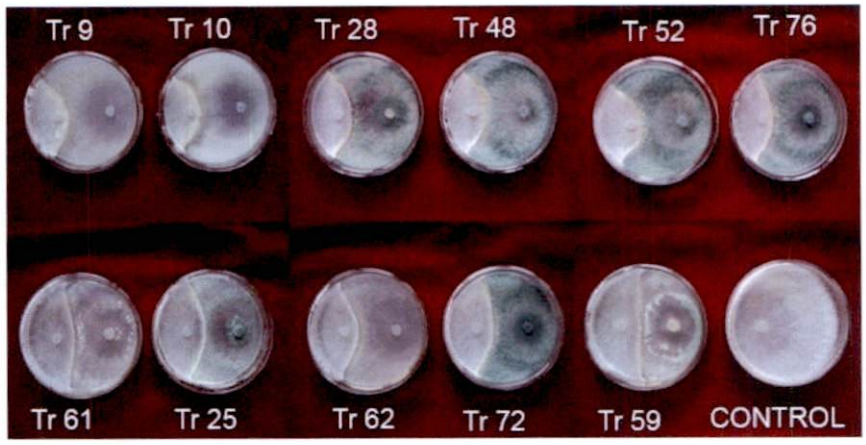
Sl. No.	Isolate of *Trichoderma* spp.	*Fusarium oxysporum* f. sp.*cubense*				*Sclerotium rolfsii*				*Rhizoctonia solani*			
		Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action
		P	T			P	T			P	T		
1	Tr 9	0.00	9.00	100.00	C	3.10	5.90	65.56	C	4.57	5.43	49.22	H
2	Tr 10	0.00	9.00	100.00	C	3.20	5.80	64.44	A	4.47	5.50	50.33	H
3	Tr 25	2.77	6.23	38.44	C	3.87	5.13	57.00	A	5.23	5.27	41.89	H
4	Tr 28	0.00	9.00	100.00	C	3.30	5.70	63.33	A	5.23	5.40	41.89	H
5	Tr 48	0.00	9.00	100.00	C	3.50	5.50	61.11	C	5.23	5.17	41.89	H
6	Tr 52	0.00	9.00	100.00	C	3.50	5.50	61.11	A	4.93	5.37	45.22	H
7	Tr 59	3.00	6.00	33.33	A	4.00	5.00	55.56	A	5.23	4.60	41.89	H
8	Tr 61	3.07	5.93	31.78	C	3.70	5.30	58.89	A	5.4	4.50	40.00	H
9	Tr 62	2.50	6.50	44.44	C	3.67	5.33	59.22	A	5.23	5.00	41.89	H
10	Tr 72	0.00	9.00	100.00	C	3.67	5.33	59.22	A	5.17	4.93	42.56	H
11	Tr 76	0.00	9.00	100.00	C	3.37	5.63	62.56	A	5.17	5.30	42.56	H
12	*T. harzianum*	3.00	6.00	33.33	C	5.83	3.17	35.22	C	5.30	7.50	41.11	H
13	*T. viride*	1.73	7.27	61.56	O	6.47	2.53	28.11	C	5.00	7.50	44.44	H
14	CONTROL	4.50				9.00				9.00			

* Mean of three replications
P : Pathogen
T : Antagonist
PIOC : Per cent inhibition over control
C : Cessation of the growth
A : Aversion
H : Homogenous growth
O : Overgrowth of antagonist over pathogen

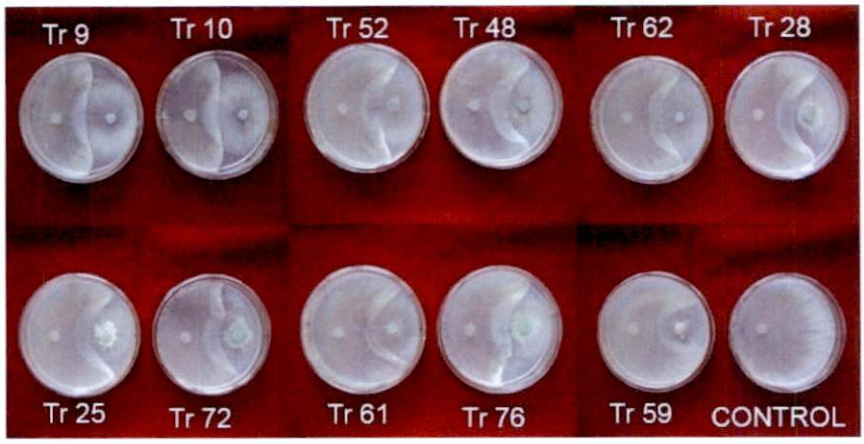
Plate 3. *In vitro* evaluation of antagonism of selected isolates of *Trichoderma* spp. from Northern zone of Kerala – II



a. **Antagonism against *F. oxysporum* f. sp. *cubense***



b. **Antagonism against *S. rolfsii***



c. **Antagonism against *R. solani***

evaluated for their antagonistic potential against the six soil borne fungal pathogens by dual culture technique.

4.6.2.1. *Pythium aphanidermatum*

The result of dual culture experiment revealed that all isolates of *Trichoderma* spp. except Tr 20 (54.44%) exhibited > 80 per cent of inhibition over the growth of pathogen, *Pythium aphanidermatum* (Table 12) which were higher than that of reference culture, *T. viride.* Among them, Tr 58 recorded cent per cent inhibition over the pathogen. It was followed by Tr 14 (94.44%), Tr 78 (93.33%) and Tr 43 (88.89%).

All the native isolates except Tr 20 and the reference cultures exhibited over growth type of mode of action against the pathogen. Isolate, Tr 20 expressed aversion type of reaction by developing a clear zone of inhibition (Plate 4a and 9a).

4.6.2.2. *Phytophthora capsici*

Except Tr 57 (89.67%), all native isolates of *Trichoderma* spp. and the reference cultures recorded cent per cent inhibition against *P. capsici* and all including Tr 57 exhibited over growth type of antagonism against the pathogen (Table 12, Plate 4b and 9b).

4.6.2.3. *Ganoderma lucidum*

All native isolates of *Trichoderma* spp. except Tr 23 (55.50%) recorded > 60 per cent inhibition over the growth of pathogen, *G. lucidum* and that was less than that of reference culture, *T. viride* (60%). All native isolates showed higher antagonistic efficiency than *T. harzianum* (52.12%). Among

the native isolates, Tr 58 showed highest percentage of inhibition of 83.83 followed by Tr 14 (83.33%), Tr 43 (75%), Tr 78 and Tr 81 (75%) (Table 12).

The isolates expressed overgrowth as mechanism of antagonism (Plate 4c and 9f).

4.6.2.4. *Fusarium oxysporum* f. sp. *cubense*

Cent per cent inhibition against the pathogen was recorded by native isolates *viz.*, Tr 2, Tr 14, Tr 43, Tr 86 and Tr 121 (Table 13). The other native isolates showed inhibition in the range of 68.09 to 89.36 per cent against the pathogen. The reference culture, *T. harzianum* recorded the minimum inhibition of 33.33 per cent.

All isolates except reference culture, *T. harzianum* showed over growth type of antagonism (Plate 5a and 9c).

4.6.2.5. *Sclerotium rolfsii*

The per cent of inhibition recorded by the native isolates of *Trichoderma* spp. against the pathogen, *S. rolfsii* was ranged from 35.22 (Tr 20) to 55.56 (Tr 14 and Tr 43) (Table 13) which was higher than that of *T. viride* (28.11%).

Both the native isolates and the reference cultures of *Trichoderma* spp. expressed cessation type of antagonism by ceasing the mycelial growth of the pathogen at line of contact (Plate 5b and 9d).

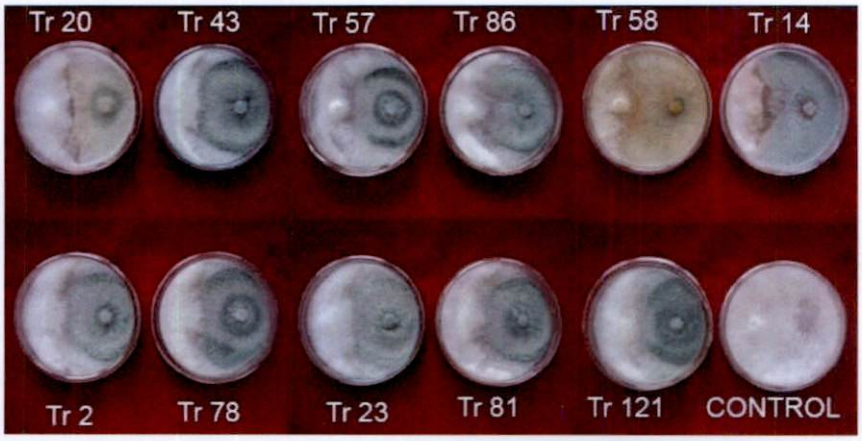
Table 12. Evaluation of antagonistic efficiency of isolates of *Trichoderma* spp. from central zone against *Pythium aphanidermatum*, *Phytophthora capsici* and *Ganoderma lucidum*

Sl. No.	Isolate of *Trichoderma* spp.	*Pythium aphanidermatum*				*Phytophthora capsici*				*Ganoderma lucidum*			
		Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action
		P	T			P	T			P	T		
1	Tr 2	1.13	7.87	87.44	O	0.00	9.00	100.00	O	2.03	6.97	66.17	O
2	Tr 14	0.50	8.50	94.44	O	0.00	9.00	100.00	O	1.00	8.00	83.33	O
3	Tr 20	4.10	4.90	54.44	A	0.00	9.00	100.00	O	1.63	7.37	72.83	O
4	Tr 23	1.50	7.50	83.33	O	0.00	9.00	100.00	O	2.67	6.33	55.50	O
5	Tr 43	1.00	8.00	88.89	O	0.00	9.00	100.00	O	1.50	7.50	75.00	O
6	Tr 57	1.73	7.27	80.78	O	0.93	8.07	89.67	O	2.00	7.00	66.67	O
7	Tr 58	0.00	9.00	100.00	O	0.00	9.00	100.00	O	0.97	8.03	83.83	O
8	Tr 78	0.60	8.40	93.33	O	0.00	9.00	100.00	O	1.50	7.50	75.00	O
9	Tr 81	1.17	7.83	87.00	O	0.00	9.00	100.00	O	1.50	7.50	75.00	O
10	Tr 86	1.17	7.83	87.00	O	0.00	9.00	100.00	O	2.30	6.70	61.67	O
11	Tr 121	1.73	7.27	80.78	O	0.00	9.00	100.00	O	2.00	7.00	66.67	O
12	*T. harzianum*	1.63	7.37	81.89	O	0.00	9.00	100.00	O	2.63	6.37	52.12	O
13	*T. viride*	2.47	6.53	72.56	O	0.00	9.00	100.00	O	2.20	6.80	60.00	O
14	CONTROL	9.00				9.00				6.00			

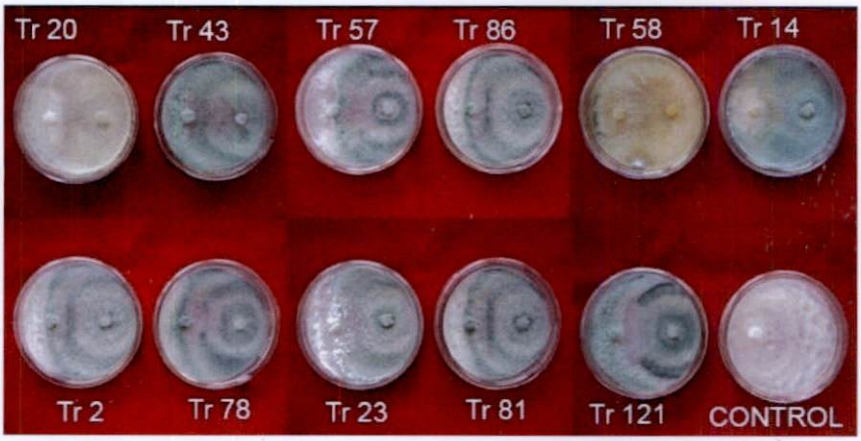
* Mean of three replications P : Pathogen T : Antagonist

PIOC : Per cent inhibition over control O : Overgrowth of antagonist over pathogen A : Aversion

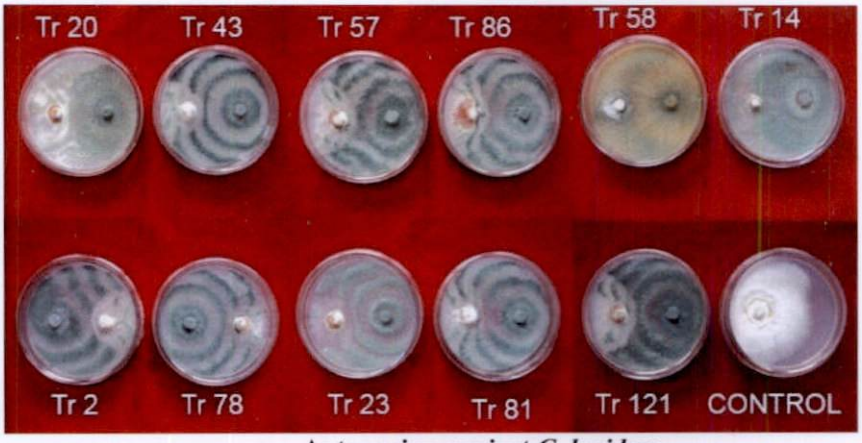
Plate 4. *In vitro* evaluation of antagonism of selected isolates of *Trichoderma* spp. from Central zone of Kerala – I



a. Antagonism against *P. aphanidermatum*



b. Antagonism against *P. capsici*



c. Antagonism against *G. lucidum*

4.6.2.6. *Rhizoctonia solani*

More than 40 per cent inhibition was recorded by all native isolates and reference cultures of *Trichoderma* spp. over the pathogen, *R. solani* and all isolates showed homogenous type of reaction by freely intermingle with the hyphae of pathogen (Table 13, Plate 5c and 9e). Among them, the highest inhibition (44.44%) was recorded by *T. viride* and the lowest inhibition (40.00%) was recorded by Tr 20.

4.6.3. Evaluation of antagonistic efficiency of isolates of *Trichoderma* spp. from southern region of Kerala

19 isolates of *Trichoderma* spp. selected from south zone after preliminary screening were further evaluated for the antagonistic potential against all the six pathogens. The results are given in Table 14 and 15.

4.6.3.1. *Pythium aphanidermatum*

All, except three isolates of *Trichoderma* spp. *viz.,* Tr 90 (55.22%), Tr 112 (62.22%) and Tr 127 (66.67%) recorded > 70 per cent of inhibition over pathogen, *P. aphanidermatum* (Table 14). The maximum inhibition on the growth of pathogen was recorded by Tr 41 (95.22%). Five isolates of *Trichoderma* spp. *viz.*, Tr 31, Tr 34, Tr 90, Tr 112 and Tr 127 exhibited less antagonistic property than the reference cultures, *T. viride* and *T. harzianum*. The isolate, Tr 90 showed the lowest inhibition against the pathogen.

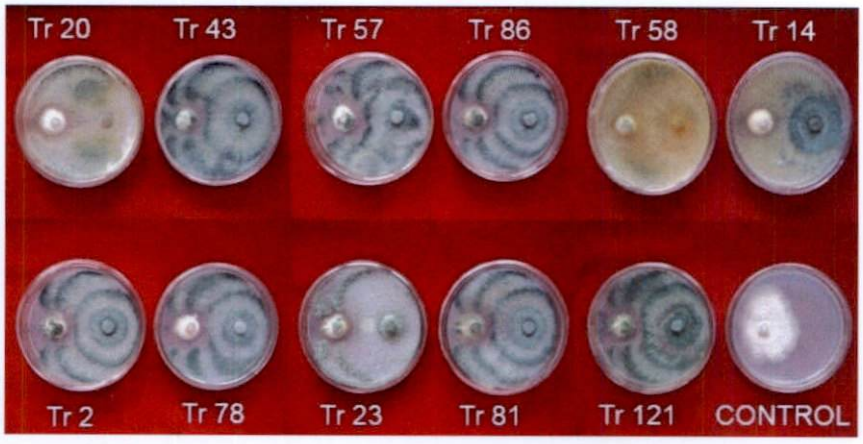
All isolates exhibited over growth as mechanism of antagonism (Plate 6a and 9a).

Table 13. Evaluation of antagonistic efficiency of isolates of *Trichoderma* spp. from central zone against *Fusarium oxysporum* f. sp. *cubense*, *Sclerotium rolfsii* and *Rhizoctonia solani*

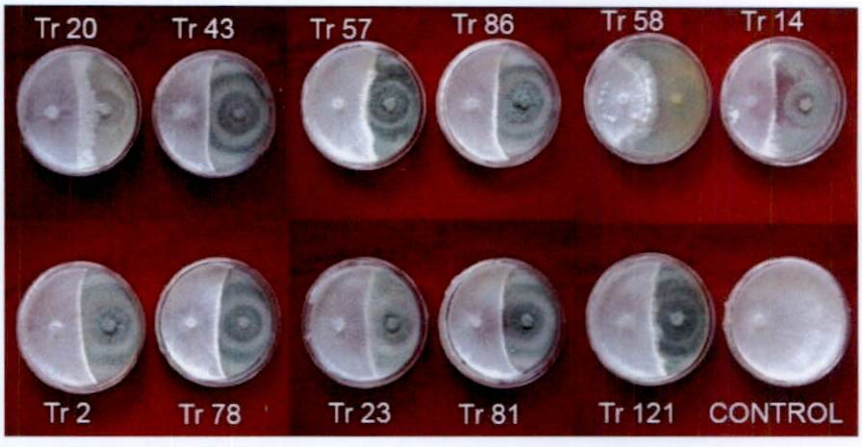
Sl. No.	Isolate of *Trichoderma* spp.	*Fusarium oxysporum* f. sp. *cubense*				*Sclerotium rolfsii*				*Rhizoctonia solani*			
		Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action
		P	T			P	T			P	T		
1	Tr 2	0.00	9.00	100.00	O	4.33	4.67	51.89	C	5.30	5.27	41.11	H
2	Tr 14	0.00	9.00	100.00	O	4.00	5.00	55.56	C	5.20	5.27	42.22	H
3	Tr 20	1.37	7.63	70.85	O	5.83	3.17	35.22	C	5.40	5.03	40.00	H
4	Tr 23	1.50	7.50	68.09	O	5.07	3.93	43.67	C	5.20	5.20	42.22	H
5	Tr 43	0.00	9.00	100.00	O	4.00	5.00	55.56	C	5.20	5.60	42.22	H
6	Tr 57	1.50	7.50	68.09	O	4.97	4.03	44.78	C	5.27	5.40	41.44	H
7	Tr 58	0.50	8.50	89.36	O	4.97	4.03	44.78	C	5.17	4.93	42.56	H
8	Tr 78	1.50	7.50	68.09	O	4.47	4.53	50.33	C	5.13	5.63	43.00	H
9	Tr 81	1.50	7.50	68.09	O	4.33	4.67	51.89	C	5.20	5.20	42.22	H
10	Tr 86	0.00	9.00	100.00	O	4.20	4.80	53.33	C	5.20	5.00	42.22	H
11	Tr 121	0.00	9.00	100.00	O	4.83	4.17	46.33	C	5.20	5.47	42.22	H
12	*T. harzianum*	3.00	6.00	33.33	C	5.83	3.17	35.22	C	5.30	7.50	41.11	H
13	*T. viride*	1.73	7.27	61.56	O	6.47	2.53	28.11	C	5.00	7.50	44.44	H
14	CONTROL	4.70				9.00				9.00			

* Mean of three replications
P : Pathogen
T : Antagonist
PIOC : Per cent inhibition over control
O : Overgrowth of antagonist over pathogen
C : Cessation of growth
H: Homogenous growth

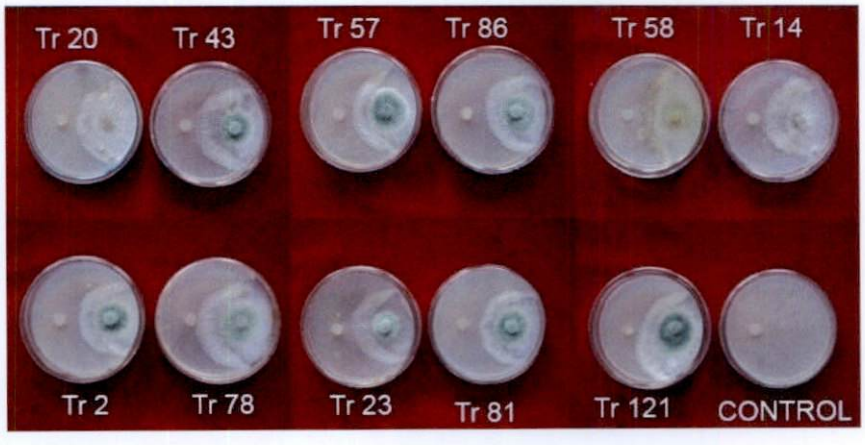
Plate 5. *In vitro* evaluation of antagonism of selected isolates of *Trichoderma* spp. from Central zone of Kerala – II



a. **Antagonism against *F. oxysporum* f. sp. *cubense***



b. **Antagonism against *S. rolfsii***



c. **Antagonism against *R. solani***

4.6.3.2. *Phytophthora capsici*

More than 77 per cent inhibition was recorded by the isolates of *Trichoderma* spp. against pathogen, *P. capsici.* Among them, eight native isolates exhibited cent per cent of inhibition (Table 14) along with the reference cultures. The lowest inhibition of 77.78 per cent was recorded by Tr 32, Tr 104 and Tr 119.

Over growth type of antagonism was shown by isolates of *Trichoderma* spp. (Plate 6b and 9b).

4.6.3.3. *Ganoderma lucidum*

The antagonistic efficiency of native isolates of *Trichoderma* spp. was recorded in the range of 54.55 to 81.82 per cent. The isolates tested were found to be more efficient than *T. harzianum* (52.12%). The highest inhibition of 81.82 per cent was recorded by native isolates *viz.,* Tr 34, Tr 41, Tr 90, Tr 97 and Tr 104.

All the isolates of *Trichoderma* spp. showed over growth type of reaction except Tr 112 which exhibited cessation type of antagonism (Table 14, Plate 7a and 9f).

4.6.3.4. *Fusarium oxysporum* f. sp. *cubense*

Out of 19, nine isolates of *Trichoderma* spp. *viz.,* Tr 34, Tr 42, Tr 90, Tr 97, Tr 101, Tr 104, Tr 109, Tr 114 and Tr 126 recorded cent per cent of inhibition against *F. oxysporum* f. sp. *cubense* followed by Tr 118 (96.38%), Tr 36 (88.72%) and Tr 41 (78.72%) (Table 15). All the isolates were found to be efficient than the reference culture, *T. harzianum* which recorded 33.33 per cent. *T. viride* recorded 61.56 per cent inhibition against the pathogen.

Table 14. Evaluation of antagonistic efficiency of isolates of *Trichoderma* spp. from southern zone against *Pythium aphanidermatum*, *Phytophthora capsici* and *Ganoderma lucidum*

Sl. No.	Isolate of *Trichoderma* spp.	*Pythium aphanidermatum*				*Phytophthora capsici*				*Ganoderma lucidum*			
		Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action
		P	T			P	T			P	T		
1	Tr 31	2.60	6.40	71.11	O	0.00	9.00	100.00	O	1.30	9.00	76.36	O
2	Tr 32	2.07	6.93	77.00	O	2.00	7.00	77.78	O	2.00	9.00	63.64	O
3	Tr 34	2.50	6.50	72.22	O	0.00	9.00	100.00	O	1.00	9.00	81.82	O
4	Tr 36	1.83	7.17	79.67	O	0.00	9.00	100.00	O	1.50	9.00	72.73	O
5	Tr 41	0.43	8.57	95.22	O	0.00	9.00	100.00	O	1.00	9.00	81.82	O
6	Tr 42	1.50	7.50	83.33	O	0.00	9.00	100.00	O	1.50	9.00	72.73	O
7	Tr 90	4.03	4.97	55.22	O	0.00	9.00	100.00	O	1.00	9.00	81.82	O
8	Tr 97	1.00	8.00	88.89	O	1.50	7.50	83.33	O	1.00	9.00	81.82	O
9	Tr 101	1.70	7.30	81.11	O	1.00	8.00	88.89	O	1.70	9.00	69.09	O
10	Tr 104	1.00	8.00	88.89	O	2.00	7.00	77.78	O	1.00	8.00	81.82	O
11	Tr 107	1.50	7.50	83.33	O	1.00	8.00	88.89	O	1.50	7.50	72.73	O
12	Tr 109	1.00	8.00	88.89	O	0.53	8.47	94.11	O	1.50	7.50	72.73	O
13	Tr 111	1.63	7.37	81.89	O	1.23	7.77	86.33	O	2.50	8.17	54.55	O
14	Tr 112	3.40	5.60	62.22	O	1.43	7.57	84.11	O	2.47	6.07	55.09	C

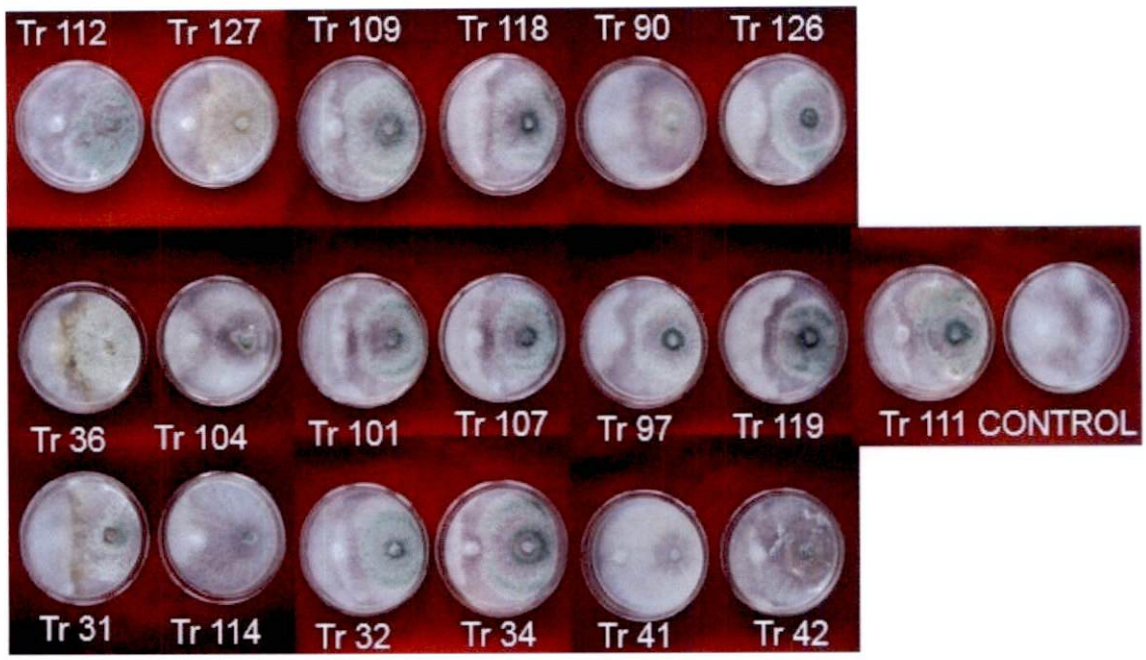
Contd ...

15	Tr 114	0.97	8.03	89.22	O	0.00	9.00	100.00	O	2.30	9.00	58.18	O
16	Tr 118	1.30	7.70	85.56	O	1.00	8.00	88.89	O	1.50	9.00	72.73	O
17	Tr 119	2.10	6.90	76.67	O	2.00	7.00	77.78	O	2.50	7.30	54.55	O
18	Tr 126	1.70	7.30	81.11	O	1.00	8.00	88.89	O	1.50	9.00	72.73	O
19	Tr 127	3.00	6.00	66.67	O	0.00	9.00	100.00	O	2.50	8.50	54.55	O
20	*T. harzianum*	1.63	7.37	81.89	O	0.00	9.00	100.00	O	2.63	6.37	52.12	O
21	*T. viride*	2.47	6.53	72.56	O	0.00	9.00	100.00	O	2.20	6.80	60.00	O
22	CONTROL	9.00				9.00				5.50			

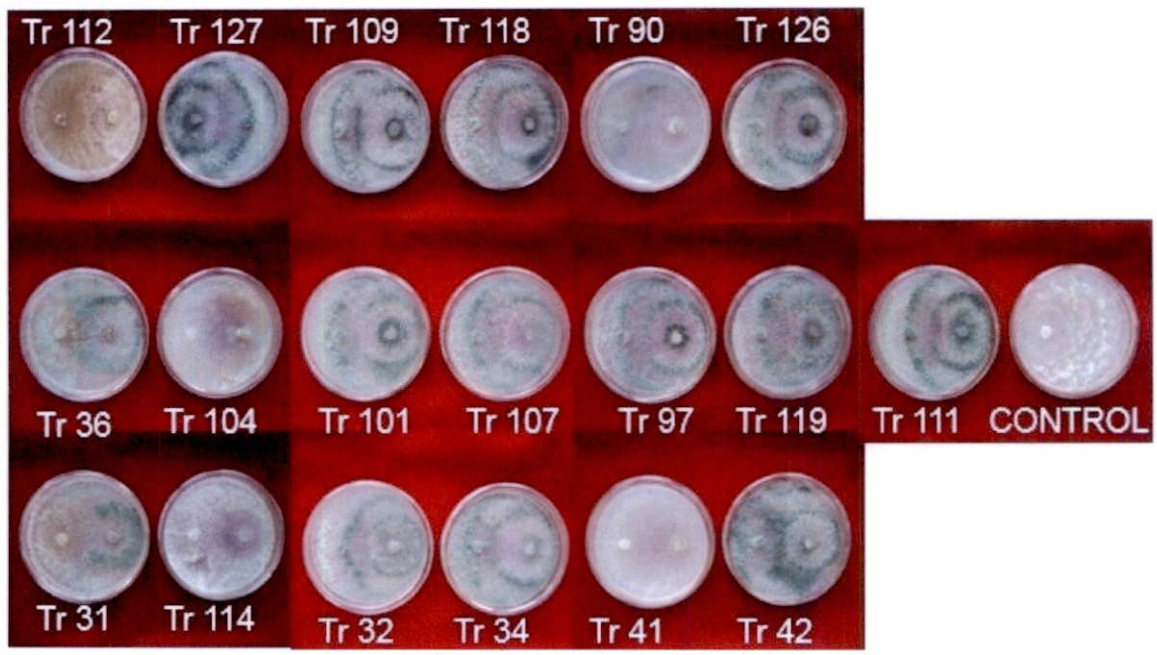
* Mean of three replications P : Pathogen T : Antagonist

PIOC : Per cent inhibition over control O : Overgrowth of antagonist over pathogen C : Cessation of growth

Plate 6. *In vitro* evaluation of antagonism of selected isolates of *Trichoderma* spp. from Southern zone of Kerala – I

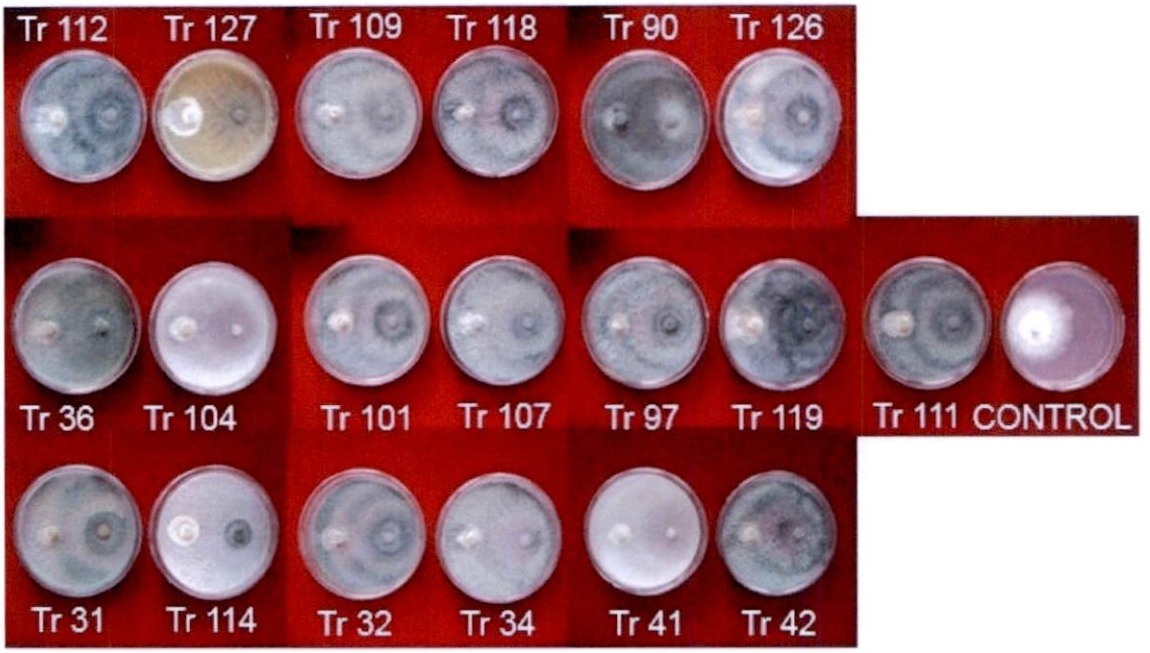


a. Antagonism against *P. aphanidermatum*

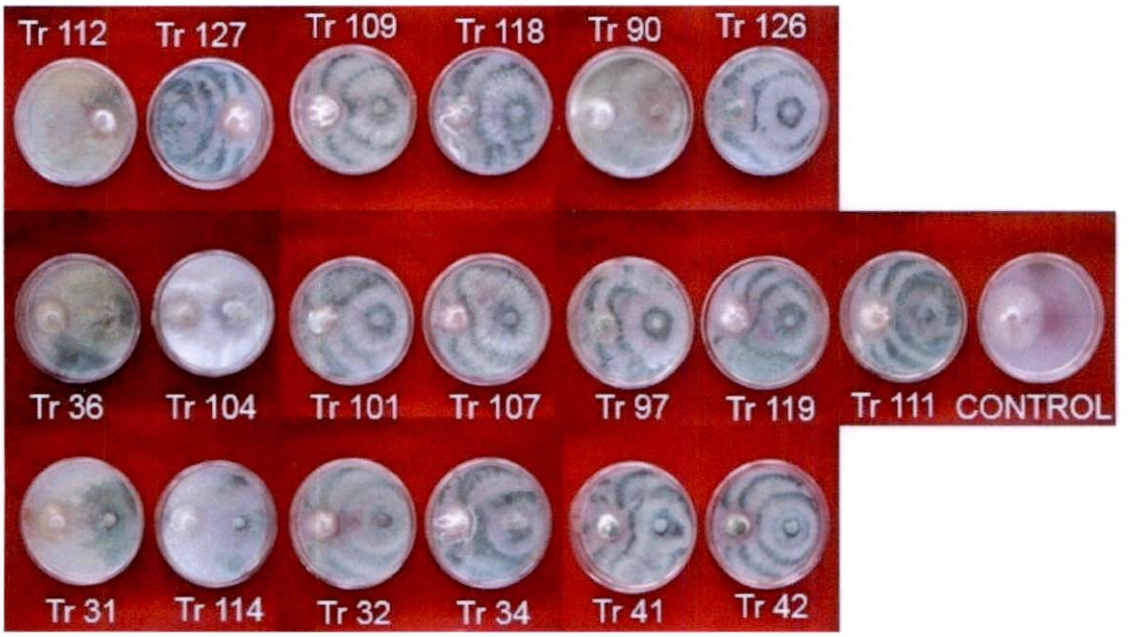


b. Antagonism against *P. capsici*

Plate 7. *In vitro* evaluation of antagonism of selected isolates of *Trichoderma* spp. from Southern zone of Kerala – II



a. **Antagonism against *G. lucidum***



b. **Antagonism against *F. oxysporum* f. sp. *cubense***

Over growth type of mechanism of antagonism was seen in case of all native isolates. The reference culture, *T. harzianum* exhibited cessation type of antagonism (Plate 7b and 9c).

4.6.3.5. *Sclerotium rolfsii*

The isolates of *Trichoderma* spp. recorded the antagonistic efficiency against the pathogen, *S. rolfsii* in the range of 15.22 (Tr 127) to 57.00 (Tr 31) (Table 15). The reference cultures *viz., T. viride* and *T. harzianum* recorded 28.11 and 35.22 per cent inhibition respectively against the pathogen.

Fifteen out of 19 native isolates of *Trichoderma* spp. as well as the reference cultures expressed cessation type of reaction. The isolates Tr 127, Tr 90 and Tr 36 showed homogenous type of antagonism and Tr 32 exhibited aversion type of antagonism (Plate 8a and 9d).

4.6.3.6. *Rhizoctonia solani*

The highest inhibition percentage of 50.00 was recorded by Tr 41 followed by isolates Tr 114 (49.22%), Tr 104 (48.89%) and Tr 97 (48.11%) (Table 15). The native isolate Tr 127 recorded the lowest inhibition of 22.22 per cent against the pathogen.

All the isolates including the reference cultures exhibited homogenous type of reaction (Plate 8b and 9e).

Based on the results of *in vitro* evaluation of 41 native isolates of *Trichoderma* spp. against the six soil borne pathogens, 20 isolates which recorded the highest antagonistic property against all the six pathogens were selected for further study. This 20 isolates included 5 isolates from northern zone, 6 from central and 9 from southern zone of Kerala.

Table 15. Evaluation of antagonistic efficiency of isolates of *Trichoderma* spp. from southern zone against *Fusarium oxysporum* f. sp. *cubense*, *Sclerotium rolfsii* and *Rhizoctonia solani*

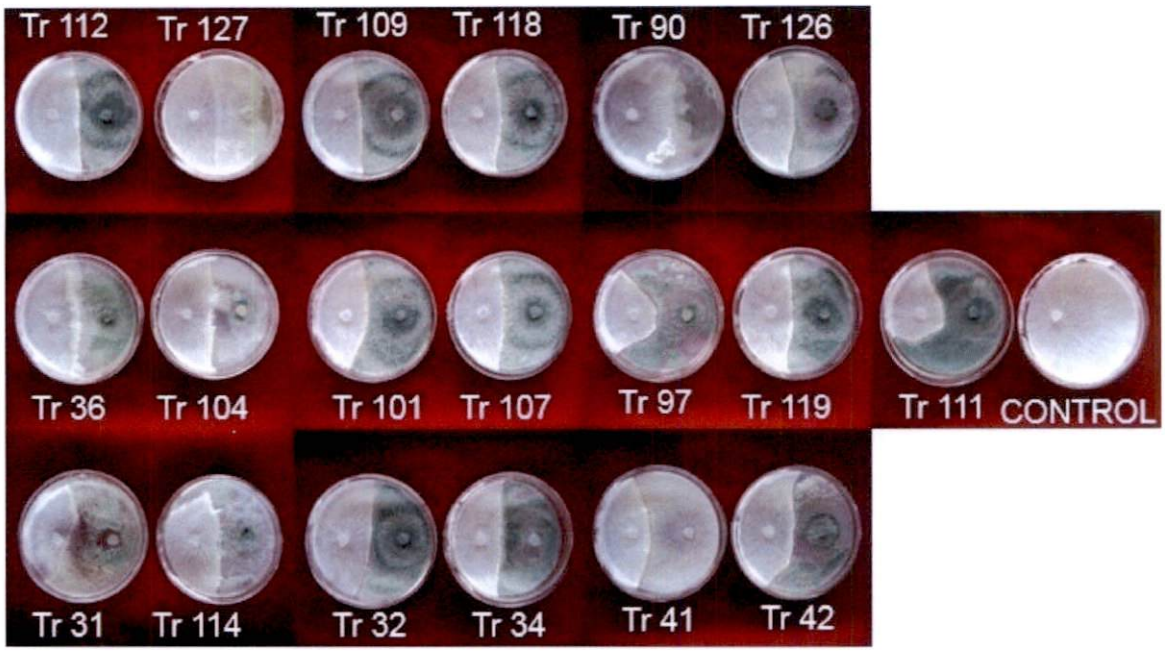
Sl. No.	Isolate of *Trichoderma* spp.	*Fusarium oxysporum* f. sp. *cubense*				*Sclerotium rolfsii*				*Rhizoctonia solani*			
		Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action
		P	T			P	T			P	T		
1	Tr 31	2.17	6.83	53.83	O	3.87	5.13	57.00	C	5.00	8.00	44.44	H
2	Tr 32	2.00	7.00	57.45	O	4.53	4.47	49.67	A	5.00	7.47	44.44	H
3	Tr 34	0.00	9.00	100.00	O	3.90	5.10	56.67	C	5.00	7.47	44.44	H
4	Tr 36	0.53	8.47	88.72	O	5.37	5.00	40.33	H	5.00	8.00	44.44	H
5	Tr 41	1.00	8.00	78.72	O	3.90	5.10	56.67	C	4.50	8.03	50.00	H
6	Tr 42	0.00	9.00	100.00	O	4.03	4.97	55.22	C	5.00	7.47	44.44	H
7	Tr 90	0.00	9.00	100.00	O	6.60	4.20	26.67	H	5.00	7.50	44.44	H
8	Tr 97	0.00	9.00	100.00	O	4.10	4.90	54.44	C	4.67	7.50	48.11	H
9	Tr 101	0.00	9.00	100.00	O	4.07	4.93	54.78	C	5.00	7.47	44.44	H
10	Tr 104	0.00	9.00	100.00	O	5.00	4.47	44.44	C	4.60	8.00	48.89	H
11	Tr 107	2.10	6.90	55.32	O	4.20	4.80	53.33	C	5.00	7.47	44.44	H
12	Tr 109	0.00	9.00	100.00	O	4.00	5.00	55.56	C	5.00	7.10	44.44	H
13	Tr 111	3.00	6.00	36.17	O	4.10	4.90	54.44	C	5.00	7.50	44.44	H
14	Tr 112	3.00	6.00	36.17	O	4.40	4.60	51.11	C	5.00	7.43	44.44	H
15	Tr 114	0.00	9.00	100.00	O	4.53	4.47	49.67	C	4.57	8.00	49.22	H

Contd ...

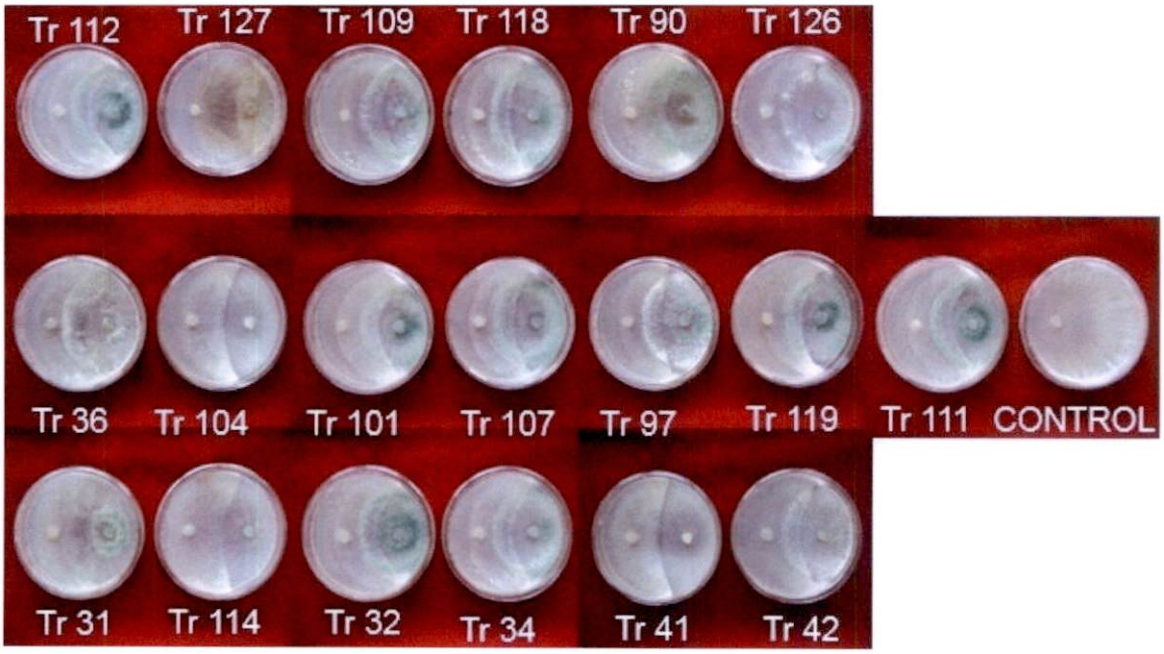
16	Tr 118	0.17	8.83	96.38	O	4.33	4.67	51.89	C	4.80	7.73	46.67	H
17	Tr 119	3.10	5.90	34.04	O	4.20	4.80	53.33	C	5.00	7.03	44.44	H
18	Tr 126	0.00	9.00	100.00	O	4.27	4.73	52.56	C	5.00	7.53	44.44	H
19	Tr 127	2.00	7.00	57.45	O	7.63	4.97	15.22	H	7.00	6.47	22.22	H
20	*T. harzianum*	3.00	6.00	33.33	C	5.83	3.17	35.22	C	5.30	7.50	41.11	H
21	*T. viride*	1.73	7.27	61.56	O	6.47	2.53	28.11	C	5.00	7.50	44.44	H
22	CONTROL	4.70				9.00				9.00			

* Mean of three replications
P : Pathogen
T : Antagonist
PIOC : Per cent inhibition over control
O : Overgrowth of antagonist over pathogen
C : Cessation of growth
A : Aversion
H : Homogenous growth

Plate 8. *In vitro* evaluation of antagonism of selected isolates of *Trichoderma* spp. from Southern zone of Kerala – III

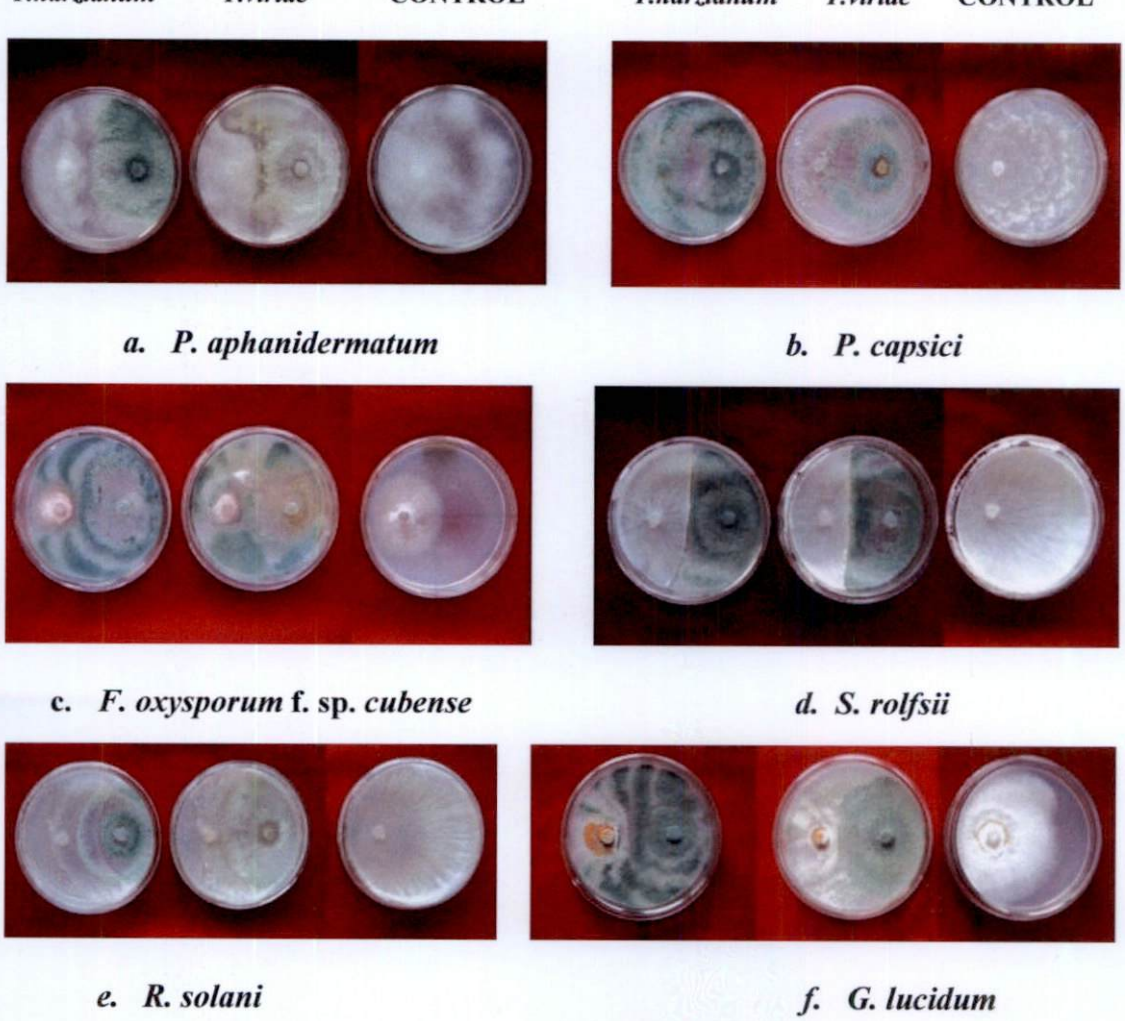


a. **Antagonism against *S. rolfsii***



b. **Antagonism against *R. solani***

Plate 9. *In vitro* evaluation of antagonism of reference cultures of *Trichoderma* spp.



a. P. aphanidermatum

b. P. capsici

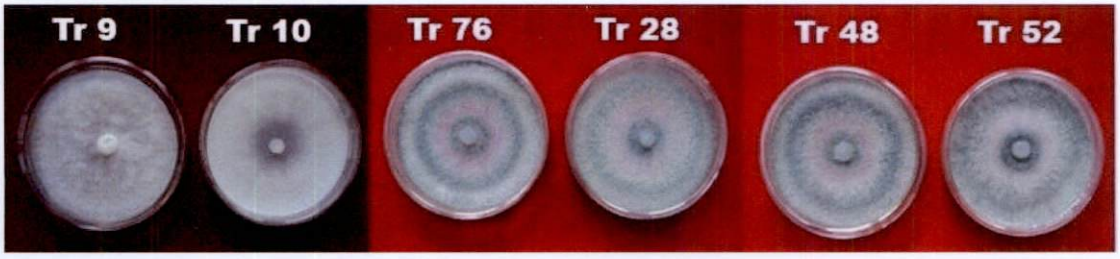
c. F. oxysporum f. sp. *cubense*

d. S. rolfsii

e. R. solani

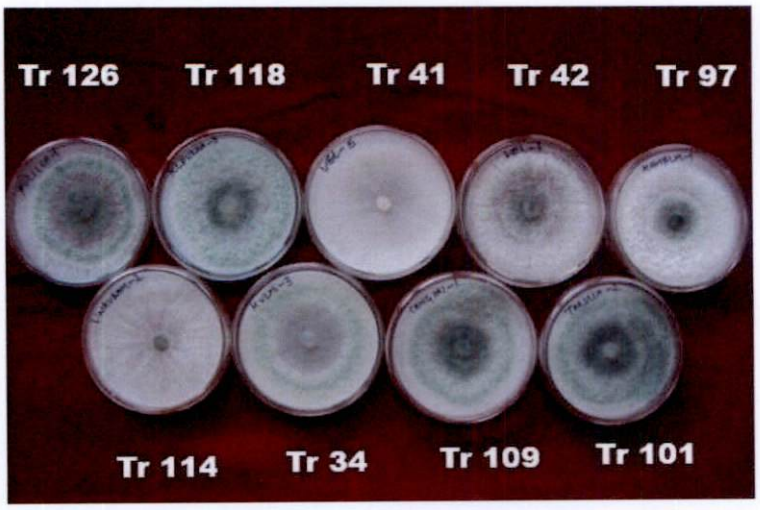
f. G. lucidum

Plate 10. Selected isolates of *Trichoderma* spp. from dual culture experiment



Isolates from northern zone of Kerala

Isolates from central zone of Kerala



Isolates from southern zone of Kerala

4.7. *IN VITRO* EVALUATION OF NATIVE *Trichoderma* spp. FOR THE CELLULASE ACTIVITY

The selected 20 isolates of *Trichoderma* spp. (Plate 10) were evaluated for the cellulose degrading efficiency under *in vitro* condition. The results of the evaluation of cellulase activity are furnished in Table 16; Fig. 1 to 3 and Plate 11.

4.7.1. Cellulase activity of isolates of *Trichoderma* spp. from northern zone of Kerala

The result of initial observation (2 DAI of isolate) revealed that there was significant difference among the treatments in the production of enzyme cellulase. Among five isolates of *Trichoderma* spp., Tr 9 recorded the highest hydrolyzing capacity of 0.99 followed by Tr 76 (0.70) and Tr 48 (0.60) (Table 16; Fig. 1 and Plate 11a). The hydrolyzing capacity of all the isolates of *Trichoderma* spp. was found to be increased in 3DAI. On that day, the highest hydrolyzing capacity of 1.00 was recorded by Tr 9 followed by Tr 52 and Tr 28 which were statistically on par (0.83) with each other.

4.7.2. Cellulase activity of isolates of *Trichoderma* spp. from central zone of Kerala

The highest hydroysing capacity was recorded by Tr 14 (0.99) followed by Tr 78 (0.76) and Tr 43 (0.66) on the 2nd day of inoculation of isolates of *Trichoderma* spp. (Table 16; Fig. 2 and Plate 11b). The isolates Tr 2, Tr 81 and Tr 86 did not show any hydrolyzing activity. Though the isolate, Tr 86 could not produce the enzyme cellulase initially, it recorded the highest hydrolyzing activity of 0.77 on 3DAI followed by 0.61 (Tr 14) and 0.54 (Tr 43).

4.7.3. Cellulase activity of isolates of *Trichoderma* spp. from southern zone of Kerala

Out of nine, only four isolates of *Trichoderma* spp. showed hydrolyzing capacity and were statistically different (Table 16; Fig. 3 and Plate 11c). Among them, Tr 114 recorded the highest hydrolyzing capacity of 0.99 on 2nd day of inoculation followed by Tr 41 (0.50), Tr 97 (0.45) and Tr 34 (0.34). By showing a hydrolyzing capacity of 0.91 on 3DAI, the isolate Tr 97 recorded the highest cellulase activity and was followed by Tr 114 (0.90), Tr 41 (0.89) and Tr 34 (0.55).

4.8. POT CULTURE EVALUATION ON ANTAGONISTIC EFFICACY OF SELECTED ISOLATES OF *Trichodema* spp.

A pot culture experiment was conducted to evaluate the antagonistic efficacy of selected isolates of *Trichoderma* spp. (Plate 12a) in plant growth promotion and management of disease in comparison with standard cultures of bio control agents along with a chemical fungicide, copper hydroxide. All treatments except fungicide were given as soil application @ 50 ml/ plant at the time of planting and 40 DAP (days after planting). Observations on the earliness of germination, germination percentage, pre-emergence rot, plant biometric characters and disease incidence were recorded at different intervals. The results are presented in Table 17 to 21.

The treatments T1 - T4:- Selected isolates of *Trichoderma* spp. from northern zone (Tr 76, Tr 48, Tr 52 and Tr 9)

T5 - T8:- Selected isolates of *Trichoderma* spp. from central zone (Tr 14, Tr 2, Tr 43 and Tr 86)

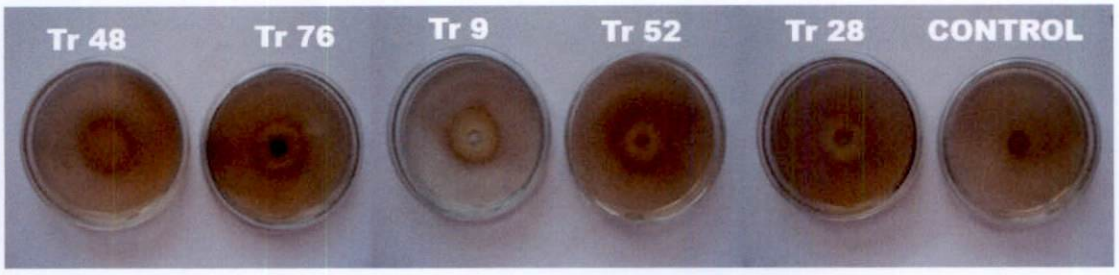
T9 – T12:- Selected isolates of *Trichoderma* spp. from southern zone (Tr 109, Tr 41,Tr 97 and Tr 34)

Table 16. Cellulase activity of selected native isolates of *Trichoderma* spp.

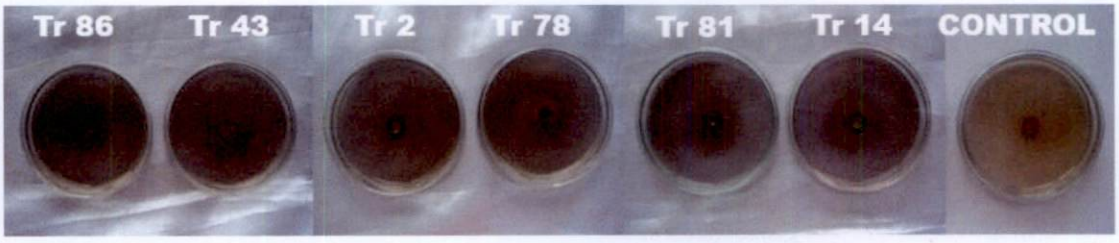
Sl. No.	Isolate of *Trichoderma* spp.	Hydrolysing capacity *	
		2DAI	3DAI
North zone isolates			
1	Tr 9	0.99^{a}	1.00^{a}
2	Tr 28	0.59^{cd}	0.83^{b}
3	Tr 48	0.60^{c}	0.70^{d}
4	Tr 52	0.57^{d}	0.83^{b}
5	Tr 76	0.70^{b}	0.71^{c}
Central zone isolates			
6	Tr 2	0.00^{d}	0.00^{e}
7	Tr 14	0.99^{a}	0.61^{b}
8	Tr 43	0.66^{c}	0.54^{c}
9	Tr 78	0.76^{b}	0.46^{d}
10	Tr 81	0.00^{d}	0.00^{e}
11	Tr 86	0.00^{d}	0.77^{a}
South zone isolates			
12	Tr 34	0.34^{d}	0.55^{c}
13	Tr 41	0.50^{b}	0.89^{b}
14	Tr 42	0.00^{e}	0.00^{d}
15	Tr 97	0.45^{c}	0.91^{a}
16	Tr 101	0.00^{e}	0.00^{d}
17	Tr 109	0.00^{e}	0.00^{d}
18	Tr 114	0.99^{a}	0.90^{ab}
19	Tr 118	0.00^{e}	0.00^{d}
20	Tr 126	0.00^{e}	0.00^{d}
21	Control	0.00	0.00

*Mean of three replications
DAI : Days After Inoculation
In each column figures followed by same letter do not differ significantly according to DMRT

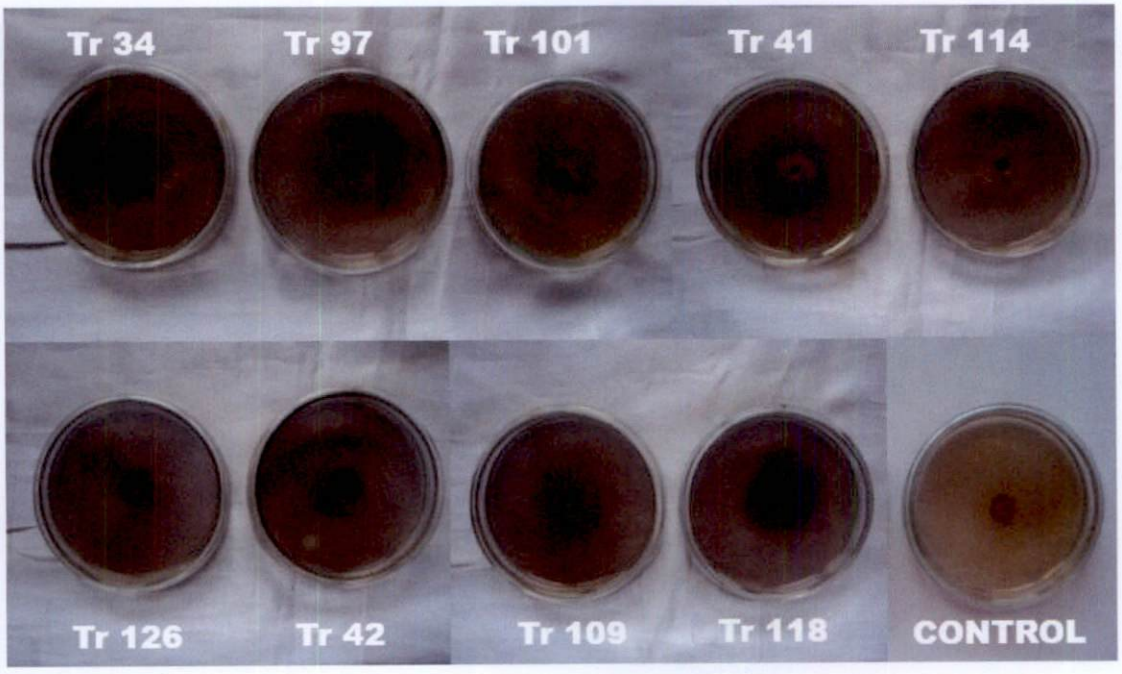
Plate 11. Evaluation of cellulose degrading activity of selected isolates of *Trichoderma* spp.



a. Isolates from northern zone of Kerala



b. Isolates from central zone of Kerala



c. Isolates from southern zone of Kerala

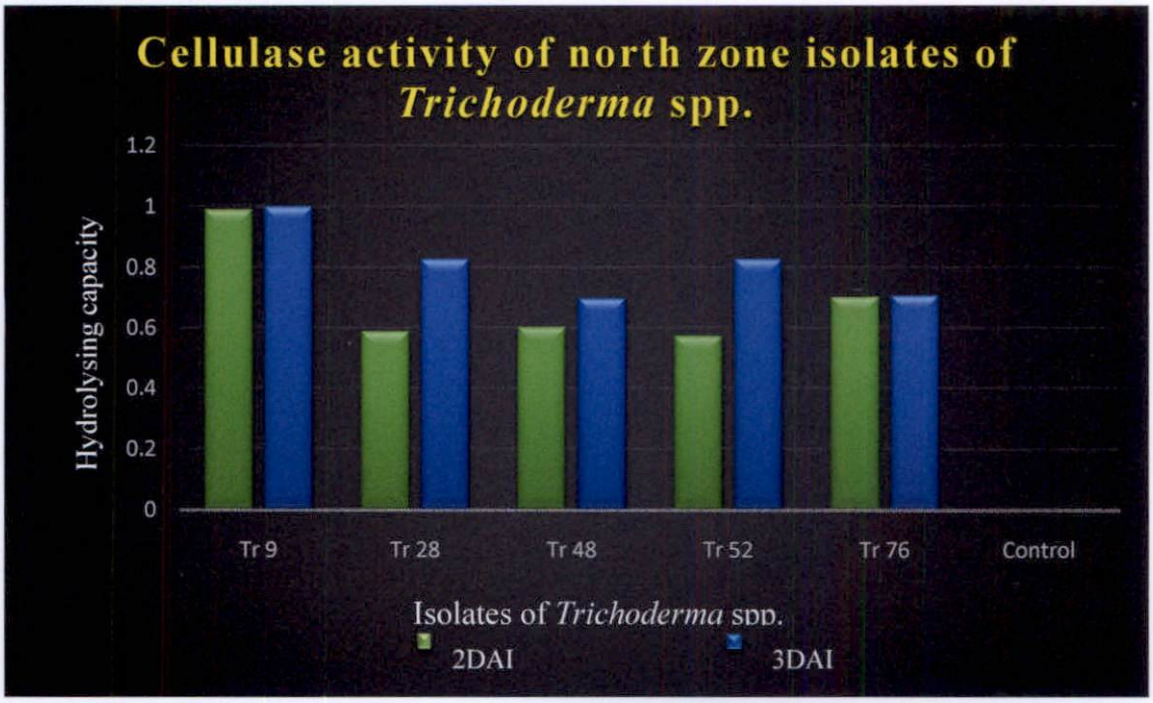


Fig. 1. Evaluation of cellulase activity of isolates of *Trichoderma* spp. from northern region of Kerala

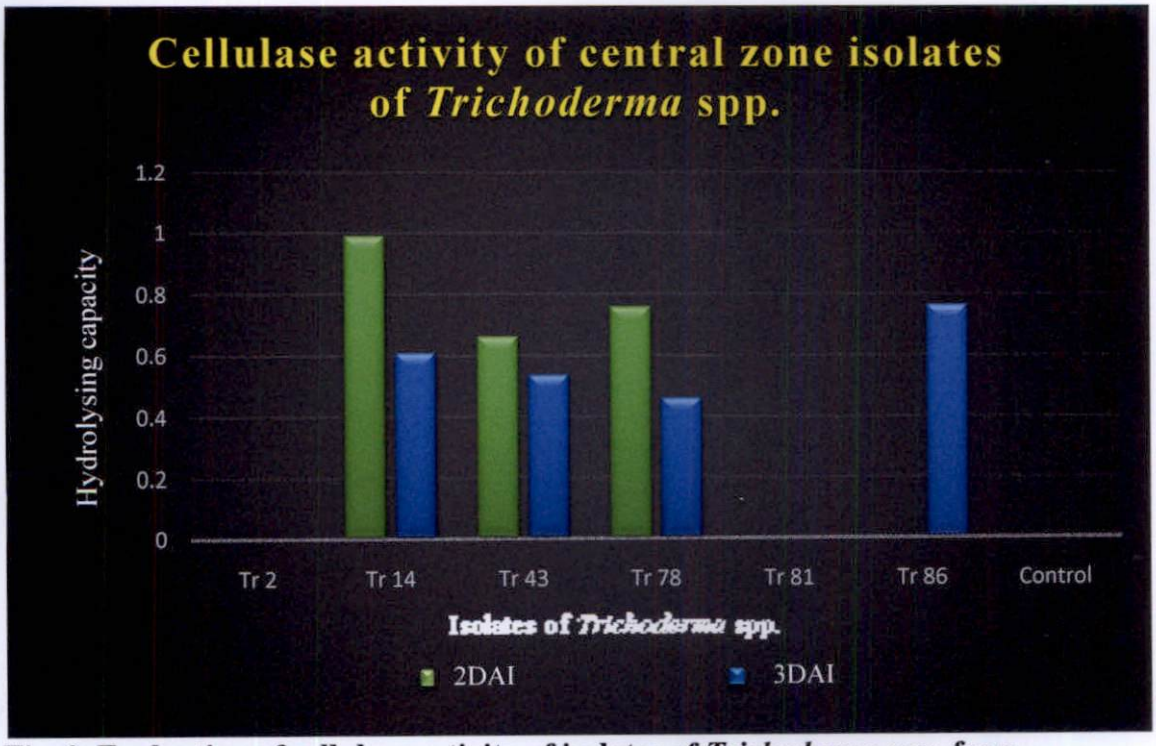


Fig. 2. Evaluation of cellulase activity of isolates of *Trichoderma* spp. from central region of Kerala

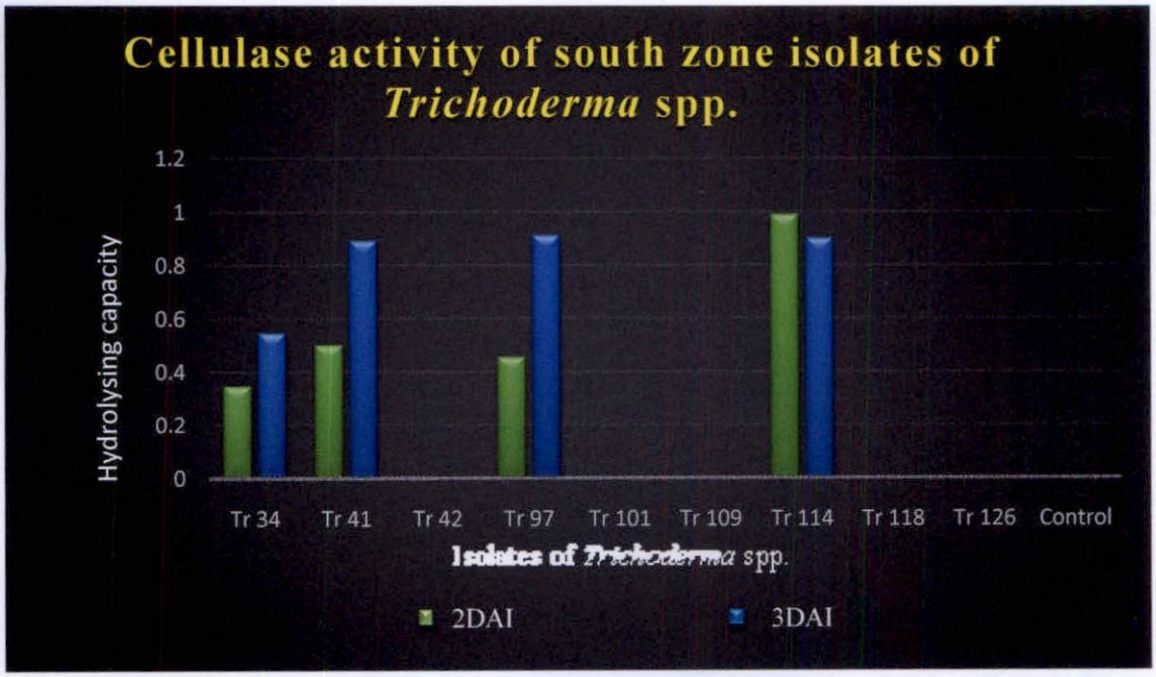


Fig. 3. Evaluation of cellulase activity of isolates of *Trichoderma* spp. from southern region of Kerala

4.8.1. Effect of selected native isolates of *Trichoderma* spp. on germination and pre emergence rot of ginger

The observation on earliness of germination was recorded from 15 DAP. Early germination of rhizomes was observed in the treatments T4 (Tr 9), T5 (Tr 14), T7 (Tr 43), T8 (Tr 86), T11 (Tr 97) and T15 (*Pseudomonas fluorescens*).

One month after planting (MAP), the number of rhizomes germinated was recorded and the per cent of germination was calculated. Statistically there was no significant difference observed among the treatments on germination percentage. All the treatments showed > 72 per cent of germination (Table 17). Among all, the treatment T9 (Tr 109) recorded highest germination percentage of 94.44.

The result of pre emergence rot of rhizomes recorded at 1MAP, revealed that there was significant difference among the treatments. The data given in the Table 17, showed a range of 5.55 (T9 – Tr 109) to 27.78 (T5 – Tr 14, T 15 – *P. fluorescens* and T 16 – Copper hydroxide) per cent of pre emergence rot. Statistically T9 was on par with T4, T7 and T11.

4.8.2. Effect of selected native isolates of *Trichoderma* spp. on plant biometric characters

The observations on bio metric characters of plant *viz.*, on number of tillers / plant, height of the plant and the number of leaves / tiller were recorded thrice at 30 days interval from 40 DAP to 100 DAP. The results are furnished in Table 18 to 20.

Table 17. Pot experiment I :- Effect of treatments on germination and pre emergence rot of ginger

Sl. No.	Tr. No.	Treatments	Germination (%) *	Pre emergence rot (%) *
1	T 1	Tr 76	75.00	25.00[a]
2	T 2	Tr 48	75.00	25.00[a]
3	T 3	Tr 52	80.55	19.45[ab]
4	T 4	Tr 9	88.89	11.11[bc]
5	T 5	Tr 14	72.22	27.78[a]
6	T 6	Tr 2	75.00	25.00[a]
7	T 7	Tr 43	86.11	13.89[bc]
8	T 8	Tr 86	75.00	25.00[a]
9	T 9	Tr 109	94.44	5.55[c]
10	T 10	Tr 41	80.56	19.48[ab]
11	T 11	Tr 97	91.67	8.33[c]
12	T 12	Tr 34	80.56	19.48[ab]
13	T 13	*Trichoderma viride*	75.00	25.00[a]
14	T 14	*Trichoderma harzianum*	75.00	25.00[a]
15	T 15	*Pseudomonas fluorescens*	72.22	27.78[a]
16	T 16	Copper hydroxide (0.2%)	72.22	27.78[a]
17	T 17	Control (With pathogen)	75.00	25.00[a]
18	T 18	Absolute control (without pathogen)	80.55	19.45[ab]

* Mean of three replications

Figures followed by same letter do not differ significantly according to DMRT

4.8.2.1. Number of tillers / plant

The data given in the Table 18 revealed that there was significant difference among the treatments in the number of tillers / plant. After 40 days of planting, the highest number of tillers / plant was recorded by treatment, T11 (Tr 97) as 7.32 followed by treatments T9 (Tr 109), T3 (Tr 52), T4 (Tr 9) and T7 (Tr 43) which were statistically on par with each other. Based on the second observation taken at 70 DAP, the highest (15) number of tillers per plant was observed in the treatments T7 (Tr 43) and T9 (109). 100 DAP, the highest record of 127.00 number of tillers / plant was shown by plants in the absolute control. This was followed by treatment T 7 (Tr 43) with 45 number of tillers / plant. At that time there was no plants in the treatments T11 (Tr 97), T 13 (*T. viride*), T 15 (*P. fluorescens*) and T 17 (control).

4.8.2.2. Height of plant

Throughout the crop period, it was observed that the treatments have an effect on the height of the plant. The maximum height was recorded by treatment, T8 (Tr 86) as 22.43 cm and 42.73 at 40 DAP and 70 DAP respectively (Table 19). Initially (40DAP), the lowest height of the plant was recorded by T 13 (*T. viride*) as 12.68 cm at 40 DAP and T 17 (control) as 23.73 cm at 70 DAP. The final observation at 100 DAP revealed that it was the treatment, T 18 (Absolute control) which recorded the maximum height of 41.01cm followed by T 7 (Tr 43) which recorded 19.25 cm.

4.8.2.3. Number of leaves / tiller

At 40 DAP, the observations on the number of leaves per plant showed no significant difference between the treatments. Even then, the plants applied with native isolate of *Trichoderma* spp., Tr 86 (T8) recorded the highest number of leaves / tiller as 5.82 (Table 20). The lowest number of leaves (4.0) was observed in T 17 (control).

Table 18. Pot experiment I :- Effect of treatments on number of tillers

Sl. No.	Tr. No.	Treatments	No. of tillers / plant *		
			40 DAP	70 DAP	100 DAP (surviving sprouts)
1	T1	Tr 76	4.08^{f}	7.50^{e}	13.00^{d} (3.659)
2	T2	Tr 48	4.92^{def}	8.67^{cde}	5.67^{e} (2.476)
3	T3	Tr 52	6.58^{ab}	13.58^{ab}	1.00^{gh} (1.225)
4	T4	Tr 9	6.42^{ab}	13.50^{abc}	17.33^{c} (4.220)
5	T5	Tr 14	4.92^{def}	9.67^{bcde}	4.33^{ef} (2.181)
6	T6	Tr 2	6.33^{abc}	12.67^{abcd}	1.00^{gh} (1.171)
7	T7	Tr 43	6.42^{ab}	15.00^{a}	45.00^{b} (6.743)
8	T8	Tr 86	4.17^{ef}	10.00^{bcde}	2.33^{fgh} (1.642)
9	T9	Tr 109	6.92^{ab}	15.00^{a}	1.67^{gh} (1.440)
10	T10	Tr 41	6.00^{bcd}	12.83^{abc}	16.67^{c} (4.140)
11	T11	Tr 97	7.32^{a}	14.08^{ab}	0.00^{h} (0.707)
12	T12	Tr 34	5.92^{bcd}	10.92^{abcde}	2.67^{fg} (1.739)
13	T13	*Trichoderma viride*	4.08^{f}	7.75^{e}	0.00^{h} (0.707)
14	T14	*Trichoderma harzianum*	5.00^{def}	7.75^{e}	1.67^{gh} (1.440)
15	T15	*Pseudomonas fluorescens*	4.08^{f}	6.50^{e}	0.00^{h} (0.707)
16	T16	Copper hydroxide (0.2%)	4.33^{ef}	7.83^{de}	0.33^{gh} (0.880)
17	T17	Control (With pathogen)	5.01^{def}	10.17^{abcde}	0.00^{h} (0.707)
18	T18	Absolute control (without pathogen)	5.25^{cde}	12.67^{abcd}	127.00^{a} (11.291)

* Mean of three replications

DAP : Days After Planting

In each column figures followed by same letter do not differ significantly according to DMRT

Figures in parenthesis are square root transformed values

Table 19. Pot experiment I :- Effect of treatments on height of the plant

Sl. No.	Tr. No.	Treatments	Height of plant (cm) *		
			40 DAP	70 DAP	100 DAP (surviving sprouts)
1	T1	Tr 76	14.31^{cde}	26.27^{ef}	10.62^{de} (3.332)
2	T2	Tr 48	16.60^{bcd}	32.13^{bcde}	10.18^{de} (3.251)
3	T3	Tr 52	14.96^{cde}	37.07^{abcd}	12.00^{cde} (3.532)
4	T4	Tr 9	15.86^{cde}	40.33^{ab}	12.49^{cd} (3.602)
5	T5	Tr 14	15.44^{cde}	31.20^{cdef}	9.62^{def} (3.176)
6	T6	Tr 2	15.89^{cde}	32.53^{bcde}	8.17^{ef} (2.615)
7	T7	Tr 43	16.46^{bcd}	38.60^{abc}	19.25^{b} (4.439)
8	T8	Tr 86	22.43^{a}	42.73^{a}	9.90^{de} (2.223)
9	T9	Tr 109	16.91^{bc}	38.07^{abc}	5.53^{fg} (2.456)
10	T10	Tr 41	15.35^{cde}	32.27^{bcde}	16.11^{bc} (4.066)
11	T11	Tr 97	17.14^{bc}	36.87^{abcd}	0.00^{h} (0.707)
12	T12	Tr 34	19.68^{ab}	39.67^{ab}	10.00^{de} (3.239)
13	T13	*Trichoderma viride*	12.68^{e}	28.93^{def}	0.00^{h} (0.707)
14	T14	*Trichoderma harzianum*	13.04^{de}	28.87^{def}	9.06^{def} (3.088)
15	T15	*Pseudomonas fluorescens*	14.90^{cde}	26.60^{ef}	0.00^{h} (0.707)
16	T16	Copper hydroxide (0.2%)	14.50^{cde}	27.33^{ef}	3.33^{gh} (1.552)
17	T17	Control (With pathogen)	12.96^{de}	23.73^{f}	0.00^{h} (0.707)
18	T18	Absolute control (without pathogen)	14.56^{cde}	35.87^{abcd}	41.01^{a} (6.441)

* Mean of three replications

DAP : Days After Planting

In each column figures followed by same letter do not differ significantly according to DMRT

Figures in parenthesis are square root transformed values

Later, the plants showed significant difference in the number of leaves among the treatments. One month after first observation (70 DAP), the treatments T 8 (Tr 86), T 9 (Tr 109) and T 12 (Tr 34) recorded the highest number of leaves / tiller and were statistically on par with each other. At 100 DAP, the plants in the treatment T18 recorded the maximum number of leaves (9.70). It was followed by treatments, T 7 (Tr 43) and T 4 (Tr 9) which recorded number of leaves as 6.72 and 6.6 respectively and were found to be statistically on par with each other.

4.8.3. Effect of selected native isolates of *Trichoderma* spp. on incidence of disease

The initial symptom of yellowing of lower leaves of plants was observed one week after inoculation of the pathogen (Plate 12b). The data furnished in Table 21 revealed that at the initial stage, the plants in the treatments T4 (Tr 9), T 7 (Tr 43), T 9 (Tr 109), T 13 (*T. viride*) and T18 (Absolute control) did not show any symptoms of disease. At that time more occurrence of disease was observed in treatments T17 (control) and T12 (Tr 34). 30 DAI, the disease incidence was more in T14 (*T. harzianum*) which recorded 76.67 per cent and the treatment T9 (Tr 109) showed the least incidence of disease after absolute control by recording 14.44 per cent. 60 days after the inoculation of pathogen, the treatment T1 (Tr 76) showed 40.12 per cent lesser incidence of disease than the control followed by treatments T 7 (Tr 43), T 4 (Tr 9), T 10 (Tr 41) and T 2 (Tr 48) recorded 32.33, 25.17, 20.1 and 12.17 percentage less incidence of disease respectively. These five native isolates of *Trichoderma* spp. were taken for further research.

Table 20. Pot experiment I :- Effect of treatments on number of leaves

Sl. No.	Tr. No.	Treatments	No. of leaves *		
			40 DAP **	70 DAP	100 DAP (surviving sprouts)
1	T1	Tr 76	4.54	8.13^{bc}	4.14^{cd} (2.154)
2	T2	Tr 48	4.50	10.13^{ab}	3.37^{ef} (1.968)
3	T3	Tr 52	4.64	9.07^{abc}	2.76^{fgh} (1.804)
4	T4	Tr 9	5.13	9.20^{abc}	6.60^{b} (2.664)
5	T5	Tr 14	5.13	7.47^{c}	2.47^{h} (1.725)
6	T6	Tr 2	4.65	9.00^{abc}	3.23^{efg} (1.931)
7	T7	Tr 43	5.17	9.40^{abc}	6.72^{b} (2.687)
8	T8	Tr 86	5.82	10.87^{a}	3.56^{de} (2.014)
9	T9	Tr 109	5.44	10.67^{a}	2.41^{h} (1.705)
10	T10	Tr 41	4.86	8.47^{bc}	4.31^{c} (2.192)
11	T11	Tr 97	5.11	9.93^{ab}	0.00^{j} (0.707)
12	T12	Tr 34	5.13	10.67^{a}	2.63^{gh} (1.768)
13	T13	*Trichoderma viride*	4.28	8.73^{abc}	0.00^{j} (0.707)
14	T14	*Trichoderma harzianum*	4.23	7.60^{c}	2.77^{fgh} (1.809)
15	T15	*Pseudomonas fluorescens*	4.18	8.93^{abc}	0.00^{j} (0.707)
16	T16	Copper hydroxide (0.2%)	4.81	8.33^{bc}	0.77^{i} (1.030)
17	T17	Control (With pathogen)	4.00	7.60^{c}	0.00^{j} (0.707)
18	T18	Absolute control (without pathogen)	4.38	9.07^{abc}	9.70^{a} (3.192)

* Mean of three replications ** Non significant

DAP : Days After Planting

In each column figures followed by same letter do not differ significantly according to DMRT

Figures in parenthesis are square root transformed values

Table 21. Pot experiment I :- Effect of treatments on incidence of disease

Sl. No.	Tr. No.	Treatments	Disease incidence *					
			7 DAI		30 DAI		60 DAI	
			Per cent incidence	PROC	Per cent incidence	PROC	Per cent incidence	PROC
1	T1	Tr 76	7.41	67.77	48.15	21.80	59.88	40.12
2	T2	Tr 48	11.20	51.28	62.59	-1.66	87.83	12.17
3	T3	Tr 52	10.37	54.89	38.15	38.04	96.60	3.4
4	T4	Tr 9	0.00	100.00	22.12	64.07	74.83	25.17
5	T5	Tr 14	7.87	65.77	43.98	28.57	93.82	6.18
6	T6	Tr 2	11.36	50.59	16.67	72.93	95.79	4.21
7	T7	Tr 43	0.00	100.00	24.24	60.63	67.67	32.33
8	T8	Tr 86	3.70	83.91	46.85	23.91	100.00	0.00
9	T9	Tr 109	0.00	100.00	14.44	76.55	100.00	0.00
10	T10	Tr 41	3.70	83.91	25.93	57.89	79.90	20.1
11	T11	Tr 97	3.03	86.82	15.15	75.39	100.00	0.00
12	T12	Tr 34	21.64	5.87	61.91	-0.55	93.02	6.98
13	T13	*Trichoderma viride*	0.00	100.00	50.95	17.25	100.00	0.00
14	T14	*Trichoderma harzianum*	15.37	33.15	76.67	-24.53	93.83	6.17
15	T15	*Pseudomonas fluorescens*	7.87	65.77	43.06	30.06	100.00	0.00
16	T16	Copper hydroxide (0.2%)	7.04	69.38	71.64	-16.36	100.00	0.00
17	T17	Control (With pathogen)	22.99		61.57		100.00	
18	T18	Absolute control (without pathogen)	0.00		0.00		0.00	

* Mean of three replications
DAI: Days After Inoculation
PROC: Per cent Reduction Over Control - No reduction

Plate 12. Pot culture experiment – I

a. View of pot experiment

b. Incidence of disease

4.9. EVALUATION OF COMPATIBILITY OF SELECTED NATIVE ISOLATES OF *Trichoderma* spp. WITH SELECTED FUNGICIDES

A total of five isolates of *Trichoderma* spp. *viz.*, Tr 9, Tr 41, Tr 43, Tr 48 and Tr 76 selected from the pot culture experiment were taken for the evaluation of compatibility with the selected fungicides *viz.,* carbendazim (0.1%), mancozeb (0.3%), bordeaux mixture (1%) and copper hydroxide (0.15%). The results of the experiment are furnished in the Table 22, Plate 13 and Fig 4. The data revealed cent per cent inhibition on the growth of all isolates in the plates poisoned with the fungicides, carbendazim and bordeaux mixture. The per cent of inhibition by mancozeb was ranged from 7.44 (Tr 9) to 55.22 (Tr 76). The fungicide, copper hydroxide showed > 72 per cent inhibition on the growth of the isolates of *Trichoderma* spp. Among them, Tr 9 recorded maximum percentage of 83.

4.10. EVALUATION OF COMPATIBILITY OF SELECTED ISOLATES OF *Trichoderma* spp. WITH SELECTED INSECTICIDES

The result of the evaluation on compatibility of selected five isolates of *Trichoderma* spp. with the selected insecticides *viz.*, malathion (0.1%), dimethoate (0.05%), quinalphos (0.05%), chlorpyriphos (0.25%) and flubendiamide (0.01%) was presented in the Table 22, Plate 14 and Fig. 5. From the data, it is evident that all the tested isolates of *Trichoderma* spp. were cent per cent compatible with the insecticide, fame and were cent per cent inhibited by the insecticide, quinalphos. It was seen that > 83 and > 85 per cent inhibition on the growth of all isolates in the plates poisoned with the insecticides, dimethoate and chlorpyriphos respectively. The isolates were found to be less inhibited (<15 %) by malathion and among them, the isolate Tr 9 did not show inhibition on the growth.

Table 22. Evaluation on compatibility of selected isolates of *Trichoderma* spp. with fungicides and insecticides

Sl. No.	Fungicides	Isolates of *Trichoderma* spp.									
		Tr 9		Tr 41		Tr 43		Tr 48		Tr 76	
		Radial growth (cm) *	PIOC	Radial growth (cm) *	PIOC	Radial growth (cm) *	PIOC	Radial growth (cm) *	PIOC	Radial growth (cm) *	PIOC
1	Carbendazim (0.1%)	0	100	0	100	0	100	0	100	0	100
2	Mancozeb (0.3%)	8.33	7.44	7.43	17.44	4.60	48.89	4.10	54.44	4.03	55.22
3	Bordeaux mixture (1%)	0	100	0	100	0	100	0	100	0	100
4	Copper hydroxide (0.15%)	1.53	83.00	2.50	72.22	2.00	77.78	2.03	77.44	2.00	77.78
5	Control	9.00		9.00		9.00		9.00		9.00	
	Insecticides										
6	Malathion (0.1%)	9.00	0	8.47	5.89	7.93	11.89	7.73	14.11	8.00	11.11
7	Dimethoate 0.05%)	1.50	83.33	0	100	1.40	84.44	1.30	85.56	1.30	85.56
8	Quinalphos (0.05%)	0	100	0	100	0	100	0	100	0	100
9	Chlorpyriphos (0.25%)	1.30	85.56	0	100	1.17	87.00	1.20	86.67	1.13	87.44
10	Flubendiamide (0.01%)	9.00	0	9.00	0	9.00	0	9.00	0	9.00	0
11	Control	9.00		9.00		9.00		9.00		9.00	

* Mean of three replications

PIOC : Per cent inhibition over control

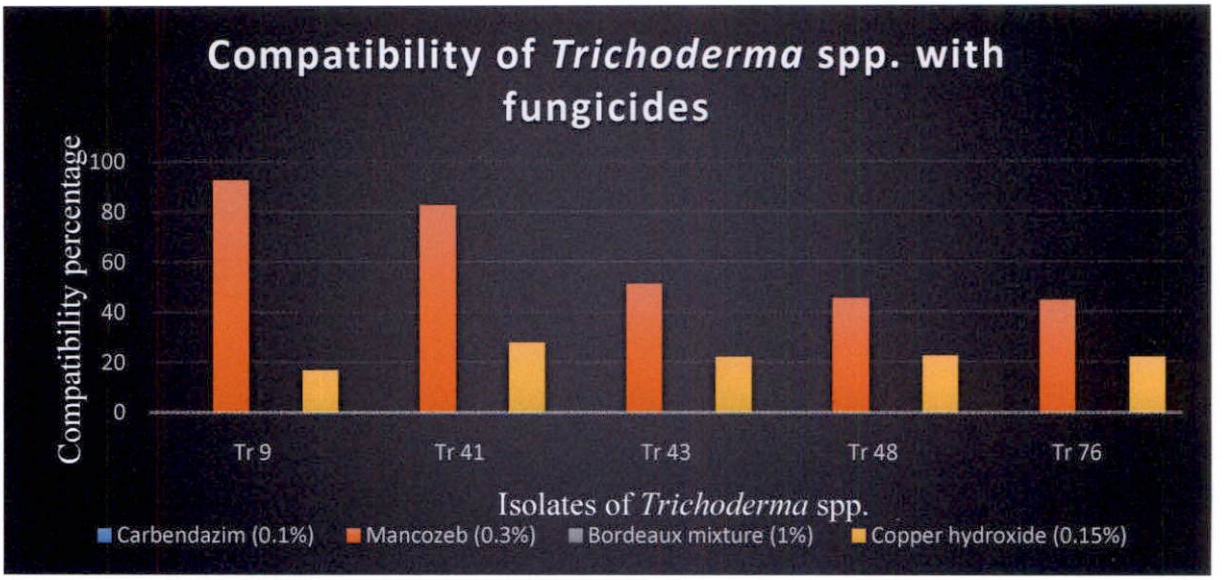


Fig 4. Evaluation on compatibility of *Trichoderma* spp. with the selected fungicides

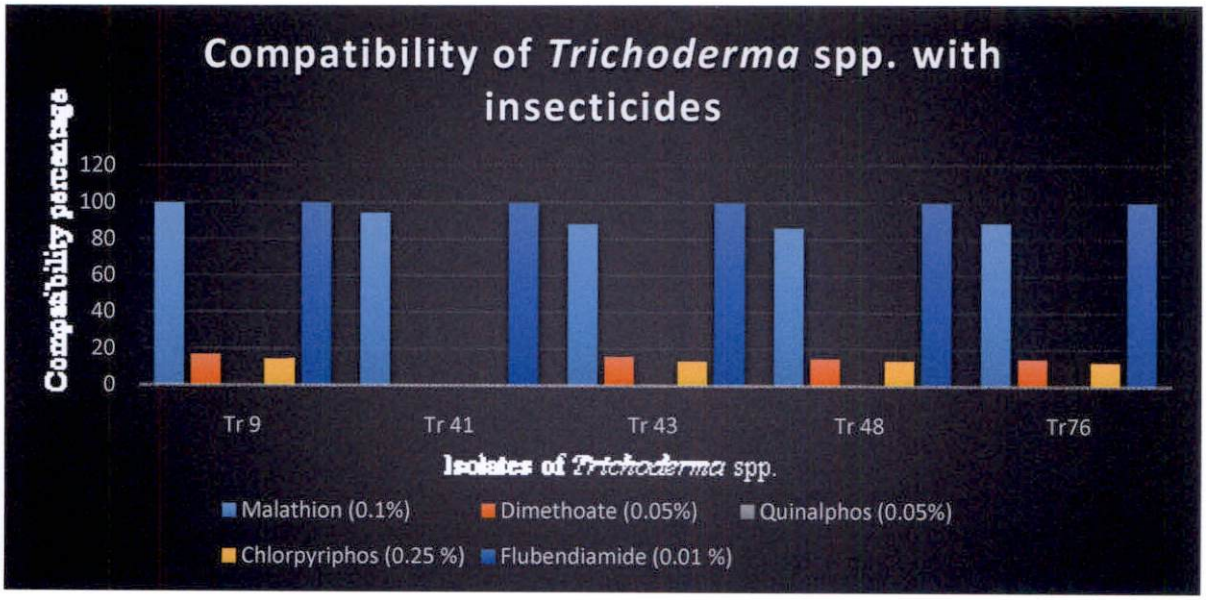
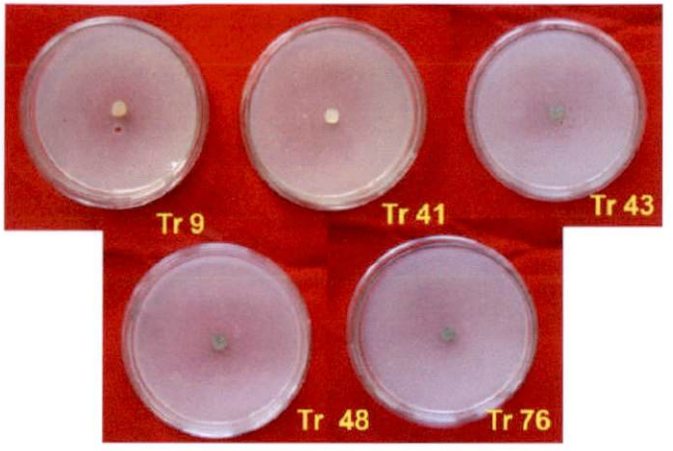
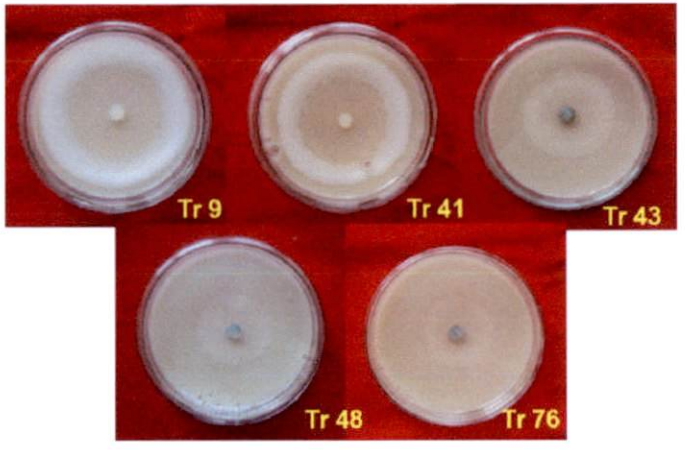


Fig 5. Evaluation on compatibility of *Trichoderma* spp. with the selected insecticides

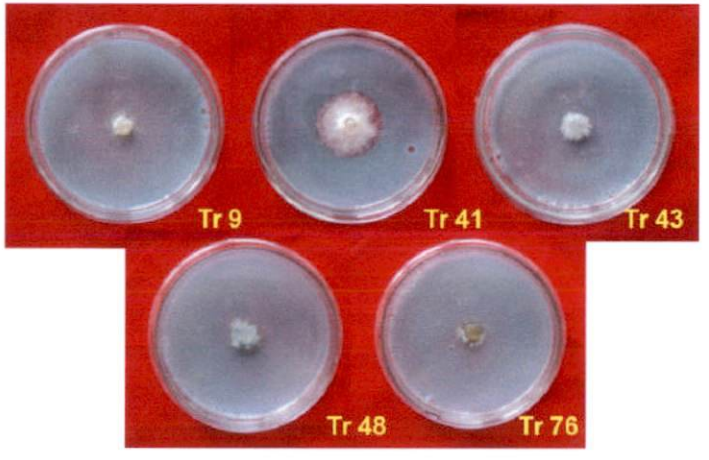
Plate 13. Evaluation on compatibility of *Trichoderma* spp. with selected fungicides



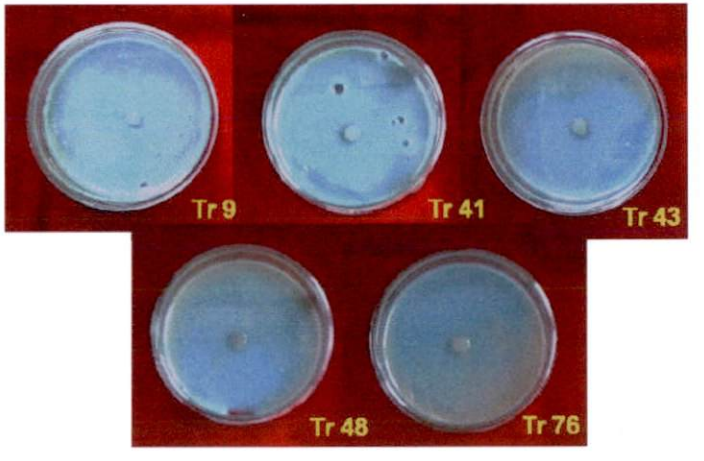
a. Carbendazim (0.1 %)



b. Mancozeb (0.3 %)

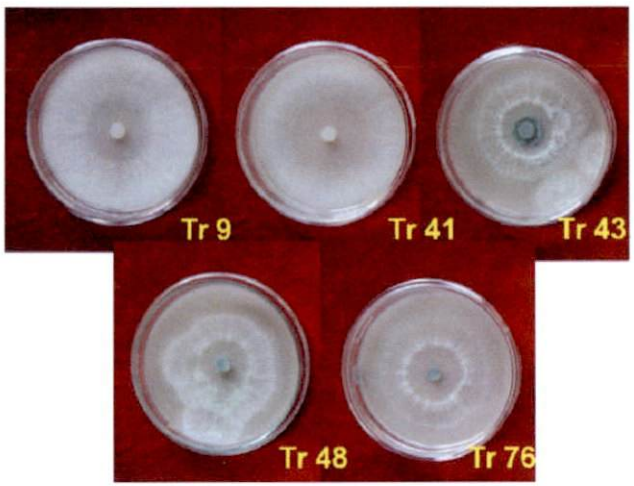


c. Copper hydroxide (0.15 %)

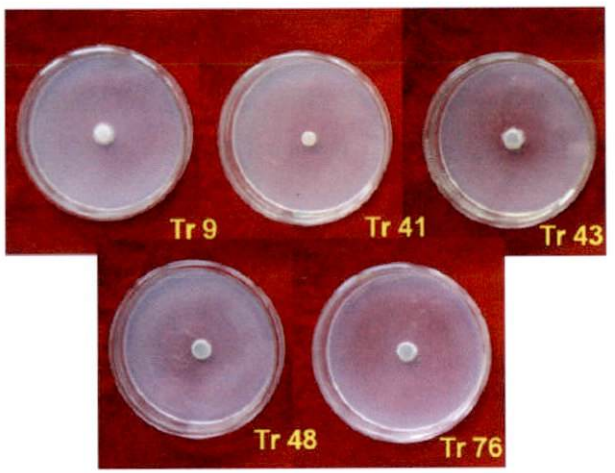


d. Bordeaux mixture (1 %)

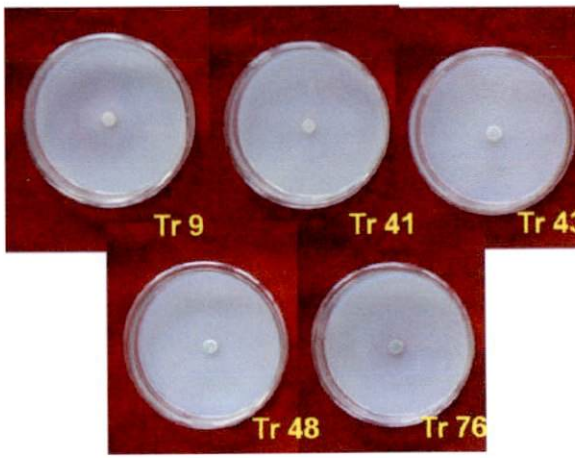
Plate 14. Evaluation on compatibility of *Trichoderma* spp. with selected insecticides



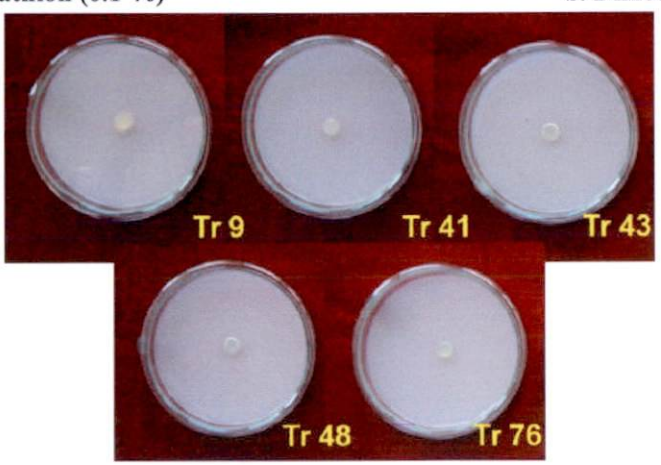
a. Malathion (0.1 %)



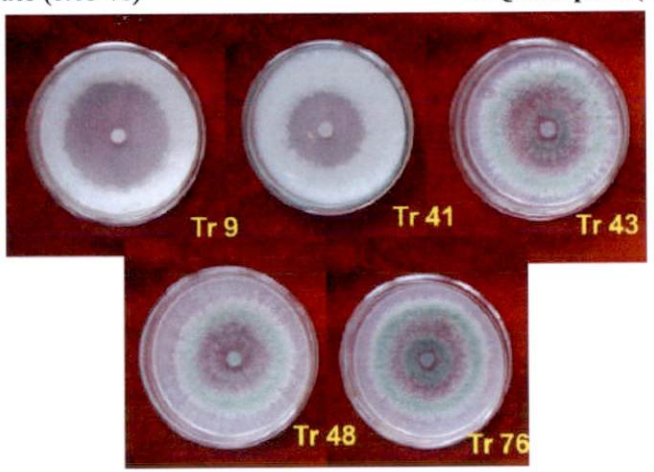
b. Dimethoate (0.05 %)



c. Quinalphos (0.05 %)



d. Chlorpyriphos (0.25 %)



e. Flubendiamide (0.01 %)

4.11. EVALUATION ON ANTAGONISTIC EFFICACY OF SELECTED ISOLATES OF *Trichodema* spp. UNDER FIELD CONDITION

The five selected isolates of *Trichoderma* spp. *viz.*, Tr 9, Tr 41, Tr 43, Tr 48 and Tr 76 from the pot culture experiment (Plate 15) were taken for the evaluation of antagonistic efficiency in plant growth promotion and management of disease in comparison with standard cultures of bio control agents along with a chemical fungicide, copper hydroxide (Plate 16). The results of the experiment are presented in the Table 23 to 27.

4.11.1. Effect of selected isolates of *Trichoderma* spp. on germination and pre emergence rot of ginger

The observation on earliness of germination was taken from 15 DAP. The earliness in germination of rhizomes was observed in the treatments T1 (Tr 48), T3 (Tr 9), T2 (Tr 76), T7 (*T. harzianum*) and T4 (Tr 43).

Germination of seedlings was completed in all the treatments within 1MAP. There was significant difference in the germination and pre emergence rot of ginger among the treatments (Table 23). All the treatments showed > 69 per cent germination at 1MAP. Out of ten, the treatments T8 (*P. fluorescens*) and T3 (Tr 9) recorded the highest per cent of germination of 82.29 and 81.25 respectively which are found statistically on par. The treatments T4 (Tr 43) and T7 (*T. harzianum*) showed a germination percentage of 80.21 when the control (T10) recorded 69.79 per cent of germination.

In case of pre emergence rot of rhizomes, all the treatments were found to be significantly different and recorded < 31 per cent of pre emergence rot. Among them, the least per cent of rot was shown by T8 (*P. fluorescens*) followed by T3 (Tr 9) recorded 17.71 per cent and 18.75 per cent of rot respectively.

Plate 15. Native isolates of *Trichoderma* spp. selected for field experiment – I

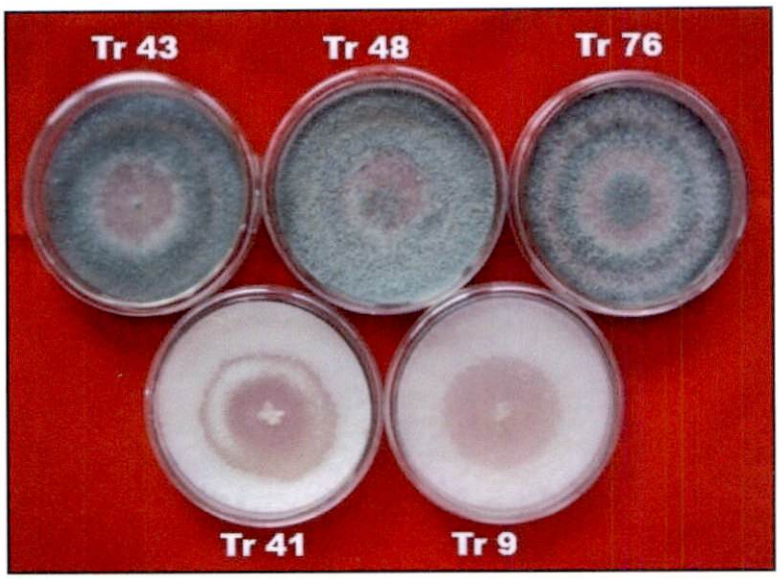


Plate 16. View of field experiment – I

Table 23. Field experiment I :- Effect of treatments on the germination and pre emergence rot of ginger

Sl. No.	Tr. No.	Treatments	Germination (%) *	Pre-emergence rot (%) *
1	T1	Tr 48	73.96^{bc}	26.04^{ab}
2	T2	Tr 76	69.79^{c}	30.21^{a}
3	T3	Tr 9	81.25^{a}	18.75^{c}
4	T4	Tr 43	80.21^{ab}	19.79^{bc}
5	T5	Tr 41	77.09^{ab}	22.92^{bc}
6	T6	*T. viride*	76.04^{abc}	23.96^{abc}
7	T7	*T. harzianum*	80.21^{ab}	19.79^{bc}
8	T8	*P. fluorescens*	82.29^{a}	17.71^{c}
9	T9	Copper hydroxide	79.17^{ab}	20.84^{bc}
10	T10	Control	69.79^{c}	30.21^{a}

* Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

4.11.2. Effect of selected isolates of *Trichoderma* spp. on plant bio metric characters

The bio metric observations on number of tillers / plant, height of the plant and the number of leaves / tiller were recorded thrice at 30 days interval from 40 DAP to 100 DAP. The results are furnished in Table 24 to 26.

4.11.2.1. Number of tillers / plant

The initial observation on 40DAP revealed that the treatments have no significant effect on the number of tillers in a plant (Table 24). Based on the second and third observations on 70 DAP and 100 DAP, it is clear that the treatment T4 (Tr 43) recorded the highest number of tillers of 7.27 and 8.71 respectively. This was followed by treatments T7 (*T. harzianum*), T3 (T 9) and T6 (*T. viride*) which were found to be statistically on par with each other.

4.11.2.2. Height of the plant

There is no significant difference in height of the plant among the treatments at 40 DAP (Table 25). The treatment T3 (Tr 9) showed maximum height of 29.72 cm and 34.02 cm at 70 DAP and 100 DAP, respectively. This was followed by treatments T4 (Tr 43) and T6 (*T. viride*) recorded 28.95 cm and 28.74 cm at 70 DAP and 33.64 cm and 32.67 cm at 100 DAP, respectively.

4.11.2.3. Number of leaves

Data in the Table 26, showed that number of leaves in a tiller is not significantly differed among the treatments at 40 DAP. Later, one month after first observation, highest number of leaves of 7.33/tiller was recorded by T3 (Tr 9) followed by T4 (Tr 43), T7 (*T. harzianum*), T2 (Tr 76) and T1 (Tr 48) with record of 7.03, 6.78, 6.51 and 6.35/tiller respectively.

Table 24. Field experiment I:- Effect of treatments on number of tillers / plant

Sl. No.	Tr. No.	Treatments	No. of tillers / plant *		
			40 DAP**	70 DAP	100 DAP (surviving sprouts)
1	T1	Tr 48	3.40	4.13^{d}	4.91^{e}
2	T2	Tr 76	4.20	5.73^{bc}	6.29^{cd}
3	T3	Tr 9	3.93	6.53^{ab}	8.13^{ab}
4	T4	Tr 43	3.00	7.267^{a}	8.71^{a}
5	T5	Tr 41	3.93	6.00^{abc}	7.29^{bc}
6	T6	*T. viride*	3.87	6.33^{ab}	7.60^{ab}
7	T7	*T. harzianum*	4.07	6.87^{ab}	8.40^{ab}
8	T8	*P. fluorescens*	3.47	5.60^{bc}	5.83^{de}
9	T9	Copper hydroxide	4.20	6.13^{ab}	7.29^{bc}
10	T10	Control	4.07	4.60^{cd}	4.78^{e}

* Mean of three replications ** Non significant

DAP : Days After Planting

In each column figures followed by same letter do not differ significantly according to DMRT

Table 25. Field experiment I:- Effect of treatments on height of the plant

Sl. No.	Tr. No.	Treatments	Height of tiller (cm) *		
			40 DAP**	70 DAP	100 DAP (surviving sprouts)
1	T1	Tr 48	18.40	25.60^{def}	28.42^{d}
2	T2	Tr 76	14.65	26.52^{cde}	32.30^{abc}
3	T3	Tr 9	17.30	29.72^{a}	34.02^{a}
4	T4	Tr 43	18.07	28.95^{ab}	33.64^{ab}
5	T5	Tr 41	17.69	23.80^{fg}	27.66^{d}
6	T6	*T. viride*	18.48	28.74^{abc}	32.67^{abc}
7	T7	*T. harzianum*	19.00	28.20^{abc}	31.56^{bc}
8	T8	*P. fluorescens*	19.11	22.78^{g}	26.80^{d}
9	T9	Copper hydroxide	18.39	26.86^{bcd}	30.62^{c}
10	T10	Control	14.59	24.32^{cfg}	28.00^{d}

* Mean of three replications ** Non significant

DAP : Days After Planting

In each column figures followed by same letter do not differ significantly according to DMRT

Table 26. Field experiment I :- Effect of treatments on number of leaves

Sl. No.	Tr. No.	Treatments	No. of leaves *		
			40 DAP**	70 DAP	100 DAP (surviving sprouts)
1	T1	Tr 48	4.707	6.35^{abcd}	7.20^{bcd}
2	T2	Tr 76	4.197	6.51^{abcd}	8.16^{abc}
3	T3	Tr 9	4.547	7.33^{a}	8.82^{a}
4	T4	Tr 43	4.993	7.03^{ab}	8.46^{ab}
5	T5	Tr 41	4.97	5.60^{d}	6.20^{d}
6	T6	*T. viride*	4.677	6.20^{bcd}	7.46^{abcd}
7	T7	*T. harzianum*	5.16	6.78^{abc}	8.12^{abc}
8	T8	*P. fluorescens*	5.203	5.58^{d}	6.98^{cd}
9	T9	Copper hydroxide	5.163	6.18^{bcd}	7.65^{abc}
10	T10	Control	4.067	5.82^{cd}	6.23^{d}

* Mean of three replications ** Non significant

DAP : Days After Planting

In each column figures followed by same letter do not differ significantly according to DMRT

As in case of second observation, the treatment T3 (Tr 9) itself recorded the highest number of leaves (8.82) at 100 DAP also and it was followed by treatments T4 (Tr 43 - 8.46), T2 (Tr 76 - 8.16), T7 (*T. harzianum* – 8.12) and T9 (copper hydroxide – 7.65).

4.11.3. Effect of selected isolates of *Trichoderma* spp. on incidence of disease

Results of evaluation on disease incidence in plants under different treatments are furnished in the Table 27. Natural infection of the pathogen, *P. aphanidermatum* was first observed on 45 DAP in the treatments, T1 (Tr 48), T2 (Tr 76), T7 (*T. harzianum*), T8 (*P. fluorescens*) and T10 (Control). At that time the treatments, T3 (Tr 9), T4 (Tr 43), T5 (Tr 41), T6 (*T. viride*) and T9 (Kocide 0.2%) did not show any symptom of yellowing of lower leaves. Per cent disease incidence and variation of disease incidence in each treatment over control were evaluated at 45 DAP, 75 DAP and 105 DAP. In all the observations least disease incidence over control was noticed in treatment, T3 (Tr 9) which recorded 0, 44.94 and 53.79 per cent followed by T4 (Tr 43) which recorded 0, 49.39 and 58.49 per cent at the initial stage of symptom appearance, 75 DAP and 105 DAP respectively.

4.12. IDENTIFICATION OF PROMISING ISOLATES OF *Trichoderma* spp.

Two promising isolates of *Trichoderma* spp. *viz.*, Tr 9 and Tr 43, selected from the field experiment based on the biometric characters and the reduction in the disease incidence, were characterized based on their cultural, morphological and molecular characters. The results are presented in the Table 28 to 30.

4.12.1. Cultural characters of *Trichoderma* spp.

The characters *viz.*, growth rate, colour, shape, texture and sporulation of colony showed by the promising isolates, Tr 9 and Tr 43 grown on three

Table 27. Field experiment I:- Effect of treatments on incidence of disease

Sl. No.	Tr. No.	Treatments	Disease Incidence					
			45 DAP		75 DAP		105 DAP	
			Per cent incidence *	PROC	Per cent incidence *	PROC	Per cent incidence *	PROC
1	T1	Tr 48	1.33	76.59	64.41	-5.56	74.36	0.28
2	T2	Tr 76	3.06	46.13	62.79	-2.90	72.75	2.44
3	T3	Tr 9	0	100	44.94	26.35	53.79	27.87
4	T4	Tr 43	0	100	49.39	19.06	58.49	21.56
5	T5	Tr 41	0	100	57.98	7.57	76.08	-2.02
6	T6	*T. viride*	0	100	55.30	9.37	65.49	12.18
7	T7	*T. harzianum*	1.23	78.35	60.01	1.66	72.36	2.96
8	T8	*P. fluorescens*	2.67	52.99	65.75	-7.75	76.84	-3.04
9	T9	Copper hydroxide	0	100	61.49	-0.77	73.90	0.90
10	T10	Control	5.68		61.02		74.57	

* Mean of three replications
DAP : Days After Planting
PROC : Per cent Reduction Over Control - No reduction

different media, Potato dextrose agar (PDA), Rose bengal agar (RBA) and *Trichoderma* selective media (TSM) were studied and the results are presented in Table 28 and 29.

In general, both the isolates showed almost similar type of growth on PDA and RBA (Table 28). The mycelial growth and sporulation were visible in both the media. The isolate, Tr 9 exhibited feeble mycelial growth in the medium TSM, which was even unable to see. Moreover, it failed to sporulate in the same medium. In contrast, though the isolate, Tr 43 did not exhibit much mycelial growth in TSM, it showed sporulation within 2 days of inoculation. The isolate, Tr 9 showed light yellow colour on the back side of the Petri plate in both PDA and RBA whereas, Tr 43 did not show any colour on the reverse side of the media.

As per the data in Table 29, the isolates Tr 9 and Tr 43 have taken 4 days to complete the growth in the Petri plate containing PDA and RBA. But in TSM, Tr 43 attained the growth of 9cm within 4 days whereas, Tr 9 took one extra day for the completion of growth.

4.12.2. Morphological characters of selected isolates of *Trichoderma* spp.

The result of the morphological identification is furnished in the Table 30 and Plate 17. The isolate Tr 43 showed a hyphal width of 4.31µm whereas Tr 9 exhibited 3.66µm. The shape of the phialide was pin shaped in Tr 9 and in Tr 43, it was flask shaped, narrower at the base, widening above the middle, attenuated into long neck. Compared to Tr 9, the size of the phialide was biggest in isolate Tr 43. The size and shape of the conidia were almost similar in both the isolates.

The pure cultures of Tr 9 and Tr 43 were sent to ITCC (Indian Type Culture Collection) IARI, New Delhi and were identified as *Trichoderma* sp. and *T. asperellum* respectively (I. D No. 9778.15 and 9782.15).

Table 28. Cultural characters of selected isolates of *Trichoderma* spp.

Sl. No.	Isolate	Characters	Media used for the study		
			PDA	RBA	TSM
1	Tr 9	1.**Mycelium**			
		Colour	White	White	Hyaline
		Texture	Medium fluffy	Highly fluffy	Very feeble
		Growth pattern	Circular	Circular	-
		2.**Spore**			
		Colour	Light green	Whitish green	-
		Sporulation	Sparse	Sparse	-
		Time of sporulation	2 weeks	2 weeks	-
		Pattern of sporulation	Irregular	Irregular	-
		3. **Colour on reverse side of plate**	+	+	-
2	Tr 43	1.**Mycelium**			
		Colour	White	White	Hyaline
		Texture	Medium fluffy	Highly fluffy	Feeble
		Growth pattern	Circular	Circular	-
		2.**Spore**			
		Colour	Dark green	Dark green	Dark green
		Sporulation	Thick	Very thick	Sparse
		Time of sporulation	48 h	48 h	48 h
		Pattern of sporulation	Plenty at margin	Uniform	In rings
		3. **Colour on reverse side of plate**	-	-	-

\+ Present
\- Absent
PDA : Potato Dextrose Agar
RBA : Rose Bengal Agar
TSM : *Trichoderma* selective medium

Table 29. Growth rate of selected isolates of *Trichoderma* spp. in different media

Sl. No.	Isolate	Radial growth (cm) DAI												
		PDA				RBA				TSM				
		1 *	2 *	3 *	4 *	1 *	2 *	3 *	4 *	1 *	2 *	3 *	4 *	5 *
Selected isolates of *Trichoderma* spp.														
1	Tr 9	2.97	5.67	7.8	9.0	2.87	5.83	8.0	9.0	1.87	3.27	5.2	7.07	9.0
2	Tr 43	3.33	6.97	9.0	9.0	2.9	4.5	7.27	9.0	2.37	4.5	7.7	9.0	-

* Mean of three replications

PDA : Potato Dextrose Agar
RBA : Rose Bengal Agar
TSM : *Trichoderma* Selective Medium

DAI : Days After Inoculation

Table 30. Morphological characters of *Trichoderma* spp.

Sl. No.	Isolate	Morphological characters *				
		Hypha	Phialide		Conidia	
		Width (µm)	Shape	Size (µm)	Shape	Size (µm)
Parental isolates of *Trichoderma* spp.						
1	Tr 9	3.66	Pin shaped	9.23 × 3.2	Oval	3.91 × 5.06
2	Tr 43	4.31	Flask shaped	10.46 × 2.4	Oval	3.69 × 5.23

*Mean of twenty replications

4.12.3. Molecular characterization of selected native isolates of *Trichoderma* spp.

The molecular characterization of isolates of *Trichoderma* spp. *viz.,* Tr 9 and Tr 43 was conducted by ITS – PCR and RAPD assay.

4.12.3.1. Isolation of genomic DNA

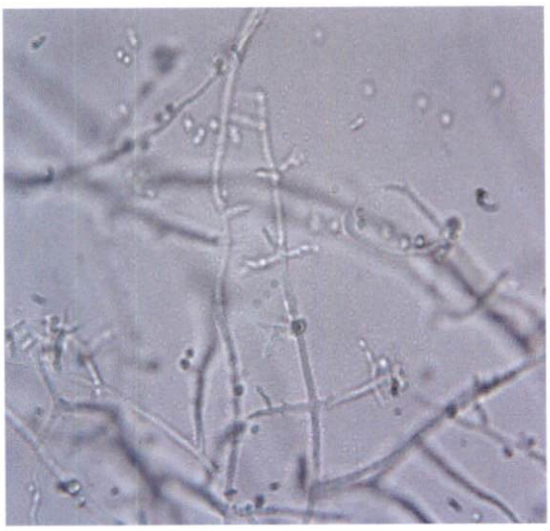
The protocol for the extraction of genomic DNA from the selected *Trichoderma* spp. *viz.,* Tr 9 and Tr 43 was standardized in the Dept. of Plant Pathology, Banana Research Station, Kannara.

The genomic DNA was isolated from three days old mycelial mat of isolates as per the procedure explained in the section 3.11.3.2. and obtained intact bands when they resolved at 0.8 per cent agarose gel. The spectrophotometric analysis of genomic DNA showed the UV absorbance ratio, A_{260}/A_{280} as 1.9 (Table 31) indicated the good quality genomic DNA.

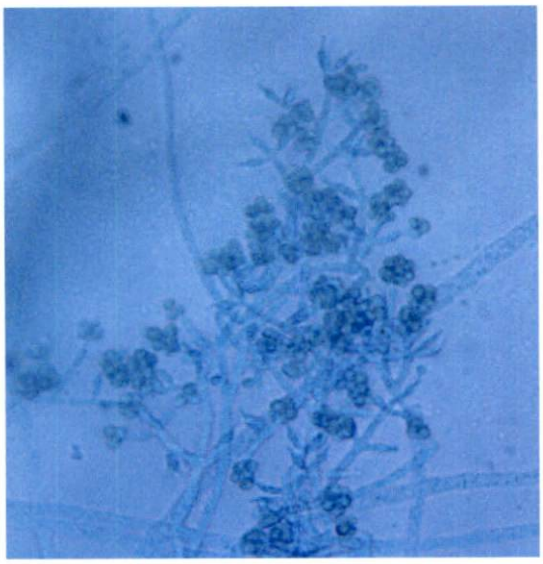
Table 31. Quality and quantity analysis of DNA by nanodrop method

Sl. No.	*Trichoderma* spp.	A_{260}/A_{280}	Quantity (ng/µl)
1	Tr 9	1.99	278.1
2	Tr 43	1.90	395.7

Plate 17 : Microphotographs of selected native isolates of *Trichoderma* spp.



a) Isolate, Tr 9 (100 X)



b) Isolate, Tr 43 (100 X)

4.12.3.2. PCR amplification of genomic DNA

4.12.3.2.1. PCR amplification by ITS primers

The amplification of ITS region of genomic DNA isolated from the isolates *viz.*, Tr 9 and Tr 43 was carried out by following the procedure described in section 3.11.3.4. The amplicons were observed as clear sharp bands of size ≈ 600 bp (Plate 18) upon resolving at 1 per cent agarose gel. Then, the PCR products were sent to SciGenom, Cochin for sequencing. The sequences generated are presented in Plate 19. The homology of sequences with other reported sequences available in NCBI (National centre for biotechnology information) data bank was analyzed. The sequences of Tr 9 and Tr 43 showed significant homology to genes of *T. erinaceum* and *T. asperellum* respectively, deposited in the domain data base. The blast results showed 100 per cent query coverage and 99 per cent identity in both the cases.

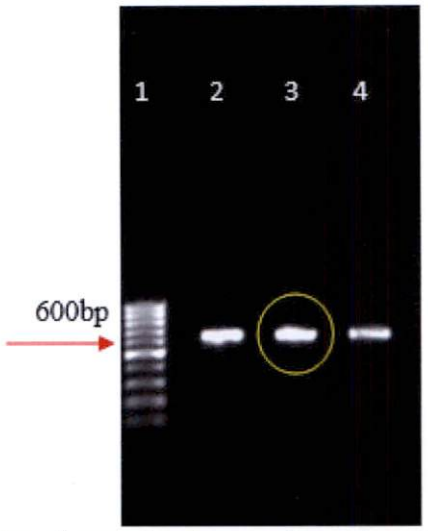
The result of molecular characterization confirms the identification based on cultural and morphological characters and hence the isolates, Tr 9 and Tr 43 were identified as *T. erinaceum* and *T. asperellum,* respectively.

4.12.3.2.2. RAPD assay

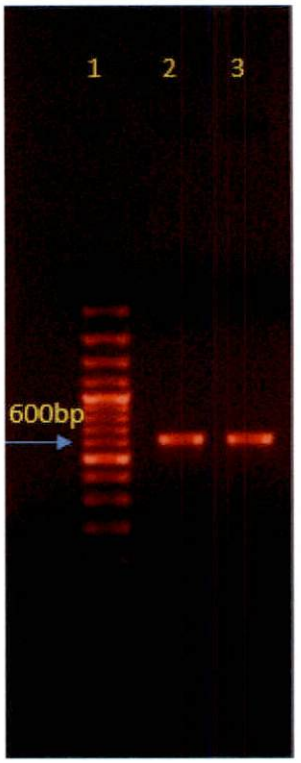
The genetic variability among the isolates, Tr 9 and Tr 43 was analyzed by RAPD as per the protocol explained in the section 3.11.3.5. Six random primers *viz.*, OPA 1, OPA 3, OPA 4, OPA 5, OPA 8 and OPA 9 were used to observe the polymorphism among the isolates under the study. The results (Plate 20) showed that all the primers tested generated reproducible polymorphism between the isolates.

PCR amplification with all the six primers was repeated before scoring for presence and absence of bands. Number of amplification products obtained was specific to each primer. The primers used in the present analysis showed 100 per cent polymorphism.

Plate 18. Amplification of genomic DNA of selected isolates of *Trichoderma* spp. by ITS-PCR



a. Standardization of annealing temperature. Lane 1 : 1 Kb ladder Lane 2 to 4 : Amplicons at annealing temperatures 52.1°C, 55.2°C and 59.4°C



b. Amplicons of ITS region of DNA of isolates of *Trichoderma* spp. Lane 1: 1 Kb ladder, Lane 2: Amplicon of Tr 9, Lane 3: Amplicon of Tr 43

Plate 19. Sequences generated from the amplicons of ITS – PCR

>Tr 43_ITS.1_20618-3_7367, Trimmed Sequence (529 bp)

GTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGCAGCCCCGGAAC
CAGGCGCCCGCCGGAGGAACCAACCAAACCCTTTCTGTAGTCCCCTCGCGGACGTAT
TTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCTCT
TGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGC
ATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGGG
ATCGGGACCCCTCACACGGGTGCCGGCCCCGAAATACAGTGGCGGTCTCGCCGCAGC
CTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCGTCCACGTCCGT
AAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAAC
TTAAGCATATCAA

>Tr 43_ITS.4_20618-4_7367, Trimmed Sequence (539 bp)

GAGGTCACATTTCAGAAAGTTGGGTGTTTTACGGACGTGGACGCGCCGCGCTCCCGG
TGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCACTGTATTT
CGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCGATCCCCCGGAGGGGT
TCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATACTGGCGGGCGCAA
TGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCAT
TTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATT
CATTTTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGGGACTACAGAAA
GGGTTTGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCACCCGGGG
CGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAGTTGTAA
ACTCGTAATGATCCCTCCGCAGG

>Tr 9_ITS.1_20618-1_7367, Trimmed Sequence (533 bp)

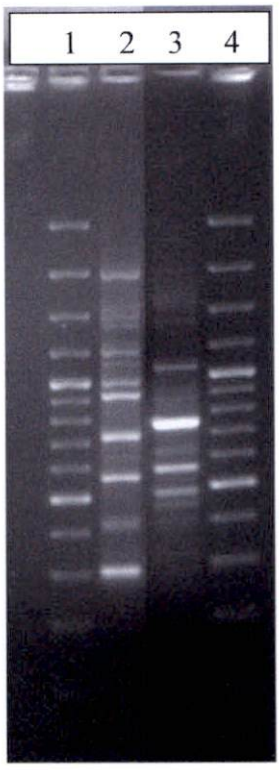
ATACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGCAGCCCCGGAACC
AGGCGCCCGCCGGAGGGACCAACCAAACTCTTTACTGTAGTCCCCTCGCGGACGTTA
TTTCTTACAGCTCTGAGCAAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGG
CATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGTCCGGCGTTGGGG
ATCGGGAACCCCTCAGACGGGATCCCGGCCCCGAAATACAGTGGCGGTCTCGCCGCA
GCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCGTCCACGTC
CGTAAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTG
AACTTAAGCATATCAA

>Tr 9 _ITS.4_20618-2_7367, Trimmed Sequence (556 bp)

GGTCACATTTCAGAAAGTTGGGTGTTTTACGGACGTGGACGCGCCGCGCTCCCGGTGCG
AGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCACTGTATTTCGGGG
CCGGGATCCCGTCTGAGGGGTTCCCGATCCCCAACGCCGGACCCCCGGAGGGGTTCGA
GGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGC
GTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTG
CGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTGAA
TTTTTTGCTCAGAGCTGTAAGAAATAACGTCCGCGAGGGGACTACAGTAAAGAGTTTGG
TTGGTCCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCACCCGGGGCGTGACCCCG
CCGAGGCAACAGTTTGGTATGGTTCACATTGGGTTTGGGAGTTGTAAACTCGGTAATGA
TCCCTCCGCAGGTCACCCCTACGGAA

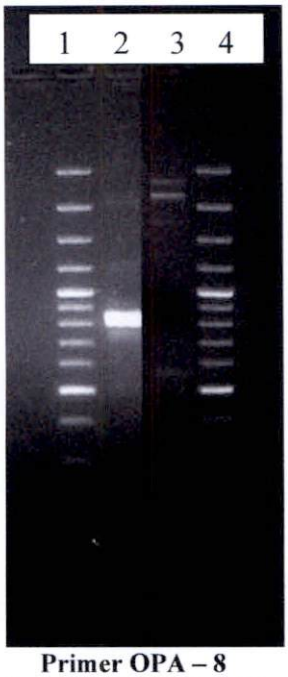
Plate 20. RAPD profiles of selected isolates of *Trichoderma* spp.

Primer OPA – 3

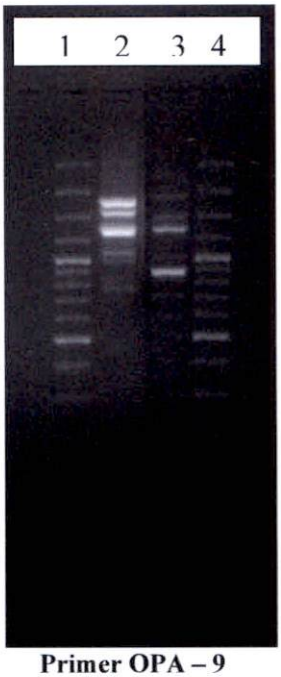


Primer OPA – 4

Primer OPA - 5



Primer OPA – 8



Primer OPA – 9

Primer OPA – 1

Lane 1 and 4 : 1 Kb ladder, Lane 2: banding pattern of Tr 9, Lane 3: banding pattern of Tr 43

4.13. STUDY ON THE STRAIN VARIATION OF *Trichoderma* spp.

Two promising native isolates of *Trichoderma* spp. *viz.,* Tr 9 (*T. erinaceum)* and Tr 43 (*T. asperellum)* selected from the field experiment (Plate 21) were taken for further enhancing their bioefficacy by mutation and protoplast fusion techniques and here after these are called as parental isolates.

4.13.1. Induction of strain variation by mutation

It was observed that in case of both the isolates (Tr 9 and Tr 43), the number of colonies developed on the media after the UV treatment was found to be inversely proportional to the exposure time (Table 32 and Plate 22). The result revealed that the isolate, Tr 9 yielded 20, 3, 0 and 0 and the isolate Tr 43 yielded 23, 20, 18 and 13 number of colonies on the media after exposure to UV- light for a period of 20, 40, 60 and 80 minutes respectively. The isolate, Tr 9 did not yield any colonies under UV light with exposure time period of 60 and 80 minutes.

Table 32. Effect of exposure time of UV light on number of colonies of *Trichoderma* spp.

Sl. No.	Period of UV exposure	Mutant isolates of Tr 9		Mutant isolates of Tr 43	
		No. of colonies	Name	No. of colonies	Name
1	20 min.	20	M20M1 to M20M20	23	K20M1 to K20M23
2	40 min.	3	M40M1 to M40M3	20	K40M1 to K40M20
3	60 min.	0	-	18	K60M1 to K60M18
4	80 min.	0	-	13	K80M1 to K80M13
	Total	23		74	

4.13.1.1. Preliminary screening of UV exposed isolates

Preliminary screening of all the 97 isolates obtained after UV exposure of native parental isolates, Tr 9 and Tr 43 against the pathogen, *Sclerotium rolfsii* resulted in the selection of three out of 23 mutant isolates of the parent, Tr 9 and six out of 74 mutant isolates of the parent, Tr 43 which showed better antagonism than their parents and reference cultures.

Plate 21: Parental cultures selected for genetic improvement after field experiment

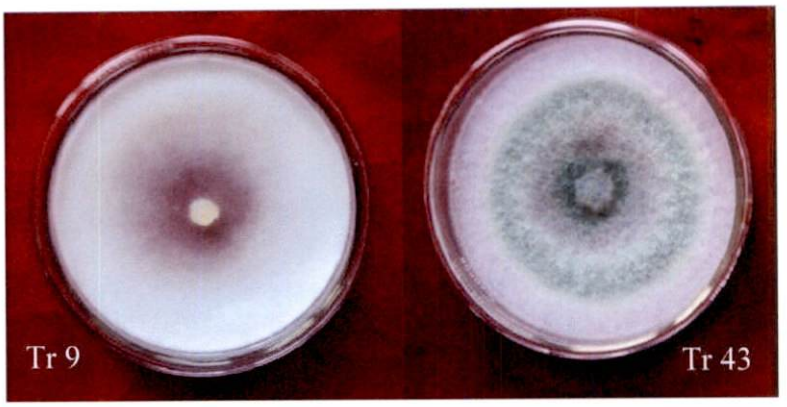
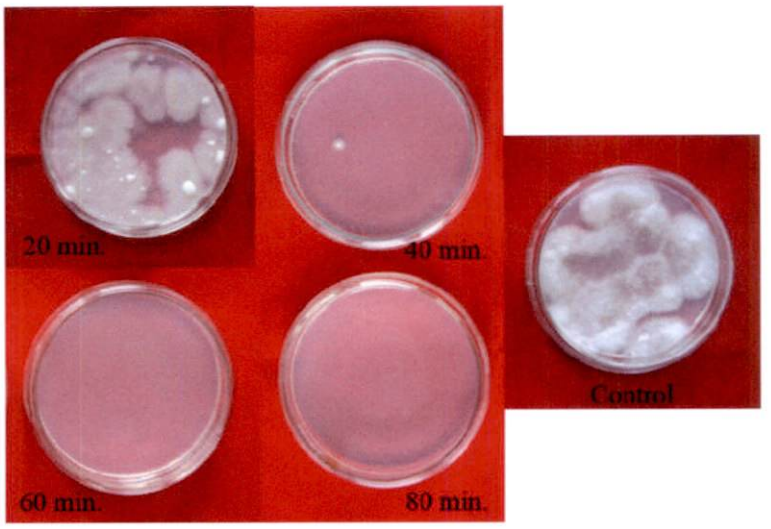
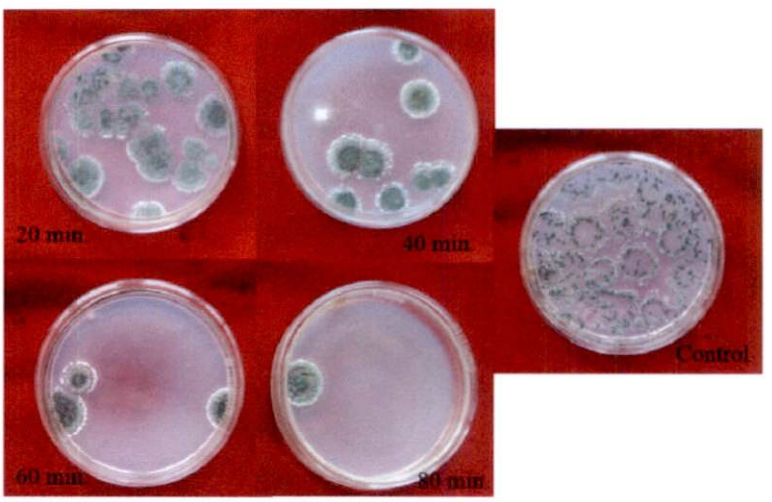


Plate 22: UV irradiated plates of parental isolates a) Tr 9 and b) Tr 43 for different time periods

a)



b)



4.13.1.2. *In vitro* evaluation on antagonistic efficiency of selected mutants of Tr 9 and Tr 43

The data of the *in vitro* evaluation on antagonistic efficiency of selected nine mutants along with their parents and reference cultures are furnished in the Table 33 and 34.

The results in the Table 33 and Plate 23a revealed that the mutant isolate, M40M3 recorded 92.55 per cent of inhibition on the growth of pathogen, *P. aphanidermatum* which was greater than its parent isolate, Tr 9. All the mutants except K60M3 recorded > 85.22 per cent of inhibition, recorded by its parent isolate, Tr 43. The mutants of Tr 9 and the mutants *viz.*, K60M1 and K80M13 of Tr 43 showed better antagonism than the reference cultures (> 90.33 %). The parental isolates, all the mutants and reference cultures showed over growth as mechanism of antagonism against *P. aphanidermatum.*

The antagonism against *Phytophthora capsici* (Table 33 and Plate 23b) revealed that only M40M3 and K80M13 and their parents recorded cent per cent inhibition on the pathogen. The antagonistic property of reference cultures *T. viride* and *T. harzianum* was observed to be 87.44 and 83.33 per cent respectively. All the isolates exhibited over growth as mechanism of antagonism.

The mutated isolate, M40M3 recorded a percentage of inhibition of 52.56 against the pathogen, *Rhizoctonia solani* which was greater than that of its parental isolate and reference cultures. Except K40M6 and K60M1, all the mutants of Tr 43 showed better antagonism (≥ 43.00) than the parent isolate and the reference cultures. All exhibited homogenous type of antagonism (Table 33 and Plate 23c).

The antagonistic efficiency of all the mutated isolates was ranged from 38.89 to 63.88 per cent (Table 34 and Plate 24a). Among them M40M3,

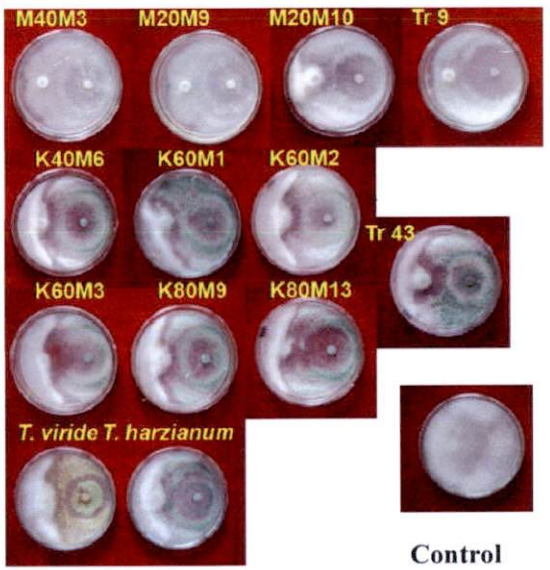
Table 33. ***In vitro*** **evaluation on antagonistic efficiency of selected mutants of** ***Trichoderma*** **spp. against** ***Pythium aphanidermatum, Phytophthora capsici*** **and** ***Rhizoctonia solani***

Sl. No.	Isolates of *Trichoderma* spp.	*P. aphanidermatum*				*P. capsici*				*R. solani*			
		Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action
		P	T			P	T			P	T		
1	Tr 9	0.73	8.27	91.89	O	0	9.00	100	O	4.47	5.53	50.33	H
2	M 40 M 3	0.67	8.33	92.55	O	0	9.00	100	O	4.27	6.00	52.56	H
3	M 40 M 9	0.80	8.20	91.11	O	1.80	7.20	80.00	O	5.00	5.00	44.44	H
4	M 40 M10	0.83	8.17	90.78	O	1.40	7.60	84.44	O	4.73	5.27	47.44	H
5	Tr 43	1.33	7.67	85.22	O	0	9.00	100	O	5.13	5.43	43.00	H
6	K 40 M 6	0.97	8.03	89.22	O	1.40	7.60	84.44	O	5.23	5.33	41.89	H
7	K 60 M 1	0.70	8.30	92.22	O	0.17	8.83	98.11	O	5.20	5.50	42.22	H
8	K 60 M 2	1.07	7.93	88.11	O	1.43	7.57	84.11	O	5.13	5.27	43.00	H
9	K 60 M 3	1.53	7.47	83.00	O	0.90	8.10	90.00	O	5.13	5.33	43.00	H
10	K 80 M 9	1.00	8.00	88.89	O	1.50	7.50	83.33	O	5.10	5.53	43.33	H
11	K 80 M 13	0.63	8.37	93.00	O	0	9.00	100	O	5.00	5.50	44.44	H
12	*T. viride*	0.87	8.13	90.33	O	1.13	7.87	87.44	O	5.50	5.30	38.89	H
13	*T. harzianum*	0.77	8.23	91.44	O	1.50	7.50	83.33	O	5.50	6.10	38.89	H
14	CONTROL	9.00				9.00				9.00			

* Mean of three replications P : Pathogen T : Antagonist

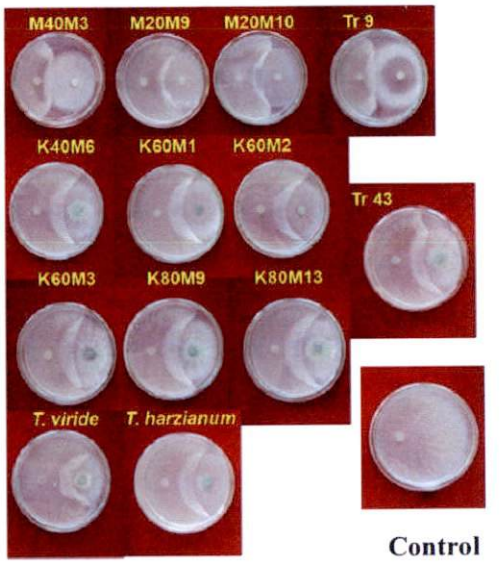
PIOC : Per cent inhibition over control O : Overgrowth of antagonist over pathogen H : Homogenous growth

Plate 23. ***In vitro*** **evaluation on antagonism of selected mutants of** ***Trichoderma*** **spp. against** ***P. aphanidermatum, P. capsici*** **and** ***R. solani***



a. Antagonism against *P. aphanidermatum*

b. Antagonism against *P. capsici*



c. Antagonism against *R. solani*

K40M6, K60M1, K80M9 and K80M13 recorded 61.11, 59.44, 56.67, 61.44 and 63.88 per cent of inhibition respectively and showed high per cent of inhibition on the growth of *Sclerotium rolfsii* than their parents and reference cultures. There was variation in the mode of antagonism among the isolates.

Data in Table 34 and Plate 24b proved that all the isolates tested *viz.*, the parents, mutants and the reference cultures recorded cent per cent inhibition by showing over growth as mechanism of antagonism against *Fusarium oxysporum* f. sp. *cubense.*

The mutant, M40M3 of Tr 9 and the mutant, K80M13 of Tr 43 showed better antagonistic property than their parents as well as the reference cultures of *Trichoderma* spp. against the growth of fungal pathogens *viz., P. aphanidermatum, P. capsici, R. solani, S. rolfsii* and *F. oxysporum* f. sp. *cubense* were selected for further research (Plate 28a).

4.13.2. Induction of strain variation by protoplasmic fusion

4.13.2.1. Formation and release of protoplast

For the strain improvement by protoplasmic fusion, the protoplasts of the parental isolates (Tr 9 and Tr 43) were isolated upon lytic digestion of mycelia using a lytic enzyme, Glucanex. It was observed that the release of protoplast was very much depended upon the age of the fungal mycelia used. In this study, the highest number of protoplast was derived from 24 hour old culture of *Trichoderma* spp. The mycelial pellet of each parental isolates suspended separately in the protoplasting buffer containing the enzyme glucanex was observed under phase contrast microscope. The observations on protoplast formation and protoplast release were taken under microscope continuously after the addition of the enzyme (Plate 25 I – a and b).

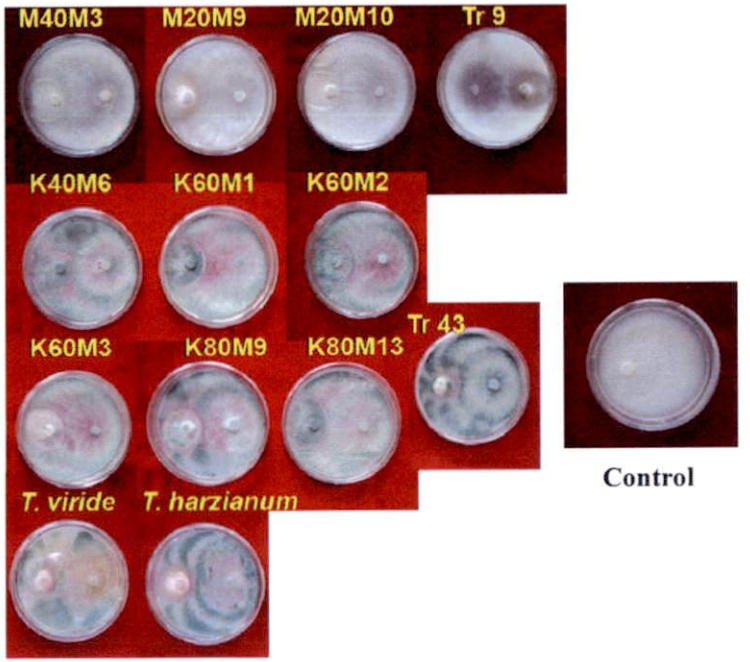
Table 34. ***In vitro*** **evaluation on antagonistic efficiency of selected mutants of** ***Trichoderma*** **spp. against** ***Sclerotium rolfsii*** **and** ***Fusarium oxysporum*** **f. sp.** ***cubense***

Sl. No.	Isolate of *Trichoderma* spp.	*S. rolfsii*				*F. oxysporum* f. sp. *cubense*			
		Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action
		P	T			P	T		
1	Tr 9	3.67	4.83	59.22	A	0	9.00	100	O
2	M 40 M 3	3.50	5.70	61.11	O	0	9.00	100	O
3	M 40 M 9	5.50	4.25	38.89	P on A	0	9.00	100	O
4	M 40 M10	3.80	5.20	57.78	O	0	9.00	100	O
5	Tr 43	4.20	4.60	53.33	A	0	9.00	100	O
6	K 40 M 6	3.65	5.35	59.44	O	0	9.00	100	O
7	K 60 M 1	3.90	4.90	56.67	A	0	9.00	100	O
8	K 60 M 2	4.50	4.30	50.00	A	0	9.00	100	O
9	K 60 M 3	4.20	5.00	53.33	O	0	9.00	100	O
10	K 80 M 9	3.47	5.53	61.44	C	0	9.00	100	O
11	K 80 M 13	3.25	5.87	63.88	O	0	9.00	100	O
12	*T. viride*	6.20	2.80	31.11	C	0	9.00	100	O
13	*T. harzianum*	4.15	4.65	53.89	A	0	9.00	100	O
14	CONTROL	9.00				9.00			

* Mean of three replications PIOC : Per cent inhibition over control P on A : Pathogen over grew on antagonist
O : Overgrowth of antagonist over pathogen C : Cessassion of growth A : Aversion P : Pathogen T : Antagonist

Plate 24. *In vitro* evaluation on antagonism of selected mutants of *Trichoderma* spp. against *S. rolfsii* and *F. oxysporum* f. sp. *cubense*

a. Antagonism against *S. rolfsii*



b. Antagonism against *F. oxysporum* f. sp. *cubense*

The quantity of the enzyme to be used for the release of maximum number of protoplast was standardized as 20 mg/ml. The degradation of fungal cell wall was observed in the first 30 min in case of both parents. The bulging of cell membrane was seen prior to protoplast release. The protoplasts were found to be released from Tr 43 after two hours of incubation. At the same time Tr 9 took 30 min. more for the release of protoplasts than Tr 43. The released protoplasts were seen as small sized for the first half an hour (Table 35) and later the size of protoplast was enlarged. The maximum number of protoplasts in the optimum size was obtained in the fourth hour of incubation in protoplasting buffer. The prolonged incubation resulted in the bursting of protoplasts (Plate 25 III).

Table 35. Protoplast formation and its release according to time of incubation

Time after incubation	Parental isolates of *Trichoderma* spp.			
	Tr 9		Tr 43	
	Enzyme added	Control	Enzyme added	Control
0 min	Mycelial mat	Mycelial mat	Mycelial mat	Mycelial mat
30 min	Mycelial mat + debris	Mycelial mat	Mycelial mat + debris	Mycelial mat
1 h	Mycelial mat + debris	Mycelial mat	Mycelial mat + debris	Mycelial mat
1.30 h	Mycelial mat + debris	Mycelial mat	Mycelial mat + debris	Mycelial mat
2 h	Mycelial mat + debris	Mycelial mat	Very few	Mycelial mat
2.30 h	Very few	Mycelial mat	few	Mycelial mat
3 h	Plenty	Mycelial mat	Plenty	Mycelial mat

4.13.2.2. Viability check of protoplast

The viability of released protoplasts was checked using one per cent Evan's blue dye. The viable protoplasts of both the isolates were seen colourless under micro scope. The dead cell wall debris was observed in blue colour (Plate 25 I - c).

4.13.2.3. Regeneration of protoplast

The hyphal growth from the protoplast in the regeneration medium indicated their regeneration capacity and confirmed their viability (Plate 25 I - d).

4.13.2.4. Isolation of protoplast

The released protoplasts were isolated after four hours of incubation in the pure form by straining through syringe filter with a pore size of five micro metre.

4.13.2.5. Protoplast fusion

In the present study, poly ethylene glycol (PEG) 6000 was used for fusing the viable protoplasts from the parent, Tr 9 and Tr 43. The fusion of protoplasts was observed within 20 minutes of incubation in fusion buffer. At the beginning, the protoplasts were observed to be come in contact at a point and later resulted in the degradation of cell membrane at the contact point. This lead to the mixing up of protoplasm of each parent with a common outer wall and resulted in the formation of one fused protoplast (Plate 25 II). The size of this fused protoplast was found to be large compared to the parental protoplast.

4.13.2.6. Regeneration of fusant

The fused protoplasts were spread plated on malt extract agar medium for the regeneration (Plate 25 IV). The colonies regenerated on the medium were sub cultured on PDA slants for further studies. A total of 15 colonies were regenerated on the medium and were taken for further research.

4.13.2.7. Preliminary screening of fusants

A preliminary screening of 15 regenerated fusants for the evaluation of antagonistic efficiency against *S. rolfsii*, was carried out and five promising fusants of *Trichoderma* spp. *viz.*, F1, F2, F3, F4 and F6 were selected for further evaluation.

4.13.2.8. *In vitro* evaluation on antagonistic efficiency of fusants

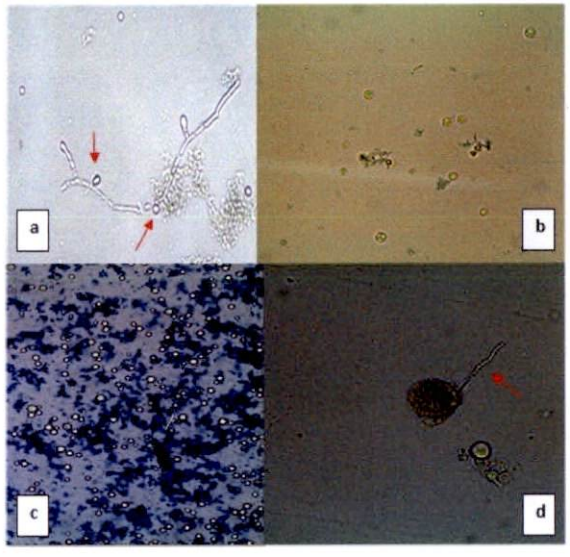
The results of the evaluation on antagonistic efficiency of fusants against soil borne fungal pathogens are furnished in Table 36 and 37.

All the fusants except F3, recorded high per cent of inhibition (> 92%) against *P. aphanidermatum* than the parents and reference cultures of *Trichoderma* spp. Among them, F2 showed cent per cent inhibition on the growth of pathogen. All isolates exhibited over growth as mechanism of antagonism (Table 36 and Plate 26a).

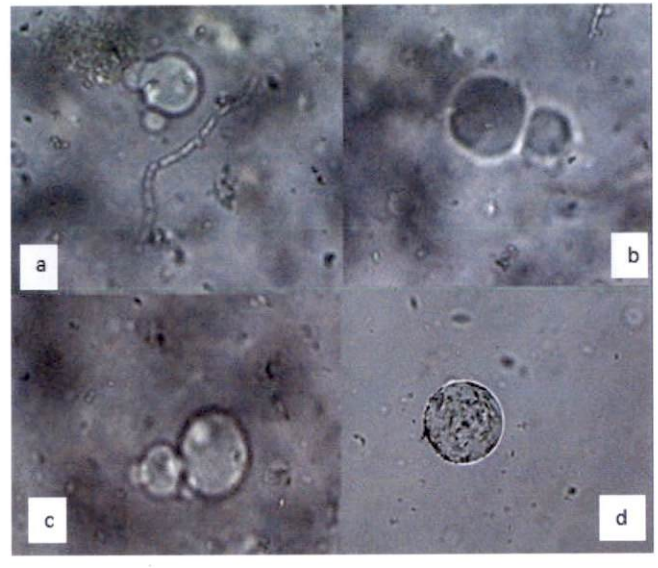
As if their parents, the fusants, F2 and F4 recorded cent per cent inhibition over the pathogen, *Phytophthora capsici* (Table 36 and Plate 26b). All the fusants were found to be antagonistically efficient than the reference cultures (> 87.44%). The parents, reference cultures and the fusants showed over growth as mode of antagonism.

In case of antagonism against *Rhizoctonia solani*, the fusants, F2 and F6 showed better antagonistic property than the parent Tr 43 (43 %) and only F2

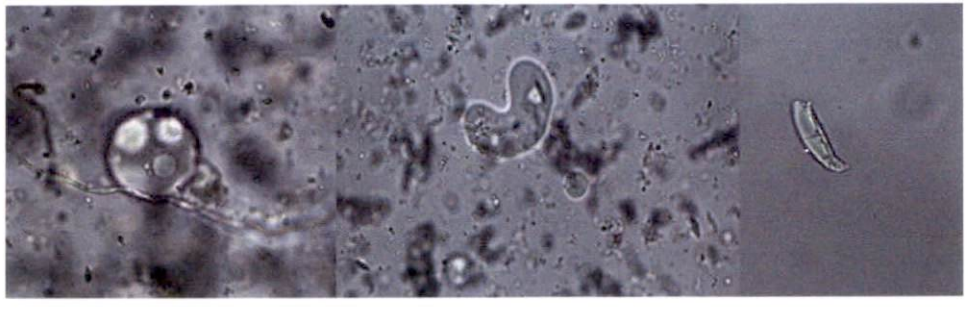
Plate 25. Strain improvement of selected isolates of *Trichoderma* spp. by protoplast fusion



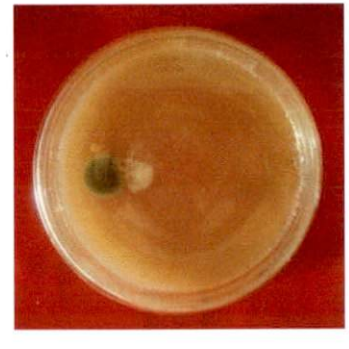
I. (a) Release of protoplast (b) Released protoplasts after 3 hours
(c) Viability check of protoplast in Evans blue dye (d) Regeneration of protoplast



II. (a,b,c) Stages of protoplast fusion (d) Fused protoplast



III. Bursting of protoplast



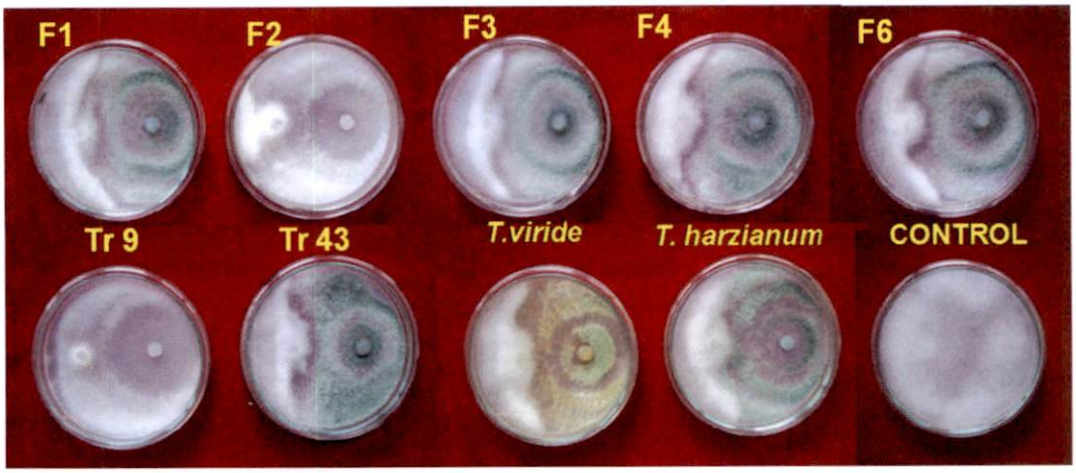
IV. Regeneration of fusant

Table 36. ***In vitro*** **evaluation on antagonistic efficiency of selected fusants against** ***Pythium aphanidermatum, Phytophthora capsici*** **and** ***Rhizoctonia solani***

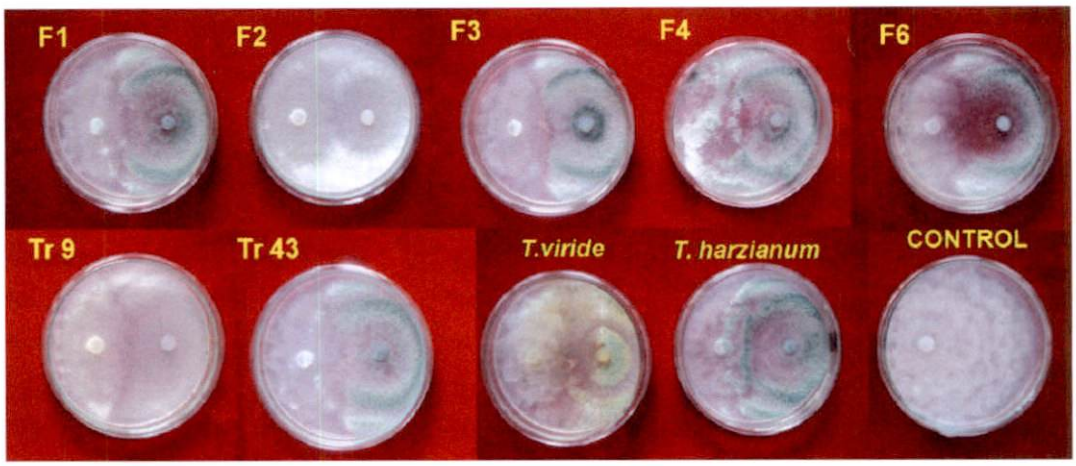
Sl. No.	Isolates of *Trichoderma* spp.	*P. aphanidermatum*				*P. capsici*				*R. solani*			
		Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action
		P	T			P	T			P	T		
1	Tr 9	0.73	8.27	91.89	O	0	9.00	100	O	4.47	5.53	50.33	H
2	Tr 43	1.33	7.67	85.22	O	0	9.00	100	O	5.13	5.43	43.00	H
3	F1	0.53	8.47	94.11	O	0.50	8.50	94.44	O	5.23	6.50	41.89	H
4	F2	0	9.00	100	O	0	9.00	100	O	4.40	7.00	51.11	H
5	F3	1.20	7.80	86.67	O	1.10	7.90	87.78	O	5.33	6.37	40.78	H
6	F4	0.60	8.40	93.33	O	0	9.00	100	O	5.20	6.33	42.22	H
7	F6	0.67	8.33	92.56	O	0.30	8.70	96.67	O	5.07	6.50	43.67	H
8	*T. viride*	0.87	8.13	90.33	O	1.13	7.87	87.44	O	5.50	5.30	38.89	H
9	*T. harzianum*	0.77	8.23	91.44	O	1.50	7.50	83.33	O	5.50	6.10	38.89	H
10	CONTROL	9.00				9.00				9.00			

* Mean of three replications
P : Pathogen
T : Antagonist
PIOC : Per cent inhibition over control
O : Overgrowth of antagonist over pathogen
H : Homogenous growth

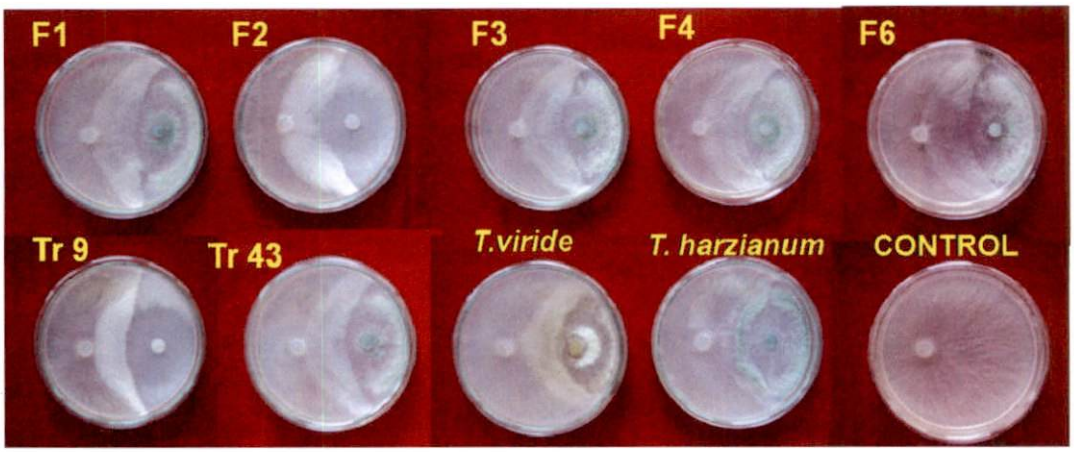
Plate 26. ***In vitro*** **evaluation on antagonism of selected fusants of** ***Trichoderma*** **spp. against** ***P. aphanidermatum, P. capsici*** **and** ***R. solani***



a. Antagonism against *P. aphanidermatum*



b. Antagonism against *P. capsici*



c. Antagonism against *R. solani*

(51.11%) recorded high inhibition percentage than the parent, Tr 9 (Table 36 and Plate 26c). All the fusants showed high inhibition percentage (> 39%) than the reference cultures. Homogenous type of antagonism was observed in all cases.

From the Table 37, it is revealed that out of five, 2 fusants *viz.*, F2 and F4 recorded higher antagonism (> 60%) than the parent, Tr 9 against *Sclerotium rolfsii* whereas all the six fusants showed better antagonism (> 54%) than the parent, Tr 43 as well as reference cultures of *Trichoderma* spp. by showing aversion type of antagonism (Plate 27a).

All the fusants, their parents and the reference cultures recorded cent per cent inhibition over the pathogen, *Fusarium oxysporum* f. sp. *cubense* by showing over growth as mechanism of antagonism (Table 37 and Plate 27b).

The results of the evaluation on antagonistic efficiency of the selected fusants against the pathogens showed that the fusant F2 recorded cent per cent inhibition on the growth of *P. aphanidermatum, P. capsici* and *F. oxysporum* f. sp. *cubense* and 66.67 per cent and 51.11 per cent against *S. rolfsii* and *R. solani* respectively. On comparing the antagonistic efficiency of five fusants, their parents and reference cultures, it was found that the fusant F2 showed > 50 per cent inhibition on the growth of all five soil borne fungal pathogens. It was followed by the fusant F4 which recorded > 50 per cent inhibition against all the pathogens except *R. solani* which recorded only 42.22 per cent but it was greater than that of the reference cultures (38.89 %). Based on these, two fusants *viz.,* F2 and F4 were selected for further work (Plate 28b).

4.14. CHARACTERIZATION OF PROMISING MUTANTS AND FUSANTS

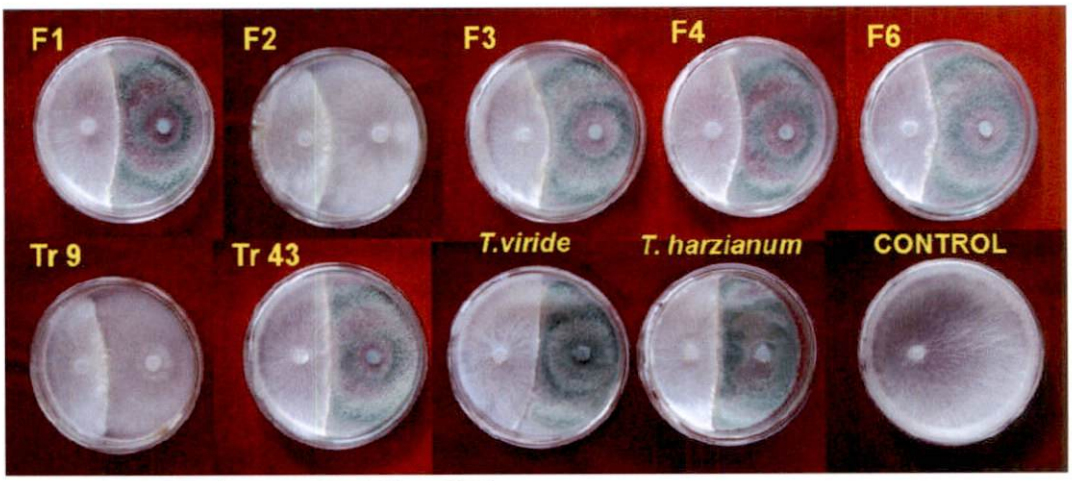
Identification of promising mutants *viz.,* M40M3 and K80M13 and fusants *viz.*, F2 and F4 selected after the dual culture experiment, was carried out based on their cultural, morphological and molecular characters.

Table 37. ***In vitro*** **evaluation on antagonistic efficiency of fusants against** ***Sclerotium rolfsii*** **and** ***Fusarium oxysporum*** **f. sp.** ***cubense***

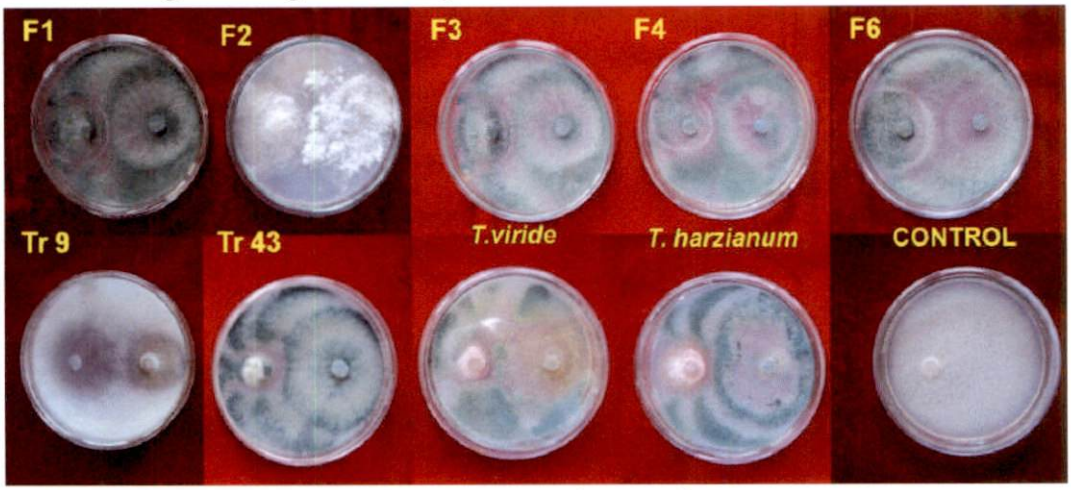
Sl. No.	Isolate of *Trichoderma* spp.	*S. rolfsii*				*F. oxysporum* f. sp. *cubense*			
		Radial growth (cm) *		PIOC	Mode of action	Radial growth (cm) *		PIOC	Mode of action
		P	T			P	T		
1	Tr 9	3.67	4.83	59.22	A	0	9.00	100	O
2	Tr 43	4.20	4.60	53.33	A	0	9.00	100	O
3	F1	3.73	5.07	58.56	A	0	9.00	100	O
4	F2	3.00	5.80	66.67	A	0	9.00	100	O
5	F3	4.00	4.80	55.56	A	0	9.00	100	O
6	F4	3.40	5.10	62.22	A	0	9.00	100	O
7	F6	3.90	4.80	56.67	A	0	9.00	100	O
8	*T. viride*	6.20	2.80	31.11	C	0	9.00	100	O
9	*T. harzianum*	4.15	4.65	53.89	A	0	9.00	100	O
10	CONTROL	9.00				9			

* Mean of three replications
P : Pathogen
T : Antagonist
PIOC : Per cent inhibition over control
A : Aversion
C : Cessation of growth
O : Overgrowth of antagonist over pathogen

Plate 27. ***In vitro*** **evaluation on antagonism of selected fusants of** ***Trichoderma*** **spp. against** ***S. rolfsii*** **and** ***F. oxysporum*** **f. sp.** ***cubense***

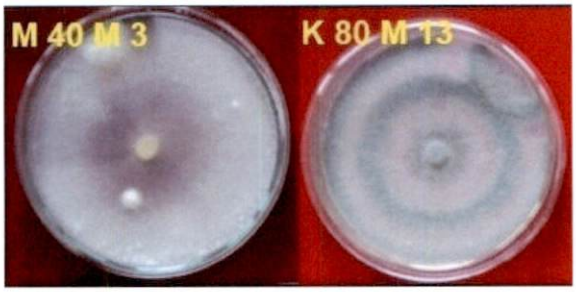


a. Antagonism against *S. rolfsii*

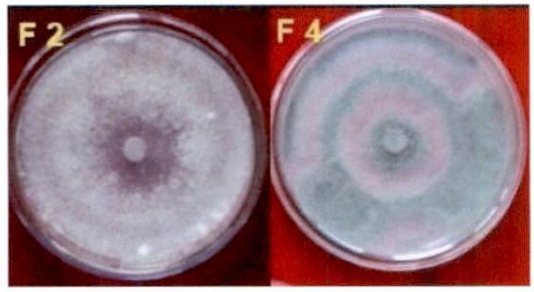


b. Antagonism against *F. oxysporum* f. sp. *cubense*

Plate 28. Selected mutants and fusants of ***Trichoderma*** **spp. for the pot culture experiment**



a. Mutants



b. Fusants

4.14.1. Cultural characterization of promising mutants and fusants

The study on cultural characters of mutants and fusants of *Trichoderma* spp. in three different media Potato Dextrose Agar, Rose Bengal Agar and *Trichoderma* Selective Medium revealed that there was remarkable variation in the growth characters and sporulation. As per the data furnished in Table 38, muants and fusants exhibited similar type of growth characters in PDA and RBA and all expressed feeble type of growth in TSM. It was also observed that all except F 2, showed sporulation in TSM.

As far as the growth rate in PDA and RBA is concerned, the mutants M40M3 and K80M13 were found to be attained full growth of 9.0 cm after four days and three days of inoculation respectively (Table 39). It was also found that both of them took one day extra for the same in TSM. Similarly the fusants, F2 and F4 gained the full plate growth in RBA and TSM within four days of inoculation. In PDA, the fusant F2 showed the growth of 9.0 cm in four days whereas F4 took only three days for the same.

4.14.2. Morphological characterization of promising mutants and fusants

The result of morphological characterization of mutants and fusants are depicted in the Table 40 and Plate 29.

Among the mutants, M40M3 recorded highest hyphal width of 4.19 µm. The other recorded the hyphal width as 3.84 µm. The phialide of M40M3 was seen in pin shape with a size of 8.69 ×2.65 µm and that of K80M13 was flask shaped with a size of 10.32 ×2.56 µm. The shape of the conidia was oval in both the cases but the size of M40M3 and K80M13 was recorded as 2.99 ×4.66 µm and 3.84 ×4.31 µm respectively.

Table 38. Cultural characters of selected mutants and fusants

Sl. No.	Isolate	Characters	Media used for the study		
			PDA	RBA	TSM
Mutants					
1	**M40M3**	1.**Mycelium**			
		Colour	Creamish white	White	Hyaline
		Texture	Low fluffy	Highly fluffy	Very feeble
		Growth pattern	Circular	Circular	-
		2.**Spore**			
		Colour	Light green	Whitish green	Green
		Sporulation	Sparse	Sparse	Sparse
		Time of sporulation	5 days	5 days	10 days
		Pattern of sporulation	Irregular	Spores at centre	Irregular
2	**K80M13**	1.**Mycelia**			
		Colour	White	White	Hyaline
		Texture	Medium fluffy	Highly fluffy	Feeble
		Growth pattern	Circular	Circular	-
		2.**Spore**			
		Colour	Dark green	Dark green	Dark green
		Sporulation	Thick	Very thick	Sparse
		Time of sporulation	3 days	3 days	3 days
		Pattern of sporulation	Plenty at margin	Uniform sporulation	Plenty at centre
Fusants					
3	**F 2**	1.**Mycelium**			
		Colour	White	White	Hyaline
		Texture	Medium fluffy	Highly fluffy	Very feeble

Sl. No.	Isolate	Characters	Media used for the study		
			PDA	RBA	TSM
		Growth pattern	Circular	Circular	-
		2.**Spore**			
		Colour	Light green	Whitish green	-
		Sporulation	Sparse	Sparse	Sparse
		Time of sporulation	2 weeks	2 weeks	-
		Pattern of sporulation	Irregular	Irregular	Irregular
4	**F 4**	1.**Mycelium**			
		Colour	White	White	Hyaline
		Texture	Medium fluffy	Highly fluffy	Feeble
		Growth pattern	Circular	Circular	-
		2.**Spore**			
		Colour	Dark green	Dark green	Dark green
		Sporulation	Thick	Very thick	Sparse
		Time of sporulation	3 days	3 days	3 days
		Pattern of sporulation	In rings at centre and margin	Uniform sporulation	Plenty at centre

PDA : Potato Dextrose Agar RBA : Rose Bengal Agar
TSM : *Trichoderma* selective medium

Table 39. Growth rate of selected mutants and fusants in different media

Sl. No.	Isolate	Radial growth (cm) DAI												
		PDA				RBA				TSM				
		1 *	2 *	3 *	4 *	1 *	2 *	3 *	4 *	1 *	2 *	3 *	4 *	5 *
Mutants														
3	M40M3	1.5	3.47	6.57	9.0	2.27	4.3	7.5	9.0	1.2	3.3	5.63	7.8	9.0
4	K80M13	3.63	7.3	9.0	-	3.0	6.03	9.0	-	2.37	5.07	8.77	9.0	-
Fusants														
5	F 2	1.87	3.43	6.87	9.0	2.6	4.73	8.17	9.0	1.8	3.57	6.2	9.0	-
6	F 4	3.4	7.2	9.0	-	2.7	5.07	8.5	9.0	2.2	4.5	7.67	9.0	-

* Mean of three replications

PDA : Potato Dextrose Agar
RBA : Rose Bengal Agarose
TSM : *Trichoderma* Selective Medium

DAI : Days After Inoculation

The fusant F2 recorded a hyphal width of 4.63µm whereas F4 recorded it as 3.56µm. The fusants F2 and F4 produced flask shaped phialide with the size of 10.25 ×2.84 µm and 9.82 × 3.47 µm respectively. The fusants showed almost similar sized oval shaped conidia.

Table 40. Morphological characters of selected mutants and fusants

Sl. No.	Isolate	Morphological characters *				
		Hypha	Phialide		Conidia	
		Width (µm)	Shape	Size (µm)	Shape	Size (µm)
Mutants						
3	M40M3	4.19	Pin shaped	8.69 × 2.65	Oval	2.99 × 4.66
4	K80M13	3.84	Flask shaped	10.32 × 2.56	Oval	3.84 × 4.31
Fusants						
5	F 2	4.63	Flask shaped	10.25 × 2.84	Oval	3.27 × 4.35
6	F 4	3.56	Flask shaped	9.82 × 3.47	Oval	3.26 × 4.65

* Mean of twenty replications

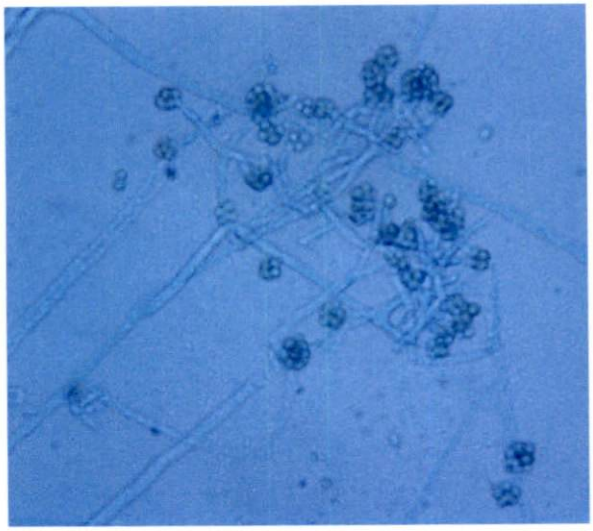
4.14.3. Molecular characterization of promising mutants and fusants

By using the biotechnological tools, ITS – PCR and RAPD, the molecular characterization of promising mutants (M40M3, K80M13) and fusants (F2, F4) developed from the parental isolates Tr 9 and Tr 43 was conducted and the results are presented below.

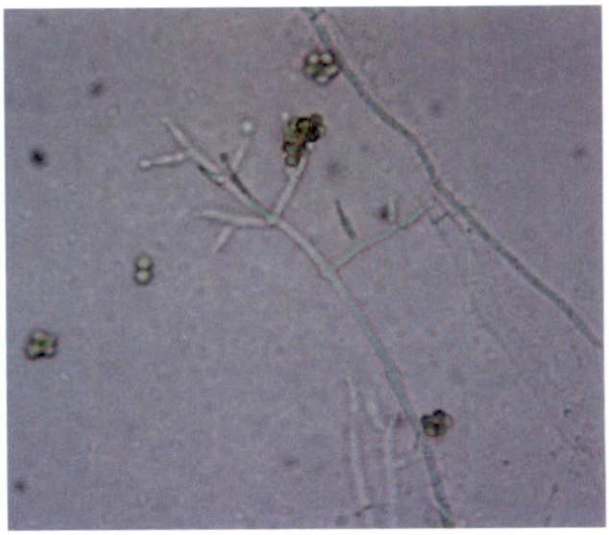
4.14.3.1. Isolation of genomic DNA

Genomic DNA of the mutants and fusants was extracted based on the protocol explained in section 3.13.3. The separation of genomic DNA on 0.8 per cent agarose gel yielded sharp clear bands indicated the good quality of DNA. It was also confirmed by spectrophotometric analysis which showed UV absorbance ratio, A_{260}/A_{280} in a range of 1.87 to 2.14 (Table 41).

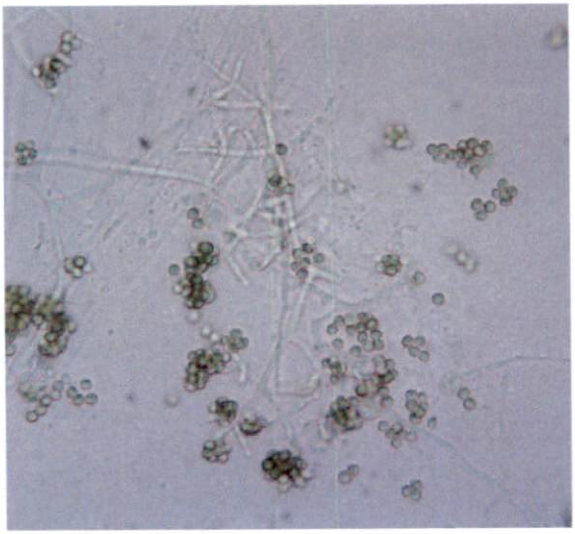
Plate 29. Microphotographs of selected mutants and fusants



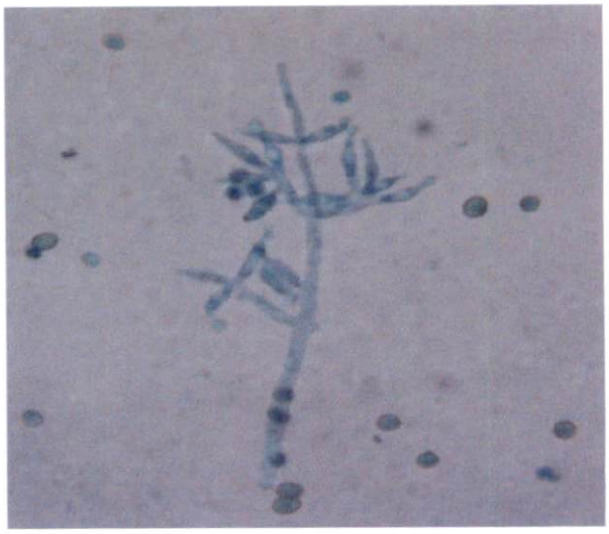
a. Mutant, K80M13 (100 X)



b. Mutant, M40M3 (100 X)



c. Fusant, F2 (100X)



d. Fusant, F4 (100X)

Table 41. Quality and quantity analysis of DNA of mutants and fusants by nanodrop method

Sl. No.	Promising mutants and fusants	A_{260}/A_{280}	Quantity (ng/µl)
1	M40M3 (Mutant)	2.09	505.5
2	K80M13 (Mutant)	1.87	434.7
3	F2 (Fusant)	2.11	805.9
4	F4 (Fusant)	2.14	451.3

4.14.3.2. PCR amplification of genomic DNA

4.14.3.2.1. PCR amplification by ITS primers

As per the procedure explained in section 3.13.3., the ITS region of genomic DNA isolated from the promising mutants and fusants was amplified using ITS -1 and ITS -4 as forward and reverse primers. The amplicons were obtained as intact bands of size ≈ 600 bp on 1 per cent agarose gel during gel electrophoresis (Plate 30a). For sequencing, the PCR products were sent to SciGenom, Cochin. The homology of sequences with other reported sequences available in NCBI (National centre for biotechnology information) data bank was analyzed.

The result of analysis revealed that the sequences of mutant, M40M3 and the fusant, F2 showed significant homology to genes of *T. erinaceum* as if the parental isolate, Tr 9. At the same time, the sequences of mutant, K80M13 and the fusant, F4 exhibited significant homology to genes of *T. asperellum* deposited in the domain data base. In all the cases the blast results showed 100 per cent query coverage and 99 per cent identity.

4.14.3.2.2. RAPD assay

The genetic variability among the mutants and the fusants in comparison with their parental isolates of *Trichoderma* spp., Tr 9 and Tr 43 was analyzed by RAPD as per the protocol explained in 3.13.3.

The random primers in the OPA series *viz.*, OPA 1, OPA 3, OPA 4, OPA 5, OPA 8 and OPA 9 were used for the observation of polymorphism among the mutants and fusants. It was observed that no primers generated reproducible polymorphism among the mutant and fusant developed from the same parent. The banding pattern exhibited by the mutant, M40M3 and the fusant, F2 were similar to that of the parent, Tr 9. In the similar way, the mutant, K80M13 and the fusant, F4 expressed the banding pattern homologous to the parent, Tr 43. As the amplified products with above mentioned six primers showed monomorphic banding pattern, it gives the indication of no genetic diversity among them (Plate 30b).

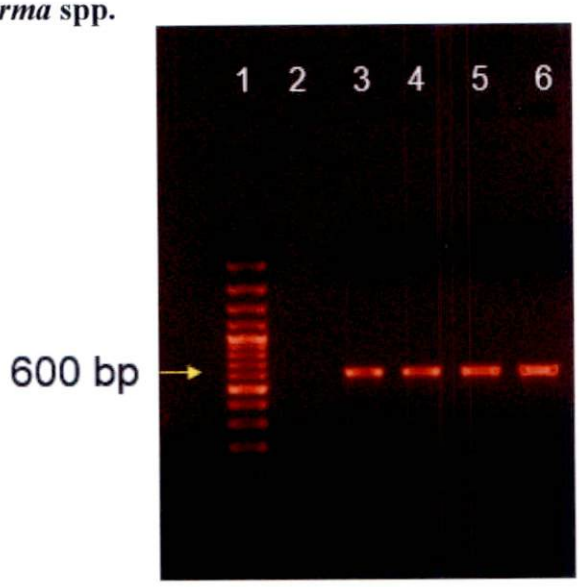
4.15. POT CULTURE EVALUATION OF BIOCONTROL EFFICIENCY OF SELECTED MUTANTS AND FUSANTS OF *Trichoderma* spp.

The selected mutants and fusants after the *in vitro* evaluation on antagonism were under gone a pot culture experiment by taking ginger as test crop and *P. aphanidermatum* as test pathogen (Plate 31). The result of the experiment is depicted in Table 42 to 47.

4.15.1. Effect of selected mutants and fusants on germination and pre emergence rot of plants

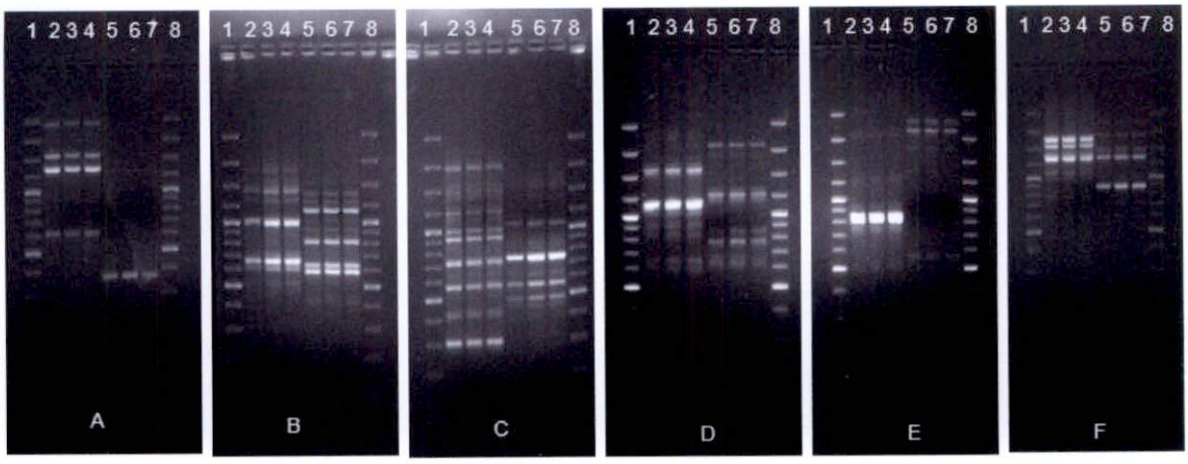
According to data in the Table 42, > 80 per cent of germination of rhizomes was observed in all the treatments. Among them, it is the treatment T1 (Tr 9) which recorded highest percentage of germination followed by T8 (*T. harzianum*), T9 (*P. fluorescens*) and T4 (K80M13) recorded 91.67 each.

Plate 30. Molecular characterization of selected mutants and fusants of *Trichoderma* spp.



a.Amplicons of ITS region of DNA of mutants and fusants of *Trichoderma* spp.

Lane 1: 1 Kb ladder, Lane 3: Amplicon of K80M13, Lane 4: Amplicon of M40M3 Lane 5: Amplicon of F2, Lane 6: Amplicon of F4



b.Amplicons of DNA of mutants and fusants of *Trichoderma* spp. by RAPD

A. OPA-1, B.OPA-3, C. OPA-4, D. OPA-5, E. OPA-8, F. OPA-9
Lane 1&8 – 1 kb plus gene ruler, Lane 2 – Tr 9, Lane 3 – M 40M 3,
Lane 4 – F 2, Lane 5 – Tr 43, Lane 6 – K 80 M13, Lane 7 - F 4

Table 42. Pot experiment II :- Effect of mutants and fusants on germination and pre emergence rot of ginger

Sl. No	Tr. No.	Treatments	Germination (%) *	Pre-emergence rot (%) *
1	T1	Tr 9	97.22^{a}	2.78^{d}
2	T2	Tr 43	80.55^{d}	19.45^{a}
3	T3	M40M3	83.33^{cd}	16.67^{ab}
4	T4	K80M13	91.67^{ab}	8.33^{cd}
5	T5	F2	80.55^{d}	19.45^{a}
6	T6	F4	86.11^{bcd}	13.89^{abc}
7	T7	*T. viride*	88.89^{bc}	11.11^{bc}
8	T8	*T. harzianum*	91.67^{ab}	8.33^{cd}
9	T9	*P.fluorescens*	91.67^{ab}	8.33^{cd}
10	T10	Copper hydroxide	86.11^{bcd}	13.89^{abc}
11	T11	Control	88.89^{bc}	11.11^{bc}
12	T12	Absolute control	88.89^{bc}	11.11^{bc}

* Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

Pre emergence rot of rhizomes was observed to be highest in treatments T5 (F2) and T2 (Tr 43) with a per cent rot of 19.45 (Table 42). The treatments T1 (Tr 9 - 2.78) and T4 (K80M13 - 8.33) showed least percentage of pre emergence rot.

4.15.2. Effect of selected mutants and fusants on bio metric characters of plants

The observations on bio metric characters *viz.*, number of tillers, height of the plant and number of leaves taken on 40, 70 and 100 DAP were presented in the Table 43 to 45.

4.15.2.1. Number of tillers / plant

Even though, there is no significant variation observed in the number of tillers of plants in each treatment at 40 DAP, the highest number of tillers 4.55 was recorded by plants in the treatment T4 (K80M13) (Table 43). One month after this the average number of tillers was observed to be highest in T12 (Absolute control) followed by T3 (M40M3) and T4 (K80M13) which recorded 36.31, 30.47 and 28.26 respectively. In the observation at 100 DAP also, the plants in the absolute control showed highest number of tillers by recording 44.33. This was followed by 34.92, 30.67 and 30.17 number of tillers per plant shown by T3 (M40M3), T2 (Tr 43) and T4 (K80M13).

4.15.2.2. Height of the plant

As far as height of the plant is concerned, there is no much variation observed among the plants in different treatments at 40DAP. But later at 70 DAP, the data in Table 44 showed significant variations among treatments.

Table 43. Pot experiment II :- Effect of mutants and fusants on number of tillers

Sl. No	Tr. No.	Treatments	No. of tillers / plant *		
			40 DAP **	70 DAP	100 DAP (surviving sprouts)
1	T1	Tr 9	3.82	18.08^{cd}	22.50^{bcde}
2	T2	Tr 43	3.97	25.15^{bc}	30.67^{abc}
3	T3	M40M3	3.91	30.47^{ab}	34.92^{ab}
4	T4	K80M13	4.55	28.26^{ab}	30.17^{bc}
5	T5	F2	3.64	24.25^{bc}	26.00^{bcde}
6	T6	F4	3.72	24.68^{bc}	27.08^{bcd}
7	T7	*T. viride*	3.37	17.82^{cd}	19.33^{cde}
8	T8	*T. harzianum*	4.40	10.91^{d}	15.00^{de}
9	T9	*P. fluorescens*	4.31	11.13^{d}	13.42^{de}
10	T10	Copper hydroxide	4.08	10.44^{d}	12.47^{e}
11	T11	Control	3.87	17.67^{cd}	18.92^{cde}
12	T12	Absolute control	3.77	36.31^{a}	44.33^{a}

* Mean of three replications ** Non significant

DAP : Days After Planting

In each column figures followed by same letter do not differ significantly according to DMRT

Table 44. Pot experiment II :- Effect of mutants and fusants on height of the plant

Sl. No	Tr. No.	Treatments	Height of tiller (cm) *		
			40 DAP **	70 DAP	100 DAP (surviving sprouts)
1	T1	Tr 9	16.55	25.79^{c}	28.29^{d}
2	T2	Tr 43	17.07	29.16^{b}	33.68^{c}
3	T3	M40M3	12.22	33.33^{a}	36.20^{ab}
4	T4	K80M13	12.84	33.78^{a}	36.63^{a}
5	T5	F2	14.82	29.64^{b}	33.76^{c}
6	T6	F4	13.67	30.05^{b}	35.19^{abc}
7	T7	*T. viride*	17.12	25.94^{c}	29.20^{d}
8	T8	*T. harzianum*	14.75	22.56^{d}	26.43^{e}
9	T9	*P. fluorescens*	14.80	24.62^{cd}	28.28^{d}
10	T10	Copper hydroxide	13.69	24.48^{cd}	28.25^{d}
11	T11	Control	14.34	28.95^{b}	33.80^{c}
12	T12	Absolute control	15.83	30.33^{b}	34.71^{bc}

* Mean of three replications ** Non significant

DAP : Days After Planting

In each column figures followed by same letter do not differ significantly according to DMRT

At that time, two treatments *viz.*, T4 (K80M13) and T3 (M40M3) recorded highest average height of the plant which was found to be statistically on par and most of the plants expressed their height in a range of 28.95 to 30.33. At 100 DAP, there was much variation in the height of the plants. Among them, the treatment, T4 (K80M13) recorded 36.63 was found to be highest among all the treatments.

4.15.2.3. Number of leaves

At the beginning, as in the case of tillers and height of the plants, the treatments could not make any significant effect on the number of leaves in plants. At 70 DAP, the average number of leaves in a plant ranged between 6.08 (T1 – Tr 9) and 8.25 (T2 – Tr 43). The data in Table 45 showed that it is the treatment, T2 (Tr 43) recorded 9.65 number of leaves, was found to be highest compared to other treatments. This was followed by T3 (M40M3) and T4 (K80M13) by recording 9.32 and 8.9 number of leaves respectively.

4.15.3. Effect of selected mutants and fusants on incidence of disease

The first symptom of yellowing of lower leaves was observed one week after challenge inoculation of the pathogen, *Pythium aphanidermatum*, in all the treatments except T12 (Absolute control) and T4 (K80M13) (Table 46). In other treatments the per cent of disease incidence was varied between 3.00 and 9.00. 30 days after inoculation of the pathogen, the treatment T4 (K80M13) recorded least per cent disease incidence of 3.00 while the others expressed the same in a range of 10.00 to 46.00. 60 DAI, the largest incidence of disease was observed in plants in the treatment, T9 (*P. fluorescens*) which recorded 94 per cent and was followed by T8 (*T. harzianum*), T 10 (Copper hydroxide) and T1 (Tr 9) by recording 70, 68 and 63 percentage of incidence of disease. The incidence of disease was observed to be less (27 %) in the treatment T4 (K80M13).

Table 45. Pot experiment II :- Effect of mutants and fusants on number of leaves

Sl. No	Tr. No.	Treatments	Number of leaves *		
			40 DAP **	70 DAP	100 DAP (surviving sprouts)
1	T1	Tr 9	5.04	6.08^{d}	6.77^{f}
2	T2	Tr 43	4.84	8.25^{a}	9.65^{a}
3	T3	M40M3	4.05	7.73^{abc}	9.32^{ab}
4	T4	K80M13	4.38	7.14^{abcd}	8.90^{abc}
5	T5	F2	5.07	7.03^{bcd}	7.68^{def}
6	T6	F4	4.08	7.13^{abcd}	8.18^{bcde}
7	T7	*T. viride*	4.96	7.53^{abc}	7.86^{cdef}
8	T8	*T. harzianum*	4.44	6.75^{cd}	7.08^{ef}
9	T9	*P. fluorescens*	4.52	6.87^{cd}	7.26^{ef}
10	T10	Copper hydroxide	4.11	7.20^{abcd}	7.75^{cdef}
11	T11	Control	4.09	7.46^{abc}	8.07^{cde}
12	T12	Absolute control	4.35	8.08^{ab}	8.69^{abcd}

* Mean of three replications ** Non significant

DAP : Days After Planting

In each column figures followed by same letter do not differ significantly according to DMRT

Table 46. Pot experiment II :- Effect of mutants and fusants on incidence of disease

Sl. No.	Tr. No.	Treatments	Disease Incidence					
			7 DAI		30 DAI		60 DAI	
			Per cent Incidence *	PROC	Per cent Incidence *	PROC	Per cent Incidence *	PROC
1	T1	Tr 9	3.00	66.67	31.00	24.39	53.00	10.17
2	T2	Tr 43	3.00	66.67	21.00	48.78	35.00	40.68
3	T3	M40M3	3.00	66.67	10.00	75.61	30.00	49.15
4	T4	K80M13	0	100	3.00	92.68	27.00	54.24
5	T5	F2	3.00	66.67	24.00	41.46	45.00	23.73
6	T6	F4	3.00	66.67	26.00	36.59	39.00	33.90
7	T7	*T. viride*	6.00	33.33	34.00	17.07	63.00	-6.78
8	T8	*T. harzianum*	6.00	33.33	42.00	-2.44	70.00	-18.64
9	T9	*P. fluorescens*	6.00	33.33	46.00	-12.20	94.00	-59.32
10	T10	Copper hydroxide	3.00	66.67	39.00	4.88	68.00	-15.25
11	T11	Control	9.00	0	41.00	0	59.00	0

* Mean of three replications
DAI : Days After Inoculation
PROC : Per cent Reduction Over Control - No reduction

On comparison with the other treatments, T4 recorded least incidence of disease throughout the crop period. Compared to the treatment with the reference culture, *T. viride* of KAU, recorded 57.14 per cent less incidence of disease and hence taken as the best treatment among all the treatments.

4.15.4. Effect of selected mutants and fusants on yield of the plant

The plants in T12 (Absolute control) recorded highest yield of 700g compared to other treatments. It was followed by T4 (K80M13) and T3 (M40M3) which recorded 616.67 and 583.33 g of fresh rhizome respectively and was found to be 124.24 and 112.12 per cent more yield of reference culture of *T. viride* (Table 47).

4.16. EVALUATION OF INDUCED SYSTEMIC RESISTANCE IN PLANTS

The estimation of defense related enzymes *viz.,* peroxidase, polyphenoloxidase and phenylalanine ammonia lyase and the total phenol was carried out in ginger plants with different treatments as well as in healthy plants to find out the induction of resistance in plants due to the attack of pathogen, application of bio control agents and the fungicide. The significant responses towards the pathogen attack in comparison with the healthy plants are depicted in Table 48 to 51.

4.16.1. Estimation of peroxidase

The changes in the production of enzyme, peroxidase (PO) due to the infection by pathogen, application of various treatments of *Trichoderma* spp. during the course of time was estimated and compared with that of the healthy plants. The results are furnished in the Table 48 and Fig. 6.

Table 47. Pot experiment II :- Effect of mutants and fusants on yield

Sl. No	Tr. No.	Treatments	Yield (g/plant) *
1	T1	Tr 9	300.00^{c}
2	T2	Tr 43	516.67^{b}
3	T3	M40M3	583.33^{ab}
4	T4	K80M13	616.67^{ab}
5	T5	F2	533.33^{b}
6	T6	F4	500.00^{b}
7	T7	*T. viride*	275.00^{cd}
8	T8	*T. harzianum*	108.33^{e}
9	T9	*P. fluorescens*	166.67^{de}
10	T10	Copper hydroxide	100.00^{e}
11	T11	Control	200.00^{cde}
12	T12	Absolute control	700.00^{a}

* Mean of three replications

Figures followed by same letter do not differ significantly according to DMRT

Plate 31. View of pot culture experiment – II

Plate 32. View of field experiment – II

It was observed that the treatment of *Trichoderma* spp. has a positive effect on the induction of peroxidase in ginger plants. Throughout the period of estimation, the healthy plants in the treatment T12 showed least production of peroxidase and it ranged from 1.23 – 1.38 min^{-1} g^{-1}. Before inoculation of the pathogen, the plants in the treatment T9 (*P. fluorescens*) recorded highest activity of PO as 2.93 min^{-1} g^{-1}. This was followed by T2 (Tr 43), T8 (*T. harzianum*) and T5 (F2) recorded the PO activity as 2.35, 2.31 and 2.03 min^{-1} g^{-1} fresh tissue respectively. The PO activity was ranged from 1.23 (T12-Absolute control) to 3.79 (T2 – Tr 43) at one day after the inoculation of pathogen. There was a profound increase in the activity of peroxidase enzyme after three days of inoculation in which the maximum activity of 5.93 min^{-1} g^{-1} was exhibited by T4 (K80M13) and was found to be 356.41 per cent more than that of healthy plants. In general, five days after inoculation the concentration of PO was found to be stabilized with slight increase. The per cent variation of PO over control was ranged from 56.52 (T7 - *T. viride*) to 329.35 (T4 – K80M13) at 5 DAI.

4.16.2. Estimation of polyphenol oxidase

The estimation on polyphenol oxidase before and after challenge inoculation with the pathogen was carried out by taking plant samples from all the treatments including the healthy plants as per the protocol described in 3.15.1.2. The results are depicted in the Table 49 and Fig. 7.

Prior to challenge inoculation of pathogen, all the treatments except T7 (*T. viride*), T8 (*T. harzianum*) and T11 (Control) recorded > 0.01 min^{-1} g^{-1} PPO activity which was found to be greater than that of healthy plants. Among them, the highest record of PPO content as 0.023 min^{-1} g^{-1} was exhibited by T2 (Tr 43) and T4 (K80M13) and was found to be 130 per cent more than that of

Table 48. Effect of different treatments on peroxidase activity in ginger

Peroxidase activity (PO) (min^{-1} g^{-1} fresh tissue) *									
Sl. No.	Treatments	Before inoculation		1 DAI		3 DAI		5 DAI	
		PO activity	POC (+/-)	PO activity	POC (+/-)	PO activity	POC (+/-)	PO activity	POC (+/-)
1	T1 (Tr 9)	1.28	+0.55	2.92	+137.98	4.40	+238.46	4.43	+220.77
2	T2 (Tr 43)	2.35	+84.34	3.79	+209.16	4.26	+227.69	4.29	+210.58
3	T3 (M40M3)	1.65	+29.35	2.05	+66.80	3.09	+137.95	3.12	+126.09
4	T4 (K80M13)	1.93	+51.35	2.91	+137.44	5.93	+356.41	5.93	+329.35
5	T5 (F2)	2.03	+59.73	2.91	+136.89	5.09	+291.28	5.09	+268.70
6	T6 (F4)	1.3	+2.12	3.01	+145.59	4.55	+250.26	4.58	+231.52
7	T7 (*T. viride*)	1.79	+40.88	2.27	+84.60	2.15	+65.15	2.16	+56.52
8	T8 (*T. harzianum*)	2.31	+81.70	2.19	+78.40	2.53	+94.85	2.55	+84.78
9	T9 (*P. fluorescens*)	2.93	+130.43	2.45	+99.67	4.24	+226.15	4.15	+200.73
10	T10 (Copper hydroxide)	1.76	+38.26	2.06	+67.65	2.52	+94.08	2.56	+85.80
11	T11 (Control)	1.65	+29.88	1.89	+54.03	2.34	+80.15	2.25	+62.80
12	T12 (Absolute control)	1.27	0.00	1.23	0.00	1.30	0.00	1.38	0.00

DAI : Days After Inoculation

POC : Per cent Over Control

* Mean of three replications

Table 49. Effect of different treatments on polyphenol oxidase activity in ginger

Polyphenol oxidase activity (PPO) (min^{-1} g^{-1} fresh tissue) *									
Sl. No.	Treatments	Before inoculation		1 DAI		3 DAI		5 DAI	
		PPO activity	POC (+/-)	PPO activity	POC (+/-)	PPO activity	POC (+/-)	PPO activity	POC (+/-)
1	T1 (Tr 9)	0.02	+70	0.02	+76.92	0.04	+300	0.08	+525
2	T2 (Tr 43)	0.02	+130	0.03	+153.85	0.04	+270	0.05	+291.67
3	T3 (M40M3)	0.02	+70	0.02	+76.92	0.05	+400	0.08	+541.67
4	T4 (K80M13)	0.02	+130	0.05	+261.54	0.07	+630	0.1	+733.33
5	T5 (F2)	0.02	+70	0.02	+30.77	0.07	+550	0.12	+900.00
6	T6 (F4)	0.01	+30	0.03	+107.69	0.03	+230	0.04	+258.33
7	T7 (*T. viride*)	0.01	-30	0.04	+207.69	0.06	+530	0.08	+575.00
8	T8 (*T. harzianum*)	0.01	-30	0.01	0	0.03	+200	0.05	+350.00
9	T9 (*P. fluorescens*)	0.02	+100	0.04	+207.69	0.06	+470	0.07	+458.33
10	T10 (Copper hydroxide)	0.01	0	0.02	+53.85	0.03	+230	0.03	+175.00
11	T11 (Control)	0.003	-70	0.03	+107.69	0.03	+230	0.05	+316.67
12	T12 (Absolute control)	0.01	0	0.01	0	0.01	0	0.01	0

DAI: Days After Inoculation

POC : Per cent over control

* Mean of three replications

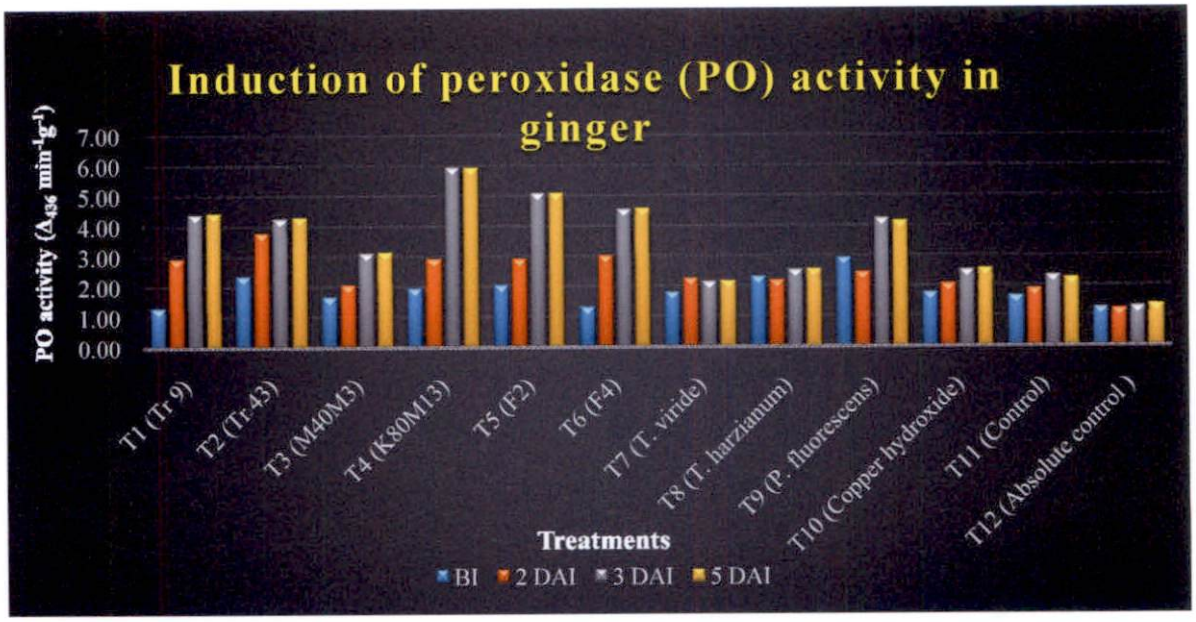


Fig. 6. Induction of peroxidase activity in ginger by *Trichoderma* spp.

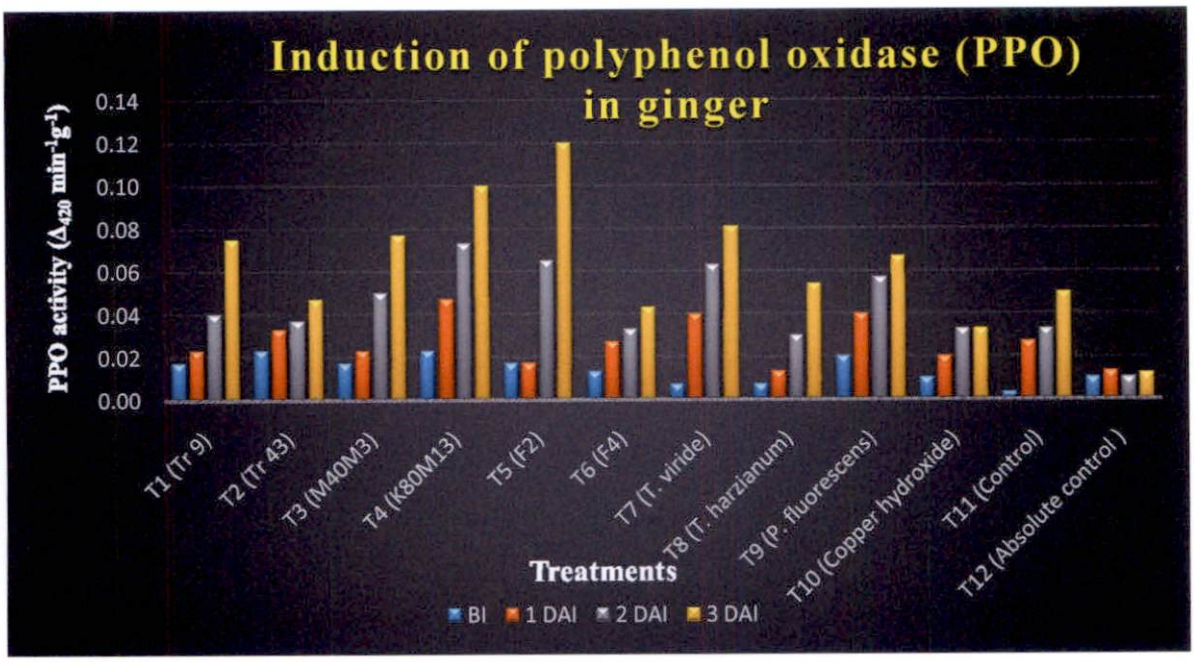


Fig. 7. Induction of polyphenol oxidase in ginger by *Trichoderma* spp.

healthy plants in the treatment, T12. After challenge inoculation of pathogen, the PPO activity was increased in all the treatments and recorded in a range of 0.013 min^{-1} g^{-1} to 0.047 in which the minimum was shown by T12 (Absolute control) and the maximum was shown by T4 (K80M13). In general, the PPO activity was noticed to be increased as the time progress. Three days after inoculation, all expressed 200 per cent more concentration of PPO than the healthy plants. In the final observation, 5 DAI the highest record of PPO was exhibited by T5 (F2) followed by T4 (K80M13) recorded 0.12 min^{-1} g^{-1} and 0.1 min^{-1} g^{-1} respectively and were found to be 900 and 733.33 per cent more than the healthy control.

4.16.3. Estimation of phenylalanine ammonia lyase

The concentration of phenylalanine ammonia lyase (PAL) occurring in the ginger plants undergone various treatments as well as the un inoculated healthy plants was estimated as per the procedure explained in section 3.15.1.3 and the results are furnished in Table 50 and Fig. 8.

According to the data, there was a visible positive effect on the production of PAL due to the application of *Trichoderma* spp. compared to healthy plants which exhibited the content of PAL in the range of 7.67 to 8.47 nmol trans cinnamic acid /g of leaf tissue before and after inoculation of the pathogen. Before challenge inoculation of pathogen, the plants contained PAL in the range of 9.56 to 15.88 nmol trans cinnamic acid /g leaf tissue which increased proportionately as the time progress. The activity of PAL one day after inoculation ranged from 11.56 to 18.09 nmol trans cinnamic acid /g leaf tissue in which the minimum value was expressed by T 11 (control) and the maximum by T8 (*T. harzianum*). Three days after inoculation, the highest activity of PAL was recorded by treatment, T9 (*P. fluorescens*) and the lowest was shown by T11 (control) which were found to be 149.36 and 82.78 per cent

Table 50. Effect of different treatments on phenylalanine ammonia lyase activity in ginger

Phenylalanine ammonia lyase activity (PAL) (nmol trans cinnamic acid g^{-1} fresh tissue) *									
		Before inoculation		1 DAI		3 DAI		5 DAI	
Sl. No.	Treatments	PAL activity	POC (+/-)	PAL activity	POC (+/-)	PAL activity	POC (+/-)	PAL activity	POC (+/-)
1	T1 (Tr 9)	9.91	+29.23	13.87	+70.44	16.80	+102.44	17.19	+102.99
2	T2 (Tr 43)	13.35	+74.08	14.10	+73.24	15.26	+83.84	17.77	+109.85
3	T3 (M40M3)	9.56	+24.73	13.05	+60.32	16.70	+101.20	18.09	+113.67
4	T4 (K80M13)	15.88	+107.14	17.77	+118.39	18.48	+122.71	29.46	+247.90
5	T5 (F2)	13.30	+73.41	14.06	+72.79	16.65	+100.58	17.19	+102.99
6	T6 (F4)	13.30	+73.41	16.27	+99.97	16.80	+102.44	17.77	+109.85
7	T7 (*T. viride*)	12.66	+65.07	14.06	+72.79	17.77	+114.12	18.09	+113.67
8	T8 (*T. harzianum*)	13.69	+78.49	18.09	+122.36	18.09	+118.02	20.69	+144.38
9	T9 (*P. fluorescens*)	16.70	+117.78	17.77	+118.39	20.69	+149.36	21.10	+159.74
10	T10 (Copper hydroxide)	11.08	+44.57	16.70	+105.20	17.19	+107.13	20.31	+139.78
11	T11 (Control)	11.46	+49.45	11.56	+42.08	15.17	+82.78	16.63	+96.40
12	T12 (Absolute control)	7.67	0.00	8.14	0.00	8.30	0.00	8.47	0.00

DAI: Days After Inoculation * Mean of three replications POC : Per cent over control

respectively, more activity of PAL in comparison with healthy plants. After five days of inoculation, a tremendous increase in the concentration of PAL was observed in T4 (K80M13) recorded 29.46 nmol trans cinnamic acid /g leaf tissue and was found to be 247.90 per cent more than that of healthy plants. All except, T11 (control) exhibited more than 102 per cent increase in the activity of PAL over control.

4.16.4. Estimation of total phenol

The total phenol content of ginger plants in each treatment including the absolute control was estimated as described in 3.15.1.4. The standard curve was prepared from which the concentration of phenol in the test samples taken before and after challenge inoculation of the pathogen, was calculated and the results are depicted in the Table 51 and Fig. 9.

As per the data, the phenol content in plants varied from 3.64 µg/g leaf tissue (T7 - *T. viride*) to 7.94 µg/g leaf tissue (T4 - K80M13) before challenge inoculation of the pathogen. They showed 12.45 per cent less and 91.01 per cent more content of phenol respectively, in comparison with the healthy plants. Except T3 (M40M3), T5 (F2) and T7 (*T. viride*) all other treatments showed positive effect on induction of phenol. One day after inoculation of pathogen, the highest content of phenol was observed in the treatment T4 (K80M13) followed by T3 (M40M3), T6 (F4), T8 (*T. harzianum*) and T1 (Tr 9) recorded 8.23, 7.63, 6.91, 6.43, 6.05 µg/g leaf tissue respectively. A range of 4.38 (Absolute control) to 9.39 (K80M13) was observed in the phenol content of plants three days after inoculation of pathogen. Even after five days of inoculation, there exists a remarkable effect by the treatments of *Trichoderma* spp. for the induction of phenols. The healthy plants recorded less content of phenol throughout the observations. Proportionate increase in the phenol content was observed in all the treatments. In the last observation 5 DAI, the

Table 51. Effect of different treatments on total phenol content in ginger

Total phenol (µg g^{-1} fresh tissue) *									
Sl. No.	Treatments	Before inoculation		1 DAI		3 DAI		5 DAI	
		Phenol µg g^{-1}	POC (+/-)	Phenol µg g^{-1}	POC (+/-)	Phenol µg g^{-1}	POC (+/-)	Phenol µg g^{-1}	POC (+/-)
1	T1 (Tr 9)	5.39	+29.86	6.05	+43.60	6.49	+47.98	8.01	+79.24
2	T2 (Tr 43)	4.84	+16.43	5.47	+29.79	6.53	+48.90	9.09	+103.50
3	T3 (M40M3)	3.79	-8.78	7.63	+81.01	8.02	+82.86	9.75	+118.32
4	T4 (K80M13)	7.94	+91.01	8.23	+95.20	9.39	+114.27	9.88	+121.20
5	T5 (F2)	3.97	-4.43	5.18	+22.94	7.76	+77.09	8.21	+83.83
6	T6 (F4)	4.87	+17.21	6.91	+63.97	7.63	+74.07	9.10	+103.77
7	T7 (*T. viride*)	3.64	-12.45	5.78	+37.12	6.88	+56.86	7.73	+73.05
8	T8 (*T. harzianum*)	4.51	+8.61	6.43	+52.55	8.12	+85.33	8.55	+91.46
9	T9 (*P. fluorescens*)	4.44	+6.87	4.90	+16.27	5.67	+29.30	6.49	+45.38
10	T10 (Copper hydroxide)	5.01	+20.69	5.81	+37.88	6.60	+50.54	6.94	+55.35
11	T11 (Control)	4.28	+3.10	4.74	+12.37	5.70	+29.94	7.63	+70.71
12	T12 (Absolute control)	4.15	0.00	4.22	0.00	4.38	0.00	4.47	0.00

DAI: Days After Inoculation * Mean of three replications POC : Per cent over control

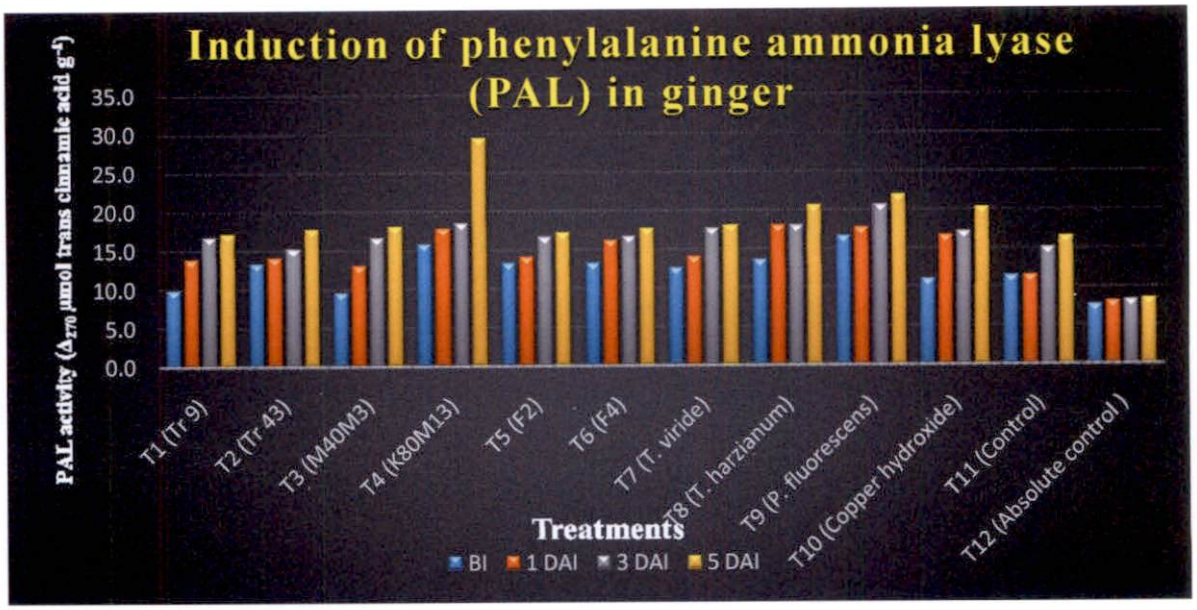


Fig. 8. Induction of phenylalanine ammonia lyase in ginger by *Trichoderma* spp.

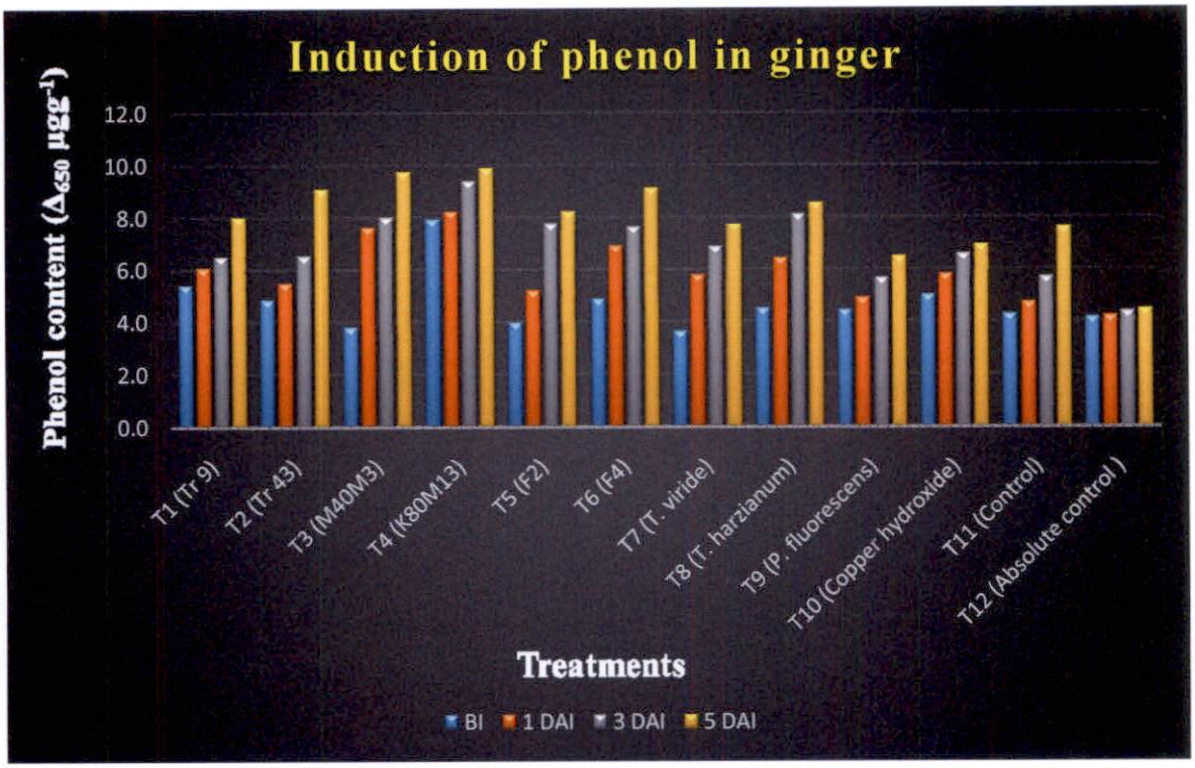


Fig. 9. Induction of phenol in ginger by *Trichoderma* spp.

treatment T4 (K80M13) recorded maximum phenol content of 9.88 µg g^{-1} closely followed by T3 (M40M3) recorded 9.75 µg g^{-1} which were found to be 121.20 per cent and 118.32 per cent more than that of healthy plants.

4.17. EVALUATION OF SELECTED MUTANTS AND FUSANTS OF *Trichoderma* spp. UNDER FIELD CONDITION

A field experiment was conducted for the performance evaluation of selected mutants and fusants under field condition and the results are presented below (Plate 32).

4.17.1. Effect of selected mutants and fusants on germination and pre emergence rot of plants

According to the data in Table 52, the germination percentage of seedlings varied in a range of 71.88 to 91.67. By showing > 91 per cent of germination, the treatment T3 (M40M3) comes first among all the treatments. In that treatment 8.34 per cent of pre emergence rot was observed. The least germination percentage was shown by treatment, T11 (Control) and hence it recorded highest per cent of pre emergence rot of 28.13.

4.17.2. Effect of selected mutants and fusants on bio metric characters of plants

Observations on bio metric characters *viz.*, number of tillers, height of the plant, number of leaves and yield and incidence of disease was taken at regular intervals and the data are depicted in Table 53 to 55.

Table 52. Field experiment II :- Effect of mutants and fusants on germination and pre emergence rot in ginger

Sl. No.	Tr. No.	Treatments	Germination (%) *	Pre-emergence rot (%) *
1	T1	Tr 9	87.50^{abc}	12.50^{cde}
2	T2	Tr 43	86.46^{abc}	13.54^{cde}
3	T3	M40M3	91.67^{a}	8.34^{e}
4	T4	K80M13	90.63^{ab}	9.38^{de}
5	T5	F2	86.46^{abc}	13.55^{cde}
6	T6	F4	88.54^{abc}	11.46^{cde}
7	T7	*T. viride*	82.29^{cd}	17.71^{bc}
8	T8	*T. harzianum*	84.38^{bc}	15.63^{cd}
9	T9	*P. fluorescens*	83.34^{c}	16.67^{c}
10	T10	Copper hydroxide	76.04^{de}	23.96^{ab}
11	T11	Control	71.88^{e}	28.13^{a}

* Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

4.17.2.1. Number of tillers / plant

The plants in the treatments T5 (F2) and T11 (Control) showed > 10 number of tillers in a plant which was found to be highest among all the treatments and are statistically on par (Table 53). This was followed by T6 (F4) and T4 (K80M13) with 9.88 and 9.61 number of tillers respectively. Again at 70 DAP and 100 DAP, the treatment, T5 (F2) itself recorded highest number of tillers of 15.0 and 44.07 respectively which was followed by T4 (K80M13) recorded 13.87 number of tillers at 70 DAP and 39.6 at 100 DAP. The treatment T7 (*T. viride*) showed least number of tillers of 7.02, 10.73 and 22.4 respectively at 40DAP, 70 DAP and 100 DAP.

4.17.2.2. Height of the plant

40 DAP, all the plants showed >18 cm height in which the plants in the treatment, T3 (M40M3) recorded highest height of 24.27 cm followed by treatments T4 (K80M13), T11 (Control) and T10 (Copper hydroxide) recorded height of 23.65, 23.15 and 21.97 cm respectively (Table 54). One month later, the plants in treatment T3 recorded 31.36 cm height which was the highest rate among all the treatments. At that time T9 (*P. fluorescens*) and T7 (*T. viride*) exhibited least height and were found to be on par. Even after 100 DAP, the plants in T3 (M40M13) come in first position as far as height of the plant is concerned by recording a height of 43.99 cm. This was followed by T 5 (F2), T 10 (Copper hydroxide) and T2 (Tr 43) recorded 39.4 cm, 39.12 and 37.38 respectively.

4.17.2.3. Number of leaves/tiller

From the Table 55, it is evident that highest number of leaves of 7.41 is seen in the plants in the treatment T3 (M40M3) followed by T4 (K80M13), T11

Table 53. Field experiment II :- Effect of mutants and fusants on number of tillers

Sl. No	Tr. No.	Treatments	No. of tillers / plant *		
			40 DAP	70 DAP	100 DAP (surviving sprouts)
1	T1	Tr 9	6.95^{e}	10.80^{e}	28.47^{bcd}
2	T2	Tr 43	8.72^{bcd}	12.87^{bcd}	36.40^{abc}
3	T3	M40M3	8.32^{d}	12.80^{bcd}	37.00^{abc}
4	T4	K80M13	9.61^{abc}	13.87^{ab}	39.60^{ab}
5	T5	F2	10.52^{a}	15.00^{a}	44.07^{a}
6	T6	F4	9.88^{ab}	13.47^{abc}	25.60^{cd}
7	T7	*T. viride*	7.02^{e}	10.73^{e}	22.40^{d}
8	T8	*T. harzianum*	8.51^{cd}	11.27^{de}	24.07^{cd}
9	T9	*P. fluorescens*	8.67^{bcd}	11.93^{cde}	30.57^{abcd}
10	T10	Copper hydroxide	8.43^{cd}	11.80^{cde}	26.13^{bcd}
11	T11	Control	10.46^{a}	12.07^{bcde}	26.47^{bcd}

* Mean of three replications

DAP : Days After Planting

In each column figures followed by same letter do not differ significantly according to DMRT

Table 54. Field experiment II :- Effect of mutants and fusants on height of tiller

Sl. No	Tr. No.	Treatments	Height of tiller (cm) *		
			40 DAP	70 DAP	100 DAP (surviving sprouts)
1	T1	Tr 9	19.71^{ef}	26.92^{bc}	32.20^{fg}
2	T2	Tr 43	20.64^{de}	26.41^{bc}	37.38^{bcd}
3	T3	M40M3	24.27^{a}	31.36^{a}	43.99^{a}
4	T4	K80M13	23.65^{ab}	27.55^{b}	36.73^{cde}
5	T5	F2	21.34^{cde}	25.33^{cd}	39.40^{b}
6	T6	F4	20.65^{de}	25.38^{cd}	34.74^{ef}
7	T7	*T. viride*	19.43^{ef}	23.47^{d}	35.84^{de}
8	T8	*T. harzianum*	20.56^{de}	25.12^{cd}	31.33^{g}
9	T9	*P. fluorescens*	18.47^{f}	24.33^{d}	36.13^{de}
10	T10	Copper hydroxide	21.97^{bcd}	27.72^{b}	39.12^{bc}
11	T11	Control	23.15^{abc}	26.85^{bc}	35.70^{de}

* Mean of three replications

DAP : Days After Planting

In each column figures followed by same letter do not differ significantly according to DMRT

Table 55. Field experiment II :- Effect of mutants and fusants on number of leaves

Sl. No	Tr. No.	Treatments	No. of leaves *		
			40 DAP	70 DAP**	100 DAP (surviving sprouts)
1	T1	Tr 9	6.12^{bcd}	7.327	10.63^{bcd}
2	T2	Tr 43	5.91^{d}	6.87	9.84^{cd}
3	T3	M40M3	7.41^{a}	8.103	12.02^{a}
4	T4	K80M13	6.83^{ab}	7.443	11.45^{ab}
5	T5	F2	6.51^{bcd}	7.247	10.85^{abcd}
6	T6	F4	6.02^{cd}	7.003	9.96^{cd}
7	T7	*T. viride*	6.12^{bcd}	7.06	10.10^{cd}
8	T8	*T. harzianum*	6.31^{bcd}	7.253	10.03^{cd}
9	T9	*P. fluorescens*	6.00^{cd}	6.877	9.65^{d}
10	T10	Copper hydroxide	6.76^{abc}	7.313	10.43^{bcd}
11	T11	Control	6.81^{ab}	7.52	11.02^{abc}

* Mean of three replications ** Non significant

DAP : Days After Planting

In each column figures followed by same letter do not differ significantly according to DMRT

(control) and T10 (copper hydroxide) which recorded number of leaves as 6.83, 6.81 and 6.76 respectively. There is no significant variation observed among the treatments in the number of leaves at 70 DAP. At 100 DAP, the average number of leaves in a plant was varied between 9.65 and 12.02. Among them, highest number of leaves (12.02) was recorded by plants in treatment, T3 (M40M3).

4.17.3. Effect of selected mutants and fusants on incidence of disease

The disease was absent in treatments *viz.*, T1 (Tr 9), T2 (Tr 43), T4 (K80M13) and T5 (F2) at the initial stage when all other treatments showed the symptom of disease in a range of 1.00 to 4.00 per cent (Table 56). Later the disease progressed to all treatments. The treatment, T4 (K80M13) recorded only 6.00 per cent of disease incidence while the treatments including parental and reference cultures exhibited > 16.00 per cent. The observation on 110 DAP, revealed that it was the treatment, T4 (K80M13) which recorded the least per cent of incidence of disease (13.00%) compared to all other treatments and was found to be 45.83 per cent less than that of reference culture, *T. viride*. The highest incidence of disease was observed in T6 (F4) recorded 38.00 per cent.

4.17.4. Effect of selected mutants and fusants on yield of the plant

The average yield of the plants was observed to be ranged from 1.5 kg to 3.7 kg (Table 57). Treatment T4 (K80M13) recorded highest yield among all the treatments showed 44.14 percentage more yield than the reference culture, *T. viride*. Out of 11, the lowest yield was recorded by treatment T6 (F4) and T1 (Tr 9) which was found statistically on par.

Table 56. Field experiment II :- Effect of mutants and fusants on incidence of disease

Sl. No	Tr. No.	Treatments	Disease Incidence					
			50 DAP		80 DAP		110 DAP	
			Per cent Incidence *	PROC	Per cent Incidence *	PROC	Per cent Incidence *	PROC
1	T1	Tr 9	0	100	19.00	-11.76	32.00	-39.13
2	T2	Tr 43	0	100	16.00	5.88	20.00	13.04
3	T3	M40M3	1.00	66.67	24.00	-41.18	24.00	-4.35
4	T4	K80M13	0	100	6.00	64.71	13.00	43.48
5	T5	F2	0	100	13.00	23.53	17.00	26.09
6	T6	F4	2.00	33.33	28.00	-64.71	38.00	-65.22
7	T7	*T. viride*	1.00	66.67	19.00	-11.77	24.00	-4.35
8	T8	*T. harzianum*	4.00	-33.33	27.00	-58.82	32.00	-39.13
9	T9	*P. fluorescens*	3.00	0	21.00	-23.53	25.00	-8.7
10	T10	Copper hydroxide	1.00	66.67	16.00	5.88	25.00	-8.7
11	T11	Control	3.00	0	17.00	0	23.00	0

* Mean of three replications
DAP : Days After Planting
PROC : Per cent Reduction Over Control - No reduction

Table 57. Field experiment II :- Effect of mutants and fusants on yield of the crop

Sl. No.	Tr. No.	Treatments	Yield (Kg / bed ($2m^2$))*
1	T1	Tr 9	1.62^{e}
2	T2	Tr 43	2.83^{b}
3	T3	M40M3	1.97^{de}
4	T4	K80M13	3.70^{a}
5	T5	F2	2.92^{b}
6	T6	F4	1.50^{e}
7	T7	*T. viride*	2.57^{bc}
8	T8	*T. harzianum*	2.30^{cd}
9	T9	*P. fluorescens*	2.85^{b}
10	T10	Copper hydroxide	2.75^{bc}
11	T11	Control	2.53^{bc}

* Mean of three replications

Figures followed by same letter do not differ significantly according to DMRT

Discussion

5. DISCUSSION

Diseases generated from the soil ecosystem in which the crop exists, have been and remain as a major constraint worldwide. The reports show that microbes *viz.*, bacteria, fungi and nematodes are the major organisms responsible for this, in which the fungal pathogens are the most devastating one. Among the fungal pathogens, *Pythium* spp., *Phytophthora* spp., *Ganoderma* spp., *Sclerotium* spp., *Rhizoctonia* spp., *Fusarium* spp., *Macrophomina* spp. *etc.* are posing serious threat to the farmers and are often potent enough to cause extensive and devastating damage to the crops. Most of them are generalistic in their host range and hence are much difficult to control by chemicals.

The research indicates that yield losses caused by pathogens, animals and weeds altogether responsible for 20 - 40 per cent in global agricultural productivity (Oerke, 2006). Savary *et al.* (2012) reported that pest and pathogens could cause short and long term consequences on crops since they could affect directly and indirectly on them.

There was a time when the farmers were very much depended upon the chemicals in the form of fertilizers and plant protection aids in order to maximize the crop yield. Now, it has been revealed and well known to everyone that excessive and unscientific use of chemicals have been resulted not only the deterioration of physical, chemical and biological health of the soil but also eliminates the ecologically beneficial microbes from it. Moreover, it created a path to generate more resistance in the pathogens. These have been grown as a major, complex problem that can affect the air, land, and water. Therefore, attention has to be focused on nonhazardous, environment friendly, sustainable techniques.

Biocontrol offers a chance to improve the crop production within the existing resources, and helps to overcome the growing problems of resistance of chemical pesticides to the target pathogen population. It has been proven that some biological control agents can successfully control plant diseases and can positively affect both on pathosystem and growth components. These positive effects include slowdown of incubation period, decreasing disease intensity and reducing the pathogenic population density in soil as well as increasing plant height, fresh and dry weight, and production (Soesanto *et al.*, 2003; Santoso *et al.*, 2007). With the increasing interest in developing alternatives to chemical fungicides, the development of bio protectants has become the focal point for research and development.

Trichoderma, an Ascomycetes cosmopolitan fungus is mostly present in soil, manure and decaying plant tissues. Although there have been a number of biopesticides in use, it is the most exploited fungal biocontrol agent in the world. In 1985, Papavizas commended on *Trichoderma* spp. as the most widely studied biocontrol agent against plant pathogens because of the ability to reduce the population of soil borne plant pathogens. The cosmopolitan nature, fast multiplication rate and broad spectrum activity build *Trichoderma* spp. as the most successful biocontrol agent. The ability to produce a variety of antibiotics, cell wall degrading enzymes and the generation of specialized structures attributes its bio control efficiency. According to Harman *et al.* (2004) *Trichoderma* spp. present in plant root systems were the most frequently isolated soil fungi. The antagonism by *Trichoderma* spp. against many soil borne plant pathogens has been well established (Harman *et al.*, 2004; Bhagat and Pan, 2007; Bhagat and Pan, 2008).

However, still interest exists in seeking more efficient *Trichoderma* spp., which considerably varies in their biocontrol efficacy. With this intension, a purposive soil sample survey was conducted in 51 different locations of Kerala

state covering all the districts. A total of 128 isolates of *Trichoderma* spp. were isolated from the collected rhizospheric soils. Like most of the researchers, the isolation was conducted on Potato dextrose agar medium, Rose bengal agar medium and *Trichoderma* selective medium, using serial dilution technique (Asran-Amal *et al.*, 2005; Gil *et al.*, 2009, Kumar *et al.,* 2012; Terna *et al.*, 2016 and Kamaruzzaman *et al.*, 2016a).

In order to reduce the number of isolates taken for further studies, a preliminary screening of all the 128 isolates of *Trichoderma* spp. was carried out with a fast growing pathogen, *Sclerotium rolfsii* and a slow growing pathogen *Fusarium oxysporum* f. sp. *cubense.* A total of 41 isolates of *Trichoderma* spp. selected from the preliminary screening were taken for further evaluating their antagonism against six important soil borne fungal pathogens, *viz., Pythium aphanidermatum, Phytophthora capsici, Ganoderma lucidum, Sclerotium rolfsii, Rhizoctonia solani,* and *Fusarium oxysporum* f. sp. *cubens* by dual culture experiments. This resulted in the selection of 20 isolates of *Trichoderma* spp. which showed the per cent inhibition in a range of 72.22 - 100; 83.33 - 100; 44.85 - 100; 49.67 - 65.56; 41.11 – 50.33 and 68.09 - 100 on the growth of pathogens *viz., P.aphanidermatum, P.capsici, G.lucidum, S.rolfsii, R.solani,* and *F.oxysporum* f. sp. *cubense* respectively. The results of dual culture experiments conducted by Gomathi and Ambikapathy (2011), Muthukumar *et al.* (2011) and Patil *et al.* (2012) proved the antagonistic potential of *Trichoderma* spp. against *Pythium* spp. Fatima *et al.* (2015) and Ambuse and Bhale (2015) were succeeded in showing the antagonistic efficacy of *Trichoderma* spp. on the growth of *Phytophthora* spp. Hence results of the present study are in confirmation with the findings of earlier researchers. Widyastuti *et al.* (2003) recorded 56.4 – 100 per cent of inhibition on *Ganoderma* isolates; Kumar *et al.* (2012) showed > 50 per cent inhibition exhibited by *Trichoderma* spp. on the growth of *Sclerotium rolfsii*; Al – qaysi and Alwan (2016) recorded 42.2 to 50 per cent inhibition on *Rhizoctonia solani*

and Kamaruzzaman *et al.* (2016b) reported the highest inhibition per cent of 99.58 on the pathogen, *Fusarium oxysporum.*

The complex process of mycoparasitism exhibited by *Trichoderma* spp. includes secretion of various types of cell wall degrading enzymes. These enzymes are able to detect the presence of other microbes by sensing the molecules released from them by enzymatic degradation (Harman *et al.*, 2004; Lorito *et al.*, 2006 and Woo and Lorito, 2007). Kumar *et al.* (2012) suggested that the potentiality of *Trichoderma* spp. to secrete extra cellular lytic enzymes can be considered as important criteria for the selection of potential antagonists. In the present investigation, apart from the efficiency in dual culture experiments, the ability to produce cellulase enzyme has also been taken as one of the criteria for the selection of potential *Trichoderma* spp. In the present study, all the 20 tested isolates, except Tr 2 and Tr 109, recorded cellulase activity of 0.5 to 1.0. Based on the above characters, 12 native isolates of *Trichoderma* spp. *viz.,* Tr 9, Tr 48, Tr 52 and Tr 76 from northern zone, Tr 2, Tr 14, Tr 43 and Tr 86 from central zone and Tr 34, Tr 41, Tr 97, and Tr 109 from southern zone, were selected after the experiment.

The finding of most potential *Trichoderma* spp. cannot be ended with *in vitro* experiments alone. It is essential to determine the effect of changing environmental conditions on biological control before assessing the practical implementation of a biocontrol agent through its antagonistic activity (Tapwal *et al.*, 2011).

Hence a pot culture experiment became necessary for the performance evaluation of the selected isolates of *Trichoderma* spp.. For this, ginger, an important spice crop of Kerala, infected by most of the soil borne fungal pathogens *viz., Pythium aphanidermatum, Sclerotium rolfsii, Rhizoctonia solani* and *Fusarium* spp. in natural condition was taken as test crop. Among them, the

most devastating pathogen, *P. aphanidermatum* was selected as the test pathogen. It was suggested that *Trichoderma* spp. can colonize the root system of the plant and can promote the plant growth (Harman, 2000; Shanmugaiah *et al.,* 2009). To take this also into consideration, observations on earliness of germination, germination percentage, pre-emergence rot and plant biometric characters were recorded periodically (40 DAP, 70 DAP and 100 DAP) along with the observation on disease intensity. In the present study, all the treatments recorded > 72 per cent of germination at one month after planting. A range of 5.55 (Tr 109) to 27.78 (Tr 14, *P. fluorescens* and copper hydroxide) per cent of pre emergence rot was observed. It was noticed that the plants treated with the isolates Tr 97, Tr 43 and the plants in absolute control showed highest number of tillers / plant with records of 7.32, 15.00 and 127.00 at 40 DAP, 70 DAP and 100 DAP respectively. Similarly, maximum height was noticed in the plants treated with the isolate, Tr 86 as 22.43cm and 42.73 cm in the first two observations and at the final stage it was the plants in absolute control which showed maximum height of 41.01 cm. In this study, maximum number of leaves / tiller was observed in a range of 5.82 (Tr 86) to 9.70 (absolute control) throughout the crop period. Among the 17 treatments, the plants treated with Tr 76 showed the lowest incidence of rhizome rot (59.88 %). The plants in the treatment with isolates Tr 86, Tr 109, Tr 97, *T. viride, P. fluorescens*, copper hydroxide and the control plants showed 100 per cent incidence of disease. This result was in agreement with the statement of Stirling *et al.* (2009) who reported that rhizome rot of ginger, one of the most destructive diseases can sometimes resulted in a crop loss of 50 – 90 per cent in tropical regions of India. They indicated that ginger crop in Kerala and Tamil Nadu are sometimes almost totally destroyed by rhizome rot. From this experiment, five isolates, Tr 9, Tr 41, Tr 43, Tr 48 and Tr 76 which showed better biometric characters with less incidence of disease than the control plants and that of reference cultures, *T. viride* and *T.harzianum* were selected for the field evaluation. Similar type of study was conducted by Kamala and Indira (2011) in which they evaluated three

indigenous isolates of *Trichoderma* spp. (T 73, T 80 and T 105) against damping-off diseases of common beans caused by *P. aphanidermatum.* They reported that the isolate T-105 could significantly reduce the pre- and post-emergence damping-off disease incidence and showed highest reduction in disease incidence of 82.86 per cent and 90.72 per cent respectively. The disease management ability of *Trichoderma* spp. against soil borne fungal pathogens was also reported by many earlier workers (Morsy *et al.,* 2009; Radheshyam *et al.,* 2012 and Surekha *et al.*, 2013).

The evaluation on the compatibility of *Trichoderma* spp. with the commonly used fungicides and insecticides was also needed before they were taken to the field. The compatibility of five isolates of *Trichoderma* spp. *viz.,* Tr 9, Tr 41, Tr 43, Tr 48 and Tr 76, with the fungicides *viz.,* carbendazim (0.1%), mancozeb (0.3%), Bordeaux mixture (1%) and copper hydroxide (0.15%) was tested under *in vitro* condition. In the present study, cent per cent inhibition on growth of all the isolates was noticed in the plates poisoned with carbendazim and Bordeaux mixture. This was in confirmation with the findings of Gowdar *et al.* (2006) who reported complete inhibition (100%) of *Trichoderma* sp. with carbendazim at 0.1 and 0.2 per cent concentrations. Gupta and Sharma (2004) also reported the complete inhibitory effect of carbendazim on the growth of biocontrol agents such as *T. viride, T. harzianum, Gliocladium virens* and *Enterobacter aerogenes.* Bhai and Thomas (2010) found that Bordeaux mixture (1%) was 100 per cent inhibitory to *T. harzianum.* In 2014, a similar study was also conducted by Thoudam and Dutta, and reported that except at 1ppm, carbendazim showed cent per cent inhibition in all other concentrations of 10, 50 and 100 ppm on the growth of *T. atroviride.* In this study, the fungicide, mancozeb 0.3 per cent showed an inhibition per cent in the range of 7.4 - 55.2 against the isolates. As per the finding of Gupta and Sharma (2004), mancozeb was least inhibitory to *Trichoderma* spp. at 200 ppm. Compatibility of mancozeb with *Trichoderma* spp. was also reported by several earlier workers

(Vijayaraghavan and Abraham, 2004; Saju, 2005; Bagwan, 2010; Bhai and Thomas, 2010). The present investigation showed > 72 per cent inhibitory effect of copper hydroxide against all the isolates, among which, the isolate Tr 9 showed highest inhibition per cent of 83.

In the similar way, the compatibility of all the five isolates of *Trichoderma* spp. with the insecticides, *viz.*, malathion (0.1%), dimethoate (0.05%), quinalphos (0.05%), chlorpyriphos (0.25%) and flubendiamide (0.01%) was also investigated and it was found that all the isolates exhibited cent per cent compatibility with flubendiamide and cent per cent incompatibility with quinalphos. The plates amended with dimethoate and chlorpyriphos showed > 83 and > 85 per cent inhibition on the growth of all the isolates respectively. The isolates were found less inhibited by malathion (<15%) and among which the isolate Tr 9 was not at all inhibited by it. These results are in agreement with Bhai and Thomas (2010) who reported the incompatibility of quinalphos with *T. harzianum* with a maximum inhibition per cent of 55.84. But in contrast, their study revealed that the insecticide, chlorpyriphos was found compatible with *T. harzianum* .

To evaluate the antagonistic efficacy of native isolates *viz.*,Tr 9, Tr 41, Tr 43, Tr 48 and Tr 76 in natural condition, a field experiment was conducted with ginger as test crop and *P. aphanidermatum* as test pathogen. All the treatments showed > 69 per cent germination of rhizome on one month after planting. The treatments, *P. fluorescens* and Tr 9 recorded the highest per cent of germination of 82.29 and 81.25 respectively which were found statistically on par with each other. The treatments were not significantly differed in their biometric characters at 40 DAP. The plants treated with Tr 43 recorded highest number of tillers of 7.27 and 8.71 respectively at 70 DAP and 100 DAP. Maximum height of 34.02 cm was recorded by plants in treatment Tr 9 at 100 DAP. The maximum number of leaves in a tiller was observed in plants treated

with Tr 9 as 7.33 and 8.82 at 70 DAP and 100 DAP respectively; which was followed by Tr 43 recorded 7.03 and 8.46 leaves / tiller. The lowest incidence of rhizome rot (53.79 %) was observed in plants in the treatment Tr 9 which was followed by Tr 43 with a record of 58.49 per cent. The studies which proved the effectiveness of *Trichoderma* spp. as a potential biocontrol agent for the management of various soil-borne plant pathogenic fungi under greenhouse and field conditions (Chakraborty, 2005; Morsy *et al.*, 2009) confirmed the result of the present investigation. It is proven that some strains of *Trichoderma* spp. can promote plant growth and reduce disease incidence in plants (Harman *et al.*, 2004; Vinale *et al.,* 2008). Similar trend was observed in the current study. Tanwar *et al.* (2010) reported that the application of *T. harzianum* increased fresh and dry shoot weights, root length and leaf area in tomato plants. In field condition, the integrated application of *T. viride* and *Bacillus subtilis* recorded a survival rate of 86.7 – 90 per cent from the wilt in tomato plants. From this experiment, the isolates Tr 9 and Tr 43, which recorded better germination percentage, better biometric characters and less incidence of disease than the control plants, were selected for further study.

Summing up the results up to the field evaluation of the native isolates of *Trichoderma* spp., the following conclusions are recorded. The native isolates, Tr 9 and Tr 43 finally selected from the field evaluation were found to be the most effective among the 128 isolates originally isolated from different rhizosphere soils of Kerala. They exhibited cent per cent inhibition on the growth of pathogens, *P. capsici* and *F. oxysporum* f. sp. *cubense* and recorded > 88, > 70, > 55 and > 42 per cent inhibition on the growth of *P. aphanidermatum, G. lucidum, S. rolfsii* and *R. solani* respectively. In the test for cellulase activity, both the isolates showed hydrolysing capacity of > 0.5. These isolates were found to be compatible with the insecticides malathion (0.1%) and flubendiamide (0.01%). The isolate, Tr 9 exhibited compatibility with the fungicide, mancozeb (0.3%). Under pot culture and field conditions, these

isolates recorded > 80 per cent germination of rhizome. In the pot experiment, these treatments recorded better biometric characters of the plant. The plants under these treatments exhibited < 75 per cent of incidence of rhizome rot. In the field experiment, the plants treated with these isolates recorded > 8, > 33 cm and > 8 number of tillers /plant, height of the plant and number of leaves /tiller respectively. Incidence of rhizome rot disease was noted as < 54 per cent in these treatments.

Hence, the isolates, Tr 9 and Tr 43 which proved their antagonistic efficacy under *in vitro*, pot culture and field evaluations, were selected as potential biocontrol agents. However, there was still considerable interest in finding of new *Trichoderma* isolates having higher antifungal activity for development of more efficient biocontrol agents. Hence the selected isolates were taken for a further attempt to enhance their antagonistic efficacy by means of genetic manipulation, as it is the major objective of the present investigation.

Prior to take these isolates for the genetic manipulation, it is necessary to confirm their genus and species level identification. For this, morphological and molecular characterization of the isolates, Tr 9 and Tr 43 were carried out.

To confirm the identification at genus level, the observation on the cultural characters *viz.*, growth rate, colour, shape, texture of the colony and sporulation were recorded, by growing the cultures on three media *viz.*, Potato dextrose agar (PDA), Rose Bengal agar (RBA) and *Trichoderma* selective medium (TSM). The morphological characters *viz.*, micrometry of hypha, phialides and spores were also taken for genus level identification. The results indicated that in general, both the isolates showed almost similar characters in PDA and RBA media. The mycelial growth of the isolates was found feeble in TSM and the isolate Tr 9 was not able to sporulate in that medium. Both the isolates produced mycelia in white colour with medium to high fluffiness in

texture. The colour of the spore varied as light green, dark green and whitish green. Ingle and Mishra (2016) studied the cultural characters of *T. erinaceum* in which they observed the conidiation in white colour and later turned to green. In the present study, the sporulation was found fast in Tr 43 (48 h) but it was very slow in case of Tr 9 (2 weeks). The isolates took four days to complete the mycelial growth in the Petri plate containing PDA and RBA whereas in TSM the isolate, Tr 9 took one extra day for the same. The result of the present study is supported and confirmed by the work of Kamaruzzaman *et al.* (2016b) who have done the morphological characterization of 10 isolates of *Trichoderma* spp.. In their study, the mycelial growth of the isolates varied up to 72 hours. The isolates have shown regular type of growth. They reported that colour of the spores varied as light green, whitish green, dark green and yellowish green.

The micrometric characters of the isolates revealed that the hyphal width of the isolates, Tr 9 and Tr 43 was varied by recording 3.66 µm and 4.31 µm respectively. The isolate, Tr 9 produced pin shaped phialide with a size of 9.23 µm × 3.2 µm and oval shaped conidia with a size of 3.91 µm × 5.06 µm. Similarly, Tr 43 produced flask shaped phialide with 10.46 µm × 2.4 µm size and oval shaped conidia in a size of 3.69 µm × 5.23 µm. Based on the above observations, the isolates were tentatively identified and assigned to the genera *Trichoderma*.

After the cultural and morphological studies, the pure cultures of Tr 9 and Tr 43 were then sent to ITCC (Indian Type Culture Collection) IARI, New Delhi for further confirmation, and the isolates Tr 9 and Tr 43 were morphologically identified as *Trichoderma* sp. and *T. asperellum* respectively (I. D. No. 9778.15 and 9782.15).

To confirm the species level identification, molecular characterization of the isolates, Tr 9 and Tr 43 were carried out using the nucleic acid detection methods, ITS - PCR and RAPD, which were proven as the common tools for the identification of microbial communities. For this, genomic DNA of the isolates was isolated from mycelia by adopting the protocol of Chakraborty *et al.* (2010) with slight modifications. The spectrophotometric analysis of genomic DNA showed UV absorbance ratio, A260/A280 as 1.9, indicated the presence of good quality genomic DNA. Using the primers, ITS 1 and ITS 4, the amplification of the ITS region of genomic DNA was carried out. The amplicons were observed as clear sharp bands of size ~ 600 bp upon resolving at 1 per cent agarose gel. The PCR products were sent to SciGenom, Cochin for sequencing. The homology of the generated sequences were analysed with the other reported sequences available in NCBI data bank. From that, it was revealed that the sequences of Tr 9 and Tr 43 showed significant homology to sequences of *T.erinaceum* and *T.asperellum* respectively with 100 per cent query coverage and 99 per cent identity. Similar type of works have been done by Herath *et al.* (2015) who used ITS – PCR technique for the identification of *Trichoderma* spp. with ITS 1 and ITS 4 as forward and reverse primers. In their study, the amplicons were received at 600 bp and the homology of the sequences obtained was analysed with the sequences available in NCBI databank. The sequences were found 100 per cent homologous to the sequences of *T.erinaceum* and the isolate was identified as *T.erinaceum*. This result is also in agreement with the findings of Kamala *et al.* (2015). They also used ITS 1 and ITS 4 as primers for the molecular characterization of 65 isolates of *Trichoderma* spp. in which one isolate was identified as *T. erinaceum* and one isolate as *T. asperellum*.

Even though there are several biotechnological methods to study the genetic variability among the isolates, RAPD was found to be simplest and cheapest method (Skoneczny *et al.*, 2015). Considering this, to study the genetic

variability among the isolates, Tr 9 and Tr 43, RAPD analysis of their genetic DNA was carried out using the oligonucleotide primers OPA 1, OPA 3, OPA 4, OPA 5, OPA 8 and OPA 9. The present study showed cent per cent polymorphism between the isolates with all the primers tested. This result was in confirmation with the result of Gupta *et al.* (2010) who conducted RAPD-PCR analysis of seven isolates of *Trichoderma* spp. by using 10 RAPD oligonucleotides (OPA 1 –OPA 10). The result showed that out of ten, seven markers OPA 1, 3, 5, 7, 8, 9 and 10 efficiently differentiated the isolates of *Trichoderma* spp. by showing reproductive banding patterns. Several other researchers were also proved the efficacy of RAPD method in the study of genetic variability of *Trichoderma* spp. (Venkateswarlu *et al.*, 2008).

In the present study, based on the cultural, morphological and molecular characterization, the isolates, Tr 9 and Tr 43 were identified and confirmed as *T. erinaceum* and *T. asperellum* respectively. These isolates were already proved their antagonistic efficiency under *in vitro* and *in vivo* conditions. Moreover, since these isolates exhibited entirely different cultural, morphological and molecular characters and were confirmed their identity as different species, these isolates were taken as parental cultures for the genetic improvement.

The reports showed that the application of biotechnological tools in fungi can be effectively used for the improvement of their character and among which, mutation and protoplast fusion techniques are found to be the rapid and simplest. Taking this in mind, in this study, the genetic improvement of the characters of the selected isolates Tr 9 and Tr 43 was carried out through mutation and protoplast fusion.

A combination of UV light and sodium nitrate treatment for a period of 20, 40, 60 and 80 minutes was used to mutate the parental isolates, Tr 9 and Tr 43. Here, the development of mutants revealed that the time of exposure is inversely proportional to the number of mutants developed. The result indicated

that the isolate, Tr 9 yielded 20, 3, 0, 0 colonies and the isolate Tr 43 yielded 23, 20, 18, 13 colonies on Czapek – Dox Agar medium after exposure to UV- light for a period of 20, 40, 60 and 80 minutes respectively. Mohamed and Haggag (2010) used a combination of UV rays and $NaNO_2$ treatment for mutating *T. koningii* and *T. reesei* and they have isolated a total of seven mutants. Radha *et al.* (2012) also practiced the physical method of UV irradiation for the development of mutants from *Aspergillus niger*. They irradiated the fungal suspension for varying time period ranging from five to 120 minutes and developed nine mutants. As in case of present study, they also observed the low survival rate of fungus with increased exposure time.

A preliminary screening of all the UV exposed isolates obtained from the parental isolates, Tr 9 and Tr 43 was carried out using a virulent pathogen, *S. rolfsii*. This experiment resulted in the selection of three out of 23 mutants of Tr 9 and six out of 74 mutants of Tr 43 which showed better antagonism than their parents and reference cultures. To check the antagonistic property of the selected mutants, an *in vitro* experiment was conducted using the soil borne fungal pathogens *viz., P.aphanidermatum, P.capsici, S.rolfsii, R.solani,* and *F.oxysporum* f. sp. *cubense*. The result of the experiment revealed that the mutants M40M3 and K80M13 showed better antagonism against all the pathogens *viz., P.aphanidermatum, P.capsici, S.rolfsii, R.solani,* and *F.oxysporum* f. sp. *cubense* by recording > 92.55, 100, > 61.11, > 44.44 and 100 per cent respectively. These improved mutants, expressed better antagonism than their parents and the reference cultures, were selected for further study. The enhanced antagonistic property of the mutants than their parental isolates has already been reported (Mohamed and Haggag, 2010). In their study, seven mutants developed from the parental isolates, *T. koningii* (3mutants) and *T. reesei* (4 mutants) showed superiority in the growth rate and antagonistic activity. The mutants caused higher reduction rate ranged between 92.6 - 98.3, 98.28 - 99.8, 89 - 98.3 and 73.3 - 99.2 per cent against *Fusarium* spp., *Pythium*

spp., *Sclerotium* spp. and *Sclerotinia* spp. respectively than their parents. Similarly, Mukherjee *et al.* (2004) reported that TgaA mutants they developed found completely pathogenic against *R. solani* than their parents.

Protoplast fusion technology, one of the successful tools to enhance the antagonistic effects of *Trichoderma* spp. against fungal plant pathogens is also used in the present study. The process of development of fusants includes the isolation of protoplast and fusion of protoplast. The isolation of protoplasts from the selected isolates, Tr 9 and Tr 43 was carried out by lytic digestion of their 24 hour old mycelia using an enzyme, glucanex. In the present study, the optimum quantity of the enzyme for the maximum release of viable protoplasts was standardized as 20 mg/ml. The degradation of the cell wall was observed in the first 30 min. followed by the bulging of cell membrane prior to release of protoplast. The isolate, Tr 43 released protoplasts two hours after incubation whereas Tr 9 took 30 min. more for the same. Patil *et al.* (2014) also reported that the release of protoplast was observed only after 2 hours of incubation in case of *T. harzianum* and *Aspergillus oryzae*. The released protoplasts were appeared in small size for the first half an hour and later the size was found increased. It was observed that the protoplasts in the optimum size were obtained in the fourth hour of incubation in the protoplasting buffer, which was supported by the findings of Balasubramanian *et al.* (2003) who showed the same in *T. roseum*. The prolonged incubation resulted in the bursting of protoplast. In this study, the protoplasts appeared colourless in 1 % Evan's blue dye, proved their viability. The dead cell wall debris was observed in blue colour. The hyphal growth from the protoplasts in the regeneration medium also proved their viability. For the isolation of the viable protoplasts, a syringe filter with 5 µm pore size was used.

The result of the present experiment is supported by the finding of Naseema *et al.* (2008) who had practiced the protoplast fusion technology in

Fusarium pallidoroseum, a potential biocontrol agent of water hyacinth. In their study they used lytic enzyme from *T. harzianum* for the lysis of cell wall of fungus. The quantity of the enzyme was standardized as 80 mg/ 4 ml of buffer which was in confirmation with the present study. Like in the current study, they observed complete lysis of the mycelium after 4 hours of incubation in the buffer and also used Evan's blue dye for checking the viability of the protoplast and observed viable cells as hyaline. In the initial stage of the protoplast release, the swelling and rounding up of cell content was also observed by Prabavathy *et al.* (2006a). The protoplasts released initially were smaller in size but later they enlarged to spherical structures (Prabavathy *et al.*, 2006a; Balasubramanian and Lalithakumari, 2008). They also reported the bursting of protoplast at increased concentration of enzyme.

The next stage of this technique is protoplast fusion and the fusion of the protoplasts was observed within 20 min. of incubation in fusion buffer containing poly ethylene glycol (PEG) 6000. Perusal of literature revealed that in most of the cases, PEG was used as fusogen (Prabavathy *et al.*, 2006a; Patil *et al.*, 2014). The quantity of the fusogen is critical for the effective fusion of protoplasts (Prabavathy *et al.*, 2006a). Hence, the quantity of the fusogen, PEG was standardized as 30 per cent. At first, the protoplasts were come in contact at a point and then the cell membrane at that point was found degraded. This yielded in the mixing up of protoplasm of each parent with a common outer wall and resulted in the formation of one fused protoplast. Perusal of literature revealed that protoplast fusion is a physical phenomenon, during which two or more protoplasts come in contact and adhere with one another either spontaneously or in presence of fusion inducing agents (Prabavathy *et al.*, 2006a; Patil *et al.*, 2014). As in case of present study, they also reported the attraction of protoplasts in the PEG solution which was followed by the disintegration of plasma membrane at the contact point and resulted in the fusion of protoplasmic contents. The fused

protoplast was found in comparatively large size and a total of 15 fusants were regenerated on malt extract agar medium. Similar type of work has already been conducted by Mohamed and Haggag (2010). They carried out the inter-specific protoplast fusion between *T. koningii* and *T. reesei* and developed 13 fusants.

The preliminary screening of all the fusants against the pathogen, *S. rolfsii* resulted in the selection of 5 fusants which were further screened against the pathogens, *viz., P.aphanidermatum, P.capsici, S.rolfsii, R.solani,* and *F.oxysporum* f. sp. *cubense.* The results revealed that the fusants F2 and F4 recorded per cent of inhibition of > 93.33, 100, > 62.22, > 42.22 and 100 against the pathogens *viz., P.aphanidermatum, P.capsici, S.rolfsii, R.solani,* and *F.oxysporum* f. sp. *cubense* respectively. These were found antagonistically potential than their parents and the reference cultures and hence were selected for further experiment. The improved antagonistic efficacy of the fusants was already reported (Mohamed and Haggag, 2010). They revealed that in dual culture, the fusants showed highest reduction rate ranged from 96.4 - 98.6, 90.4 - 94.6, 90.2 - 98.2 and 92.8 - 99.3 per cent against the pathogens, *Fusarium oxysporum, Pythium ultimum, Sclerotium rolfsii* and *Sclerotinia sclerotiorum* respectively compared to their parents. The reports indicated that five self-fusant strains of *T. harzianum viz.,* SFTh2, SFTh8, SFTh10, SFTh12 and SFTh13 were able to show cent per cent inhibition on the growth of the pathogen, *R. solani* when their parent strain showed an inhibition of 67.6 per cent (Prabavathy *et al.,* 2006b). The enhancement in the antagonism of fusants of *T. harzianum* and *T. viride* than the parents was also reported by Hassan (2014). They proved that fusant 15 showed the highest inhibition percentage of 92.8 per cent against the growth of the pathogens *Macrophomina phaseolina* and *Pythium ultimum* which was found to be greater than that of parental cultures. Similarly, fusant 9 showed the highest inhibition percentage of 98.2 per cent against the growth of *S. rolfsii.*

It is necessary to carry out the cultural, morphological and molecular characterization of the improved strains of the parental cultures, Tr 9 and Tr 43. The cultural characters of the mutants, M40M3 and K80M13 and the fusants, F2 and F4 were studied on media, *viz.*, potato dextrose agar, rose bengal agar and *Trichoderma* selective medium which revealed that there was significant variation in the growth characters and sporulation. Generally, the mutants and the fusants exhibited similar type of growth in PDA and RBA and all showed feeble growth in TSM. Except F2, all the isolates showed sporulation in TSM. The mutant, M40M3 could produce light green spores five days after inoculation where as, its parent, Tr 9 took two weeks for sporulation. At the same time the mutant of Tr 43, K80M13 and the fusant, F4 took one day extra than the parental isolate Tr 43 for sporulation. The mutant, M40M3 attained full plate growth within four days and K80M13 within three days in media PDA and RBA; whereas both took one extra day for the same in TSM. Similarly, both the fusants took four days for full plate growth in RBA and TSM. The fusant, F2 completed 9.0 cm growth within 4 days in PDA but F4 took only three days for the same in that medium.

The morphological characterization of the mutants and fusants showed that the hyphal width of the isolates ranged from 3.56 µm to 4.63 µm. The phialide of M40M3 was seen in pin shape with a size of 8.69 × 2.65 µm and that of K80M13 was flask shaped with a size of 10.32×2.56 µm. In both the cases the shape of the conidia was oval. The conidia of M40M3 and K80M13 were recorded as 2.99 ×4.66 µm and 3.84 × 4.31 µm size respectively. The fusnats, F2 and F4 produced phialide in flask shape with size of 10.25 × 2.84 µm and 9.82 × 3.47 µm respectively. The conidia of them were appeared almost in same size and in oval shape.

Molecular characterization of the promising mutants (M40M3, K80M13) and the fusants (F2, F4) was conducted using the biotechnological tools, ITS-PCR and RAPD. In ITS – PCR, ITS 1 and ITS 4 were used as

primers for the amplification of ITS region of the genomic DNA. The separation of the genomic DNA on agarose gel yielded sharp clear bands indicated the good quality DNA. It was confirmed by spectrophotometric analysis of the gel, which gave UV absorbance ratio, A_{260}/A_{280} in a range of 1.87 to 2.14. After PCR, the amplicons were observed as intact bands of size ~ 600 bp in 1 per cent gel. The amplicons were further sent to Sciegenome, Cochin for sequencing. The sequences generated were blasted with the sequences available at NCBI data bank. The result of analysis revealed that the mutant, M40M3 and the fusant, F2 showed significant homology to *T. erinaceum* and that of K80M13 and F4 exhibited homology to *T. asperellum* with cent per cent query coverage and 99 per cent identity. It was already proved that the ITS region of the genomic DNA is one of the most reliable loci for the identification of a strain at a species level (Kullnig *et al.*, 2002; Shahid *et al.*, 2014).

The RAPD analysis of the promising mutants and the fusants were carried out using OPA primers, OPA - 1, 3, 4, 5, 8 and 9. The result revealed that no primers generated reproducible polymorphism among the mutant and the fusant developed from the same parent. The banding pattern of M40M3 and F2 showed similarity to that of parent Tr 9 and the banding pattern of K80M13 and F4 exhibited similarity to the parent, Tr 43. Since the RAPD markers are dominant in nature, Patil *et al.* (2014) suggested that RAPD-PCR is not able to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous. In this study, the parental cultures used are coming under one genus, *Trichoderma*. This may be the reason for the failure of this biotechnological tool for distinguishing the fusants and the mutants from their parents in the present study. In contrast to this, Patil *et al.* (2014) used denaturing gradient gel electrophoresis (DGGE) which is an electrophoresis technique used to identify the single base changes in segment of DNA, for the characterization of fusants developed from *T. harzianum* and *Aspergillus oryzae*.

To check the feasibility of mutants and fusants in natural condition it was necessary to evaluate their antagonistic potential under *in vivo* condition. Hence, the mutants, M40M3 and K80M13and the fusants, F2 and F4 of *Trichoderma* spp., which proved their antagonistic efficiency under *in vitro* condition were taken for pot culture and field experiment. Both the experiments were conducted with ginger as test crop and *P. aphanidermatum* as test pathogen.

In the pot culture experiment, > 80 per cent of germination of rhizomes was observed in all the treatments on 1MAP. The plants treated with K80M13 showed a germination percentage of 91.67. Highest number of tillers was observed in the plants which were not challenge inoculated with pathogen. This was followed by the treatments M40M3, Tr 43 and K80M13 recorded 34.92, 30.67 and 30.17 respectively. The highest height of 36.63 cm was recorded by the plants in treatment, K80M13. The average number of leaves / tiller was observed to be highest in the treatment, Tr 43 (9.65) followed by treatments, M40M3 and K80M13 with records of 9.32 and 8.9 respectively. Compared to other treatments, the plants treated with K80M13 showed lowest incidence of disease of 27%. After the plants in absolute control, it was the treatment K80M13 gave the highest yield of rhizome (616.67g/plant).

All plants are having endogenous defense mechanisms that can be induced in response to attack by insects and pathogens (Bostock *et al.*, 2001). Selvaraj and Ambalavanan (2013) suggested that application of biocontrol agents can elevate the production of defense related enzymes *viz.*, peroxidase (PO), polyphenoloxidase (PPO) and phenylalanine ammonia lyase (PAL) and accumulation of phenols in plants. So, estimation of defense related enzymes *viz.*, peroxidase, polyphenoloxidase and phenylalanine ammonia lyase and total phenol was carried out from the pot culture experiment, to investigate the effect of *Trichoderma* spp. in the induction of systemic resistance in ginger. For the estimation, the leaf samples were collected from all the treatments including the

healthy plants on the day of inoculation as well as at one, three and five days of inoculation of the pathogen, *P. aphanidermatum*.

Peroxidase is an already proven key enzyme involved in the phenyl propanoid pathway which is associated with disease resistance in plants. In the present study, the estimation of peroxidase revealed that there was a profound increase in the activity of peroxidase enzyme at 3 days of inoculation with a maximum activity of 5.93 min^{-1} g^{-1} in the plants treated with K80M13 and was found to be 356.41 per cent more than that of healthy plants. At five days of inoculation, the per cent variation of PO over control was ranged from 56.52 (T7 - *T. viride*) to 329.35 (T4 – K80M13). Similar research works have been conducted by Vivekananthan (2003) who reported the presence of four isoforms *viz.*, PO 1 to PO 4 in mango fruits which were pre treated with FP7 strain of *P. fluorescens* followed by challenge inoculation with *C. gloeosporioides*. This indicated the elevation in the activity of PO in plants upon treatment with bio control agent.

Search of literature revealed that PPO is a copper containing enzyme that oxidizes phenolics to highly toxic quinines which are responsible for the terminal oxidation of diseased plant tissue there by attributing to the resistance. In this study, the estimation of polyphenol oxidase revealed that there was increase in the PPO activity after challenge inoculation of pathogen in all the treatments. The minimum activity of 0.013 min^{-1} g^{-1} was recorded in T12 (Absolute control) and the maximum of 0.047 min^{-1} g^{-1} in T4 (K80M13). In general, the PPO activity was noticed to be increased with the time. The plants in treatment F2 showed the highest record of PPO (0.12 min^{-1} g^{-1}) at five DAI, followed by T4 (K80M13) with 0.1 min^{-1} g^{-1}, thus exhibiting 900 and 733.33 per cent more than the healthy control.

Perusal of literature revealed that PAL is the first enzyme of phenyl propanoid metabolism in higher plants and it has a significant role in the

regulation of accumulation of phenolics, phytoalexins and lignins, the key factors responsible for the disease resistance. The estimation of phenylalanine ammonia lyase revealed that the content of PAL was found in a range of 7.67 to 8.47 nmol trans cinnamic acid /g of leaf tissue before and after inoculation of the pathogen. A tremendous increase in the concentration of PAL (29.46 nmol trans cinnamic acid /g leaf tissue) was observed in plants treated with K80M13 at five DAI. It was found to be 247.90 per cent more than that of healthy plants. This result is in agreement with the findings of Vivekananthan *et al.* (2004) and Manjunath (2009) who have already reported the induction of PAL by the treatment of fluorescent pseudomonads in mango and noni against the attack of pathogen, *Colletotrichum gloeosporioides*.

It was revealed that phenolics are fungi toxic in nature and could increase the physical and mechanical strength of the host cell wall. As per the data obtained from the estimation of total phenol, less phenol content was observed in the healthy plants throughout the observation period. In all the treatments, there was proportionate increase in the phenol content. At five DAI, the plants treated with K80M13 recorded maximum phenol content of 9.88 µg g^{-1} closely followed by treatment with M40M3 (9.75 µg g^{-1}) which were found 121.20 and 118.32 per cent more than that of healthy plants respectively.

In brief, the present study revealed that there was increase in the production of defense related enzymes *viz.*, PO, PPO and PAL and total phenols in ginger plants after the challenge inoculation with the pathogen, *P. aphanidermatum* compared to that of healthy plants. A gradual increase in their production with time was observed. Moreover, there was variation in the content of these defense related products in different treatments of *Trichoderma* spp. and it was also observed that the treatment of biocontrol agents could elevate the production of these defense molecules in plants compared to the attack of pathogen alone. Similar results were reported by several researchers. Selvaraj and Ambalavanan (2013) conducted a pot culture experiment in

anthurium to study the induction of various defense enzymes and phenols by two isolates of three biocontrol agents *viz., Pseudomonas fluorescens, Bacillus subtilis* and *Trichoderma viride*. Their study revealed that the application of biocontrol agents triggered the activity of these enzymes and phenols upon inoculation with the pathogen. They observed the peak activity of these compounds at six days after the inoculation of the pathogen. Native PAGE analysis of the enzymes showed the presence of additional three isoforms (PO 1 to PO 3) of PO and four isoforms (PPO 1 to PPO 4) of PPO which were absent in healthy plants. The present study is also supported by the findings of Parmar and Subramanian (2012) who conducted an experiment in tomato challenge inoculated with the pathogen, *Fusarium oxysporum* f. sp. *lycopersici*. They also reported the increased production of defense related enzymes *viz.*, PO, PPO and PAL and total phenol in plants inoculated with the pathogen than the healthy plants.

To evaluate the performance of mutants, M40M3 and K80M13 and fusants, F 2 and F 4 under natural condition, a field experiment was conducted with ginger as test crop and *P. aphanidermatum* as test pathogen. In the field condition, the germination percentage of rhizomes varied in a range of 71.88 (control) to 91.67 (M40M3) at 1 MAP. The plants in the treatment, F2 showed highest number of tillers/plant (44.07) followed by the treatments, K80M13 and M40M3 recorded 39.6 and 37.00 respectively. It was the treatment M40M3 which showed maximum height and number of leaves / tiller by recording 43.99 cm and 12.02 respectively. In the field condition, plants in the treatment, K80M13 recorded least per cent of incidence of disease (13%). The yield of the crop was found to be highest in the treatment, K80M13 (3.7 Kg / bed of $2m^2$).

On concluding the results of the study discussed so far, it was revealed that the mutants (M40M3 and K80M13) and the fusants (F2 and F4) and their native parental isolates of *Trichoderma* spp. (Tr 9 and Tr 43) were found promising antagonists with significant disease suppression and increased plant

growth. Among them, the mutant K80M13 which was identified as *T. asperellum* proved its highest antagonistic efficiency both under *in vitro* and *in vivo* condition. In the pot culture and field experiments, the treatment of *T. asperellum* showed least incidence of disease by recording 27 and 13 per cent respectively and were found to be 54.24 and 43.48 per cent less than that of control plants. This same treatment gave highest yield of 616.67 g/plant and 3.7 Kg/bed in the pot and field conditions. More over, the highest content of PO, PAL and total phenol was noticed in plants with the treatment of *T. asperellum,* recorded 5.93 min^{-1} and 29.46 nmol trans cinnamic acid and 9.88 µg per g of fresh tissue respectively. Eventhough, highest content of PPO (0.12min^{-1}/g) was noticed in fusant F2, it was closely followed by K80M13 with a record of 0.1min^{-1}/g. Hence the treatment K80M13 (*T. asperellum*) was considered as the most promising one among all the treatments. This improved isolate can be released as efficient biocontrol agent in future after conducting proper multilocational trials and toxicological studies.

Summary

SUMMARY

The present investigation on 'Enhancing bio-efficacy of *Trichoderma* spp. for the management of soil borne fungal pathogens' was conducted in the Department of Plant Pathology, College of Horticulture, Vellanikkara to evaluate the antagonistic efficiency of native and induced strains of *Trichoderma* spp. and to study the induced systemic resistance in plants.

Purposive sample surveyes were carried out in different locations of northern, central and southern zones of Kerala, and collected 51 soil samples for the isolation of *Trichoderma* spp.. A total of 128 isolates of *Trichoderma* spp. were obtained and were named as Tr1 to Tr 128. The observation on the effect of soil pH on the survival of *Trichoderma* spp. revealed that majority of the isolates (51%) were obtained from soil samples having pH range of 5.5 - 6.5 and moreover, it was found that they can survive even at a pH range of 2.5 - 3.5. Six important soil borne fungal pathogens *viz., Pythium aphanidermatum, Phytophthora capsici, Fusarium oxysporum* f. sp. *cubense, Sclerotium rolfsii, Ganoderma lucidum* and *Rhizoctonia solani* were isolated from the diseased specimens of host plants *viz.,* ginger, pepper, banana, pepper, coconut and rice respectively.

Preliminary screening on antagonistic efficiency of the isolates of *Trichoderma* spp. under *in vitro* condition resulted in the selection of 41 isolates of *Trichoderma* spp. which when screened against all the six pathogens, 20 isolates of *Trichoderma* spp. were found to have antagonistic efficiency by recording higher per cent of inhibition in comparison with the reference cultures of biocontrol agents *viz., T. viride* and *T. harzianum. In vitro* evaluation on the cellulose degrading efficacy of the selected isolates of *Trichoderma* spp. was carried out and all isolates except Tr 2 and Tr 109, recorded hydrolyzing efficacy of 0.5 to 1.0. Based on the results of dual culture experiments and the cellulose

degrading ability, 12 native isolates of *Trichoderma* spp. *viz.*, Tr 9, Tr 48, Tr 52 and Tr 76 from northern zone, Tr 2, Tr 14, Tr 43 and Tr 86 from central zone and Tr 34, Tr 41, Tr 97 and Tr 109 from southern zone, were selected for the evaluation of their antagonistic efficiency and the plant growth improvement under pot culture experiment.

The pot culture experiment, conducted with ginger as test crop and *P. aphanidermatum* as test pathogen, included the observations on earliness of germination, germination percentage, pre - emergence rot, number of tillers/plant, height of the tiller, number of leaves / tiller and the disease incidence which were taken at 40DAP, 70DAP and 100 DAP. All the treatments recorded > 72 per cent of germination and a range of 5.55 (Tr 109) to 27.78 (Tr 14, *P. fluorescens* and Copper hydroxide) per cent of pre emergence rot at one month after planting. After challenge inoculation of the pathogen at 45DAP, disease incidence was observed in all the treatments except absolute control. The treatments T8, T9, T11, T13, T15, T16 and T17 showed 100 per cent incidence of disease. Among the 17 treatments (except absolute control) the lowest disease incidence was observed in T1 (59.88%). Out of 12 native isolates of *Trichoderma* spp., 5 isolates *viz.*, Tr43, Tr76, Tr9, Tr41 and Tr48 exhibited higher antagonistic efficiency over the reference cultures of *T. viride* and *T. harzianum* and were selected for the field experiment.

The compatibility of these selected native isolates with the selected fungicides and insecticides and the phytotoxicity on ginger plant were evaluated and the results showed that none of the fungicides were compatible with the isolates tested. The compatibility test of these isolates with the selected insecticides revealed cent per cent compatibility with flubendiamide (0.01%) and cent per cent incompatibility with quinalphos (0.05%) and partial compatibility with malathion (0.1%).

Phytotoxicity of these selected isolates of *Trichoderma* spp. on ginger plant was tested under *in vitro* condition by inoculating the ginger rhizomes with the culture of each isolates in different ways and found that the inoculated rhizomes were found uninfected even after one week of incubation.

In the field experiment, all the treatments showed > 69% germination at 1 MAP, in which the treatment, *P. fluorescens* showed the highest germination percentage (82.29%) followed by Tr 9 (81.25%). Pre emergence rot was found to be ranged from 17.71 per cent (*P. fluorescens*) to 30.21 per cent (Tr 76 and Control). At 45 DAP, when the natural infection of the pathogen, *P. aphanidermatum* was first observed in plants under the treatments Tr 48, Tr 76, *T. harzianum*, *P. fluorescens* and control. But, treatments, T3 (Tr 9), T4 (Tr 43), T5 (Tr 41), T6 (*T. viride*) and T9 (Kocide 0.2%) did not show any symptom of disease. Based on the biometric observations and the incidence of disease, 2 native isolates of *Trichoderma* spp. (Tr 9 and Tr 43) were selected as promising isolates and by considering the difference in their morphological characters also, were taken as parent cultures for the strain improvement.

These promising native isolates of *Trichoderma* spp. (Tr 9 and Tr 43) selected from the field experiment were identified at the genus level, based on the observations on their cultural characters *viz.*, growth rate, colour, shape, texture and sporulation of colony and the morphological characters *viz.*, micrometry of hypha, phialides and spores. The cultures were further sent to ITCC (Indian Type Culture Collection, IARI, New Delhi) for the confirmation of the identification and Tr 9 and Tr 43 were identified and confirmed as *Trichoderma* sp. and *T. asperellum* respectively with I. D No. 9778.15 and 9782.15.

To study the molecular characteristics of the isolates Tr 9 and Tr 43, DNA was isolated using the protocol given by Chakraborty *et al.*, 2010 with

slight modifications. The exhibition of clear sharp bands on agarose gel and recording UV absorbance ratio, A_{260}/A_{280} as 1.9, indicated sufficient amount of good quality DNA. The amplification of ITS region of DNA was carried out by ITS- PCR using the primers ITS -1 and ITS-4 with an annealing temperature of 56°C. The amplicons developed of size ≈ 600 bp were sent to Sciegenome, Cochin for sequencing. The homology of sequences generated was then analyzed with the reported sequences available in NCBI (National centre for biotechnology information) data bank. The blast results of Tr 9 and Tr 43 showed 99% similarity to *T. erinaceum* and *T. asperellum* respectively which confirmed the result of morphological identification. The RAPD analysis of the isolated DNA was carried out using the primers in OPA series *viz.*, OPA 1, OPA 3, OPA 4, OPA 5, OPA 8 and OPA 9. All the primers produced reproducible and scorable polymorphic bands and showed 100 per cent polymorphism among the isolates.

An attempt to induce strain variation for enhancing bioefficacy of the two promising native isolates of *Trichoderma* spp. (Tr 9 and Tr 43) selected from the field experiment was carried out by mutation and protoplast fusion techniques as per the standard protocol of Mohamed and Haggag, 2010. In case of both the isolates (Tr 9 and Tr 43), the number of colonies developed on the Czapek dox agar medium after the UV treatment was found to be inversely proportional to the exposure time. The isolate, Tr 9 did not yield any colonies under UV light with exposure time period of 60 and 80min. For the strain improvement by protoplasmic fusion, the protoplasts of each isolates (Tr 9 and Tr 43) were isolated upon lytic digestion of mycelia using a lytic enzyme, Glucanex. The PEG (poly ethylene glycol) mediated fusion of the protoplasts yielded 15 fusants. .

The preliminary screening of mutants and fusants of *Trichoderma* spp. with the pathogen *Sclerotium rolfsii* resulted in the selection of nine mutants

and 5 fusants. Among them, the two mutants *viz.*, M40M3 and K80M13 and two fusants *viz.*, F2 and F4 recorded better antagonistic efficiency against the five soil borne fungal pathogens than their parental as well as reference cultures of *Trichoderma* spp.,were selected for pot culture and field evaluation.

The cultural and morphological characterization of the selected mutants and fusants was carried out by studying their cultural characters *viz.*, growth rate, colour, shape, texture and sporulation of colony and morphological characters *viz.*, micrometry of hypha, phialides and spores. The genomic DNA was isolated from these strains for the molecular characterization which was done by ITS-PCR and RAPD. The clear sharp bands produced on 0.8% agarose gel and the UV absorbance ratio, A_{260}/A_{280} in the range of 1.87 to 2.14 qualitatively and quantitatively proved the quality of DNA as good. The amplicons developed of size ≈ 600bp with the ITS primers ITS-1 and ITS-4 were sent to Sciegenome, Cochin and the sequences obtained were blasted with the other reported sequences in the NCBI data bank. The results revealed that the sequences of mutant M40M3 and the fusant F2 showed 99 per cent homology with that of the parent Tr 9 while the mutant K80M13 and the fusant F4 exhibited 99 per cent identity with the parent Tr 43. The RAPD analysis of the genomic DNA with six OPA primers OPA 1, OPA 3, OPA 4, OPA 5, OPA 8 and OPA 9 was carried out and it was noticed that no primers generated reproducible polymorphism among the mutant and the fusant developed from the same parent.

In the pot culture experiment, conducted for the evaluation of selected mutants and fusants, all the treatments recorded > 80 % of germination of rhizomes at 1 MAP and a range of 2.78 - 19.45 per cent pre emergence rot. The plants in absolute control showed highest number of tillers of 36.31 and 44.33 at 70 DAP and 100 DAP respectively and the treatment K80M13 recorded statistically maximum height throughout the crop period. The highest number of

leaves per tiller was observed in the treatment Tr 43 followed by M40M3 and K80M13 recorded 9.65, 9.32 and 8.90 respectively. The least incidence of disease was noticed in K80M13 which recorded 27 per cent disease incidence and was 54.24 % less than that of control plants. Apart from the absolute control, the highest yield of 616.67 g/plant was noticed in K80M13.

The analysis of total phenol and defense related enzymes *viz.*, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase was carried out in ginger plants to find out the effect of different treatments on the induction of resistance in plants and it was noticed that the concentration of these enzymes and phenol was increased as the time proceeds. In general, the concentration of defense compounds was almost uniform throughout the experiment in healthy plants where as a proportionate increase was observed in plants under different treatments which were artificially inoculated with pathogen. The treatment with mutant K80M13 showed highest content of PO, PAL and total phenol and recorded 5.93mt^{-1}, 29.46nmol trans cinnamic acid and 9.88 µg per g of fresh tissue respectively in the final stage. The highest content of PPO was observed in plants treated with the fusant F2 (0.12) which was closely followed by that of mutant isolate K80M13 (0.1).

In the field experiment, conducted for the evaluation of selected mutants and fusants in natural condition, the germination percentage of seedlings varied in a range of 71.88 to 91.67 and the pre emergence rot ranged from 8.34 - 28.13 per cent at 1MAP. Throughout the crop period significant variation was noticed in the number of tillers/plant among the treatments. The treatment F 2 exhibited highest number of tillers per plant of 44.07 which was followed by K80M13 recorded 39.6 number of tillers/plant. As far as plant height is concerned, mutant isolate M40M3 showed maximum height of 43.99 cm followed by fusant F 2 recorded a height of 39.4 cm. The treatment, M40M3 recorded more number of leaves/tiller than that of other treatments followed by K80M13 by

recording 12.02 and 11.45 respectively. The incidence of disease was found to be less in K80M13 (13%) followed by F 2 (17%) which was found to be in order 43.48 and 26.09 per cent less than the control plants. A yield of 3.7 Kg/bed recorded by K80M13 was observed to be the highest among all the treatments.

Summarizing the results with respect to enhance the biocontrol efficacy of native isolates of *Trichoderma* spp. by the induction of resistance in plants and evaluation of mutants and fusants with their parents and reference cultures under *in vitro* and *in vivo* conditions, it is evident that the mutant, K80M13 recorded the highest efficiency for the management of soil borne fungal pathogens and was selected as the best among all the treatments. This isolate showed 99 per cent identity with the parent Tr 43 which was identified as *Trichoderma asperellum*. It may be concluded that after confirming the biocontrol efficacy of this mutant (K80M13) by multi locational trials and toxicological studies, it can be released as a safe biocontrol agent in future

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Appendices

APPENDIX - I

MEDIA COMPOSITION

1. Carboxy Methyl Cellulose (CMC) Agar

Carboxy methyl cellulose	: 5.00 g
Sodium nitrate	: 1.00 g
Dipotassium phosphate	: 1.00 g
Potassium chloride	: 1.00 g
Magnesium sulphate	: 0.50 g
Yeast extract	: 0.50 g
Glucose	: 1.00 g
Agar	: 15.00 g
Distilled water	: 1000 ml

2. Czapek's (Dox) Agar

Sucrose	: 30.00 g
Sodium nitrate	: 2.00 g
Dipotassium phosphate	: 1.00 g
Magnesium sulphate	: 0.50 g
Potassium chloride	: 0.50 g
Ferrous sulphate	: 0.01 g
Agar	: 20.00 g
Distilled water	: 1000 ml

3. Malt Extract Agar

Malt extract	: 15.00 g
Agar	: 20.00 g
Distilled water	: 1000 ml

4. Martin's Rose Bengal Agar

Dextrose	: 10.00 g
Peptone	: 5.00 g
KH_2PO_4	: 1.00 g
$MgSO_4$	: 0.50 g
Agar	: 20.00 g
Rose Bengal	: 0.03 g
Streptomycin	: 30.00 mg (added aseptically)
Distilled water	: 1000 ml

5. Nutrient Broth (NB)

Peptone	: 5.00 g
Beef extract	: 1.00 g
Sodium chloride	: 5.00 g
Distilled water	: 1000 ml
pH	: 6.5 to 7

6. **Potato Dextrose Agar (PDA)**

Potato	: 200.00 g
Dextrose	: 20.00 g
Agar	: 20.00 g
Distilled water	: 1000 ml

7. Potato Dextrose Broth (PDB)

Potato	: 200.00 g
Dextrose	: 20.00 g
Distilled water	: 1000 ml

8. ***Trichoderma*** **Selective Medium (TSM)**

$MgSO_4$	: 2.00 g
K_2HPO_4	: 0.90 g
NH_4NO_3	: 1.00 g
KCL	: 0.15 g
Glucose	: 3.00 g
Metalaxyl	: 0.30 g
PCNB	: 0.20 g
Rose Bengal	: 0.15 g
Chloramphenicol	: 0.25 g
Agar	: 20.00 g
Distilled water	: 1000 ml

APPENDIX -II

LIST OF PUBLICATIONS

- Hima, V. M. and Beena, S. 2013. *Hypocrea koningii* – Teleomorph of *Trichoderma koningii*, first report from India. *J. Mycol. Plant Pathol.* 43 (2): 257
- Hima, V. M. and Beena, S. 2016. *In vitro* antagonism of *Trichoderma* spp. against six soil borne fungal pathogens. *International journal of applied and pure science and agriculture.* 2 (3): 45 – 48.
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- Hima, V. M. and Beena, S. 2014. Cultural and antagonistic variability among indigenous isolates of *Trichoderma* spp. from Kerala [abstract]. In: Abstracts, 26 Kerala Science Congress, 28-31, January, 2014, Wayanad. Kerala State Council for Science, Technology and Environment, Trivandrum. p.12.
- Hima, V. M., Beena, S. and Cherian, A. K. 2016. Protoplasmic fusion: A biotechnological tool for the enhancement of bio-efficacy of native isolates of *Trichoderma* spp. In: Abstracts, 6th International conference on Plant, pathogens and people, 23-27, February, 2016, New Delhi. Indian Phytopathological Society, New Delhi. 6th International conference on Plant, pathogens and people, 23-27, February, 2016. 512 – 513.
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ENHANCING BIO - EFFICACY OF *Trichoderma* spp. FOR THE MANAGEMENT OF SOIL BORNE FUNGAL PATHOGENS

By

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ABSTRACT OF THE THESIS

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ABSTRACT

A study on 'Enhancing bioefficacy of *Trichoderma* spp. for the management of soil borne fungal pathogens' was carried out in the Department of Plant Pathology, College of Horticulture, Vellanikkara, during the period 2011-2016.

Soil borne fungal pathogens are the major threat to the farmers for the cultivation of the crops. Estimate showed that 37 per cent of the crop loss was due to pests of which 12 per cent was due to pathogens (Radheshyam *et al.*, 2012). Eventhough, there are various mechanisms including chemicals for the management of soil borne diseases, biocontrol approach is getting wide acceptance because of its broad spectrum activity and eco friendly nature. The ascomycetous fungus, *Trichoderma* spp. based bio control products share 60 per cent of all fungal based products in the global market. Taking the high potentiality of this fungus in the management of soil borne fungal pathogens, the present study was conducted.

Purposive sample surveys were conducted in Kerala state and collected 51 rhizosphere soil samples from different locations of northern, central and southern zones of Kerala including Wayanad. A total of 128 isolates of *Trichoderma* spp. were isolated from the collected soil samples and were named as Tr 1 to Tr 128. The estimation on soil pH revealed that the occurrence of *Trichoderma* spp. was more in the pH range of 5.5 - 6.5. Six important soil borne fungal pathogens *viz.*, *Pythium aphanidermatum*, *Phytophthora capsici*, *Fusarium oxysporum* f. sp. *cubense, Sclerotium rolfsii, Ganoderma lucidum* and *Rhizoctonia solani* were isolated from the diseased specimens of ginger, pepper, banana, pepper, coconut, rice respectively and were selected to study the antagonistic activity.

After preliminary screening and dual culture experiments 20 isolates of *Trichoderma* spp. showed antagonistic efficiency against all the six pathogens were selected. The antagonistic efficiency of native *Trichoderma* isolates was compared with that of reference biocontrol agents *T. viride* and *T. harzianum* released from KAU. All the isolates, except Tr 2 and Tr 109, recorded cellulase activity of 0.5 to 1.0. Based on the above characters, 12 native isolates of *Trichoderma* spp., Tr 9, Tr 48, Tr 52 and Tr 76 from northern zone, Tr 2, Tr 14, Tr 43 and Tr 86 from central zone and Tr 34, Tr 41, Tr 97 and Tr 109 from southern zone, were selected for the evaluation of their antagonistic efficiency and the plant growth promoting efficiency under pot culture experiment, where ginger was taken as the test crop and *P. aphanidermatum* as the test pathogen.

In the pot experiment, all the treatments recorded > 72 per cent of germination on 1 MAP. After absolute control, plants treated with Tr 43 showed highest number of tillers/plant, height of the plant and the number of leaves/tiller with records of 45, 19.25cm and 6.72 respectively. Compared to control plants, the least incidence of disease was noticed in the treatment Tr 76 (40.12%) which was followed by Tr 43 (32.33%). Out of 12 *Trichoderma* isolates, five isolates named Tr 43, Tr 76, Tr 9, Tr 41 and Tr 48, expressed better plant growth promotion and disease suppression activity in the pot culture experiment were selected for further study.

The compatibility study of these five isolates with the selected fungicides and insecticides revealed that all the isolates tested were found incompatible with fungicides, carbendazim (0.1%), Bordeaux mixture (1%) and the insecticide quinalphos (0.05%). Only the isolate, Tr 9 was found compatible with the fungicide, mancozeb (0.3%). None of the isolates was inhibited by the insecticide, flubendiamide (0.01%).

The field evaluation of these isolates was carried out by taking ginger as test crop and *P. aphanidermatum* as test pathogen. One month after planting, > 69 per cent of germination was noticed in all the treatments. The treatment Tr 43 recorded highest number of tillers of 8.71. The maximum height of tiller (34.02 cm) and number of leaves (8.82) were recorded by the treatment Tr 9. The least incidence of disease over control was recorded by Tr 9 (27.87 %). Among the five isolates, two isolates (Tr 9 and Tr 43) which showed highest bio-efficacy were selected for strain improvement by mutation and protoplast fusion technics.

The parental cultures, Tr 9 and Tr 43 were morphologically characterized and were identified from ITCC (Indian Type Culture Collection) IARI, New Delhi as *Trichoderma* sp. and *T. asperellum* respectively (I. D No. 9778.15 and 9782.15). The molecular characterization (RAPD and ITS - PCR) of these isolates was carried out and were identified and confirmed as *T. erinaceum* and *T. asperellum* respectively.

The mutation of parental cultures Tr 9 (*T. erinaceum*) and Tr 43 (*T. asperellum*) conducted by the combination of treatment with UV rays and sodium nitrate, yielded 97 isolates. Preliminary screening of these isolates with the pathogen, *S. rolfsii* resulted in the selection of three out of 23 mutants of Tr 9 and six out of 74 mutants of Tr 43. The protoplast fusion of Tr 9 and Tr 43 was carried out by using glucanex as lytic enzyme and poly ethylene glycol as fusagent. Out of 15, five fusant isolates were selected after the preliminary screening with *S. rolfsii*. The antagonistic efficiency of the selected nine mutants and five fusants were further evaluated with soil borne fungal pathogens, *Pythium aphanidermatum*, *Phytophthora capsici*, *Fusarium oxysporum* f. sp. *cubense*, *Sclerotium rolfsii* and *Rhizoctonia solani*. The two mutants, M40M3 and K80M13 and the two fusants, F2 and F4 which showed better antagonism than their parents and the KAU reference cultures, were further evaluated under the pot and field experiments.

In the pot culture experiment, the treatment with the mutant K80M13 exhibited better plant growth by recording 91.67 per cent of germination, 30.17 tillers/plant, 36.63 cm height of tiller and 8.9 leaves /tiller. The least incidence of rhizome rot (27 %) was noticed in the plants treated with K80M13 with a maximum yield of 616.67 g/plant.

The estimation of total phenols and defense related enzymes *viz.*, peroxidase (PO), poly phenol oxidase (PPO), and phenyl alanine ammonia lyase (PAL) revealed that there was significant increase in the production of defense molecules among the treatments. The highest content of PO, PAL and total phenol was recorded in the plants treated with mutant K80M13 showed 5.93 min^{-1} and 29.46 nmol trans cinnamic acid and 9.88 µg per g of fresh tissue respectively. The highest content of PPO was observed in plants treated with the fusant F2 (0.12) which was closely followed by that of mutant isolate K80M13 (0.1).

The mutant, K80M13 showed better biometric characters of plants in the field condition. It recorded 90.63 per cent of germination, 39.6 tillers/plant, 36.73 cm height of tiller and 11.45 leaves/tiller. Among all the treatments, K80M13 recorded lowest disease incidence of 13 per cent. The maximum record of yield (3.7 Kg/bed) was also noticed in the treatment K80M13.

The morphological and molecular characterization of the mutants (M40M3 and K80M13) and the fusants (F2 and F4) was conducted. The results revealed that the sequences of M40M3 and F2 showed significant homology to genes of *T. erinaceum* and that of K80M13 and F4 showed homology to genes of *T. asperellum.*

The studies revealed that, the mutants M40M3 (*T. erinaceum*) and K80M13 (*T. asperellum*); the fusants F2 (*T. erinaceum*) and F4 (*T. asperellum*) and their parents Tr 9 (*T. erinaceum*) and Tr 43 (*T. asperellum*) were the

promising candidates with significant disease suppression and increased plant growth. Among them, the mutant K80M13 which was identified as *Trichoderma asperellum* recorded maximum disease suppression (45.83%) and highest yield (43.97%) compared to the reference culture of KAU under field condition. This improved mutant *Trichoderma asperellum* can be released as an efficient bio-control agent in future after conducting multi-locational trials and toxicological studies.