MOLECULAR CHARACTERIZATION OF PROMISING ISOLATES OF *Trichoderma* spp. AND THEIR FIELD EVALUATION AGAINST FUSARIUM WILT OF VEGETABLE COWPEA

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(2020-11-135)

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM - 695 522
KERALA, INDIA

2023

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by

JEEVIDHA M.

(2020-11-135)

THESIS

Submitted in partial fulfilment of the

requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM – 695 522

KERALA, INDIA

2023

DECLARATION

I, hereby declare that this thesis entitled **"Molecular characterization of promising isolates of *Trichoderma* spp. and their field evaluation against Fusarium wilt of vegetable cowpea"** is a bonafide record of research work done by me during the course of research and that 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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Place : Vellayani

Date : 07.08.2023

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CERTIFICATE

Certified that this thesis entitled **"Molecular characterization of promising isolates of *Trichoderma* spp. and their field evaluation against Fusarium wilt of vegetable cowpea"** is a record of research work done independently by **Mrs. Jeevidha M. (2020-11-135)** under my guidance and supervision and that it has not previously formed the basis for any degree, diploma, fellowship or associateship to her.

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ACKNOWLEDGEMENT

I bow to the feet of the almighty whose grace had endowed me with the inner strength, patience, willpower and health to complete this endeavor successfully.

Words fail to express my indebtedness with veneration to ***Dr. Sible George Varghese****, Professor and Head, Department of Plant Pathology, College of Agriculture, Vellanikkara, Chairman of my advisory committee, for his inspiring guidance, constant supervision, affectionate advice, timely help and encouragement throughout the study period and during the thesis preparation.*

I owe my sincere thanks to ***Dr. N.V. Radhakrishnan****, Professor and Head, Department of Plant Pathology, College of Agriculture, Vellayani and member of my advisory committee for his expert advice, constant inspiration and constructive criticisms provided throughout the research work.*

I am ineffable in expressing my deep sense of gratitude to ***Dr. Joy M.****, Professor and Head, Coconut Research Station and member of my advisory committee for the valuable guidance, close scrutiny, precious suggestions and help rendered all throughout my study.*

I take this opportunity to express my heartfelt gratitude to ***Dr. Susha S. Thara****, Assistant Professor, Department of Plant Pathology, College of Agriculture, Vellayani and member of my advisory committee for her suggestions, constant encouragement and support at various stages of my work.*

I express my heartiest thankfulness to ***Dr. Anuradha T.****, Assistant Professor, Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani and member of my Advisory Committee for her valuable suggestions, expert guidance and support to complete the research work.*

I deeply express my special whole hearted thanks to ***Dr. Sreeja S. J.****, Assistant Professor and Head, Rice Research Station for her ever-willing help, valuable guidance and creative suggestions all throughout the conduct of my study.*

I am so much grateful to Dr. Ayisha R., Assistant Professor, and Department of Plant Pathology for the valuable suggestions and advice throughout the study period. I also express my gratitude to Dr. Radhika N. S., Dr. Heera G., Dr. Krishnapriya P. J., Mrs. Sherin A. Salam, Mrs. Divya S., and Dr. Pramod S., Assistant Professors, Department of Plant Pathology for their moral support.

I also avail this opportunity to express my thankfulness to Dr. Rajeev Kumar Sukumaran, Senior Principal scientist, Microbial Processes and Technology Division, for extending all the facilities to carry out the enzyme study at CSIR- National Institute for Interdisciplinary Science and Technology.

I wish to express my sincere gratefulness to Dr. Meera Christoper, Senior Research Fellow, Department of Microbial Processes and Technology Division, at CSIR- National Institute for Interdisciplinary Science and Technology for the guidance and support to complete the enzyme analysis of my research data.

I express my heartfelt gratitude and indebtedness to Jayakumar uncle and Sujin chettan, Department of Plant Pathology who helped me unconditionally throughout the study period.

I extend my gratitude to the non-teaching staff, College of Agriculture, Vellayani who have helped and supported me in one or the other way throughout this journey.

Words fall short as I place on record my indebtedness to Abirami G., Vyshnavi A. S., Amelinora Tariang and Vijesh M. for their untiring support and encouragement throughout my PG life.

It gives me great pleasure to express my immense gratitude to my selfless friends Pratiksha I., Sushmitha T., Asmitha Takale, Anjali S., Arathy, Amudhayazhmozhi kural, Harini, Devika and Kani. I also extend my thanks to all my batchmates, Amritha, Navya, Sreekanth, Ramseena, Aswathy and Anukrishna for their support and help whenever needed.

I also express my gratitude towards my lovable seniors Athira Nair, Siva M., Vijeth, Amitha chechi, Mary Sharmila, Aswathy chechi, Ritesh chettan, Ananthu chettan, Arun chettan, Deepa chechi, Amritha chechi, Josiya chechi, Sushitha chechi, Elizbeth chechi, Manjushri chechi for their support, timely advice and help during the study period. I extend my thanks to all my juniors for their support and help.

My heartfelt words cannot be expressed in words for the constant support and helping hands of Rahul anna, Keerthu, Yoki anna, Sangavi, Sahaa anna, Prashanth anna, Sharan anna and Ragavi

Above all, I am forever beholden to my loving parents, sister, in-laws and grandparents for their affection, prayers, sacrifice and inspiration which has always melted the hurdles on my way to success.

Last but not least; I am deeply indebted to my loving husband for his unwavering support, care and sincere encouragement which greatly helped me in the successful completion of this endeavor.

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ACKNOWLEDGEMENT

I bow to the feet of the almighty whose grace had endowed me with the inner strength, patience, willpower and health to complete this endeavor successfully.

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gratitude to ***Dr. Radhika N. S., Dr. Heera G., Dr. Krishnapriya P. J., Mrs. Sherin A. Salam, Mrs. Divya S.,*** *and* ***Dr. Pramod S.,*** *Assistant Professors, Department of Plant Pathology for their moral support.*

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Jeevidha M.

CONTENTS

Sl. No.	Chapter	Page No.
1	INTRODUCTION	1`
2	REVIEW OF LITERATURE	3
3	MATERIALS AND METHODS	15
4	RESULTS	33
5	DISCUSSION	55
6	SUMMARY	63
7	REFERENCES	65
	APPENDICES	79
	ABSTRACT	82

LIST OF TABLES

Table No.	Title	Page No.
1	Primers used in PCR	23
2	*In vitro* activity of chitinase, protease and lipase by isolates of *Trichoderma* spp. grown in potato dextrose broth	34
3	Colonization by *Trichoderma* spp. on paddy straw bits in soil inoculated with spore suspensions of different isolates under study	35
4	Radial growth of the *Trichoderma* in dual culture on PDA medium at seven DAI	36
5	Probable interaction of different *Trichoderma* isolates in dual culture graded by visual observation	37
6	*In vitro* efficacy of selected isolates of *Trichoderma* sp. against *F. oxysporum*	39
7	Antagonistic characters of selected isolates of *Trichoderma* sp. against *Fusarium oxysporum*	40
8	Molecular characterization of isolates of *Trichoderma* spp. by ITS-PCR	43
9	Efficacy of *Trichoderma* isolates against Fusarium wilt of vegetable cowpea in pot culture	46

10	Population of *Trichoderma* spp. in soil from different treatments of the pot culture experiment	48
11	Efficacy of *Trichoderma* isolates against Fusarium wilt of vegetable cowpea under field conditions	51
12	Population of *Trichoderma* spp. in soil from different treatments of the field experiment	53

LIST OF FIGURES

Figure No.	Title	Between pages
1	BLAST analysis of ITS sequences of TRMW-2	42-43
2	BLAST analysis of ITS sequences of TRKR-2	42-43
3	BLAST analysis of ITS sequences of TRPN-3	42-43
4	BLAST analysis of ITS sequences of TRPN-11	42-43
5	BLAST analysis of ITS sequences of TRPN-17	42-43
6	BLAST analysis of TvP sequences of TRMW-2	42-43
7	BLAST analysis of TvP sequences of TRKR-2	42-43
8	BLAST analysis of TvP sequences of TRPN-3	42-43
9	BLAST analysis of TvP sequences of TRPN 11	42-43
10	BLAST analysis of TvP sequences of TRPN 17	42-43
11	Phylogenetic tree generated from ITS-rDNA sequences of *Trichoderma* isolates by Neighbor-Joining Tree (NJT) using MEGA 11	42-43
12	Phylogenetic tree generated from TvP-rDNA sequences of *Trichoderma* isolates by Neighbor-Joining Tree (NJT) using MEGA 11	42-43
13	Absorbance value of chitinase activity of *Trichoderma* isolates	56-57

14	Absorbance value of protease activity of *Trichoderma* isolates	56-57
15	Absorbance value of lipase activity of *Trichoderma* isolates	56-57
16	Per cent colonization of straw bits by *Trichoderma* isolates	56-57
17	Per cent inhibition of selected isolates of *Trichoderma* sp. against *Fusarium oxysporum*	56-57

LIST OF PLATES

Plate No.	Title	Between Pages
1	Isolates of *Trichoderma* spp. on PDA medium	34-35
2	Growth of *Trichoderma* spp. on TSM (five DAI) from straw bits taken from soil inoculated with isolate TRMW-2	34-35
3	Growth of *Trichoderma* spp. on TSM (five DAI) from straw bits taken from soil inoculated with isolate TRKR-2	34-35
4	Growth of *Trichoderma* spp. on TSM (five DAI) from straw bits taken from soil inoculated with isolate TRPN-3	34-35
5	Growth of *Trichoderma* spp. on TSM (five DAI) from straw bits taken from soil inoculated with isolate TRPN-3	34-35
6	Growth of *Trichoderma* spp. on TSM (five DAI) from straw bits taken from soil inoculated with isolate TRPN-17	34-35
7	Fungal growth on TSM (five DAI) from straw bits taken from soil (Control)	34-35
8	Assessment of compatibility of different *Trichoderma* isolates by dual culture	36-37
9	Assessment of compatibility of different *Trichoderma* isolates by dual culture	36-37
10	Assessment of compatibility of different *Trichoderma* isolates by dual culture	36-37
11	Dual culture assay of *Trichoderma* isolates TRMW-2, TRKR-2, TRPN-3 and TRPN-11 against *F. oxysporum*	38-39
12	Dual culture assay of *Trichoderma* isolates TRPN-17, *T. asperellum* (KAU strain) and *T. harzianum* (NBAIR strain) against *F. oxysporum*	38-39
13	PCR amplification pattern of *Trichoderma* isolates using ITS primers	42-43

14	PCR amplification pattern of *Trichoderma* isolates using genus-specific primers TvPF and TvPR	42-43
15	PCR amplification of *Trichoderma* isolates using species-specific primer tef 1A	42-43
16	PCR amplification of *Trichoderma* isolates using species-specific primers tef 1B, tef 1C, tef 1D and rpb2	42-43
17	PCR amplification of *Trichoderma* isolates using species-specific primers at gradient temperature 55 °C - 65 °C.	42-43
18	PCR amplification of *Trichoderma* isolates using species-specific primers with MgCl2	42-43
19	PCR amplification of *Trichoderma* isolates using 2 µl of species-specific primers	42-43
20	Sequences of amplified ITS region of isolates TRMW-2, TRKR-2 and TRPN-3	42-43
21	Sequences of amplified ITS region of isolates TRPN-11 and TRPN-17	42-43
22	Amplified sequences of isolates TRMW-2, TRKR-2 and TRPN-3 using genus specific primers TvPF and TvPR	42-43
23	Amplified sequences of isolate TRPN-11 and TRPN-17 using genus specific primers TvPF and TvPR	42-43
24	Field evaluation of selected *Trichoderma* isolates against Fusarium wilt of vegetable cowpea in sick plot	52-53

LIST OF APPENDICES

Sl. No.	Title	Page No.
1	Composition of Media used	79
2	List of buffers used	80

LIST OF ABBREVIATIONS AND SYMBOLS USED

ANOVA	Analysis of Variance
Bp	Base pair
BLAST	Basic Local Alignment Search Tool
CD	Critical difference
CMCase	Carboxymethyl cellulose enzyme
Cm	Centimetre
CRD	Completely Randomized Design
CSA	Competitive saprophytic ability
DAI	Days After Inoculation
DAS	Days After Sowing
DMAB	p-dinitro methyl amino benzaldehyde
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetraacetic acid
EF1	alpha elongation factor
et al.	And co-workers
FCR	Fusarium Crown Rot
FHB	Fusarium Head Blight
Foc TR4	*Fusarium oxysporum* f. sp. *cubense* tropical race 4
H	Hour
ITS	Internal Transcribed Spacer region
Kg	Kilogram
KAU	Kerala Agricultural University
Mm	Millimetre
Ml	Millilitre
mM	Millimolar
min.	Minute
MEGA	Molecular Evolutionary Genetics Analysis
M	Molar

NBAIR	National Bureau of Agricultural Insect Resources
NCBI	National Center for Biotechnology Information
NJT	Neighbor-Joining Tree
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PCR	Polymerase chain reaction
PI	Per cent inhibition
RBD	Randomized Block Design
Rpb2	RNA polymerase B II
rpm	Revolutions per minute
SAP	Shrimp Alkaline Phosphatase
SD	Standard deviation
spp.	Species
s	Seconds
TBE	Tris-Borate EDTA
TE	Tris EDTA
TSM	*Trichoderma* Selective Medium
tef1	Translation elongation factor 1-alpha
UV	Ultra violet
var.	Variety
viz.	Namely
i.e.	That is
@	At the rate of
%	Per cent
°C	Degree Celsius
µl	Microlitre
µm	Micrometre
w/w	Weight/Weight
g l^{-1}	Gram/litre
g kg^{-1}	Gram/kilogram
cfu/g	Colony forming units/ gram

Introduction

1. INTRODUCTION

Vegetable cowpea or yard long bean (*Vigna unguiculata* subsp. *sesquipedalis* (L.) Verdcourt) is one of the economically important legume crops that originated in Africa and was later introduced in other tropical countries. It is a widely cultivated vegetable crop in the wetland fallows of Kerala due to its favourable agro-climatic conditions as it fits well in the multi-farming system. Green pods and seeds are consumed as fresh vegetables and the plants can also be used as fodder, green manure, or cover crop. The tender pods are an inexpensive and rich source of digestible protein (28 %), iron (2.5 mg/100 g), calcium (80 mg/100 g), phosphorus (74 mg/100 g), vitamin A (941 IU/100 g), vitamin C (13 mg/100g), and dietary fiber (2 g/100 g), which makes it an outstanding vegetable crop (Singh *et al*, 2001). The crop has become very important in agriculture due to its ability to fix atmospheric nitrogen through its well-branched root structure. It reduces soil erosion by enhancing the soil binding effect.

Soil-borne diseases, such as Fusarium wilt, collar rot, web blight, etc., have a significant impact on the crop at various stages of its development. In recent years, Fusarium wilt has become one of the most devastating crop diseases in Kerala (Reghunath *et al*., 1995; Kumar, 2003). The management of Fusarium wilt commercially relies on the use of fungicides. The necessity to find effective natural biocontrol agents in integrated crop management programs has arisen due to the negative impact of fungicides and market regulations. The use of bioagents as part of integrated disease management has also been investigated.

Although many other biocontrol strategies have been explored, one of the most promising ways to both decrease fungicide use and guarantee safe food production is to deploy more potent strains of *Trichoderma*. It is a predominantly used fungal biocontrol agent against soil-borne pathogens due to its capacity to effectively counteract soil-borne fungal infections through several mechanisms viz., mycoparasitism, antibiosis, competition for nutrient and space, induced resistance, tolerance to stress through enhanced root and plant development, solubilisation and sequestration of inorganic nutrients and inactivation of pathogen enzymes (Harman, 2000).

Morphological traits alone are insufficient for identifying *Trichoderma* spp. It is crucial to characterize them on a molecular level in order to comprehend their antagonistic activity against a variety of plant diseases. Molecular identification of *Trichoderma* spp. has become very much essential in addition to morphological identification in recent times for knowing the identity up to species level for which amplification of specific gene sequences in fungi is being employed. In addition, knowledge on biocontrol attributes like competitive saprophytic ability, enzyme production etc. of *Trichoderma* isolates also helps in selecting the best candidate for effective disease management. Field evaluation of promising bioagents will help in carrying forward new strains to combat soil-borne diseases.

The present study was undertaken with the following objectives:

1. Molecular characterization of promising isolates of *Trichoderma* spp. obtained from virgin forest soils of Kerala.
2. Field evaluation of the promising isolates of *Trichoderma* spp. against Fusarium wilt of vegetable cowpea.

Review of Literature

2. REVIEW OF LITERATURE

Trichoderma spp. has been used in a wide range of agro-climatic conditions for the control of soil-borne plant diseases. Rice, tomato, cowpea, and other crops has benefited from the application of *T. viride* and *T. harzianum* for disease management (de Franca *et al.,* 2015; Kipngeno *et al.,* 2015).

2.1. ASSESSMENT OF THE BIOCONTROL ATTRIBUTES OF *TRICHODERMA* ISOLATES

2.1.1. Assay of activity of cell wall degrading enzymes of *Trichoderma* isolates

Trichoderma species compete with other fungi for resources and space through mycoparasitism, antibiosis, and other modes of antagonism. In addition to these, induced systemic or localised resistance and the synthesis of antifungal enzymes such as chitinases and β-1, 3 glucanases also contribute to pathogen suppression (Harman, 2006). Some genes such as *chit36Y*, responsible for the production of chitinase can be expressed in the absence of direct interaction with the pathogen. Overexpression of the *chit36*, *chit42*, and *Chit33* genes in *Trichoderma* spp. during mycoparasitism provides evidence for the importance of chitinases in disease management. It is widely known that β-1, 3 glucan is a major structural component of fungal cell walls and that the β -1, 3 glucanase enzyme degrades glucan in the cell walls of pathogenic fungus. While several β-1, 3-glucanases have been discovered in recent years, only a small number of genes like *bgn13.1* have been cloned (Benitez *et al*., 1998; Khatri *et al*., 2017)

According to Geraldine *et al.* (2013), *Trichoderma* species can limit the growth of common bean white mold by using enzymes such as N-acetylglucosaminidase, acid phosphatase, β-1,3-glucanases, glucosidases, lipases, proteases, and chitinases. Cherkupally *et al.* (2017) isolated seven species of *Trichoderma i.e*. *T. viride, T. harzianum, T. virens, T. atroviride, T. koningii, T. pseudokoningii* and *T. reesei*. They assessed the antagonistic activity of the isolates with the production of extracellular enzymes on solid media by plate assay method against several fungal pathogens and observed the presence of cellulase, amylase, pectinase, protease, and chitinase activity. Boat *et al*. (2019) identified six strains of *Trichoderma* isolates namely *T. asperellum*

It-13, *T. koningiopsis* It-21, *T. erinaceum* It-58, *T. gamsii* It-62, *T. afroharzianum* P-8, and *T. harzianum* P-11. The identified isolates were shown to release hydrolytic enzymes (chitinase, cellulase, protease and lipase), as well as volatile and non-volatile inhibitory metabolites, with *T. gamsii* It-62 displaying the highest enzyme secretion.

Baiyee *et al.* (2019) treated lettuce plants infected by *Corynespora cassiicola* and *Curvularia aeria* with *T. asperellum* spore suspension and found that cell wall degrading enzymes β-1,3-glucanase, chitinase; peroxidase, and polyphenol oxidase were higher at 24 and 48 h after treatment. Vyawahare *et al.* (2019) have shown enhanced productivity of the chitinase enzyme of *T. viride* mutants. The mutant isolates TvME-4, TvMH-9 and TvME-3 produced a maximum of 0.64, 0.63 and 0.62 enzyme units respectively compared to wild-type strains. Ghasemi *et al.* (2019) demonstrated that ninety-five per cent of mutants of *T. harzianum* had higher chitinase expression than the wild strain. The mutant isolates T. h M8 (42.48 U mg^{-1}) and T. h M15 (38.25 U mg^{-1}) exhibited highest chitinase activity.

Reghmit *et al.* (2021) examined the biocontrol effectiveness of fifteen native isolates of *Trichoderma* spp. using a solid medium. The isolates T2 and T6 exhibited maximum chitinolytic activity of 0.043 mol ml^{-1} min^{-1} and a maximum proteolytic activity of 0.019 mol ml^{-1} min^{-1} respectively. Under *in vitro,* the isolates T2, T6 and T12 were observed to be the most effective *Trichoderma* isolates against the olive wilt pathogen *Verticillium dahliae*. *T. asperelloides* PSU-P1, as shown by Intana *et al.* (2022), mitigated gummy stem blight in muskmelon plants by upregulation of PR genes and increased enzyme activity. Muskmelon plants treated with *T. asperelloides* PSU-P1 revealed seven to ten times the increased expression of chitinase (0.15 to 0.284) and β-1,3 -glucanase (0.343 to 0.681 U ml^{-1}) genes compared to the control. Scanning electron microscopy confirmed the activity of cell wall degrading enzymes in crude metabolites isolated from *T. asperelloides* PSU-P1 treated muskmelon plants, causing wilting and lysis of *Stagonosporopsis cucurbitacearum* hyphae.

2.1.2. Competitive Saprophytic Ability (CSA)

Nakkeeran *et al.* (2005) screened the antagonistic efficacy of *Trichoderma viride* mutants and found that four mutants MG6 (91.6 %), MG3 (83.3 %), UV10 (75 %), and MNT7 (68.3 %) recorded higher CSA than the parent. The antagonistic potential of *T. viride* mutants corresponded to its increased CSA against the fungal pathogen. Four treatments were used by Sarrocco *et al*. (2009) to test the efficacy of their CSA against *Rhizoctonia solani* using wheat straw bits *viz*., substrate inoculated with *R. solani* alone, substrate inoculated with *R. solani* and *T. asperellum* I252, substrate inoculated with *R. solani* and *T. virens* I10 and substrate inoculated with *R. solani* and both *Trichoderma* isolates. They observed that both the antagonists superseded *R. solani* but their potential CSA seemed to be time-bound.

Khare *et al.* (2010) assayed CSA of wild-type and mutant strains of *Trichoderma viride* 1433 and found that the colonization of the mutant strains *viz*., Tv m6 (75.0 %), Tv m13 (68.3 %), Tv m21 (61.4 %) and Tv m9 (56.6 %) were more than that of the parent strain *T. viride* 1433 (46.4 %). According to Mahalakshmi and Raja (2013), there is a positive correlation between the CSA and per cent colonization of the isolates of *T. viride* (Tv1, Tv2, Tv3 and Tv4), *T. harzianum* (Th1, Th2, Th3, Th4, Th5, Th6, Th7 and Th8), and *T. viride* (Tr1, Tr2, Tr3, Tr4 and Tr5). Of all other isolates, Th2 and Th6 had the highest CSA, with percentage colonization of 61.84 and 59.12 per cent, respectively.

Paramasivan *et al.* (2014) assessed CSA of *T. viride* isolates namely TVB1, TVB2, TVB3, TVB4, TVB6, TVB11, TVB12, TVB14, TVB20 and TVB31 using paddy straw bits and noticed that TVB1 (91.01 %) had the highest per cent colonization followed by TVB2 (85.09 %), TVB3 (71.04 %), TVB6 (69.07 %), TVB4 (61.18 %), TVB31 (66.11 %), TVB12 (60.19 %), TVB11 (59.20 %), TVB20 (54.27 %) and TVB14 (53.28 %). Singh *et al.* (2016) have shown enhanced CSA of *T. harzianum* and *T. atroviride* mutants than that of the wild strains. Compared to the other mutant strains, Th-m_1 had a considerably greater percentage of colonies at all population densities on all incubation days in addition to increased enzyme activities. Th-m_1 was found to be effective for the management of chickpea collar rot caused by *Sclerotium rolfsii*. Nair

et al. (2020) analyzed the rhizosphere competence of three *Trichoderma* species namely *T. harzianum, T. koningii* and *T. pseudokoningii in vitro* using spore suspension containing 2 x 10^6 spores g^{-1}. Good saprophytic and colonization ability was noticed in *T. harzianum* and *T. koningii* with colonization frequency of 100 and 88 per cent respectively, followed by *T. pseudokoningii* with 38 per cent.

2.1.3. *In vitro* antagonistic ability of *Trichoderma* spp. against soil borne fungal pathogens

The high antagonistic and mycoparasitic ability of *Trichoderma* spp. has been shown to lower the severity of plant diseases by suppressing phytopathogens (Shang *et al*., 2020; Diaz-Gutierrez *et al*., 2021). Ten isolates from each of the three *Trichoderma* species (*T. viride, T. harzianum,* and *T. virens*) were tested by Dubey *et al*. (2007) against four isolates of the *F. oxysporum* f.sp. *ciceris*, which represented four divergent races from locations *viz.,* Dharwad, Kanpur, Ludhiana, and Delhi (race 4). The pathogen's mycelial development was most effectively suppressed by *T. viride* isolates from Ranchi and Delhi. Siameto *et al.* (2010) studied sixteen isolates of *T. harzianum* selected from different land use types in Embu, Kenya for their antagonistic activity in dual culture against soil-borne phytopathogenic fungi such as *Fusarium graminearum*, *F. oxysporum* f. sp. *phaseoli, F. oxysporum* f. sp. *lycopersici, Rhizoctonia solani* and *Pythium* spp. They found that the isolates 015E and 05 IE showed inhibition against all five pathogens amidst all the other selected isolates of *T. harzianum.* Based on the ability to suppress *F. oxysporum* f. sp. *pisi*, Sharma (2011) evaluated eighteen *Trichoderma* isolates (T1 - T18) for their antagonistic potential. The best activity was demonstrated by *T. atroviride* (isolate T1) followed by *T. harzianum* isolates (T8 to T11).

The efficacy of nineteen isolates of *Trichoderma* that belongs to three species *viz.*, *T. harzianum* (Th), *T. viride* (Tv) and *T. koningii* (Tk) were investigated *in vitro* by dual culture method by Choudhary and Mohanka (2012). Among all the isolates, Th-5 caused highest inhibition of 82.8 per cent followed by Th-7 (82.3 %), Tv-2 (79.2 %), Tv-18 (74.4 %) and Tk-9 (71 %). Belete *et al.* (2015) reported that isolates of *Trichoderma* spp. exhibited potent biological control action against *F. solani*. In dual

culture, *Trichoderma* inhibited *F. solani* mycelial growth to a degree ranging from 33.9 to 67.0 per cent. Rai and Maurya (2021) evaluated *T. asperellum* against seven species of *Fusarium viz., F. oxysporum* f. sp. *ciceris*, *F. oxysporum* f. sp. *cubense* (Tropical complex), *F. oxysporum* f. sp. *cubense* (Tropical Race 2), *F.* oxysporum f. sp. *cubense* (Tropical Race 1), *F.* oxysporum f. sp. *lycopersici* and *F. solani*. The strain was found to be most effective against *F. oxysporum* f. sp. *lycopersici* on tomato with an antagonistic efficacy of 73.91 per cent.

The antagonistic efficacy of *T. harzianum* and *T. viride* were evaluated by Yassin *et al*. (2021) by dual culture technique against *F. verticillioides* and *F. proliferatum*. *T. viride* showed better inhibition by suppressing the mycelial growth of *F. proliferatum* at 80.17 per cent and *F. verticillioides* at 70.46 per cent which was followed by *T. harzianum* at the rate of 68.38 per cent and 60.64 per cent respectively. Under *in vitro* conditions, Chen *et al.* (2021) observed seven isolates of three *Trichoderma* species (*T. harzianum, T. asperelloides,* and *T. rugulosum)* from the *Radix pseudostellariae* rhizosphere against *F. oxysporum.* Among these isolates, *T. harzianum* (ZC5) demonstrated the highest per cent inhibition (47.19). Alwadai *et al.* (2022) isolated 48 isolates of *Trichoderma* from six locations in Abha, Saudi Arabia and assessed their antagonistic potential by dual culture assay against *F. oxysporum*, *Alternaria alternata* and *Helminthosporium rostratum*. They reported that *Trichoderma* A (1) 2.1 showed maximum inhibition against *F. oxysporum* (82 %) while *Trichoderma* A (6) 2.2 T against *H. rostratum* (77 %).

Nguyen *et al*. (2023) screened the antagonistic ability of twenty-six isolates of *Trichoderma* spp. isolated from twenty soil samples by dual culture against *F. oxysporum* F.28.1A, the causal agent of wilt disease on sesame. The antagonistic efficacy of isolates of *Trichoderma* spp. ranged between 42.3 to 65.5 per cent. The highest efficacy was recorded by T-18B2 (65.5 %) followed by T-28B1 (62.7 %), T-02B1 (61.9 %), and T-20B1 (61.3 %) after 72 h of inoculation. Katyayani *et al.* (2020) examined the mycoparasitism inhibitory effect of three *Trichoderma* spp. *viz., T. harzianum, T. viride and T. koningii* against *Fusarium oxysporum* f.sp. *ciceri*, the causal agent of Fusarium wilt of chickpea at different time intervals of 48 h, 72 h and 96 h. At 48 h, *T. koningii* was found to be the most efficient at suppressing *F. oxysporum* f. sp.

ciceri, with an inhibition of 79.28 per cent, followed by *T. viride* (77.14 %) and *T. harzianum* (74.28 %). At 72 h, *T. harzianum* was most effective, with an inhibition of 83.87 per cent whereas *T. viride* and *T. koningii* suppressed *F. oxysporum* f.sp. *ciceri*, with an inhibition of 60.00 per cent and 50.32 per cent, respectively. At 96 h, *T. harzianum* had the maximum inhibition growth formation (85.55 %) against *F. oxysporum* f. sp. *ciceri*, followed by *T. viride* (69.55 %) and *T. koningiii* (62.66 %).

2.2. MOLECULAR CHARACTERIZATION OF PROMISING *TRICHODERMA* ISOLATES

The ability to use variable portions of the *rpb1* and *rpb2* genes, which encode the two biggest subunits of RNA polymerase II, in conjunction with ITS sequences to deduce the phylogeny of the mushroom-forming fungus *Cortinarius* sp., was established by Froslev *et al*. (2005). The oligonucleotide primer set (TvPF and TvPR) developed by Prameeladevi *et al.*(2011) was shown to be effective in amplifying a specific 245 base pair (bp) region of ribosomal DNA only with the genus *Trichoderma*. The DNA sequence data analysis of ITS1, ITS2, and *tef1* by Prameeladevi *et al*. (2012a, b) confirmed the morphological and cultural identification of 72 isolates of *Trichoderma* spp. into four species namely *T. virens*, *T. harzianum*, *T. longibrachiatum* and *T. asperellum*, and also observed that the amplicon size of the ITS sequence generally varied from 550 to 600 bp while for species-specific primer *tef1* amplicon size varied from 330 to 452 bp depending on the species. The greater transition/transversion ratio of 1.32 and evolutionary divergence of 1.965 makes *tef1* a preferable marker to differentiate the species of *Trichoderma*.

Savitha and Sriram (2015) identified ten isolates of *Trichoderma* as *T. asperellum, T. harzianum,* and *T. virens* by amplifying and analyzing their ITS 1 and 2 and translation elongation factor 1-alpha encoding gene (*tef1*). The most effective strain of *Trichoderma* T14 against four strains of *Fusarium culmorum* namely FC11, FC2, FC4, and FC20,l causing Fusarium crown rot and head blight of durum wheat was characterized as *T. afroharzianum* based on translation elongation factor1-alpha (*tef1*) and internal transcribed spacers rDNA (ITS1) primers by Bouanaka *et al.* (2021). Asis *et al.* (2021) utilized molecular methods based on a single gene to multiple genes

approach to describe the genetic diversity among forty-three strains of *Trichoderma*. They were identified as *T. asperellum* (81 %), *T. harzianum* (17 %), and *T. reesei* (2 %), based on the sequencing of the ITS region, *TEF1α* and CAL genes. These findings supported the ability of molecular techniques to distinguish between *Trichoderma* species and identify diverse isolates at a specific level.

Using four sets of species-specific primers from *tef1* and *rpb2* genes, Prabhakaran *et al.* (2014) characterized *T. asperellum* (*tef1* gene), *T. harzianum* (*rpb2* gene), *T. longibrachiatum* (*tef1* gene) and *T. virens* (*tef1* gene) with the help of multiplex PCR assay and obtained distinct amplicons of 507, 824, 452, and 330 bp, respectively. Based on the DNA barcoding using ITS (1 and 2), *tef*1, *chi*18-5 and *rpb*2 genes Oskiera *et al.* (2015) grouped 104 *Trichoderma* strains into the following groups; *T. atroviride* (38 %), *T. harzianum* (21 %), *T. lentiforme* (9 %), *T. virens* (9 %), and *T. simmonsii* (6 %) and the single strains belonging to *T. atrobrunneum*, *T. citrinoviride*, *T. crassum*, *T. gamsii*, *T. hamatum*, *T. spirale*, *T. tomentosum*, and *T. viridescens.* Three strains of *Trichoderma* were identified as *T. harzianum* by sequencing the amplified tef1 gene, according to Vazquez *et al.* (2015). Pandian *et al.* (2016) suggested a combination of multi-gene phylogeny and morphological characteristics for the identification of *Trichoderma* at the species level. He characterized *T. asperellum* strain Ta13 based on morphology and molecular analysis using genes such as ITS, *tef1*, *rpb2*, *act* and *cal*.

Li *et al.* (2018) examined the taxonomic position of new species of *Trichoderma* namely *T. cyanodichotomus* by utilizing Internal transcribed spacer rDNA 7 (ITS), translation elongation factor 1-alpha (TEF1-α) and RNA polymerase II subunit B (RPB2). It showed less than 95 per cent similarity with all known *Trichoderma* spp. and without any close relative *T. cyanodichotomus* was found to have an individual subgroup of Section Pachybasium in the combined phylogenetic tree. Based on ITS and TEF-1α sequence identity, Rahman *et al.* (2021) confirmed three efficient *T. asperellum* isolates *viz.,* B1902, T2007 and C1667 that showed better antagonistic potential against *F. oxysporum* f. sp. *cubense* under *in vitro* conditions.

Twenty-four *Trichoderma* isolates from the Jammu and Kashmir apple rhizosphere were characterized by Surma *et al*. (2022) using a combination of morpho-cultural and molecular methods, including multigene sequencing based on internal transcribed spacer (ITS), alpha elongation factor (EF1-1), and RNA polymerase B II (RPB2). Based on the sequencing data obtained from the ITS region, the isolates were assigned to *T. harzianum* (16.67 %), *T. viride* (25.00 %), *T. asperelloides* (20.83 %), and *T. koningiopsis* (29.16 %); two (8.33%) belonged to separate lineages. In contrast, 24 *Trichoderma* isolates were split into six different sub-clades *viz., T. asperelloides*, *T. asperellum*, *T. hamatum*, *T. viride*, *T. koningiopsis*, and *T. harzianum* based on the EF1-1 and RPB2 genes. As it was impossible to achieve the same outcomes using only morpho-cultural or molecular characterization, 24 *Trichoderma* isolates were finally identified using a combined morpho-cultural and molecular approach, and these were classified into six groups *viz., T. koningiopsis* (PTi1, PR3, NT1, NT2, Z2), *T. viride* (PNi2, SS, TB1, NT3, Z3), *T. asperellum* (Psh1, PNi3, TB3), *T. asperelloides* (PTi3, PNi1, PR1, TB2), *T. hamatum* (PR2, SR, SG) and *T. harzianum* (Psh2, Psh3, PTi2, Z1) indicating the presence of six species of *Trichoderma* in apple rhizosphere in the temperate region of Jammu and Kashmir.

2.3. *EFFICACY OF TRICHOERMA SPP AGAINST SOIL BORNE DISEASES*

Paulina *et al.* (2019) evaluated eight *Trichoderma* spp. isolates (T0, T2, T3, T4, T7, T8, T9 and T10) against net blotch in barley caused by *Pyrenophora teres* isolates (Pt A, Pt C and Pt M) for their biocontrol potential and plant growth-promoting efficiency and observed that under *in vivo* conditions, the disease incidence of the *Trichoderma* treated plants were reduced up to 55 per cent on barley seedlings with a significant increase in aerial dry weight (20 %) and radicular dry weight (15 %). In addition, treated plants showed an increase of chlorophyll by up to 9 per cent than that of the control plants. Anjum *et al.* (2020) analyzed the antifungal activity of five *Trichoderma* spp. *viz.*, *T. atroviride*, *T. hamatum*, *T. harzianum*, *T. longibrachiatum,* and *T. viride* against Fusarium wilt of chilli caused by *F. oxysporum* f. sp. *capsici* under *in vivo* conditions by using spore suspension (1 x 10^6 conidia per ml) of the antagonist. Among all treatments, *T. longibrachiatum* reduced the disease severity to 24.7 per cent and also enhanced the length and weight of the root and shoot of the diseased plants

under greenhouse conditions. *T. hamatum* showed the highest mycelial inhibition of 70.15 per cent followed by *T. longibrachiatum* (69.46 %), *T. harzianum* (68.75 %), *T. atroviride* (67.18 %) and *T. viride* (66.75 %).

The biocontrol ability of 15 isolates of *Trichoderma* (T1 to T15), derived from various rhizosphere soils and ecosystems in Algeria, was assessed by Bouanaka *et al.* (2021) against four strains of *F. culmorum* (FC11, FC2, FC4, and FC20), the primary causative agent of Fusarium Crown Rot (FCR) and Fusarium Head Blight (FHB) of wheat. The *in vitro* results revealed a maximum inhibition percentage of T9 (81.81 %), T12 (77.27 %), and T14 (80.68 %) through direct and indirect confrontations. The tube and pot experiment revealed that T14 treatment reduced disease severity by 50 to 63.63 per cent respectively against FCR. All treated durum wheat cultivars exhibited decreased FHB infection with a significant increase in the yield. Hasan *et al.* (2021) reported that *T. asperellum* strain B1092 was effective in the management of the Fusarium wilt of tomato under both *in vitro* and *in vivo* conditions.

Medeiros *et al.* (2022) reported high *in vivo* antagonistic efficacy of *Trichoderma* strains against *F. oxysporum* in common beans. In addition, *Trichoderma* inoculum acts as a plant growth promoter as well. The bio-potential of five *T. viride* strains (ITCC 6889, ITCC 7204, ITCC 7764, ITCC 7847, and ITCC 8276) for the management of Fusarium wilt of chickpea was examined by Pradhan *et al.* (2022) under *in vivo* conditions. The seeds treated with a dustable powder formulation comprising spores of the antagonist suppressed the pathogen's growth and development with an enhanced germination per cent of 93.33 per cent followed by carbendazim (80.33 %) and talc-based *T. viride* formulation (83.33 %). Compared to plants treated with carbendazim and talc-based formulations, the wilting per cent was significantly lower in plants treated with dustable *T. viride* powder formulation.

Mukherjee *et al.* (2019) demonstrated that the mutant (G2) of *T. virens* developed using gamma rays under extensive field evaluation against collar rot caused by *S. rolfsii* in chickpea and lentil over multiple locations consistently enhanced seed germination, decreased seedling mortality, and enhanced plant growth and yield in addition to growth promotion, improved pod bearing, and early flowering (7 to 10

days). The mutant-based formulation named TrichoBARC outperformed the parent strain. According to Mao *et al.* (2020), *T. hamatum* (MHT1134), a phosphate-solubilizing strain of *Trichoderma*, was antagonistic to *Phytophthora capsici, Colletotrichum capsici, Verticillium dahliae, V. alboatrum, Botryosphaeria dothidea, Pyricularia grisea,* and *Phoma lingam*. The results of the field tests demonstrated that strain MHT1134 significantly increased pepper yield while inhibiting pepper Fusarium wilt with a control effect that was only marginally inferior to that of the chemical hymexazol. As a result, MHT1134 is suggested as a promising biocontrol fungus against pepper wilt.

According to Damodaran *et al*. (2020), *T. reesei* isolate CSR-T-3 formulation showed excellent control against Fusarium wilt of banana caused by *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4) under *in vitro* and field conditions. By increasing the activity of defense enzymes such as *β*-1,3-glucanase, peroxidase, chitinase, polyphenol oxidase, and phenylalanine ammonia lyase with increased phenol levels as well as the enhanced production of antifungal substances, it lowers the disease severity. Under field conditions, treated plants showed a high phenological growth and yield with a disease severity score of 1.14.

In an experiment against Fusarium wilt of tomato induced by *Fusarium oxysporum* f. sp. *lycopersici*, Jamil (2020) employed *T. harzianum* and *T. viride* and assessed the severity of the disease and the properties of these species that promote plant growth. Under field conditions, tomato plants treated with *T. viride* showed the best growth and yield, the least amount of disease severity, the highest physiological activity, and the highest biochemical and antioxidant contents. Jambhulkar *et al.* (2021) evaluated 27 strains of *Trichoderma* against *F. verticillioides* and selected three effective indigenous strains namely *T. harzianum* BThr29, *T. asperellum* BTas25, and *T. erinaceum* BTer43 based on per cent inhibition in dual culture assay. The selected strains were tested for the management of post-flowering stalk rot (PFSR) under field conditions through soil application, seed treatment and soil drenching. *T. harzianum* BThr29 treatment was found to be on par with the fungicidal application and it also minimized disease severity by 62.37 per cent, crop lodging by 71.1 per cent and grain

discoloration to less than 50 per cent. Hence, *T. harzianum* BThr29 was recommended for the biological control of PFSR in maize plants.

Venkataramanamma *et al.* (2022) isolated 21 isolates of *Trichoderma* from the rhizosphere soils of chickpea plants. Based on *in vitro* dual culture assays, four effective isolates (Tr-20, Tr-6, Tr-1 and Tr-10) were selected that produced IAA, HCN, chitinases and cellulases against *F. oxysporum* f.sp. *ciceris*. The field evaluation was done with Tr-6 isolate, fungicides and fluorescent Pseudomonad. Among all the treatments, a combination of Tr-6, fungicide and fluorescent Pseudomonad outperformed others with increased yield and reduced disease severity comparatively. *T. harzianum* (T-H4) and *T. koningiopsis* (T-K11) were tested for their ability to inhibit *F. solani* (MX-MIC798) growth during the germination and establishment of chilli Miahuateco seedlings by Miguel-Ferrer *et al.* (2021), *T. harzianum* T-H4 treatment increased the germination rate of MX-MIC 798 infected seeds by 82 per cent. Root rot reached severity level 4.0 and mortality rate of 70 per cent in untreated plants infected with *F. solani.*

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled "Molecular characterization of promising isolates of *Trichoderma* spp. and their field evaluation against Fusarium wilt of vegetable cowpea" was conducted in the Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala during the period 2020-2022. The study aimed at the molecular characterization of promising isolates of *Trichoderma* spp. obtained from virgin forest soils of Kerala and their field evaluation against the soil borne disease, wilting and basal swelling of vegetable cowpea incited by *Fusarium oxysporum, F. equiseti* and *F. solani.* The following is a description of the materials used and the procedures followed.

3.1. COLLECTION AND MAINTENANCE OF *TRICHODERMA* ISOLATES AND ASSESSMENT OF THEIR BIOCONTROL ATTRIBUTES

Five promising isolates of *Trichoderma* spp. *viz.,* TRMW-2, TRKR-2, TRPN-3, TRPN-11 and TRPN-17 from the previous study (Cyriac, 2020; Cyriac *et al*., 2021), collected earlier from virgin forest soils of Kerala and found effective in *in vitro* and pot culture experiments against *Fusarium* wilt of vegetable cowpea were used in the present study. In addition, *Trichoderma asperellum* (KAU strain) was also used. All six isolates were sub-cultured and maintained in Potato Dextrose Agar (PDA) medium.

3.1.1. Assay of activity of cell wall degrading enzymes of *Trichoderma* isolates

3.1.1.1. Preparation of culture filtrate for enzyme assay of *Trichoderma* spp. isolates

Culture filtrate was prepared by transferring the actively growing mycelial bits of the *Trichoderma* isolates from a three-day-old Petri plate to freshly prepared 100 ml Potato Dextrose Broth (PDB) in 250 ml conical flasks. One per cent of either chitin, casein or triolein was used for enzyme induction and incubated at room temperature (28 ± 2°C) for five days. Using Whatman No. 1 filter paper, the contents in the conical flask were filtered and centrifuged at 9000 rpm at 4°C for 10 min for clarification. The supernatant was collected and 2X volume of ice-cold acetone was added for protein

precipitation and incubated overnight at 4°C. Then it was again centrifuged at 9000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was air-dried. Then the pellet was dissolved in the appropriate buffer (sodium acetate buffer for chitinase; Tris HCL for lipase and protease) and the resulting solution was used as the culture filtrate for the enzyme assay.

3.1.1.2. Chitinase

The chitinase activity was determined using the method described by Gajera and Vakharia (2012). Colloidal chitin (0.5 %) was prepared using 10 mM of sodium acetate buffer (pH 5.2). Two hundred µl of 0.5 per cent colloidal chitin and 100 µl of culture filtrate were used to prepare the reaction mixture which was incubated at 50°C for one hour. For a known volume of the reaction mixture, an equal volume of 120 mM of potassium borate buffer was added to the test tube and boiled vigorously in the water bath for three min. Then three ml p-dinitro methyl amino benzaldehyde (DMAB) reagent was added to each test tube and incubated at 38°C for 20 min. Then the tubes were allowed to cool and absorbance was measured at 544 nm using a spectrophotometer. Standard was prepared using N-acetyl glucosamine in borate buffer and the above procedure was carried out. The amount of N-acetyl glucosamine was measured. A blank of 200 µl of water was used instead of the colloidal chitin.

3.1.1.3. Protease

The protease activity was calculated using the protocol given by Cupp-Enyard, (2008). Casein solution of 0.65 per cent was prepared using 50 mM of potassium phosphate buffer at 85°C for about 10 min of gentle stirring. Five ml of 0.65 percent was added to the test tube and incubated at 37°C for 5 min using water bath. One ml of enzyme solution was added to the tubes except to the blank. It was then mixed properly and incubated at 37°C for 10 min. After 10 min, a 5 ml of 110 mM trichloro acetic acid was added to each tube to stop the reaction and incubated at 37°C for 30 min. The absorbance was read at 660 nm. The standard used was tyrosine. To varying concentration of 1.1 mM tyrosine, distilled water was added to make up the volume to one millilitre and a blank was maintained. Then it was incubated for 30 min. Using

syringe filter of size 0.45 µm the samples were filtered. To the filtrate 5 ml of sodium carbonate was added followed by 1ml of 0.5 M Folins reagent to measure the activity of free protease by colour change and the drop in pH was adjusted using drops of sodium carbonate. The samples are then incubated at 37°C for 30 min and then the absorbance for the standard was measured at 660 nm using spectrophotometer.

3.1.1.4. Lipase

The lipase enzyme activity was assayed using the methodology given by Gupta *et al.* (2002). Ten ml of isopropanol was used to dissolve 30 mg of p-Nitro phenyl palmitate (p-NPP) to make the stock solution. Then the working sample was prepared by taking 0.2 mg ml^{-1} of the stock which was diluted with distilled water five times. The reaction mixture consisted of 100 µl of culture filtrate and 500 µl of the substrate, which was incubated at 23°C for 30 min. Four hundred µl of Tris HCl was added and the absorbance reading was taken at 410 nm with help of a spectrophotometer. Only Tris HCl without substrate served as a blank.

3.1.2. Assessment of competitive saprophytic ability (CSA) of *Trichoderma* isolates

CSA was assessed based on the method given by Nakkeeran *et al.* (2005). Isolates of *Trichoderma* spp. were grown on PDA and incubated at room temperature (28 ± 2°C) for seven days. The plates were flooded with sterile distilled water, and the conidia were gently freed from the culture. The conidial count was made using a haemocytometer. The conidial suspension was then inoculated in unsterilized soil to obtain a desired concentration of 10^6 per gram of soil. Twenty pieces of one-centimetre matured, clean and polished paddy straw bits were randomly placed into each pot. Control pots were maintained without the addition of the conidial suspension. The pots were covered with a plastic cover to conserve moisture and kept randomly in dark for seven days. All the 20 paddy straw bits from each pot were removed, washed with tap water and sterilized using a mixture of one per cent sodium hypochlorite solution and five per cent ethanol for five min. Bits were plated onto *Trichoderma* selective medium (TSM) and incubated at room temperature (28 ± 2°C) for five days. The number of bits colonized and the percentage colonization of *Trichoderma* were assessed.

$$\text{Per centage colonization} = \frac{\text{Number of colonized straw bits x 100}}{\text{Total number of straw bits observed}}$$

3.1.3. Compatibility of different *Trichoderma* isolates by dual culture

The compatibility of *Trichoderma* isolates was assessed by dual culture technique. Three-day-old *Trichoderma* cultures grown on PDA plates were used for the study. With a cork borer, a five-millimetre diameter mycelial disc was cut from a three-day-old culture of one *Trichoderma* isolate and placed at one side of the Petri plate containing PDA at two centimetres from the periphery. On the other end, another isolate of *Trichoderma* was placed leaving two cm from the periphery to maintain a five cm gap between the isolates. The plates were incubated at room temperature (28 ± 2°C) for three days. Radial growth of both the isolates was measured until the seventh day and antagonistic properties like antibiosis by pigmentation at the area of interaction of the mycelia, overgrowth of the mycelia on one another and lysis with clear demarcation zone were observed and recorded.

3.1.4. Dual culture assay of *Trichoderma* isolates against *F. oxysporum*

In vitro screening of *Trichoderma* isolates against *F. oxysporum* was performed based on the dual culture method described by Skidmore and Dickinson (1976). From the actively growing tip of the nine-day-old culture of the pathogen, a five-millimetre mycelial disc was cut using a cork borer and was placed at one end of the Petri plate at two cm away from the periphery. An actively growing three-day-old mycelial disc of *Trichoderma* was placed at the opposite end of the Petri plate at two cm from the periphery, on the fifth day of pathogen inoculation. The control was maintained with the monoculture of the pathogen placed at one end of the Petri plate. Triplicates were maintained for each treatment. Until the control plate showed full growth, the radial growth (cm) of the pathogen and antagonist was measured.

Per cent inhibition (PI) between the growth of control and treatment plates was calculated using the formula given by Vincent (1927).

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

Where,

C = Radial growth of pathogen (cm) in the control plate

T= Radial growth of pathogen (cm) in the treatment plate

3.2. MOLECULAR CHARACTERIZATION OF PROMISING *TRICHODERMA* ISOLATES

3.2.1. Isolation of genomic DNA

The genomic DNA isolation of the *Trichoderma* isolates was carried out based on the Cetyl Trimethyl Ammonium Bromide (CTAB) method of extraction described by Culling (1992) with partial modification. A three-day-old *Trichoderma* mycelial mat grown in PDB was filtered using Whatman filter paper and dried overnight in a bell jar. Around 200 mg of the sample was taken in a sterile mortar and ground with the pestle using liquid nitrogen for homogenization. Two ml of pre-warmed CTAB extraction buffer (100 mM Tris HCl, 25 mM EDTA, 2 M NaCl, 2% CTAB, 3% Polyvinyl pyrrolidone and 0.2% β-mercaptoethanol) was added followed by the addition of RNAse at the rate of 2 µl per 2ml of CTAB. The homogenate was incubated at 65°C in the water bath for 20 to 30 min with intermittent mixing. After incubation, samples were centrifuged at 13000 rpm at 25°C for 10 min and the supernatant was transferred to a fresh centrifuge tube. An equal volume of Chloroform: Isoamyl alcohol (C:I) 24:1 v/v was added to each tube and vortexed briefly. The samples were again centrifuged at 13000 rpm at 25°C for 10 min. Three distinct layers were formed and the upper aqueous layer with supernatant was collected in a fresh centrifuge tube without disturbing the middle protein layer. Again C:I wash was given and centrifuged at 13000 rpm at 25°C for 10 min. The supernatant was collected in a fresh tube and an equal volume of 100 per cent pre-chilled isopropanol was added for precipitation. It was

incubated overnight at 20°C. After incubation, the samples were centrifuged at 13000 rpm at 4°C for 10 min to obtain the pellet. The supernatant was discarded and the pellets were washed with 70 per cent ethanol by centrifuging at 7000 rpm at 4°C for 10 min. The ethanol was discarded completely and the pellets were air-dried. After drying, the pellets were dissolved in TE buffer depending on the size of the pellet and subsequently stored at -20°C.The quantification of the DNA was done using a spectrophotometer.

3.2.2. Agarose gel electrophoresis

The quality of the isolated DNA was checked using agarose gel electrophoresis. The agarose gel of 0.8 per cent was prepared in 1X Tris-Borate-EDTA (TBE) buffer containing 0.5 µg L^{-1} of ethidium bromide and casted into the gel tank with the comb. After the solidification of the gel, the comb was removed carefully and 2 µl of the sample, 3 µl of distilled water and 1 µl of 6X loading dye (0.25 % bromophenol blue, 30 per cent sucrose in TE buffer pH-8.0) was loaded into the well. The electrophoresis was carried out using 1X TBE buffer at 75 V cm^{-1} until the bromophenol dye had migrated a three-fourth distance of the gel. After the completion of the gel run, the gel was visualized under a UV transilluminator (Genei) and images were documented using the Gel documentation system (Bio-Rad).

3.2.3 Polymerase chain reaction (PCR) analysis

3.2.3.1. Reaction mixtures

3.2.3.1.1. General concentration of reaction mixture used:

Taq mix 2X (G bioscience) - 10 µl

DNA (40 ng/µl) - 1 µl

Forward primer 0.2 µM - 0.5 µl

Reverse primer 0.2 µM - 0.5 µl

ddH_2O - 8 µl

Total reaction volume - 20 µl

3.2.3.1.2. Reaction mixture with increased primer concentration used for species-specific primers

Taq mix 2X (G bioscience) - 12.5 µl

DNA (40 ng/µl) - 1 µl

Forward primer 0.2 µM - 2 µl

Reverse primer 0.2 µM - 2 µl

ddH_2O - 7.5 µl

Total reaction volume - 25 µl

3.2.3.1.3. Reaction mixture used for species-specific primers with the addition of $MgCl_2$

Taq mix 2X (G bioscience) - 10 µl

DNA (40 ng/µl) - 1 µl

Forward primer 0.2 µM - 0.5 µl

Reverse primer 0.2 µM - 0.5 µl

ddH_2O - 7.5 µl

$MgCl_2$ - 0.5 µl

Total reaction volume - 20 µl

3.2.3.2. Primers

The isolates of *Trichoderma* spp. were subjected to PCR analysis using four sets of species-specific primers for the *tef1* gene of *T. asperellum, T. longibrachiatum, T. virens* and *rpb2* gene of *T. harzianum*.

3.2.3.3. Thermal profile used for the amplification of ITS region

The reaction conditions were maintained as follows:

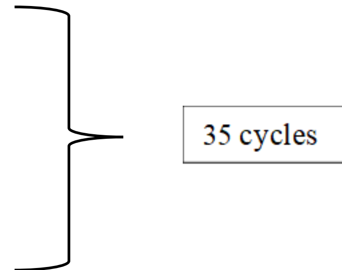
Initial denaturation – 94°C for 1 min.

Annealing – 57°C for 1 min.

First extension – 72°C for 1 min.

Final extension – 72°C for 10 min.

Final denaturation – 94°C for 35 s



Hold – 4°C

3.2.3.4. Thermal profile used for genus-specific primers

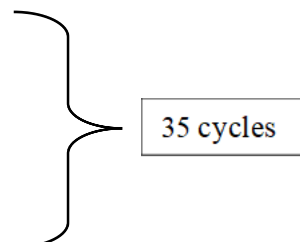
The reaction conditions were maintained as follows:

Initial denaturation – 94°C for 3 min.

Final denaturation – 94°C for 1 min.

Annealing – 60°C for 30 s

First extension – 72°C for 1 min.



Final extension – 72°C for 3 min.

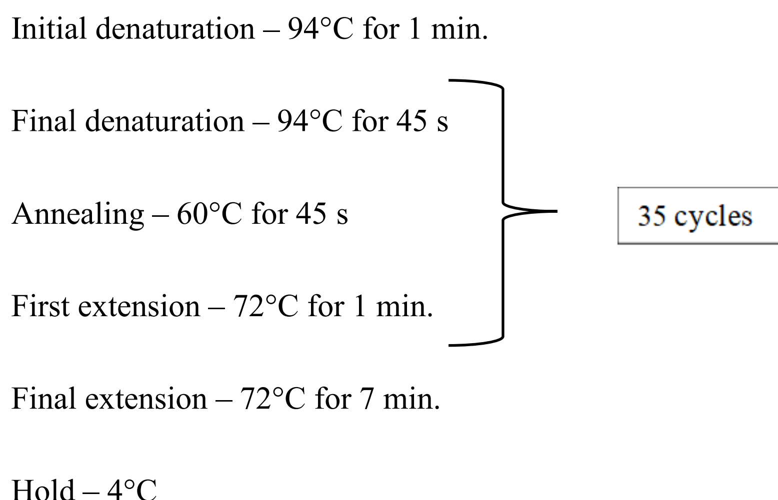
Hold – 4°C

Table 1. Primers used for PCR

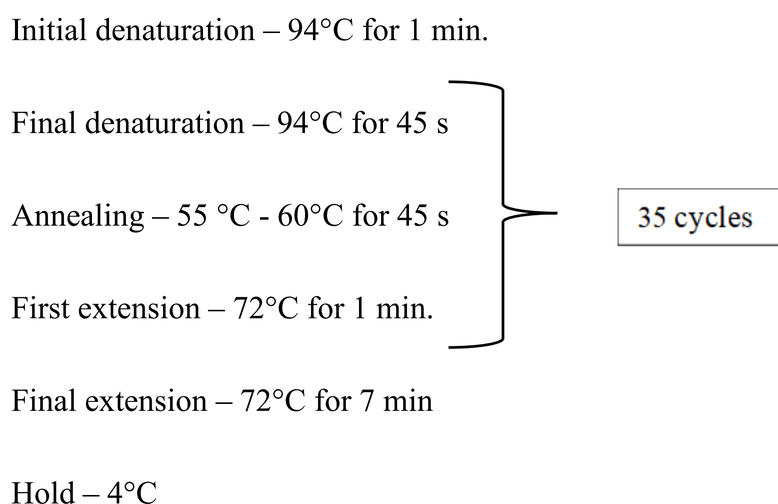
Primer	Direction	Sequence	Purpose	Reference
ITS1	Forward	5′- TCTGTAGGTGAACCTGCGG -3′	Amplification of ITS region	Savitha and Sreeram, (2015)
ITS4	Reverse	5′- TCCTCCGCTTATTGATATGC -3′		
TvPF	Forward	5′- CCGCCGGAGGACCAACCAA -3′	Genus-specific identification	Prameeladevi *et al.*, (2011)
TvPR	Reverse	5′- GACAGGCATGCCCGCCAGAA -3′		
tef1 AF	Forward	5′- CTCTGCCGTTGACTGTGAACG -3′	Species-specific identification	Prabhakaran *et al.*, (2014)
tef1 AR	Reverse	5′-CGATAGTGGGGTTGCCGTCAA -3′		
tef1 BF	Forward	5′- CCGTGAGTACACACCGAGCTT-3′		
tef1 BR	Reverse	5′- CGGCTTCCTGTTGAGGGGA -3′		
tef1 CF	Forward	5′- CCGTTTGATGCGGGGAGTCTA-3′		
tef1 CR	Reverse	5′- GGCAAAGAGCAGCGAGGTA-3′		
rpb2F	Forward	5′-TTGCATGGGTTCGCTAAAGG-3′		
rpb2R	Reverse	5′-TCTTGTCAGCATCATGGCCGT-3′		

3.2.3.5. Thermal profile used for species-specific primers

The reaction conditions were maintained as follows



The reaction conditions were maintained as follows for gradient PCR:



3.2.4. Agarose gel electrophoresis of PCR products

The agarose gel of one per cent was prepared using 1X TBE buffer containing 0.5 µg/ml of ethidium bromide to check the PCR products. Ten microliters of PCR product along with 2µl of 6X loading dye were loaded. The molecular standard of a two-log DNA ladder (NEB) was used. The electrophoresis was carried out using 1X TBE buffer at 75 V cm^{-1} for about one to two hours. The gel was visualized under a UV transilluminator (Genei) and images were documented using a Gel documentation system (Bio-Rad).

3.2.5. ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) has two hydrolytic enzymes, exonuclease I and shrimp alkaline phosphatase (SAP), in a specially prepared buffer to remove the unwanted primers and dNTPs from a PCR product mixture without interfering with any subsequent applications. ExoSAP-IT of 0.5 µl was mixed with 5 µl of PCR product and then incubated at 37°C for 15min. followed by enzyme inactivation at 85°C for 5 min.

3.2.6. Sequencing using BigDye Terminator v3.1

The sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following the manufacturer's protocol.

The sequencing PCR mix had the following components:

Distilled water	-	6.6 µl
Sequencing Buffer 5X	-	1.9 µl
Forward primer	-	0.3 µl
Reverse primer	-	0.3 µl
Sequencing Mix	-	0.2 µl
Exosap-treatedPCR product	-	1 µl

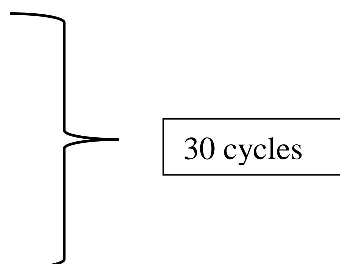
3.2.6.1. Sequencing PCR amplification profile

Initial denaturation - 96°C for 2 min

Final denaturation - 96°C for 30 s

Annealing -50°C for 40 s

Extension - 60°C for 4 min



Hold- 4°C- ∞

3.2.6.2. Post-Sequencing PCR Clean up

Distilled water	-	5 µl
Sodium Acetate 3M	-	1 µl
EDTA	-	0.1 µl
Ethanol 100%	-	44 µl

A mixture of distilled water, 125 mM EDTA, 3 M sodium acetate (pH 4.6) and ethanol was prepared and mixed thoroughly. Fifty microlitres of the mix was then added to each well of the sequencing plate with the sequencing PCR product and vortexed by Mixmate vortex. Then it was incubated at room temperature (28 ± 2°C) for 30 min and centrifuged at 3700 rpm for 30 min. The supernatant was discarded and 50 µl of 70 per cent ethanol was added to each well. The sample was centrifuged at 3700 rpm for 20 min. The 70 per cent ethanol wash was repeated after discarding the supernatant followed by centrifugation at 3700 rpm for 20 min. The supernatant was decanted and the pellet was air-dried. The cleaned, air-dried pellet was sequenced in ABI 3500 DNA analyzer (Applied Biosystems).

3.2.6.3. Sequence Analysis

Sequence Scanner Software v1 (Applied Biosystems) was used to check the quality of the sequence. Geneious Pro v5.1 was used for sequence alignment and required editing of the obtained sequences (Drummond *et al*., 2010). The sequence data obtained for the conserved ITS region, TvP, TvF, *tef1* gene and *rpb2* gene of the *Trichoderma* isolates were assembled and analyzed by using the Basic Local Alignment Search Tool (BLAST) and the sequences were compared with the current sequence database for species confirmation at the National Centre for Biotechnology Information (NCBI) database.

3.2.7. Phylogenetic tree

Comparisons were made between the data set based on the ITS-DNA region, TvP, *tef1*gene and *rpb2* gene of the five *Trichoderma* isolates and other reference sequences obtained from the NCBI Genbank database (USA). Multiple sequence alignment was

carried out using Clustal omega and the phylogenetic tree was built using the Neighbor-Joining Tree (NJT) method of analysis with bootstrap of 1000 using Molecular Evolutionary Genetics Analysis (MEGA) 11 software.

3.3. *IN VIVO* TESTING OF THE EFFICACY OF SELECTED ISOLATES OF *TRICHODERMA* SPP. AGAINST FUSARIUM WILT OF VEGETABLE COWPEA

3.3.1. Mass multiplication of *Trichoderma* spp. isolates

3.3.1.1. Preparation of formulations of individual isolates

All five isolates of *Trichoderma* spp. *viz* TRMW-2, TRKR-2, TRPN-3, TRPN-11 and TRPN-17 were grown in 300 ml of PDB individually and incubated at room temperature (28 ± 2°C) for seven days. It was thoroughly mixed with one kilogram of sterilized talc powder. The formulation was shade dried to maintain a moisture content of seven to eight per cent. The population of *Trichoderma* in the formulations was confirmed to be a minimum of 2×10^6 cfu g^{-1} before use.

3.3.1.2. Preparation of consortial formulations of *Trichoderma* spp.

Mycelial bits of three-day-old *Trichoderma* isolates used in consortia *viz.,* TRKR-2, TRPN-3 and TRPN-17 were aseptically transferred to a conical flask containing pre-sterilized 300 ml of PDB and incubated at room temperature (28 ± 2°C) for seven days. One kg of talc powder was autoclaved at 121°C at 15 psi for 30 min for three consecutive days. The seven-day-old mycelial mat of one isolate from PDB was filtered using Whatman No.1 filter paper and kept. It was then mixed with seven-day-old growth of another isolate in 300 ml PDB (without filtration) which was in turn thoroughly mixed with one kilogram of autoclaved talc. The mixture was shade dried for five days. The mass-multiplied talc-based formulation was used for the seed treatment and soil drenching at specified intervals.

3.3.2. Mass multiplication of *Fusarium* spp.

Sand-maize mixture was used for the mass multiplication of *Fusarium* by the modified method given by Lewis and Papavizas (1984). Sand and maize were mixed thoroughly in a ratio of 9:1 and were moistened sufficiently to increase the fungal growth. The mixture was autoclaved in a conical flask. Then actively growing mycelial bits of

Fusarium were transferred to it in an aseptic condition. Then it was incubated at room temperature (28 ± 2°C) for 15 days.

3.3.3. Pot culture experiment

A pot culture experiment was carried out at the Department of Plant Pathology, College of Agriculture, Vellayani to evaluate the efficacy of the five *Trichoderma* isolates against the Fusarium wilt of vegetable cowpea. The experiment consisted of eight treatments and three replications in a Completely Randomized Design (CRD) and the variety used was Githika.

The details of the treatment were as follows:

T1: *Trichoderma* isolate TRKR2

T2: *Trichoderma* isolate TRPN17

T3: *Trichoderma* isolate TRPN3

T4: Consortia of *Trichoderma* isolates TRPN 17 and TRKR 2

T5: Consortia of *Trichoderma* isolates TRPN 17 and TRPN 3

T6: KAU strain of *Trichoderma* sp.

T7: Standard check (fungicide)

T8: Control

Sand, soil and cow dung were mixed in a ratio of 1:1:1 as a potting mixture and it was fumigated with five per cent formaldehyde. The potting mixture was filled into medium-sized pots. The cowpea seeds (Var. Githika) after seed treatment were sown in the pots. The package of practice was adopted as per the Package of Practice Recommendations: Crops (KAU, 2011)

3.3.3.1. Method of application:

Pathogen inoculation: Pathogen multiplied in the sand-maize mixture (9:1) was applied to the root zone and incorporated to soil @ 0.2 % (w/w) in pots at 20 days after sowing (DAS) (Sreeja, 2014)

Trichoderma spp.: Seed treatment of talc-based formulation @ 20 g kg^{-1} of seed followed by soil drenching @ 2 per cent at 20, 40 and 60 DAS.

Standard check: Seed treatment with carbendazim @ 2g kg^{-1} of seed followed by soil drenching @ 0.2 per cent at 20 and 40 DAS.

Biometric observations like the number of plants infected, days for flowering, number of pods per plant, length of the pod, number of seeds per pod and yield were recorded. Disease incidence was calculated using the formula

$$\text{Disease incidence} = \frac{\text{Total number of plants infected x 100}}{\text{Total number of plants observed}}$$

The total microbial population of *Trichoderma* spp. was enumerated at 30, 60 and 90 DAS by serial dilution technique (Jhonson and Curl, 1972). A sample of 10 g of soil was collected from each treatment and mixed individually with 90 ml of sterilized distilled water. One ml of the aliquot from this suspension was mixed with nine ml of sterilized distilled water in a fresh test tube making 10^{-3} dilutions and continued up to 10^{-6} dilutions. From 10^{-3} and 10^{-4} dilutions, one ml of the suspension was plated into three Petri plates containing TSM media by pour plate method and swirled for uniform mixing of the suspension and the media. It was incubated at room temperature (28 ± 2°C) and the colonies were counted after three to four days.

3.3.4. Field evaluation of *Trichoderma* isolates against Fusarium wilt of vegetable cowpea

A field experiment was conducted to determine the efficacy of the selected isolates of *Trichoderma* spp. in the management of Fusarium wilt. The experiment was laid out in

Randomized Block Design (RBD) with eight treatments and three replications and the variety used was Githika.

The details of the treatment were as follows:

T1: *Trichoderma* isolate TRKR2

T2: *Trichoderma* isolate TRPN17

T3: *Trichoderma* isolate TRPN3

T4: Consortia of *Trichoderma* isolates TRPN 17 and TRKR 2

T5: Consortia of *Trichoderma* isolates TRPN 17 and TRPN 3

T6: KAU strain of *Trichoderma* sp.

T7: Standard check (fungicide)

T8: Control

3.3.4.1. Method of application:

Trichoderma spp.: Seed treatment of talc-based formulation @ 20g kg^{-1} of seed followed by soil drenching @ 2 per cent at 20, 40 and 60 DAS.

Standard check: Seed treatment with carbendazim @ 2g kg^{-1} of seed followed by soil drenching @ 0.2 per cent at 20 and 40 DAS.

Liming was done at the time of land preparation for all the treatments. The other package of practices was adopted as per the standard and recommended Package of Practices (KAU, 2011). Then the biometric observations were recorded as mentioned earlier in 3.3.3.1

3.3.5. Statistical analysis

The data obtained from the experiments was used to calculate Analysis of variance (ANOVA) and Critical Difference (CD) at a five per cent level of significance to compare the means of the treatments. The analysis was performed using KAU GRAPES 1.0.0 and standard error as well as standard deviation was also calculated for all the treatment means.

Results

4. RESULTS

The present study entitled "Molecular characterization of promising isolates of *Trichoderma* spp. and their field evaluation against Fusarium wilt of vegetable cowpea" was conducted during the period 2020-2022 at the Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala to identify potential *Trichoderma* isolates for the management of Fusarium wilt of vegetable cowpea under *in vivo* condition. The experimental results obtained are presented below.

4.1. COLLECTION AND MAINTENANCE OF *TRICHODERMA* ISOLATES AND ASSESSMENT OF THEIR BIOCONTROL ATTRIBUTES

Five promising isolates of *Trichoderma* spp. *viz.,* TRMW-2, TRKR-2, TRPN-3, TRPN-11 and TRPN-17 (Plate 1) collected earlier from virgin forest soils of Kerala were obtained and used for further study along with a reference strain *viz.,Trichoderma asperellum* (KAU strain). In the previous study, the isolate TRMW-2 was obtained from the Central Zone and isolates TRKR-2, TRPN-3, TRPN-11 and TRPN-17 were obtained from the Southern Zone of Kerala. These isolates were selected based on their *in vitro* antagonistic efficacy against the *Fusarium* spp. causing wilt disease in vegetable cowpea.

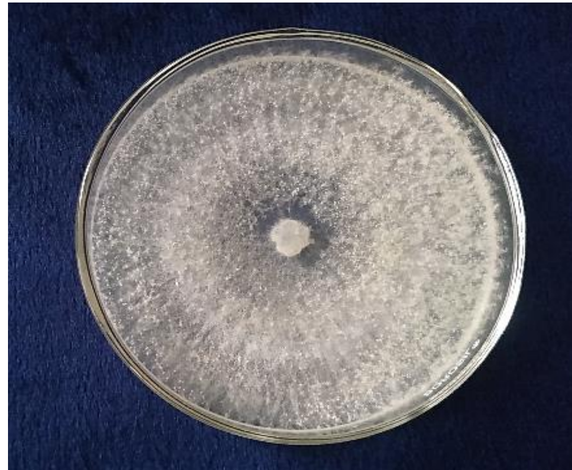
4.1.1. Assay of activity of cell wall degrading enzymes of *Trichoderma* isolates

The data presented in Table 2 shows that the chitinase enzyme activity of the isolate TRPN-17 was observed to be the highest (88.39 U ml^{-1}) followed by the isolates TRPN-3 (76.75 U ml^{-1}) and TRKR-2 (71.06 U ml^{-1}) while the highest protease activity was recorded by the isolate TRPN-11 (165.86 U ml^{-1}) followed by TRPN-17 (92.90 U ml^{-1}) and TRPN-3 (74.80 U ml^{-1}). The isolate TRPN-3 had the highest (4.86 U ml^{-1}) lipase enzyme activity followed by the isolates TRKR-2 (4.20 U ml^{-1}) and TRPN-11 (3.14 U ml^{-1}).

TRMW-2

TRKR-2

TRPN-3



TRPN-11

TRPN-17

Plate 1. Isolates of *Trichoderma* spp. on PDA mediu

Table 2. ***In vitro*** **activity of chitinase, protease and lipase by isolates of *Trichoderma* spp. grown in potato dextrose broth**

Sl. No.	Isolate	Chitinase (U ml^{-1})*	Protease (U ml^{-1})*	Lipase (U ml^{-1})*
1	TRMW-2	61.82 ± 0.18[d]	64.43 ± 0.23[d]	2.69 ± 0.006[e]
2	TRKR-2	71.06 ± 0.40[c]	48.68 ± 0.12[e]	4.20 ± 0.010[b]
3	TRPN-3	76.75 ± 0.32[b]	74.80 ± 0.14[c]	4.86 ± 0.008[a]
4	TRPN-11	55.68 ± 0.11[e]	165.86 ± 0.19[a]	3.14 ± 0.010[c]
5	TRPN-17	88.39 ± 0.18[a]	92.90 ± 0.14[b]	2.83 ± 0.005[d]
	SE(m)	0.157	0.096	0.005
	CD (0.05)	0.40	0.28	0.01

*Mean ± SD of four replications; In a column, means followed by common letters are not significantly different from each other.

4.1.2. Assessment of competitive saprophytic ability (CSA) of *Trichoderma* isolates

Table 3 represents the mean number of straw bits colonized out of 20 bits in each treatment and the per cent colonization. It was found that all the isolates effectively colonized the straw bits in unsterilized soil compared to the uninoculated control. Among them, TRKR-2 showed the highest per cent colonization of 67.50 per cent followed by TRPN-3 and TRPN-17 (56.25 %). Isolates TRMW-2 and TRPN-11 exhibited 50.00 and 48.75 per cent colonization respectively (Plates 2, 3, 4, 5, 6 and 7).

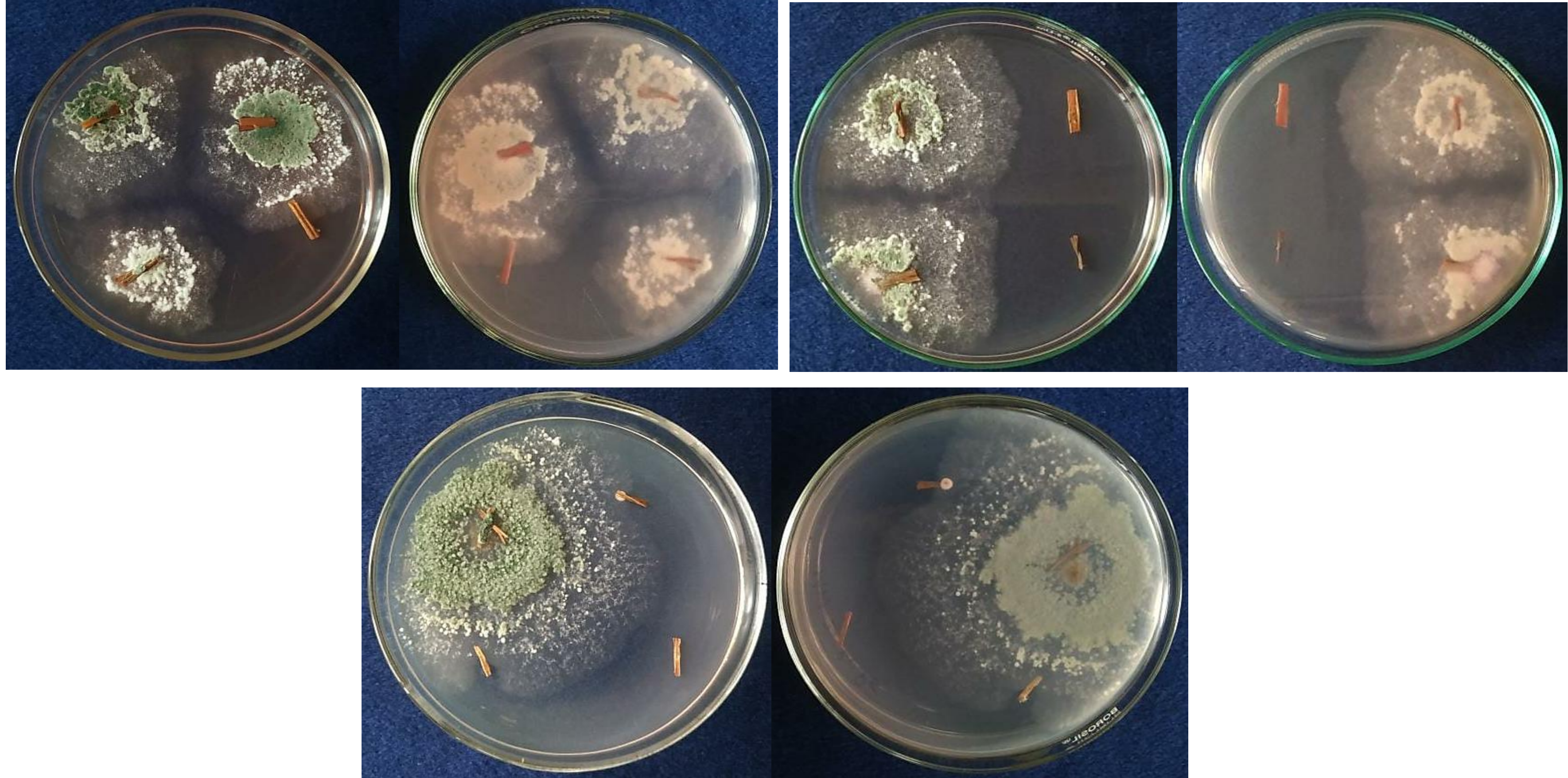


Plate 2. Growth of *Trichoderma* spp. on TSM (five DAI) from straw bits taken from soil inoculated with isolate TRMW-2

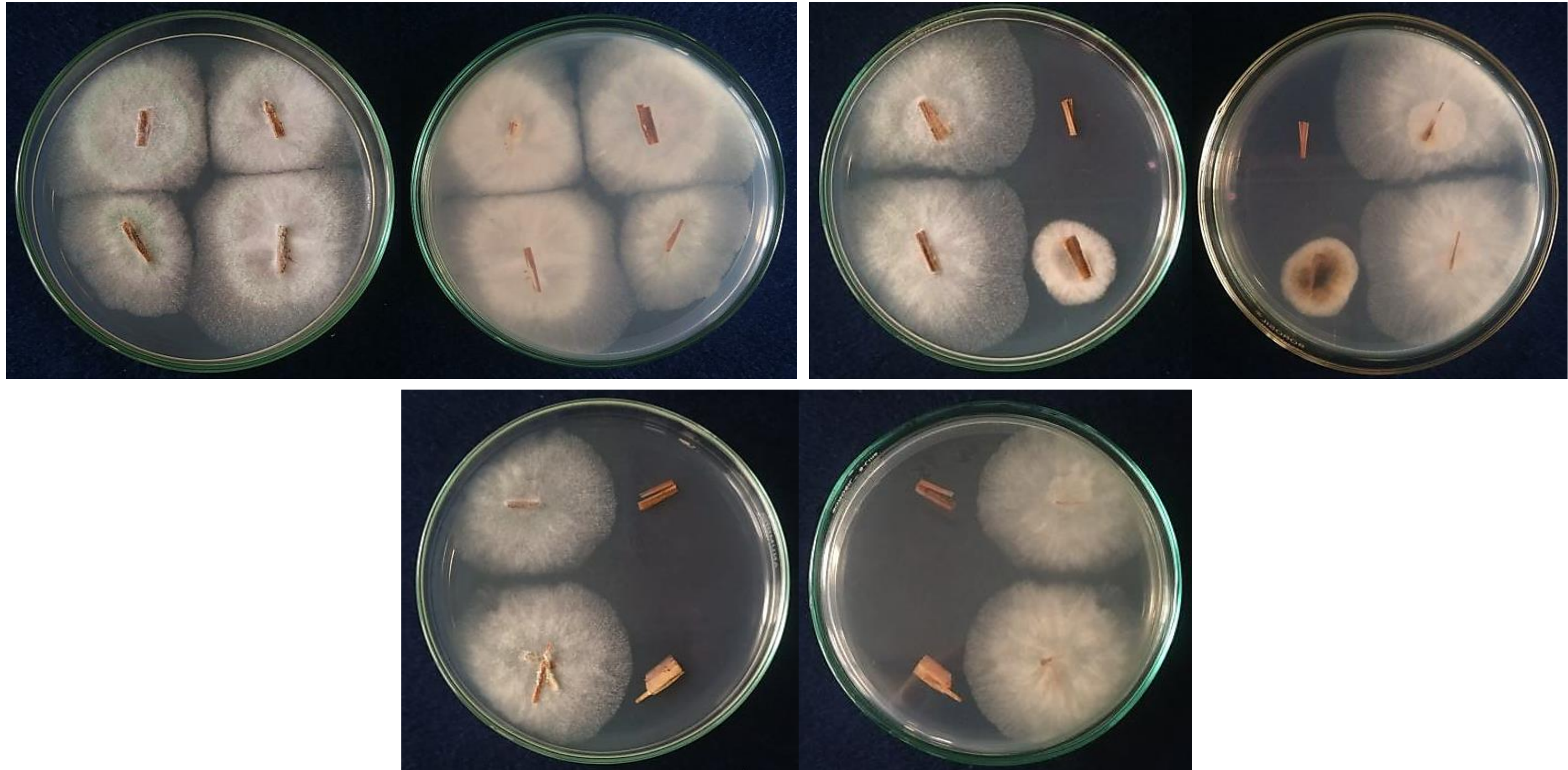


Plate 3. Growth of *Trichoderma* spp. on TSM (five DAI) from straw bits taken from soil inoculated with isolate TRKR-2

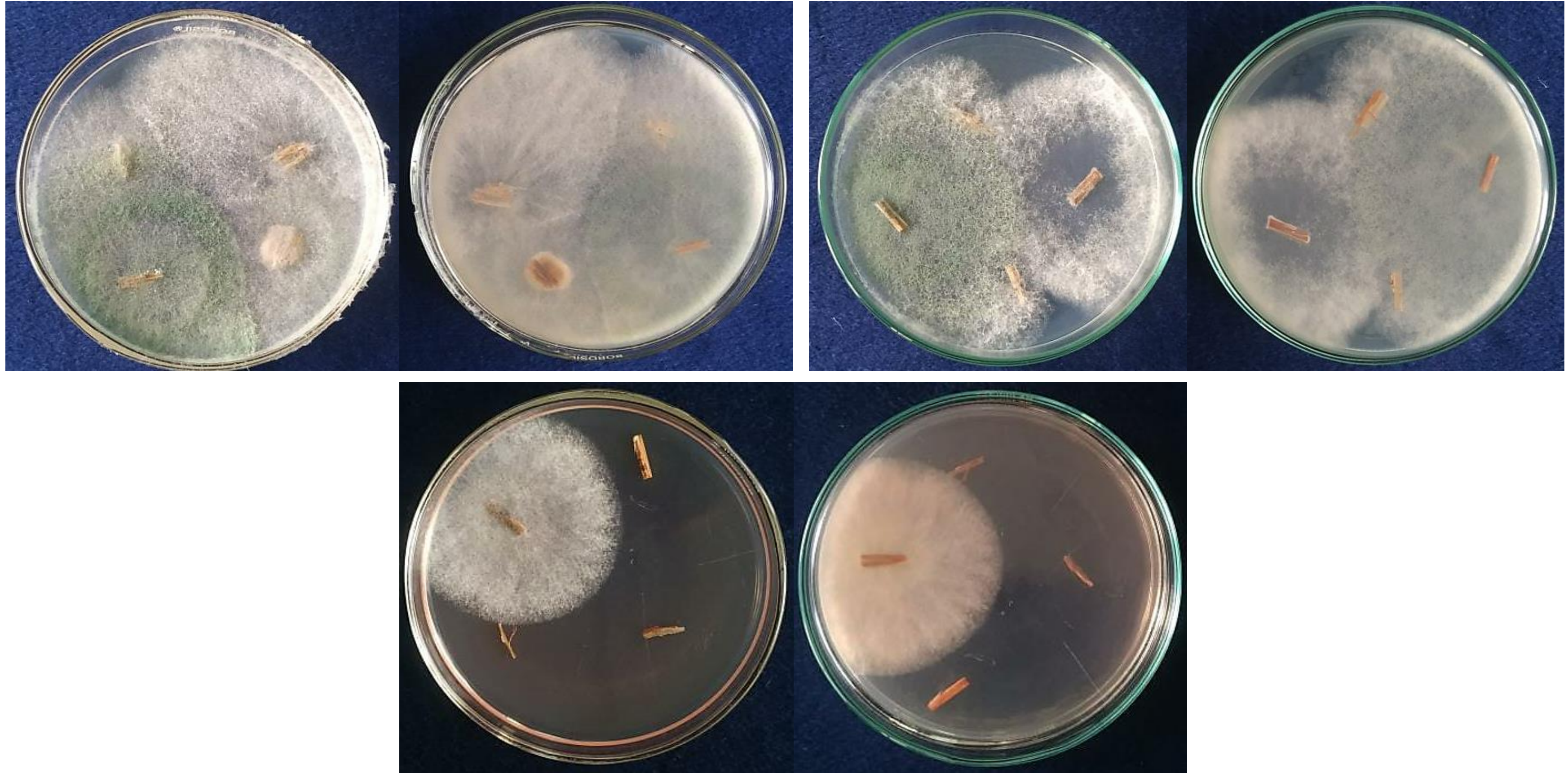


Plate 4. Growth of *Trichoderma* spp. on TSM (five DAI) from straw bits taken from soil inoculated with isolate TRPN-3

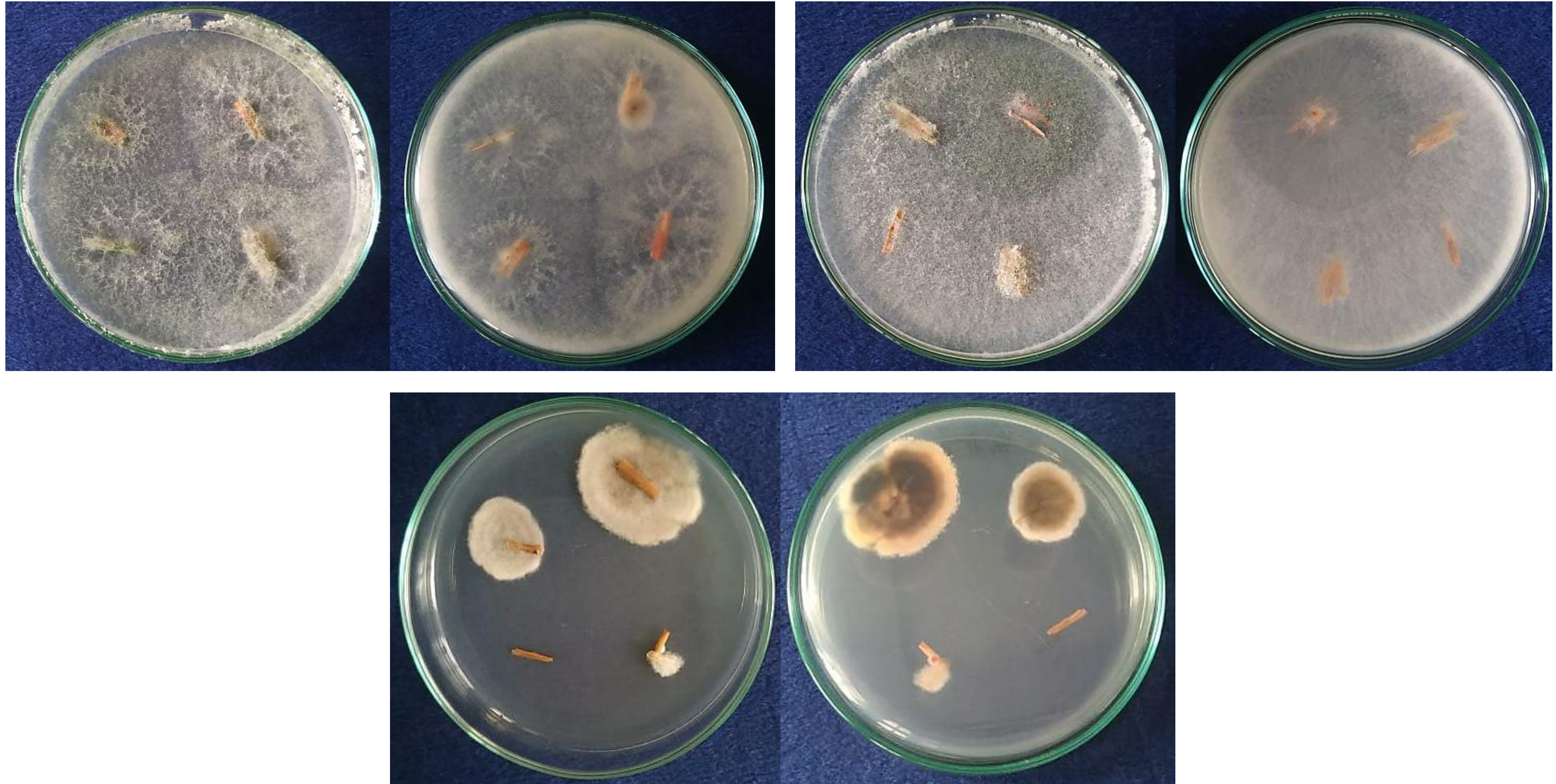


Plate 5. Growth of *Trichoderma* spp. on TSM (five DAI) from straw bits taken from soil inoculated with isolate TRPN-11

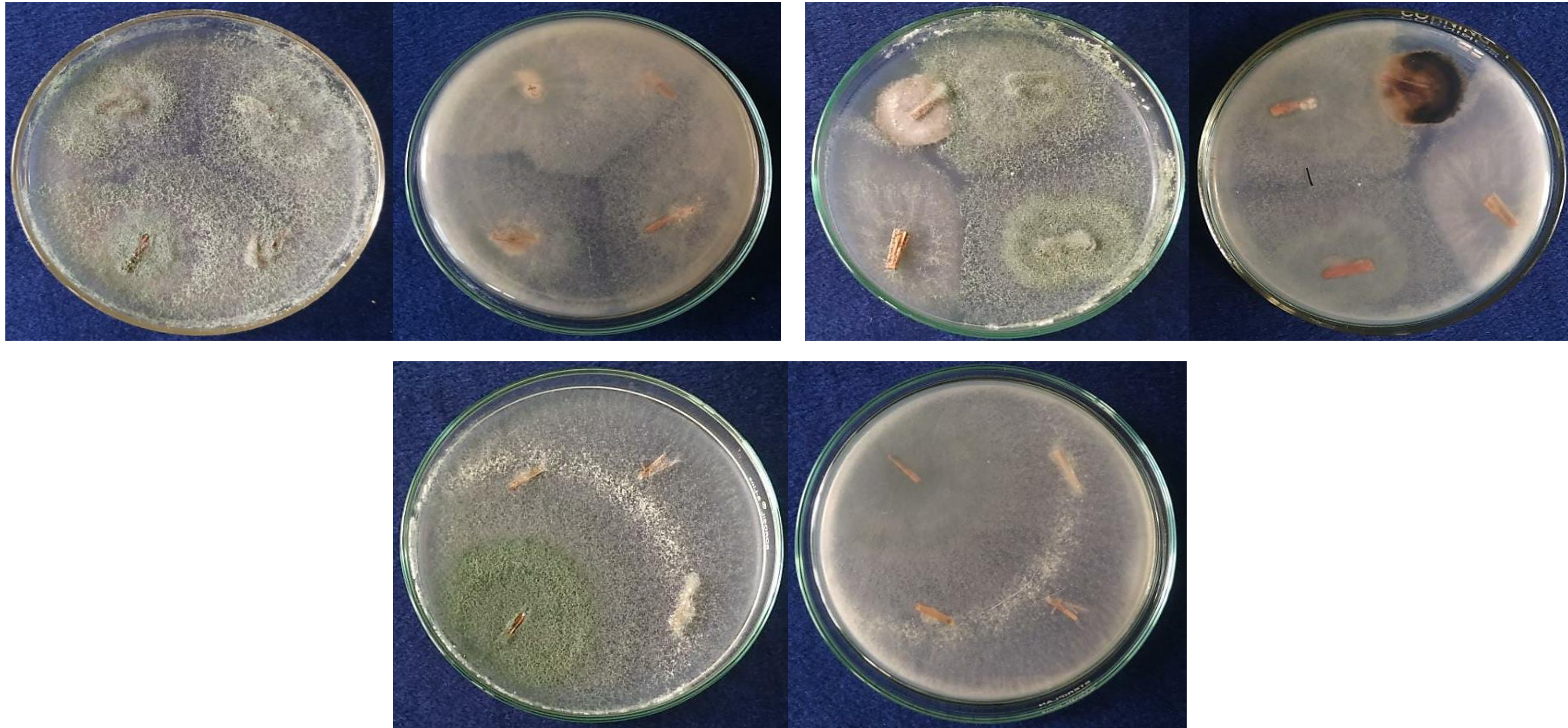


Plate 6. Growth of *Trichoderma* spp. on TSM (five DAI) from straw bits taken from soil inoculated with isolate TRPN-17

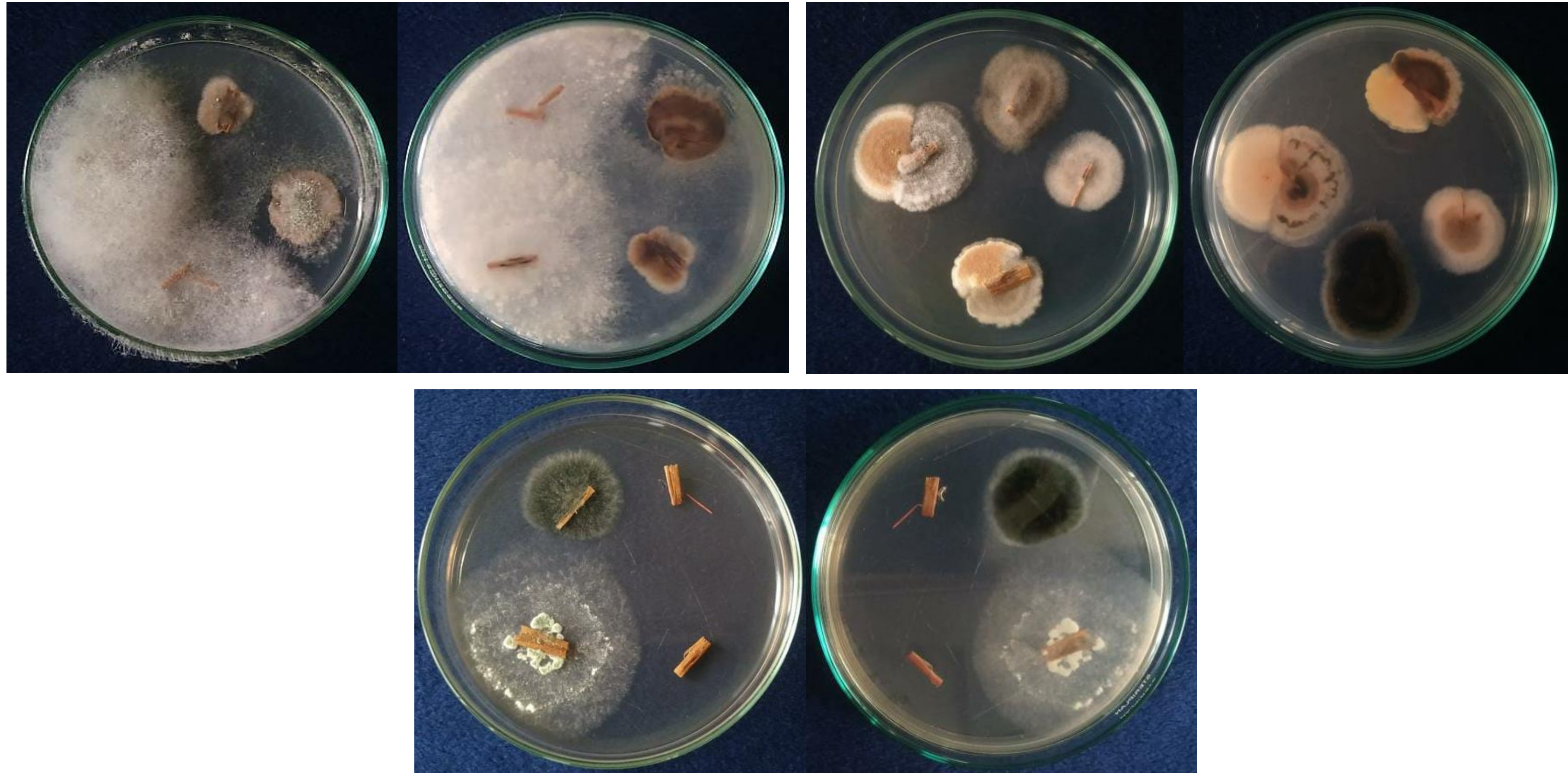


Plate 7. Fungal growth on TSM (five DAI) from straw bits taken from soil (Control)

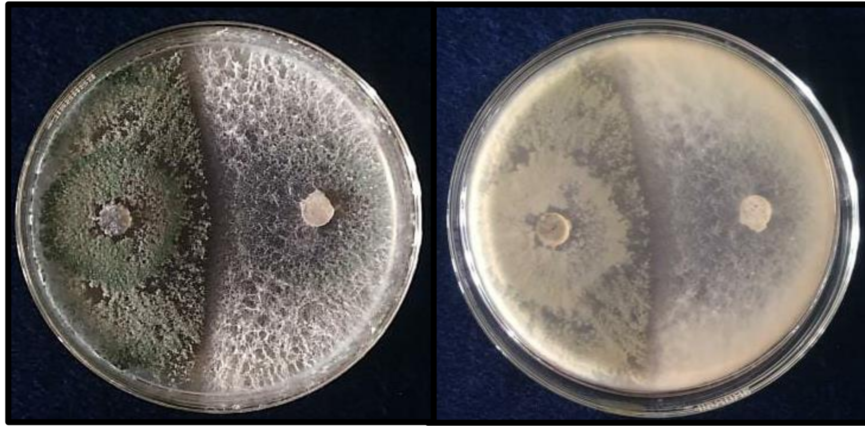
Table 3. Colonization by *Trichoderma* spp. on paddy straw bits in soil inoculated with spore suspensions of different isolates under study

Sl. No.	Isolate	No. of straw bits colonized**	Per cent colonization*
1.	TRMW-2	10.00 (3.22)[a]	50.00 (45.00)[a]
2.	TRKR-2	13.50 (3.73)[a]	67.50 (55.46)[a]
3.	TRPN- 3	11.25 (3.37)[a]	56.25 (48.81)[a]
4.	TRPN-11	9.75 (3.12)[a]	48.75 (45.03)[a]
5.	TRPN-17	11.25 (3.42)[a]	56.25 (48.62)[a]
6.	Control	1.00 (1.13)[b]	5.00 (8.92)[b]
	SE(m)	0.28	5.78
	CD (0.05)	0.84	17.18

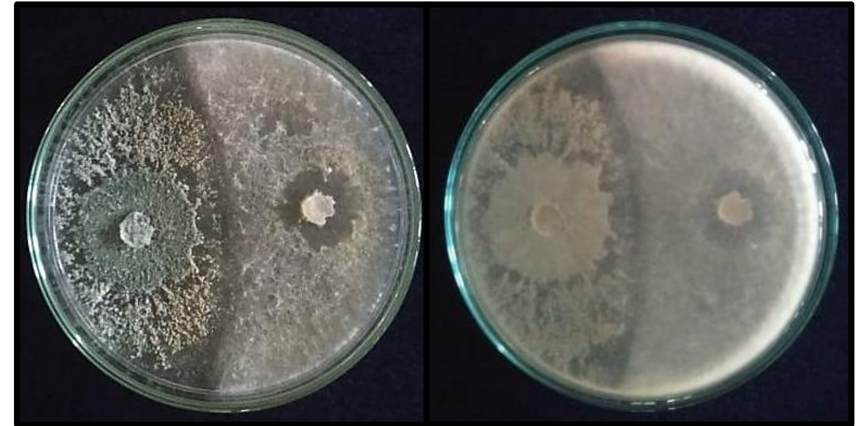
Mean value of three replication; *Values in the parenthesis are square root transformed ** Values in the parenthesis are arcsine transformed; In a column, means followed by common letters are not significantly different from each other.

4.1.3. Compatibility of different *Trichoderma* isolates by dual culture

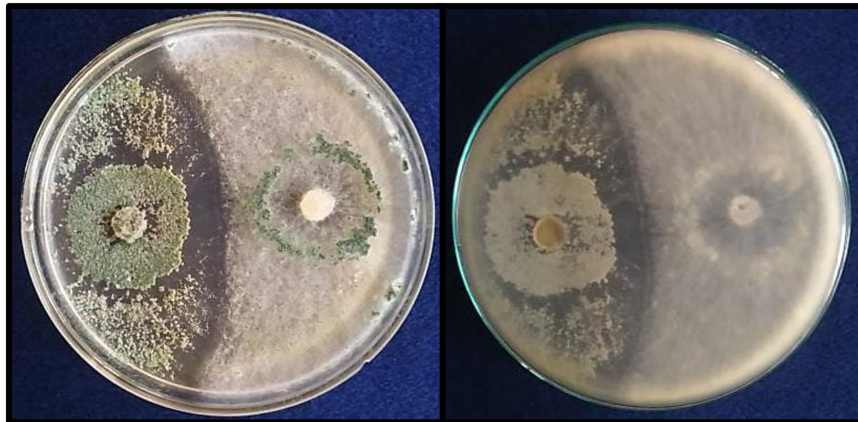
The compatibility study between the five different isolates of *Trichoderma* spp. was done by dual culture (Plate 8 to 10). The radial growth of individual isolates recorded after three days of incubation is given in Table 4. The types of interactions recorded on visual observation of the growth on Petri plates are listed in Table 5. It was found that lysis was observed only in the interaction of the TRMW-2 isolate with other isolates (Plate 8). Two antagonistic characters *viz.,* antibiosis and overgrowth were seen among most of the interactions except between TRPN-3 and TRPN-11 in which only overgrowth was observed (Plate 9). Only a mild level of antibiosis and overgrowth was reported between the interaction of TRKR-2 and TRPN-17 (Plate 9).



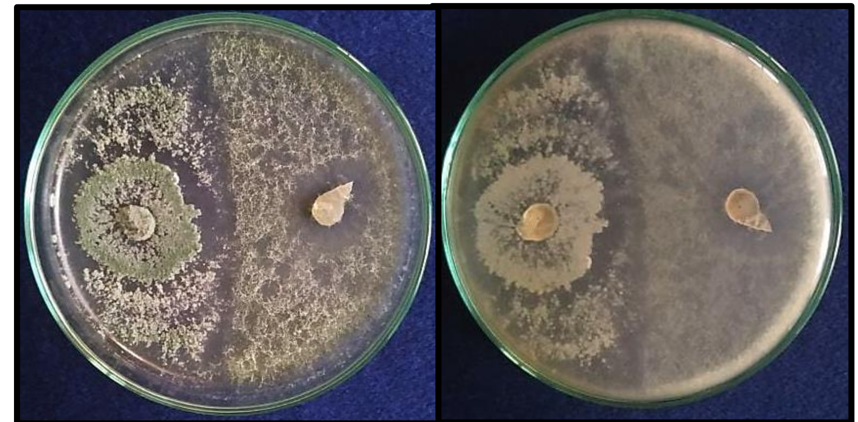
TRMW-2 vs TRKR-2



TRMW-2 vs TRPN-3



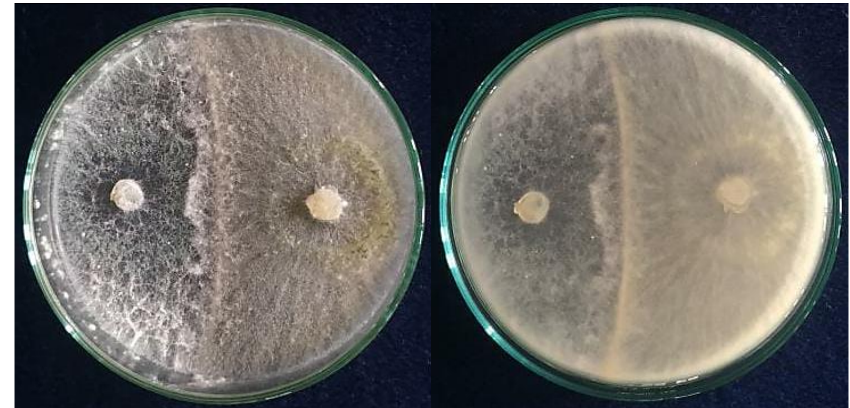
TRMW-2 vs TRPN-11



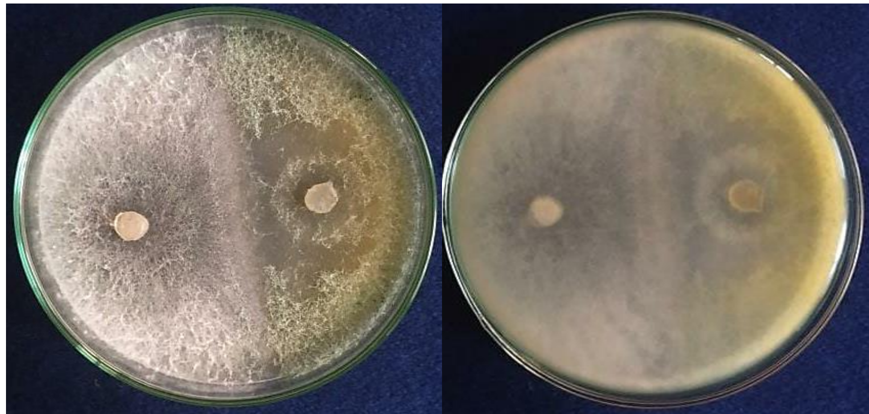
TRMW-2 vs TRPN-17

Plate 8. Assessment of compatibility of different *Trichoderma* isolates by dual culture

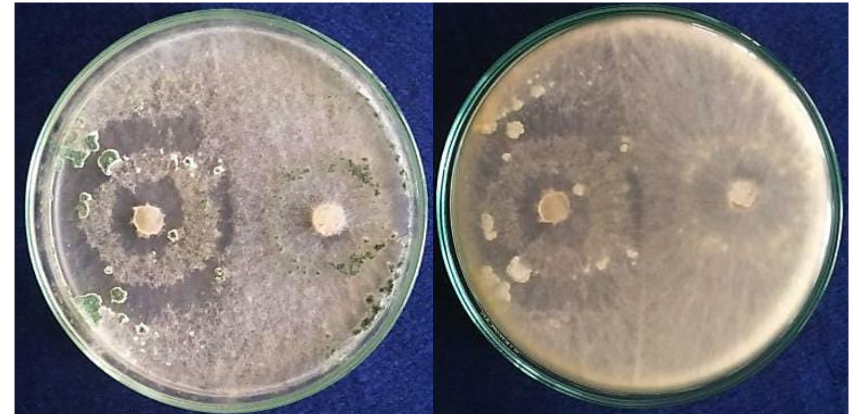
TRKR-2 vs TRPN-3



TRKR-2 vs TRPN-11

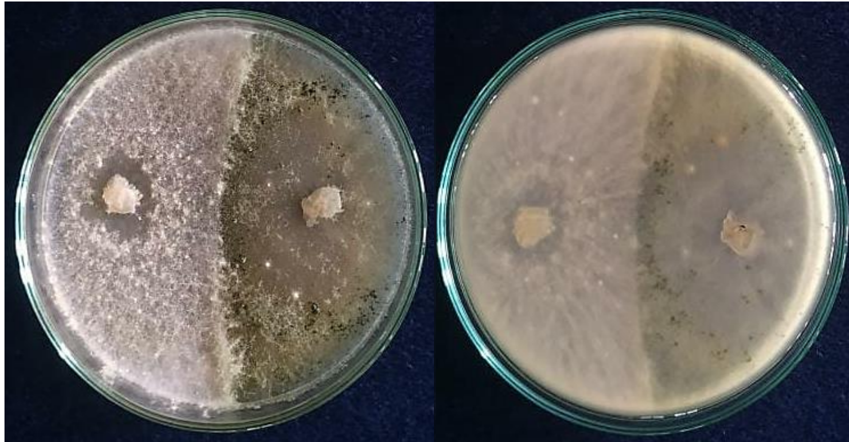


TRKR-2 vs TRPN-17



TRPN-3 vs TRPN-11

Plate 9. Assessment of compatibility of different *Trichoderma* isolates by dual culture



TRPN-3 vs TRPN-17

TRPN-11 vs TRPN-17

Plate 10. Assessment of compatibility of different *Trichoderma* isolates by dual culture

Table 4. Radial growth of the *Trichoderma* in dual culture on PDA medium at seven DAI

Sl. No.	Isolate pairs tested	Radial growth (cm)*
1	TRMW-2 / TRKR-2	2.1 (TRMW-2)
		2.6 (TRKR-2)
2	TRMW-2 / TRPN-3	2.3 (TRMW-2)
		2.3 (TRPN-3)
3	TRMW-2 / TRPN-11	2.1 (TRMW-2)
		2.5 (TRPN-11)
4	TRMW-2 / TRPN-17	2.3 (TRMW-2)
		2.6 (TRPN-17)
5	TRKR-2 / TRPN-3	2.5 (TRKR-2)
		2.5 (TRPN-3)
6	TRKR-2 / TRPN-11	2.4 (TRKR-2)
		2.7 (TRPN-11)
7	TRKR-2 / TRPN-17	2.4 (TRKR-2)
		2.4 (TRPN-17)
8	TRPN-3 / TRPN-11	2.2 (TRPN-3)
		2.4 (TRPN-11)
9	TRPN-3/ TRPN-17	2.4 (TRPN-3)
		2.5 (TRPN-17)
10	TRPN-11/ TRPN-17	2.6 (TRPN-11)
		2.5 (TRPN-17)

*Mean value of three replication

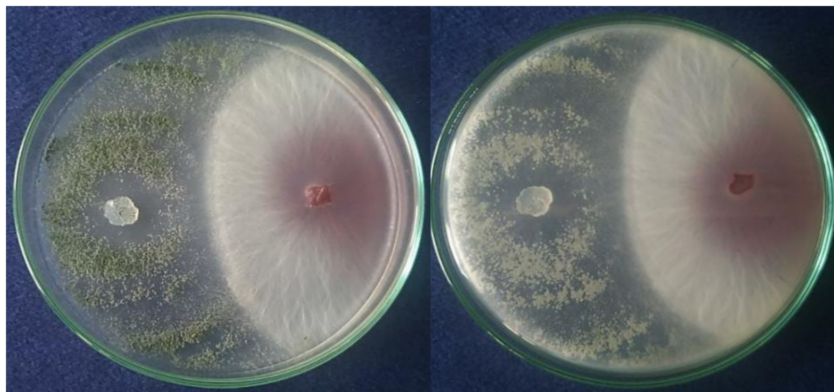
Table 5. Probable interaction of different *Trichoderma* isolates in dual culture graded by visual observation

Sl. No.	Isolate pairs	Type of interaction		
		Antibiosis	Lysis	Overgrowth
1	TRMW-2 on TRKR-2	-	++	-
2	TRKR-2 on TRMW-2	-	-	-
3	TRMW-2 on TRPN-3	-	++	-
4	TRPN-3 on TRMW-2	-	-	-
5	TRMW-2 on TRPN-11	-	+++	-
6	TRPN-11 on TRMW-2	-	-	-
7	TRMW-2 on TRPN-17	-	-	+
8	TRPN-17 on TRMW-2	+	-	+
9	TRKR-2 on TRPN-3	+	-	++
10	TRPN-3 on TRKR-2	-	-	++
11	TRKR-2 on TRPN-11	+	-	+++
12	TRPN-11 on TRKR-2	-	-	+++
13	TRKR-2 on TRPN-17	+	-	+
14	TRPN-17 on TRKR-2	-	-	+
15	TRPN-3 on TRPN-11	-	-	++
16	TRPN-11 on TRPN-3	-	-	++
17	TRPN-3 on TRPN-17	-	-	+++
18	TRPN-17 on TRPN-3	+	-	+++
19	TRPN-11 on TRPN-17	-	-	+++
20	TRPN-17 on TRPN-11	+	-	+++

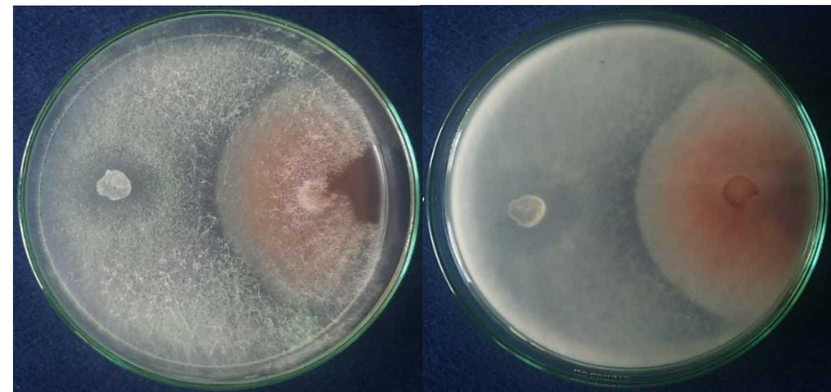
- Absence ; + Low ; ++ Medium ; +++ High

4.1.4. Dual culture assay of *Trichoderma* isolates against *Fusarium* spp.

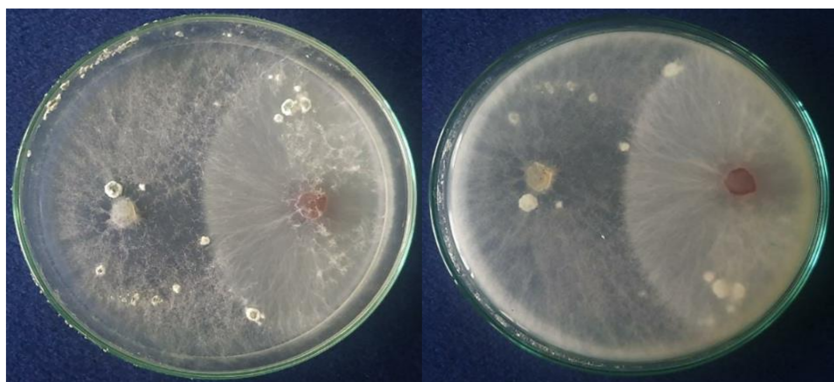
Table 6 represents the *in vitro* efficacy of *Trichoderma* isolates against *Fusarium* spp. tested by dual culture method (Plates 11 and 12). Among the tested isolates, TRPN-17 caused the highest per cent inhibition (63.71 %) of the mycelial growth and differed significantly from other isolates. It was followed by TRKR-2 (58.76 %), TRPN-3 (58.21 %), TRMW-2 (57.10 %) and TRPN-11 (56.56 %) with no significant difference. The reference strains *T. asperellum* (KAU strain) and *T. harzianum* (NBAIR strain) had the per cent inhibition of 73.04 per cent and 53.26 per cent respectively. Table 7 shows the antagonistic characters *viz.,* antibiosis, lysis and overgrowth of *Trichoderma* isolates against *Fusarium* spp. Almost all the isolates exhibited all three antagonistic characters except *Trichoderma* sp. (KAU strain) and *T. harzianum* (NBAIR strain) in which only lysis and overgrowth was observed. TRKR-2 recorded only antibiosis and overgrowth against the pathogen.



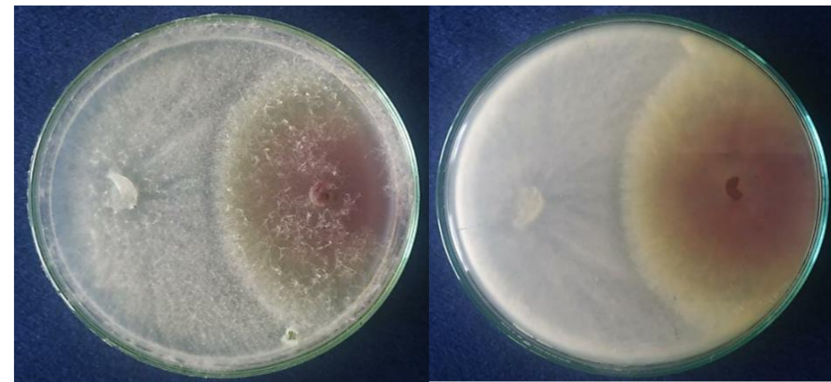
TRMW-2



TRKR-2

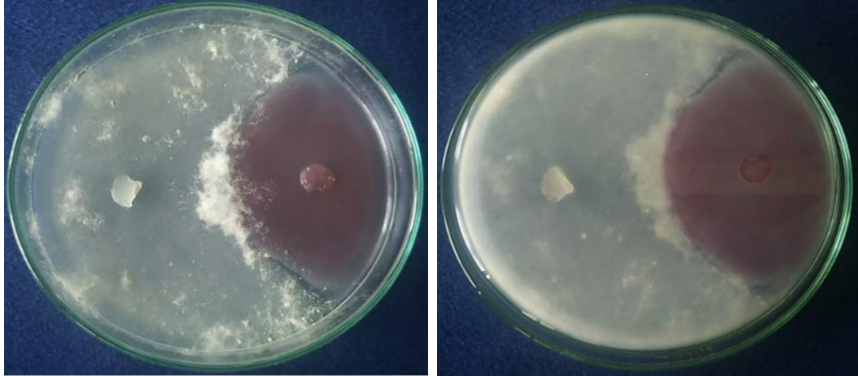


TRPN-3

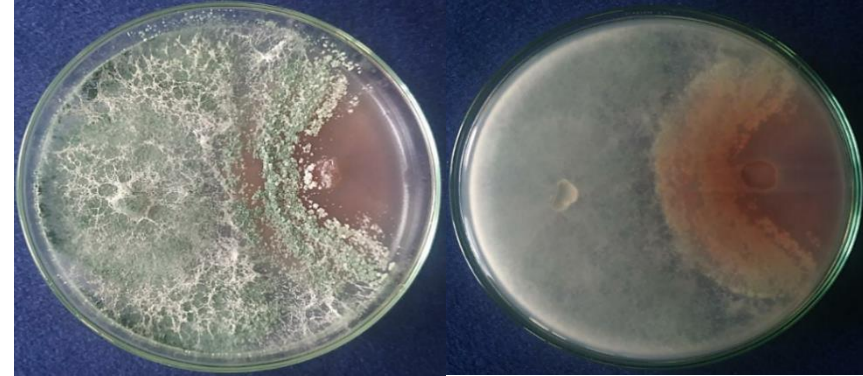


TRPN-11

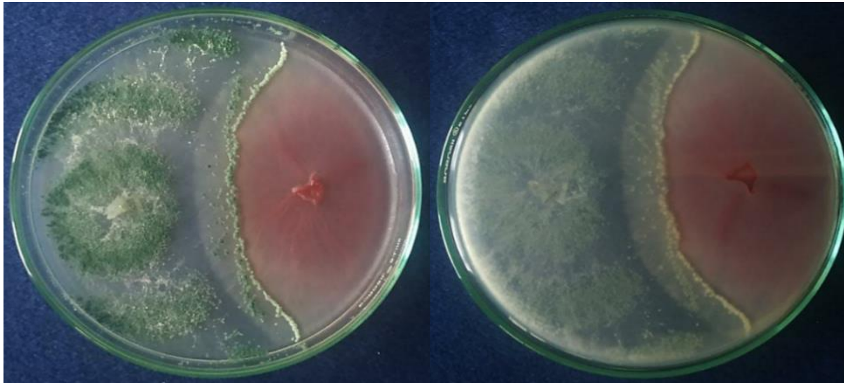
Plate 11. Dual culture assay of *Trichoderma* isolates TRMW-2, TRKR-2, TRPN-3 and TRPN-11 against *F. oxysporum*



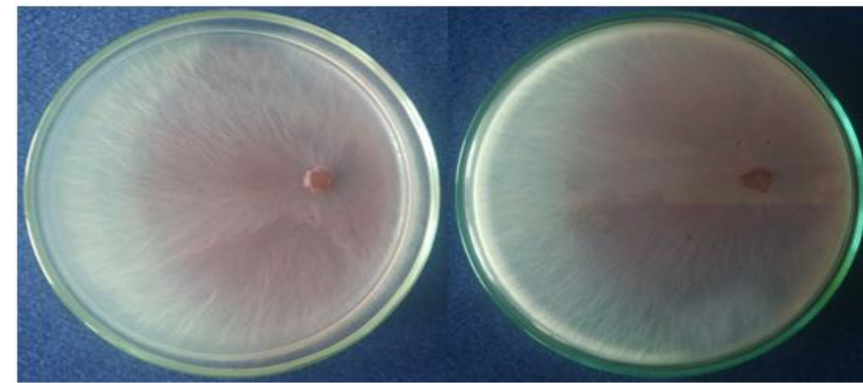
TRPN-



T. asperellum (KAU



T. harzianum (NBAIR



Control (*F. oxysporum*)

Plate 12. Dual culture assay of *Trichoderma* isolates TRPN-17, *T. asperellum* (KAU strain) and *T. harzianum* (NBAIR strain) against *F. oxysporum*

Table 6. *In vitro* efficacy of selected isolates of *Trichoderma* sp. against *F. oxysporum*

Sl. No.	Isolate	Radial growth of pathogen (cm)*	Inhibition (%)**
1.	TRMW-2	2.60±0.26[bc]	57.10 (49.10)[bc]
2.	TRKR-2	2.50±0.00[bc]	58.76 (50.05)[bc]
3.	TRPN-3	2.53±0.15[bc]	58.21 (49.73)[bc]
4.	TRPN-11	2.63±0.15[bc]	56.56 (48.78)[bc]
5.	TRPN-17	2.20±0.00[c]	63.71 (52.96)[b]
6.	*Trichoderma* sp. (KAU strain)	1.60±0.59[d]	73.04 (58.94)[a]
7.	*T. harzianum* (NBAIR strain)	2.80±0.05[b]	53.26 (46.87)[c]
8.	Control	6.07±0.21[a]	0 (0)[d]
	SE (m)	0.14	1.50
	CD (0.05)	0.43	4.52

*Mean ± SD of three replication ** Values in the parenthesis are arcsine transformed; In a column, means followed by common letters are not significantly different from each other.

Table 7. Antagonistic characters of selected isolates of *Trichoderma* sp. against *Fusarium oxysporum*

Sl. No.	Isolate	Antibiosis	Lysis	Overgrowth
1.	TRMW-2	+	+	++
2.	TRKR-2	+	-	++
3.	TRPN-3	+	+	++
4.	TRPN-11	+	+	+
5.	TRPN-17	+	++	++
6.	*Trichoderma* sp. (KAU strain)	-	++	++
7.	*T. harzianum* (NBAIR strain)	-	++	+

- Absence; + Low; ++ Medium; +++ High

4.2. MOLECULAR CHARACTERIZATION OF PROMISING *TRICHODERMA* ISOLATES

4.2.1. Isolation of genomic DNA

The genomic DNA was isolated from all the *Trichoderma* isolates using the CTAB method and its concentration was found to be 10 µg/µl using a spectrophotometer.

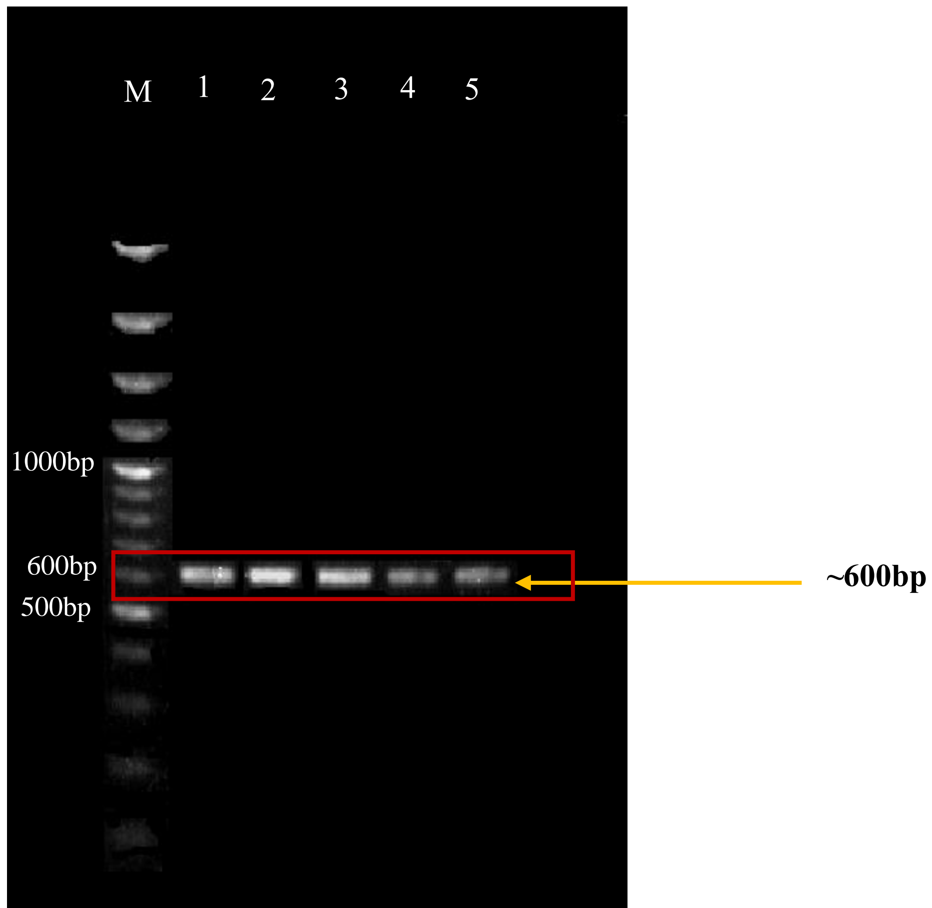
4.2.2. Agarose gel electrophoresis

Agarose gel electrophoresis revealed good-quality DNA samples with intact bands free from RNA, protein or any form of contamination.

4.2.3 Polymerase chain reaction (PCR) analysis

4.2.3.1. PCR amplification using ITS primers

PCR was done using the primers for Internal Transcribed Spacer (ITS) regions 1 and 4. The isolated genomic DNA was used as the template in PCR and a bright intact amplicon of size 600 bp approximately (Plate 13) was yielded at an annealing temperature of 57°C.



Lane M-Ladder 100bp, Lane 1-TRMW-2, Lane 2-TRKR-2, Lane 3-TRPN-3, Lane 4-TRPN-11 and Lane 5-TRPN-17

Plate 13. PCR amplification of ~600 bp rDNA-ITS region of *Trichoderma* isolates using ITS primers.

4.2.3.2. PCR amplification using genus-specific primer

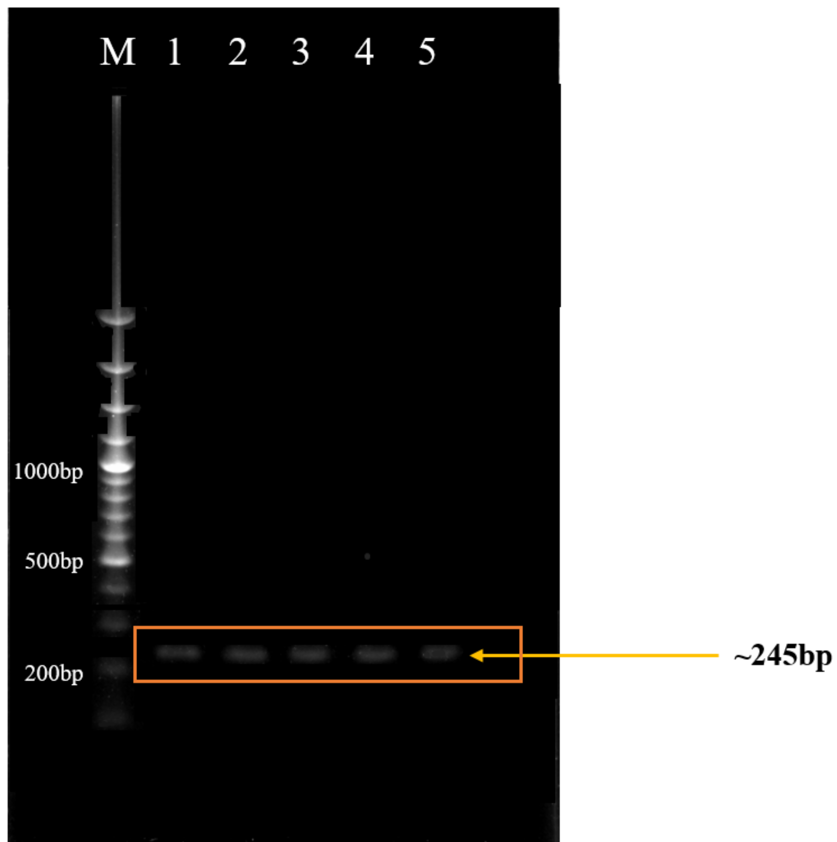
Genus-specific identification of the isolated genomic DNA was done using the oligonucleotide set of genus-specific primer TvPF (forward) and TvPR (reverse). A PCR product of size approximately 245 bp at an annealing temperature of 60°C was obtained for all five isolates (Plate 14).

4.2.3.3. PCR amplification using species-specific primers

The species-specific identification was tried with translational elongation factor- 1 (*tef1)* and RNA polymerase II (*rpb2)* gene specific primers under different conditions (Plates 15, 16, 17, 18 and 19) which did not yield any amplicon indicating that the isolates may not belong to the species *T. virens*, *T. longibrachiatum*, *T. harzianum* and *T. asperellum.*

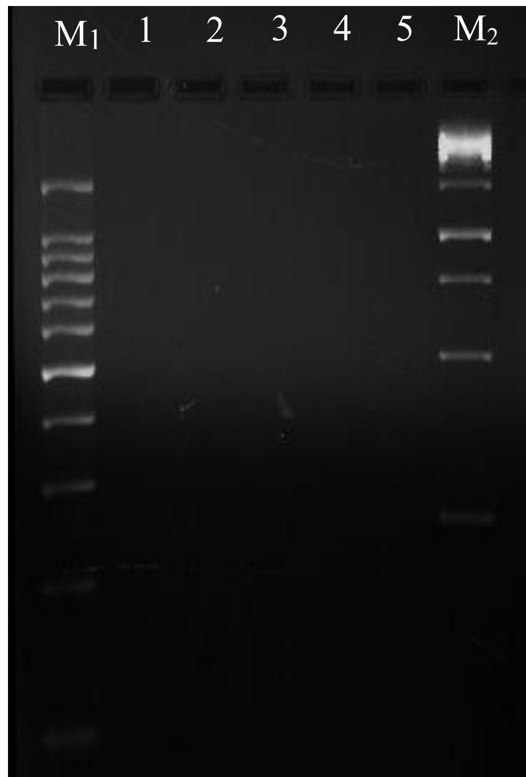
4.2.4. Sequence analysis of the PCR products

The nucleotide sequences of all the ITS-PCR products (Plate 20, 21) and TvP-PCR products (Plate 22, 23) obtained from the five isolates were confirmed through bidirectional sequencing using the Sangers DNA sequencing. The best match of the amplified sequences of ITS region obtained from the GenBank database using NCBI-BLAST based on per cent similarity was shown in Table 8. The results of NCBI-BLAST analysis of the amplified ITS sequences of the five isolates are given in Fig. 1 to 5. The isolate TRMW-2 showed a similarity of 99.42 per cent with the *T. brevicompactum* isolate, *Trichoderma* sp. isolate SDAS203739 and *T. deliquescens***.** Isolate TRKR-2 was found to be 99.65 per cent similar to *T. koningiopsis* isolate and *T. ovalisporum* strain. TRPN-3 isolate resembled 99.82 per cent to *T. harzianum* clone, *T. asperellum* isolate and *T. lixii* isolate. The isolate TRPN-11 was 98.31 per cent similar to *T. effusum voucher, Trichoderma* sp. Isolate SDAS203144, *T. cf harzianum voucher* and *T. lixii* strain. The isolate TRPN-17 resembled 98.70 per cent *T. harzianum* isolate and *T. asperellum* isolate. Using genus-specific primers for TvP region, the DNA barcoding of the amplicon confirmed the genus identity of all the isolates as *Trichoderma* (Fig. 6, 7, 8, 9 and 10).



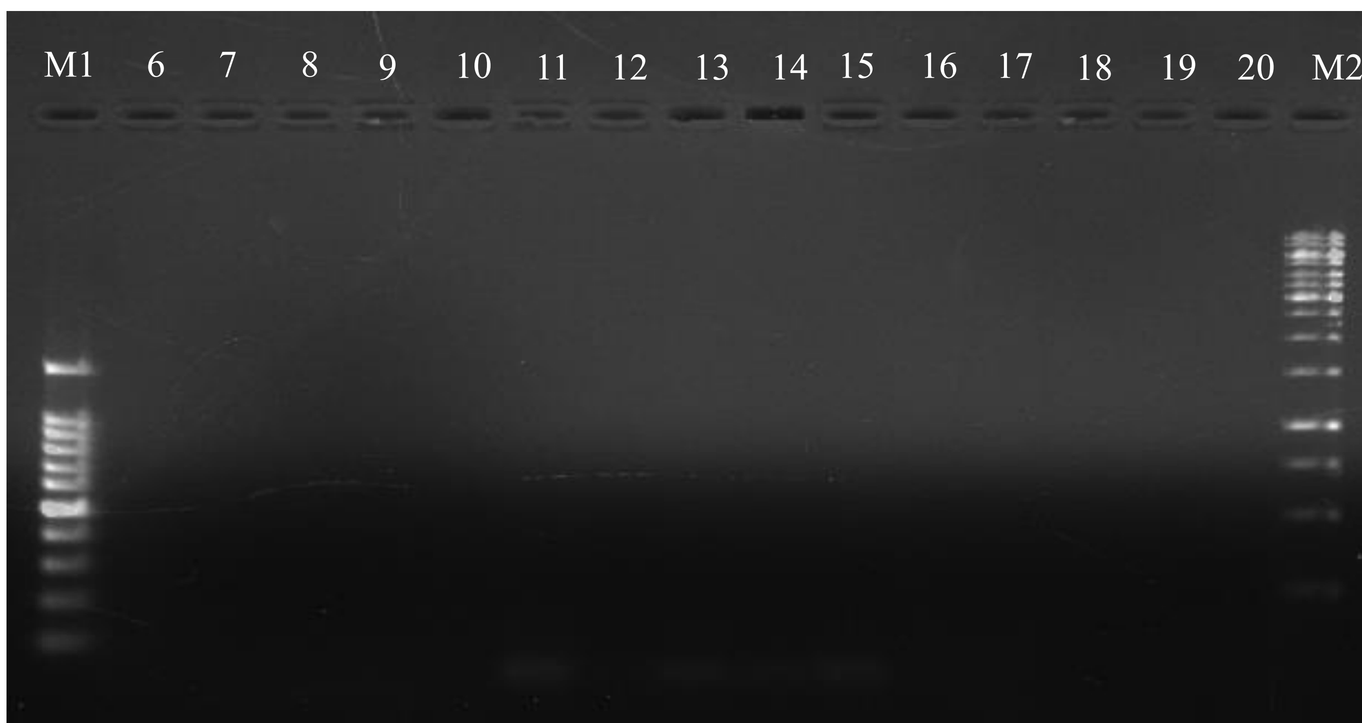
Lane M-Ladder 100bp, Lane 1-TRMW-2, Lane 2-TRKR-2, Lane 3-TRPN-3, Lane 4-TRPN-11 and Lane 5-TRPN-17

Plate 14. PCR amplification of ~245 bp rDNA region of *Trichoderma* isolates using genus-specific primers TvPF and TvPR



Lane M1-Ladder 100bp, Lane 1-TRMW-2, Lane 2-TRKR-2, Lane 3-TRPN-3, Lane 4-TRPN-11, Lane 5-TRPN-17 and Lane M2-Ladder 1kb

Plate 15. PCR amplification of rDNA region of *Trichoderma* isolates using species-specific primer *tef* 1A

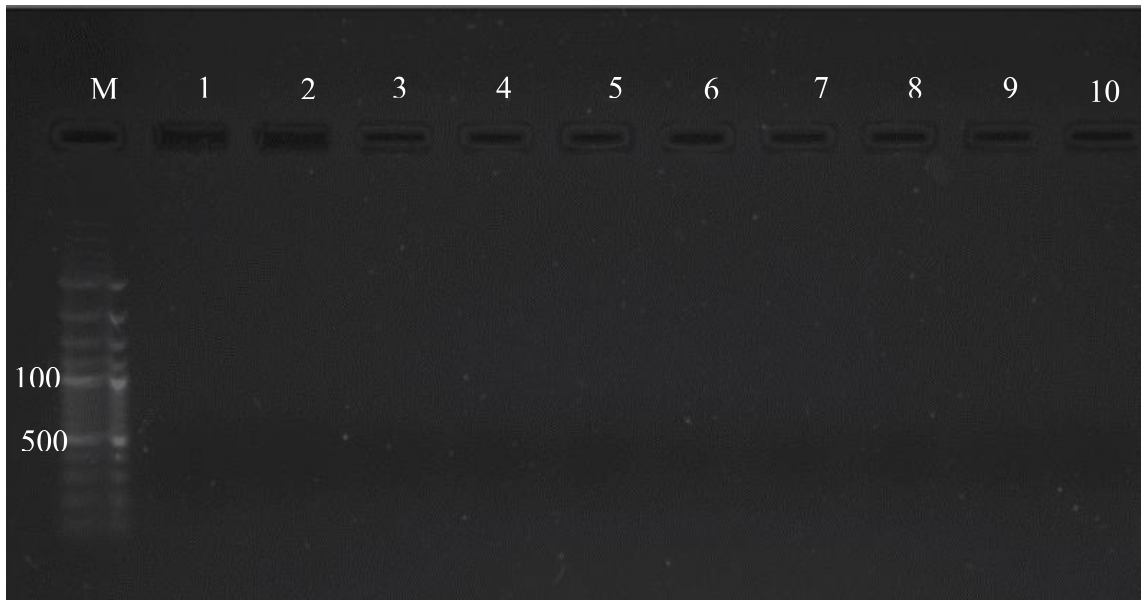


Lane M1-Ladder 100bp, Lane M2-Ladder 1kb, Lane 6, 11and 16-TRMW-2, Lane 7, 12 and 17-TRKR-2, Lane 8, 13 and 18-TRPN-3, Lane 9, 14 and 19-TRPN-11, Lane 10, 15 and 20-TRPN-17; Lane 6 to 10 –*tef*1B, Lane 11 to 15-*tef*1C and Lane 16 to 20-*tef*1D

Plate 16. PCR amplification of rDNA region of *Trichoderma* isolates using species-specific primers *tef* 1B, *tef* 1C, *tef* 1D and *rpb2*

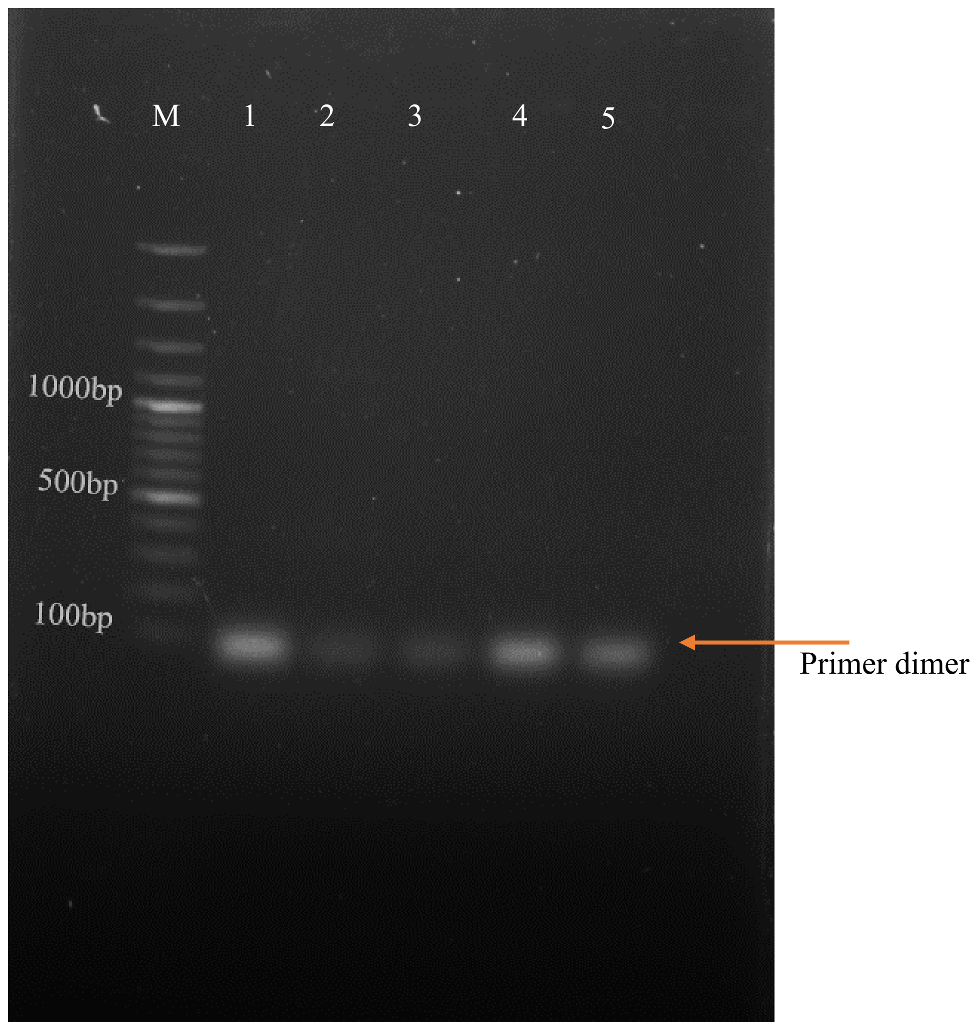
Lane M-Ladder 100bp, Lane 1 to 6 - *tef1* A primer with TRMW-2, Lane 7 to 12 – *rpb2* primer with TRMW-2, Lane A-65 ℃, Lane C – 63 ℃, Lane D – 61.1 ℃, Lane E – 58.8 ℃, Lane F – 56.9 ℃ and Lane G – 55.7 ℃

Plate 17. PCR amplification of rDNA region of *Trichoderma* isolates using species-specific primers at gradient temperature 55 ℃ - 65 ℃.



Lane M- Ladder 1kb, Lane 1 to 5 - *rpb2* primer with $MgCl_2$, Lane 6 to 10 – *rpb2* primer without $MgCl_2$, Lane 1,6 – TRMW-2; Lane 2,7 – TRKR-2; Lane 3,8 – TRPN-3; Lane 4,9 – TRPN-11and Lane 5,10 – TRPN-17.

Plate 18. PCR amplification of rDNA region of *Trichoderma* isolates using species-specific primers with $MgCl_2$



Lane M-Ladder 100bp, Lane 1-TRMW-2, Lane 2-TRKR-2, Lane 3-TRPN-3, Lane 4-TRPN-11 and Lane 5-TRPN-17.

Plate 19. PCR amplification of rDNA region of *Trichoderma* isolates using 2 µl of species-specific primers

a) TRMW2- ITS

5’TGCCCCGGGCGCGTCGCAGCCCCGGACCAAGGCGCCCGCCGGAGGACCAATTTACA
AACTCTTTTGTATATCCCATCGCGGATTCTTTACATTGTGAGCTTTCTCGGCGCTCCTAG
CGAGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
GAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCG
TCATTTCAACCCTCGAGCCCCTCCGGGGGGTCGGCGTTGGGGATCGGCACTTACCTGC
CGGCCCCGAAATACAGTGGCGGTCTCGCCACAGCCTCTCCTGCGCAGTAGTTTGCACA
CTCGCACCGGGAGCGCGGCGCGTCCACGGCCGTAAAACAACCCAAACTTCTGAAATG
TTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAG3’

b) TRKR2- ITS

5’CTTCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCC
AATGTGAACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCGC
AGCCCCGGACCAAGGCGCCCGCCGGAGGACCAACCAAAACTCTTTTTGTATACCC
CCTCGCGGGTTTTTTATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAA
ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA
GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
ACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCA
ACCCTCGAACCCCTCCGGGGGGTCGGCGTTGGGGATCGGCCCTGCCTCTTGGCGG
TGGCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTT
TGCACACTCGCATCGGGAGCGCGGCGCGTCCACAGCCGCTAAACACT3’

c) TRPN3- ITS

5’TTACAACTCCCAAACCCAATGTGAACCATACCAAACTGTTGCCTCGGCGGGGTCAC
GCCCCGGGTGCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGGACCAACCAAAC
TCTTTCTGTAGTCCCCTCCGGCGGACGTTATTTCTTACAGCTCTGAGCAAAAATTCAAA
ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC
GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTC
GAACCCCTCCGGGGGGTCGGCGTTGGGGATCGGGAACCCCTAAGACGGGATCCCGGC
CCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTC
GCACCGGGAGCGCGGCGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTGA
CCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA3’

Plate 20. Sequences of amplified ITS region of isolates TRMW-2, TRKR-2 and TRPN-3

d) TRPN11- ITS

5’GGGAAAGGGGTTTACAACTCCCAAACCCAATGTGAACGTTACCAAACTGTTGCCTC
GGACGGGATCTCTGCCCCGGGCTGCGTCGCAGCCCCGGACCAAGGAACGCCCGCCG
GAGGACCAACAGCAAAACTCTTTTTGTATACCCCCTCGCGGGTTTTTTATAATCTGAG
CCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGAT
CTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC
AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCG
GGCAGGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGTGGGCATTG
GGGATCGGCCCTGCCTCTTGGCGGTGGCCGTCTCCGAAATACAGTGGCGGTCTCGCC
GCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGGGAGCGCGGCGCGTCCACA
AGCCGTTAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCT
GAACTTAAGCATATCAATAAGCGGAGGAAC3’

e) TRPN17- ITS

5’TTTCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCA
ATGTGAACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCGCAGC
CCCGGACCAAGGCGCCCGCCGGAGGACCAACCAAAACTCTTTTTGTATACCCCCTCG
CGGGTTTTTTATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATC
AAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGC
GCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCC
TCCGGGGGGTCGGCGTTGGGGATCGGCCCTGCCTCTTGGCGGTGGCCGTCTCCGAAA
TACAGGGGCGGTCTCGCCGCAGCCTATCATGGGCAGTAGTTTGCACACTGGCATCGGG
AGCGCGGCGTATCCACAGGAACATAACAAGCAAACTGAAAAATCCAAAGCGAGGAC
CACCCGCCGCACCAAACAACCCAAC3’

Plate 21. Sequences of amplified ITS region of isolates TRPN-11 and TRPN-17

a) TRMW2-TvP

5’TTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGAACCAAGAGATCC
GTTGTTGAAAGTTTTGATTCTTTTTCGAAACGCCTACGAGAGGCGCCGAGAAGGCT
CAAATTATAAAAAACCCGCGAGGGGGTATACAAAAAGAGTTTTGGTTGGTCCTCCG
GCGGGAACACTTACGACAGTCTTTGACTTACAACAA3’

b) TRKR2-TvP

5’TTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATC
CGTTGTTGAAAGTTTTGATTCATTTTCGAAACGCCTACTAGAGGCGCCGAGAAGGCT
CAAATTATAAAAAACCCGCGAGGGGGTATACAAAAAGAGTTTTGGTTGGTCCTCCGG
CGGGAACACTTACGACAG3’

c) TRPN3-TvP

5’ACCAACCAAAACTCTTTTTGTATACCCCCTCGCGGGTTTTTTATAATTTGAGCCTTC
TCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCT
TGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCACCAGTATTCTGGCGGGC

Plate 22. Amplified sequences of isolates TRMW-2, TRKR-2 and TRPN-3 using genus specific primers TvPF and TvPR

d) TRPN11-TvP

5’TTCCGCCGGAGGACCAACCAAAACTCTTTTTGTATACCCCCTCGCGGGTTTTTAT
AATTTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTTC
AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT
AATGTGAATTGCAAAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCA
CCAGTATTCTGGCGGGCATGCCTGTCAAAA3’

e) TRPN17-TvP

5’CTAAGATTCCCCCTCGCGGGTTTTTATAATCTGAGCCTTCTCGGCGCCTCTCGTA
GGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAATCTTTGAACGCACATTGCGCCCGCC3’

Plate 23. Amplified sequences of isolate TRPN-11 and TRPN-17 using genus specific primers TvPF and TvPR

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Trichoderma brevicompactum isolate RL806 internal transcribed spacer 1, partial sequence; 5.8S ribosomal R...	Trichoderma bre...	942	942	100%	0.0	99.42%	600	MT557327.1
Trichoderma brevicompactum isolate RL177 internal transcribed spacer 1, partial sequence; 5.8S ribosomal R...	Trichoderma bre...	942	942	100%	0.0	99.42%	700	MT557156.1
Trichoderma sp. isolate SDAS203739 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma sp.	942	942	100%	0.0	99.42%	590	MK871076.1
Trichoderma sp. isolate SDAS203682 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma sp.	942	942	100%	0.0	99.42%	590	MK871068.1
Trichoderma sp. isolate SDAS203879 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma sp.	942	942	100%	0.0	99.42%	620	MK870985.1
Trichoderma sp. isolate SDAS203876 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma sp.	942	942	100%	0.0	99.42%	617	MK870976.1
Trichoderma sp. isolate SDAS203861 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma sp.	942	942	100%	0.0	99.42%	619	MK870927.1
Trichoderma sp. isolate SDAS203798 small subunit ribosomal RNA gene, partial sequence; internal transcrib...	Trichoderma sp.	942	942	100%	0.0	99.42%	634	MK870908.1
Trichoderma sp. isolate SDAS203912 small subunit ribosomal RNA gene, partial sequence; internal transcrib...	Trichoderma sp.	942	942	100%	0.0	99.42%	614	MK870758.1
Trichoderma sp. isolate SDAS204104 small subunit ribosomal RNA gene, partial sequence; internal transcrib...	Trichoderma sp.	942	942	100%	0.0	99.42%	614	MK870717.1
Trichoderma sp. isolate SDAS203899 small subunit ribosomal RNA gene, partial sequence; internal transcrib...	Trichoderma sp.	942	942	100%	0.0	99.42%	614	MK870714.1
Trichoderma sp. isolate SDAS203898 small subunit ribosomal RNA gene, partial sequence; internal transcrib...	Trichoderma sp.	942	942	100%	0.0	99.42%	614	MK870706.1
Trichoderma sp. isolate SDAS203897 small subunit ribosomal RNA gene, partial sequence; internal transcrib...	Trichoderma sp.	942	942	100%	0.0	99.42%	614	MK870705.1
Trichoderma sp. isolate SDAS203895 small subunit ribosomal RNA gene, partial sequence; internal transcrib...	Trichoderma sp.	942	942	100%	0.0	99.42%	614	MK870703.1
Trichoderma sp. isolate SDAS203891 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma sp.	942	942	100%	0.0	99.42%	588	MK870667.1
Trichoderma sp. isolate SDAS203890 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma sp.	942	942	100%	0.0	99.42%	588	MK870663.1
Trichoderma sp. isolate SDAS203818 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma sp.	942	942	100%	0.0	99.42%	589	MK870125.1

Fig. 1. BLAST analysis of ITS sequences of TRMW-2

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Trichoderma koningiopsis isolate X2P6Tk small subunit ribosomal RNA gene, partial sequence; internal transc...	Trichoderma kon...	1044	1044	100%	0.0	99.65%	608	ON920714.1
Trichoderma ovalisporum strain LIPIMC0571 18S ribosomal RNA gene, partial sequence; internal transcribed ...	Trichoderma ova...	1044	1044	100%	0.0	99.65%	614	KC847168.1
Uncultured fungus clone HI38 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ...	uncultured fungus	1042	1042	99%	0.0	99.65%	643	JX457015.1
Trichoderma koningiopsis isolate upm9 small subunit ribosomal RNA gene, partial sequence; internal transcrib...	Trichoderma kon...	1038	1038	100%	0.0	99.48%	622	MK027312.1
Trichoderma afroharzianum voucher research collection Farrer lab 245 internal transcribed spacer 1, partial se...	Trichoderma afr...	1037	1037	100%	0.0	99.48%	1131	MN644679.1
Trichoderma koningiopsis isolate XXTF5 small subunit ribosomal RNA gene, partial sequence; internal transcri...	Trichoderma kon...	1037	1037	100%	0.0	99.48%	606	MN602617.1
Trichoderma koningiopsis strain SKCGW010 internal transcribed spacer 1, partial sequence; 5.8S ribosomal R...	Trichoderma kon...	1037	1037	100%	0.0	99.48%	744	MG940962.1
Trichoderma koningiopsis strain U small subunit ribosomal RNA gene, partial sequence; internal transcribed s...	Trichoderma kon...	1037	1037	100%	0.0	99.48%	610	MK791712.1
Trichoderma koningiopsis isolate QA-3 small subunit ribosomal RNA gene, partial sequence; internal transcrib...	Trichoderma kon...	1037	1037	100%	0.0	99.48%	606	MF616361.1
Ascomycota sp. isolate LTS615 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and...	Ascomycota sp.	1037	1037	100%	0.0	99.48%	580	MH430770.1
Trichoderma sp. isolate Cg05 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer...	Trichoderma sp.	1037	1037	100%	0.0	99.48%	674	MF101380.1
Fungal sp. voucher ARIZ:PS0981 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a...	fungal sp.	1037	1037	100%	0.0	99.48%	1045	KU978005.1
Trichoderma sp. isolate KOFO small subunit ribosomal RNA gene, partial sequence; internal transcribed spac...	Trichoderma sp.	1037	1037	100%	0.0	99.48%	639	KY312024.1
Trichoderma koningiopsis strain CN018D3 small subunit ribosomal RNA gene, partial sequence; internal trans...	Trichoderma kon...	1037	1037	100%	0.0	99.48%	933	ON074847.1
Trichoderma koningiopsis isolate Tc26 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma kon...	1037	1037	100%	0.0	99.48%	576	KP898755.1
Trichoderma sp. isolate N29 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and int...	Trichoderma sp.	1037	1037	100%	0.0	99.48%	1004	OP556606.1
Trichoderma sp. isolate N10 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and int...	Trichoderma sp.	1037	1037	100%	0.0	99.48%	1017	OP556600.1
Hypocreaceae sp. isolate GW20_581 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Hypocreaceae sp.	1037	1037	100%	0.0	99.48%	1113	MZ423777.1

Fig. 2. BLAST analysis of ITS sequences of TRKR-2

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Trichoderma harzianum isolate Thar14 18S ribosomal RNA gene, partial sequence; internal transcribed spacer ...	Trichoderma har...	1005	1005	99%	0.0	99.82%	592	KU317846.1
Trichoderma asperellum isolate Thar2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer ...	Trichoderma asp...	1005	1005	99%	0.0	99.82%	592	KT426894.1
Trichoderma harzianum strain C113N small subunit ribosomal RNA gene, partial sequence; internal transcribed...	Trichoderma har...	1005	1005	99%	0.0	99.82%	621	OP237474.1
Fungal sp. isolate ZX-G-6 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5...	fungal sp.	1005	1005	99%	0.0	99.82%	568	OL376656.1
Trichoderma harzianum clone HC-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed s...	Trichoderma har...	1003	1003	99%	0.0	99.82%	619	MK552405.1
Trichoderma harzianum strain NECC30437 small subunit ribosomal RNA gene, partial sequence; internal trans...	Trichoderma har...	1003	1003	99%	0.0	99.82%	619	MH153638.1
Trichoderma harzianum strain NECC30248 small subunit ribosomal RNA gene, partial sequence; internal trans...	Trichoderma har...	1003	1003	99%	0.0	99.82%	620	MH153617.1
Trichoderma harzianum strain ZG-2-2-1 18S ribosomal RNA gene, partial sequence; internal transcribed space...	Trichoderma har...	1003	1003	99%	0.0	99.82%	620	KT192387.1
Trichoderma harzianum isolate HB11C small subunit ribosomal RNA gene, partial sequence; internal transcribe...	Trichoderma har...	1003	1003	99%	0.0	99.82%	600	MZ569503.1
Fungal sp. isolate ZX-G-5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5...	fungal sp.	1003	1003	99%	0.0	99.82%	567	OL376655.1
Fungal sp. isolate ZX-B-3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 a...	fungal sp.	1003	1003	99%	0.0	99.82%	562	OL376648.1
Hypocrea lixii isolate T94a 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribos...	Trichoderma lixii	1003	1003	99%	0.0	99.82%	594	FJ459968.1
Colletotrichum gloeosporioides isolate T166 18S ribosomal RNA gene, partial sequence; internal transcribed sp...	Colletotrichum gl...	1003	1003	99%	0.0	99.82%	619	FJ459934.1
Trichoderma sp. strain HZ0025 small subunit ribosomal RNA gene, partial sequence; internal transcribed space...	Trichoderma sp.	1002	1002	99%	0.0	99.64%	597	OP269824.1
Trichoderma harzianum isolate FL8 small subunit ribosomal RNA gene, partial sequence; internal transcribed s...	Trichoderma har...	1000	1000	99%	0.0	99.64%	577	MT229305.1
Fungal sp. strain UBDFT12 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...	fungal sp.	1000	1000	99%	0.0	99.63%	620	MK116422.1
Fungal sp. strain UBDFT02 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...	fungal sp.	1000	1000	99%	0.0	99.64%	621	MK116418.1
Trichoderma harzianum strain HNC12-106 small subunit ribosomal RNA gene, partial sequence; internal transc...	Trichoderma har...	1000	1000	99%	0.0	99.63%	581	KX867536.1

Fig. 3. BLAST analysis of ITS sequences of TRPN-3

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Irpex laceratus voucher research collection Farrer lab 267 internal transcribed spacer 1, partial sequence; 5.8...	Irpex laceratus	1033	1033	98%	0.0	98.31%	1153	MN644789.1
Trichoderma effusum voucher research collection Farrer lab 265 internal transcribed spacer 1, partial sequenc...	Trichoderma eff...	1033	1033	98%	0.0	98.31%	1125	MN644616.1
Trichoderma sp. isolate SDAS203144 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma sp.	1033	1033	98%	0.0	98.31%	620	MK870343.1
Trichoderma sp. isolate yi1600_1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a...	Trichoderma sp.	1033	1033	98%	0.0	98.31%	620	MH284171.1
Trichoderma cf. harzianum voucher XZ N201-2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal...	Trichoderma cf. ...	1033	1033	98%	0.0	98.31%	622	MF109004.1
Trichoderma cf. harzianum voucher PDA N181-1 internal transcribed spacer 1, partial sequence; 5.8S ribosom...	Trichoderma cf. ...	1033	1033	98%	0.0	98.31%	615	MF108997.1
Trichoderma cf. harzianum voucher PDA N102-3 internal transcribed spacer 1, partial sequence; 5.8S ribosom...	Trichoderma cf. ...	1033	1033	98%	0.0	98.31%	618	MF108967.1
Trichoderma cf. harzianum voucher PDA N29-2 internal transcribed spacer 1, partial sequence; 5.8S ribosoma...	Trichoderma cf. ...	1033	1033	98%	0.0	98.31%	616	MF108937.1
Trichoderma harzianum isolate CTCCSJ-F-KZ40631 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Trichoderma har...	1033	1033	98%	0.0	98.31%	615	KY750427.1
Trichoderma harzianum isolate FIS21 voucher MBL201621 small subunit ribosomal RNA gene, partial sequen...	Trichoderma har...	1033	1033	98%	0.0	98.31%	647	KY378955.1
Fungal endophyte isolate 4954 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and ...	fungal endophyte	1033	1033	98%	0.0	98.31%	606	KR015919.1
Trichoderma harzianum strain ZG-2-2-1 18S ribosomal RNA gene, partial sequence; internal transcribed spac...	Trichoderma har...	1033	1033	98%	0.0	98.31%	620	KT192387.1
Trichoderma harzianum strain C113N small subunit ribosomal RNA gene, partial sequence; internal transcribe...	Trichoderma har...	1033	1033	98%	0.0	98.31%	621	OP237474.1
Trichoderma sp. 3 DoF17 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribo...	Trichoderma sp. ...	1033	1033	98%	0.0	98.31%	622	JQ388262.1
Trichoderma sp. SQR582 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribo...	Trichoderma sp. ...	1033	1033	98%	0.0	98.31%	621	GQ497169.1
Hypocrea lixii strain CPK 2616 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S...	Trichoderma har...	1033	1033	98%	0.0	98.31%	603	FJ412026.1
Trichoderma harzianum clone HC-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed ...	Trichoderma har...	1031	1031	98%	0.0	98.31%	619	MK552405.1
Trichoderma harzianum strain Cef-B4 small subunit ribosomal RNA gene, partial sequence; internal transcribe...	Trichoderma har...	1031	1031	98%	0.0	98.31%	643	KX960805.1
Trichoderma harzianum strain Edf-12 small subunit ribosomal RNA gene, partial sequence; internal transcribe...	Trichoderma har...	1031	1031	98%	0.0	98.31%	643	KX960797.1

Fig. 4. BLAST analysis of ITS sequences of TRPN-11

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Trichoderma harzianum isolate Thar14 18S ribosomal RNA gene, partial sequence; internal transcribed spacer ...	Trichoderma har...	953	953	89%	0.0	98.70%	592	KU317846.1
Trichoderma asperellum isolate Thar2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer ...	Trichoderma asp...	953	953	89%	0.0	98.70%	592	KT426894.1
Trichoderma harzianum strain C113N small subunit ribosomal RNA gene, partial sequence; internal transcribed...	Trichoderma har...	952	952	89%	0.0	98.69%	621	OP237474.1
Fungal sp. isolate ZX-G-6 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5...	fungal sp.	952	952	89%	0.0	98.69%	568	OL376656.1
Trichoderma harzianum clone HC-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed s...	Trichoderma har...	950	950	89%	0.0	98.69%	619	MK552405.1
Trichoderma harzianum strain NECC30437 small subunit ribosomal RNA gene, partial sequence; internal trans...	Trichoderma har...	950	950	89%	0.0	98.69%	619	MH153638.1
Trichoderma harzianum strain NECC30248 small subunit ribosomal RNA gene, partial sequence; internal trans...	Trichoderma har...	950	950	89%	0.0	98.69%	620	MH153617.1
Trichoderma harzianum strain ZG-2-2-1 18S ribosomal RNA gene, partial sequence; internal transcribed space...	Trichoderma har...	950	950	89%	0.0	98.69%	620	KT192387.1
Trichoderma harzianum isolate HB11C small subunit ribosomal RNA gene, partial sequence; internal transcribe...	Trichoderma har...	950	950	89%	0.0	98.69%	600	MZ569503.1
Fungal sp. isolate ZX-G-5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5...	fungal sp.	950	950	89%	0.0	98.69%	567	OL376655.1
Fungal sp. isolate ZX-B-3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 a...	fungal sp.	950	950	89%	0.0	98.69%	562	OL376648.1
Hypocrea lixii isolate T94a 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribos...	Trichoderma lixii	950	950	89%	0.0	98.69%	594	FJ459968.1
Colletotrichum gloeosporioides isolate T166 18S ribosomal RNA gene, partial sequence; internal transcribed sp...	Colletotrichum gl...	950	950	89%	0.0	98.69%	619	FJ459934.1
Trichoderma harzianum isolate FL8 small subunit ribosomal RNA gene, partial sequence; internal transcribed s...	Trichoderma har...	948	948	89%	0.0	98.51%	577	MT229305.1
Fungal sp. strain UBDFT12 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...	fungal sp.	946	946	89%	0.0	98.51%	620	MK116422.1
Fungal sp. strain UBDFT02 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...	fungal sp.	946	946	89%	0.0	98.51%	621	MK116418.1
Trichoderma harzianum strain HNC12-106 small subunit ribosomal RNA gene, partial sequence; internal transc...	Trichoderma har...	946	946	89%	0.0	98.51%	581	KX867536.1

Fig. 5. BLAST analysis of ITS sequences of TRPN-17

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Uncultured Trichoderma clone O2-17 18S ribosomal RNA gene, partial sequence; internal transcribed spacer ...	uncultured Trich...	307	307	84%	4e-79	98.30%	428	GQ255689.1
Trichoderma sp. isolate ZDT5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	305	305	84%	2e-78	98.29%	641	OQ560491.1
Trichoderma harzianum isolate NIMM800013 internal transcribed spacer 1, partial sequence; 5.8S ribosomal ...	Trichoderma har...	305	305	84%	2e-78	98.29%	567	OQ552744.1
Trichoderma tomentosum isolate 54_ITS1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Trichoderma to...	305	305	84%	2e-78	98.29%	565	OQ519912.1
Trichoderma harzianum isolate 70.LE.3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g...	Trichoderma har...	305	305	84%	2e-78	98.29%	662	OQ456219.1
Trichoderma harzianum isolate 45.SH.2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g...	Trichoderma har...	305	305	84%	2e-78	98.29%	604	OQ456218.1
Trichoderma harzianum isolate IKT-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed...	Trichoderma har...	305	305	84%	2e-78	98.29%	624	OQ398549.1
Trichoderma atrobrunneum isolate UMBmyc4-2018BPs internal transcribed spacer 1, partial sequence; 5.8S ri...	Trichoderma atr...	305	305	84%	2e-78	98.29%	579	OQ378927.1
Trichoderma harzianum strain KACC410239 small subunit ribosomal RNA gene, partial sequence; internal tran...	Trichoderma har...	305	305	84%	2e-78	98.29%	627	OQ271299.1
Trichoderma lixii isolate SST5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma lixii	305	305	84%	2e-78	98.29%	592	OQ244432.1
Trichoderma sp. strain YN-1-2-L internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene an...	Trichoderma sp.	305	305	84%	2e-78	98.29%	566	OQ195117.1
Trichoderma simmonsii strain TAA11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gen...	Trichoderma sim...	305	305	84%	2e-78	98.29%	601	OQ161062.1
Trichoderma simmonsii strain TAA9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	305	305	84%	2e-78	98.29%	583	OQ161060.1
Trichoderma simmonsii strain TAA5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	305	305	84%	2e-78	98.29%	594	OQ161056.1
Trichoderma simmonsii strain TAA4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	305	305	84%	2e-78	98.29%	592	OQ161055.1
Trichoderma simmonsii strain TAA2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	305	305	84%	2e-78	98.29%	592	OQ161053.1
Trichoderma sp. isolate ZDG6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	305	305	84%	2e-78	98.29%	627	OP984873.1
Trichoderma sp. strain Tlix01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and in...	Trichoderma sp.	305	305	84%	2e-78	98.29%	576	OP970989.1
Trichoderma sp. isolate ZDG1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	305	305	84%	2e-78	98.29%	625	OP872734.1

Fig. 6. BLAST analysis of TvP sequences of TRMW-2

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Trichoderma sp. FLS-2010 isolate FS-3.9(2) 18S ribosomal RNA gene, partial sequence; internal transcribed s...	Trichoderma sp. ...	318	318	92%	2e-82	99.43%	483	HQ023045.1
Trichoderma sp. isolate ZDT5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	313	313	92%	9e-81	98.86%	641	OQ560491.1
Trichoderma harzianum isolate NIMM800013 internal transcribed spacer 1, partial sequence; 5.8S ribosomal ...	Trichoderma har...	313	313	92%	9e-81	98.86%	567	OQ552744.1
Trichoderma tomentosum isolate 54_ITS1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Trichoderma to...	313	313	92%	9e-81	98.86%	565	OQ519912.1
Trichoderma harzianum isolate 70.LE.3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g...	Trichoderma har...	313	313	92%	9e-81	98.86%	662	OQ456219.1
Trichoderma harzianum isolate 45.SH.2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g...	Trichoderma har...	313	313	92%	9e-81	98.86%	604	OQ456218.1
Trichoderma harzianum isolate IKT-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed...	Trichoderma har...	313	313	92%	9e-81	98.86%	624	OQ398549.1
Trichoderma atrobrunneum isolate UMBmyc4-2018BPs internal transcribed spacer 1, partial sequence; 5.8S ri...	Trichoderma atr...	313	313	92%	9e-81	98.86%	579	OQ378927.1
Trichoderma harzianum strain KACC410239 small subunit ribosomal RNA gene, partial sequence; internal tran...	Trichoderma har...	313	313	92%	9e-81	98.86%	627	OQ271299.1
Trichoderma lixii isolate SST5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma lixii	313	313	92%	9e-81	98.86%	592	OQ244432.1
Trichoderma sp. strain YN-1-2-L internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene an...	Trichoderma sp.	313	313	92%	9e-81	98.86%	566	OQ195117.1
Trichoderma simmonsii strain TAA11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gen...	Trichoderma sim...	313	313	92%	9e-81	98.86%	601	OQ161062.1
Trichoderma simmonsii strain TAA9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	313	313	92%	9e-81	98.86%	583	OQ161060.1
Trichoderma simmonsii strain TAA5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	313	313	92%	9e-81	98.86%	594	OQ161056.1
Trichoderma simmonsii strain TAA4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	313	313	92%	9e-81	98.86%	592	OQ161055.1
Trichoderma simmonsii strain TAA2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	313	313	92%	9e-81	98.86%	592	OQ161053.1
Trichoderma sp. isolate ZDG6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	313	313	92%	9e-81	98.86%	627	OP984873.1
Trichoderma sp. strain Tlix01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and in...	Trichoderma sp.	313	313	92%	9e-81	98.86%	576	OP970989.1
Trichoderma sp. isolate ZDG1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	313	313	92%	9e-81	98.86%	625	OP872734.1

Fig. 7. BLAST analysis of TvP sequences of TRKR-2

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Trichoderma sp. isolate ZDT5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	429	429	100%	1e-115	99.16%	641	OQ560491.1
Trichoderma harzianum isolate NIMM800013 internal transcribed spacer 1, partial sequence; 5.8S ribosomal ...	Trichoderma har...	429	429	100%	1e-115	99.16%	567	OQ552744.1
Trichoderma tomentosum isolate 54_ITS1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Trichoderma to...	429	429	100%	1e-115	99.16%	565	OQ519912.1
Trichoderma harzianum isolate 70.LE.3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g...	Trichoderma har...	429	429	100%	1e-115	99.16%	662	OQ456219.1
Trichoderma harzianum isolate 45.SH.2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g...	Trichoderma har...	429	429	100%	1e-115	99.16%	604	OQ456218.1
Trichoderma harzianum isolate IKT-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed...	Trichoderma har...	429	429	100%	1e-115	99.16%	624	OQ398549.1
Trichoderma atrobrunneum isolate UMBmyc4-2018BPs internal transcribed spacer 1, partial sequence; 5.8S ri...	Trichoderma atr...	429	429	100%	1e-115	99.16%	579	OQ378927.1
Trichoderma harzianum strain KACC410239 small subunit ribosomal RNA gene, partial sequence; internal tran...	Trichoderma har...	429	429	100%	1e-115	99.16%	627	OQ271299.1
Trichoderma lixii isolate SST5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma lixii	429	429	100%	1e-115	99.16%	592	OQ244432.1
Trichoderma sp. strain YN-1-2-L internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene an...	Trichoderma sp.	429	429	100%	1e-115	99.16%	566	OQ195117.1
Trichoderma simmonsii strain TAA11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gen...	Trichoderma sim...	429	429	100%	1e-115	99.16%	601	OQ161062.1
Trichoderma simmonsii strain TAA9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	429	429	100%	1e-115	99.16%	583	OQ161060.1
Trichoderma simmonsii strain TAA5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	429	429	100%	1e-115	99.16%	594	OQ161056.1
Trichoderma simmonsii strain TAA4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	429	429	100%	1e-115	99.16%	592	OQ161055.1
Trichoderma simmonsii strain TAA2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	429	429	100%	1e-115	99.16%	592	OQ161053.1
Trichoderma sp. isolate ZDG6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	429	429	100%	1e-115	99.16%	627	OP984873.1
Trichoderma sp. strain Tlix01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and in...	Trichoderma sp.	429	429	100%	1e-115	99.16%	576	OP970989.1
Trichoderma sp. isolate ZDG1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	429	429	100%	1e-115	99.16%	625	OP872734.1
Trichoderma harzianum isolate MM53 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma har...	429	429	100%	1e-115	99.16%	567	OP809564.1

Fig. 8. BLAST analysis of TvP sequences of TRPN-3

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Trichoderma sp. isolate SDAS204036 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma sp.	448	448	97%	3e-121	99.19%	590	MK870500.1
Trichoderma harzianum isolate CTCCSJ-F-KZ23376 18S ribosomal RNA gene, partial sequence; and internal ...	Trichoderma har...	448	448	97%	3e-121	99.19%	553	MF408593.1
Trichoderma sp. isolate yi0158_1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene an...	Trichoderma sp.	448	448	97%	3e-121	99.19%	590	MH284352.1
Hypocrea sp. TWP1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal...	Trichoderma sp. ...	448	448	97%	3e-121	99.19%	566	GQ426041.1
Trichoderma sp. AH-Group-1 isolate 21/LC/June2004 18S ribosomal RNA gene, partial sequence; internal tran...	Trichoderma sp. ...	444	444	97%	4e-120	98.79%	554	DQ993557.1
Trichoderma sp. isolate ZDT5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	442	442	97%	1e-119	98.79%	641	OQ560491.1
Trichoderma harzianum isolate NIMM800013 internal transcribed spacer 1, partial sequence; 5.8S ribosomal ...	Trichoderma har...	442	442	97%	1e-119	98.79%	567	OQ552744.1
Trichoderma tomentosum isolate 54_ITS1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Trichoderma to...	442	442	97%	1e-119	98.79%	565	OQ519912.1
Trichoderma harzianum isolate 70.LE.3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g...	Trichoderma har...	442	442	97%	1e-119	98.79%	662	OQ456219.1
Trichoderma harzianum isolate 45.SH.2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g...	Trichoderma har...	442	442	97%	1e-119	98.79%	604	OQ456218.1
Trichoderma harzianum isolate IKT-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed...	Trichoderma har...	442	442	97%	1e-119	98.79%	624	OQ398549.1
Trichoderma atrobrunneum isolate UMBmyc4-2018BPs internal transcribed spacer 1, partial sequence; 5.8S ri...	Trichoderma atr...	442	442	97%	1e-119	98.79%	579	OQ378927.1
Trichoderma harzianum strain KACC410239 small subunit ribosomal RNA gene, partial sequence; internal tran...	Trichoderma har...	442	442	97%	1e-119	98.79%	627	OQ271299.1
Trichoderma lixii isolate SST5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma lixii	442	442	97%	1e-119	98.79%	592	OQ244432.1
Trichoderma sp. strain YN-1-2-L internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene an...	Trichoderma sp.	442	442	97%	1e-119	98.79%	566	OQ195117.1
Trichoderma simmonsii strain TAA11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gen...	Trichoderma sim...	442	442	97%	1e-119	98.79%	601	OQ161062.1
Trichoderma simmonsii strain TAA9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	442	442	97%	1e-119	98.79%	583	OQ161060.1
Trichoderma simmonsii strain TAA5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	442	442	97%	1e-119	98.79%	594	OQ161056.1
Trichoderma simmonsii strain TAA4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	442	442	97%	1e-119	98.79%	592	OQ161055.1

Fig. 9. BLAST analysis of TvP sequences of TRPN 11

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Trichoderma sp. isolate ZDT5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	351	351	97%	2e-92	99.48%	641	OQ560491.1
Trichoderma harzianum isolate NIMM800013 internal transcribed spacer 1, partial sequence; 5.8S ribosomal ...	Trichoderma har...	351	351	97%	2e-92	99.48%	567	OQ552744.1
Trichoderma tomentosum isolate 54_ITS1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Trichoderma to...	351	351	97%	2e-92	99.48%	565	OQ519912.1
Trichoderma harzianum isolate 70.LE.3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g...	Trichoderma har...	351	351	97%	2e-92	99.48%	662	OQ456219.1
Trichoderma harzianum isolate 45.SH.2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g...	Trichoderma har...	351	351	97%	2e-92	99.48%	604	OQ456218.1
Trichoderma harzianum isolate IKT-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed...	Trichoderma har...	351	351	97%	2e-92	99.48%	624	OQ398549.1
Trichoderma atrobrunneum isolate UMBmyc4-2018BPs internal transcribed spacer 1, partial sequence; 5.8S ri...	Trichoderma atr...	351	351	97%	2e-92	99.48%	579	OQ378927.1
Trichoderma harzianum strain KACC410239 small subunit ribosomal RNA gene, partial sequence; internal tran...	Trichoderma har...	351	351	97%	2e-92	99.48%	627	OQ271299.1
Trichoderma lixii isolate SST5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma lixii	351	351	97%	2e-92	99.48%	592	OQ244432.1
Trichoderma sp. strain YN-1-2-L internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene an...	Trichoderma sp.	351	351	97%	2e-92	99.48%	566	OQ195117.1
Trichoderma simmonsii strain TAA11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gen...	Trichoderma sim...	351	351	97%	2e-92	99.48%	601	OQ161062.1
Trichoderma simmonsii strain TAA9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	351	351	97%	2e-92	99.48%	583	OQ161060.1
Trichoderma simmonsii strain TAA5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	351	351	97%	2e-92	99.48%	594	OQ161056.1
Trichoderma simmonsii strain TAA4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	351	351	97%	2e-92	99.48%	592	OQ161055.1
Trichoderma simmonsii strain TAA2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene ...	Trichoderma sim...	351	351	97%	2e-92	99.48%	592	OQ161053.1
Trichoderma sp. isolate ZDG6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	351	351	97%	2e-92	99.48%	627	OP984873.1
Trichoderma sp. strain Tlix01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and in...	Trichoderma sp.	351	351	97%	2e-92	99.48%	576	OP970989.1
Trichoderma sp. isolate ZDG1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Trichoderma sp.	351	351	97%	2e-92	99.48%	625	OP872734.1
Trichoderma harzianum isolate MM53 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma har...	351	351	97%	2e-92	99.48%	567	OP809564.1

Fig. 10. BLAST analysis of TvP sequences of TRPN 17

4.2.5. Phylogenetic tree

A phylogenetic tree was constructed using Neighbor Joining Tree (NJT) method by taking similar sequences of the *Trichoderma* isolates from the NCBI BLAST for both ITS and TvP primer sequences with bootstrap 1000 using MEGA 11 software. The phylogenetic analysis of ITS grouped *Trichoderma* into two clusters (Fig. 11). Cluster one included TRPN-3, TRPN-11 and TRPN-17 with other referral sequences while cluster two included the isolates TRMW-2 and TRKR-2. The phylogenetic tree of TvP analysis grouped the isolates of *Trichoderma* (Fig. 12) into two clusters with TRPN-3 and TRPN-17 in one cluster and another cluster with the isolates TRMW-2, TRKR-2 and TRPN-11 with other referral sequences.

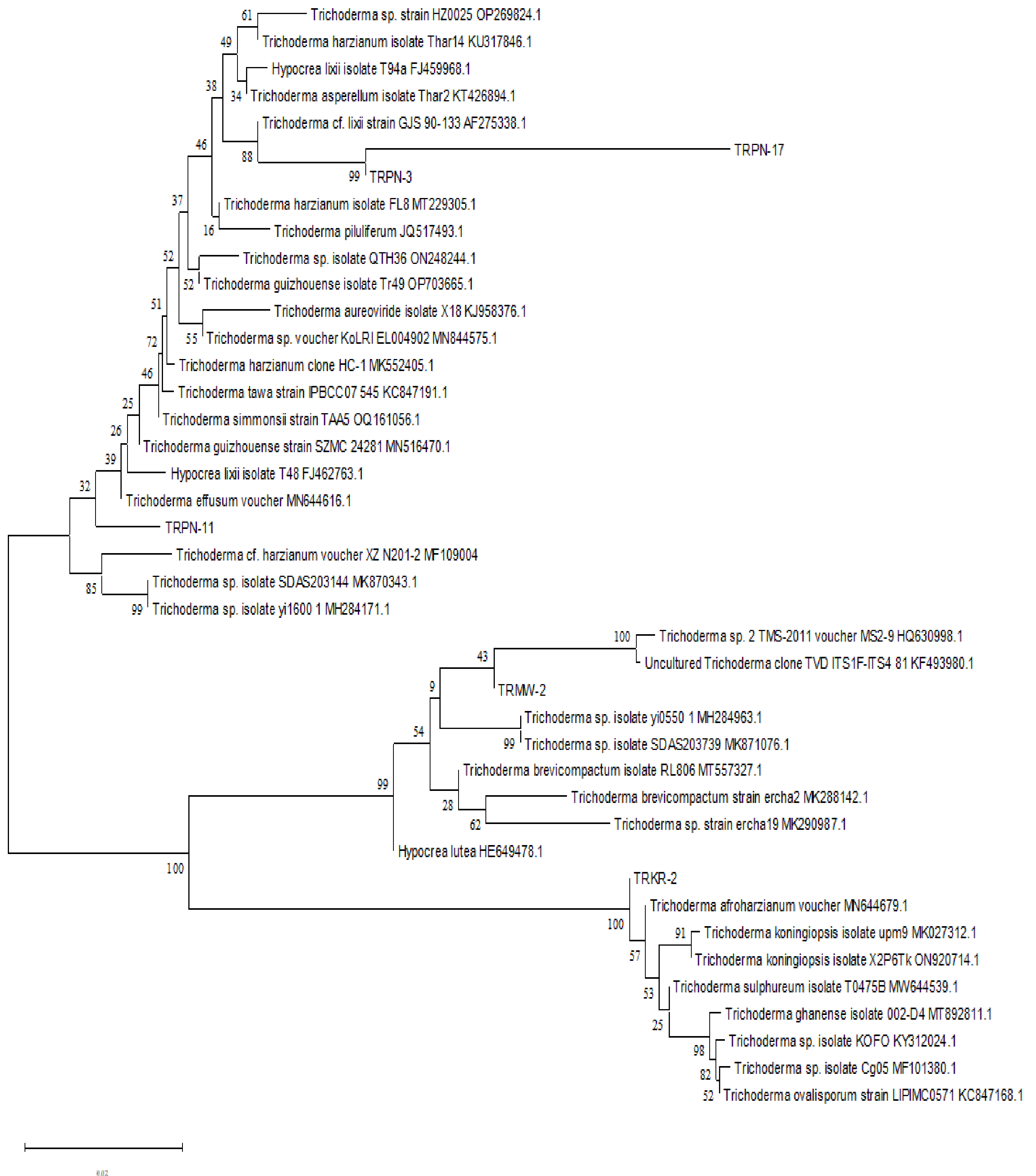


Fig. 11. Phylogenetic tree generated from ITS-rDNA sequences of *Trichoderma* isolates by Neighbor-Joining Tree (NJT) using MEGA 11

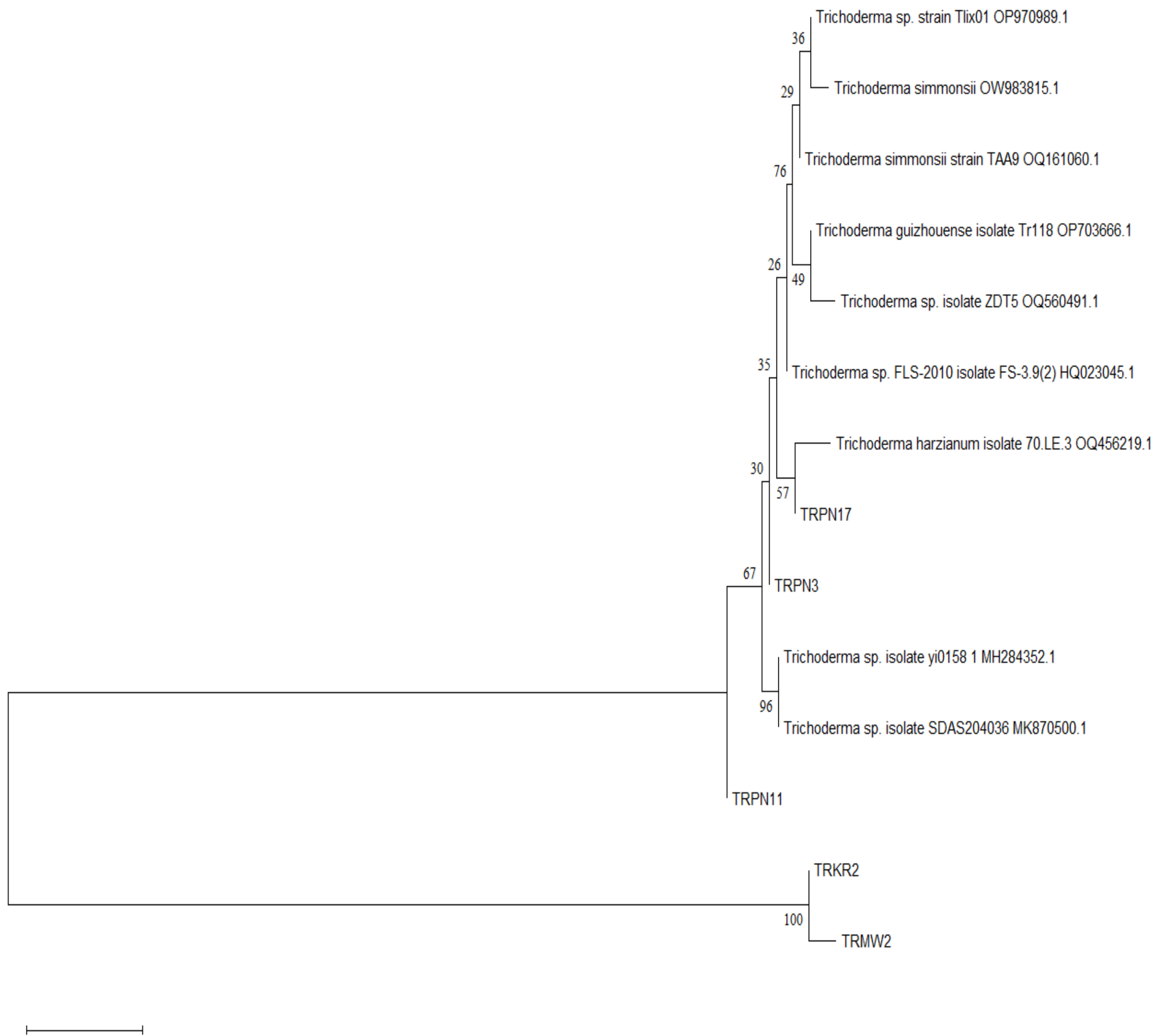


Fig. 12. Phylogenetic tree generated from TvP-rDNA sequences of *Trichoderma* isolates by Neighbor-Joining Tree (NJT) using MEGA 11

Table 8. Molecular characterization of isolates of *Trichoderma* spp. by ITS-PCR

Sl. No.	Isolate	Best match in Genbank data base	Per cent similarity	Accession number
1	TRMW-2	*Trichoderma brevicompactum* isolate RL806 *Trichoderma* sp. isolate SDAS203739 *Trichoderma deliquescens* (*Hypocrea lutea*)	99.42%	MT557327.1 MK871076.1 HE649478.1
2	TRKR- 2	*Trichoderma koningiopsis* isolate X2P6Tk *Trichoderma ovalisporum* strain LIPIMC0571	99.65%	ON920714.1 KC847168.1
3	TRPN- 3	*Trichoderma harzianum* clone HC-1 *Trichoderma asperellum* isolate Thar2 *Hypocrea lixii* isolate T94a	99.82%	MK552405.1 KT426894.1 FJ459968.1
4	TRPN- 11	*Trichoderma effusum* voucher *Trichoderma* sp. isolate SDAS203144 *Hypocrea lixii* strain CPK 2616	98.31%	MN644616.1 MK870343.1 FJ412026.1
5	TRPN- 17	*Trichoderma harzianum* isolate Thar14 *Trichoderma asperellum* isolate Thar2	98.70%	KU317846.1 KT426894.1

4.3. *IN VIVO* TESTING OF THE EFFICACY OF SELECTED ISOLATES OF *TRICHODERMA* SPP. AGAINST FUSARIUM WILT OF VEGETABLE COWPEA

Based on the enzyme activity, *in vitro* efficacy and the antagonistic characters three isolates *viz.,* TRKR-2, TRPN-3, TRPN-17 and their consortia were used for the *in vivo* evaluation against the Fusarium wilt of vegetable cowpea under both pot culture and field conditions.

4.3.1. Pot culture studies

A pot culture study was conducted with eight treatments and three replications against Fusarium wilt of vegetable cowpea in Completely Randomized Design (CRD) using variety Githika. Observations such as the number of plants infected, days for flowering, number of pods per plant, length of the pod, number of seeds per pod and yield are represented in Table 9.

4.3.1.1. Disease incidence

It was observed that all the treatments had a prominent effect on the reduction in wilt incidence compared to control pots. The application of talc-based formulation of the isolates TRKR-2, TRPN-3, consortium of TRPN-3 and TRPN-17 and KAU strain as seed treatment @ of 20g kg^{-1} of seed followed by soil drenching @ 2 per cent at 20, 40 and 60 DAS along with the fungicide treatment completely controlled the wilt incidence and the results were found to be on par. This was followed by the treatments involving TRPN-17 alone (25 %) and a consortium of isolates TRKR-2 and TRPN-17 (8.33 %).

4.3.1.2. Days for flowering

Early flowering was observed when the plants were treated with the isolate TRPN-3 (31 days) which was significantly different from all other treatments. This was followed by the plants treated with the isolate TRMW-2 (33.33 days), fungicide-treated plants (33.33 days), the consortium treatment of the isolates TRKR-2 and TRPN-17 (33.67 days), the consortium treatment of the isolates TRPN-3 and TRPN-17 (33.67 days) and KAU strain (33.67 days) with no notable difference.

4.3.1.3. Number of pods per plant

The plants treated with the isolate TRPN-3 produced the highest number of pods per plant (19.00) which was significantly different from all the other treatments. This was followed by the fungicide treatment (15.00). Compared to control, significantly higher number of pods was obtained on treatments with isolate TRKR-2 (12.33), KAU strain (12.00), consortia of TRKR-2 and TRPN-17 (9.67), and TRPN-3 and TRPN-17 (11.33).

4.3.1.4. Pod length

All the treatments resulted in significant effect on pod length. The length of the pods produced by the plants treated with the consortium of the isolates TRKR-2 and TRPN-17 (43.85 cm),TRPN-3 (43.61 cm),TRKR-2 (43.55 cm), a consortium of the isolates TRPN-3 and TRPN-17 (43.35 cm), TRPN-17 (42.37 cm), fungicide treated plants (40.69 cm) and KAU strain (40.13 cm) were found to be on par.

4.3.1.5. Number of seeds per pod

There was no significant difference in the number of seeds per pod between the different treatments.

4.3.1.6. Yield

With regards to yield, the plants treated with TRPN-3 brought about the highest yield of 369.57 g plant^{-1} followed by consortium of TRPN-3 and TRPN-17 (344.52 g plant^{-1}) which were on par. The carbendazim-treated plants produced 310.99 g plant^{-1} of pod yield. This was followed by treatments with isolates TRKR-2 (261.50 g plant^{-1}), KAU strain (246.79 g plant^{-1}), consortium of TRKR-2 and TRPN-17 (218.27 g plant^{-1}), and TRPN-17 alone (186.35 g plant^{-1}) which were all significantly different from control.

Table 9. Efficacy of *Trichoderma* isolates against Fusarium wilt of vegetable cowpea in pot culture

Sl. No.	Treatment	DI (%)**	Days for flowering*	No. of pods / plant*	Pod length (cm)*	No. of seeds/ pod*	Yield (g/plant)*
1	T1- (TRKR-2)	0.00 (4.05)[c]	33.33 ± 0.58[b]	12.33 ± 1.53[c]	43.55 ± 4.18[a]	20.57 ± 1.74	261.60 ± 42.35[bc]
2	T2- (TRPN-17)	25.00(4.92)[b]	33.00 ± 1.00[b]	11.33 ± 1.53[cd]	42.37 ± 0.60[a]	20.39 ± 0.18	186.35 ± 21.69[d]
3	T3- (TRPN-3)	0.00 (4.05)[c]	31.33 ± 1.16[c]	19.00 ± 1.00[a]	43.61 ± 2.23[a]	21.05 ± 0.66	369.57 ± 21.53[a]
4	T4- (TRKR-2 + TRPN-17)	8.33 (4.35)[bc]	33.67 ± 0.58[b]	9.67 ± 1.53[d]	43.85 ± 2.59[a]	20.88 ± 1.13	218.27 ± 15.47[cd]
5	T5- (TRPN-3 + TRPN-17)	0.00 (4.05)[c]	33.67 ± 0.58[b]	11.33 ± 1.16[cd]	43.35 ± 1.58[a]	20.36 ± 1.01	344.52 ± 41.39[a]
6	T6- (KAU)	0.00 (4.05)[c]	33.67 ± 0.58[b]	12.00 ± 1.00[c]	40.13 ± 1.91[a]	20.37 ± 1.30	246.79 ± 33.28[bcd]
7	T7- (Carbendazim)	0.00 (4.05)[c]	33.33 ± 0.58[b]	15.33 ± 1.16[b]	40.69 ± 4.59[a]	19.71 ± 1.79	310.99 ± 75.80[ab]
8	T8- (Control)	100(7.03)[a]	35.33 ± 0.58[a]	5.67 ± 1.53[e]	35.29 ± 0.78[b]	18.20 ± 0.51	105.57 ± 42.83[e]
	SE (m)	0.204	0.43	0.76	1.54	0.67	23.6
	CD (0.05)	0.61	1.27	2.29	4.63	NS	16.00

*Mean ± SD of three replication; **Values in the parenthesis are arcsine transformed; NS = Not significant; In a column, means followed by common letters are not significantly different from each other.

4.3.1.7. Population of *Trichoderma* spp.in soil from different treatment of pot culture experiment

The population of *Trichoderma* isolates were recorded at 30, 60 and 90 DAS. It was found that in all the treatments at 30, 60 and 90 DAS, the colony count of *Trichoderma* spp. was significantly higher than that of fungicide treatment and control. Table 10 depicts that initially at 30 DAS the consortium of TRPN-3 and TRPN-17 exhibited 8.33 x 10^4 cfu g^{-1} followed by TRPN-3 (6.67 x 10^4 cfu g^{-1}) and TRKR-2 (6.33 x 10^4 cfu g^{-1}). At 60 DAS, population of *Trichoderma* sp. was found to be highest in case of treatment with isolate TRPN 3 (11.67 ×10^4 cfu g^{-1}) followed by KAU strain (9.67 x 10^4 cfu g^{-1}) and the consortium of the isolates TRPN-3 and TRPN-17 (9.00 x 10^4 cfu g^{-1}). The treatment with isolate TRPN-3 (7.00 ×10^4 cfu g^{-1}) showed the highest population of *Trichoderma* sp. even at 90 DAS. It was followed by consortia treatment of TRKR-2 and TRPN-17 (4.33 x 10^4 cfu g^{-1}) and isolates of TRPN-3 and TRPN-17 (4.00 x 10^4 cfu g^{-1}). At 30, 60 and 90 DAS the population of *Trichoderma* sp. was not recovered from the soil of fungicide-treated plants whereas only at 90 DAS the control revealed a population of 0.33 x 10^4 cfu g^{-1}.

Table 10. Population of *Trichoderma* spp. in soil from different treatments of the pot culture experiment

Sl. No.	Treatment	Population in cfu g^{-1} at 10^{-4} dilution*		
		30 DAS	**60 DAS**	**90 DAS**
1	T1- (TRKR-2)	6.33 (0.83)^{ab}	8.33 (0.94)^{abc}	3.33 (0.57)^{bc}
2	T2- (TRPN-17)	3.67 (0.58)^{b}	6.33 (0.83)^{bc}	1.33 (0.14)^{cd}
3	T3- (TRPN-3)	6.67 (0.85)^{ab}	11.67 (1.08)^{a}	7.00 (0.86)^{a}
4	T4- (TRKR-2 + TRPN-17)	5.00 (0.64)^{ab}	6.33 (0.81)^{c}	4.33 (0.67)^{ab}
5	T5- (TRPN-3 + TRPN-17)	8.33 (0.93)^{a}	9.00 (0.97)^{abc}	4.00 (0.61)^{bc}
6	T6- (KAU)	6.00 (0.78)^{ab}	9.67 (0.99)^{ab}	3.67 (0.60)^{bc}
7	T7- (Carbendazim)	0.00 (-0.30)^{c}	0.00 (-0.30)^{d}	0.00 (-0.30)^{d}
8	T8- (Control)	0.00 (-0.30)^{c}	0.00 (-0.30)^{d}	0.33 (-0.14)^{d}
	SE(m)	1.26	1.18	0.91
	CD (0.05)	0.33	0.30	0.38

Mean value of three replication; *Values in parentheses are log transformed; In a column, means followed by common letters are not significantly different from each other.

4.3.2. Field evaluation of *Trichoderma* isolates against Fusarium wilt of vegetable cowpea

Field evaluation was conducted in a sick plot to determine the efficacy of *Trichoderma* isolates against Fusarium wilt of vegetable cowpea with eight treatments (Plate 24) and three replication laid out in Randomized Block Design (RBD) and the variety used was Githika. Observations such as the number of plants infected, days for flowering, number of pods per plant, length of the pod, number of seeds per pod and yield arerepresented in Table 11.

4.3.2.1. Disease incidence

Application of talc-based formulations of the isolates TRKR-2, TRPN-3, KAU strain and consortium of TRKR-2 and TRPN-17 as seed treatment @ of 20g kg^{-1} of seed followed by soil drenching @ 2 per cent at 20, 40 and 60 DAS effectively controlled the incidence of Fusarium wilt of vegetable cowpea in field conditions and was found to be statistically on par with the consortium treatment of the isolates TRPN-3 and TRPN-17 (11.11 %). This was followed by treatment with isolate TRPN-17 alone (44.44 %). Thus, a notable reduction in the disease incidence was recorded by all the treated plants compared to control plants.

4.3.2.2. Days for flowering

Treatment of the plants with the isolate TRPN-3 induced early flowering (31.00 days). It was followed by the fungicide treatment with carbendazim (32.33 days). Plants treated with the isolates TRKR-2, a consortium of TRKR-2 and TRPN-17, a consortium of TRPN-3 and TRPN-17 and KAU strain were found to on par (33 days).

4.3.2.3. Number of pods per plant

All the treatments resulted in significantly higher number of pods per plant compared to control.The highest number of pods per plant was produced by the plants treated with the isolate TRPN-3 (19) followed by fungicide treatment with carbendazim (14.22). The treatment with a consortium of TRPN-3 and TRPN-17 (10.11) and plants

Plate 24. Field evaluation of selected *Trichoderma* isolates against Fusarium wilt of vegetable cowpea in sick plot

treated with KAU strain (11.11) was found to be on par but significantly different from control. There was no significant difference in number of pods in the treatments with isolate TRKR-2, TRPN-17 and consortium of TRKR-2 and TRPN-17.

4.3.2.4. Pod length

A consortium of TRPN-3 and TRPN-17 produced the lengthy pods of 48.16 cm and was found to be on par with the treatment TRPN-3 (47.53 cm). It was followed by the isolates TRKR-2 (46.19 cm), the consortium of TRKR-2 and TRPN-17 (45.61 cm), TRPN-17 (44.58 cm) and KAU strain (43.45 cm)

4.3.2.5. Number of seeds per pod

Application of consortium of the isolates TRPN-3 and TRPN-17 registered the highest number of seeds per pod of 21.73 followed by the fungicide treatment with carbendazim (21.34). The treatment with the isolates TRPN-3 (20.85), KAU strain (20.67), and the consortium of TRKR-2 and TRPN-17 (20.16) were also found to be significantly different from the control treatment.

4.3.2.6. Yield

The highest yield of 1172.63 g per plant was in plants treated with the isolate TRPN-3 followed by fungicide treatment with carbendazim (929.06 g plant^{-1}). Application of a consortium of the isolates TRPN-3 and TRPN-17 (658.88 g plant^{-1}) was found to be on par with the plants treated with the KAU strain (653.08 g plant^{-1}). Treatment with consortium of TRKR-2 and TRPN-17 (358.94 g plant^{-1}), TRKR-2 alone (352.53 g plant^{-1}) and TRPN-17 (301.44 g plant^{-1}) although yielded higher than control plants (260.98 g plant^{-1}) were found not found to be significant.

Table 11. Efficacy of *Trichoderma* isolates against Fusarium wilt of vegetable cowpea under field conditions

Sl. No.	Treatment	DI (%)	Days for flowering	No. of pods/ plant	Pod length (cm)	No. of seeds/ pod	Yield / plant (g)
1	T1- (TRKR-2)	0.00 (4.05)[c]	33.00±0.00[c]	6.11±1.39[d]	46.19±0.41[abc]	19.79±0.63[c]	352.53±108.71[d]
2	T2- (TRPN-17)	44.44(5.54)[b]	34.00±0.00[b]	5.89±0.70[d]	44.58±2.26[bc]	20.28±0.74[bc]	301.44±29.90[d]
3	T3- (TRPN-3)	0.00 (4.05)[c]	31.00±0.00[e]	19.00±0.88[a]	47.53±0.52[ab]	20.85±0.60[abc]	1172.63±59.86[a]
4	T4- (TRKR-2 + TRPN-17)	0.00 (4.05)[c]	33.00±0.00[c]	6.11±1.71[d]	45.61±2.17[abc]	20.16±0.61[c]	358.94±76.63[d]
5	T5- (TRPN-3 + TRPN-17)	11.11(4.44)[c]	33.00±0.00[c]	10.11±0.84[c]	48.16±3.26[ab]	21.73±0.94[a]	658.88±45.84[c]
6	T6- (KAU strain)	0.00 (4.05)[c]	33.00±0.00[c]	11.11±0.51[c]	43.45±2.33[cd]	20.67±0.37[abc]	653.08±33.20[c]
7	T7- (Carbendazim)	0.00 (4.05)[c]	32.33±0.57[d]	14.22±1.35[b]	48.90±1.37[a]	21.34±0.66[ab]	929.06±38.20[b]
8	T8- (Control)	88.88(6.70)[a]	34.67±0.57[a]	4.56±0.51[d]	39.78±3.20[d]	18.51±1.17[d]	260.98±24.75[d]
	SE(m)	0.21	0.17	0.63	1.24	0.35	33.65
	CD (0.05)	0.65	0.54	1.92	3.77	1.07	102.09

Mean value of three replication; *Values in parentheses are log-transformed; In a column, means followed by common letters are not significantly different from each other

4.3.2.7. Population of *Trichoderma* spp. in soil from different treatment of field experiment

The population of *Trichoderma* isolates were recorded at 30, 60 and 90 DAS. It was found that in all the treatments at 30, 60 and 90 DAS, the colony count of *Trichoderma* spp. was significantly higher than that of fungicide treatment and control. Table 12. shows that at 30 DAS the highest population of *Trichoderma* spp. was recorded by the consortium treatment of isolates TRPN-3 and TRPN-17 (9 x 10^4 cfu g^{-1}) followed by TRPN-3 (7.67 x 10^4 cfu g^{-1}) and KAU strain (6.67 x 10^4 cfu g^{-1}). The treatment with consortium of isolates TRPN-3 and TRPN-17 (10.67 x 10^4 cfu g^{-1}) was found to be highest at 60 DAS. It was followed by the isolates TRPN-3 (9x 10^4 cfu g^{-1}), KAU strain (8.67x 10^4 cfu g^{-1}) and isolate TRKR-2 (7.33 x 10^4 cfu g^{-1}). At 90 DAS the treatment with the isolate TRPN-3 exhibited the highest population of 4.33 x 10^4 cfu g^{-1} followed by KAU strain (4 x 10^4 cfu g^{-1}) and the consortium of TRPN-3 and TRPN-17 (3.67 x 10^4 cfu g^{-1}).Only during the initial period of 30 DAS *Trichoderma* sp was found to exist in the control (0.33 x 10^4 cfu g^{-1}).

Table 12. Population of *Trichoderma* spp. in soil from different treatments of the field experiment

Sl. No.	Treatment	Population in cfu g^{-1} at 10^{-4} dilution		
		30 DAS	60 DAS	90 DAS
1	T1- (TRKR-2)	5.00 (0.72)^{b}	7.33 (0.88)^{ab}	1.67 (0.30)^{abc}
2	T2- (TRPN-17)	5.00 (0.74)^{b}	6.33 (0.82)^{b}	1.33 (0.14)^{bc}
3	T3- (TRPN-3)	7.67 (0.91)^{ab}	9.0 (0.97)^{ab}	4.33 (0.64)^{a}
4	T4- (TRKR-2 + TRPN-17)	5.33 (0.71)^{b}	6.67 (0.84)^{b}	2.00 (0.26)^{abc}
5	T5- (TRPN-3 + TRPN-17)	9.00 (0.97)^{a}	10.67 (1.04)^{a}	3.67 (0.60)^{ab}
6	T6- (KAU strain)	6.67 (0.83)^{ab}	8.67 (0.95)^{ab}	4.00 (0.62)^{ab}
7	T7- (Carbendazim)	0.00 (-0.30)^{c}	0.00 (-0.30)^{c}	0.33 (-0.14)^{c}
8	T8- (Control)	0.33 (-0.14)^{c}	0.00 (-0.30)^{c}	0.00 (-0.30)^{c}
	SE(m)	1.18	1.15	0.90
	CD (0.05)	0.31	0.18	0.52

Mean value of three replication; *Values in parentheses are log transformed; In a column, means followed by common letters are not significantly different from each other

Discussion

5. DISCUSSION

Management of Fusarium wilt is primarily dependent on the use of fungicides. The usage of such chemicals has a detrimental effect on human health and other living beings (Carvalho 2017; Kohl *et al*. 2019). In addition, the high expense of developing, producing, and registering new synthetic pesticides as well as the strong development of pathogen resistance has sparked interest in developing alternative disease management strategies (Matson *et al*. 2015; Bedine *et al.* 2022). The amendment of the soil with biocontrol agents is an alternative to manage soil-borne plant pathogens through parasitism, the synthesis of antagonistic compounds, competition for hosts and the development of resistance in plants against the phytopathogens. The mycoparasitic nature and the ability to secrete a huge quantity of fungi-toxic enzymes make *Trichoderma* an effective biocontrol agent against several foliar and soil-borne plant diseases (Shafique *et al.* 2016; Panth *et al.* 2020).

5.1. ASSESSMENT OF THE BIOCONTROL ATTRIBUTES OF *TRICHODERMA* ISOLATES.

To effectively lyse the cell wall of pathogens, antagonists secrete enzymes that hydrolyse the chitin, glucan, lipids and proteins that make up the skeleton of fungal cells (Khatri *et al*., 2017). Moreover, *Trichoderma* spp. suppress hyphal growth of the fungal pathogens by antibiosis by releasing antimicrobial chemicals in the culture (Gajera *et al*. 2020).

In the present study, the cell wall degrading enzymes *viz.,* chitinase, protease and lipase were evaluated and found that the chitinase enzyme activity of the isolate TRPN-17 was observed to be the highest (88.39 U ml^{-1}) followed by the isolates TRPN-3 (76.75 U ml^{-1}) and TRKR-2 (71.06 U ml^{-1}) while the highest protease activity was recorded by the isolate TRPN-11 (165.86 U ml^{-1}) followed by TRPN-17 (92.90 U ml^{-1}) and TRPN-3 (74.80 U ml^{-1}). The isolate TRPN-3 had the highest (4.86 U ml^{-1}) lipase enzyme activity followed by the isolates TRKR-2 (4.20 U ml^{-1}) and TRPN-11 (3.14 U ml^{-1}).

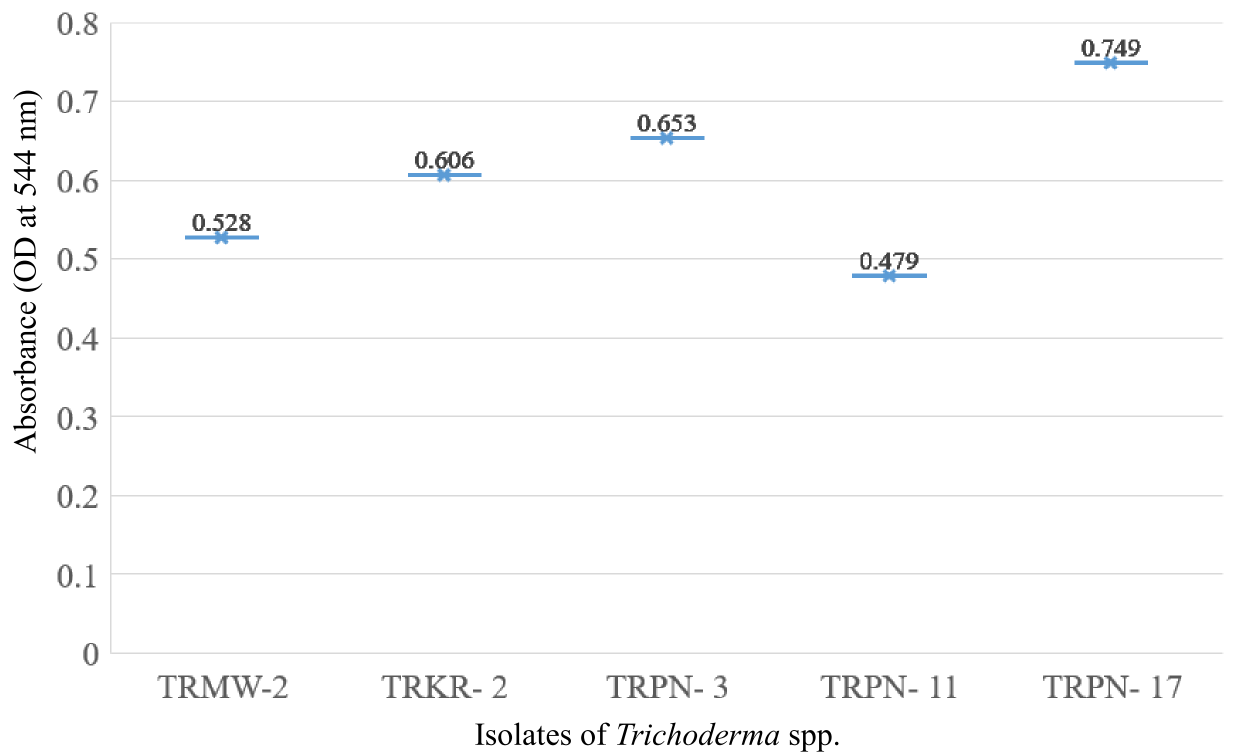


Fig. 13. Absorbance value of chitinase activity of *Trichoderma* isolates

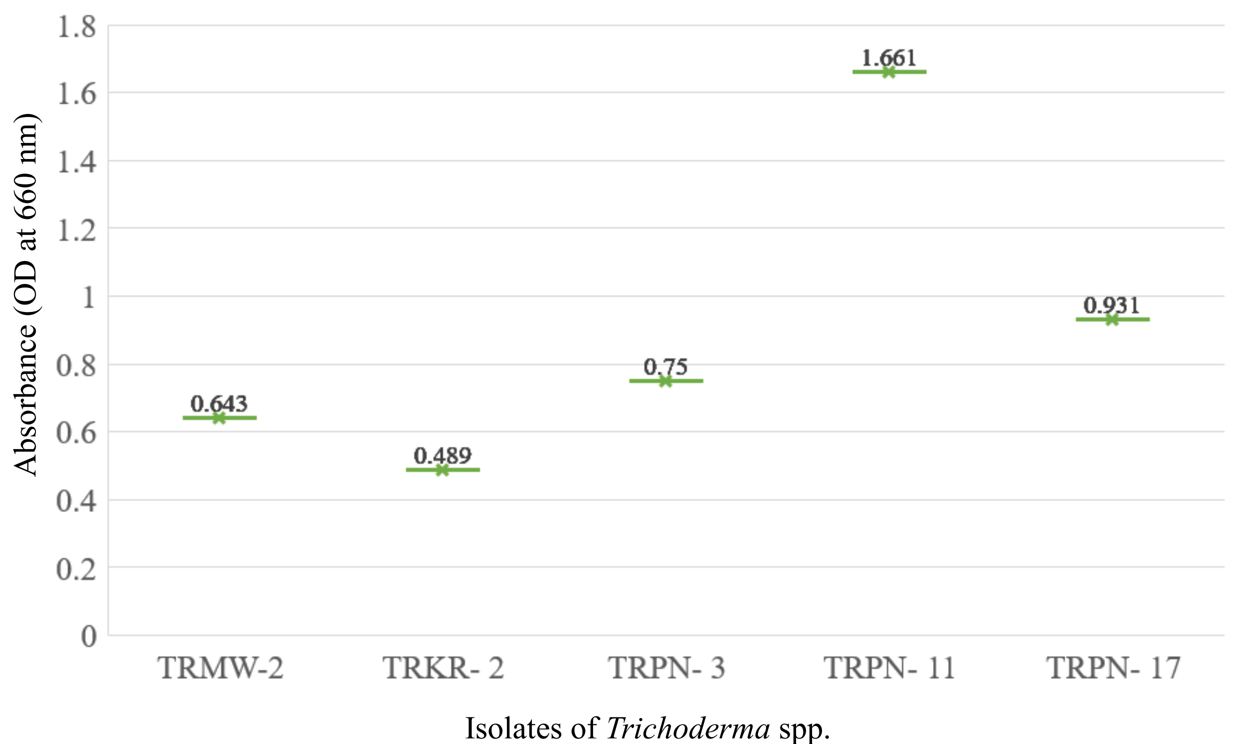


Fig. 14. Absorbance value of protease activity of *Trichoderma* isolates

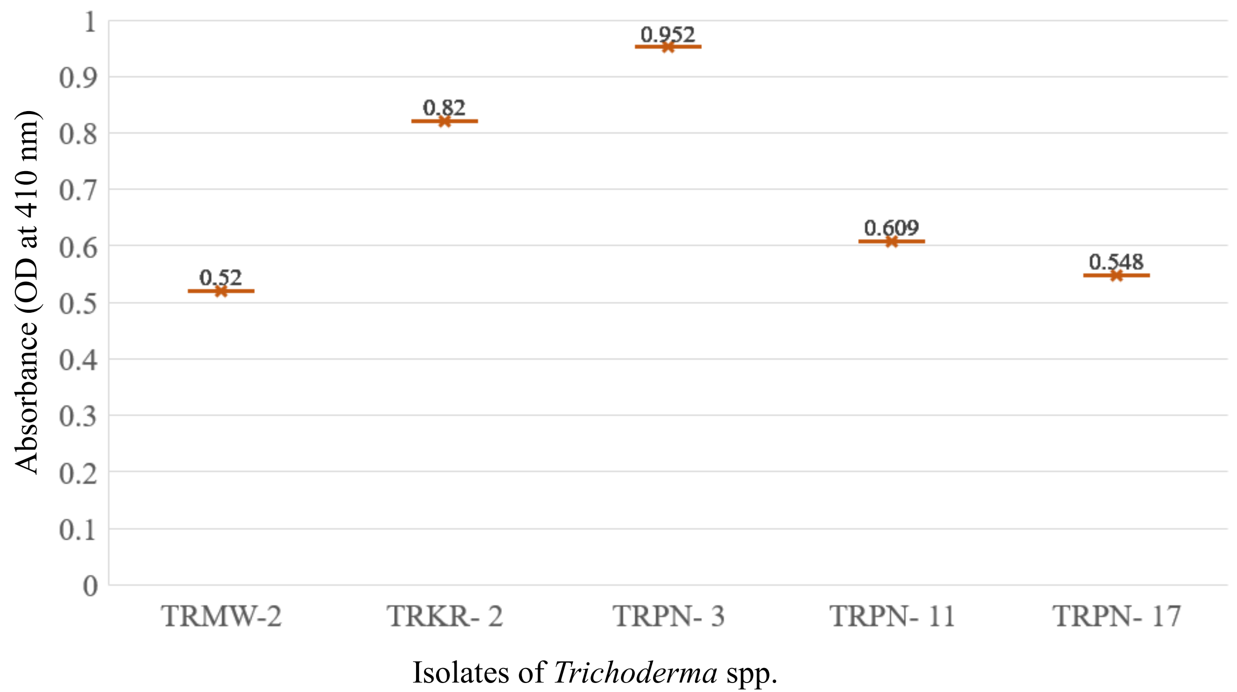
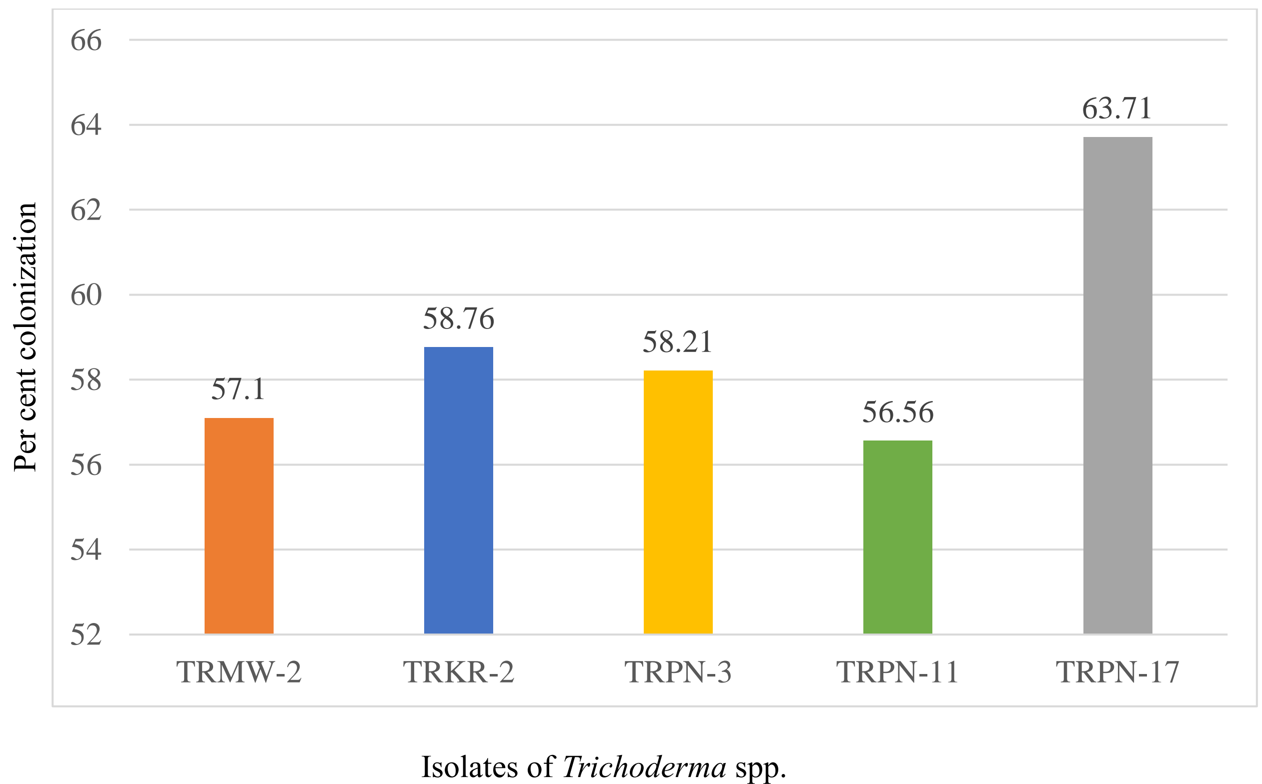
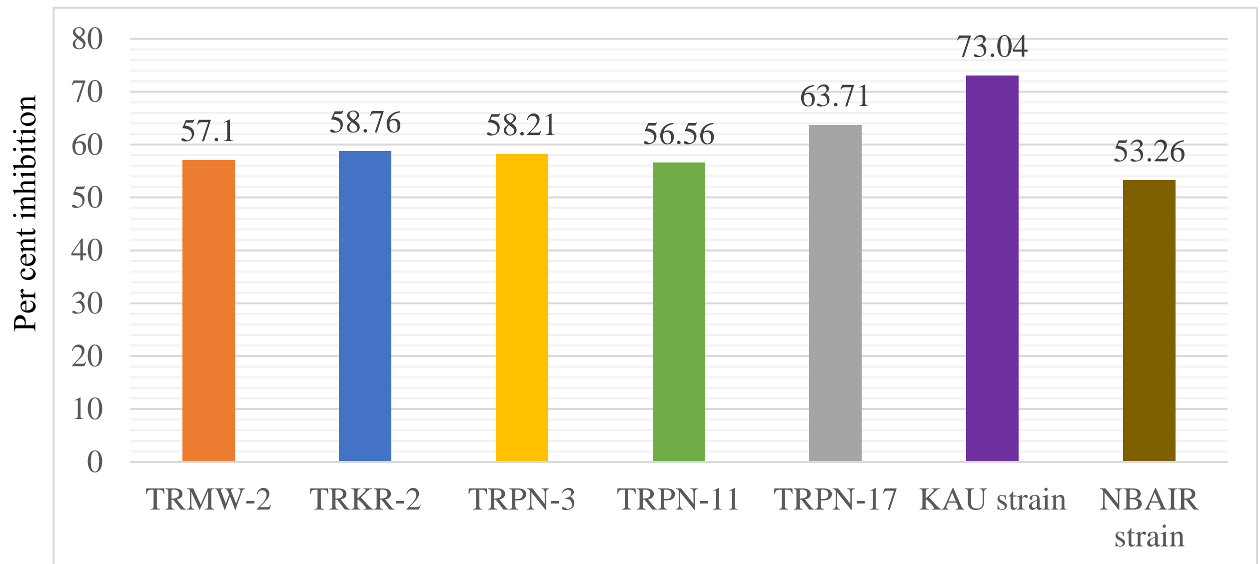


Fig. 15. Absorbance value of lipase activity of *Trichoderma* isolates



Isolates of *Trichoderma* spp.

Fig. 16. Per cent colonization of straw bits by *Trichoderma* isolates



Isolates of *Trichoderma* spp.

Fig. 17. Per cent inhibition of selected isolates of *Trichoderma* sp. against *Fusarium oxysporum*

Asad *et al.* (2015) reported that after 96 h of growth at 25 °C, *T. asperellum* reached its maximum chitinolytic activity (173 U ml^{-1}), which was the highest of all of the isolates tested; after 72 h of culture at the same temperature, *T. harzianum* reached 117 U ml^{-1}. Chitinase from several *Trichoderma* species has distinct properties and antifungal action. Two strains of *S. rolfsii* and *Colletotrichum sp*. are both inhibited by *T. asperellum* PQ34 chitinase (Loc *et al*., 2020). Chitin and glucan are the two major constituents of fungal cell walls, which are vulnerable to chitinase, N-acetyl-*β*-glucosaminidase, and β-glucosidase (Karlicic *et al*., 2021). Using RAPD analysis, Gajera and Vakharia (2010) correlated the biocontrol efficacy of 12 *Trichoderma* isolates against *Aspergillus niger* to the production of cell wall-degrading enzymes such chitinase, *β*-1, 3 glucanase, and others during mycoparasitism. In terms of increased production of extracellular enzymes including chitinase, *β*-1,3 glucanases, protease, cellulose, and pectinase, *Trichoderma* isolates (UNT68, NAT70, UNT38, UNS63, UNT13, UNS30, and DET02) displayed a mycotrophic mode of antagonism that may be effective against *Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria alternata*, *Colletotrichum gloeosporoides* and *Rhizoctonia solani* (Rai, 2016). According to Solanki *et al*. (2011), the superior chitinase producing fungi may not always show a higher level of *β* -1,3 glucanases and vice versa. This provides evidence for the possibility that each given metabolite or biomolecule is strain or species-specific in its production.

The per cent colonization and saprophytic competence of the *Trichoderma* isolates were assessed using paddy straw bits. Among all the isolates, TRKR-2 showed the highest per cent colonization of 67.50 % followed by TRPN-3 (56.25 %), TRPN-17 (56.25 %), TRMW-2 (50 %) and TRPN-11 (48.75 %) with no significant difference at the concentration of 10^6 cfu g^{-1} of soil. Our result accords with Mahalakshmi and Raja (2013) who showed a positive correlation between the CSA and per cent colonization of the isolates of *T. viride* (Tv1, Tv2, Tv3 and Tv4), *T. harzianum* (Th1, Th2, Th3, Th4, Th5, Th6, Th7 and Th8), and *T. viride* (Tr1, Tr2, Tr3, Tr4 and Tr5). Of all other isolates, Th2 and Th6 had the highest CSA, with percentage colonization of 61.84 per cent and 59.12 per cent, respectively. Nakkeeran *et al.* (2005) revealed a positive relationship between cellulose activity and CSA. Similar observations were

recorded by Khare and Upadhyay (2009), and Khare *et al.* (2010). Thus, effective biocontrol agents could be selected based on the nature of cellulose exploitation (Sarrocco *et al*., 2009).

Rashmi *et al.* (2016) studied the CSA of *Trichoderma* spp. *viz*., *T. harzianum*-143, *T. atroviride, Th*-m_1 and *T. atroviride* m_1 and found that the mutant strain *Th*-m_1 has enhanced CSA activity than the parent strain. They also concluded that the CSA activity is responsible for the potential disease control of *Th*-m_1 against chickpea collar rot caused by *Sclerotium rolfsii* with increased cellulolytic activity. Thiruvudainambi *et al*. (2010) demonstrated that *T. viride* with the highest CSA inhibited the mycelial growth of soil-borne pathogen *Sclerotium rolfsii* efficiently. The strains with CSA can survive better in the soil ecosystem.

Sarrocco *et al.* (2021) evaluated the competitive ability of *T. gamsii* T6085, alone and in the presence of *F. oxysporum* 7121, against *F. graminearum* ITEM 124 causal agent of Fusarium head blight using wheat straw bits and they found that the presence of *T. gamsii* T6085 significantly reduced perithecium development. Even in the presence of an *F. oxysporum* isolate, one of the principal natural competitors of *F. culmorum* and *F. graminearum* for wheat straw, *T. gamsii* is able to restrict *F. graminearum* growth and perithecium development on wheat straw.

5.2. *IN VITRO* EFFICACY OF TRICHODERMA ISOLATES AGAINST *FUSARIUM* SPP

In the present study, the efficacy of *Trichoderma* isolates against *Fusarium* spp. was tested by the dual culture method. Among the tested isolates, TRPN-17 caused the highest per cent inhibition (63.71 %) of the mycelial growth and differed significantly from other isolates. It was followed by TRKR-2 (58.76 %), TRPN-3 (58.21 %), TRMW-2 (57.10 %) and TRPN-11 (56.56 %) with no significant difference. The reference strains *T. asperellum* (KAU strain) and *T. harzianum* (NBAIR strain) brought about per cent inhibition of 73.04 per cent and 53.26 per cent respectively. The antagonistic growth characters were recorded visually and almost all the isolates exhibited all three antagonistic characters except *Trichoderma* sp. (KAU strain) and *T.*

harzianum (NBAIR strain) in which only lysis and overgrowth was observed. TRKR-2 recorded only antibiosis and overgrowth against the pathogen.

The findings concur with Fan *et al.,* (2020) who reported that the *T. citrinoviride* strain Snef1910 suppressed the growth of *F. graminearum* by 60.76 per cent, *F. oxysporum* by 49.28 per cent, *F. moniliforme* by 21.73 per cent, and *F. roseum* by 25.20 per cent. Bastakoti *et al.,* (2017) reported that *Trichoderma* isolate TS 215 showed per cent inhibition of more than 60 per cent against *F.* solani. *T. harzianum* effectively inhibited the mycelial growth of *Fusarium* spp. (Nwankiti and Gwa, 2018; Yassin *et al.,* 2021). The isolates of *Trichoderma viz.,* TRSN-1, TRMW-2, TRKR-2, TRPN-3, TRPN-7, TRPN-11, TRPN-14, TRPN-15, TRPN-17, TRPN-18 and TRML-1 were tested *in vit*ro for their ability to inhibit the growth of two pathogens, *Pythium aphanidermatum* and *F. oxysporum* and were found to exhibit per cent inhibition of more than 57 per cent and 45 per cent, respectively (Nair, 2022).

Awad-Allah *et al*., (2022) investigated the antagonistic activity of *Trichoderma* isolates *viz., T. viride and T. harzianum* against *F. solani* which causes wilt disease in cherry tomatoes. They found that 10 days post-inoculation, *T. harzianum* suppressed *F. solani* mycelial growth by 78.0 per cent, while *T. viride* inhibited it by 61.2 per cent. Scanning electron microscopy (SEM) analysis also showed that *Trichoderma* isolates coiled themselves around *F. solani* hyphae, degraded and deformed pathogen hyphae, and grew in concordance with the Fusarium wilt pathogen. These results suggest that the antagonistic *Trichoderma* isolates studied here could provide a source of novel biological fungicides that avoid the harmful effects of chemical fungicides, particularly against the Fusarium wilt pathogen *F. solani*.

5.3. MOLECULAR CHARACTERIZATION OF PROMISING *TRICHODERMA* ISOLATES

Primers specific to regions 1 and 4 of the Internal Transcribed Spacer (ITS) were used in the present PCR experiment. Using the extracted genomic DNA as a template in PCR, an intact amplicon of around 600 bp was obtained at annealing temperature to 57°C. The NCBI-BLAST programme was used to find the closest match in the

GenBank database to the amplified sequences. Using the oligonucleotide set of genus-specific primer TvP, genus-specific PCR was performed to obtain an amplicon of about 245 bp in size. DNA barcoding of the amplicon verified that all of the isolates belonged to the genus *Trichoderma*.

Identifying *Trichoderma* spp. correctly is crucial for studying their detailed mechanism of antagonism and developing efficient management measures against soil-borne fungal diseases (Mokhtari *et al*. 2017). Ribosomal DNA gene repeats, specifically the ITS regions, are thought to undergo rapid evolution, making it possible for their length and sequences to differ. The ITS region is amplified quickly and easily because of its high copy number (up to 30,000 per cell), modest size (600-700 bp), and the presence of universal PCR primers built from highly conserved areas. The ITS region and the ribosomal DNA gene sequence are the most commonly used markers for molecular typing because of these benefits. Since the ITS sequence is less variable from one species to another, it is used as the basis for a more stringent method in the present investigation (Balasubramani *et al.,* 2010)

Prameeladevi *et al*. (2012a, b) used ITS1, ITS2, and *tef1* DNA sequence data to confirm the morphological and cultural identification of 72 isolates of *Trichoderma* spp. into four species: *Trichoderma virens, T. harzianum, T. longibrachiatum*, and *T. asperellum*. They also found that, while ITS sequence amplicon sizes generally ranged from 550 to 600, the higher transition/transversion ratio (1.32) and evolutionary divergence (1.965) of *tef1* make it a superior marker for distinguishing *Trichoderma* species. A 245-bp fragment of ribosomal DNA was successfully amplified using an oligonucleotide primer combination (TvPF and TvPR) designed by Prameeladevi *et al*.(2011) and shown to be exclusive to the genus *Trichoderma*. The present study also confirms the identity of the isolates as belonging to the genus *Trichoderma* based on the amplification with primers TvPF and TvPR. Species of *Trichoderma* can be identified by a combination of multi-gene phylogeny and morphological traits, as proposed by Pandian *et al.* (2016). They used the morphological and molecular study of genes including ITS, *tef1*, *rpb2*, *act*, and *cal* to define *T. asperellum* strain Ta13. In the present study, attempts at species-specific identification using gene-specific primers

for translation elongation factor-1 (*tef1*) and RNA polymerase II (*rpb2*) failed to produce any amplicon, thus giving an indication that the isolates might not belong to the species *viz.,T. virens, T. longibrachiatum, T. harzianum,* or *T. asperellum*. Consequently, further investigations are required to confirm the species identity of the isolates.

5.4. EFFICACY OF *TRICHODERMA* SPP. AGAINST SOIL-BORNE DISEASES

The application of chemical pesticides is progressively reoriented using biological control methods to manage crop diseases. *Trichoderma* spp. has prominent attention among various fungal and bacterial biocontrol agents due to their abilities to establish a large rhizosphere population and suppress diseases of treated plants. It is used to control various soil-borne plant pathogens like *Fusarium, Rhizoctonia, Pythium, Phytophthora,* etc. It is also used against some bacterial and viral infections. The endorsement of *Trichoderma* as a biocontrol agent overcomes several adverse effects of chemical pesticides in an eco-friendly manner.

In the present study, application of talc-based formulations of the isolates TRKR-2, TRPN-3, KAU strain and consortium of TRPN-3 and TRPN-17 as seed treatment @ of 20 g kg^{-1} of seed followed by soil drenching @ 2 per cent at 20, 40 and 60 DAS effectively reduced the incidence of Fusarium wilt of vegetable cowpea in pot culture study. The application of isolate TRPN-3 produced highest pod number (19) in pot culture experiment and also yield per plant (369.57 g) on par with the yield per plant produced by consortia application of TRPN-3 and TRPN-17 (344.52 g). Under field conditions, the disease was most effectively reduced by isolates TRKR-2, TRPN-3, KAU strain and consortium of TRKR-2 and TRPN-17. Lengthy pods (48.16 cm) and highest number of seeds per pod (21.73) were recorded by the application of consortium of TRPN-3 and TRPN-17 whereas application of TRPN-3 resulted in highest yield (1172.63 g/plant), number of pods per plant (19) and reduced days of flowering. Enumeration of population of *Trichoderma* spp. from soil in different treatments at 90 DAS revealed that, highest population was in treatment with isolate TRPN 3 under pot culture (7×10^4 cfu g^{-1} soil) and field conditions (4×10^4 cfu g^{-1} soil).

These findings are in agreement with Pradhan *et al*. (2022) investigated the *in vivo* bio-potential of five *T. viride* strains (ITCC 6889, ITCC 7204, ITCC 7764, ITCC 7847, and ITCC 8276) for the control of Fusarium wilt of chickpea. When compared to seeds treated with carbendazim (80.33 %) and a talc-based *T. viride* formulation (83.33 %), seeds treated with a dustable powder formulation comprising spores of the antagonist suppressed the growth and development of the pathogen, resulting in an increased germination percentage of 93.33 per cent. The wilting percentage of plants treated with the dustable *T. viride* powder formulation was much lower than that of plants treated with carbendazim and talc-based formulations. Our findings are consistent with those of Fang-Fang *et al*. (2016) and Luo *et al*. (2016), who also found that *Trichoderma* increases growth and biomass accumulation in plants. The use of *Trichoderma* strains *viz., T. harzianum* and *T. viride* against the wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici* led to a decrease in disease severity, an increase in fresh and dry weights, plant length, and yield in the field through the production of cell wall degrading enzymes and improved physiological activity (Jamil, 2021).

Excellent control against Fusarium wilt of banana induced by *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4) was observed using a formulation of the *T. reesei* isolate CSR-T-3, as reported by Damodaran *et al*. (2020) in both under *in vitro* and field conditions. Enhanced production of antifungal compounds and higher activity of defense enzymes such as β-1,3-glucanase, peroxidase, chitinase, polyphenol oxidase, and phenylalanine ammonia lyase reduce disease severity. In the field, plants that were given the treatment had a high phenological growth rate and yield, and they only had a disease severity score of 1.14.

Trichoderma acts as a biocontrol agent that is both effective and environmentally safe, in addition to being a beneficial symbiotic organism for plants in the management of soil-borne plant diseases. Further characterization of the species identity of the isolates needs to be carried out. The field level efficacy of these isolates against other soil-borne pathogens of vegetable cowpea also need to be explored for their successful use by the farming community.

Summary

6. SUMMARY

The study entitled "Molecular characterization of promising isolates of *Trichoderma* spp. and their field evaluation against Fusarium wilt of vegetable cowpea" was conducted in the Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram during 2020-2022 with the aim of molecular characterization of potential *Trichoderma* isolates and their evaluation against Fusarium wilt of vegetable cowpea under field conditions.

Five potent isolates of *Trichoderma viz*., TRMW-2, TRKR-2, TRPN-3, TRPN-11 and TRPN-17 were obtained from the previous study along with a reference strain *viz., Trichoderma asperellum* (KAU strain). The isolates TRPN-17 (88.39 U ml-1), TRPN-3 (76.75 U ml^{-1}), and TRKR-2 (71.06 U ml^{-1}) displayed the highest chitinase activity. The isolates TRPN-11 (165.86 U ml^{-1}), TRPN-17 (92.90 U ml^{-1}), and TRPN-3 (74.80 U ml^{-1}) had the highest levels of protease activity. The isolate TRPN-3 (4.86 U ml-1) had the highest level of lipase activity, followed by TRKR-2 (4.20 U ml^{-1}) and TRPN-11 (3.14 U ml^{-1}). Assessment of competitive saprophytic ability revealed that TRKR-2 isolate had the highest colonisation rate (67.50 %), followed by the TRPN-3 and TRPN-17 isolates with 56.25 per cent each. The isolates TRPN-17 (63.71 %), TRKR-2 (58.78 %), and TRPN-3 (58.21 %) showed the highest percentage inhibition *in vitro* against *F. oxysporum*.

Compatibility studies of the isolates revealed that, with the exception of the combination involving TRPN-11 and TRPN-3, all others showed antibiosis, lysis, and overgrowth. When TRPN-3 and TRPN-11 were combined, neither antibiosis nor lysis was evident, however when TRKR-2 and TRPN-17 were combined, only a modest degree of antibiosis was noticed. For the *in vivo* evaluation against Fusarium wilt of vegetable cowpea, the effective isolates TRKR-2, TRPN-17, TRPN-3, consortia of isolates TRKR-2 and TRPN-17, and consortia of isolates TRPN-3 and TRPN-17 were chosen based on the *in vitro* investigations.

Molecular characterization of all the five isolates using ITS primers revealed the best matches from the GenBank database of NCBI based on per cent similarity. The

isolate TRMW-2 showed a similarity of 99.42 per cent with the *T. brevicompactum* isolate, *Trichoderma* sp. isolate SDAS203739 and *T. deliquescens.* Isolate TRKR-2 was found to be 99.65 per cent similar to *T. koningiopsis* isolate and *T. ovalisporum* strain. TRPN-3 isolate resembled 99.82 per cent to *T. harzianum* clone, *T. asperellum* isolate and *T. lixii* isolate. The isolate TRPN-11 was 98.31 per cent similar to *T. effusum voucher, Trichoderma* sp. Isolate SDAS203144, *T. cf harzianum voucher* and *T. lixii* strain. The isolate TRPN-17 resembled 98.70 per cent *T. harzianum* isolate and *T. asperellum* isolate. Using genus-specific primers for TvP region, the DNA barcoding of the amplicon confirmed the genus identity of all the isolates as *Trichoderma.* The species-specific identification tried with translational elongation factor- 1 (*tef1)* and RNA polymerase II (*rpb2)* gene specific primers did not yield any amplicon indicating that the isolates may not belong to the species *T. virens*, *T. longibrachiatum*, *T. harzianum* and *T. asperellum.*

Individual treatment with the isolates TRKR 2, TRPN 3 and KAU strain were found to be effective for the management of Fusarium wilt of vegetable cowpea under both pot culture and field conditions. Among the consortia, the application of a combination of TRPN-3 and TRPN-17 was effective under pot culture conditions whereas that of isolates TRKR-2 and TRPN-17 was effective under field conditions. In pot culture, treatment with TRPN 3 recorded the highest number of pods (19) and yield (369.57 g). Under field conditions also the same isolate could bring about highest number of pods (19) and yield (1172.63 g). Application of consortium of isolates TRKR 2 and TRPN 17 resulted in maximum pod length (43.85 cm) under pot culture while consortium of TRPN 3 and TRPN 17 could result in maximum pod length (48.16 cm) in field conditions. Highest number of seeds per pod (21.73) was obtained on application of consortium of the isolates TRPN 3 and TRPN 17 under field conditions. At 90 DAS, population of *Trichoderma* sp. was found to be highest in case of treatment with isolate TRPN 3 under pot culture (7×10^4 cfu g^{-1}) and field conditions (4.33×10^4 cfu g^{-1}).

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Appendices

APPENDIX – I

COMPOSITION OF MEDIA USED

Potato Dextrose Agar Medium (PDA)

Potato	: 200 g
Dextrose	: 20 g
Agar	: 20 g
Distilled water	: 1 L

Potato Dextrose Broth (PDB)

Potato	: 200 g
Dextrose	: 20 g
Distilled water	: 1 L

Trichoderma Selective Medium (TSM)

$MgSo_4.7H_2O$	: 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
Distilled water	: 1 L

APPENDIX – II

LIST OF BUFFERS USED

Reagents and buffers used for genomic DNA isolation from *Trichoderma* spp.

CTAB Extraction buffer

Chloroform: Isoamyl Alcohol (24:1)

100% Isopropanol

70% ethanol

TE buffer

Components of CTAB Extraction buffer

Tris HCl – 100mM

EDTA – 25mM

NaCl – 2M

CTAB – 2%

Polyvinyl pyrrolidone – 3%

β- mercapta ethanol – 0.2%

Components of TE buffer

Tris HCl – 10mM

EDTA- 1mM

Reagents and buffers used in agarose gel electrophoresis

10X TBE buffer (Tris Borate EDTA buffer)

Agarose – 1%

Ethidium bromide – 0.5 μg/ml

Sample loading dye (6X)

Components of 10X TBE buffer

Tris HCl – 108g

Boric acid - 55g

0.5M EDTA – 4ml

Components of sample loading dye (6X)

Bromophenol blue- 0.25%

Xylene cyanol- 30%

Glycerol- 40%

Abstract

MOLECULAR CHARACTERIZATION OF PROMISING ISOLATES OF *Trichoderma* spp. AND THEIR FIELD EVALUATION AGAINST FUSARIUM WILT OF VEGETABLE COWPEA

by

JEEVIDHA M.

(2020-11-135)

Abstract of Thesis

submitted in partial fulfilment of the

requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM – 695 522

KERALA, INDIA

2023

ABSTRACT

The present study entitled "Molecular characterization of promising isolates of *Trichoderma* spp. and their field evaluation against Fusarium wilt of vegetable cowpea" was conducted at the Department of Plant Pathology, College of Agriculture, Vellayani during 2020-2022. The study aimed at molecular characterization of potential *Trichoderma* isolates and their evaluation against Fusarium wilt of vegetable cowpea under field conditions. Five potent isolates of *Trichoderma viz*., TRMW-2, TRKR-2, TRPN-3, TRPN-11 and TRPN-17 were obtained from the previous study.

The analysis of *Trichoderma* isolates for cell wall degrading enzymes like chitinase, protease and lipase revealed that TRPN-17 recorded the highest chitinase activity of 88.38 U ml^{-1}. Protease activity was found to be higher in TRPN-11 (165.86 U ml^{-1}) whereas highest lipase activity was exhibited by TRPN-3 (4.86 U ml^{-1}). The compatibility test among the five isolates showed that all the combinations exhibited antibiosis, lysis and overgrowth except the isolates TRPN-3 and TRPN-11 which displayed only overgrowth. A mild level of antibiosis and overgrowth was observed in the combination of the isolates TRKR-2 and TRPN-17.

Assessment of competitive saprophytic ability of the *Trichoderma* isolates showed that per cent colonization of TRKR-2 was found to be higher (67.50 %) than other isolates and was followed by TRPN-3 and TRPN-17 (56.25 %) with no significant difference among all five isolates. *In vitro* screening of *Trichoderma* isolates by dual culture technique revealed that the per cent inhibition of TRPN-17 (63.71 %) was found to be higher, followed by TRKR-2 (58.76 %) and TRPN-3 (58.21 %). Based on the *in vitro* studies the effective isolates TRKR-2, TRPN-17, TRPN-3, consortia of isolates TRKR-2 and TRPN-17 and consortia of isolates TRPN-3 and TRPN-17 were selected for the *in vivo* evaluation against Fusarium wilt of vegetable cowpea.

Molecular characterization of the isolates were done using the primers for Internal Transcribed Spacer (ITS) regions 1 and 4. All the five isolates produced a single, intact amplicon of size 600 bp approximately. The nucleotide sequences of all the ITS PCR products obtained from the five isolates were confirmed through bidirectional sequencing using the Sangers DNA sequencing. The best match of the

amplified sequences was obtained from the GenBank database using NCBI-BLAST and their phylogenetic positions were explored along with the referral sequences from BLAST analysis. The oligonucleotide set of genus-specific primer TvP was used for genus-specific PCR analysis and an amplicon of approximately 245 bp was obtained. The DNA barcoding of the amplicon confirmed the genus identity of all the isolates as *Trichoderma.* The species-specific identification tried with translational elongation factor- 1 (*tef1)* and RNA polymerase II (*rpb2)* gene specific primers did not yield any amplicon indicating that the isolates did not belong to the species *T. virens*, *T. longibrachiatum*, *T. harzianum* and *T. asperellum.*

Application of talc-based formulations of the isolates TRKR-2, TRPN-3, KAU strain and consortium of TRPN-3 and TRPN-17 as seed treatment @ of 20 g kg^{-1} of seed followed by soil drenching @ 2 per cent at 20, 40 and 60 DAS effectively reduced the incidence of Fusarium wilt of vegetable cowpea in pot culture study. The application of isolate TRPN-3 produced highest pod number (19) in pot culture experiment and also yield per plant (369.57 g) on par with the yield per plant produced by consortia application of TRPN-3 and TRPN-17 (344.52 g). Under field conditions, the disease was most effectively reduced by isolates TRKR-2, TRPN-3, KAU strain and consortia of TRKR-2 and TRPN-17. Lengthy pods (48.16 cm) and highest number of seeds per pod (21.73) were recorded by the application of consortium of TRPN-3 and TRPN-17 whereas application of TRPN-3 resulted in highest yield (1172.63 g/plant), number of pods per plant (19) and reduced days of flowering. Enumeration of population of *Trichoderma* spp. from soil in different treatments at 90 DAS revealed that, highest population was in treatment with isolate TRPN 3 under pot culture (7×10^4 cfu g^{-1}) and field conditions (4×10^4 cfu g^{-1}) .

In the present study, *Trichoderma* isolates TRKR-2, TRPN-3, and consortium of TRPN-3 and TRPN-17 were found effective against Fusarium wilt of vegetable cowpea under field conditions. The genus of the isolates under study was identified as *Trichoderma*. The efficacy of the isolates against other soil-borne diseases of vegetable cowpea and species level identification need to be explored.

സംഗ്രഹം

"ട്രൈക്കോഡെർമ സ്പീഷീസിലെ മികച്ച ഐസൊലേറ്റുകളുടെ തന്മാത്രാ സ്വഭാവ പഠനവും വള്ളിപ്പയറിന്റെ ഫ്യൂസേറിയൽ വാട്ട നിയന്ത്രണത്തിൽ അവയുടെ ഉപയോഗവും" എന്ന വിഷയത്തിൽ 2021-22 കാലയളവിൽ വെള്ളായണി കാർഷിക കോളേജിൽ പ്ലാന്റ് പാത്തോളജി വിഭാഗത്തിൽ നടത്തിയ ഗവേഷണത്തിന്റെ സംക്ഷിപ്ത രൂപം ഇവിടെ ഉൾപ്പെടുത്തിയിരിക്കുന്നു. മുൻ പഠനത്തിൽ മികച്ച ഗുണങ്ങൾ കാണിച്ച ട്രൈക്കോഡെർമയുടെ ഐസൊലേറ്റുകളായ ടി. ആർ. എം. ഡബ്ള്യൂ- 2, ടി. ആർ. കെ. ആർ- 2, ടി. ആർ. പി. എൻ.- 3, ടി. ആർ. പി. എൻ-11, ടി. ആർ. പി. എൻ- 17 എന്നിവ പഠനം നടത്തുവാനായി തിരഞ്ഞെടുത്തു.

കോശ ഭിത്തി നശിപ്പിക്കുന്ന എൻസൈമുകളായ കൈറ്റിനേസ്, പ്രോട്ടിയേസ്, ലിപേസ് എന്നിവയുടെ വിശകലനത്തിൽ ടി. ആർ. പി. എൻ-17 ഉയർന്ന കൈറ്റിനേസ് പ്രവർത്തനവും (88.38 യൂണിറ്റ്/മി. ലി.), ടി. ആർ. പി. എൻ-11 ഉയർന്ന പ്രോട്ടിയേസ് പ്രവർത്തനവും (165.86 യൂണിറ്റ്/മി. ലി.), ടി. ആർ. പി. എൻ-3 ഉയർന്ന ലിപേസ് പ്രവർത്തനവും (4.86 യൂണിറ്റ്/മി. ലി.) കാണിക്കുന്നതായി കണ്ടെത്തി.

പഠനത്തിനായി ഉപയോഗിച്ച അഞ്ച് ഐസൊലേറ്റുകളുടെ പരസ്പര അനുയോജ്യതാ പരിശോധനയിൽ ടി. ആർ. പി. എൻ-3 , ടി. ആർ. പി. എൻ-11 എന്നിവ ഒഴികെയുള്ള എല്ലാ സംയോജനങ്ങളും ആന്റിബയോസിസ്, ലൈസിസ്, ക്രമാതീതമായ വളർച്ച എന്നിവ കാണിച്ചു. ടി. ആർ. കെ. ആർ-2 , ടി. ആർ. പി. എൻ-17 എന്നിവ മിതമായ നിരക്കിൽ ആന്റിബയോസിസ് രേഖപ്പെടുത്തി.

ട്രൈക്കോഡെർമ ഐസൊലേറ്റുകളുടെ കോമ്പറ്റിറ്റീവ് സാപ്രൊഫയ്റ്റിക് എബിലിറ്റി വിശകലനത്തിൽ ടി. ആർ. കെ. ആർ-2 ന്റെ കോളനൈസേഷൻ നിരക്ക് (67.50 %) താരതമ്യേന കൂടുതൽ ആയി കണ്ടെത്തി. ടി. ആർ. പി. എൻ-17 , ടി. ആർ. പി. എൻ-3 എന്നിവ 56.25 ശതമാനം കൊളനൈസേഷനാണ് പ്രദർശിപ്പിച്ചത്. ഡ്യുവൽ കൾച്ചർ പ്രക്രിയ വഴിയുള്ള ഇൻവിട്രോ പഠനത്തിൽ ടി. ആർ. പി. എൻ-17 ഉയർന്ന പ്രതിരോധം (63 .71%) രേഖപ്പെടുത്തി. തുടർന്ന് ടി. ആർ. കെ. ആർ-2 (58.76 %), ടി. ആർ. പി. എൻ-3 (58.21 %) എന്നിവയാണ് മികച്ച പ്രതിരോധ നിരക്ക്

രേഖപ്പെടുത്തിയത്. ഇൻ വിറ്റ്റോ പഠനങ്ങളുടെ അടിസ്ഥാനത്തിൽ മികച്ച ഐസൊലേറ്റുകളായ ടി. ആർ. കെ. ആർ-2, ടി. ആർ. പി. എൻ- 17 , ടി. ആർ. പി. എൻ-3 എന്നിവയും ടി. ആർ. കെ. ആർ-2 , ടി. ആർ. പി. എൻ- 17 എന്നിവയുടെ സംയോജിത രൂപവും ടി. ആർ. പി. എൻ-3 ടി. ആർ. പി. എൻ-17 എന്നിവയുടെ സംയോജിത രൂപവും വള്ളിപ്പയറിന്റെ വാട്ടരോഗത്തിനു എതിരെ പ്രയോഗിക്കാൻ തിരഞ്ഞെടുത്തു.

ഐ. ടി. എസ്- 4, ഐ. ടി. എസ്- 1 എന്നീ പ്രൈമറുകൾ ഉപയോഗിച്ച് തന്മാത്രാ സ്വഭാവ പഠനം നടത്തി. എല്ലാ ഐസൊലേറ്റുകളും ഏകദേശം 600 ബേസ് പെയറുകളുള്ള ആംപ്ലികോൺ ഉത്പാദിപ്പിച്ചു. പി. സി. ആർ വഴി ലഭിച്ച എല്ലാ സീക്വൻസുകളും സാങ്കേർസ് ഡി. എൻ. എ. സീക്വൻസിങ് പ്രക്രിയ വഴി ദ്വി ദിശാ സീക്വൻസിങ്ങ്ന് വിധേയമാക്കി. പ്രസ്തുത ഡി. എൻ. എ. സീക്വൻസുകളെ എൻ.സി.ബി.ഐ ബ്ളാസ്റ്റ് ഉപയോഗിച്ചു ജീൻ ബാങ്ക് ഡാറ്റ ബേസിൽ ഉള്ള മറ്റ് സാമ്യതയുള്ള സീക്വൻസുകളുമായി താരതമ്യം ചെയ്ത് ഫൈലോജനറ്റിക് സ്ഥാനം കണ്ടെത്തി. ജീനസ് നിർദ്ദിഷ്ടമായ പി. സി. ആർ വിശകലനത്തിന് ടി. വി. പി. പ്രൈമർ ഉപയോഗിക്കുകയും എല്ലാ ഐസൊലേറ്റുകൾക്കും ഏകദേശം 245 ബേസ് പെയറുകൾ ലഭിക്കുകയും ചെയ്തു. ഇത്തരത്തിൽ ലഭിച്ച ആംപ്ലിക്കോണുകളുടെ ഡി. എൻ. എ ബാർകോഡിങ് വഴി ഇവ ട്രൈക്കോഡെർമ ജീനസിൽ ഉൾപെട്ടവയാണെന്ന് സ്ഥിരീകരിച്ചു.

ടെഫ്-1, ആർ. പി. ബി-2 എന്നീ പ്രൈമറുകൾ ഉപയോഗിച്ചുള്ള സ്പീഷീസ് നിർദ്ദിഷ്ട തിരിച്ചറിയലിൽ ആംപ്ലിക്കോണുകൾ ഒന്നും തന്നെ ലഭിക്കാത്തതിനാൽ പ്രസ്തുതത ഐസൊലേറ്റുകൾ ട്രൈക്കോഡെർമ വൈറൻസ്, ട്രൈക്കോഡെർമ ലോങിബ്രാക്കിയേറ്റം, ട്രൈക്കോഡെർമ ഹാർസിയാനം, ട്രൈക്കോഡെർമ ആസ്പെറില്ലം എന്നിവയിൽ ഉൾപെട്ടതായിരിക്കില്ല എന്ന നിഗമനത്തിൽ എത്തിച്ചേർന്നു.

വള്ളിപ്പയറിന്റെ രോഗങ്ങൾക്കെതിരെയുള്ള പോട്ട് കൾച്ചർ പഠനത്തിൽ ടി. ആർ. കെ. ആർ-2 ടി. ആർ. പി. എൻ-3, കെ. എ. യു. സ്ട്രെയിൻ, ടി. ആർ. പി. എൻ-3, ടി.ആർ. പി. എൻ- 17 എന്നിവയുടെ സംയോജിത രൂപം തുടങ്ങിയവയുടെ ടാൽക് അധിഷ്ഠിത ഫോർമുലേഷനുകൾ 20 ഗ്രാം /കി. ഗ്രാം എന്ന

തോതിൽ വിത്തുപചാരത്തിനും വിത്ത് നട്ട് 20, 40, 60 ദിവസങ്ങൾക്ക് ശേഷം രണ്ട് ശതമാനം നിരക്കിൽ മണ്ണ് നനക്കലിനും പ്രയോഗിച്ചത് വഴി ഫ്യുസേറിയൽ വാട്ടരോഗം നിയന്ത്രിക്കുവാൻ സാധിച്ചു. ടി. ആർ. പി. എൻ-3-ന്റെ പ്രയോഗം നടത്തിയ ചെടികളിൽ കൂടുതൽ കായ ഉത്പാദനം (19 എണ്ണം) ഉയർന്ന വിളവ് (369.57 ഗ്രാം/ചെടി) എന്നിവ രേഖപ്പെടുത്തി. താരതമ്യേന സാമ്യമായ വിളവ് ടി. ആർ. പി. എൻ-3, ടി. ആർ. പി. എൻ-17 എന്നിവയുടെ സംയോജിത രൂപം പ്രയോഗിച്ച ചെടികളിൽ രേഖപ്പെടുത്തി.

ഫീൽഡ് പഠനത്തിലും ടി. ആർ. കെ. ആർ-2, ടി. ആർ. പി. എൻ-3 , കെ. എ. യു. സ്ട്രെയിൻ, ടി. ആർ. കെ. ആർ-2, ടി.ആർ. പി. എൻ-17 എന്നിവയുടെ സംയോജിത രൂപം എന്നിവ വള്ളിപ്പയറിന്റെ ഫ്യുസേറിയൽ വാട്ടരോഗം വലിയ തോതിൽ നിയന്ത്രിച്ചു. ടി. ആർ. കെ. ആർ-2, ടി.ആർ. പി. എൻ-17 എന്നിവയുടെ സംയോജിത രൂപം പ്രയോഗിച്ച ചെടികളിൽ നീളം കൂടിയ കായ്കളും കൂടുതൽ വിത്തുകളും ഉത്പാദിപ്പിച്ചതായി കണ്ടെത്തി. കൂടുതൽ എണ്ണം കായ്കൾ പരമാവധി വിളവ് (1172.63 ഗ്രാം/ചെടി) എന്നിവ, ടി. ആർ. പി. എൻ-3 പ്രയോഗിച്ച ചെടികളിൽ രേഖപ്പെടുത്തി. പൂക്കുന്നതിനുള്ള സമയം ടി. ആർ. പി. എൻ-3 പ്രയോഗിച്ച ചെടികളിൽ താരതമ്യേന കുറവ് രേഖപ്പെടുത്തി. വിത്ത് നട്ട് 90 ദിവസങ്ങൾക്ക് ശേഷം വിവിധ ട്രീറ്റ്മെന്റുകൾ നടത്തിയ സ്ഥലങ്ങളിലെ മണ്ണ് പരിശോധിച്ചതിൽ പോട്ട് കൾച്ചറ് പഠനത്തിലും ഫീൽഡ് പഠനത്തിലും ടി. ആർ. പി. എൻ-3 ഐസൊലേറ്റ് പ്രയോഗിച്ച ഇടങ്ങളിൽ ട്രൈക്കോഡെർമയുടെ അളവ് വളരെ കൂടുതലായി കണ്ടെത്തി.

ഈ പഠന ഫലങ്ങൾ, വള്ളിപ്പയറിന്റെ ഫ്യൂസേറിയൽ വാട്ടരോഗ നിയന്ത്രണത്തിന് ട്രൈക്കോഡെർമ ഐസൊലേറ്റുകളായ ടി. ആർ. കെ. ആർ-2, ടി. ആർ. പി. എൻ-3, ടി. ആർ. പി. എൻ-3, ടി.ആർ. പി. എൻ- 17 എന്നിവയുടെ സംയോജിത രൂപം എന്നിവ വളരെ മികച്ചതാണെന്ന് തെളിയിക്കുന്നു. മണ്ണിലൂടെ ഉണ്ടാകുന്ന വള്ളിപ്പയറിന്റെ മറ്റ് രോഗങ്ങളുടെ നിയന്ത്രണത്തിന് മേൽ പറഞ്ഞ ഐസൊലേറ്റുകളുടെ ഉപയോഗം, ഇവയുടെ സ്പീഷീസ് നിർദ്ദിഷ്ട തിരിച്ചറിയൽ എന്നിവയിൽ തുടർന്നും ഗവേഷണം നടത്തേണ്ടതുണ്ട്.