Strain improvement of *Trichoderma* spp. by protoplast fusion

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(2018-11-017)

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE

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by

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2020

DECLARATION

I, hereby declare that this thesis entitled **"Strain improvement of *Trichoderma* spp. by protoplast fusion"** 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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Certified that this thesis entitled **"Strain improvement of *Trichoderma* spp. by protoplast fusion"** is a record of research work done independently by Ms. Anit Cyriac 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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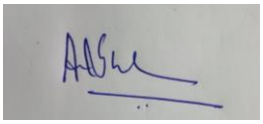

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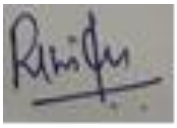

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Anit Cyriac

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
µg	Microgram
µL	Microlitre
µm	Micrometer
^{0}C	Degree Celsius
CD	Critical difference
CRD	Completely Randomized Design
cm	Centimeter
DAI	Days after inoculation
et al	And co workers
g	Gram
h	Hour
L	Litre
M	Molar
mM	Millimolar
ml	Milliliter
mg	Milligram
mm	Millimeter
PB	Protoplast buffer
PDA	Potato dextrose agar
PEG	Poly Ethylene Glycol
SE (m)±	Standard error of mean
TSM	Trichoderma selective medium
viz.,	Namely

Introduction

1. INTRODUCTION

The genus *Trichoderma* was described by Persoon in 1794 but captured the attention of agriculturists only after the discovery of one species of the genus could kill other fungi and control plant diseases (Weindling, 1932). *Trichoderma* species are widely used in agriculture and industry as biopesticides and sources of enzymes, respectively. The mechanisms of antagonism of *Trichoderma* spp. include mycoparasitism, antibiosis and competition for space and nutrients. Apart from these, production of antifungal enzymes including chitinases, β-1,3glucanases and induced systemic or localized resistance also contributed to pathogen suppression (Harman, 2006). Genus *Trichoderma* had been known for their ability to act as biocontrol agents against plant pathogens such as *Rhizoctonia*, *Fusarium*, *Alternaria*, *Colletotrichum* and *Helminthosporium*, which caused detrimental effects on crops of economic importance (Amin *et al*., 2010)

Several species of *Trichoderma* like *T. viride* and *T. harzianum* have been successfully used in the management of plant diseases. These include *T. harzianum* and *T. viride* against rice sheath blight (Bhat *et al*., 2009), *T. harzianum* against rotting of ginger (Gupta *et al*., 2010) and collar rot of cowpea (Pan and Das, 2011), *T. flavofuscum and T. viride* used against *Pythium* damping off in tomato (Patil *et al.,* 2012), and *T. harzianum* against *Phytophthora infestans* in potato and tomato (Fatima *et al*., 2015).

Although chemical pesticides play a significant role in disease management, their continuous use results in residual toxicity, phytotoxicity, environmental pollution and human health hazards. The use of biocontrol agents have helped to reduce these impacts (Vinale *et al*., 2008). But inconsistency and low efficacy of biocontrol agents under varied environmental conditions are the major hurdles in their wide spread use in plant disease management. To overcome this, the improvements of existing strains of biocontrol agents have evolved as a major strategy. Few methods of strain improvement employed for *Trichoderma* spp. are mutation, protoplast fusion and genetic recombination (Raghuchander *et al*., 2011).

Protoplast fusion is applied for developing intraspecific, interspecific and intrageneric suprahybrids. Intrastrain protoplast fusion had been attempted in

T. harzianum PTh18 and it resulted in two- fold increase in chitinase production and biocontrol activity (Prabavathy *et al*., 2006a). Intraspecific fusant of *T. koningii* showed better bio-control activity to *Rhizoctonia solani* (Hanson andHowell, 2002). Interspecific protoplast fusion of Tr 9 (*T. erinaceum*) and Tr 43 (*T. asperellum*) showed increased enzyme activity *viz,* cellulase, *β* -1,3 glucanse and enhanced antagonistic activity against soil borne pathogens *viz, Pythium aphanidermatum, Phytophthora capsici, Fusarium oxysporum f. sp. cubense, Sclerotium rolfsii, Ganoderma lucidum* and *R. solani* (Hima, 2017).

In this context, the present study entitled 'Strain improvement of *Trichoderma* spp. by protoplast fusion' was undertaken with the following objectives.

1. Isolation of *Trichoderma* spp. from soil and to study the cultural characters of isolated *Trichoderma* spp.
2. To evaluate the antagonistic property of isolated *Trichoderma* spp. against *Rhizoctonia solani* and *Pythium aphanidermatum*
3. Protoplast fusion of best isolates of *Trichoderma* spp.
4. Evaluate the antagonistic property of fusant strains of *Trichoderma* spp.

Review of Literature

2. REVIEW OF LITERATURE

The genus *Trichoderma* is a diverse group of free-living fungi, belonging to the family Hypocreaceae, commonly found in all soils. These ascomycetes fungi also inhabit root ecosystems and are parasitic on several other groups of fungi (Shah and Afiya, 2019).

2.1. Characteristics of *Trichoderma* spp.

Shalini and Kotasthane (2007) studied the morphological characters of isolated *Trichoderma* strains from Raipur and Bilaspur in potato dextrose agar (PDA), Trichoderma selective medium (TSM), corn meal dextrose agar (CMDA), oat meal agar (OMA) and special nutrient agar (SNA) media. Based on the visual and microscopic observations which include growth pattern, sporulation, size and shape conidiophores, size and shapes of philades and pigmentation, they were identified as *T. viride, T. harzianum* and *T. aureoviride.*

Savitha and Sriram (2015) studied the morphological characters of *Trichoderma* isolates. Most of the isolates showed colony colour ranging from white to green. The conidial shape of *T. harzianum* and *T. asperellum* were sub-globose while in *T. virens* isolates, conidia were ellipsoidal. The relatively small sized conidia (2.9 - 3.1 µm) were the key to identification of *T. harzianum* isolates from other *Trichoderma* species. The branching pattern of conidiophore also served as a distinguishing character to identify *Trichoderma* species. *Trichoderma sp.* isolated from the *Pleurotus sp*. was identified as *T. harzianum*, *T. viride* and *T. pseudokoningii*. The cultural studies on six media showed resemblance of *T. harzianum* and *T. viride* in appearance, morphology and with sufficient conidiation whereas *T. pseudokoningii* appeared whitish with no conidiation and pigmentation (Shah *et al.,* 2012).

Sharma and Singh (2014) reported all the isolates of *Trichoderma* in their study were fast growing with 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. Conidial formation was found in most isolates by 48 h and conidial colour changed to varying shades of green within 72 h. Growth characteristics of *Trichoderma* isolates were studied in three different media like

PDA, TSM, and OMA. Growth rate at two days after inoculation was 6.93 cm to 9.00 cm in PDA, 5.33 cm to 7.40 cm on TSM and 6.93 cm to 9.00 cm on OMA (Devi and Sinha, 2014).

2.2. Occurrence of *Trichoderma* in relation to soil pH

Kredics *et al*. (2003) reported the effects of pH on mycelial growth and enzyme involved in mycoparasitism. Okoth *et al.* (2007) found that *Trichoderma sp.* was tolerant to soil acidity since it was found in soils under different land use systems in Kenya. Garcia-Nunez *et al*. (2012) reported that native strains of *Trichoderma* were found in acidic soils obtained from seven locations in Mexico. Hima (2017) found that majority (50.78 %) of isolates of *Trichoderma* spp. were obtained in soil samples having pH of 5.5 - 6.5. About 26.56 per cent and 14.06 per cent of *Trichoderma* isolates in the study were obtained at pH range of 4.5 - 5.5 and 6.5 - 7.5 respectively.

2.3 *Trichoderma* strains against fungal pathogens

Grondona *et al*. (1997) reported the *in vitro* antibiotic activity of the *T. harzianum* isolates against 10 isolates of five different soil borne fungal plant pathogens such as *Aphanomyces cochlioides*, *Rhizoctonia solani*, *Phoma betae*, *Acremonium cucurbitacearum*, and *Fusarium oxysporum f. sp. radicis lycopersici.*. Kumar *et al*. (2012) found that seven isolates of *Trichoderma* from Jharkand soils showed strong antagonistic potential against the damping off pathogen *R. solani*. *In vitro* inhibition of *Trichoderma* isolates from different geographical regions of Nepal was 29.07 - 66.08 per cent against *R. solani* and 84 - 100 per cent against *Sclerotium rolfsii* (Bastakoti *et al*., 2017).

T. asperellum strain AFP, *T. asperellum* strain MC1, *T. brevicompactum* MF1 and *T. harzianum* strain CH1 were tested for their efficacy against *F. oxysporum, R. solani and P. capsici*. Among the *Trichoderma* isolates, *T. harzianum* exhibited maximum mycelial growth inhibition to *F. oxysporum* (78.3 %) and *P. capsici* (65.3 %) than *T. asperellum* and *T. brevicompactum* (Das *et al.*, 2018). A study conducted by Manandhar *et al.* (2019) showed varying degrees of antagonism to different pathogens *viz., F. solani, R. solani*, and *Sclerotinia sclerotiorum* by different *Trichoderma* isolates. The isolates *viz*., T153, T283, T285, T357, T362 and T363

showed more than 80 per cent inhibition and reduced sclerotia formation in *R. solani.* Isolate T363 was found to inhibit *S. sclerotiorum* (87.81 %), while isolate T357 was most effective against *F. solani* (84.76 %).

Culture filtrate of *Trichoderma sp.* was tested for growth inhibition and sporangium germination of *Pythium* isolate N1. The highest inhibition percent was obtained from filtrate of *T. harzianum* T-12 (96.3 %) followed by *T. koningii* T-8 (65.4 %) compared to the control (Lifshitz *et al*., 1986). Lahlali and Hijri (2010) found that *T. atroviride* showed high inhibition of *R. solani* in dual culture assays. Ekefan *et al.* (2009) showed that *T. harzianum* isolates Th-G, Th-N Th-I and Th-F suppressed the growth of *Colletotrichum capsici* and reduced the incidence of pepper anthracnose. The culture filtrate and volatile compounds of the antagonistic isolate also caused the inhibition of pathogen. Devi *et al*. (2012) reported that isolate Th12 of *T. harzianum* had inhibitory effect on growth of *F. solani* and *S. rolfsii*. *T. virens* Vn09 and Vn03 had shown inhibition against *S. rolfsii* and *F. udum* respectively. Hima (2017) observed the inhibitory effect of various *Trichoderma* isolates against *Pythium aphanidermatum* and *R. solani* in dual culture.

A study conducted by Rajan *et al.* (2002) highlighted the effect of *T. harzianum* against diseases of ginger incited by *Ralstonia solanacearum*, *Pythium* spp. and *F. oxysporum*. The isolates *viz., T. hamatum* T614, *T. hamatum* T612, *T. harzianum* T447, *T. harzianum* T969 and *T. virens* T523 and were tested against *Fusarium graminearum, R. solani* (AG4 and AG5), *Macrophomina phaseoli and Phytophtora cacturum*. All the isolates showed remarkable inhibitory effect on mycelial growth of the pathogens. Maximum inhibition occurred in *F. graminearum*-*T. hamatum* T614 interaction (Hajieghrari *et al*., 2008). Antagonistic assessment of *T. harzianum* and *T. viride* against *Alternaria alternata, A. brassicae, A. solani, F.oxysporum* and *F. solani* resulted in effective inhibition of mycelial growth of pathogens (Meena *et al.,* 2017).

Khattabi *et al*. (2004) reported 45-50 per cent inhibition of *S. rolfsii* by *Trichoderma* isolates Kb2, Kb3 and Kf1 which also exhibited good competitiveness in soil. The inhibition capacity of water-soluble and volatile metabolites of *Trichoderma* (*T. asperellum, T. harzianum* and *Trichoderma* spp.) was tested against

R. solani. About 74.4 to 67.8 and 15.3 to 10.6 per cent inhibition of pathogen growth was obtained with water soluble metabolite and volatile metabolites respectively. Among different *Trichoderma* spp., *T. asperellum* was more effective in reducing *R. solani* infection in beans under green house conditions (Asad *et al.,* 2014).

2.4. Mechanisms of antagonism in *Trichoderma*

Antagonistic properties of *Trichoderma* strains depend on several mechanisms *viz.,* indirect mechanisms which include competition for space and nutrients and activation of host defence mechanisms, and the direct mechanisms like mycoparasitisim and antibiosis.

Parasitisim of *R. solani* with *T. harzianum* was studied by hyphal interaction. After 24 hours of incubation of *T. harzianum* with *R. solani* on PDA, early stages of parasitism was observed. The parasitic hyphae reached the host hyphae; and started coiling and penetrating the cell wall directly using lytic enzymes, which are capable of degrading the cell wall (Melo and Faull, 2000).

Mycoparasitic activity of *Trichoderma* with *R. solani* showed the absence of sclerotia formation in *R. solani* and parasitisation of sclerotia by profuse sporulation of *Trichoderma*. The mechanisms involved were coiling around pathogen hyphae, penetration, and subsequent dissolution of the host cytoplasm. In addition, competition and tuft formation by *Trichoderma* also limited the growth of the pathogen (Shalini and Kotasthane, 2007).

A study conducted by Benhamou and Chet (1993) showed the structural changes of *R. solani* by the coiling action of *T. harzianum*. The close contact between two fungi resulted in cell wall degradation, plasmalemma disorganisation and cytoplasm aggregation. Mycoparasitic interaction of *T. virens* against *R. solani* was found to be host sensing, attraction, attachment, coiling around and lysis brought about by hydrolytic enzymes along with the action of secondary metabolites (Mukherjee *et al*., 2012).

Bailey *et al.,* (2008) reported the antagonistic characters *viz.,* antibiosis and mycoparasitisim of *Trichoderma* isolates against *Moniliophthora roreri*. The presence

of inhibitory metabolite and complete parasitism by *Trichoderma* isolates inhibited the growth of *M. roreri.*

Antagonism test of *Trichoderma* over *Macrophomina phaseolina* revealed the mechanisms of competition, hyperparasitisim, antibiosis (Mendoza *et al*., 2015). Competition occurred by the formation of barrier by *T. harzianum* and *T. virens* that prevented the growth of *M. phaseolina*. Antibiosis was observed after 48 h with a colour change in the medium due the production of secondary metabolite. Hyperparasitism involved coiling and appressorium-like structure formation which helped in penetration of the host.

Dennis and Webster (1971) reported the production of trichodermin and peptide antibiotics by *Trichoderma sp*. Many microbes produce and secrete one or more compounds with antibiotic activity (Yoshihisa *et al.,* 1989). Role of antibiosis in antagonistic activity of *Gliocladium virens* against *R. solani* was studied by Aluko and Hering (1970) where antibiotics like gliotoxin and viridin were responsible for the inhibition. Antibiotic produced from *T. koningii* (4.8-dihydroxy-2-(l-hydroxyheptyl)-3,4,5,6,7,8-hexahydro-ZH-1-benzopyran-5one) inhibited the growth of soil borne pathogens such as *G. graminis* var. *tritici, R. solani, P. cinnamomi, P. middletonii, F. oxysporum* and *Bipolaris mrokiniana* (Dunlop *et al.,* 1989). Benítez *et al., (*2004) showed inhibition of fungal pathogens due to production of metabolites which include harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins and heptadilic acid.

In vitro interaction between *Trichoderma* spp. and *Pythium sp*. (isolate N1) studied on cellophane showed no coiling or lysis after 24 h. At later stages *Trichoderma* had overgrown *Pythium* and sporulated at areas of hyphal interaction. Certain isolates also formed chlamydospores (Lifshitz *et al*., 1986).

El-Debaiky (2017) studied the *in vitro* hyphal interaction between *Aspergillus piperis* and *T. harzianum* with pathogens *A. alternata, A. solani, Botrytis cinerea, Sclerotium cepivorum* and *S. sclerotiorum*. The mechanisms observed were mycoparasitism (coiling and penetration of the hyphae) and antibiosis (lysis of the hyphal cells and spores, denaturation and breaking of the hyphae). Strains of *T. atroviride*, *T. koningiopsis* and *T. stilbohypoxyli* studied by Lopez *et al*. (2019)

showed high antagonistic index where hyphae of phytopathogens (*C. gigasporum, F. oxysporum A. destruens, Phoma destructive, Phoma sp.,* and *Pilidium concavum*) were surrounded by spore aggregate antagonists. Jash and Pan (2007) found that *T. viride* T3 and *T. roseum* T9 overgrew *R. solani* within three days of incubation in dual culture.

2.5. Strain improvement in *Trichoderma*

T. viride Tv-6 isolate was subjected to mutagens *viz*., UV rays, gamma rays and nitrosoguanidine. A few mutants *viz*., MG 3, MG 6, UV 10 and MNT 7 showed high antagonistic index as revealed by their competitive saprophytic ability in soil, and other parameters like percent inhibition, colonization behavior, inhibition zone, speed of overgrowth and propagule lysis to the cotton root rot pathogen, *R. solani* (Nakkeeran *et al*., 2005). Antibiotic-producing mutants of *Coniothyrium minitans* B21 obtained after UV treatment displayed high infection rates of parasitism of sclerotia, resulting in low viability of sclerotia. Three of those mutans (CM4b, CM4c and CM23) were destructive parasites of *S. sclerotiorum* by killing more than 80 per cent of sclerotia (Melo *et al*., 2011). Conidial suspension of *T. viride* FCBP-142 were subjected to UV irradiation and chemical mutagen, ethyl methane sulfonate (EMS) for enhancement of celluolytic activity. UV treated strain Tv-UV-5.6 exhibited maximum cellulase activity of 87 IU/ml at exposure time of 25 min. EMS treatment resulted in even higher levels of enzyme production (122.66 IU/ml) by the strain Tv-Ch-4.3 (Shafique *et al*., 2011).

Trichoderma mutants obtained after gamma irradiation were selected based on the consistent expression of the phenotypic characters up to four generations (Mukherjee and Mukhopadhyay, 1993). Strains of *T. harzianum, T. viride* and *T. koningii* were subjected to gamma radiation and the mutants *viz.,* TH508, TK509, TK508 and TV208 exhibited fast growth on PDA on comparison with the parental strains. Strains TH508, TK509 and TK508 could cause high inhibition of *S. cepivorum*, the causal agent of onion white rot disease. Mutant strains TH508 and TK509 were found to produce enhanced levels of chitinase and β-1, 3-glucanase enzymes. Seed treatment and soil application in onion with mutant strains TH508,

TK509 and TK508 could bring about complete reduction of white rot disease under field conditions (Haggag and Mohamed, 2002).

Mutagenesis of *T. harzianum*-1432 and *T. atroviride* with N′-methyl-N′-nitro-N′-guanidine (NTG) resulted in high cellulase and *β*-1, 3-glucanase production by *T. harzianum* mutant *Th*-m_1. The mutant also possessed enhanced competitive saprophytic ability. In field experiments, 82.9 per cent control of chick pea collar rot caused by *S. rolfsii* was obtained upon soil application of the mutant strain.

Combined treatment of chemical mutagen sodium nitrate ($NaNO_3$) and UV irradiation in *T. koningii* and *T. reesei* yielded potential mutants having increased antagonistic activity against root rot and damping off pathogens (Mohamed and Haggag, 2010).

Attempts have been made in *Trichoderma sp*. for constitutive over expression of genes encoding hydrolytic enzymes. Djonovic *et al.* (2007) generated transformants of *T. virens* in which *β*-1,3- and *β*-1,6-glucanase genes, *TvBgn2* and *TvBgn3* respectively were constitutively co-expressed in the same genetic *T. virens* Gv29.8 wild-type background. Although the transformants grew and sporulated slower, higher levels of enzyme production and enhanced protection of cotton seedlings against *P. ultimum, R. solani* and *Rhizopus oryzae* was obtained.

2.6. Protoplast fusion in *Trichoderma*

Protoplast fusion is an important tool in strain improvement for bringing genetic recombination and developing hybrid strains in filamentous fungi. It involves release of protoplasts by using cell wall degrading enzyme and then fusion by addition of polyethylene glycol (PEG) in the presence of calcium. Fusion of auxotrophs and selection of somatic hybrid colonies on medium deficient in needed nutrients is the commonly followed strategy (Stasz, 1990).

Protoplast production, regeneration and factors for maximum release of protoplast were earlier studied in fungi such as *Phanerochaete chrysosporium, Postia placenta, Gloeophyllum trabeum* and *Trametes versicolor* (Rui and Morrell, 1993). The important step of protoplast isolation was lysis of cell wall and other

factors for effective release of protoplast were age of culture, enzyme combination, incubation time and presence of suitable osmotic stabilizer (Lalithakumari, 1996).

Fusion of protoplast from antagonistic *T. harzianum* strains T252, T253 and T259 iso.lated from sclerotia of *Sclerotina minor* was carried out with their complim.entary auxotrophs (Pecchia and Anne, 1989). The results revealed variations existing among the strains and their auxotrophs in the susceptibility to different enzyme mixtures. Protoplast fusion of auxotrophic mutants of *T. koningii* was carried out in which exposure of protoplasts to PEG at 30 per cent concentration for 10 min was sufficient to induce heterokaryon formation. Optimum pH of 5.5 and presence of 1 mM calcium chloride in fusion mixture enhanced protoplast fusion frequency (Hong *et al.,* 1984). Balasubramanian and Lalithakumari (2008) standardized methods for isolation, fusion and regeneration of protoplasts from *T. harzianum* and *T. viride*. Novozyme 234 (5 mg ml^{-1}) was used as lytic enzyme, potassium chloride as osmotic stabilizer and PEG as fusogen. The protoplast fusion frequency for inter-specific fusion was 1.92 per cent. For intra-specific fusion it was 6.2 per cent for *T. harzianum* and 7.2 per cent for *T. viride*. El-bondkly *et al.* (2011) compared electrofusion and PEG mediated fusion for creating fusants of *T. harzianum* isolates. The electrofusion method was found more effective for obtaining fusants with high β-glucosidase activity.

Intra-strain protoplast fusion of *T. harzianum* was carried out by Sharma *et al.* (2016). Most of the fusants exhibited fast and vigorous growth. One of the fusant *Th3fu5* showed highest inhibition against the pathogens *viz., S. sclerotiorum, F. oxysporum* and *A. brassicicola.*

2.7. Performance of protoplast fusants of *Trichoderma* spp.

Peer and Chet (1990) studied the antagonistic ability of protoplast fusants derived from auxotrophic mutants of *T. harzianum* against *R. solani, Sclerotium rolfsii* and *P. aphanidermatum*. The prototrophic strain A2 overgrew all pathogens compared to parental strains and also controlled *Rhizoctonia* damping off of cotton seedlings under greenhouse conditions. Protoplast fusion between *T. harzianum* and *T. longibrachiatum* resulted in fusants having characters of both parents *viz.,* growth pattern and pigmentation of *T. harzianum* and carbendazim and

copper sulphate tolerance of *T. longibrachiatum* (Lalithakumari *et al.,* 1996). These fusants also yielded best results in pot culture experiments. Prabhavathy *et al.* (2006a) reported two-fold increase of carboxy methyl cellulase activity in protoplast fusants SFTr2 and SFTr3 of *T. reesei* strain PTr2.

Out of 201 fusants obtained on intergeneric protoplast fusion between *T. reesei* QM 9414 and *Saccharomyces cerevisiae* NCIM 328, 107 fusants were found stable and two of them *viz.*, M14 and M62 exhibited enhanced ethanol production (Kumari and Panda, 1994).

Migheli *et al.* (1995) reported inter- and intra-strain protoplast fusion of hygromycin B and propiconazole resistant UV-induced mutants of two British and two Italian strains of *Trichoderma*. A high degree of variability in the biocontrol and mycoparasitic ability against *P. ultimum* on lettuce and *B. cinerea* on grape was found but without any significant increase in those traits.

Self-fusion of *T. harzianum via* protoplast carried out using PEG resulted in increased chitinase activity in most of the fusants. Four fusants exhibited two-fold increase in chitinase activity compared to parents (El-Bondkly and Talkhan, 2007). Mohamed and Haggag (2010) reported interspecific protoplast fusion of *T. reesei* and *T. koningii* for enhancement of biocontrol abilities. The fusion frequency was 1.25 per cent with 6.035×10^5 fusants. The mutants could bring about reduction in growth of *F. oxysporum* up to 98.6 per cent and *P. ultimum* up to 94.6 per cent. Most of the fusant isolates obtained from protoplast fusion of *T. harzianum* NBAII Th1 and *T. viride* NBAII Tv23 produced high antagonistic activity against *F. oxysporum* (Lakhani *et al*., 2016).

Hima (2017) carried out mutation and protoplast fusion of *T. erinaceum* Tr 9 and *T. asperellum* Tr 43. Mutation was done by the combination of UV treatment and sodium nitrate; and fusion using glucanex as lytic enzyme and PEG as fusogen. It was revealed that the mutants M40M3 (*T. erinaceum*) and K80M3 (*T. asperellum*); fusants F2 (*T. erinaceum*) and F4 (*T. asperellum*) were capable of bringing about significant disease suppression against soil borne pathogens of ginger.

The diploid strain D1 of *T. harzianum* obtained by protoplast fusion of *T. harzianum* wild strain CB-Pin-01 and a benomyl resistant mutant m1-100-32 exhibited high inhibition of *F. oxysporum* f. sp. *raphani* and *Pyricularia oryzae* in dual culture. It was also found that the fusant D1 strain produced enhanced levels of chitinase and *β*-1,3-glucanase. Soil inoculation of the fusant D1 strain of *T. harzianum* improved germination of radish plants and protected them against yellows disease incited by *F. oxysporum* f. sp. *raphani* (Ogawa *et al*., 2000). Five fusants from protoplast fusion of two fungicide tolerant mutants PTv-V of *T. viride* and PTz-F of *T. harzianum* showed inhibition of growth of *R. solani*, *F. oxysporum* and *P. ultimum in vitro*. One of the fusants, Fus 7 alone and in combination with fungicides Thiophanate-methyl and Vitavax could reduce damping off disease in tomato (Fahmi *et al.,* 2012).

Hassan (2014) had demonstrated the fusion of high chitinase producing *T. harzianum* and high cellulase producing *T. viride* strains. It was found that there was about three-fold increase in chitinase and *β*-1, 3-glucanase production among the fusants. In dual culture assays conducted against grapevine soil pathogens, one of the fusants, *Fus. 15* exhibited 92.8 per cent inhibition against *M. phaseolina* and *P. ultimum* and another fusant *Fus. 9* showed 98.2 per cent inhibition against *S. rolfsii*.

El- Bondkly and Talkhan (2007) observed that the self fusants *viz.*, ATh1/9, ATh1/12, ATh1/14 and ATh1/17 exhibited two-fold increased chitinase activity and complete *in vitro* inhibition over *Cephalosporium acremonium* and *R. solani* than the parent strain ATh1 of *T. harzianum*. Montealegre *et al.* (2014) reported that protoplast fusants of *T. harzianum* ThF2-1, ThF3-3, ThF4-15 and ThF5-8 could inhibit *R. solani in vitro* by 60 to 91 per cent. In glass house experiments, ThF2-1 and ThF5-8 could significantly reduce tomato canker caused by *R. solani.*

In vitro efficacy of *Trichoderma* fusants F1, F2, F3, F4, and F6 tested against *P. aphanidermatum* and *R. solani* showed high percentage of inhibition than parents. Among them, F2 showed high inhibition (92 %) against *P. aphanidermatum* and F2 and F6 exhibited better antagonistic property against *R. solani.* Most of the fusants showed over growth as mode of antagonisim (Hima, 2017). Chandrappa and

Basavarajappa (2017) studied the antagonistic activity of inter- and intra-specific protoplast fusants of *T. harzianum, T. virens, and T. viride* against *F. oxysporum f. sp. udum* and *F. solani*, the causal agents of wilt disease in pigeon pea and acid lime. Among eight intra-fusants, fusant *Tvdsf-2* recorded highest per cent inhibition (93.70 %) against *F. oxysporum* f. sp. *udum* and *Tvssf-1* (86.70 %) against *F. solani.* Among the inter-specific fusants, the maximum per cent inhibition of mycelial growth was observed in Tvs×Tas-1 (94.07 %) and Tvd×Tas-1 (94.07 %) against *F. oxysporum f. sp. udum* and Tvd×Tas-1 (89.63 %) against the *F. solani*.

Srinivasan *et al.* (2009) reported that the strains evolved out of inter-specific protoplast fusion of *T. harziaum* and *T. reesei* displayed 40-50 per cent increased cellulose production and 10-20 per cent increased chitinase production than the parent strains. The antagonistic activity of the fusants against *R. solani* was also enhanced by seven to eight per cent. Dolatabad *et al*. (2019) studied the fusant Fu3 obtained from protoplast fusion of *T. harzianum* TH5-1-2 and TH10-2-2 exhibited significant *in vitro* inhibition of *R. solani, S. sclerotiorum* and *Aspergillus flavus.* Fusants Fu3 and Fu5 showed high chitinase activity. Under liquid chromatography-mass spectrometry (LC-MS) study, the antifungal secondary metabolites harzianopyridone and chrysophanic acid compounds were identified in the fusant F3.

Materials and Methods

3. MATERIALS AND METHODS

3.1. Collection and isolation of different isolates of *Trichoderma* from five agro climatic zones

The soil samples were collected from virgin forest area located in Northern, Central, High range, Problem area and Southern zone of Kerala coming under the districts of Malappuram, Idukki, Wayanad, Kottayam, Pathanamthitta and Thiruvananthapuram.

3.1.1. pH of collected soil samples

The pH of collected soil samples was determined by potentiometric method using a pH meter. Soil samples were shade dried separately and sieved through a mesh size of 2 mm. The dried soil samples each with 10 g 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 pH was recorded using a pH meter.

3.1.2. Trichoderma enumeration

Trichoderma spp. were isolated from virgin forest soils collected from five agro climatic zones of Kerala *viz*., Northern, Central, High range, Problem area and Southern zone. The collected soil samples were pooled separately, shade dried and the total microbial population of *Trichoderma* spp. was estimated by serial dilution plate technique (Johnson and Curl, 1972). Microbial count of *Trichoderma* spp. was estimated at 10^{-3} and 10^{-4} dilutions on Trichoderma Selective Medium (TSM). The composition of TSM included $MgSo_4.7H_2O$: 0.2 g, K_2HPO_4: 0.9 g, KCl: 0.15 g, NH_4NO_3: 1.0 g, Glucose: 3.0 g, Chloramphenicol: 0.25 g, Rose Bengal: 0.15 g, Agar: 20 g/l.

Ten grams of shade dried soil was transferred to 90 ml of sterilized water in 250 ml conical flask and shaken well for 10 min. in a shaker to get 10^{-1} dilution and again 1 ml from 10^{-1} was transferred to 9 ml sterile distilled water to make 10^{-2} dilution. The serial dilutions were continued up to 10^{-4} dilution. One ml each from 10^{-3} and 10^{-4} dilutions were transferred separately to sterilized Petri dishes, TSM was poured on the transferred dilutions 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. were recorded. The colonies showing the cultural characters of *Trichoderma* spp. were transferred to potato dextrose agar (PDA) slants. These isolates were purified and sub cultured and maintained as pure culture for further work.

3.1.3. Radial growth of *Trichoderma* isolates

The *Trichoderma* isolates were transferred into Petri dish with PDA medium using cork borer. The growth of colonies was observed at 24 h interval. Radial growth (cm) was measured at third, fifth and seventh day of inoculation (DAI).

3.1.4. Colony characters of *Trichoderma* isolates

The cultural and morphological characters of 31 *Trichoderma* isolates were studied on PDA medium. From the actively growing edge of a fresh colony, a cut was made using sterile cork borer and placed on the petriplates containing PDA medium. Three replications of each isolate were maintained. Petri dishes were incubated at room temperature and the colonies were examined at 24 h intervals for the colour of mycelium, texture of colony and growth pattern.

3.2. Isolation of different pathogens

Pythium aphanidermatum was isolated from damping-off infected tomato seedlings collected from Regional Agricultural Research Station, Kumarakom and *Rhizoctonia solani* from collar rot infected cowpea plants at College of Agriculure, Vellayani.

The infected tissue was cut into small bit (2-4 mm) with a margin of healthy tissue around it, surface sterilized with 0.1 per cent mercuric chloride solution for one min and then rewashed in two to three changes of sterile distilled water. These bits were placed on PDA medium in sterilized Petri dishes. The dishes were incubated at room temperature (28 ± 2 ºC). When the growth of the fungus was visible, mycelial bits were transferred aseptically to PDA slants. The slants were incubated at room temperature.

3.3. *In vitro* screening of *Trichoderma* isolates against fungal pathogens

Isolates were tested for their antagonistic efficacy against *P. aphanidermatum* and *R. solani*. The antagonistic index was calculated using parameters like overgrowth, lysis and antibiosis.

3.3.1. Dual culture assay of *Trichoderma* isolates against *P. aphanidermatum* and *R. solani*

The isolates of *Trichoderma* spp. were preliminary screened for their antagonistic efficacy against two important soil-borne fungal pathogens, *viz*., *P. aphanidermatum* and *R. solani* by dual culture method outlined by Skidmore and Dickinson (1976) under *in vitro* condition on PDA medium. From actively grown culture of pathogens, 8 mm mycelial disc was cut off with the help of cork borer and placed at 2 cm away from edge of PDA medium in Petri dish. Mycelial disc of 8 mm diameter of each isolate of *Trichoderma* spp. was transferred to the same plate and was placed at 2 cm away from the opposite side. Three replications were maintained for each treatment. The pathogen grown as monoculture on one side 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.

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

$$PI = [C\text{-}T/C] \times 100$$

Where, C = Radial growth of pathogen (cm) in control

T= Radial growth of pathogen (cm) in treatment

3.3.2. Assessment of zone of lysis, antibiosis and overgrowth (Antagonistic index)

The modes of antagonism of like lysis, antibiosis and overgrowth of isolates were tested against phytopathogens. A similar method as mentioned in section 3.3.1. was followed. Observations were recorded when the control plate exhibited full

growth. Overgrowth of isolates of *Trichoderma* spp, against phytopathogens after 72 h was recorded. The lysis between pathogen and *Trichoderma* spp. was observed as the region where pathogen was wiped out leading to formation of clear zone during the pathogen-antagonist interaction. Antibiosis could be observed at the point of interaction between the pathogen and antagonist which was observed as pigmented region.

Antagonistic index is multiple of overgrowth of *Trichoderma* spp., lysis of pathogen and antibiosis. Three isolates of *Trichoderma* spp. were selected for further studies based on *in vitro* inhibition and the antagonistic index parameters.

3.4. Strain improvement of *Trichoderma* isolates through protoplast fusion

3.4.1. Testing the sensitivity of selected *Trichoderma* isolates against fungicide

The three isolates *Trichoderma* spp. *viz.*, TRPN14, TRSN1 and TRMW2 were screened for tolerance against fungicide carbendazim on PDA medium by poisoned food technique (Zentmeyer, 1955).

PDA medium of 100 ml was sterilized in 250 ml conical flask. Different concentrations (0.1%, 0.2% and 0.4%) of carbendazim was mixed separately with the sterilized medium in conical flask, shaken well and poured into sterilized Petri plates @ 20 ml per plate. Different isolates of *Trichoderma* spp. from actively growing culture with 8 mm diameter was placed on Petri dish containing the poisoned medium. Six replications were maintained for each concentration of fungicide amended medium and a control plate without fungicide was maintained. All the dishes were incubated at room temperature at 28 ± 2°C. The radial growth of each isolate of *Trichoderma* spp. was recorded daily till the control plate was fully covered with the growth of the isolate. The per cent inhibition on the growth of isolates was calculated by using the formula mentioned in 3.3.1.

3.4.2. Isolation and purification of protoplasts

Protoplast were isolated and purified from selected isolates of *Trichoderma* spp. as per standard procedure described by Gnanam (2013). Conidial suspension was prepared by conidia collected from seven- day-old culture plate of *Trichoderma* spp. The surface of culture plate was gently scratched with inoculation

loop and resulting suspension was collected using micropipette. One millilitre of conidial suspension of *Trichoderma* isolates were inoculated in 250 ml conical flask containing 100 ml sterile PD broth and incubated at 28 ± 2 °C in a shaker of 120 rpm for 24 h. The young mycelia (100 mg) was harvested and filtered through Whatman filter paper No.1, washed thrice with 5 ml sterile distilled water followed by three washes with osmotic stabilizer (KCl 0.6M with pH 6.5). The washed mycelium of *Trichoderma* isolates were transferred to sterile centrifuge tube containing two millilitre of enzyme mixture.

Enzymatic mixture/ Protoplasting buffer (PB): The parental isolates were inoculated in PB consists of osmotic stabilizer (KCl 0.6 M with pH 6.5) containing 0.7 M NaCl, 0.2 M $CaCl_2$ and lytic enzyme Glucanex (12 mg ml^{-1}, Sigma-Aldrich, L1412) and incubated in room temperature (28 ± 2^0 C) in a shaker for 4 h at 200 rpm The enzymatic mixture was sterilized using sterilized filter disc assembly just before use. The released protoplasts were observed under high resolution compound microscope (Leica, USA) at an interval of every 30 min.

Purification of protoplast was carried out according to Gnanam (2013). The release of maximum number of mature protoplasts was completed at four hours and the crude protoplast suspension was filtered through six layers of sterile cheese cloth to remove the hyphal debris. Protoplast were then collected and sedimented from the filtrate by centrifugation at 2000 rpm for 15 min. The supernatant was carefully removed with micropipette and resuspended in five millilitre of the same osmotic stabilizer.

3.4.3. Protoplasmic fusion

Fusion of isolated protoplasts will be carried out by PEG method according to Anne and Peberdy (1975) with slight modifications. The purified protoplasts of *Trichoderma* spp. was kept in osmotic stabilizer (KCl 0.6M with pH 6.5). Equal volume of protoplast suspension of two parental isolates (1 µl each) was mixed with 1 µl of fusion buffer in sterile centrifuge tube. The mixture was incubated at room temperature for 20 min by shaking the tube manually at every 5 min. The fusion buffer (FB) consisted of phosphate buffer (0.06 M, pH 6.5) containing 30 per cent poly ethylene glycol (PEG) (molecular weight 4000), 50 mM $CaCl_2$ and 0.7 M NaCl.

After uniform mixing, the mixture was centrifuged at 2000 rpm for 10 min. Pelleted protoplasts were washed twice with the osmotic stabilizer and resuspended in 5 ml of osmotic stabilizer. The fusion process was examined by observing the aliquots (10 µl) of fusion suspension under high resolution compound microscope (Leica, USA).

3.4.4. Selection of fusants

Malt extract agar medium used for the regeneration medium for the protoplast fusant. 100 µl of PEG-treated fused protoplast was plated on to petri dish containing malt extract agar medium amended with fungicide carbendazim (0.2 %) and incubated at room temperature for three days. Colonies growing on the surface of the plates were considered as fusants. Single colonies of three fusants were isolated from the selection media.

3.5. Screening of protoplast fusants against soil-borne fungal plant pathogens

3.5.1. Radial growth of fusants

A similar method as mentioned in section 3.1.4 was followed. Radial growth (cm) of parental *Trichoderma* isolates and fusants (F1, F2 and F3) was measured at third, fifth and seventh DAI.

3.5.2. Colony characters of fusants

The cultural and morphological characters of fusant isolates were compared with the parental isolates on PDA. A similar method as mentioned in section 3.1.4 was followed.

3.5.3. *In vitro* screening of fusant Trichoderma isolates against *P. aphanidermatum* and *R. solani*

The fusant isolates of screened for their antagonistic efficacy against *P. aphanidermatum* and *R. solani.* A similar method as mentioned in section 3.3.1 was followed.

3.5.4. Assessment of zone of lysis, antibiosis and overgrowth (Antagonistic index)

The modes of antagonism like lysis, antibiosis and overgrowth of the fusant isolates were assessed against *P. aphanidermatum* and *R. solani*. A similar method as mentioned in section 3.3.2 was followed.

3.6 Statistical analysis

Analysis of variance was done on the data collected using statistical package OPSTAT. Multiple comparisons among the treatments were done by using WASP 2.0.

Results

4. RESULTS

The present study on "Strain improvement of *Trichoderma* spp. by protoplast fusion" was conducted during the period of 2018-2020 at the Department of Plant Pathology, College of Agriculture, Vellayani, for enhancing the biocontrol efficacy of isolated *Trichoderma* spp. against important soil borne fungal pathogens by protoplast fusion.

4.1. Collection and isolation of different isolates of *Trichoderma* from five agroclimatic zones

The soil samples were collected from virgin forest soils of five agroclimatic zones of Kerala *viz*, Northern, Central, High range, Problem area and Southern zone (Plate 1). The list of places for sample collection is given in Table 1. A total of 11 soil samples were collected from different locations.

4.1.1. pH of collected soil samples

The pH of soil samples collected from different locations was estimated and pH of each location is listed in Table 2. The pH range between 3 and 7 was observed. The least pH was recorded in sample from Idukki (3.8) and highest from Thiruvananthapuram (6.8). The influence of pH on the occurrence of *Trichoderma* spp. is listed in Table 3. It was found that majority of isolates (70.9 %) occurred in pH range of 6-7.

4.1.2. Trichoderma enumeration

The isolation of *Trichoderma* spp. from collected soil samples was done by serial dilution technique on Trichoderma selective medium (TSM) at 10^3 and 10^4 dilutions. The microbial colonies were observed from two days after inoculation. The data of enumeration of *Trichoderma* spp. is given in Table 4. A total of 31 isolates were obtained; one from Northern zone, nine from Central zone, four from High range zone, five from Problem area zone and 12 from Southern zone. The isolates were named as TRML1 (Northern zone), TRSN1, TRSN2, TRMW1, TRMW2, TRMW3, TREN1, TREZ1, TREZ2, TREZ3 (Central zone), TRLL1, TRTL2, TRTL5, TRTL6 (High range zone), TRRN1, TRRN2, TRKM1, TRKM2, TRIL1 (Problem

area zone), TRPN3, TRPN7, TRPN9, TRPN10, TRPN11, TRPN14, TRPN15, TRPN 17, TRPN18, TRKR1, TRKR2 and TRKR3 (Southern zone)

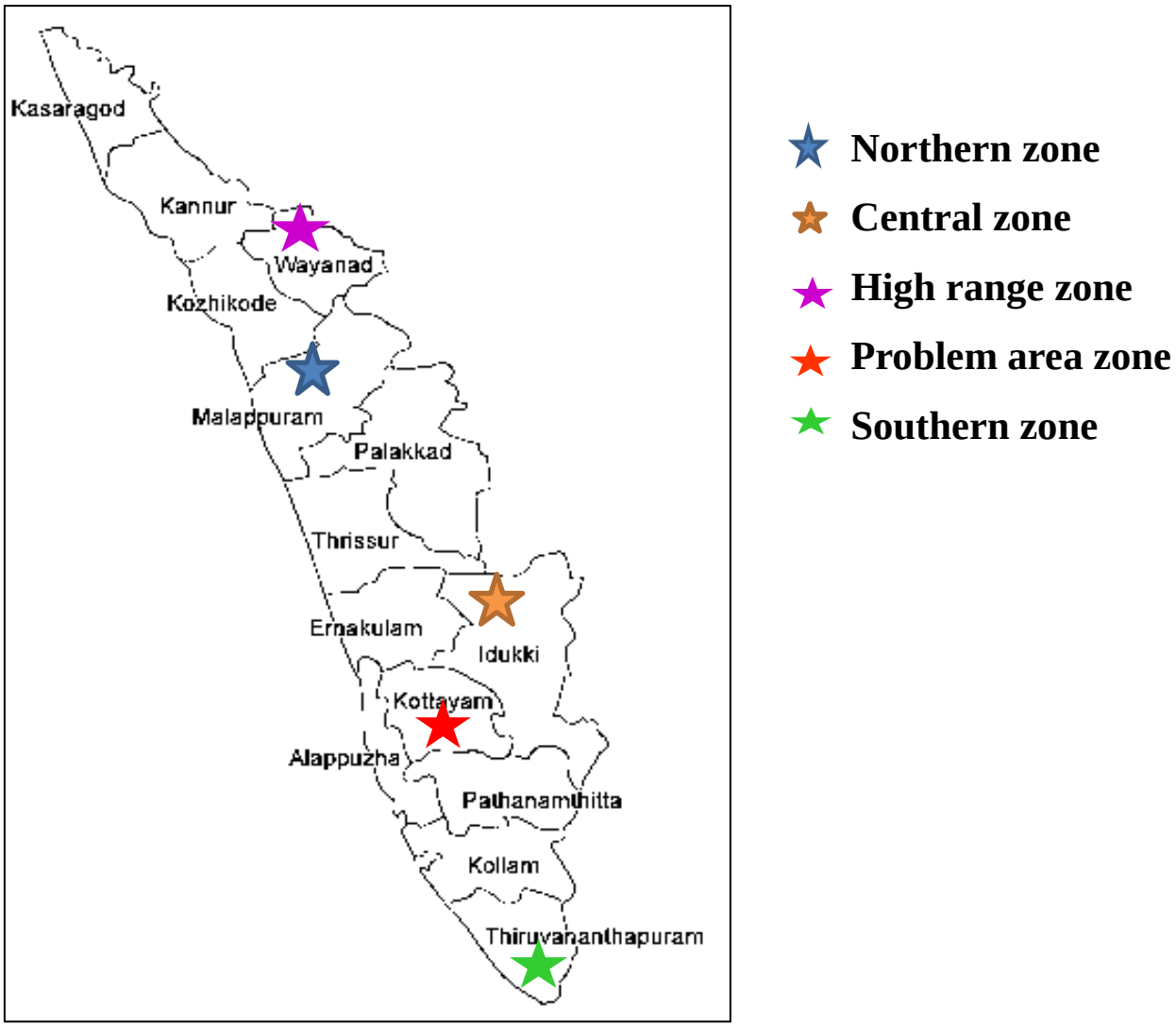


Plate 1. Map showing survey locations of soil sample collection in five agroclimatic zones of Kerala

Table 1. Survey locations of soil sample collection from five different agroclimatic zones of Kerala

Sl. No.	Soil sample	District	Location
1	M1ARM	Malappuram	Arimbra `
2	I1SNP	Idukki	Sholay National park
3	I2MWD	Idukki	Munnar wild life Division
4	I3ENP	Idukki	Eravikulam National Park
5	I4EZM	Idukki	Ezhumalai
6	W1THR	Wayanad	Thirunelli
7	K1KMK	Kottayam	Kottayam
8	K2ILK	Kottayam	Illickal
9	P1RNI	Pathanamthitta	Ranni
10	T1PND	Thiruvananthapuram	Ponmudi
11	T2KTR	Thiruvananthapuram	Kottur

Table 2. pH of soil samples collected from different locations

Sl. No.	Soil sample	District	pH of the soil
1	M1ARM	Malappuram	6.50
2	I1SNP	Idukki	4.90
3	I2MWD	Idukki	3.83
4	I3ENP	Idukki	5.57
5	I4EZM	Idukki	5.25
6	W1THR	Wayanad	6.38

7	K1KMK	Kottayam	6.23
8	K2ILK	Kottayam	6.30
9	P1RNI	Pathanamthitta	6.50
10	T1PND	Thiruvananthapuram	6.50
11	T2KTR	Thiruvananthapuram	6.80

Table 3. Influence of pH on occurrence of different isolates of *Trichoderma* spp. in soil

Sl. No.	pH range	Soil Sample	No. of *Trichoderma* isolates	Per cent
1	3 - 4	I2MWD	3	9.6
2	4 - 5	I1SNP	2	6.4
3	5 - 6	I3ENP, I4EZM	4	12.9
4	6 - 7	W1THR, M1ARM, T1PND, T2KTR, K1RN1, K2KMK, K3ILK	22	70.9
	Total		31	

Table 4. Population of *Trichoderma* spp. in soil samples collected from different survey locations

Sl. No.	Soil sample	Population of *Trichoderma* spp. (cfu g^{-1}) grown on TSM		Number of isolates
		10^3	10^4	
1	W1THR	8	3	4
2	M1ARM	10	3	1
3	I1SNP	14	4	2
4	I2MWD	6	7	3
5	I3ENP	3	3	1

6	I4 EZM	5	3	3
7	T1PND	23	20	9
8	T2KTR	20	11	3
9	K1RNI	17	4	2
10	K2KMK	34	12	2
11	K3ILK	24	17	1

4.1.3. Radial growth of *Trichoderma*

Trichoderma isolates were inoculated on PDA media and the radial growth of isolates was recorded. Most of the isolates were fast growers. Table 5 shows the radial growth of Trichoderma from North and Central zone. The TRML1 isolate from Northern zone showed fast growth with thick green sporulation and completed growth within six days. Similarly isolates from Central zone also exhibited fast growth. Isolate TRMW2, TREN1, TREZ1, TREZ2 and TREZ3 attained complete growth at 5 DAI. All the isolates from High range zone attained full plate growth at seven days after inoculation on PDA (Table 6). The isolates TRRN1 and TRRN2 from Problem area zone exhibited complete mycelial growth within five days after inoculation while isolates TRKM1, TRMM2 and TRIL1 reached complete growth at seven days after inoculation. The radial growth of isolates obtained from Problem Area Zone is detailed in Table 7. The radial growth of isolates from Southern zone showed in Table 8. Isolates TRKR1, TRPN3, TRPN7, TRPN10 and TRPN18 completed growth by 4 DAI.

Table 5. Radial growth of different isolates of *Trichoderma* spp. from Northern and Central zone

Isolate	**Radial growth on PDA medium**		
	3 DAI (cm)	**5 DAI (cm)**	**7 DAI (cm)**
Northern zone			
TRML 1	5.66	8.66	9.00
Central zone			

TRSN1	3.20 ± 0.11[bc]	8.03 ± 0.08[b]	9.00
TRSN2	4.03 ± 0.08[a]	8.06 ± 0.03[ab]	9.00
TRMW1	4.60 ± 0.11[a]	8.53 ± 0.08[a]	9.00
TRMW2	6.17 ± 0.12[bc]	9.00 [c]	9.00
TRMW3	3.27 ± 0.17[c]	7.13 ± 0.08 [c]	9.00
TREN1	6.03 ± 0.14[c]	9.00 [c]	9.00
TREZ1	5.96 ± 0.05[c]	9.00 [c]	9.00
TREZ2	6.30 ± 0.05 [c]	9.00 [c]	9.00
TREZ3	5.90 ± 0.88[c]	9.00 [c]	9.00
CD (0.05)	0.33	0.15	
SE (m)	0.11	0.05	

Mean ± SD of nine replications

Values followed by similar superscripts are not significantly different at 5% level

Table 6. Radial growth of different isolates of *Trichoderma* spp. from High range zone

Isolate	**Radial growth on PDA medium**		
	3 DAI (cm)	**5 DAI (cm)**	**7 DAI (cm)**
TRTL1	5.40 ± 0.05[a]	6.63 ± 0.30[a]	9.00
TRTL2	5.93 ± 0.03[b]	7.26 ± 0.03[b]	9.00
TRTL5	6.03 ± 0.14[b]	7.16 ± 0.08[b]	9.00
TRTL6	5.10 ± 0.11[b]	6.83 ± 0.2[b]	9.00
CD (0.05)	2.78	3.86	
SE (m)	0.09	0.19	

Mean ± SD of five replications

Values followed by similar superscripts are not significantly different at 5% level

Table 7. Radial growth of different isolates of *Trichoderma* spp. from Problem area zone

Isolate	Radial growth on PDA medium		
	3 DAI (cm)	5 DAI (cm)	7 DAI (cm)
TRRN1	6.28 ± 0.12 [a]	9.00 [a]	9.00
TRRN2	5.23 ± 0.14 [a]	9.00 [a]	9.00
TRKM1	5.73 ± 0.08 [b]	8.96 ± 0.03[b]	9.00
TRKM2	5.26 ± 0.12[b]	8.36 ± 0.08[b]	9.00
TRIL1	5.26 ± 0.14[b]	8.80 ± 0.05[b]	9.00
CD (0.05)	2.61	3.97	
SE (m)	0.12	0.04	

Mean ± SD of four replications

Values followed by similar superscripts are not significantly different at 5% level

Table 8. Radial growth of different isolates of *Trichoderma* spp. from Southern zone

Isolate	Radial growth on PDA medium		
	3 [rd] day (cm)	5[th] day (cm)	7[th] day (cm)
TRPN3	6.90 ± 0.05[ab]	9.00 [b]	9.00
TRPN7	6.16 ± 0.06[c]	9.00 [c]	9.00
TRPN9	4.60 ± 0.05[c]	8.56 ± 0.08[c]	9.00
TRPN 10	6.36 ± 0.08[c]	9.00 [c]	9.00
TRPN11	3.56 ± 0.08[c]	7.36 ± 0.08[c]	9.00
TRPN 14	4.50 ± 0.05[c]	6.90 ± 0.03[c]	9.00
TRPN 15	5.00 ± 0.05[c]	8.73 ± 0.03[c]	9.00
TRPN 17	3.76 ± 0.08[c]	6.90 ± 0.05[c]	9.00

TRPN 18	6.70 ± 0.11[c]	9.00 [c]	9.00
TRKR1	5.93±0.12[ab]	9.00 [a]	9.00
TRKR2	6.00±0.01[a]	8.96 ± 0.03[a]	9.00
TRKR3	5.13 ± 0.08[b]	8.73 ± 0.03[b]	9.00
CD (0.05)	1.05	0.73	
SE (m)	0.08	0.06	

Mean ± SD of four replications

Values followed by similar superscripts are not significantly different at 5% level

4.1.4. Colony characters of *Trichoderma* isolates

The cultural characters of isolates were evaluated by subculturing the isolates on PDA medium. The mycelial growth started from 2 DAI. The cultural characters of the isolates are shown in Table 9. Growth pattern of isolates from each zone was observed after completion of mycelial growth on the petriplate (Plates 2 to 6).

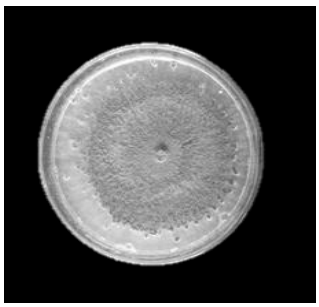
Table 9. Colony characters of different isolates of *Trichoderma* spp. grown on PDA medium at 7 days after inoculation

Sl. No.	Isolate	Colour of mycelium	Texture of colony	Sporulation/Growth Pattern
1	TRML1	Dark Green	Medium Fluffy	Circular dark green sporulation
2	TRSN1	White	Highly Fluffy	Less sporulation
3	TRSN2	White	Highly fluffy	No sporulation
4	TRMW1	Light green	Smooth	Circular green sporulation
5	TRMW2	Dark green	Highly fluffy	Circular sporulation
6	TRMW3	White	Fluffy	Circular sporulation
7	TREN1	Green	Fluffy	Sporulation as thick ring
8	TREZ1	Green	Smooth	Green sporulation at centre
9	TREZ2	Green	Fluffy	Circular sporulation
10	TREZ3	White	Smooth	High sporulation

11	TRTL1	Green	Fluffy	Circular sporulation
12	TRTL2	Dark Green	Fluffy	Circular sporulation
13	TRTL5	Dark Green	Fluffy	Circular sporulation
14	TRTL6	Green	Fluffy	Circular sporulation
15	TRRN1	Dark green	Raised	Circular sporulation
16	TRRN2	Dark green	Fluffy	Circular sporulation
17	TRKM1	White	Smooth	Green ring type sporulation
18	TRKM2	White	Smooth	Light yellow sporulation
19	TRIL1	Dark green	Smooth	Circular sporulation
20	TRKR1	Dark green	Spreading smooth	Thick circular sporulation
21	TRKR2	Dark green	Spreading	Circular sporulation
22	TRKR3	Dark green	Smooth	Sporulation as ring
23	TRPN3	White	Fluffy raised	No sporulation
24	TRPN7	White	Fluffy raised	No sporulation
25	TRPN9	Dark green	Spreading smooth	Sporulation at centre
26	TRPN 10	White	Fluffy	Yellow sporulation at centre
27	TRPN11	Green	Spreading	Circular sporulation
28	TRPN 14	Green	Fluffy	Less sporulation
29	TRPN 15	Green	Spreading	Circular sporulation
30	TRPN 17	Green	Fluffy raised	Sporulation at centre
31	TRPN 18	Dark green	Fluffy raised	Less sporulation

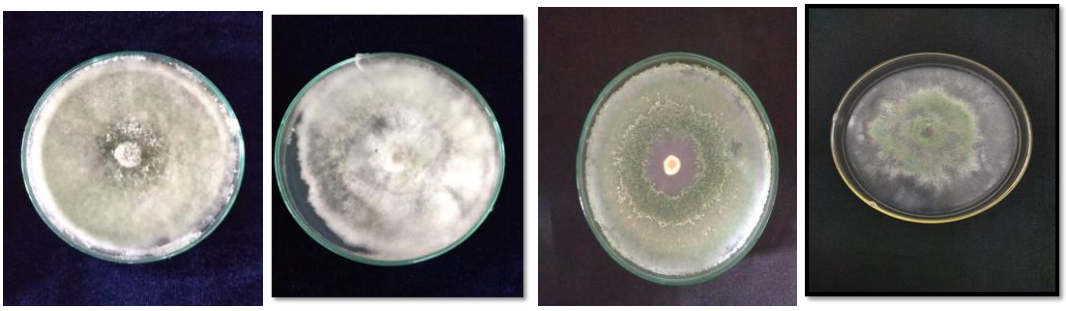
4.1.4.1. Growth Pattern

Varying degree of growth pattern from no sporulation to heavy circular green sporulation was observed. Sporulation patterns with thick dense, sporulation at centre or circular, spreading sporulation were seen. Most of the isolates produce green sporulation except isolates like TRSN2, TRPN3 and TRPN7. Dark green sporulation was observed in isolate TRML while it was yellow in isolates TRKM2 and TRP

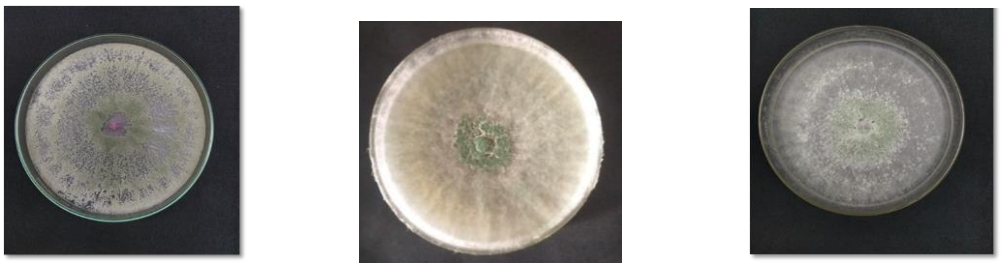


TRML1

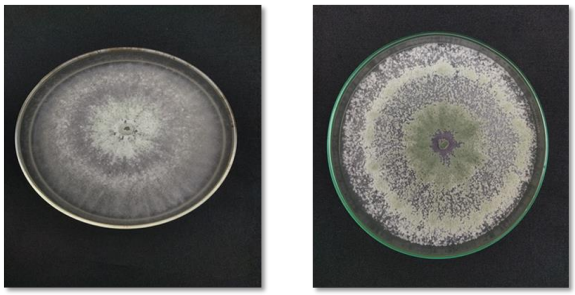
Plate 2. Isolates of *Trichoderma* spp. obtained from Northern zone of Kerala



TRSN1 **TRSN2** **TREN1** **TRMW1**

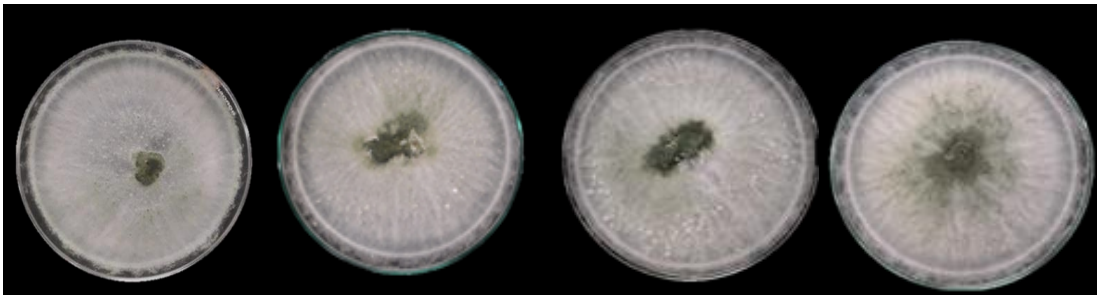


TRMW2 **TRMW3** **TREZ1**



TREZ2 **TREZ3**

Plate 3. Isolates of *Trichoderma* spp. obtained from Central zone of Kerala



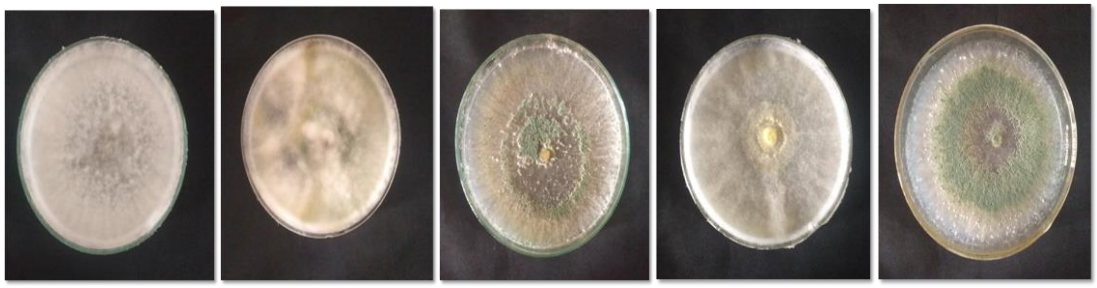
TRTL1 **TRTL2** **TRTL5** **TRTL6**

Plate 4. Isolates of *Trichoderma* spp. obtained from High range zone of Kerala

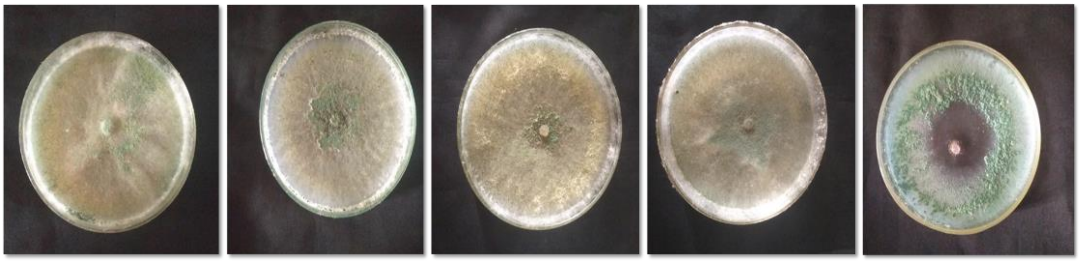
TRKM1 **TRKM2** **TRIL1**

TRRN1 **TRRN2**

Plate 5. Isolates of *Trichoderma* spp. obtained from Problem area zone of Kerala



TRPN3 **TRPN7** **TRPN9** **TRPN 10** **TRPN11**



TRPN 14 **TRPN 15** **TRPN 17** **TRPN 18** **TRKR 1**

TRKR2 **TRKR3**

Plate 6. Isolates of *Trichoderma* spp. obtained from Southern zone of Kerala

4.1.4.2. Texture of isolates

Texture of isolates showed variations like smooth, fluffy and spreading type. Majority of the isolates showed fluffy type. The isolates TRSN1, TRSN2 and TRMW2 exhibited highly fluffy mycelial growth while isolates TRMW1, TREZ1, TREZ3, TRKM1, TRKM2, TRIL1 and TRKR3 displayed smooth mycelial growth.

4.1.4.3. Colour of isolates

Colour of culture differed with different isolates of *Trichoderma* spp. They exhibited wide ranges of green colour from light green to dark green. Some isolates showed white colour (TRSN1, TRSN2, TRMW3, TREZ3, TRKM1, TRKM2, TRPN3, TRPN7 and TRPN10).

4.2 Radial growth of different pathogens

The major soil borne fungal pathogens *viz., P. aphanidermatum* and *R. solani* associated with the tomato and cowpea, respectively were isolated on PDA medium. The pathogens were purified and maintained as pure culture. The pathogen cultures were inoculated into PDA medium on Petri plates. Both the pathogens were fast growing. The mycelium of *R. solani* completely covered the plate within three days and formed sclerotia from fifth day. *P. aphanidermatum* completed its mycelial growth within four to five days.

4.3. *In vitro* screening of *Trichoderma* isolates against fungal pathogens

4.3.1. Dual culture assay of *Trichoderma* isolates against *P. aphanidermatum* and *R. solani*

The *in vitro* evaluation of *Trichoderma* isolates was carried out by dual culture method against fungal pathogens *P. aphanidermatum* and *R. solani.* The percent inhibition of pathogen by the antagonist isolates is presented in Tables 10 to 12.

4.3.1.1. Evaluation of antagonistic efficacy of *Trichoderma* isolates from Northern and Central zone

The result of evaluation of isolates against *P. aphanidermatum* is given in Table 10. All the isolates exhibited high antagonistic activity. Highest inhibition percent against *P. aphanidermatum* was observed in isolate TRML1 (97.40) followed by TRSN1 (95.18) and TRSN2 (90.37) (Plate 7).

Against *R. solani,* complete inhibition (cent per cent inhibition) was observed in isolates TREN1, TRMW2, TREZ1 and TREZ2. All the isolates exhibited more than 80 per cent inhibition (Plate 8 and 9). Majority of isolates exhibited high inhibition percent compared to the KAU strain of *Trichoderma.*

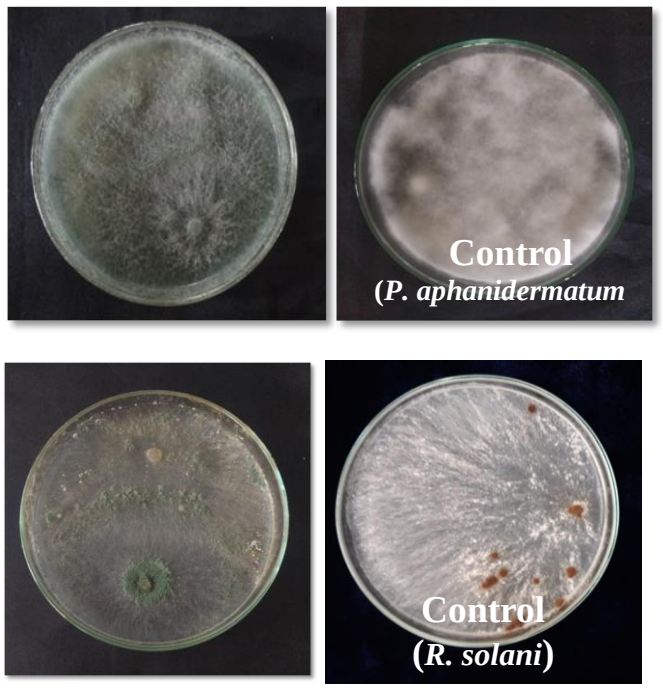
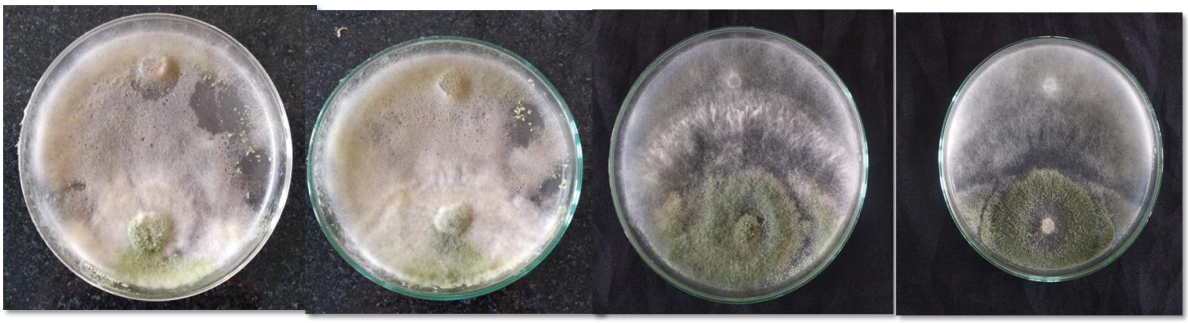


Plate 7. Overgrowth by *Trichoderma* isolate TRML1

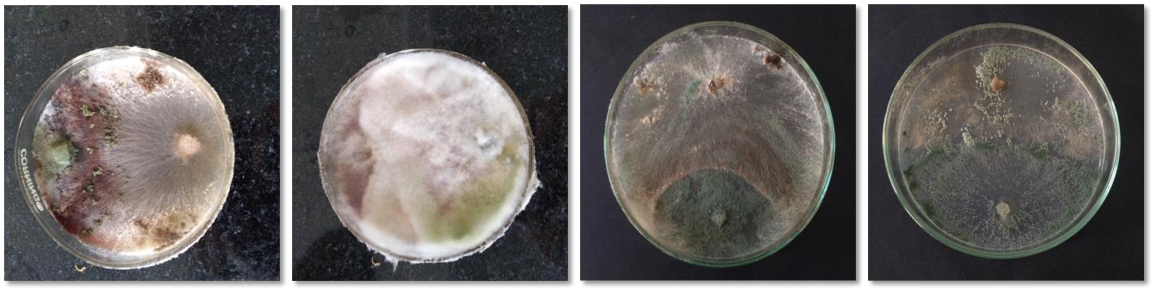


TRSN1 **TRSN2** **TRMW1** **TRMW2**

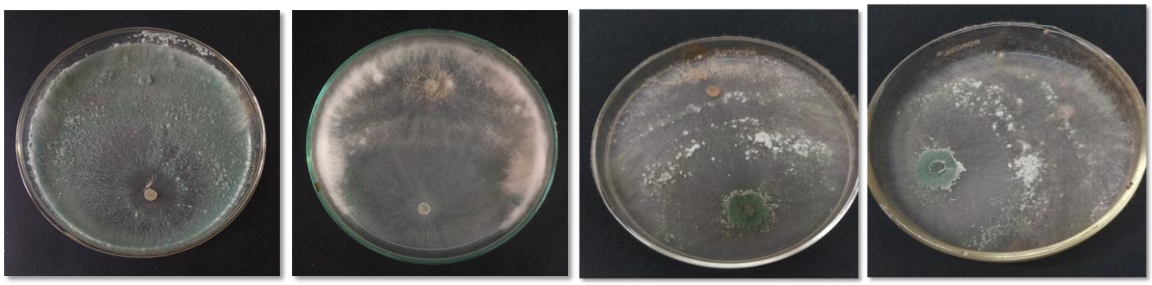
TRMW3 **TREN1** **TREZ1** **TREZ2**

TREZ3 ***T.viride*** **(KAU strain)** **Control**

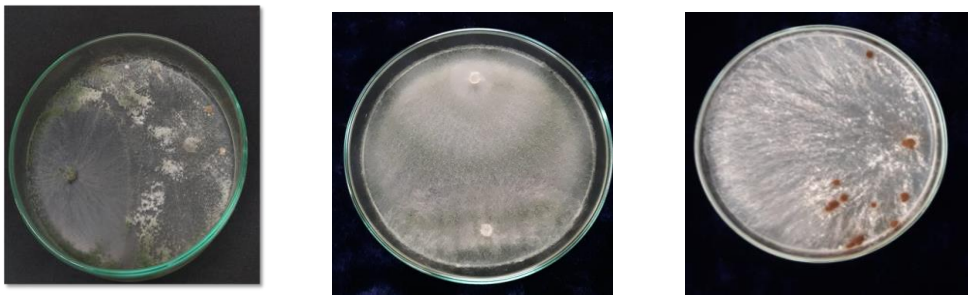
Plate 8. Inhibition of mycelial growth of *P. aphanidermatum* by *Trichoderma* isolates from Central zone



TRSN1 **TRSN2** **TRMW1** **TRMW2**



TRMW3 **TREN1** **TREZ1** **TREZ2**



TREZ3 ***T. viride* (KAU)** **Control**

Plate 9. Inhibition of mycelial growth of *R. solani* by *Trichoderma* isolates from Central zone

Table 10. Per cent inhibition of *P. aphanidermatum* and *R. solani* by isolates of *Trichoderma* spp. from Northern and Central zone

Zone	Isolate	*P. aphanidermatum*		*R. solani*	
		Radial growth (cm)*	***Inhibition (%)*** **	***Radial growth (cm)****	***Inhibition (%)*** **
Northern zone	TRML1	10.10	97.40 (80.64)	10.06	98.50 (84.26)
Central zone	TREN1	22.00	75.18^{c} (60.11)	10.00	100^{a} (89.71)
	TRSN1	4.30	95.18^{a} (77.28)	10.04	98.88^{b} (84.90)
	TRSN2	8.60	90.37^{b} (71.91)	10.07	98.14^{b} (83.45)
	TRMW1	18.60	79.25^{c} (47.78)	11.37	58.88^{d} (50.00)
	TRMW2	43.00	52.22^{g} (46.23)	10.00	100^{a} (89.71)
	TRMW3	25.00	72.22^{d} (58.195)	10.09	97.77^{b} (81.45)
	TREZ1	34.60	61.48^{ef} (51.63)	10.00	100^{a} (89.71)
	TREZ2	33.30	62.96^{f} (52.5)	10.00	100^{a} (89.71)
	TREZ3	29.00	67.77^{e} (55.30)	10.54	85.18^{c} (67.34)
T. viride (KAU Strain)		26.00	71.10^{d} (57.25)	10.68	80.7^{d} (64.00)
Control		9.00		9.00	
CD (0.05)		0.45	18.70	0.32	23.90

*Log transformed; ** Values in parenthesis are arcsine transformed.

Values followed by similar superscripts are not significantly different at 5% level

4.3.1.2. Evaluation of antagonistic efficacy of *Trichoderma* isolates from High range zone

The results given in Table 11 indicated that the inhibition percent of *P. aphanidermatum* ranged between 40 and 60 per cent by all the isolates of *Trichoderma* spp. from High range zone (Plate 10). The highest inhibition per cent of *R. solani* was observed with isolate TRTL2 (87.40 %), followed by TRTL5 (85.92 %) and TRTL1 (84.44 %). The lowest inhibition per cent was observed with TRTL6 isolate (62.22 %) (Plate 11). Except TRTL6, all the other isolates showed higher inhibition than KAU strain of *T. viride* against *R. solani*.

4.3.1.3. Evaluation of antagonistic efficacy of *Trichoderma* isolates from Problem area zone

More than 90 per cent of inhibition of *P. aphanidermatum* was observed in all the isolates (Plate 12). Complete inhibition (100 %) was seen with isolate TRRN1 and TRRN2 followed by TRIL1 (99.25 %), TRKM2 (98.88 %) and TRKM1 (98.51 %).

Among the 5 isolates, TRKM1 exhibited cent percent inhibition against *R. solani*. Other isolates *viz.*, TRRN2, TRRN1, TRKM2 and TRIL1 displayed inhibition per cent of 88.14, 85.18, 62.22 and 60.00 respectively (Plate 13).

Table 11. Per cent inhibition of *P. aphanidermatum* and *R. solani* by isolates of *Trichoderma* spp. from High range and Problem area zone

Zone	Isolate	*P. aphanidermatum*		*R. solani*	
		Radial growth (cm)*	***Inhibition (%)*****	***Radial growth (cm)****	***Inhibition (%)*****
High range zone	TRTL1	49.60	44.81^{b} (41.90)	14.00	84.44^{b} (66.762)
	TRTL2	39.60	55.92^{a} (48.36)	11.30	87.40^{a} (69.23)
	TRTL5	49.30	45.18^{ab} (42.20)	12.60	85.92^{b} (67.93)
	TRTL6	52.00	42.22^{c}	34.00	62.22^{c}

			(40.52)		(52.06)
	Control	9.00		9.00	
	CD (0.05)	0.18	1.17	0.25	2.33
Problem area zone	TRRN1	10.00	100.00^{a} (89.71)	10.50	85.18^{c} (67.34)
	TRRN2	10.00	100.00^{a} (89.71)	10.30	88.14^{b} (69.80)
	TRKM1	10.06	98.51^{b} (83.08)	10.00	100.00^{a} (89.71)
	TRKM2	10.04	98.88^{ab} (85.00)	10.08	62.22^{d} (52.75)
	TRIL1	10.03	99.25^{ab} (86.90)	10.01	60.00^{d} (50.75)
T. viride (KAU Strain)		10.70	71.10^{c} (57.25)	10.00	80.10^{c} (64.00)
Control		9.00		9.00	
CD (0.05)		0.007	4.82	0.03	1.44

**Log transformed; ** Values in parenthesis are arcsine transformed.*

Values followed by similar superscripts are not significantly different at 5% level

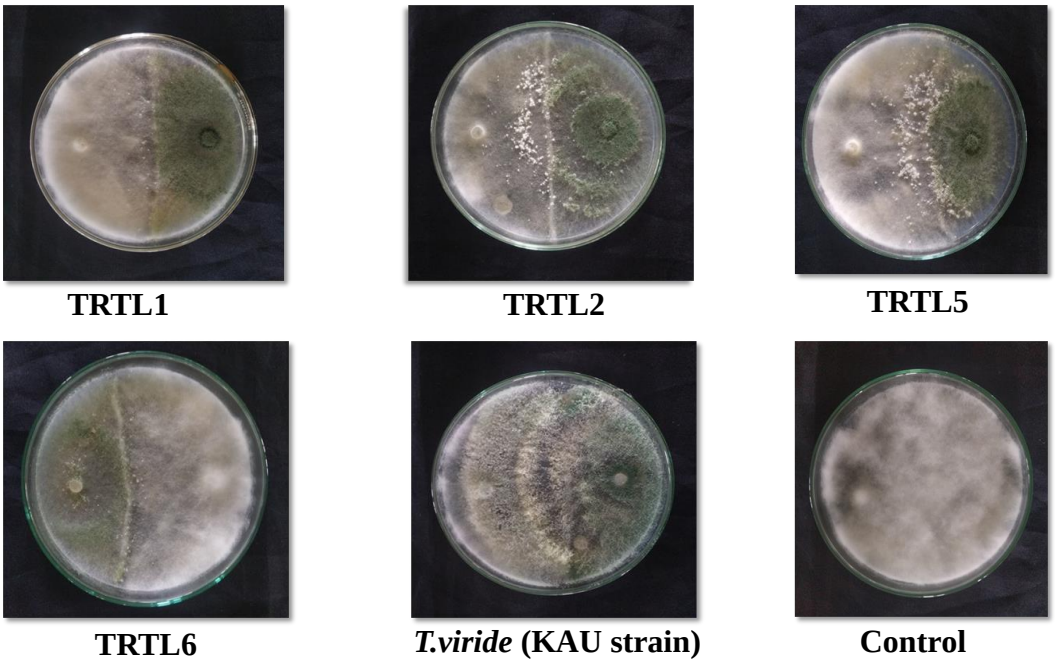


Plate 10. Inhibition of mycelial growth of *P. aphanidermatum* by *Trichoderma* isolates from High range zone

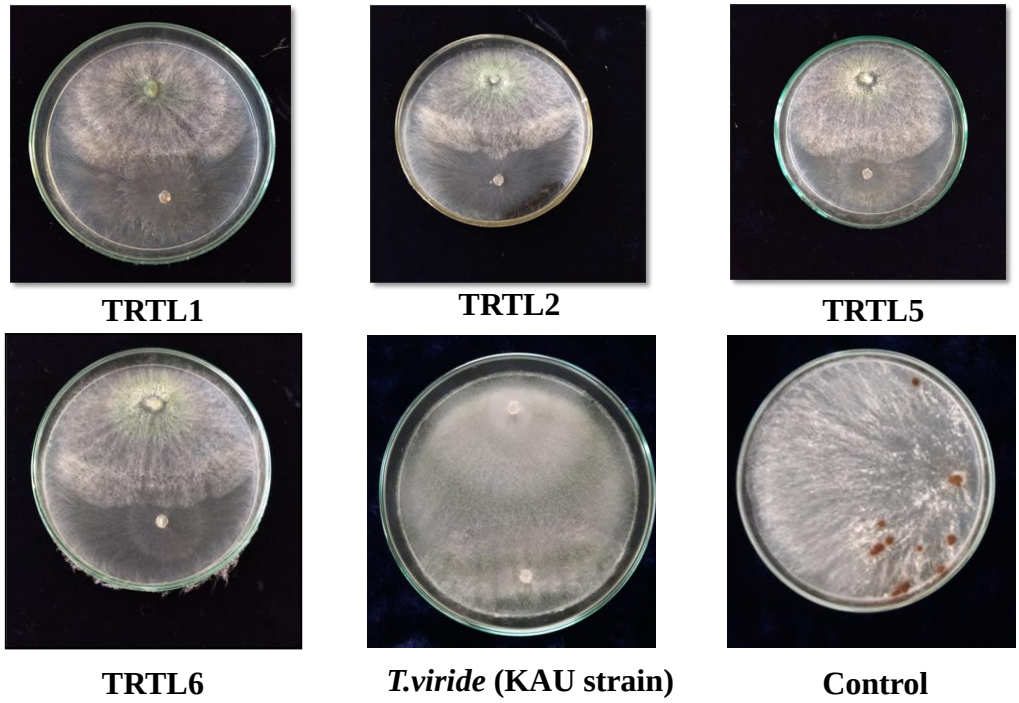


Plate 11. Inhibition of mycelial growth of *R. solani* by *Trichoderma* isolates from High range zone

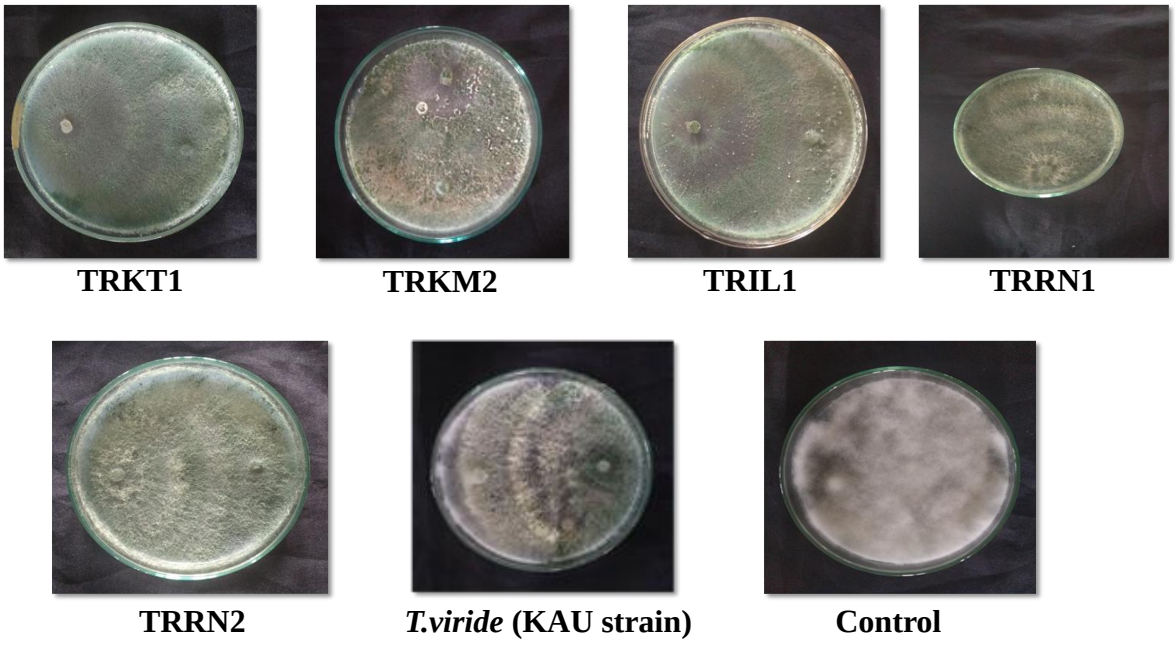


Plate 12. Inhibition of mycelial growth of *P. aphanidermatum* by *Trichoderma* isolates from Problem area zone

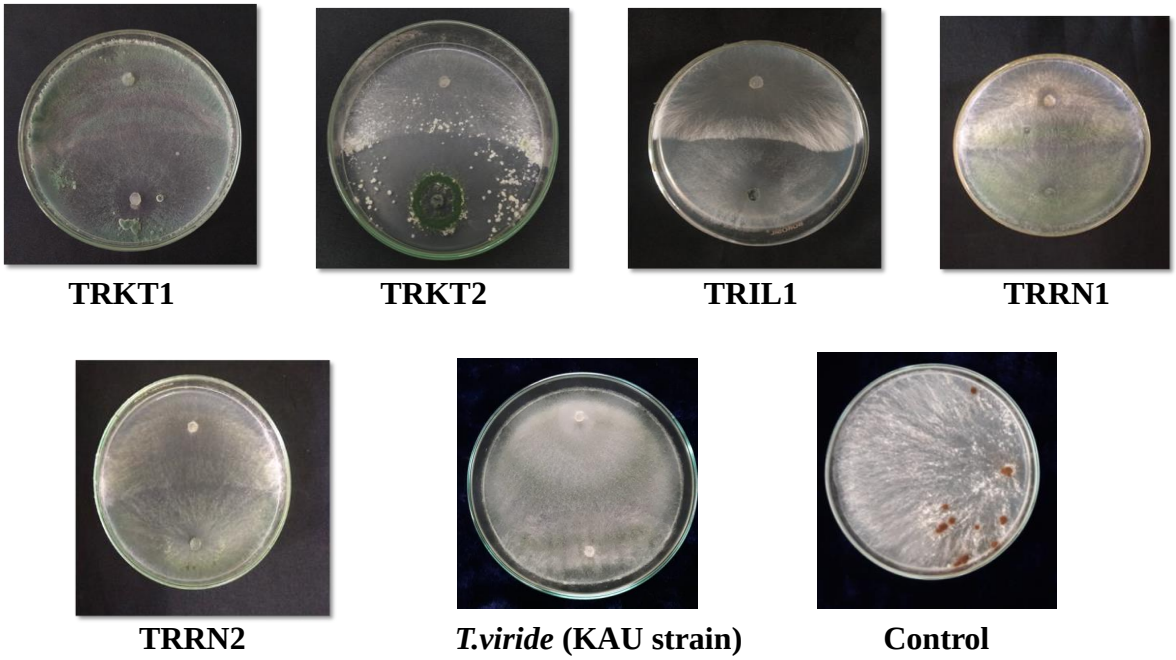


Plate 13. Inhibition of mycelial growth of *R. solani* by *Trichoderma* isolates from Problem area zone

4.3.1.4. Evaluation of antagonistic efficacy of *Trichoderma* isolates from Southern zone

The antagonistic efficacy of *Trichoderma* isolates from Southern zone is given in Table 12. Isolates which showed complete inhibition of *P. aphanidermatum* are TRPN7, TRPN11, TRPN15 and TRKR2. Majority of the isolates exhibited more than 90 per cent inhibition (Plate 14).

Against *R. solani,* isolates TRPN7, TRPN9, TRPN14, TRPN15, TRPN17, TRPN18 and TRKR2 exhibited complete inhibition. Except TRKR1 isolate all other isolates exhibited more than 90 per cent inhibition. (Plate 15). All the isolates were fast growing and overgrow above the pathogen inhibiting the growth of mycelia and sclerotial production of pathogen.

Table 12. Per cent inhibition of *P. aphanidermatum* and *R. solani* by isolates of *Trichoderma* spp. from Southern zone

Isolate	*P. aphanidermatum*		*R. solani*	
	Radial growth (cm)*	***Inhibition (%)*** **	***Radial growth (cm)****	***Inhibition (%)*** **
TRPN3	10.01	99.62^{ab} (87.71)	10.10	97.40^{bc} (82.20)
TRPN7	10.00	100^{a} (89.71)	10.00	100^{a} (89.71)
TRPN9	10.11	97.03^{de} (80)	10.00	100^{a} (89.71)
TRPN 10	10.97	72.22^{h} (58.18)	10.20	94.81^{d} (76.83)
TRPN11	10.00	100^{a} (89.71)	10.04	98.88^{b} (84.90)
TRPN 14	10.07	98.14^{cd} (83.4)	10.00	100^{a} (89.71)

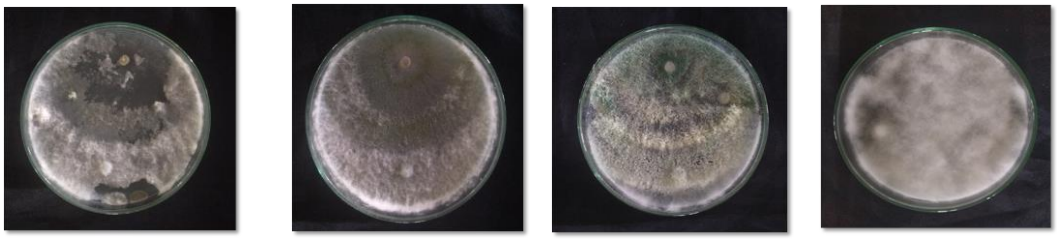
TRPN 15	10.00	100[a] (89.71)	10.00	100[a] (89.71)
TRPN 17	10.04	98.88[bc] (84.90)	10.00	100[a] (89.71)
TRPN 18	10.13	96.66[e] (79.46)	10.00	100[a] (89.71)
TRKR1	10.49	86.66[f] (68.53)	10.43	88.51[e] (70.18)
TRKR2	10.00	100[a] (89.71)	10.00	100[a] (89.71)
TRKR3	10.05	78.88[g] 62.61)	10.13	96.66[cd] (79.46)
T. viride (KAU strain)	11.00	71.11[h] (57.25)	10.07	80.7[f] (64.01)
Control	9.00		9.00	
CD (0.05)	0.28	3.70	0.27	4.37

*Log transformed; ** Values in parenthesis are arcsine transformed.

Values followed by similar superscripts are not significantly different at 5% level

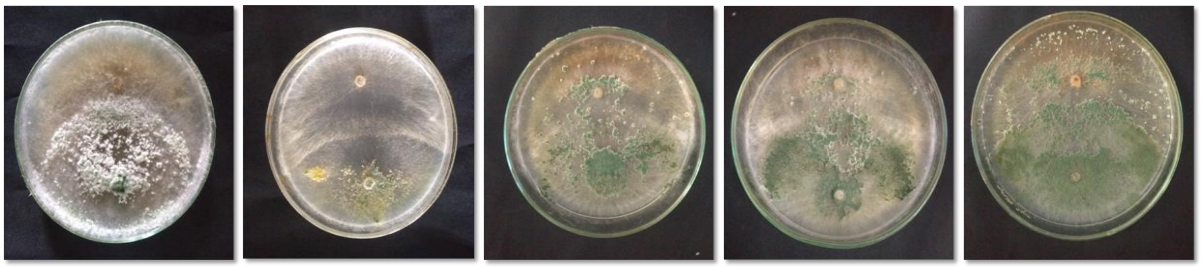
TRPN3 **TRPN7** **TRPN9** **TRPN10** **TRPN11**

TRPN14 **TRPN15** **TRPN17** **TRPN18** **TRKR1**

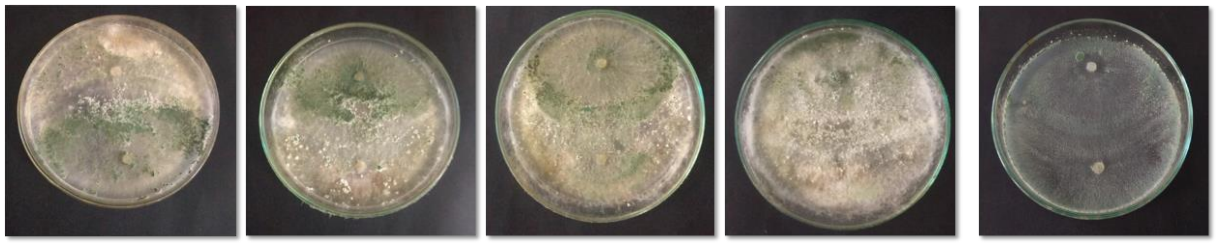


TRKR2 **TRKR3** ***T.viride*** **(KAU strain)** **Control**

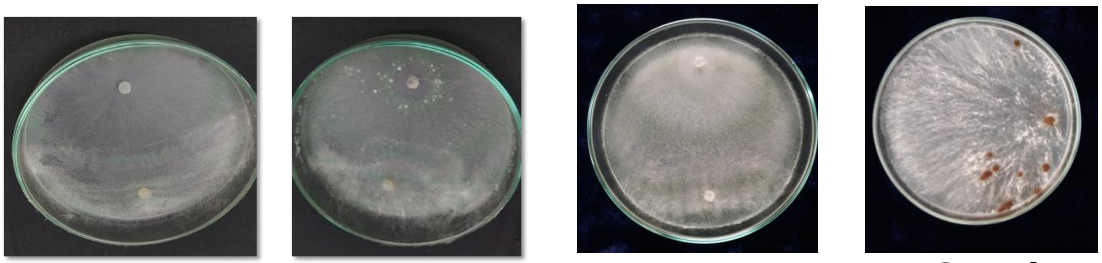
Plate 14. Inhibition of mycelial growth of *P. aphanidermatum* by *Trichoderma* isolates from Southern zone



TRPN3 **TRPN7** **TRPN9** **TRPN10** **TRPN11**



TRPN14 **TRPN15** **TRPN17** **TRPN18** **TRKR1**



TRKR2 **TRKR3** ***T.viride*** **(KAU strain)** **Control**

Plate 15. Inhibition of mycelial growth of *R. solani* by *Trichoderma* isolates from Southern zone

4.3.2. Assessment of zone of lysis, antibiosis and overgrowth (Antagonistic index)

Antagonist and pathogen interaction during dual culture shows mechanisms like antibiosis, lysis and overgrowth.

4.3.2.1. Antagonistic characters of *Trichoderma* isolates from Northern and Central zone against *P. aphanidermatum* and *R. solani*

The antagonistic characters of *Trichoderma* isolates from Northern and Central zone of Kerala are listed in Table 13. Antagonistic action like lysis and overgrowth were exhibited by isolates against *P. aphanidermatum*. It is revealed that there is absence of antibiosis during the pathogen-antagonistic interaction against *P. aphanidermatum*. The prominent mechanism observed in isolates TRML1, TRSN1 and TRSN2 was overgrowth. Except TRML1 and TRMW3 all the other isolates exhibited lysis of pathogen.

Antagonistic reaction against *R. solani* showed antibiosis, lysis and overgrowth. Presence of antibiosis was observed in isolates like TRSN1, TRSN2 moderate level in TRMW1 and low level in TRMW2. Isolates showing lysis were TRSN1, TRMW2 and TRSV1. All the isolates showed high to moderate level of overgrowth on the pathogen. *Trichoderma* isolates showing all three mechanisms against *R. solani* were TRSN1 and TRMW2.

4.3.2.2 Antagonistic characters of *Trichoderma* isolates from High range zone against *R. solani* and *P. aphanidermatum*

Effect of *Trichoderma* spp. against *P. aphanidermatum* revealed lysis (TRTL1, TRTL6) and overgrowth (TRTL2, TRTL5) (Table 13).

All the four isolates showed antibiosis and overgrowth against *R. solani*. Lysis of pathogen was observed only in isolate TRTL6.

4.3.2.3. Antagonistic characters of *Trichoderma* isolates from Problem area zone against *R. solani* and *P. aphanidermatum*

Major mechanism by the antagonists against *P. aphanidermatum* and *R. solani* during dual culture was overgrowth of antagonist. Against *P. aphanidermatum*, lysis of mycelia was observed only with TRIL1 isolate. Against *R. solani*, lysis of pathogenic mycelia was observed only on interaction with isolates TRRN1, TRRN2 and TRIL1 (Table 13).

Table 13. Antagonistic properties of isolates of *Trichoderma* spp. against *P. aphanidermatum* and *R. solani*

Zone	Isolate	Antibiosis		Lysis		Overgrowth	
		Pa	Rs	Pa	Rs	Pa	Rs
Northern zone	TRML1	-	-	-	-	+++	+++
Central zone	TRSN1	-	+++	+	+	+++	++
	TRSN2	-	+++	+	-	+++	++
	TRMW1	-	++	+	-	++	+++
	TRMW2	-	+	++	+	-	+++
	TRMW3	-	-	-	-	++	++
	TREN1	-	-	+	-	+	++
	TREZ1	-	-	+	++	++	+++
	TREZ2	-	-	+	-	+	+++
	TREZ3	-	-	+	+	+	++
High range zone	TRTL1	-	++	+	-	-	++
	TRTL2	-	++	-	-	++	++
	TRTL5	-	+	-	-	+	++
	TRTL6	-	++	+	+	-	+
Problem area zone	TRRN1	-	-	-	+	++	++
	TRRN2	-	-	-	+	+++	+++
	TRKM1	-	-	-	-	+++	++
	TRKM2	-	-	-	-	+++	++
	TRIL1	-	-	+	+	+++	++
Southern zone	TRKR1	-	-	+	+	+++	++
	TRKR2	-	-	++	+	+++	++
	TRKR3	-	-	+	+	+++	+++
	TRPN3	+	++	-	+	++	+++
	TRPN7	+	-	-	+	+	+
	TRPN9	++	-	-	+	++	+++
	TRPN 10	++	+++	-	+	++	+++
	TRPN11	++	++	-	++	++	+++
	TRPN 14	++	+++	+	+	++	+++
	TRPN 15	+++	+++	+	+	+	+
	TRPN 17	+++	+++	-	+	++	+++

+++ high ++ moderate +low - absence ; Pa- *P. aphanidermatum,* Rs – *R. solani*

4.4. Strain improvement of *Trichoderma* isolates through protoplast fusion

The parental isolates selected for protoplast fusion were TRPN14, TRMW2 and TRSN1 (Table 14, Plate 16). The selected isolates displayed high *in vitro* inhibition against *P. aphanidermatum* and *R. solani*. The isolate TRPN14 exhibited all the antagonistic properties while TRSN1 and TRMW2 exhibited lysis and overgrowth, and medium to high sporulation on culture medium. Protoplast fusion was performed between TRPN14×TRSN1, TRPN14×TRMW2 and TRSN1×TRMW2 (Plate 17).

4.4.1 Sensitivity of selected *Trichoderma* isolates against fungicide

The *Trichoderma* spp. selected based on the antagonistic properties and inhibition per cent were tested for sensitivity against the fungicide carbendazim. The results of experiment are furnished in Table 15. Isolates TRPN14, TRMW2 and TRSN1 used as parents in protoplast fusion showed complete inhibition in 0.2 per cent concentration of fungicide. Hence carbendazim amended in selection medium at 0.2 per cent concentration was selected as marker for the selection of fusant.

Table 14. Properties of *Trichoderma* isolates selected for protoplast fusion

Property	TRPN14	TRSN1	TRMW2
Per cent inhibition of *P. aphanidermatum*	98.14	95.18	52.20
Mode of antagonism	Antibiosis, Lysis, overgrowth	Lysis, Overgrowth	Lysis, Overgrowth
Per cent inhibition of *R. solani*	100.00	98.88	100.00
Mode of antagonism	Antibiosis, Lysis, Overgrowth	Antibiosis, Lysis, Overgrowth	Antibiosis, Lysis, Overgrowth
Sporulation	Medium	Medium	High

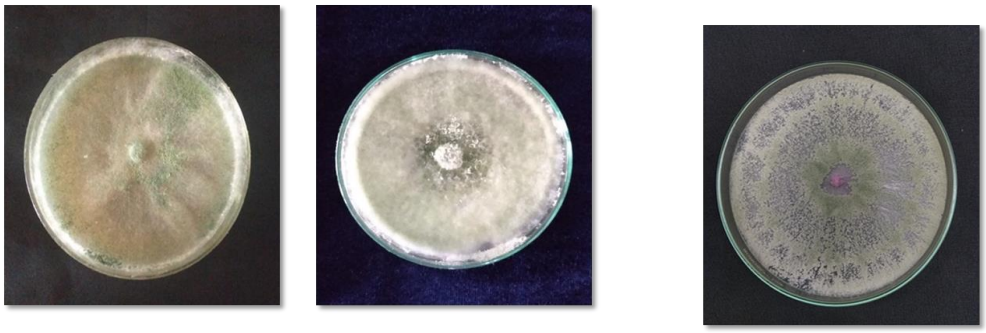


Plate 16. *Trichoderma* isolates selected for protoplast fusion

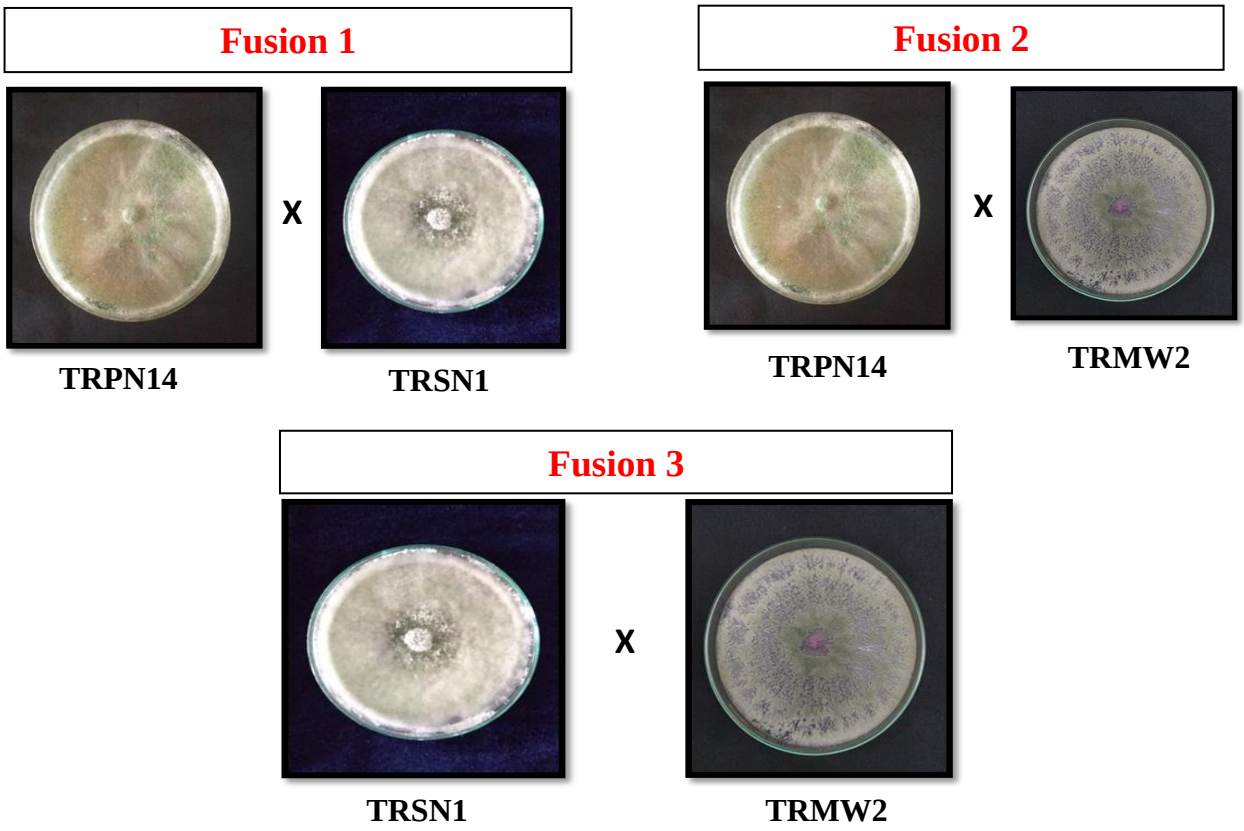


Plate 17. *Trichoderma* isolates used in protoplast fusion

Table 15. Sensitivity of selected *Trichoderma* isolates and protoplast fusants against fungicide carbendazim

Sl. No.	Isolate	Carbendazim (0.2%)	
		Radial growth (cm)	Per cent Inhibition *
1	TRPN14	0	100.00
2	TRSN1	0	100.00
3	TRMW2	0	100.00
4	F1 (TRPN1 × TRSN1)	2.50	72.20
5	F2 (TRPN1 × TRMW2)	3.26	63.70
6	F3 (TRSN1 × TRMW2)	3.43	61.80

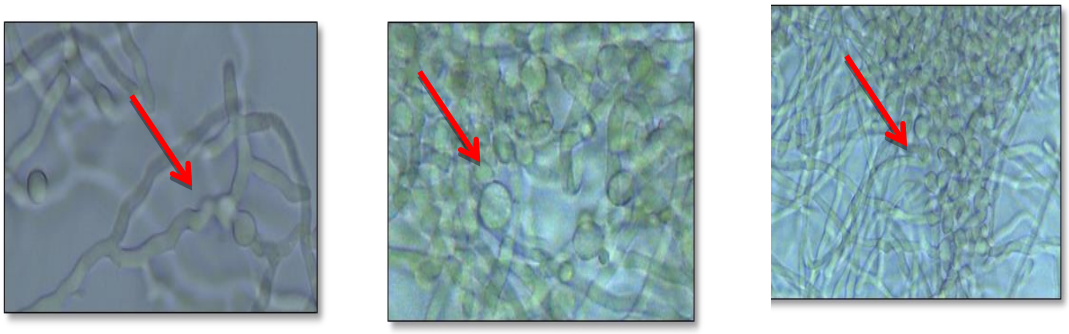
* Mean of 6 observations

4.4.2. Isolation and purification of protoplasts

Isolation of protoplast was carried out from the parental isolates (TRPN14, TRSN1 and TRMW2) using the lytic enzyme. Protoplast was derived from 20- hour old growth of *Trichoderma* spp in (PDB). The mycelia was washed and resuspended in protoplasting buffer containing the lytic enzyme glucanase. The protoplast formation and protoplast release was observed every 30 min under high resolution microscope (Leica, USA) continuously after the addition of the enzyme (Plate 18)

Addition of lytic enzymes caused the degradation of fungal cell wall of the selected parents. Before the release of protoplast, bulging of cell membrane was seen after 30 min. The observation was taken every 30 min. and maximum number of protoplast was obtained within two hours after the addition of lytic enzyme. The size of protoplast enlarged and resulted in bursting if the incubation in protoplast buffer

prolonged. The sedimented protoplasts were washed and suspended in osmotic stabilizer. This purified protoplast was used in fusion.



a **b** **c**

(a) At 30 min Swelling and rounding up of cell content, (b) Release of protoplast at 1 Hr , (c) Maximum release of protoplast after 2 Hrs.

Plate 18. Protoplast formation from isolates at different time intervals

4.4.3. Protoplast fusion

In the present study, poly ethylene glycol (PEG) 6000 was used as fusogen for the fusion of viable protoplasts from the parental isolates *viz.*, TRPN14, TRSN1 and TRMW2. The fusion was performed between parents TRPN14×TRSN1, TRPN14×TRMW2 and TRSN1×TRMW2. The purified protoplast suspension of parents were mixed in fusion buffer. The mixture was incubated in room temperature for 20 min with gentle shaking. The fusion process was observed under high resolution microscope (Leica). At the beginning, the protoplasts were observed to come in contact at a point and later resulted in the degradation of cell membrane at the contact point. This led to the mixing up of protoplasm of each parent with a common outer wall and resulted in the formation of one fused protoplast (Plate 19). It is found that the size of fused protoplast was larger compared to parental protoplast

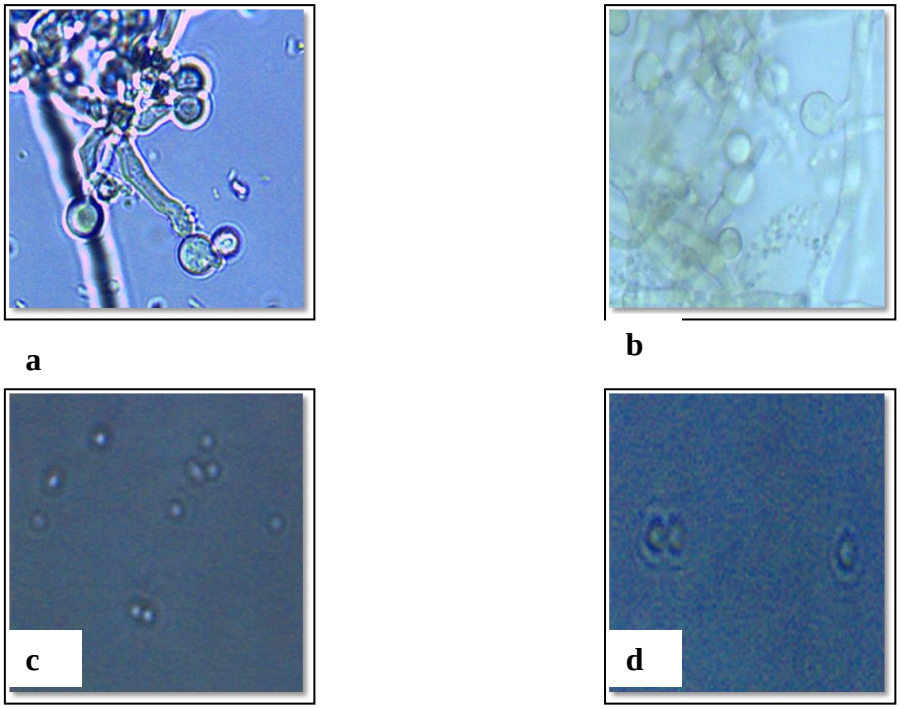


Plate 19. Protoplast fusion of *Trichoderma* spp.

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

4.4.4. Regeneration of fusant

The fused protoplasts were spread plated on malt extract agar medium amended with the fungicide carbendazim for regeneration. The colonies regenerated after 3- 4 days. The colonies that appeared in medium were sub cultured on PDA slants for further studies.

4.5. Screening of fusants against soil borne fungal pathogens

4.5.1. Colony characters of fusants

The colony characters of three fusants *viz.,* Fusant 1 (F1), Fusant 2 (F2) and Fusant 3 (F3) are given in Table 16. The fusants were dark green colour with fluffy mycelia. The sporulation varied compared to parental isolates. The fusant F1 exhibited circular green sporulation and fusant F2 scattered green sporulation (Plate 20)

4.5. Screening of fusants against soil borne fungal pathogens

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4.5.2 Radial growth of fusant

The fusants exhibited fast growth on PDA medium compared to the parents. All the isolates were completly grown five days after inoculation. (Table 17)

4.5.3. *In vitro* evaluation of fusants against soil borne pathogens

The results of evaluation of antagonistic efficacy of fusants are given in Table 18. Among the three fusants, F3 showed highest inhibition against *P. aphanidermatum* (84.44 %). Inhibition of F1 was 72.96 and F2 was 74.44 per cent. (Plate21a).

Fusant F2 showed maximum inhibition against *R. solani* (100 %) followed by F3 (70.37) and F1 (62.22) (Plate 21b).

Table 16. Colony characters of protoplast fusants of *Trichoderma* spp.

Sl. No.	Protoplast fusant isolate	Colour of mycelium	Texture of colony	Sporulation / Growth Pattern
1	F1 (TRPN14 × TRSN1)	Dark green	Fluffy	Circular green sporulation
2	F2 (TRPN14 × TRMW2)	Dark green	Medium fluffy	Scattered green sporulation at centre
3	F 3 (TRSN1 × TRMW2)	Light Green	Fluffy	Green sporulation

Table 17. Radial growth of protoplast fusants and parental isolates of *Trichoderma* spp.

Sl. No.	Isolate	Radial growth (cm) on PDA medium *			Sporulation
		3 DAI	5 DAI	7 DAI	
1	Fusant F1	6.52 ± 0.11	9.00	9.00	High
2	Fusant F2	6.22 ± 0.08	9.00	9.00	High
3	Fusant F3	6.04 ± 0.05	9.00	9.00	High
4	TRPN14	4.50 ± 0.05	6.90 ± 0.03	9.00	Medium
5	TRSN1	3.20 ± 0.11	8.03 ± 0.08	9.00	Medium
6	TRMW2	6.17 ± 0.12	9.00	9.00	High
	CD (0.05)	0.26	0.12		
	SE (m)	0..08	0.04		

*Mean ± SD of five replications

Table 18. Per cent inhibition of *P. aphanidermatum* and *R. solani* by protoplast fusants of *Trichoderma* spp.

Sl. No.	Isolate	*P. aphanidermatum*		*R. solani*	
		Radial growth (cm)	Inhibition (%)	Radial growth (cm)	Inhibition (%)
1	Fusant F1 (TRPN14 × TRSN1)	2.43	72.96	3.40	62.22
2	Fusant F2 (TRPN14 × TRMW2)	2.30	74.44	0.00	100.00
3	Fusant F3 (TRSN1 × TRMW2)	1.40	84.44	2.60	70.37
4	Control (Pathogen alone)	9.00		9.00	
	CD (0.05)	0.59		0.60	
	SE (m)	0.10		0.07	

F1 (TRPN14 x TRSN1) **F2 (TRPN14xTRMW2)** **F3 (TRSN1xTRMW2)**

Plate 20. *Trichoderma* spp. obtained after protoplast fusion

F1 **F2** **F3**

a. Inhibition of mycelial growth of *P. aphanidermatum* by *Trichoderma* fusants

F1 **F2** **F3**

b. Inhibition of mycelial growth of *R. solani* by *Trichoderma* fusants

Plate 21. *In vitro* evaluation of antagonism of protoplast fusants against *P. aphanidermatum* and *R. solani*

4.5.4. Antagonistic characters of fusants

The antagonistic character of fusants observed during *in vitro* evaluation is given in Table 19. Among the fusants, F1 exhibited antibiosis, lysis and overgrowth against *P.aphanidermatum and R. solani*. Fusants F2 and F3 exhibited lysis and overgrowth. A comparison of the parental isolates and fusants are given in Table 20. It is revealed that fusants exhibited fast growth and high sporulation. Fusants also displayed antagonistic properties equal to that of the parents.

Table 19. Antagonistic properties of protoplast fusants of *Trichoderma* spp. against *P. aphanidermatum* and *R. solani*

Protoplast fusant	Antibiosis		Lysis		Overgrowth	
	Pa	Rs	Pa	Rs	Pa	Rs
F1	+++	+	++	+++	+++	++
F2	-	-	+++	-	+++	+++
F3	-	-	+	++	+++	+++

+++ high ++ moderate +low - absence Pa- *P. aphanidermatum,* Rs – *R. solani*

Table 20. Comparison of parental *Trichoderma* isolates and their protoplast fusants

Property	TRPN14	TRSN1	TRMW2	F1	F2	F3
Per cent inhibition of *P. aphanidermatum*	98.14	95.18	52.2	72.96	74.4	84.44
Mode of antagonism	A, L, OV	L, OV	L, OV	A,L,OV	L,OV	L,OV
Per cent inhibition of *R. solani*	100	98.88	100	62.22	100	70.37
Mode of antagonism	A, L, OV	A, L,OV	A, L,OV	A,L,OV	OV	L,OV
Sporulation	Medium	Medium	High	High	High	High

Discussion

5. DISCUSSION

Trichoderma spp. are long been recognized as efficient fungal biocontrol agents for the control of plant disease and for their ability to increase plant growth and development. *Trichoderma* spp. are soil-borne, green-spored ascomycetes with broad spectrum biocontrol activities against different soil and air borne plant pathogenic fungi. Soil borne diseases are becoming severe in recent years due to poor soil management by farmers. Biological control has emerged as an important component in the integrated disease management of soil borne diseases. Different species of *Trichoderma* may not be having all attributes of an efficient biocontrol agent for the management of wide spectrum of soil borne fungal diseases. This warrants bringing the majority of the antagonistic properties of biocontrol agents in a single species or strain. The results of the study on strain improvement of *Trichoderma* spp. by protoplast fusion are discussed in this chapter.

Soil samples were collected from selected locations in Northern, Central, High range, Problem area and Southern zone of Kerala coming under the districts of Malappuram, Idukki, Wayanad, Kottayam, Pathanamthitta and Thiruvananthapuram. The pH of soil samples collected from Northern zone was 6.50. The pH of soil samples from Central, High range, Problem area and Southern Zone was in between three to seven. Majority of the *Trichoderma* isolates were found in soils having pH range of six to seven. Earlier, Okoth *et al*. (2007) had reported the tolerance of *Trichoderma* spp. to soil acidity. Native *Trichoderma* strains found in acidic soils were reported by Garcia-Nunez *et al*. (2012) and Hima (2017).

pH has influence on the occurrence of *Trichoderma* spp with enzyme production which are involved in degradation of fungi (Kredics *et al*., 2003; Samaniego, 2008). *Trichoderma* isolates obtained based on the sclerotial viability showed pH of 8.08 (Khattabi *et al*., 2004). Delgado *et al.* (2000) has reported maximum stability of β-1, 6 glucanase produced by *T. harzianum* at a pH of seven.

Enumeration of *Trichoderma* in collected soil samples was carried out in Trichoderma selective medium. A total of 31 isolates were obtained from different soil samples from five agroclimatic zone. The highest number of isolates was obtained from Southern zone. *Trichoderma* enumeration on TSM was reported by

Elad *et al*. (1981). Elad and Chet (1983) improved TSM for effective isolation of *Trichoderma* spp. from soil having *Fusarium* spp. Isolation of *Trichoderma* isolates was also carried out in PDA medium which resulted in colonies after three days of incubation (Shalini and Kotasthane, 2007). Enumeration of *Trichoderma* isolates was also performed in Rose Bengal Agar medium (Lunge and Patil, 2012).

The use of antibiotics streptomycin and chloramphenicol favoured the growth of *Trichoderma* and reduced the amount of contamination in TSM compared to other culture media (Gil *et al*., 2009). Similarly, enumeration of *Trichoderma* spp. in TSM resulted in large population of *Trichoderma* spp. (Devi and Sinha, 2014; Kale *et al*., 2018; Saravanakumar and Wang, 2020).

Variations were found in the radial growth of different isolates of *Trichoderma*. Isolates such as TRMW2, TREN1, TREZ1, TREZ2, TREZ3, TRRN1, TRRN2, TRKR1, TRPN3, TRPN7, TRPN10 and TRPN18 exhibited full growth at five days after inoculation (DAI). Radial growth of *T. harzianum* exhibited on PDA medium was 22 mm per day (Jahan *et al*., 2013). *Trichoderma* isolates exhibited faster growth rate ranging from six to nine cm at two days after inoculation on PDA medium (Devi and Sinha, 2014). Sharma and Singh (2014) also reported the fast growth of *Trichoderma* isolates with eight to nine cm after 72 h at 28 ^{0}C and four to seven cm at 35 ^{0}C.

Cultural characters like colour, texture and sporulation of *Trichoderma* spp. varied among the isolates under study. Sekhar *et al*. (2017) had observed wide variations in colony colour like white, pale yellow, bluish green and dull green in ten isolates of *Trichoderma sp*. isolated from groundnut rhizosphere. The colony colour of isolates of *T. harzianum*, *T. viride* and *T. aureoviride* exhibited variations from green to dark green (Shalini and Kotasthane, 2007). Savitha and Sriram (2015) found that colony colour of *Trichoderma* isolates which are potential biocontrol agents against *P. capsici* in bell pepper ranged from white to green.

The growth pattern of isolates of *Trichoderma* spp. in this study showed less to heavy sporulation. The sporulation pattern included circular green sporulation, sporulation at centre, and scattered and isolates with no sporulation (TRSN2, TRPN3 and TRPN7). Such variations had been noted by many workers like Shah *et al.* (2012)

who reported variations in growth pattern with *T. harzianum* forming one to two concentric rings with green conidial production denser in the centre and lighter towards margins, *T. viride* with granular green conidia distributed throughout and *T. pseudokoningii* with no conidial formation on PDA medium. Devi and Sinha (2014) studied the cultural characteristics of *T. viride, T. harziaum* and *T. hamatum.* These isolates showed fluffy to spare growth, with different range of colony colour and different patterns of sporulation.

Sharma and Singh (2014) reported all the *Trichoderma* isolates were fast growing with 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. Conidial formation occurred by 48 h with conidial colour in varying shades of green within 72 h. Growth rate of *Trichoderma* isolates in TSM and OMA medium exhibited two days after inoculation was 5.33 cm to 7.40 cm and 6.93 cm to 9.00 cm respectively (Devi and Sinha, 2014).

In vitro screening of *Trichoderma* isolates against selected pathogens *viz., P. aphanidermatum* and *R. solani* revealed that isolates *viz.,* TRML1 (Northern zone) (Fig.1), TRSN1, TRSN2 (Central zone) (Fig. 2), TRRN1, TRRN2, TRKM1 (Problem area zone) (Fig. 3), TRPN3, TRPN9, TRPN11, TRPN14, TRPN17 and TRPN18 (Southern zone) exhibited more than 90 per cent inhibition against both the pathogens. Isolates TRPN7, TRPN15 and TRKR2 from Southern zone exhibited complete inhibition against both pathogens. But the isolates from High range zone showed less than 90 per inhibition (Fig. 4). *T. virens* isolate Vn11 and Vn05 displayed highest inhibition against *R. solani* (85 %) and *P. aphanidermatum* (76 %). *T. harzianum* isolate Th01 and Th02 caused inhibition of 88 percent and 85 per cent against *R. solani* and *P. aphanidermatum* respectively (Devi *et al.,* 2012). Joshi *et al.* (2010) reported that highest inhibition per cent by *Trichoderma* spp. against *R. solani* was 72.97 %.

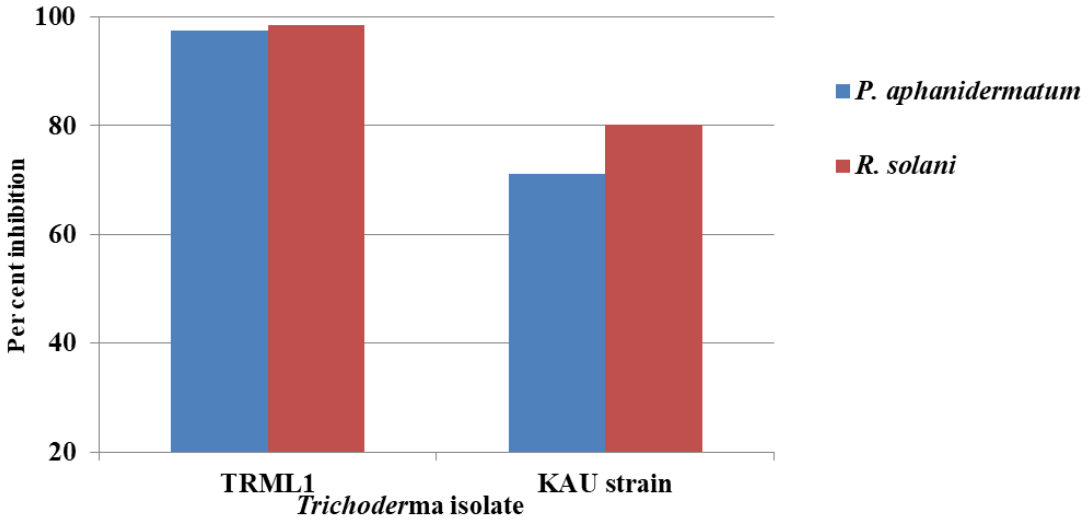

Fig 1. Per cent inhibition of *P. aphanidermatum* and *R. solani* by *Trichoderma* isolate obtained from Northern zone

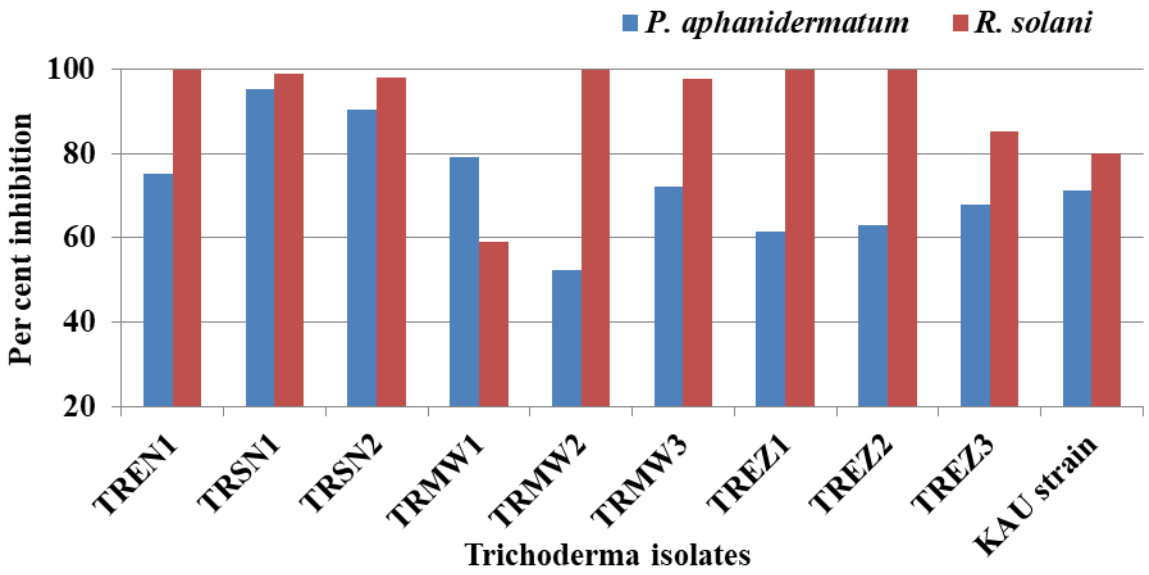

Fig 2. Per cent inhibition of *P. aphanidermatum* and *R. solani* by *Trichoderma* isolate obtained from Central zone

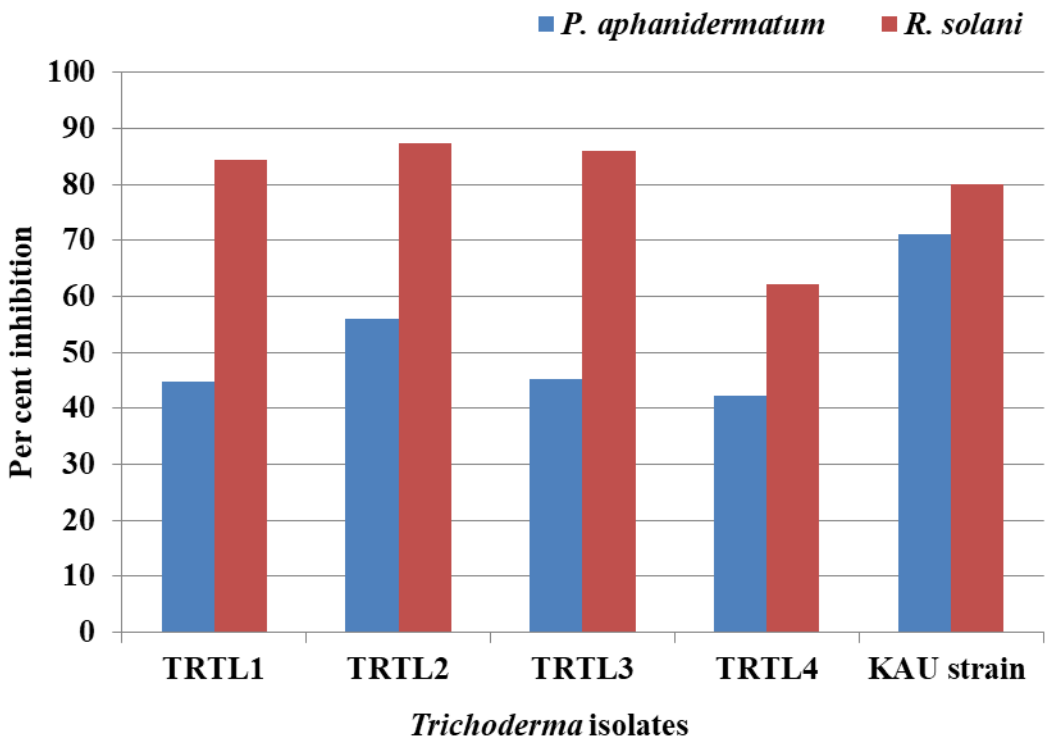

Fig 3. Per cent inhibition of *P. aphanidermatum* and *R. solani* by *Trichoderma* isolate obtained from High range zone

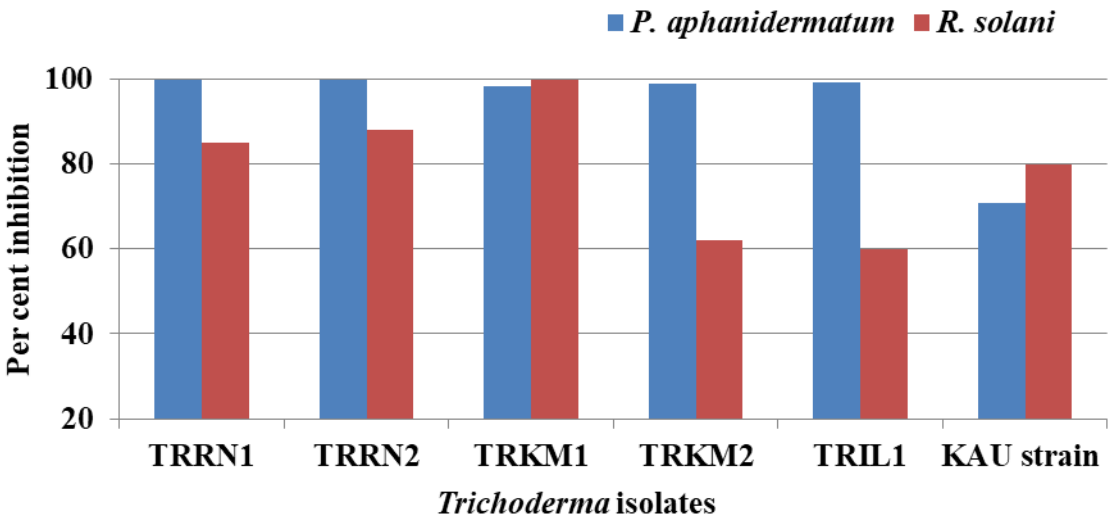

Fig 4. Per cent inhibition of *P. aphanidermatum* and *R. solani* by *Trichoderma* isolates obtained from Problem area zone

In the present study, isolates TREN1, TRMW2, TREZ1, TREZ2, TRKM1, TRPN7, TRPN9, TRPN14, TRPN15, TRPN17, TRPN18 and TRKR2 caused complete inhibition of *R. solani* due to overgrowth of antagonist. Various *Trichoderma* spp. have been found to inhibit pathogens *in vitro* by overgrowth. According to Bastakoti *et al.* (2017) the colony of *S. rolfsii* was covered by the growth of *Trichoderma sp*. on the fourth day.

Due to overgrowth of *Trichoderma* species in the plate, the growth of test fungal pathogen was highly inhibited. Manandhar *et al.* (2019) also reported that *Trichoderma* isolates showed more than 80 per cent inhibition of radial growth of *R. solani*. Some of the isolates completely overgrew the pathogen at 7 DAI. Chao *et al.* (2019) reported the variations exhibited by different isolates of *Trichoderma* spp. *viz., T. simmonsii* 8702 and *T. pyramidale* 7921 which showed highest inhibition rate (82 %), followed by *T. pyramidale* 8991 (75 %) whereas *T. atrobrunneum* 9926 and *T. paratroviride* 8997 showed very low inhibition.

Reduced sclerotia formation or overgrowth of the antagonist on sclerotia was observed during the *in vitro* assay of *Trichoderma* spp. against *R. solani*. Similarly, absence of sclerotia formation in *R. solani* by *Trichoderma* spp. was reported by Shalini and Kotasthane (2007). Mycoparasitic action including coiling around pathogen hyphae, penetration, and subsequent dissolution of the host cytoplasm leads to parasitisation of sclerotia by heavy sporulation. Seema and Devaki (2012) also reported decreased sclerotia formation of *R. solani*. *T. harzianum* and *T. viride* completely overgrew the pathogen with percentage inhibition of 67 per cent and 70 per cent respectively. Dutta and Das (2002) had reported that application of *T. harzianum*, *T. koningii*, *T. viride* along with *Sclerotium rolfsii* resulted in reduced disease incidence and sclerotial production. Maximum sclerotia reduction was observed in *T. harzianum* (94.2 %) followed by *T. viride* (86.8 %) and *T. koningii* (84.1 %).

Isolates such as TRRN1, TRRN2, TRPN3, TRPN7, TRPN11, TRPN15 and TRKR2 exhibited maximum inhibition over *P. aphanidermatum*. Mishra (2010) reported variations in inhibition per cent and the maximum inhibition of *P. aphanidermatum* was by *T. viride*-1433 (72.0 %), which was followed by

T. harzianum-4572 (69.8 %), *T. viride*-793 (62.1 %), *T. harzianum*-4532 (60.3 %) and *T. virens*-2194 (59.6 %). Kamala and Indira (2011) studied the antifungal activity of *Trichoderma* spp. against *P. aphanidermatum* under *in vitro* and *in vivo* conditions. Maximum inhibition of *P. aphanidermatum* was observed in isolate T105.

Inhibition of *P. aphanidermatum* occurs due to the inhibition of zoospore, germ tube elongation and mycelial growth of *P. aphanidermatum* (Fajola and Alasoadura, 1975). Increased culture filtrate of *Trichoderma* isolates also led to reduced mycelial growth of *P. aphanidermatum* and maximum inhibition was observed in *TVC3* (Muthukumar *et al.,* 2011).

Majority of the isolates exhibited lysis of pathogens during the antagonist-pathogen interaction. Lytic enzymes play a vital role in mycoparasitism and degrade the pathogen cell wall (Ridout *et al.,* 1986). Mycoparasitism is one of the most important mechanisms with antagonistic activity against soil-borne plant pathogenic fungi (Howell, 2003; Kubicek *et al.,* 2011), in which hydrolytic enzymes, especially chitinases and *β*-1,3-glucanases, play a crucial role. These enzymes were induced efficiently by pathogen cell walls.

Parasitic interaction between *P. aphanidermatum* and *R. solani* by *T. hamatum* was reported by Chet *et al.* (1981). During the interaction, *T. hamatum* exhibited appresorial formation, coiling and penetrating into the pathogens resulting in lysis of *P. aphanidermatum* and *R. solani*. Lysis of different pathogen such as *Fusarium oxysporurn f. sp. vasinfectum, F. oxysporum f. sp. melonis, R. solani* and *P. aphanidermatum* by *T. harzianum* was also reported by Sivan and Chet (1989). Among different pathogens, parasitism was observed in *R. solani* and *P. aphanidermatum*. Lytic activity in *R. solani* and *P. aphanidermatum* was favoured by the action of β-1,3glucanase and chitinase.

During the mycoparasitic interaction, structural changes such as cell wall degradation, plasmalemma disorganisation and cytoplasm aggregation was reported by Benhamou and Chet (1993). Parasitic interaction between *T. virens* against *R. solani* includes host sensing, attraction, attachment, coiling around and lysis brought about by hydrolytic enzymes along with the action of secondary metabolites (Mukherjee *et al*., 2012).

Isolates such as TRSN1, TRSN2, TRPN14 and TRPN15 exhibited high levels of antibiosis. Pathogen-antagonist zone displayed as dark red brown and yellow colour indicated the production of antibiotics. Antibiosis also has an important role in antagonistic activity (Aluko and Hering, 1970). Production of antibiotics during the antagonist-pathogen interaction inhibits the growth of pathogens (Dennis and Webster, 1971). Antibiotics are microbial toxins that, at low concentration can poison or kill other microorganisms (Heydari and Pessarakli, 2010). Mendoza *et al*. (2015) also reported antibiosis after 48 h with a colour change in medium due to the production of secondary metabolite. Metabolite production by *Trichoderma* strains for the inhibition of pathogens includes harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins and heptadilic acid (Benitez *et al.*, 2004). A combinations of all antagonistic properties resulted in high inhibition of both the pathogens by isolates obtained from Southern zone (Fig 5).

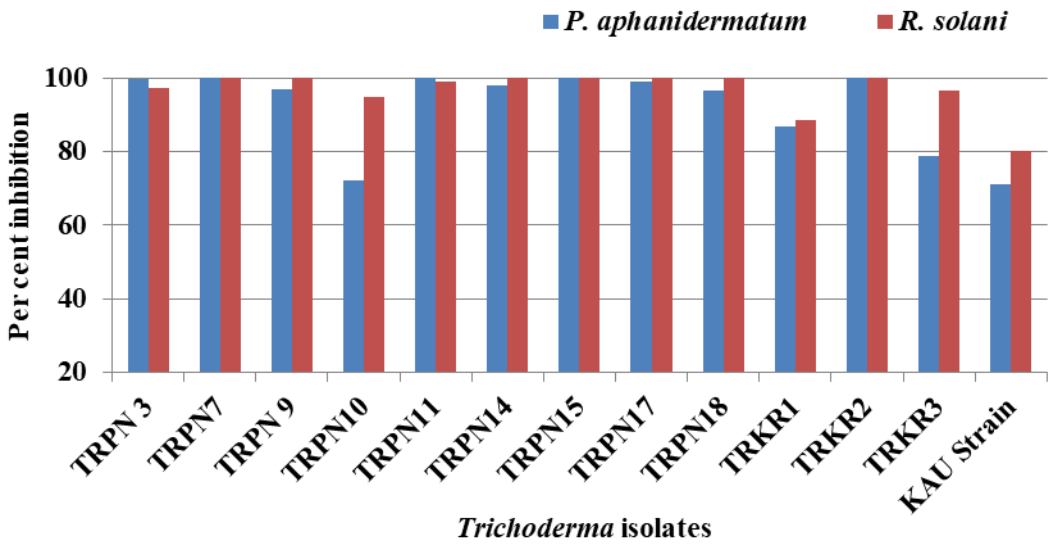

Fig 5. Per cent inhibition of *P. aphanidermatum* and *R. solani* by *Trichoderma* isolate obtained from Southern zone

Protoplast was isolated from selected *Trichoderma* isolates *viz.*, TRPN14, TRSN1 and TRMW2. Isolation of protoplast was performed using lytic enzyme, Glucanex and maximum protoplast were obtained from 20 h old mycelia for 2 h of incubation. Isolated protoplasts were fused in presence of 30 per cent PEG and the fusion was carried out between *Trichoderma* isolates. In the present study, maximum number of protoplast was obtained within 2 h after the addition of lytic enzyme.

Several workers had earlier proved the effectiveness of PEG-mediated protoplast fusion in *Trichoderma* spp. Hong *et al*. (1984) reported conditions for intra-strain protoplast fusion of *T. koningii* include18 h old mycelia, 30 per cent PEG for 10 min at pH 5.5 resulted in enhanced the fusion. Protoplast fusion of *T. harzianum* for improving antagonistic activity was carried out by Pechia and Anne (1989). Protoplasts were isolated using Novozyme 234 and fusion was performed using 30 per cent PEG. Factors for the effective isolation of protoplast from fungi were reported by Rui and Morrell (1993). It includes age of culture, enzyme combination, incubation time, osmotic stabilizer and lysis of cell wall. Balasubramaninan and Lalithakumari (2008) also standardised methods for inter-specific protoplast fusion between *T. harzianum* and *T. viride*. The protoplasts from *T. harzianum* and *T. viride* were isolated using Novozyme 234 as lytic enzyme and potassium chloride as osmotic stabilizer. The maximum number of protoplasts was obtained from 16 h old mycelium of *T. harzianum* and for *T. viride* from 14 h old mycelium at pH 5.5, for 3 h. The inter-specific and intra-specific fusion was performed using 40 per cent polyethylene glycol (PEG) as fusogen.

Fusants obtained were selected based on growth in fungicide amended medium. Carbendazim @ 0.2 per cent was used as marker for the selection. Fusant F1 exhibited highest inhibition (72 %) followed by fusant F2 (63.70 %) and F3 (61.80%). Similar studies have indicated the potential of selection of fusants in fungicide-amended media. Protoplast fusion between *T. koningii* and *T. virens* for enhancing biocontrol potential was reported by Hanson and Howell (2002). The fusion was performed in presence of 30 per cent PEG, with 0.1 M potassium phosphate buffer and 10 mM $CaCl_2$. Protoplast fusants were selected based on repeated exposure of fusants into PDA medium and back to selective medium containing fungicide. It is

revealed that after the repeated exposure these isolates retained the ability to grow on the selective medium, on which the parental isolates could not grow.

Selection of inter- and intra- specific fusants from fungicide amended media was carried out by Migheli *et al*. (1995). Fusants were selected from PDA medium containing hygromycin and propiconazole (fungicide). The selected fusants were fungicide resistant, fast-growing and genetically stable isolates. Selection of interspecific fusant was carried out after testing the fungicide resistance of individual parental isolates (Mrinalini and Lalithakumari, 1998). Hatvani *et al.* (2006) reported intra-specific fusion between carbendazim and tebuconazole-resistant mutants of *T. atroviride* isolated recombinants were resistant to both the fungicides. Similarly Balasubramanian and Lalithkumari (2008) also reported the selection of fusants of *T. harzianum* and *T. viride*. Selection of inter-specific fusants was carried out using carbendazim and copper sulphate whereas for intra-fusants, two per cent chitin and cellulose medium was used. The true fusants only could grow on fungicide-supplemented medium. Fungicide tolerance of fusants was exhibited in protoplast fusion of mutant PTv-V and PTz-F due to nuclear fusion or cytoplasmic fusion between the two fused parents (Fahmi *et al.,* 2012).

Inter-generic protoplast fusion between *Trichoderma* sp. and *Aspergilus niger* was reported by El-Bondkly (2006). Protoplast was isolated using Novozyme 234 and fused in 30 per cent PEG solution. The fusants were selected based on the resistance or sensitivity on selective medium. Similarly, Patil *et al.* (2015) carried out fusion between *T. harzianum* and *A. oryzae* using 60 per cent PEG as fusogen followed by the selection of fusants in colloidal chitin medium.

The results of the protoplast fusion and evaluation of characters of fusants *viz.*, F1, F2 and F3 in the present study revealed that the fusants exhibited faster radial growth on PDA medium. Fusants displayed complete growth on PDA medium 5 DAI. Earlier, the prototrophic strains such as A1, A2, B1, C1, C2, D2 obtained after intra-strain protoplast fusion of *T. harzianum* Rifai (ATCC 32173) resulted in faster growth and variation in colony colour compared to the parental isolates. The antagonistic activity against *R. solani*, *S. rolfsii* and *P. aphanidermatum* resulted in overgrowth of

fusants on pathogens. Prototrophic strains with lower overgrowth ability were also reported by Peer and Chet (1990).

Protoplast fusant F2 exhibited complete inhibition against *R. solani* (Fig 6). Similarly, intra-strain protoplast fusion of *T. harzianum* resulted in fusants with enhanced growth rate in PDA medium compared to the parental isolates. Antagonisitic activity in self-fusants strains against *R. solani* revealed that complete inhibition (100 %) of mycelial growth of *R. solani* was recorded with five fusants (SFTh2, SFTh8, SFTh10, SFTh12 and SFTh13) and 67.6 per cent against parental strains (Prabavathy *et al.*, 2006a). Bondkly *et al.* (2011) reported the faster mycelium growth of fusants of *T. harzianum* on PDA medium. The antagonistic activity of fusants against *R. solani* revealed maximum inhibition among isolates ATh1/9, ATh1/12, ATh1/14 and ATh1/17.

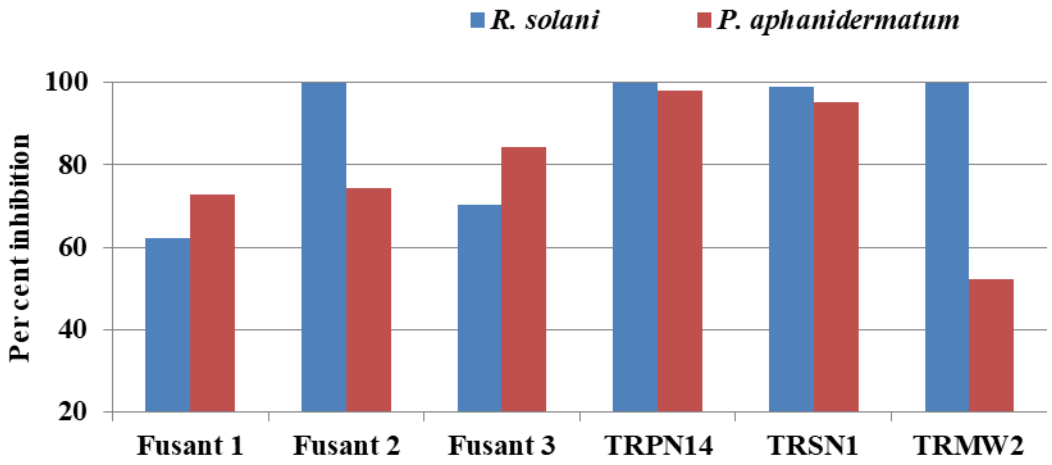

Fig 6. Inhibition of mycelial growth of *P. aphanidermatum* and *R. solani* by fusants

The antagonistic effects of fusants of *T. koningii* and *T. reesei* against pathogens *viz., F. oxysporum, P. ultimum, S. rolfsii* and *S. sclerotiorum* were demonstrated by Mohamed and Haggag (2010). Similarly, fusants of *T. viride* and *T. harzianum* were found to inhibit *R. solani, F. oxysporum* and *P. ultimum* (Fahmi *et al.,* 2012). The antagonistic effect of fusants of *T. harzianum* and *T. viride* against R. *solani, F. oxysporum, S. rolfsii* and *M. phaselona* was also reported (Lakhani *et al.,* 2016). Fusants of *Trichoderma sp.* exhibited more than 50 per cent inhibition against *P. aphanidermatum, P. capsici, F. oxysporum f. sp. cubense, S. rolfsii* and *R. solani*. (Hima, 2017). Intra- and inter- specific fusion *T. harzianum, T. virens, and T. viride* also reported high inhibition against *F. oxysporum f. sp. udum.*

All the parental isolates displayed medium to high level of sporulation, whereas the fusants exhibited heavy sporulation. The protoplast fusants obtained in the present study exhibited fast growth and high sporulation. In a similar way, Hassan (2014) reported profuse sporulation of fusants compared to parents. The colony characters of fusants ranged from dark green to off white and parental strains was dark green and yellow. The fusant strains were growing 60 to 70 per cent faster than the parental isolates. Inhibition of mycelial growth of *M. phaseolina, P. ultimum* and *S. rolfsii* by fusants was also observed. This is also in accordance of the results obtained by Prabavathy *et al.* (2006b) where protoplast fusion was employed to enhance carboxy methyl cellulase activity and most of the fusants exhibited fast growth and abundant sporulation compared to non-fusant and parental strains. Lalithakumari (1996) reported that protoplast fusion can result in changes in sporulation and sensitivity to fungicides of fusants. Fusants obtained after protoplast fusion exhibited phenotypic similarity to one parent but differed in characters such as sporulation and secondary metabolite production. Fusants also differed in characteristics such as pigmentation compared to parental isolates (Shin and Cho 1993, Kumari and Panda, 1994).

In the present study, Fusant F1 displayed all the antagonistic properties such as antibiosis, lysis and overgrowth against both the pathogens. Fusant F2 and F3 exhibited lysis and overgrowth on pathogen. Montealegre *et al.* (2014) found that protoplast fusants of *T. harzianum* showed *in vitro* inhibition against *R. solani* strain

509 (AG 2-1) and 618 (AG 4) where presence of diffusible metabolite production resulted inhibition of *R. solani*. Mycoparasitic interaction was also observed in fusants of *Trichoderma* strains where the contact zone between *M. phaseolina* and *Trichoderma* fusants exhibited coiling. Hassan (2014) had found that there was about three- fold increase in chitinase and *β*-1, 3-glucanase production among the fusants. The high sporulation and improved antagonistic properties might prove as efficient mechanisms for utilization of fusant strains of *Trichoderma* in biocontrol of plant diseases. Further evaluation of different biocontrol traits and disease control efficacy of fusants under *in vivo* conditions will help to adopt these fusant strains as an important component in integrated disease management.

Summary

6. SUMMARY

The present investigation on 'Strain improvement of *Trichoderma* spp. by protoplast fusion" was conducted in the Department of Plant Pathology, College of Agriculture, Vellayani during the year 2018-2020 to evaluate the antagonistic efficiency of screened strains of *Trichoderma* spp. by protoplast fusion for increasing the antagonistic ability and related traits against soil-borne pathogens.

A survey was conducted in five agroclimatic zones of Kerala *viz.*, Northern zone, Central zone, High range zone, Problem area zone and Southern zone for collection of soil samples forest soils. A total of 31 *Trichoderma* isolates were obtained from the soil samples using Trichoderma selective medium (TSM). The influence of pH on occurrence of *Trichoderma* spp. revealed that majority (70.9 %) of the isolates were obtained from soil samples with pH values ranging from six to seven. The radial growth and colony characters like colour of mycelium, texture of colony and sporulation pattern of isolates on PDA medium revealed that the isolated *Trichoderma* spp. differed in growth rate and colony characters. Isolates TRMW2, TREN1, TREZ1, TREZ2, TREZ3, TRRN1, TRRN2, TRKR1, TRPN3, TRPN7, TRPN10 and TRPN18 exhibited full growth at five days after inoculation. Most of the isolates displayed green mycelial colour and sporulation except isolates TRSN2, TRPN3 and TRPN7 which produce white mycelium and no sporulation.

Trichoderma spp. obtained from different zones were tested for their antagonistic efficacy against soil borne pathogens such as *P. aphanidermatum* and *R. solani* under *in vitro* conditions. Majority of the isolates displayed high inhibition per cent compared to KAU strain of *Trichoderma sp*. Isolates, TRRN1, TRRN2, TRPN3, TRPN7, TRPN11, TRPN15 and TRKR2 exhibited complete inhibition of *P. aphanidermatum* in dual culture experiment; whereas TREN1, TRMW2, TREZ1, TREZ2, TRKM1, TRPN7, TRPN9, TRPN14, TRPN15, TRPN17, TRPN18 and TRKR2 exhibited complete inhibition of *R. solani*. *Trichoderma* isolates such as TRPN7, TRPN15 and TRKR2 exhibited complete inhibition against both the pathogens.

Antagonistic properties *viz.*, antibiosis, lysis and overgrowth of *Trichoderma* isolates against *P. aphanidermatum* and *R. solani* were observed during *in vitro*

evaluation. Isolates from Northern zone exhibited overgrowth as major mode of antagonism against both pathogens. Isolates TRSN1 from Central zone displayed all the antagonistic properties. Isolates from High range zone exhibited antibiosis and overgrowth as major mode of antagonism. Isolates from Problem area zone displayed overgrowth as antagonistic property. *Trichoderma* isolates such as TRPN14 and TRPN15 from Southern zone exhibited all the antagonistic properties. During the antagonist-pathogen interaction, and isolates TRSN1, TRSN2, TRPN10, TRPN14, TRPN15, TRPN17 and TRPN18 exhibited high levels of antibiosis. Most of the isolates caused lysis of mycelium of the pathogens which resulted in formation of clear zones in dual culture. Overgrowth of the antagonist was another prominent antagonistic property observed in the majority of the isolates. Effect of the antagonistic property resulted in reduced radial growth of pathogen and absence of sclerotial production in *R. solani*.

Based on the antagonistic properties and inhibition percentage, the *Trichoderma* isolates *viz.,* TRSN1, TRMW2 and TRPN14 were selected for protoplast fusion. During the protoplast isolation, the maximum number of protoplasts was obtained after 2 h of incubation of mycelia of parental isolates with the lytic enzyme. Protoplast fusion was carried out between the selected isolates (TRSN1 × TRPN14, TRSN1 × TRMW2 and TRPN14 × TRMW2) in the presence of poly ethylene glycol (PEG 6000).

The protoplast fusants were selected using carbendazim-amended PDA medium. Fungicide concentration at 0.2 per cent (carbendazim) was selected as marker for selection of fusants. The radial growth and colony characters of the protoplast fusants were studied on PDA medium. The selected protoplast fusants displayed fast growth and completely covered the Petri dish at fifth day of growth. The colony characters such as mycelial colour varied from light to dark green mycelium with fluffy growth and scattered to circular green heavy sporulation. Fusants exhibited variations in colony characters, radial growth and enhanced sporulation in fusants compared to the parents

The antagonistic efficacy of the selected fusants was tested under *in vitro* condition against *P. aphanidermatum* and *R. solani*. It was revealed that highest

inhibition against *P. aphanidermatum* was observed with fusant 3 (84.4 %) followed by fusant 2 (74.44 %). Highest inhibition against *R. solani* was observed with fusant 2 (100 %) followed by fusant 3 (70.30 %).

Antagonistic properties *viz.*, antibiosis, lysis and overgrowth were observed in the protoplast fusants during antagonist-pathogen interaction. Among the three protoplast fusants, fusant 1 exhibited all the antagonistic properties against both the pathogens with heavy sporulation. Thus, protoplast fusion between *Trichoderma* isolates was found to increase the sporulation and antagonistic properties.

This study revealed that protoplast fusion among the selected isolates of *Trichoderma* spp. led to the production of fusants with high sporulation while compared to parents. Further studies on characterisation and *in vivo* evaluation of fusants and parental strains will be needed to utilize these strains in crop disease management.

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Appendix

APPENDIX

Composition of media

1. Trichoderma Selective Medium (1L)

$MgSo_4$. : $7H_2O$: 0.2 g

K_2HPO_4 : 0.9 g

KCl: 0.15 g

NH_4NO_3: 1.0 g

Glucose: 3.0 g

Chloramphenicol: 0.25 g

Rose Bengal: 0.15 g

Agar: 20 g

2. Potato Dextrose Agar Medium (1 L)

Potato : 200 g

Dextrrose : 20 g

Agar : 20 g

3. Malt Extract Agar medium (1L)

Malt Extract : 20 g

Dextrose : 20 g

Peptone : 6 g

Agar : 15 g

Strain improvement of *Trichoderma* spp. by protoplast fusion

by

ANIT CYRIAC

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Abstract of the thesis

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ABSTRACT

A study on "Strain improvement of *Trichoderma* spp. by protoplast fusion" was conducted at Department of Plant Pathology, College of Agriculture, Vellayani during the year 2018-2020, with the objective of improving the screened strains of *Trichoderma* spp. by protoplast fusion for increasing the antagonistic ability and related traits against soil borne pathogens.

A survey was conducted in five agro-climatic zones of Kerala *viz.*, Northern Zone, Central Zone, High Range Zone, Problem Area Zone and Southern Zone for collection of soil samples especially from forest soils. The collected soil samples were assessed for the population of *Trichoderma* spp. A total of 31 *Trichoderma* spp. isolates were obtained from the soil samples collected from five agro-climatic zones. Majority of the isolates were obtained from soils with pH of 6 to 7. Isolate TRMW2, TREN1, TREZ1, TREZ2, TREZ3, TRRN1, TRRN2, TRKR1, TRPN3, TRPN7, TRPN10 and TRPN18 exhibited full growth at four days after inoculation (DAI). The isolated *Trichoderma* spp. differed in growth rate and colony characters like colour of mycelium, texture of colony and sporulation pattern.

Isolates of *Trichoderma* spp. from different zones exhibited *in vitro* inhibition against soil borne pathogens such as *Pythium aphanidermatum* and *Rhizoctonia solani*. Majority of the isolates displayed high inhibition per cent compared to KAU strain of *Trichoderma sp*. TRRN1, TRRN2, TRPN3, TRPN7, TRPN11, TRPN15 and TRKR2 isolates exhibited complete inhibition of *P. aphanidermatum* in dual culture experiment; whereas TREN1, TRMW2, TREZ1, TREZ2, TRKM1, TRPN7, TRPN9, TRPN14, TRPN15, TRPN17, TRPN18 and TRKR2 isolates exhibited complete inhibition of *R. solani*. *Trichoderma* isolates such as TRPN7, TRPN15 and TRKR2 exhibited complete inhibition against both the pathogens. Antagonistic properties *viz.*, antibiosis, lysis and overgrowth of *Trichoderma* isolates against *P. aphanidermatum* and *R. solani* were observed. During the antagonist-pathogen interaction, isolates TRSN1, TRSN2, TRPN10, TRPN14, TRPN15, TRPN17 and TRPN18 exhibited high levels of antibiosis. Most of the isolates caused lysis of mycelium of the pathogens which resulted in formation of clear zones in dual culture. Overgrowth of the

antagonist was another prominent antagonistic property observed in the majority of the isolates.

Based on the antagonistic properties, the *Trichoderma* isolates *viz.*, TRSN1, TRMW2 and TRPN14 were selected for the protoplast fusion. During the protoplast isolation, the maximum number of protoplasts was obtained after 2 h of incubation of mycelia of parental isolates with the lytic enzyme. Protoplast fusion was carried out between the selected isolates (TRSN1 x TRPN14, TRSN1 x TRMW2, and TRPN14 x TRMW2) in the presence of poly ethylene glycol (PEG 6000). Three protoplast fusants were selected using carbendazim-amended PDA medium. The protoplast fusants displayed fast growth on PDA medium and completely covered the Petri dish at 5^{th} of growth. The colony characters of fusants varied from light to dark green mycelium with fluffy growth and scattered to circular green heavy sporulation.

In vitro screening of protoplast fusants against *P. aphanidermatum* and *R. solani* revealed that highest inhibition against *P. aphanidermatum* was observed with fusant 3 (84.4%) followed by fusant 2 (74.44%). Highest inhibition against *R. solani* was observed with fusant 2 (100%) followed by fusant 3 (70.30%). Antagonistic properties *viz.,* antibiosis, lysis and overgrowth were observed in the protoplast fusants. Among the three protoplast fusants, fusant 1 exhibited all the antagonistic properties against both the pathogens with heavy sporulation.

Thus, the present study has thrown light in understanding the potential of protoplast fusion in evolving improved strains of *Trichoderma* spp. Protoplast fusion enhanced sporulation in fusants compared to the parents. Further studies need to be conducted for the biochemical and molecular characterisation of parental isolates and fusants. The parents and protoplast fusants also have to be evaluated for their *in vivo* efficacy against soil borne pathogens in major crops of Kerala.

സംഗ്രഹം

"ട്രൈക്കോഡെർമ സ്പെഷ്സ് സ്ട്രെയിൻ മെച്ചപ്പെടുത്തൽ" എന്ന ലക്ഷ്യത്തോടെ, 2018-2020 വർഷത്തിൽ വെള്ളായണി കാർഷിക കോളേജ്, പ്ലാന്റ് പാത്തോളജി വിഭാഗം നടത്തിയ പഠനമാണ് "പ്രോട്ടോപ്ലാസ്റ് സംയോജനം വഴിയുള്ള ട്രൈകോഡർമ സ്പെഷ്സുകളുടെ സട്രെയിൻ മച്ചപ്പെടുത്തൽ". മണ്ണിൽ നിന്ന് പകരുന്ന രോഗകാരികൾക്കെതിരായി അന്റാഗോണിസ്റ്റിക് ശേഷിയും അനുബന്ധ സ്വഭാവങ്ങളും വർദ്ധിപ്പിക്കുക എന്ന ലക്ഷ്യത്തോടെയാണ് പ്രത്യേകം തിരഞ്ഞിടുത്ത ട്രൈകോഡെർമ സ്പെഷ്സുകളുടെ പ്രോട്ടോപ്ലാസ്റ്റ് സംയോജനം നടത്തിയത്.

കേരളത്തിലെ അഞ്ച് കാർഷിക കാലാവസ്ഥാ മേഖലകലായ, വടക്കൻ മേഖല, മധ്യമേഖല, ഉയർന്ന ശ്രേണി മേഖല, പ്രശ്നമേഖല, തെക്കൻ മേഖല എന്നിവിടങ്ങളിൽ നിന്ന് ഒരു സർവേ നടത്തി അവിടുത്തെ വനപ്രദേശത്തു നിന്നുള്ള മണ്ണ് സാമ്പിളുകളാണ് ശേഖരിച്ചത്. ഓരോ മണ്ണ് സാമ്പിൾലുകളിലെ ട്രൈക്കോഡെർമ സ്പെഷ്സുകളുടെ എണ്ണം ടി യസ് എം മീഡിയയിൽ വിലയിരുത്തി. അഞ്ച് കാർഷിക കാലാവസ്ഥാ മേഖലകളിൽ നിന്ന് ശേഖരിച്ച മണ്ണിന്റെ സാമ്പിളുകളിൽ നിന്ന് ആകെ 31 ട്രൈകോഡെർമ ഐസൊലേറ്റലുകൾ ലഭിച്ചു. ഭൂരിഭാഗം ഐസൊലേറ്റുകളും 6 മുതൽ 7 വരെ പി.എച്ച് ഉള്ള മണ്ണിൽ നിന്നാണ് ലഭിച്ചത്. ഐസൊലേട്ടുകളായ ടിർമഡബ്ലിയു2, ടി ർ ഇ ൻ 1, ടി ർ ഇ സ്ഡ് 1, ടി ർ ഇ സ്ഡ് 2, ടി ർ ഇ സ്ഡ് 3, ടി ആർ ആർ ൻ 1, ടി ആർ ആർ ൻ 2, ടി ആർ കെ ർ 1, ടി ർ പി ൻ 3, ടി ർ പി ൻ 7, ടി ർ പി ൻ 10, ടി ർ പി ൻ 18 എന്നിവ ഇനോകുലേറ്റ് ചെയ്ത് നാല് ദിവസത്തിനുള്ളിൽ പൂർണ്ണ വളർച്ച പ്രകടമാക്കി. ട്രൈക്കോഡെർമ സ്പെഷ്സുകളുടെ കോളനി സ്വഭാവങ്ങളായ വളർച്ചാ നിരക്ക്, മൈസീലിയത്തിന്റെ നിറം, സ്പോർലേഷൻ പാറ്റേൺ എന്നിവയിൽ വ്യത്യാസമുണ്ട്.

വിവിധ മേഖലകളിൽ നിന്നുള്ള ഐസൊലേറ്റുകൾ പിത്തിയം അഫാനിഡെർമേറ്റം, റൈസോക്റ്റോണിയ സോളാനി തുടങ്ങിയ മണ്ണിൽ നിന്നുള്ള രോഗകാരികൾക്കെതിരായി ഇൻ വിട്രോ സാഹചര്യത്തിൽ ഇൻഹിബിഷനിൽ പ്രദർശിപ്പിച്ചിരുന്നു. കേരള കാർഷിക യൂണിവേഴ്സിറ്റിയുടെ ട്രൈക്കോഡെർമ സ്പെഷ്സുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ ഭൂരിഭാഗം ഐസുലേറ്റുകൾ ഉയർന്ന ഇൻഹിബിഷൻ ശതമാണ് കാണിക്കുന്നു. ഐസൊലേടുകളായ ടി ആർ ആർ ൻ 1, ടി ആർ ആർ ൻ 2, ടി ആർ പി ൻ 3, ടി ആർ പി ൻ 7,ടി ആർ പി ൻ 11, ടി ആർ പി ൻ 15, ടി ആർ കെ ആർ 2 പിത്തിയം അഫനിഡർമേറ്റത്തിന്റെ വളർച്ചയെ പൂർണ്ണമായി ഇൻഹിബിറ്റ് ചെയ്തു. അതുപോലെ തന്നെ ഐസോ സുലേറ്റുകലായ ടി ആർ ഇ ൻ 1, ടി ആർ മ് ഡബ്ലിയു 2, ടി ആർ ഇ സ്ഡ് 1, ടി ആർ ഇ സ്ഡ് 2, ടി ആർ കെ മ് 1, ടി ആർ പി ൻ 7, ടി ആർ പി ൻ 9, ടി ആർ പി ൻ 14, ടി ആർ പി ൻ 15, ടി ആർ പി ൻ 17, ടി ആർ പി ൻ 18, ടി ആർ കെ ആർ

2 എന്നിവയും പൂർണമായ ഇൻഹിബിഷൻ ആർ . സോളാനിക്കെതിരെ പ്രകടമാക്കി. ട്രൈക്കോഡെർമ ഐസോലേറ്റുകളായ ടി ആർ പി ൻ 7, ടി ആർ പി ൻ 15, ടി ആർ കെ ആർ 2 എന്നിവ രണ്ട് രോഗകാരികൾക്കെതിരായി പൂർണ്ണമായ ഇൻഹിബിഷൻ പ്രകടമാക്കി. പി. അഫാനിഡെർമാറ്റത്തിനും ആർ. സോളാനിക്കും എതിരായി ട്രൈക്കോഡെർമ ഐസൊ സുലേറ്റുകളുടെ ആന്റിബയോസിസ്, ലിസിസ്, ഓവർ ഗ്രൗത്ത് എന്നിവ നിരീക്ഷിക്കപ്പെട്ടു. അന്റാഗോണിസ്റ്റ്-പത്തോജൻ പ്രതിപ്രവർത്തന സമയത്ത് ടി ആർ യസ് ൻ 1, ടി ആർ യസ് ൻ 2, ടി ആർ പി ൻ 10, ടി ആർ പി ൻ 14, ടി ആർ പി ൻ 15, ടി ആർ പി ൻ 17, ടി ആർ പി ൻ 18 ഐസൊ സുലേറ്റുകൾ ഉയർന്ന അളവിൽ ആന്റിബയോസിസ് പ്രദർശിപ്പിച്ചു. മിക്ക ഐസൊലേറ്റുകളും രോഗകാരികളുടെ മൈസീലിയം ലൈസിസിന് കാരണമായി, ഇത് ഡ്യൂവൽ കൾച്ചറിൽ വ്യക്തമായ സോണുകൾ രൂപപ്പെടുന്നതിന് കാരണമായി. ഭൂരിഭാഗം ഐസൊലേറ്റുകളിലും നിരീക്ഷിക്കപ്പെടുന്ന മറ്റൊരു പ്രധാന സ്വഭാവമാണ് അന്റാഗോണിസ്റ്റിന്റെ വളർച്ച.

അന്റാഗോണിസ്റ്റിക് സ്വഭാവങ്ങളെ അടിസ്ഥാനമാക്കി, പ്രോട്ടോപ്ലാസ്റ്റ് സംയോജനത്തിനായി ട്രൈക്കോഡെർമ ഐസൊലേറ്റുകൾ, ടി ആർ യസ് ൻ 1, ടി ആർ മ ഡബ്ലിയു 2, ടി ആർ പി ൻ 14 എന്നിവ തിരഞ്ഞെടുത്തു. പ്രോട്ടോപ്ലാസ്റ്റ് വേർതിരിച്ചെടുക്കുന്ന സമയത്ത്, ലൈറ്റിക് എൻസൈമിനൊപ്പം പേരെന്റ്സ് ഐസൊലേറ്റുകളുടെ മൈസീലിയ ഇൻകുബേഷൻ ചെയ്തപ്പോൾ പരമാവധി പ്രോട്ടോപ്ലാസ്റ്റുകൾ ലഭിച്ചു. പോളി എഥിലീൻ ഗ്ലൈക്കോളിന്റെ (PEG 6000) സാന്നിധ്യത്തിൽ തിരഞ്ഞെടുത്ത ഐസൊലേറ്റുകളിൽ (ടി ആർ യസ് ൻ 1 x ടി ആർ പി ൻ 14, ടി ആർ യസ് ൻ 1 x ടി ആർ മ് ഡബ്ലിയു 2, ടി ആർ പി ൻ 14 x ടി ആർ മ് ഡബ്ലിയു 2) പ്രോട്ടോപ്ലാസ്റ്റ് സംയോജനം നടത്തി. കാർബെൻഡാസിം ചേർത്ത പിഡിഎ മീഡിയം ഉപയോഗിച്ച് മൂന്ന് പ്രോട്ടോപ്ലാസ്റ്റ് ഫ്യൂസന്റുകൾ തിരഞ്ഞെടുത്തു. പ്രോട്ടോപ്ലാസ്റ്റ് ഫ്യൂസന്റുകൾ പിഡിഎ മീഡിയത്തിൽ അതിവേഗ വളർച്ച കാണിക്കുകയും വളർച്ചയുടെ അഞ്ചാം ദിവസം പെട്രിപ്ലേറ്റിൽ പൂർണ്ണമായും വളരുകയും ചെയ്തു. ഫ്യൂസന്റുകളുടെ കോളനി സ്വഭാവങ്ങളിൽ വെള്ള മുതൽ കടും പച്ച നിറമുള്ള മൈസീലിയം വരെ കാണപ്പെട്ടു.

പി. അഫാനിഡെർമാറ്റമിനും ആർ. സോളാനിക്കുമെതിരായ പ്രോട്ടോപ്ലാസ്റ്റ് ഫ്യൂസന്റുകളുടെ ഇൻ വിട്രോ സ്ക്രീനിംഗിൽ ഏറ്റവും ഉയർന്ന ഇൻഹിബിഷൻ പി. അഫനിഡർമേട്റ്റത്തിന് എതിരായി ഫ്യൂസന്റ് 3 (84.4%), ഫ്യൂസന്റ് 2 (74.44%). ആർ സോളനിക്കെതിരെ ഫ്യൂസന്റ് 2 (100%), ഫ്യൂസന്റ് 3 (70.30%) ഇൻഹിബിഷൻ നിരീക്ഷിച്ചു. പ്രോട്ടോപ്ലാസ്റ്റ് ഫ്യൂസന്റുകളിൽ അന്റാഗിണിസ്റ്റിക് സ്വഭാവങ്ങളായ, ആന്റിബയോസിസ്, ലിസിസ്, ഓവർ ഗ്രോത്ത് എന്നിവ നിരീക്ഷിക്കപ്പെട്ടു. മൂന്ന് പ്രോട്ടോപ്ലാസ്റ്റ് ഫ്യൂസന്റുകളിൽ, ഫ്യൂസന്റ് 1 രണ്ട് രോഗകാരികൾക്കെതിരെയും എല്ലാ അന്റാഗോണിസ്റ്റിക് സ്വഭാവങ്ങളും കനത്ത സ്പോർലേഷൻ പ്രദർശിപ്പിച്ചു.

അതിനാൽ, ട്രൈക്കോഡെർമ സ്പിസ്സുകളുടെ സ്ട്രെയിൻ മെച്ചപ്പെടുത്തുന്നതിൽ പ്രോട്ടോപ്ലാസ്റ്റ് സംയോജനത്തിന്റെ സാധ്യതകൾ മനസ്സിലാക്കുന്നതിൽ ഇപ്പോഴത്തെ പഠനം സഹായിക്കുന്നു. പ്രോട്ടോപ്ലാസ്റ്റ് ഫുസന്റ്സ് പേരെന്റ്സുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ ഫ്യൂസന്റുകളിൽ സ്പോർലേഷൻ വർദ്ധിപ്പിച്ചു. പരൻസിന്റെയും ഐസൊലേറ്റുകളുടെയും ഫ്യൂസന്റുകളുടെയും ബയോകെമിക്കൽ, മോളിക്യുലർ സ്വഭാവസവിശേഷതകൾക്കായി കൂടുതൽ പഠനങ്ങൾ നടത്തേണ്ടതുണ്ട്. കേരളത്തിലെ പ്രധാന വിളകളിലെ മണ്ണിൽ നിന്നുള്ള രോഗകാരികൾക്കെതിരായ എൻ വിവോ പരീക്ഷണത്തിലൂടെ പേരൻസിനെയും പ്രോട്ടോപ്ലാസ്റ്റ് ഫ്യൂസന്റുകളെയും വിലയിരുത്തേണ്ടതുണ്ട്.