MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *Trichoderma* ISOLATES FROM TUBER CROP ECOSYSTEM

By,

LINET K. JOSEPH

(2014-09-114)

THESIS

Submitted in partial fulfilment of the

requirement for the degree of

B. Sc. - M. Sc. (INTEGRATED) BIOTECHNOLOGY

Faculty of Agriculture

Kerala Agricultural University, Thrissur

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695 522

KERALA, INDIA

2019

DECLARATION

I, hereby declare that this thesis entitled **"Morphological, biochemical and molecular characterization of *Trichoderma* isolates from tuber crop ecosystem"** 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 of any degree, diploma, associate ship, fellowship or other similar title, of any other university or society.

Vellayani
16.11.2019

LINET K. JOSEPH
(2014-09-114)

स्वच्छ भारत
एक कदम स्वच्छता की ओर

भा.कृ.अनु.प- केंद्रीय कन्द फसल अनुसंधान संस्थान
(भारतीय कृषि अनुसंधान परिषद, कृषि और किसान कल्याण मंत्रालय, भारत सरकार)
श्रीकार्यम, तिरुवनन्तपुरम-695 017, केरल, भारत

ICAR- CENTRAL TUBER CROPS RESEARCH INSTITUTE
(Indian Council of Agriculture Research, Ministry of Agriculture and Farmers Welfare, Govt. of India)
Sreekariyam, Thiruvananthapuram-695 017, Kerala, India

ISO 9001:2008

CERTIFICATE

Certified that this thesis entitled "**Morphological, biochemical and molecular characterization of *Trichoderma* isolates from tuber crop ecosystem**" is a record of research work done independently by **Ms. Linet K. Joseph**. (2014-09-114) under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Dr. (Mrs.) Veena. S. S.
Principal Scientist (Plant Pathology)
Division of Crop Protection
Central Tuber Crops Research Institute
Sreekariyam, Thiruvananthapuram - 695 017
Kerala, India

16/11/19

Sreekariyam
16.11.2019

Dr. S. S. Veena
(Chairman, Advisory Committee)
Principal Scientist
Division of Crop Protection
ICAR - CTCRI,
Sreekariyam,
Thiruvananthapuram

Phone : +91-471-2598551 to 2598554
Director (Per) : +91-471-2598431
(Res) : +91-471-2597211
Sr. Admn. Officer : +91-471-2598193
Fax : +91-471-2590063
E-mail : director.ctcri@icar.gov.in
ctcritvm@gmail.com
Web : http://www.ctcri.org

CERTIFICATE

We, the undersigned members of the advisory committee of Ms. Linet K. Joseph (2014-09-114), a candidate for the degree of B. Sc. - M. Sc. (Integrated) Biotechnology, agree that the thesis entitled **"Morphological, biochemical and molecular characterization of *Trichoderma* isolates from tuber crop ecosystem"** may be submitted by Ms. Linet K. Joseph in partial fulfillment of the requirement for the degree.

Dr. S. S. Veena
(Chairman, Advisory Committee)
Principal Scientist
Division of Crop Protection
ICAR –CTCRI, Sreekariyam
Thiruvananthapuram - 695017

Dr. K. B. Soni
(Member, Advisory Committee)
Professor and Head
Dept. of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram - 695522

Dr. J. Sreekumar
(Member, Advisory Committee)
Principal Scientist
Section of ESS
ICAR – CTCRI, Sreekariyam
Thiruvananthapuram - 695017

Dr. Swapna Alex
(Member, Advisory Committee)
Professor and Course Director
B. Sc – M. Sc. (Integrated)
Biotechnology Course
Dept. of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram - 695522

Dr. Vinayaka Hegde
(External Examiner)
Principal Scientist
(Plant Pathology) & Acting Head
Division of Crop Protection
ICAR – CPCRI
Kasaragod - 671124

ACKNOWLEDGEMENT

With boundless love and appreciation, I would like to extend my heartfelt gratitude and appreciation to the people who helped me in bringing this study into reality. It is with my heartfelt feelings I express my deep sense of gratitude to my beloved advisor Dr. S. S. Veena, Principal Scientist (Division of Crop Protection, ICAR-CTCRI, Sreekariyam) for her persistent interest in my work, continuous support and constant encouragement throughout my study. I am highly indebted for her valuable guidance.

I also express my deepest gratitude to the advisory committee members, Dr. K. B. Soni (Professor and Head, Department of Plant Biotechnology, CoA), Dr. J. Sreekumar (Principal Scientist, Section of ESS, ICAR-CTCRI) and Dr. Swapna Alex (Professor and Course Director, B. Sc. – M. Sc. (Integrated) Biotechnology Course, Department of Plant Biotechnology, CoA) for all their help and encouragement during my work.

I take immense pleasure to express my deep sense of gratitude to Dr. A. Anilkumar (Dean, CoA) and Dr. Archana Mukherjee (Director, ICAR-CTCRI) for providing me all the facilities during the course of my work. I express my gratitude to Dr. C. A. Jayaprakash (Head, Division of Crop Protection, ICAR-CTCRI), Dr. T. Makesh kumar (Principal Scientist, Division of Crop Protection, ICAR-CTCRI), Dr. M. L. Jeeva (Principal scientist, Division of Crop Protection, ICAR-CTCRI) and Ms. Sangeetha B. G. (Scientist, Division of Crop Protection, ICAR-CTCRI) for their meaningful suggestions.

I would like to thank Dr. S. Karthikeyan, Dr. G. Sreelatha, Mrs. Amrutha Akhil, Ms. Jyothilekshmi O. B., Mr. Tom Cyriac, Mr. Jayakrishnan J. T., Ms. Adithya V. and Mrs. Sujina Satheesh for their constant encouragement during this project. The support extended by Mr. Shinil T. M., Mrs. Nija Sajeev and Smt. Usha throughout this work is gratefully acknowledged.

I would like to put on record my sincere thanks and gratitude to Dr. C. Mohan (Principal Scientist, Division of Crop Improvement, ICAR-CTCRI) and Dr. Senthilkumar K. M. (Scientist, Division of Crop Improvement, ICAR-CTCRI) for their comments. I express my thanks to Dr. Joy M. (Associate Professor & Head, Department of Plant Pathology, CoA) for allowing me to use the Microscope facility.

Last but not least, I thank my entire family for their support and encouragement. I would like to thank my father, K. A. Joseph and mother, Liju Joseph for their selfless sacrifice and great efforts with pain and tears and unceasing prayers which enabled me to successfully complete the research work. I would like to thank my spouse, Antony Raju for his continuous support. My special gratitude towards my brothers, Christy Joseph and Alan Joseph. I would also like to extend my gratitude towards my classmates and roommates.

Above all, I bow before your Almighty.

Linet K. Joseph

DEDICATED TO MY FAMILY

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVATIONS

%	Percentage
µl	Microlitre
µM	Micromolar
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cm	Centimetre
DNA	Deoxyribo nucleic acid
g	Gram
h	Hour
hrs	Hours
mg	Milligram
MgCl2	Magnesium Chloride
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
ng	Nanogram
nm	Nanometre
no.	Number
nos.	Numbers
°C	Degree Celsius
PCR	Polymerase Chain Reaction
PVP	Polyvinyl pyrrolidine
RNase	Ribonuclease

rpm	Revolution per minute
s	Seconds
sp	Species
TE	Tris EDTA
U	Enzyme unit
UV	Ultraviolet
VOCs	Volatile organic compounds
V	Volt

INTRODUCTION

1. INTRODUCTION

Elephant foot yam (*Amorphophallus paeonifolius* (Dennst.) Nicolson) is an important tuber crop of tropical and sub-tropical countries which offers an exceptional reach as a cash crop because of its high production potential (50-80 t/ha), market acceptability, medicinal properties and lucrative economic returns (Misra, 1997).

Amorphophallus is susceptible to a number of diseases, often causing heavy loss to the crop. The plant is affected by three main diseases in the field, *viz*., collar rot, mosaic and leaf blight. Among them, collar rot caused by *Sclerotium rolfsii* is the most destructive and common disease prevalent in the entire elephant foot yam growing areas. Collar rot causes yield loss up to 100% (Misra, 1997). In addition, it is the major cause for the post harvest loss. The heavy and widespread application of chemical pesticides has created public's growing concern for the human health conditions and the environmental pollution. Hence, managing the disease through the application of fungicides has been increasingly condensed. Biological control using antagonists has proved to be one of the most promising alternatives, either alone or as part of an integrated pest management policy to reduce pesticide use.

Trichoderma is one of the most important filamentous fungi common in soil and root ecosystems and used as an effective biocontrol agent for soil borne fungal plant pathogens and some species are also known for their abilities to enhance systemic resistance to plant diseases (Gajera *et al*., 2015).

Trichoderma is being recommended for mitigation of collar rot incidence. However, the ability of *Trichoderma* isolates to control *S. rolfsii* varies considerably. Screening and characterization of *Trichoderma* from different geographical locations provide diversity in terms of antagonistic potential and adaptation to tuber crops ecosystem, which can be exploited further for the control of collar rot.

Studies say that specific activity of cell wall degrading enzymes, chitinases and β-1,3-glucanase is positively correlated with growth inhibition of pathogen (Hirpara *et al.*, 2017). Even though the volatile organic compounds (VOCs) produced by *Trichoderma* have effect on growth, their kind, proportion and concentration vary with the producing species. In order to utilize the full potential of *Trichoderma* species in specific applications, precise identification and characterization of the fungi is vital.

In differentiating *Trichoderma* populations, simple sequence repeat (SSR) or microsatellite markers have become a preferred choice due to their multiallelic nature, co-dominant inheritance, high abundance, hyper variance, extensive genome coverage, reproducibility, and discriminatory power (Mahfooz *et al.*, 2012). This will help in identifying the best isolate and the entire process will ensure more precised and targeted application in field condition.

Hence the present study, "Morphological, biochemical and molecular characterization of *Trichoderma* isolates from tuber crop ecosystem" was carried out with specific objectives like:

1. To study the differential antagonistic potential of *Trichoderma* isolates against *Sclerotium rolfsii,* the collar rot pathogen of *Amorphophallus paeonifolius* (Dennst.).
2. To characterize the isolates using morphological, biochemical and molecular approaches.
3. To analyse the molecular diversity using SSR markers.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Root and tuber crops adorns as third important food crop of human kind after cereals and grain legumes (Edison, 2006). These crops also play a considerable role in world's food supply and contribute about 6 % of the world's dietary calories (Reddy, 2015). Tropical root and tuber crops (TRC) occur in different production systems and used as staple food in many under developed and developing countries of the world (Saranraj *et al.,* 2019). Globally, about 45 percent of root and tuber crop production is consumed as food, with the rest being used as animal feed or for industrial processing for products such as starch, distilled spirits, and a variety of minor products (Reddy, 2015). Tropical root and tuber crops consist of both dicots and monocots. Dicots include cassava and sweet potato. Whereas, yams and aroids like taro, tannia, and elephant foot yam are monocots.

According to 2012 statistics, the major root and tuber crops occupied about 53 million hectares, producing 797 million metric tons world-wide. In this, forty two percent of the contribution was from Asia and 6.45 % from India (Reddy, 2015). Cassava mosaic disease, tuber rot in cassava (*Phytophthora palmivora*), taro leaf blight (*P. colocasiae)*, mosaic diseases in taro and elephant foot yam (Dasheen mosaic virus), collar rot in elephant foot yam (*Sclerotium rolfsii),* yam anthracnose (*Colletotrichum gloeosporioides*) and viral diseases in sweet potato are the major diseases in tropical tuber crops (Reddy, 2015). Both the pre and post harvest losses of root and tuber crops as a result of pests and diseases are very high.

2.2 ELEPHANT FOOT YAM (EFY)

Elephant foot yam (*Amorphophallus paeonifolius* (Dennst.) Nicolson) is an important tuber crop of tropical and sub-tropical countries which is being considered as a cash crop because of its high production potential (50-80 t/ha), market

acceptability, medicinal properties and lucrative economic returns (Misra, 1997; Srinivas and Ramanathan, 2005 and Behera and Ray, 2016). Elephant foot yam offers excellent opportunities in world's food and nutrition security in coming years as the supply of cereals is decreasing (John *et al.*, 2015).

The crop is susceptible to many foliar pathogens as well as soil pathogens at different stages of growth and severely hampers the corm production and become serious constraint for elephant foot yam cultivation (Kumari *et al.*, 2013). Collar rot, mosaic and leaf spots are the most destructive diseases of elephant foot yam (Singh *et al.*, 2006). Among these diseases, the collar rot disease caused by *S. rolfsii* is the most ruinous and common disease in EFY growing areas which lead to yield loss up to 100% (Misra, 1997). *S. rolfsii* is a common soil borne pathogen with diverse host range which can survive both in soil and plant for long period with the help of rigid and resistant survival structures (Singh *et al.*, 2003).

2.3 COLLAR ROT DISEASE

Collar rot disease is most prevalent during rainy season followed by warm dry weather and is generally observed in the later stages of crop development, but it can infect the crop at any stages. Injury to the collar region during intercultural operations, poor drainage and water logging acts predisposing factors for infection (Kumari *et al.*, 2013). The disease causes damage by rotting the collar region which eventually leads to falling off the plant. It causes heavy reduction in yield and loss of quality of yield. Once the pathogen attacks the collar region, water soaked lesions appear on the pseudostems just above the soil surface. The leaf starts yellowing from the tip. Finally the entire plant falls due to rotting of the collar region. The white mycelial mat with lots of sclerotia of the pathogen could be seen on the collar region of the affected plant (Misra, 2008 and Reddy, 2015).

2.3.1 Disease management

S. rolfsii is a non specialized soil borne pathogen present world-wide and affects more than 500 species of economically important crops. Thus management of this pathogen with a single strategy including use of chemical fungicides seems to be difficult (Gogoi *et al.,* 2002). The present management strategy of collar rot disease includes practice of various physical, chemical and biological control methods (Punja, 1985).

The problems associated with the wide application of chemical methods are: the biomagnification of toxic compounds in food chain as well as in the environment (Cook and Baker, 1983) and the evolution of fungicide resistant pathogens (Dekker and Georgopolous, 1982). The growing apprehensions to use of hazardous fungicides and chemical fertilizers in agriculture have led several policy makers to encourage the research to develop alternative options for controlling plant diseases. In order to resolve these problems, along with physical methods an eco-friendly method was investigated. The use of biological control agents seems to be one of the promising approaches (Cook, 1985). Due to the ability of the bio-agents to provide eco-friendly disease control, bio-control of plant pathogens is regarded as the best alternative strategy in disease management (Nath *et al.,* 2014). Many bio-agents have been isolated, characterized and commercialized. The list includes *Trichoderma* species (Harman, 2006); *Verticillium* (Fenice *et al.,* 1998), *Pseudomonas, Bacillus* and *Streptomyces* (Weller, 2007; Froes *et al.*, 2012; Ashwini and Srividya, 2013). According to Harman *et al.* (2004), due to the multipronged action of *Trichoderma* against several economically important plant pathogens made the organism to gain significant attention.

2.4 *Trichoderma* AS BIOCONTROL AGENT

Among the investigated biological control agents, *Trichoderma* species have attracted special position due to the particular biological characteristics since the early 1930s (You *et al.,* 2016). *Trichoderma* is an important filamentous fungus which is common in soil and root ecosystems and is used as an effective bio-control agent for soil-borne fungal plant pathogens. Species of *Trichoderma* are known for their abilities to enhance systemic resistance to plant diseases (Gajera *et al.*, 2015). *Trichoderma* species have the ability to mitigate plant diseases and promote plant growth by multiple modes of action including systemic resistance, antibiosis, enhanced nutrient efficiency and mycoparasitism (Mathys *et al.,* 2012). *Trichoderma* are very promising bio-agents and are often added to soils to increase crop yields and control soil borne pathogen. In India alone, more than 250 *Trichoderma* based formulations are sold commercially (Lee *et al.,* 2016). *Trichoderma* species are prominent bio-agents used to control *S. rolfsii* (Rao *et al.,* 2004). *Trichoderma* which are soil-borne, free-living and non-pathogenic fungi are important in controlling several phytopathogens (Benitez *et al.,* 2004). It can colonize the rhizosphere as well as roots of many plants. Based on the report of Hjeljord and Tronsmo in 1998, several diseases in greenhouse and field conditions are also get controlled by seed treatment using *T. harzianum*.

The wide range of adaptability to different ecological niche by strains within the genus *Trichoderma* and its ability to live on diverse substrates make them competent. It was reflected in the fact that these fungi are common in soils all over the world, under diverse geographical locations. This diversity, coupled with their agreeable growth on cheapest medium, makes *Trichoderma* isolates an attractive candidate for a variety of biological control applications (Harman and Kubicek, 1998). According to reports, about 60 % of all the registered bio-fungicides across the world are *Trichoderma* based (Verma *et al.,* 2007). Harman *et al.* (2004) mentioned that

interaction of *Trichoderma* with microorganisms and plants are by diverse ways. Various mechanisms which support the striking performance of *Trichoderma* species during antagonism with plant pathogens are mycoparasitism, antibiosis, competition for nutrients and space, modification of the growing region, and/or stimulating plant growth and plant defense mechanisms (Benitez *et al.,* 2004 and Druzhinina *et al.,* 2011).

During interaction with pathogens, *Trichoderma* spp. attach *via.* coiling, hooks and appressorium-like bodies (Kubicek *et al.,* 2001). It also penetrates the host cell wall by secreting lytic enzymes (Kubicek *et al.,* 2001). Even within the *Trichoderma* genus, it exhibits numerous mechanisms during phytopathogen interaction. Thus necessitate the study of each mode of action to complete the research on biological control (John *et al.,* 2015). Hence, understanding the genetic variability of *Trichoderma* strains in terms of antagonistic potential, biological and biochemical activities are necessary to improve the selection of different isolates as biocontrol agents (Consolo *et al.,* 2012 and Sharma *et al.,* 2009).

Many workers have adopted *in vitro* screening techniques to evaluate the potential of *Trichoderma* isolates against fungal pathogens of economic importance.

2.4.1 Dual culture method

To study the direct effect of fungi on each other, inoculating them on a single PDA plate was recommended (Skidmore and Dickinson, 1976). Singly inoculated plates can serve as control (Skidmore and Dickinson, 1976). Gogoi *et al.* in 2015 adopted the dual culture method for the *in vitro* screening of *Trichoderma* isolates against fungal pathogens, *Pestalotia theae* and *Fusarium solani.* This method was also used to evaluate antagonistic activity of *Trichoderma* against pathogenic fungi of *Arachis hypogaea* (Yuzana and Thein, 2018). Dual culture method was used in studying antagonism of *T. harzianum* isolates and classifies the isolates in to

antagonism classes (Sharma *et al.*, 2009). The antagonistic potential of *T. atroviride* against *Fusarium* spp. was evaluated by dual culture technique (Srivastava *et al.*, 2012 and Singh *et al.*, 2013). Reddy *et al.* (2014) adopted dual culture technique to study the antagonistic potential of seven species of *Trichoderma* against different plant pathogenic fungi. Similarly antagonistic potential of 7 species of *Trichoderma* against the pathogen, *M. phaseolina* was studied by adopting dual culture method and then calculating *in vitro* percent growth inhibition (Gajera *et al.*, 2012). Based on the references, it was clear that dual culture method is the common and reliable method for the *in vitro* primary screening of *Trichoderma* spp. The formula used to calculate the percentage inhibition of fungal pathogen was, I = (C-T/C)×100. Where, I = percent inhibition; C = radial growth of pathogen (in mm) alone in the control plate; T = radial growth of pathogen (in mm) in the presence of *Trichoderma* isolates (Edington *et al.*, 1971).

2.4.2 Antibiosis test for production of diffusible inhibitory metabolites

During the biocontrol action of *Trichoderma* spp., antibiotics found to play an important role. Various volatile and non volatile secondary metabolites have also been characterized from *Trichoderma* spp. with high antagonistic potential and plant defense promoting activities (Brotman *et al.*, 2010). During the attempt to characterize secondary metabolites produced by *T. asperellum* isolates using GC-MS and LC-MS methods, studies have shown the existence of nine antifungal metabolites like Viridin, Viridiol, Butenolides, Harzianolides, Ferulic acid, Viridiofungin A, Cyclonerodiol, Massoilactone and Gliovirin (Srinivasa *et al.*, 2017). Nath *et al.*, 2014 and John *et al.*, 2015 used antibiosis test for production of diffusible inhibitory metabolites method to screen *Trichoderma* isolates against tuber crop pathogens.

2.4.3 Volatile compounds by *Trichoderma* isolates against pathogen

The volatile compounds produced by *Trichoderma* spp. are physiologically active and it takes part in signaling between microbes (Srinivasa *et al.*, 2017). Galindo *et al.*, in 2004, reported the volatile compound 6-pentyl-a-pyrone (6-PAP) in detail and its herbicidal and antimicrobial activity. Later it is found out as an important volatile compound synthesized by *Trichoderma* isolates and which imparts the characteristic smell of *T. asperellum* (coconut odour) (Srinivasa *et al.*, 2017). The production of VOCs by each *Trichoderma* isolates differs in quantity and quality. Based on the antagonistic potential exhibited by VOCs, a protocol was adopted (Dennis and Webster, 1971b).

2.5 BIOCHEMICAL APPROACH

Trichoderma spp. are highly valued for their mycoparasitic actions against plant pathogens of many economically important crops. The bio-control potential exhibited by this organism can occur by means of several antagonistic mechanisms such as nutrient competition, antibiotic production and mycoparasitism. Mycoparasites produce cell wall degrading enzymes and permit to extract nutrients from the host fungi (Gajera *et al.*, 2012). The ability of *Trichoderma* spp. to combat plant diseases is assigned to their direct antagonistic effects on the fungal pathogen, and mainly their ability to produce lytic enzymes *viz.*, chitinases and β-1,3-glucanases (Benítez *et al.*, 2004; Viterbo *et al.*, 2002). *Trichoderma* spp. grow on the root surface of various plants and soil and can inhibit the growth of pathogenic fungi by producing enzymes capable of degrading cell walls such as chitinase, β-1,3-glucanase, proteases and other hydrolysed enzymes (Prasetyawan, 2017). *Trichoderma* can check the growth of pathogens by producing enzymes capable of degrading cell walls such as chitinase, β-1,3-glucanase and other hydrolysed enzymes (Goldman *et al.*, 1994). Cell wall lytic enzymes are the important factor for its mycoparasitic action. Chitinases, β-1,3-glucanases and proteases are the major extracellular enzymes produced by

Trichoderma during fungal pathogen interaction (Lopes *et al.*, 2012; Geraldine *et al.*, 2013; Vos *et al.*, 2015). Gajera and Vakharia (2012) studied production of lytic enzymes by *Trichoderma* during *in vitro* antagonism and reported a significant positive correlation between percentage of growth inhibition of pathogen and lytic enzymes *viz.*, chitinase, β-1-3- glucanase and protease. Chitinase and β -1, 3-glucanase has been reported to be directly involved in the antagonism between *Trichoderma* spp. and its hosts (Kubicek *et al.*, 2001).

2.5.1 Production of chitinase enzyme

Chitin is the second most abundant polymer in nature and plays a major role in fungal cells. Chitinase is an extracellular enzyme produced by fungi and these chitin degrading enzyme breaks the β-1,4-glycosidic bonds between chitin N-acetyl glucosamine residues (Kitamura and Kamei 2003). Due to the production of variety of chitinase enzymes by *Trichoderma,* it has become a very important bio-control agent to combat fungal diseases (Agarwal and Kotasthane, 2012). Chitinases produced by *Trichoderma* spp. come under glycosyl hydrolase family and this enzyme is considered important during the bio-control action and putative antifungal activity. Chitinolytic activity of the indigenous strains of *Trichoderma* which are collected from the North Western regions of Pakistan was studied (Munir *et al.*, 2019). Tweddell *et al.* (1994) and Calistru *et al.* (1997) reported that crude culture filtrates of *T. harzianum* has β-1,3-glucanase and chitinase activities. It has the capacity to release reduced sugars (glucose, GlcNAc) from dried or fresh mycelium of pathogenic fungi. The difference in chitinase activity among five types of *Trichoderma* spp. was studied and compared and found that the activity varies greatly between strains (Prasetyawan, 2017). However, one of the studies opinioned that no correlation was found between coiling frequency of *T. harzianum* and cell wall degrading enzyme chitinase (Almeida *et al.*, 2007).

2.5.2 Production of β-1,3-glucanase enzyme

The fungal cell wall includes β-1,3-glucan as the main structural element and β-1,3-glucanase enzyme is accountable for the lysis of glucan present in the pathogenic fungal cell wall (Khatri *et al.,* 2017). Study made by Prasetyawan and Sulistyowati (2018) concluded that there was an increase in both chitinase and glucanase activity upon the addition of chitin, laminarin or cell wall of pathogenic fungi into the *Trichoderma* growing medium. This finding supports the previous findings made by Chen *et al.* in 1995. Years back Chen *et al.* (1995) mentioned the activated laminarinase (β-1,3-glucanase) enzyme production by *T. harzianum* upon the addition of cell wall of target fungus into the medium. Coiling and cell wall degrading enzyme activities were studied in connection with antagonism shown by *Trichoderma* spp. against *Macrophomina phaseolina* and found that the growth inhibition was positively correlated with coiling pattern of antagonists and induction of chitinase, β-1,3-glucanase and total phenol content (Gajera *et al.,* 2012). Eleven *Trichoderma* isolates were tested against *S. rolfsii* and found that specific activity of cell wall degrading enzymes *viz.*, chitinases and β-1,3-glucanase production capacities were positively correlated with growth inhibition of the test pathogen (Hirpara *et al.*, 2017).

2.5.3 Effect of VOCs produced by *Trichoderma* isolates on plant growth

Trichoderma species are known producers of metabolites with medical and agricultural importance (Mathivanan *et al.,* 2008; Mukherjee *et al.,* 2012). *Trichoderma* has various routes to enhance plant growth. One of the route is volatile metabolite production by *Trichoderma* isolates which contribute to the growth promotion by switching on various pathways related to auxin synthesise (Hung *et al.,* 2013). Volatile metabolites or volatile organic compounds (VOCs) are with low molecular mass, high vapour pressure, low boiling point and low polarity (Insam and Seewald, 2010). The role of microbial VOCs in direct interaction with plants was first reported by Ryu *et al.* (2003, 2004) from bacteria. Volatile metabolites play a lead

role in mycoparasitism of *Trichoderma* species and their interactions with other organisms and plants (Tabarestani *et al.*, 2016). The coconut odor volatile, 6- pentyl 2H-pyran-2-one (6PP) is the first volatile compound to be isolated and studied from *Trichoderma* (Lee *et al.*, 2016). Hung *et al.* (2013) reported the ability of mixtures of VOCs from *T. viride* to promote plant growth in the absence of pathogen attack. *Arabidopsis thaliana* exposed to VOCs from *T. viride* promoted plant size, fresh weight, chlorophyll, root growth, number of flowers even without the attack of pathogen. The volatile-mediated growth promotion depended on species of *Trichoderma*, culture, stage of the plant, duration of the exposure (Hung *et al.*, 2013; Lee *et al.*, 2015). Lee *et al.* (2016) screened 11 species of *Trichoderma* for VOC production. The results of their study pointed that, 9 out of 11 species emitted VOC mixtures which could significantly promote growth in *Arabidopsis*. Similarly, Jalali *et al.* (2017) studied beneficial effects of VOCs from 13 strains of *Trichoderma* and found that *Arabidopsis* plants responded differently to VOCs of various strains and showed no change to significant growth promotion. *T. virens* VOCs elicit both plant growth and defense programs in *Arabidopsis* (Contreras-Cornejo *et al.*, 2014). *T. asperellum* (IsmT5) increased numbers of trichomes, accumulation of defense-related compounds and expression of defense-related genes (Kottb *et al.*, 2015). *Trichoderma* strains can produce many metabolites that are similar to plant metabolites produced under stressful environment. Thus the volatile compounds released by growth inducing *Trichoderma* strains mimics plant metabolites and provide clues to plant which ultimately trigger growth of plant (Lee *et al.*, 2016).

2.6 CHARACTERIZATION OF *Trichoderma* ISOLATES

Trichoderma is a fungal genera that has more than 200 species that occur all over the world in different climatic zones (Atanasova *et al.*, 2013; Kredics *et al.*, 2014). Precise identification and characterization of *Trichoderma* isolates is essential to tap the full potential of the species in specific applications (Lieckfeldt *et al.*, 1999).

Earlier taxonomy of the species was based mostly on morphological characters (Rifai, 1969; Bissett, 1984). Later, identification of the species was done based on morphological and cultural characteristics (Shahid *et al.,* 2013). Many species of *Trichoderma* have comparable physical appearance under cultural circumstances and demonstrate comparable morphological characters, although they are entirely distinct species. It is therefore suggested that multiple approach can be implemented for the accurate detection of *Trichoderma* isolates to its species-level (Siddiquee, 2017).

2.6.1 Morphology based approach

Combinations of physiological features as well as microscopic observations still have practical and scientific significance in the examination of fungi. Microscopic observations are widely used in fungal research and commercial labs (Siddiquee, 2017). Fahmi *et al.*, 2016 reported that in their study, on the basis of morphological characteristics, 75% of isolates were identified to species level.

Hibbet *et al.* (2011) stated that, in the varied classification of fungi traditional morphology based classification is still permitted. Based on the observation by Samuels *et al.* (2002), there were slight variations in colony appearance of *Trichoderma* when cultured over Potato Dextrose Agar (PDA) and Cornmeal Dextrose Agar (CMD). *Trichoderma* species have been reported to be white on rich media such as PDA and more transparent when grown on media such as CMD (Samuels *et al.,* 2002; Shah *et al.*, 2012). Compared to other media, they also reported PDA as the better media in order to make observations such as mycelial growth and pigmentation (Samuels *et al.*, 2002). Some species of *Trichoderma* are characterized by lack of pigment production in reverse side of the colony, whereas reddish brown pigmentation occurs in some species (Gams and Bissett, 1998). Gams and Bissett (1998) also reported growth rates of isolates in culture as a better method to distinguish between morphologically similar species. *Trichoderma* colonies have many important characteristics like growth rate, growth pattern, pigmentation, pustule

formation, and odors that can be used to identify it as *Trichoderma* species (Siddiquee, 2017). Along with macro morphological characters, microscopic observations such as spores and their arrangement, conidiophores, conidia, phialide and chlamydospore are very essential in the classification of *Trichoderma* isolates (Siddiquee, 2017). Shahid *et al.* (2014) used two techniques, visual observation on Petri dishes and micro-morphological studies in slide culture for identification of *Trichoderma* species. For visual observation, *Trichoderma* was grown on PDA agar for 3-5 days. The pattern of mycelial growth, color, odor and changes of medium color for each isolate were monitored. For micro-morphological studies, a slide culture technique was used. Examination of the shape, size, arrangement and development of conidiophores or phialides provided a tentative identification of *Trichoderma* spp. The isolates under study varied in colony color, colony morphology, growth rate, colors of conidia, conidiophores branching pattern etc (Savitha and Sriram, 2015).

2.6.2 Molecular approach

Molecular biology techniques have proven to be precious tool in fungal taxonomy and its application has led to the reconsideration of several genera (LoBuglio *et al.*, 1993). Due to varied economic applications of *Trichoderma*, the right species identification is essential. Even though, the classification using morphological characters is being practiced, incorrect species identification using morphological characters is very prevalent even for professionals due to the more resemblance of morphological characters (Samuels *et al.*, 2010; Anees *et al.*, 2010). Identification, solely depending on morphological characterization was insufficient to identify *Trichoderma* species because they have comparatively few morphological characters and less variation that cause overlapping and improper identification of the isolates. Hence, it was essential to use molecular technique for précised characterization (Fahmi *et al.*, 2016). To resolve this misidentification, many

molecular techniques and recognition instruments have been created based on DNA sequence analysis. Therefore, it is now feasible to define each *Trichoderma* isolate to its species level (Druzhinina *et al.,* 2005). Application of molecular techniques in taxonomy could resolve inaccuracies related to species identification in *T. atroviride* as well as demonstrated interspecies genetic variability (Skoneczny *et al.*, 2015).

Certain molecular methods that are being used includes, sequence evaluation of the ribosomal DNA internal transcribed spacer (ITS) (ITS1—5.8S rDNA — ITS2) as well as the translation elongation factor 1-alpha (*tef*1) gene, endochitinase (*chi18-5*, earlier known as *ech42*), RNA polymerase II subunit (*rpb2*) and calmodulin (*cal1*) (Kullnig-Gradinger *et al.,* 2002; Druzhinina *et al.,* 2008). The internal Transcribed Spacer (ITS) region is the most widely sequenced region of fungal DNA. ITS is the most used gene for molecular systematic study at species level, and even within species (Ospina- Giraldo *et al.*, 1998; Fahmi *et al.*, 2016). They are appropriate for accurate diagnosis and thus allow the problem of morphology based species identification to be addressed (Gupta *et al.,* 2014). The ITS region is one of the most reliable loci for the identification of *Trichoderma* at the species level and comparison of the sequences of the 5.8S-ITS region to the sequences deposited in GenBank, all of the *Trichoderma* isolates were identified to species level with homology percentage of at least 99% (Kullnig-Gradinger, 2002). The molecular identification of *Trichoderma* isolates was done by using ITS gene 1 and 2 and *tef*1 amplification and its sequence analysis (Nath *et al.*, 2014; John *et al.*, 2015; Savitha and Sriram, 2015).

Despite the wide use of ITS region, GenBank database contain plenty of sequences of *Trichoderma* isolates which may have been wrongly identified and comes under a false name. Due to this fact, search tool named *Trich*OKEY that precisely compare ITS1 and ITS2 sequences to a specific database for *Trichoderma* generated from only verified sequences were used to assess the reliability of BLAST

results (Druzhinina and Kubicek, 2005). *Trich*OKEY was used by many researchers and resulted in precised identification of *Trichoderma* isolates (Anees *et al.,* 2010).

2.6.3 Genetic diversity study using SSR markers

Genetic diversity means any difference in nucleotides, genes, chromosomes or whole genomes of organisms (Wang *et al.,* 2009). Diversity among organisms occur mainly due to the mutations resulting from substitution of single nucleotides, insertion or deletion of DNA fragments, duplication or inversion of DNA fragments and can be due to recombination. The genetic diversity can be studied among different accessions/ individuals within same species (intra specific), among species (inter specific) and between genus and families (Mittal and Dubey, 2009).

On studying the variability in *Trichoderma* population, use of molecular markers like random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), and inter simple sequence repeats (ISSRs) have been reported (Shahid *et al.,* 2013; Shalini Devi *et al.,* 2012; Muthu Kumar and Sharma 2011; Sadfi-Zouaoui *et al.,* 2009; Sharma *et al.,* 2009; Vahabi *et al.,* 2009). These markers have many limitations and in order to overcome the limitations of RAPD, AFLP and ISSR markers in diversity studies, simple sequence repeat (SSR) markers are being recommended due to the reproducibility, multi-allelic nature, codominant inheritance, relative abundance and good genome coverage (Singh *et al.,* 2014; Kumar *et al.,* 2012; Mahfooz *et al.,* 2012). SSR markers are referred to as stretches of DNA consisting of tandemly repeating motifs of variable lengths that are distributed throughout the eukaryotic nuclear genome in both coding and non coding regions (Jarne and Lagoda, 1996). Simple sequence repeat (SSR) polymorphisms were able to amplify 202 alleles across 11 *Trichoderma* isolates. Through this, a *Trichoderma* isolate was identified with 7 unique SSR alleles amplified by 5 SSR markers (Hirpara *et al.*, 2017).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 LOCATION

The study entitled "Morphological, biochemical and molecular characterization of *Trichoderma* isolates from tuber crop ecosystem" was carried out at the Division of Crop Protection, ICAR - Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram with time duration of 11 months starting from 1st October 2018 to 5th August 2019. In this chapter, details related to the experimental materials and procedures used in the study are elaborated.

3.2 SAMPLE COLLECTION

Forty-three isolates of *Trichoderma* obtained from tuber crops ecosystem and maintained at Microbial repository, ICAR-CTCRI were used for the study. The details of locations from where the isolates were obtained are given below (Table 1).

Table 1. Details of *Trichoderma* isolates used for the study

Isolate code	Place of collection
T1	Moonnar, Kerala (Tr9)
T2	Sreekariyam,Thiruvananthapuram (Tr15)
T3	Belagavi, Karnataka
T4	Sreekariyam, Thiruvananthapuram
T5	Sreekariyam, Thiruvananthapuram
T6	Kadakkal, Kollam
T7	Sreekariyam, Thiruvananthapuram
T8	Pullad, Pathanamthitta
T9	Arunachal Pradesh
T10	Sreekariyam, Thiruvananthapuram
T11	Bhubaneswar, Odisha
T12	Belagavi, Karnataka
T13	Sreekariyam, Thiruvananthapuram
T14	Pathanamthitta
T15	Sreekariyam, Thiruvananthapuram
T16	Arunachal Pradesh
T17	Pallimukku, Kollam

T18	Kottayam
T19	Sreekariyam, Thiruvananthapuram
T20	Sreekariyam, Thiruvananthapuram
T21	Sreekariyam, Thiruvananthapuram
T22	Mavelikkara
T23	Ranni, Pathanamthitta
T24	Ranni, Pathanamthitta
T25	Neyyatinkara, Thiruvananthapuram
T26	Pathanamthitta
T27	Aranmula, Pathanamthitta
T28	Anchal, Kollam
T29	Aranmula, Pathanamthitta
T30	Thottapuzhassery, Pathanamthitta
T31	Thottapuzhassery, Pathanamthitta
T32	Punalur, Kollam
T33	Aranmula, Pathanamthitta
T34	Alangad, Ernakulam
T35	Pathanamthitta
T36	Kattappana, Idukki
T37	Kattappana, Idukki
T38	Sreekariyam, Thiruvananthapuram
T39	Kattappana, Idukki
T40	Sreekariyam, Thiruvananthapuram
T41	Sreekariyam, Thiruvananthapuram
T42	Sreekariyam, Thiruvananthapuram
T43	Sreekariyam, Thiruvananthapuram

3.3 MAINTENANCE OF *Trichoderma* ISOLATES

The isolates were sub cultured periodically and were maintained on Potato Dextrose Agar (PDA) plates and slants under the culture condition at 28±2°C.

3.4 SCREENING OF *Trichoderma* ISOLATES AGAINST *Sclerotium rolfsii*

Adopting three *in vitro* screening methods *viz.*, dual culture, production of diffusible metabolites and volatiles, the differential antagonistic potential of all the isolates were assessed.

3.4.1 Dual culture method (Skidmore and Dickinson, 1976)

Mycelial discs of 5mm diameter were taken from the actively growing culture of each isolate. These discs were used for the *in vitro* testing of antagonistic potential of the isolates. The inhibitory action was assessed by inoculating two discs on a single PDA plate, one from the pathogen and other one from *Trichoderma* isolate with a distance of 3 cm apart from each other. Three replicates were maintained for each isolate and the mycelial growth of *S. rolfsii* was measured at an interval of 24h. The plates inoculated with *S. rolfsii* alone served as the control. On day 3, mycelial growth of *S. rolfsii* completely covered 90 mm Petri-plates in control plates. The radial growth of *S. rolfsii* was measured, and percentage of inhibition was calculated in relation to growth of the controls as follows:

$$I = (C - T/C) \times 100$$

Where, I = percent inhibition; C = radial growth of pathogen (in mm) alone in the control plate; T = radial growth of pathogen (in mm) in the presence of *Trichoderma* isolates (Edington *et al.,* 1971).

3.4.2 Antibiosis test for production of diffusible inhibitory metabolites (Dennis and Webster 1971a)

This test was performed to evaluate the role of diffusible inhibitory metabolites produced by *Trichoderma* isolates on arresting the growth of *S. rolfsii*, the pathogen. In this method, *Trichoderma* discs were inoculated centrally on sterile cellophane membrane placed over the PDA medium in Petri plates. The *Trichoderma* inoculated plates were incubated for 2 days at 28±2 °C. The cellophane membranes along with mycelial growth of *Trichoderma* isolates were removed aseptically using sterile forceps. Mycelial discs (5 mm) of *S. rolfsii* were cut from actively growing cultures and were inoculated in the centre of the plates where *Trichoderma* was grown earlier on cellophane membrane. Plates without the prior inoculation of *Trichoderma* under the above-mentioned conditions served as the control. Mycelial growth of *S. rolfsii*

was measured at 24h interval. The percentage inhibition of *S. rolfsii* was calculated in relation to growth of the controls as follows:

$$I = (C - T/C) \times 100$$

Where, I = percent inhibition; C = radial growth of pathogen (in mm) alone in the control plate; T = radial growth of pathogen (in mm) on PDA plates with prior inoculation of *Trichoderma* isolates.

3.4.3 Antibiosis test for production of volatile compounds by isolates against pathogen (Dennis and Webster 1971b)

The isolates were screened by adopting this method to evaluate the potential of volatile compounds produced by *Trichoderma* isolates on checking the growth of *S. rolfsii.* The PDA plates were separately inoculated centrally with mycelial discs of *Trichoderma* isolates and *S. rolfsii.* The bottom portion of the plates with *S. rolfsii* inoculum replaced the lids of the Petri plates inoculated with *Trichoderma.* The plates were sealed using an adhesive tape and incubated for 2-3 days. The radial growth of *S. rolfsii* was measured at an interval of 24 h. The percentage inhibition of *S. rolfsii* was calculated in relation to growth of the controls as follows:

$$I = (C - T/C) \times 100$$

Where, I = percent inhibition; C = radial growth of pathogen (in mm) alone in the control plate; T = radial growth of pathogen (in mm) on PDA plates placed over the *Trichoderma* inoculated bottom dish.

3.4.4 Additive effect

To identify the most potent isolates, additive effect of the isolates (% inhibition shown by the isolate in dual culture + inhibition by diffusible metabolites + inhibition by volatile compounds) were calculated and based on the effect 26 isolates were selected for further study.

3.4.5 Statistical analysis

The data were statistically analysed using SAS statistical software (SAS 2010 – SAS Institute Inc., Cary, North Carolina, USA).

3.5 MORPHOLOGICAL CHARACTERIZATION OF *Trichoderma* ISOLATES

The details of macro and micro morphological descriptors used for the characterization of *Trichoderma* strains in PDA media are given below.

I. Macro morphological characteristics

- Mycelial growth rate
- Colony colour
- Culture smell
- Colony colour (surface and reverse)
- Mycelial form

II. Micro morphological characteristics

- Shape of conidia
- Conidiophore branching
- Size of conidia (µm)
- Size of phialides (µm)

To study the macro-morphological characteristics of selected 26 *Trichoderma* isolates, the isolates were grown on solidified PDA medium. Mycelial discs of 5mm diameter were taken from the actively growing culture of each isolate and were inoculated at the center of Petri dish that contained PDA medium. For each isolates, three replicates were maintained and the plates were incubated at 28±2°C. The radial growth of mycelium was recorded at an interval of 24h and the colony morphology was studied.

To study the micro-morpho characters, slide culture technique was adopted. For slide culture, 2% plain agar was prepared and 10 ml of the medium was poured into the half portion of sterile Petri dishes by keeping the dishes in slanting position (Fig. 1). After the solidification of medium, pre-sterilized glass slides were kept on the empty portion of Petri dish and mycelial discs were inoculated at the far end of medium. The Petri dishes were incubated at 28±2°C for 3 days. Glass slide was taken out carefully and stained using lacto phenol cotton blue. The micro-morpho characters were observed under microscopic field (40X) and photographed with image analyzer (Leica Microsystems).

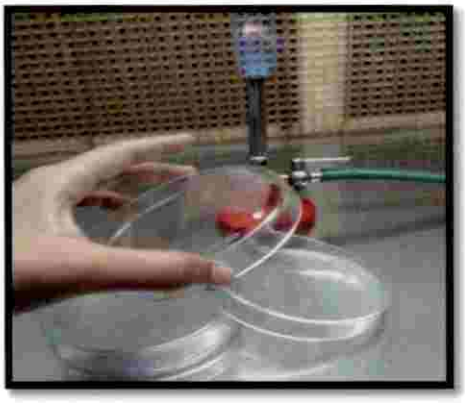


Fig. 1: Medium pouring and keeping of slides for slide culture

3.6 BIOCHEMICAL ASSAY

The efficiency of various isolates for production of chitinase and β-1, 3-glucanase was studied using the cell wall of *S. rolfsii* as carbon source. The induction of extracellular lytic enzyme production was done by culturing each *Trichoderma* isolate in Czapek's broth amended with 1% (w/v) *S. rolfsii* mycelium (Appendix I) as the carbon source (John *et al.,* 2015). Three 5mm discs of *Trichoderma* isolate were used to inoculate 250ml Erlenmeyer flask containing 75ml medium. The seeded flasks were incubated at 150rpm on a shaker for 7 days at 28°C. Then the culture filtrates were centrifuged at 5000×g for 10 min at 4°C and was stored at -20°C until the enzyme assay.

3.6.1 Chitinase enzyme assay

3.6.1.1 Preparation of colloidal chitin (Berger and Reynold, 1958)

Thirty ml of concentrated HCl was added to 5g of chitin (Sigma Aldrich) and was incubated overnight at 4°C. The mix was then added slowly to 250ml of 50% chilled ethanol by constant stirring. Pellet out the colloidal chitin by centrifugation of the mixture at 10,000 rpm at room temperature (28 ±2°C). The pellet was washed thoroughly using sterile distilled water for three times and centrifuged. The resulting pellet was saved for making 0.5% colloidal chitin in 50 mM acetate buffer of pH 5.2.

3.6.1.2 N-acetyl glucosamine (GlcNAc) standard curve preparation

N-Acetyl glucosamine (GlcNAc) stock (20 mg/ml) was prepared and it was used to make series of dilutions of GlcNAc at 0.05 mg/ml, 0.1 mg/ml, 0.15 mg/ml, 0.2 mg/ml, 0.25 mg/ml, 0.3 mg/ml. 1ml of each of the dilution was taken and 1ml of 3,5-dinitrosalicylic acid (DNS) (Appendix II) was added to it. Then it was heated at 95 °C for 10 minutes and absorbance was measured at 540 nm. This could also be used in the future to interpret chitinase activity by locating the concentration of GlcNAc corresponding to a particular absorbance value at 540 nm on the standard curve.

3.6.1.3 Chitinase enzyme assay using colorimetric method

Chitinase (EC 3.2.1.14) activity was assayed using the colorimetric method as described by Molano *et al.* (1977) with minor modifications. The assay mixture contained 1ml of 0.5% colloidal chitin (suspended in 50 mM acetate buffer pH 5.2) and 1 ml of enzyme solution. The reaction mixture was incubated for 1hr at 40 °C with shaking. Placing the vials into a boiling water bath for 5 min stopped the reaction and then 1 ml of dinitrosalicylate (Appendix II) was added to the reaction mixture. The mixture was again placed in boiling water bath for 5 min. The absorbance was

measured at 540 nm. The amount of reducing sugar released was calculated using a standard curve recorded for GlcNAc in mg/ ml. The chitinase activity was expressed in pmol GlcNAc released per sec (pmol/s). To express the activity in terms of pkat GlcNAc released per ml,

kat = mole/sec

pkat = pmole/sec

Hence, 1 pmol/sec/ml = 1pkat/ ml

One picokatal (pkat) of enzyme activity releases one pmol s^{-1} of GlcNAc equivalent under the specific assay conditions.

3.6.2 β-1, 3- glucanase enzyme assay

3.6.2.1 Standard curve preparation of glucose

Glucose stock (20 mg/ml) was prepared and it was used to make series of dilutions of glucose at 0.05 mg/ml, 0.1 mg/ml, 0.15 mg/ml, 0.2 mg/ml, 0.25 mg/ml, 0.3 mg /ml. 1 ml of each of the dilution was taken and 1 ml of 3,5-dinitrosalicylic acid (DNS) was added to it. Then it was heated at 95 °C for 10 minutes and absorbance was measured at 540 nm. This could also be used in the future to interpret β-1,3-glucanase activity by locating the concentration of glucose corresponding to a particular absorbance value at 540 nm on the standard curve.

3.6.2.2 β-1,3-glucanase enzyme assay using colorimetric method

β-1,3-glucanase (EC 3.2.1.3.9) activity was assayed by incubating 1ml enzyme solution to 2 ml of 5mg ml^{-1} laminarin (50 mM acetate buffer, pH 4.8) at 50 °C for 1hr and determination of glucose with dinitrosalicylate. The amount of reducing sugar released was calculated using a standard curve recorded for glucose (mg/ml). One nanokatal (nkat) of β-1,3-glucanase activity was defined as the amount of enzyme that releases one nmol $s^{-1}$of glucose equivalent under specific assay conditions.

To express the activity in terms of nkat glucose released per ml,

kat = mole/sec

nkat = nmole/sec

Hence, 1 nmol/sec/ml = 1nkat/ ml

3.7 EFFECT OF VOLATILE ORGANIC COMPOUNDS (VOCs) ON PLANT GROWTH

A modification of the method used by Lee *et al.* (2016) was adopted for studying the effect of VOCs produced by *Trichoderma* isolates on plant growth. For the *in vitro* study, mustard seeds were used.

3.7.1 Surface sterilization of mustard seeds

The soaked (48 h) mustard seeds were surface sterilized in 90% ethanol for 20s followed by 0.2% mercuric chloride for 8 min (Ratan *et al.,* 2001). There-after, the seeds were rinsed with sterile distilled water for four times. The excess water content of surface sterilized seeds was removed by placing it over the sterile Whatman No. 1 filter paper.

3.7.2 Inoculation of mustard seeds

Six surface sterilized mustard seeds were inoculated on Murashige and Skoog (MS) medium poured in small Petri dish (50 mm diameter). They were kept at 25±2°C, with a photoperiod of 16/8 h for 4 days prior to volatile exposure.

3.7.3 Inoculation of *Trichoderma* isolates

Petri dishes of size 20 cm were used for the study. PDA was poured to one portion of the dish and they were kept in a slanting position to ensure that the medium covers only half portion of the dish. *Trichoderma* isolates were grown in PDA for 4

days. Mycelial discs of 5 mm were cut from actively growing cultures of *Trichoderma* and placed in the centre of the media in larger Petri dishes.

3.7.4 Plant-*Trichoderma* volatile-exposure bioassay

Exposure of germinated mustard seeds to VOCs produced by *Trichoderma* isolates was carried out using a double plate-within-a-plate system (Lee *et al.*, 2016). A small Petri dish containing six germinated mustard seeds on MS medium was placed into a larger Petri dish with *Trichoderma* isolate. The large Petri dish was sealed using an adhesive tape. The exposure set up without the *Trichoderma* inoculation on PDA served as control.

3.7.5 Comparison of growth parameters among *Trichoderma* isolates

After 9 days of exposure of mustards seedlings to VOCs, the plants were gently removed from the medium, photographed, recorded measurements of shoot and root length, counted the number of leaves and shoots, and finally fresh weight of the shoot was taken.

3.8 MOLECULAR CHARACTERIZATION OF *Trichoderma* ISOLATES

3.8.1 Genomic DNA isolation

Selected twenty-six *Trichoderma* isolates were taken for genomic DNA isolation. Two mycelial discs of 5 mm diameter were taken from the actively growing culture of each isolate and were grown in 100 ml of sterile Potato Dextrose Broth (PDB) in a 250 ml Erlenmeyer flask under aseptic condition. The cultures were kept at 28 ± 2 ˚C in an orbital shaker incubator set at 150 rpm for 3 days. Mycelia were harvested by filtration through sterile Whatman-filter paper. The harvested mycelial mass was dried using another sterile Whatman-filter paper. These dried mycelia were taken for genomic DNA isolation.

3.8.2 Isolation of genomic DNA of *Trichoderma*

DNA was extracted by following the SDS method (George *et al.*, 2018) with few modifications. Dried mycelium (80 mg) was crushed into fine powder with liquid nitrogen using a pre-sterilized mortar and pestle. Powdered mycelium was mixed with 1 ml extraction buffer (Appendix III), and transferred to a sterile 2 ml micro centrifuge tube. Then 5 µl of Proteinase K (10 mg ml^{-1}) was added to each tubes and vortexed for 1 min. Tubes were then incubated at 65 °C for 45 min followed by incubation at 37 °C for 20 min after adding five µl RNase A (10mg ml^{-1}), samples were centrifuged at 12,000 rpm for 15 min, supernatant was collected in a fresh tube and pellet was discarded. To the supernatant, equal volume of phenol: chloroform: iso amyl alcohol (25:24:1) was added. For proper mixing, tubes were inverted for 30 times. After centrifugation at 12,000 rpm for 15 min, the upper aqueous layer was transferred into a fresh tube without disturbing the middle layer and the same process was repeated twice. Equal volume of chloroform: iso amyl alcohol (24:1) was added to the aqueous layer collected in fresh tube. It was followed by centrifugation at 12,000 rpm for 15 min and the aqueous layers were collected. The process was repeated twice. To the aqueous layer collected in fresh tube, 500 µl of ice-cold isopropanol was added and inverted the tubes gently to precipitate DNA. To enhance the precipitation, tubes were kept at -20 °C for 6 h. To pellet out the DNA, the tubes were centrifuged at 12,000 rpm for 15 min. Six hundred micro liter of 75 % ethanol was added to the pellet and centrifuged at 10,000 rpm for 5 min. The ethanol wash was repeated and saved the pellet of DNA. Pellet was air dried and dissolved in 20 µl of nuclease free TE buffer (Appendix III).

3.8.3 Analysis of the extracted DNA

3.8.3.1 Analysis using Nano drop spectrophotometer

The quality and quantity of DNA was measured using Thermo Scientific NanoDrop™ 1000 Spectrophotometer. TE buffer was used as the blank. The

concentration (ng/µl) of DNA sample, absorbance at 260 nm, 280 nm and the ratio of absorbance at 260 nm and 280 nm were recorded for further calculations.

3.8.3.2 Agarose gel electrophoresis

One percentage agarose gel was prepared in 1X TAE buffer (Appendix III) to check the quality of the extracted DNA. The solution was heated until the agarose get completely melted and was allowed to cool. At bearable temperature (40 °C-50 °C), 1.5 µl of Ethidium Bromide (EtBr) was added directly to the molten gel to get a final concentration of 0.5 µg/ ml and mixed gently to facilitate the visualization of DNA. It was poured into a casting tray with comb and allowed to solidify. The combs were removed and transferred the gel to electrophoresis chamber filled with 1X TAE buffer. Five microlitres (µl) of DNA samples were mixed with 2 µl of gel loading dye and was loaded in each well of the gel. Power pack (BIO RAD Power Pac HV, USA) set at 70V was used to run the gel for 25 min. The gel image was visualized using Alpha imager (Alpha Innotech,USA).

3.8.4 Molecular identification

Identification of *Trichoderma* isolate to the species level was done by DNA sequencing of two regions, the ribosomal DNA (rDNA) - ITS region and the *tef1* gene (Nath *et al.,* 2014).

3.8.4.1 PCR amplification of ITS region

The genomic DNA isolated from 26 *Trichoderma* isolates were used for the amplification of the rDNA-ITS region using ITS1, (5' TCC GTA GGT GAA CCT TGC GG 3') and ITS4, (5' TCC TCC GCT TAT TGA TAT GC 3') primers (White *et al.,* 1990). The concentration of diluted DNA used for the amplification was 50 ng/ µl. PCR amplification was carried out in a total volume of 25 µl by adding 2 µl of template DNA with 0.5 µl of each primer (10 µM stock concentration) and 12.5 µl of

2X EmeraldAmp GT PCR Master Mix. Finally, 9.5 µl of deionised water was added to the reaction mix and made up to final volume of 25 µl.

Reagent	**Volume taken in µl**
EmeraldAmp GT PCR Master Mix (2X) (Taq DNA Polymerase Reaction buffer MgCl2 dNTPs (dATP,dCTP,dGTP and dTTP))	12.5 µl
ITS1 (Forward Primer) 10µM	0.5 µl
ITS4 (Reverse Primer) 10µM	0.5 µl
Template DNA (50 ng/ µl)	2.0 µl
Deionised Water	9.5 µl
Total Volume	25 µl

After the reaction mix preparation, each vial was flashed down and placed in Agilent Technologies Sure Cycler 8800 (USA). The thermal profile for ITS 1 and 2 gene amplification was- Initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 51.6 °C for 1min and elongation at 72 °C for 1 min 30 s. The final extension was performed at 72 °C for 8 min. Amplified products were analyzed by resolving 5 µl of each sample in 1.2% agarose gel in 1X TAE buffer which was stained with 0.5 µg ml^{-1} ethidium bromide. The amplified product resolved on electrophoresis was viewed under Gel Doc System (Alpha Imager, Alpha Innotech, San Leandro, CA, USA).

3.8.4.2 DNA Sequencing

The amplified products were sequenced at the AgriGenome Labs Private Limited, Kochi, India. The obtained sequences were analyzed using Bioinformatics tool BLASTn of NCBI (Altschul *et al.*, 1990). Besides, the 5.8S-ITS sequences were

compared to a specific database for *Trichoderma* using *Trich*OKEY 2 program, which was available online from the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy. Each species were identified and each sequence was submitted in GenBank of NCBI.

3.8.4.3 PCR amplification of tef1 gene

The genomic DNA isolated from 26 *Trichoderma* isolates were used for the amplification of the *tef*1 gene. The primers used are *tef*85f and *tef*954r (John Bissett, 2009).

*tef*85f primer sequence: 5' AG GAC AAG ACT CAC ATC AAC G 3'
*tef*954r primer sequence: 5'AGT ACC AGT GAT CAT GTT CTT G 3'
The reaction mix consist of following reagents.

Reagent	**Volume taken in µl**
EmeraldAmp GT PCR Master Mix (2X)	12.5 µl
(Taq DNA Polymerase, Reaction buffer, MgCl2, dNTPs (dATP,dCTP,dGTP and dTTP))	
*tef*85f (Forward Primer) 10µM	0.5 µl
*tef*954r (Reverse Primer) 10µM	0.5 µl
Template DNA (50 ng/ µl)	2.0 µl
Deionised Water	9.5 µl
Total Volume	25 µl

After the reaction mix preparation, each vial was flashed down and placed in Agilent Technologies Sure Cycler 8800 (USA). The thermal profile for *tef*1 gene amplification was initial denaturation at 95 °C for 2 min, followed by 30 cycles of

denaturation at 95 °C for 30 s, annealing at 54.5 °C for 45 s and elongation at 72 °C for 1 min. The final extension was performed at 72 °C for 8 min. Amplified products were analyzed by resolving 5 µl of each sample in 1.2 % agarose gel in 1X TAE buffer which was stained with 0.5 µg ml^{-1} ethidium bromide. The amplified product resolved on electrophoresis was viewed under Gel Doc System (Alpha Imager, Alpha Innotech, San Leandro, CA, USA).

3.8.4.4 DNA Sequencing

The amplified products were sequenced at the AgriGenome Labs Private Limited, Kochi, India. The obtained sequences were analyzed using Bioinformatics tool BLASTn of NCBI (Altschul *et al.*, 1990). Each species were identified and each sequence was submitted to GenBank through BankIt (a World Wide Web sequence submission server available at NCBI home page).

3.9 MOLECULAR DIVERSITY ANALYSIS

Genetic diversity of 26 *Trichoderma* isolates from different parts of India was studied using the reported 10 SSR markers (Rai *et al.*, 2016).

Table 2. List of SSR primers used for the study

Forward primers	Reverse primers	Motifs
TvSSR-1f CTATGGTGCCTCTGGTCTTTTC	TvSSR-1r GATGTTGGACTTGATACCACCC	(CAT)4
TaSSR-1 f AAGCGGTCAGTTGAAAGTAACG	TaSSR-1 r AAGGGTTTTGCTTGTCCAGATA	(ATTA)3
TaSSR-4 f CTTGACCTGCTACAACAACTGC	TaSSR-4 r TGTCCATCTCTCTCTCTCTTCTCA	(TGC)4
ThSSR-1 f GCGATTGAGAGGAACGAACT	ThSSR-1 r AATCAAGTGAGGATTTGCTGCT	(CTGT)3
ThSSR-4 f GTCGTCGGCCATCATTCC	ThSSR-4 r TTTCAAGGGCAGGACTCTCTCT	(AC)6

ThSSR-5 f AGACGCAGATGAAAAGGAGC	ThSSR-5 r GTGGACCTGAGCTTTGATTGTT	(GAA)6
ThSSR-6 f GCGAATGTCACCATCATCTTC	ThSSR-6 r TGAGAGAGCCGGAGTATAGGAG	(GAAGA) 4
TvSSR-4 f CCCCTTTACGAGGAGATACGAT	TvSSR-4 r GAGAGAGAAGAAGCGAAAGCAC	(TC)9
TvSSR-5 f GGATCGGCAAGGAATATAAACA	TvSSR-5 r CAACTTCCATAAAGACCGAAGC	(TTCCA)3
TvSSR-2 f CCATCAAATCATCAAGAGTCCA	TvSSR-2 r ACAAAGGCCATGAAAGAGAAAG	(TCT)4

3.9.1 Gradient PCR for the optimization of annealing temperature

Each primer pair was subjected to gradient PCR to find out the annealing temperature for each primer pair. The temperature range given for gradient PCR was 50 °C to 60 °C (TvSSR-1, TaSSR-1, ThSSR-5, ThSSR-6, TvSSR-4 and TvSSR-5) and 45 °C to 55 °C (TaSSR-4, ThSSR-1, ThSSR-4 and TvSSR-2). The amplified products were analysed after agarose gel electrophoresis and noted the annealing temperature at which it gave clear band.

3.9.2 PCR using SSR primers

The PCR programme was set with initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing temperature which was found to be optimum for each primers for 45 s and elongation at 72 °C for 1 min. The final extension was performed at 72 °C for 8 min. The amplified products were then subjected to electrophoresis in an agarose gel of 2% concentration. Also run the Gene Ruler 1kb plus DNA ladder (Fermentas) along with the amplified products by using 60V for about 1 and half hours. It was then visualized under UV light and image was documented in Gel Doc System (Alpha imager, Alpha Innotech, USA).

3.9.3 Band scoring

All SSR gel profiles were taken and scored each reproducible and clear band. Binary scoring was done by assigning "1" for the presence of a particular band and "0" for the absence of the band. The scored details were entered in MS Excel and it was further used as an input for cluster analysis and other statistical analysis.

RESULTS

4. RESULTS

The results of the study entitled "Morphological, biochemical and molecular characterization of *Trichoderma* isolates from tuber crop ecosystem" carried out at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2018-2019 are given in this chapter.

4.1 SCREENING OF *Trichoderma* ISOLATES AGAINST *Sclerotium rolfsii*

Total of 43 *Trichoderma* isolates were screened against *S. rolfsii* by adopting three different methods *viz.*, dual culture method/ direct confrontation method, antibiosis test for production of diffusible inhibitory metabolites and test for production of volatile compounds by isolates against pathogen to select the most potent isolate. The percentage inhibition of mycelial growth of *S. rolfsii* by *Trichoderma* isolates in three different methods of screening is given below.

4.1.1 Dual culture method

All the 43 isolates could restrict the mycelial growth of *S. rolfsii* (Table 3 and Plate 1-3). The isolates showed differential inhibition potential in suppressing the mycelial growth of the pathogen. Maximum percentage inhibition was shown by the isolate, T32 (82.32 %). It was followed by T36 (75.85 %), T38 (74.13 %), T34 (69.38 %) and T40 (62.73 %). Most of the isolates exhibited high inhibition towards the collar rot pathogen. Out of 43 isolates, 74.4 % of the isolates showed more than 50 % inhibition. The majority of isolates (27 isolates) showed 50 % to 60 % inhibition (Fig. 2). The least percentage inhibition was shown by T26 (9.44 %). A curve in the contact region of pathogen and *Trichoderma* was observed in majority of isolates. T31 isolate showed an exception by showing straight line at the contact region.

Table 3. Mycelial growth inhibition of *S. rolfsii* by *Trichoderma* isolate in dual culture (3 DAI)

Isolate code	Mycelial growth (mm)	Percentage inhibition
T1	38.67^{bc}	49.98 (0.52*)^{ef}
T2	36.33 ^{bcd}	53.00 (0.56) ^{def}
T3	35.33 ^{bcd}	54.29 (0.57) ^{def}
T4	42.00 ^{b}	45.67(0.47) ^{ef}
T5	35.33 ^{bcd}	54.29 (0.58) ^{def}
T6	32.33 ^{bcde}	58.17 (0.62) ^{def}
T7	40.00 ^{bc}	48.25(0.50) ^{ef}
T8	37.00 ^{bc}	52.13(0.55) ^{ef}
T9	39.67 ^{bc}	48.68(0.51) ^{ef}
T10	38.67 ^{bc}	49.98(0.52) ^{ef}
T11	32.67 ^{bcde}	57.74 (0.62)^{def}
T12	41.67 ^{bc}	46.10(0.48$^{)ef}$
T13	39.00 ^{bc}	49.55(0.52) ^{ef}
T14	32.67 ^{bcde}	57.74(0.62) ^{def}
T15	35.33 ^{bcd}	54.29(0.57) ^{def}
T16	35.67 ^{bcd}	53.86(0.57) ^{def}
T17	35.33 ^{bcd}	54.29(0.57) ^{def}
T18	33.33 ^{bcd}	56.88(0.61) ^{def}
T19	37.67 ^{bc}	51.27(0.54) ^{ef}
T20	34.67 ^{bcd}	55.15(0.58) ^{def}
T21	32.33 ^{bcde}	58.17(0.62) ^{def}
T22	37.67 ^{bc}	51.27(0.54) ^{ef}
T23	40.00 ^{bc}	48.25(0.50) ^{ef}
T24	42.67 ^{b}	44.80(0.46) ^{f}
T25	37.67 ^{bc}	51.27(0.54) ^{ef}
T26	70.00 ^{a}	9.44(0.10) ^{g}
T27	32.67 ^{bcde}	57.74(0.62) ^{def}
T28	31.67 ^{bcdef}	59.0(0.63)3 ^{cdef}
Table 3 continued		

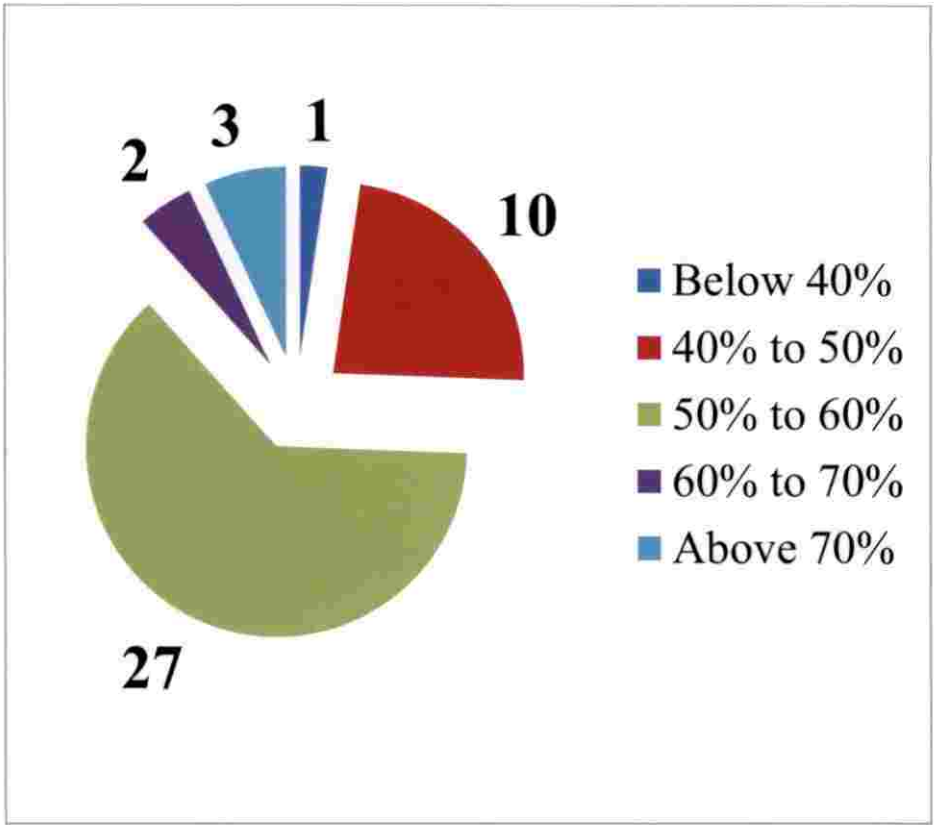


Fig. 2. Number of isolates in each range of % inhibition by dual culture method

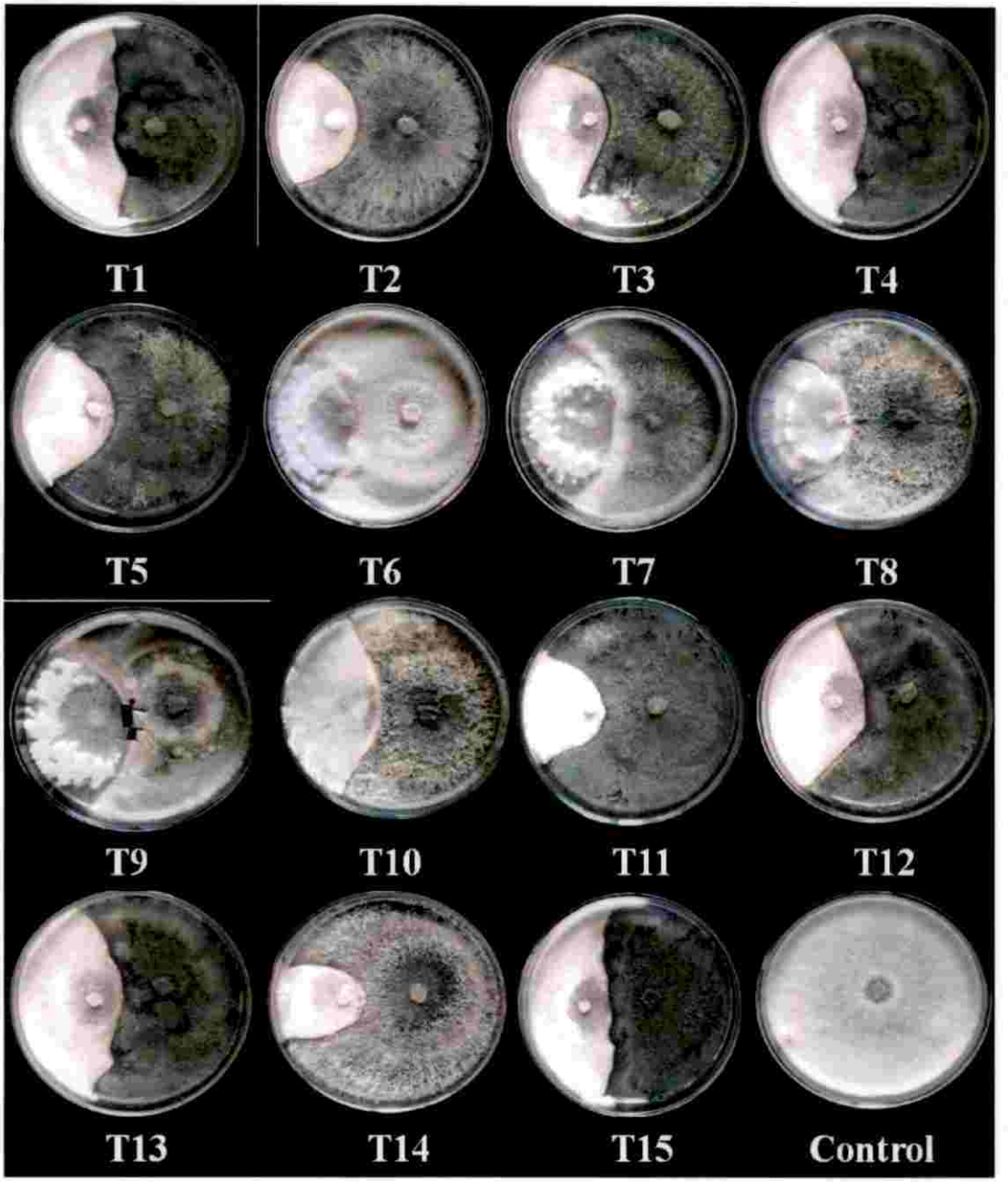


Plate 1. Mycelial growth inhibition of pathogen by *Trichoderma* isolates (T1-T15) in dual culture (4DAI) and control

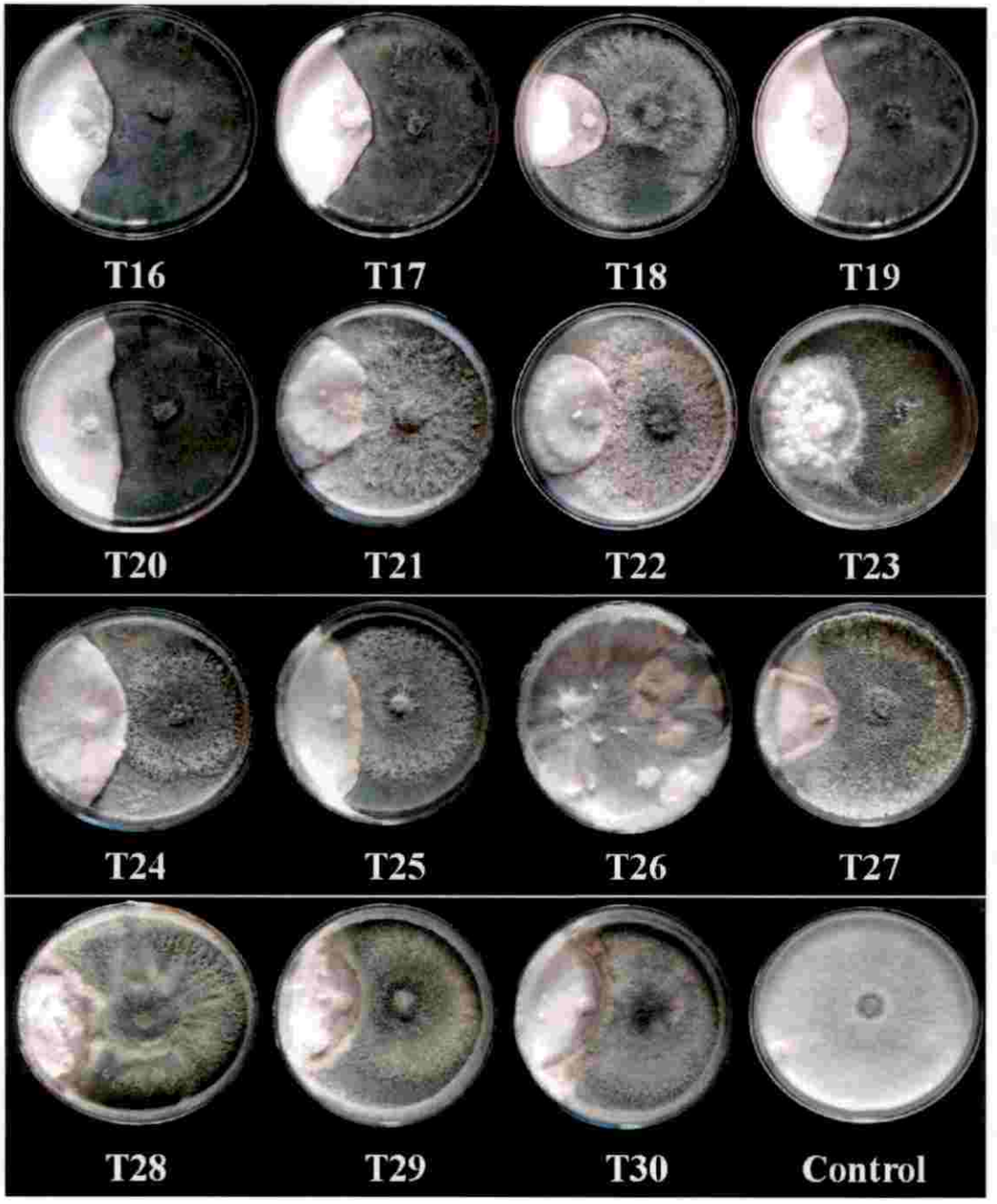


Plate 2. Mycelial growth inhibition of pathogen by *Trichoderma* isolates (T16-T30) in dual culture (4DAI) and control

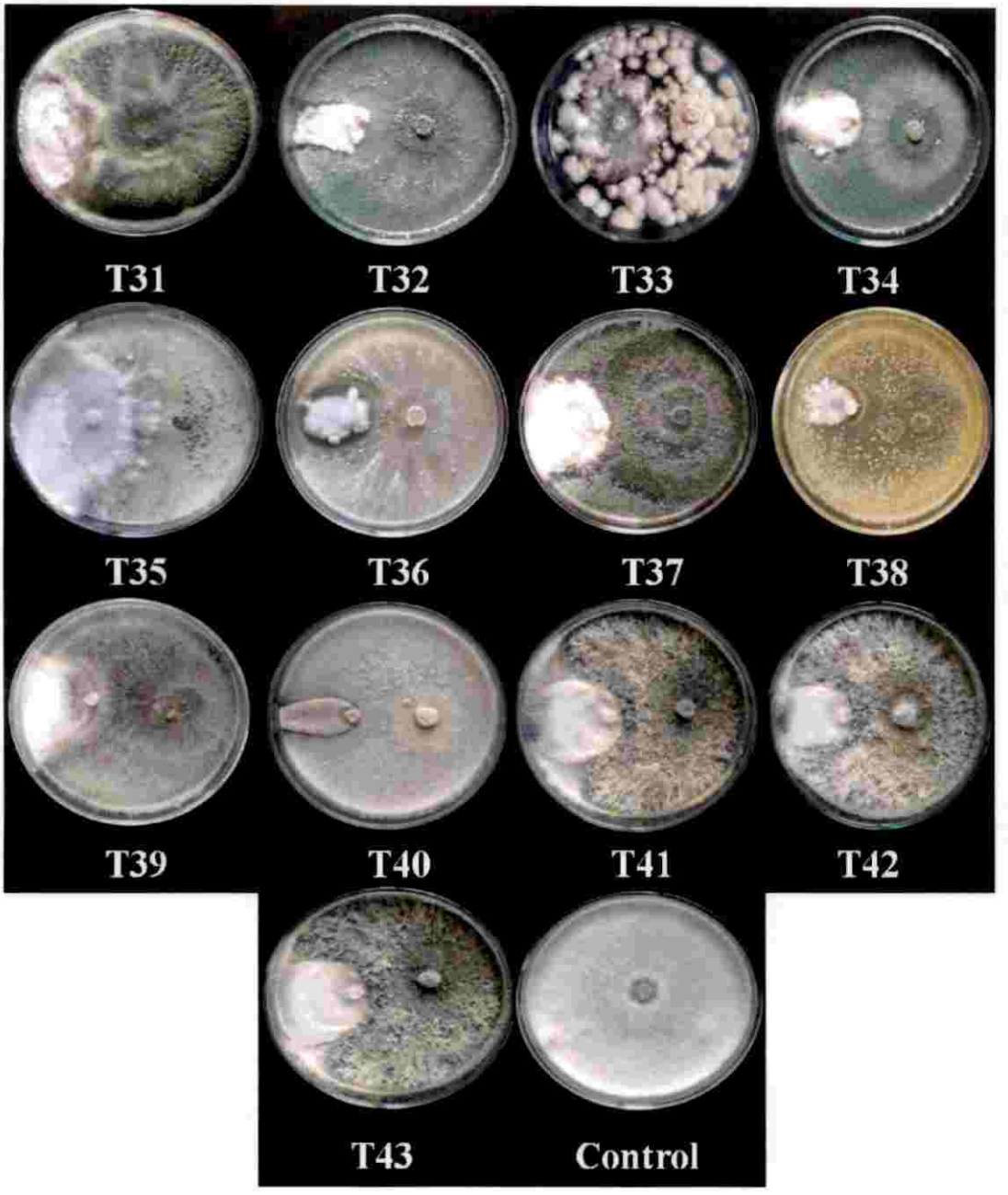


Plate 3. Mycelial growth inhibition of pathogen by *Trichoderma* isolates (T31-T43) in dual culture (4DAI) and control

Table 3 continued		
Isolate code	**Mycelial growth (mm)**	**Percentage inhibition**
T29	32.33 ^{bcde}	58.17(0.62) ^{def}
T30	37.00 ^{bc}	52.13(0.55) ^{ef}
T31	38.33 ^{bc}	50.41(0.53) ^{ef}
T32	13.67 ^{g}	82.32(0.97) ^{a}
T33	35.67 ^{bcd}	53.86(0.57) ^{def}
T34	23.67 ^{defg}	69.38(0.77) ^{abcd}
T35	39.67 ^{bc}	48.68(0.51) ^{ef}
T36	18.67 ^{fg}	75.85(0.86) ^{ab}
T37	34.67 ^{bcd}	55.15(0.59) ^{def}
T38	20.00 ^{efg}	74.13(0.84) ^{abc}
T39	33.33 ^{bcd}	56.88(0.61) ^{def}
T40	28.70 ^{cdef}	62.73(0.68) ^{bcde}
T41	36.17 ^{bcd}	53.03(0.56) ^{def}
T42	32.63 ^{bcde}	57.62(0.61) ^{def}
T43	35.40 ^{bcd}	54.03 (0.57) ^{def}
Control	77.33 ^{a}	
Tukey HSD at 1%	13.196	

*Values in parentheses are values after arcsine transformation. Means with at least one letter common are not statistically significant using TUKEY's Honest Significant Difference.

4.1.2 Antibiosis test for production of diffusible inhibitory metabolites

Most of the isolates showed 100 % inhibition of mycelial growth by this method (Table 4 and Plate 4-6). In this screening method, 69.76 % of the isolates (30 *Trichoderma* isolates) showed 100 % inhibition of pathogen. It suggests the involvement of lytic enzymes and other metabolites in pathogen suppression. Lowest inhibition was shown by the isolate T31 (3.70 %) followed by T26 (5.93 %). The isolate, T26 showed least inhibition in dual culture method also.

Table 4. Mycelial growth inhibition of *S. rolfsii* by diffusible inhibitory metabolites of *Trichoderma* isolates (3 DAI)

Isolate code	Mycelial growth (mm)	Percentage inhibition
T1	0.0^{i}	100.00(1.57*)^{a}
T2	0.0^{i}	100.00(1.57)^{a}
T3	0.0^{i}.	100.00(1.57)^{a}
T4	0.0^{i}	100.00(1.57)^{a}
T5	0.0^{i}	100.00(1.57)^{a}
T6	0.0^{i}	100.00(1.57)^{a}
T7	0.0^{i}	100.00(1.57)^{a}
T8	44.0^{cdef}	51.11(0.74)^{bcde}
T9	17.5^{ghi}	80.56(0.94)^{bc}
T10	36.2^{cdefgh}	59.72(0.64)^{cdefg}
T11	0.0^{i}	100.00(1.57)^{a}
T12	0.0^{i}	100.00(1.57)^{a}
T13	0.0^{i}	100.00(1.57)^{a}
T14	0.0^{i}	100.00(1.57)^{a}
T15	0.0^{i}	100.00(1.57)^{a}
T16	0.0^{i}	100.00(1.57)^{a}
T17	0.0^{i}	100.00(1.57)^{a}
T18	0.0^{i}	100.00(1.57)^{a}
T19	0.0^{i}	100.00(1.57)^{a}
T20	0.0^{i}	100.00(1.57)^{a}
T21	52.3^{cd}	41.85(0.44)^{ef}
T22	33.5^{defgh}	62.78(0.70)^{cde}
T23	60.0^{bc}	33.33(0.34)^{fg}
T24	29.6^{defgh}	67.04(0.76)^{bcde}
T25	47.5^{cde}	47.22(0.50)^{ef}
T26	84.6^{ab}	5.93(0.06)^{g}
T27	42.5^{cdefg}	52.78(0.56)^{def}
T28	0.0^{i}	100.00(1.57)^{a}
Table 4 continued		

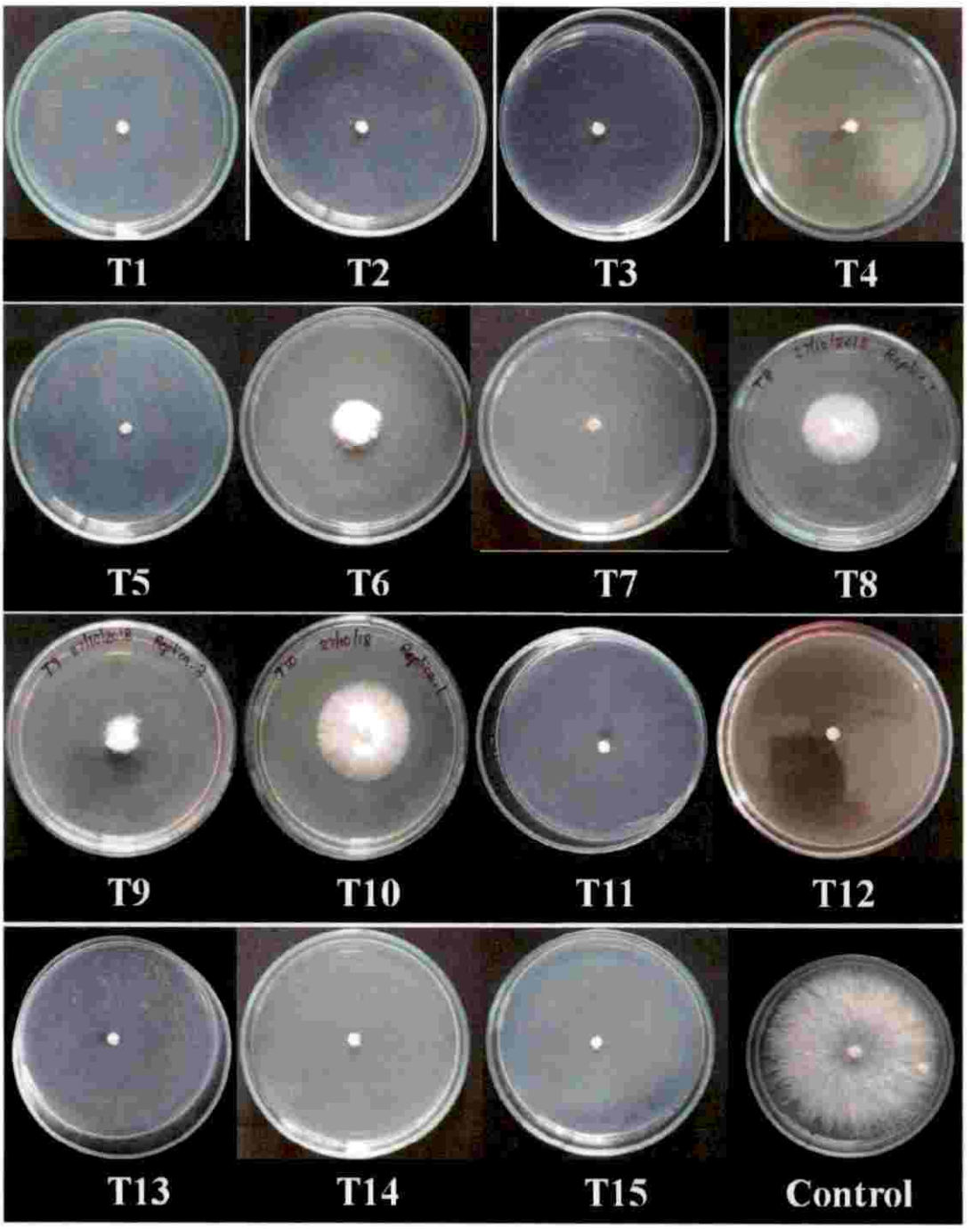


Plate 4. Mycelial growth inhibition of *S. rolfsii* by diffusible inhibitory metabolites of *Trichoderma* isolates, T1-T15 and control

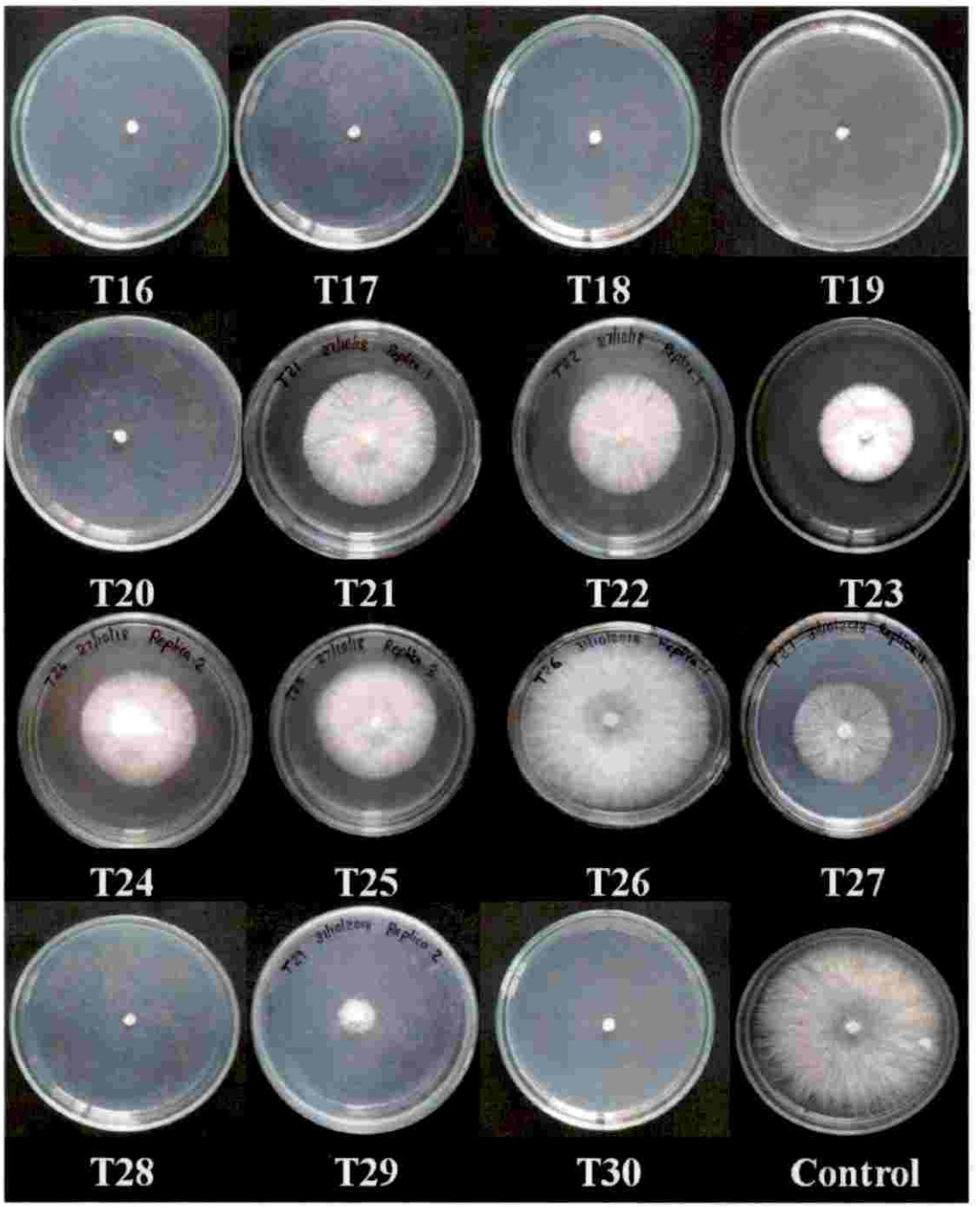


Plate 5. Mycelial growth inhibition of *S. rolfsii* by diffusible inhibitory metabolites of *Trichoderma* isolates, T16-T30 and control

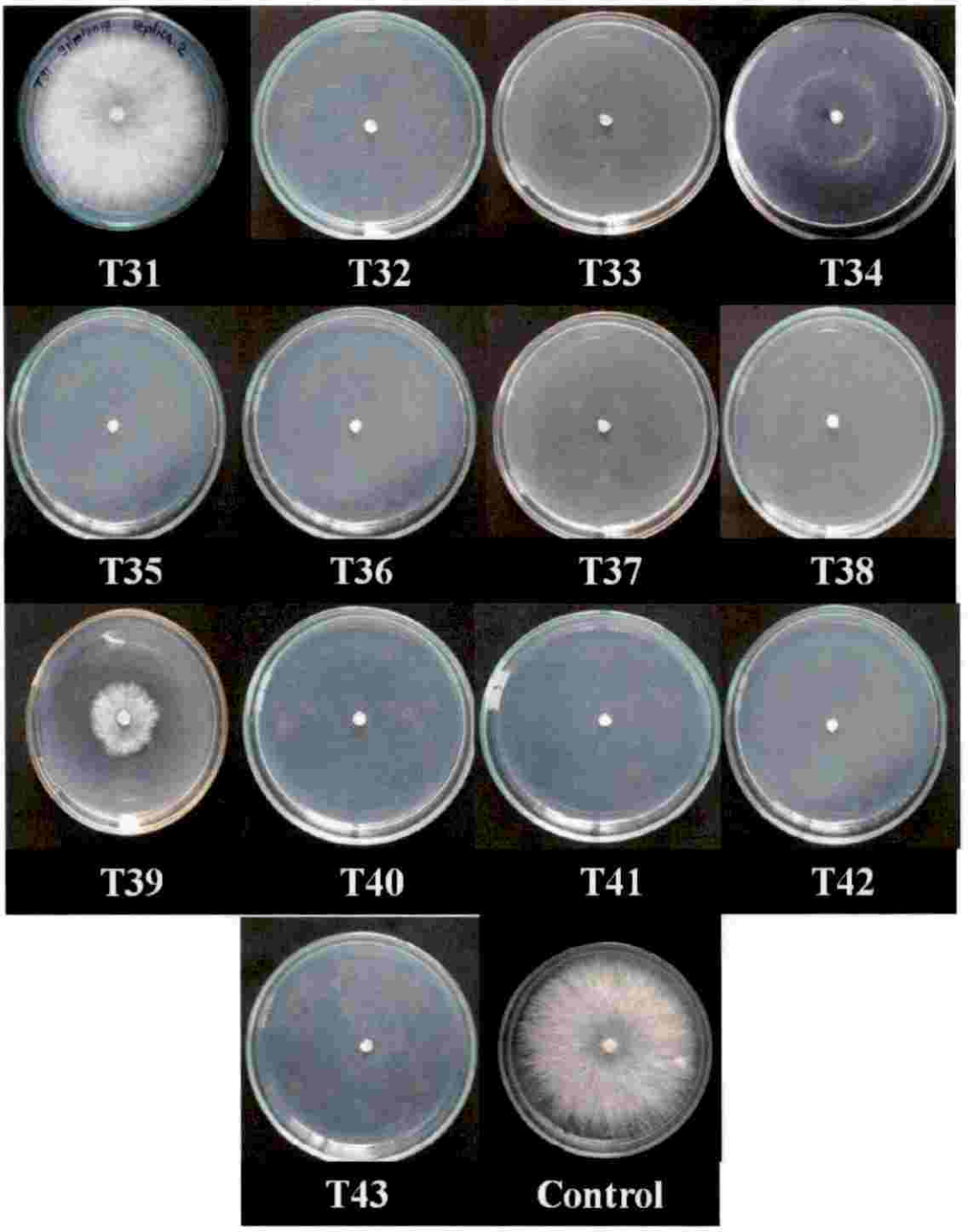


Plate 6. Mycelial growth inhibition of *S. rolfsii* by diffusible inhibitory metabolites of *Trichoderma* isolates, T31-T43 and control

Table 4 continued		
Isolate code	**Mycelial growth (mm)**	**Percentage inhibition**
T29	12.0h[i]	86.67(1.06)[b]
T30	0.0[i]	100.00(1.57)[a]
T31	86.8[a]	3.70(0.53)[def]
T32	0.0[i]	100.00(1.57)[a]
T33	0.0[i]	100.00(1.57)[a]
T34	0.0[i]	100.00(1.57)[a]
T35	0.0[i]	100.00(1.57)[a]
T36	0.0[i]	100.00(1.57)[a]
T37	0.0[i]	100.00(1.57)[a]
T38	0.0[i]	100.00(1.57)[a]
T39	20.7[fghi]	76.92(0.88)[bcd]
T40	0.0[i]	100.00(1.57)[a]
T41	0.0[i]	100.00(1.57)[a]
T42	0.0[i]	100.0(1.57)[a]
T43	0.0[i]	100.00(1.57)[a]
Control	90.0[a]	

*Values in parentheses are values after arcsine transformation. Means with at least one letter common are not statistically significant using TUKEY's Honest Significant Difference.

4.1.3 Antibiosis test for production of volatile compounds by isolates against pathogen

Thirty eight isolates could inhibit the mycelial growth significantly. The volatiles of isolates T20, T25, T26, T33 and T37 could not restrict the mycelial growth of *S. rolfsii* (Table 5 and Plate 7-9). The inhibitory action by volatile metabolites was found to be relatively less. Isolate, T31 showed maximum percentage inhibition (42.96 %) followed by the isolate T24 (40.16 %). The least inhibition was with T26 (15.38 %) and this isolate showed least inhibition in all three methods

adopted for screening the isolates. Among 43 isolates, 18 isolates showed more than 30 % inhibition of mycelial growth.

Table 5. Mycelial growth inhibition of *S. rolfsii* by volatile compounds produced by *Trichoderma* isolates (3 DAI)

Isolate code	Mycelial growth (mm)	Percentage inhibition
T1	67.5 bcdefgh	24.96(0.25*) abcdefg
T2	56.9 defgh	36.70(0.38) abcdef
T3	69.8 bcdefg	22.41(0.23) bcdefg
T4	73.9 abcd	17.82(0.18) efg
T5	72.1 bcdef	19.86(0.20) cdefg
T6	56.5 efgh	37.19(0.38) abcde
T7	63.8 bcdefgh	29.12(0.30) abcdefg
T8	65.1 bcdefgh	27.77(0.28) abcdefg
T9	68.24 bcdefgh	24.18(0.24) abcdefg
T10	62.5 bcdefgh	30.46(0.31) abcdefg
T11	67.1 bcdefgh	25.47(0.26) abcdefg
T12	67.9 bcdefgh	24.45(0.25) abcdefg
T13	64.7 bcdefgh	28.02(0.28) abcdefg
T14	62.9 bcdefgh	30.07(0.31) abcdefg
T15	57.4 cdefgh	36.19(0.37) abcdef
T16	62.4 bcdefgh	30.58(0.31) abcdefg
T17	67.8 bcdefgh	24.63(0.25) abcdefg
T18	68.0 bcdefgh	24.41(0.25) abcdefg
T19	67.0 bcdefgh	25.53(0.26) abcdefg
T20	72.6 abcde	19.25(0.19) defg
T21	68.2 bcdefgh	24.18(0.24) abcdefg
T22	58.4 cdefgh	35.02(0.36) abcdefg
T23	58.4 cdefgh	35.02(0.36) abcdefg
T24	53.8 gh	40.16(0.41) ab
T25	72.6 abcde	19.25(0.19) defg
Table 5 continued		

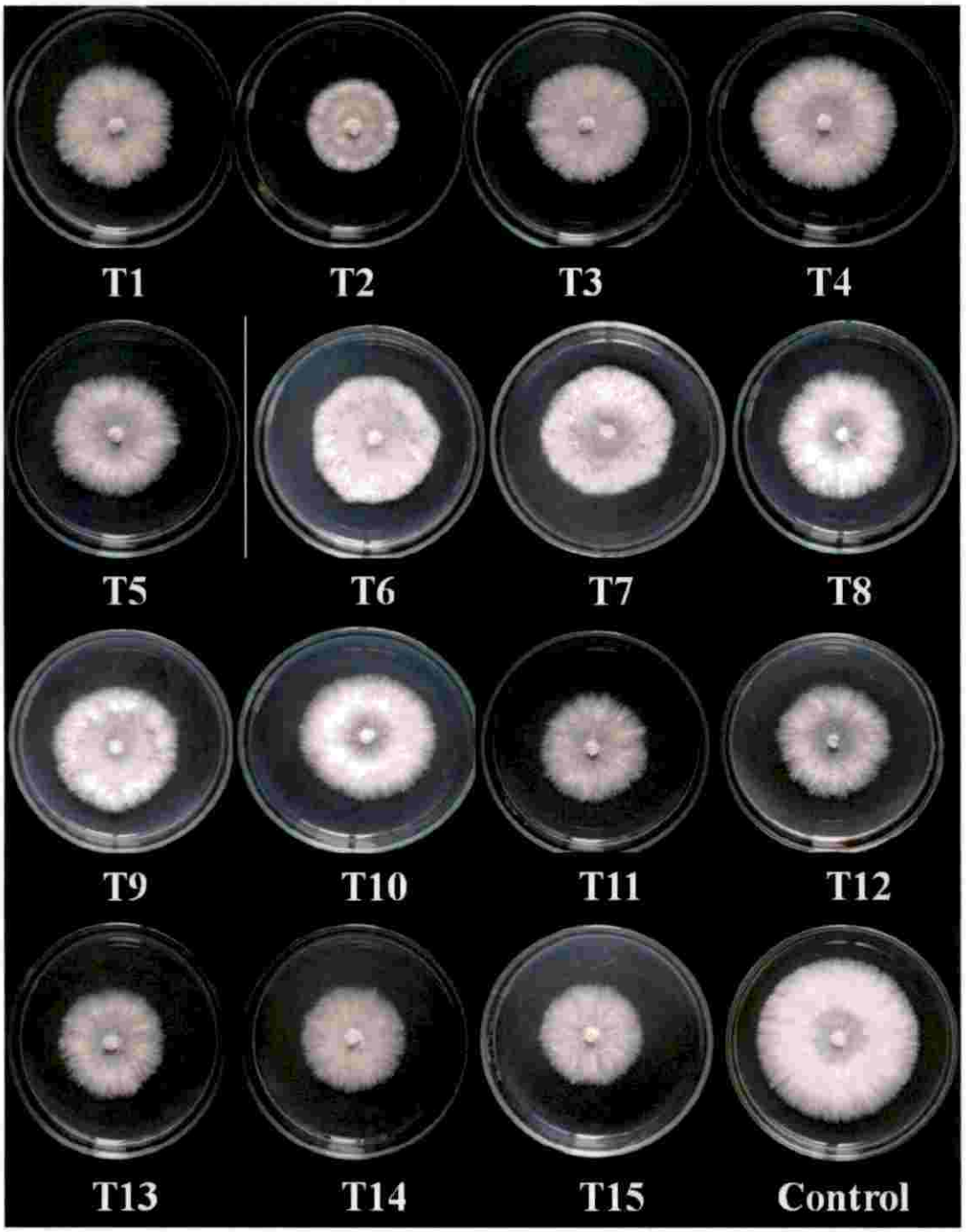


Plate 7. Mycelial growth inhibition of *S. rolfsii* by volatile compounds produced by *Trichoderma* isolates, T1-T15 and Control

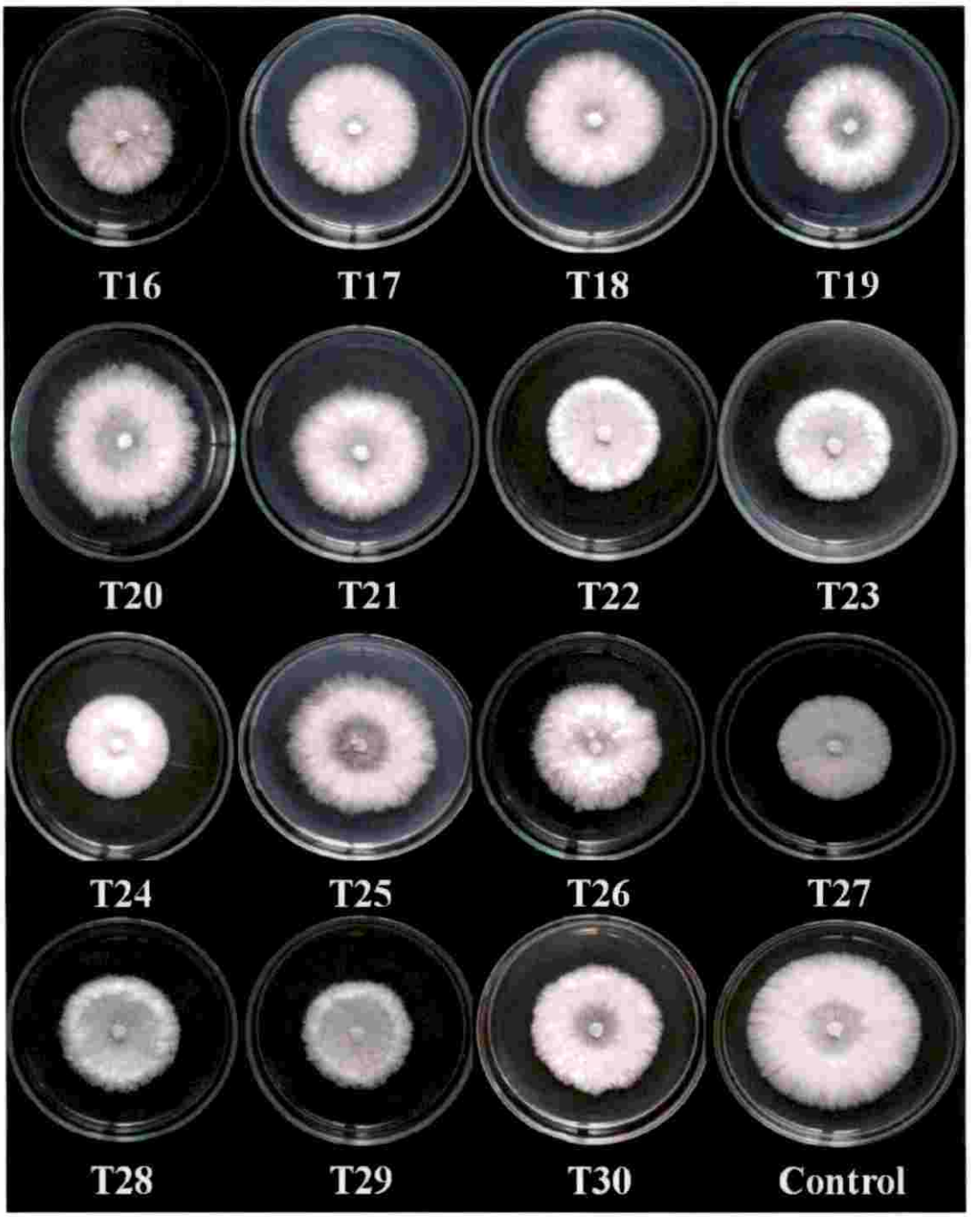


Plate 8. Mycelial growth inhibition of *S. rolfsii* by volatile compounds produced by *Trichoderma* isolates, T16-T30 and Control

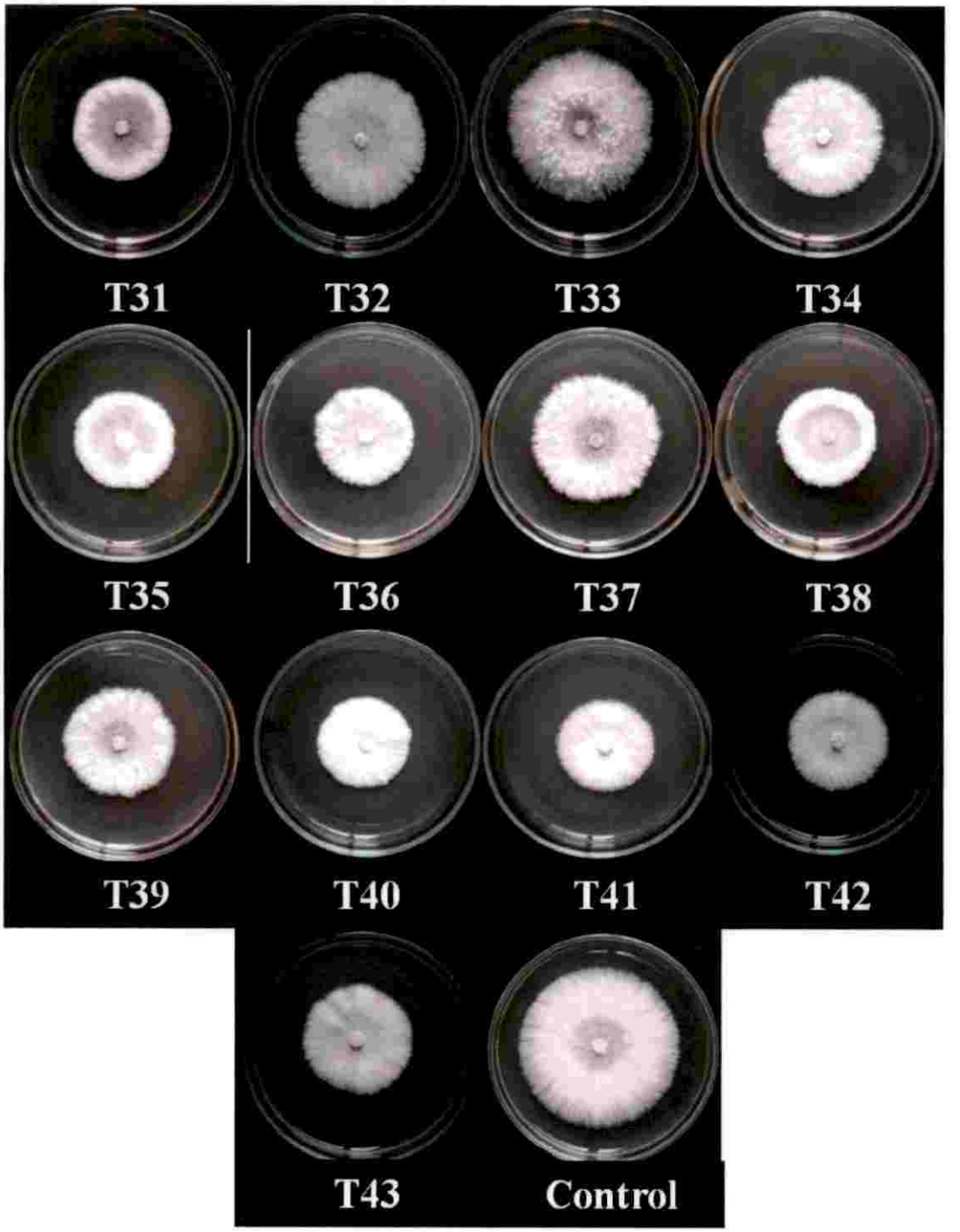


Plate 9. Mycelial growth inhibition of *S. rolfsii* by volatile compounds produced by *Trichoderma* isolates, T31-T43 and Control

Table 5 continued		
Isolate code	**Mycelial growth (mm)**	**Percentage inhibition**
T26	76.1 ^{ab}	15.38(0.15) ^{g}
T27	57.6 ^{cdefgh}	35.95(0.37) ^{abcdef}
T28	69.8 ^{bcdefgh}	22.39(0.23) ^{bcdefg}
T29	62.6 ^{bcdefgh}	30.34(0.31) ^{abcdefg}
T30	70.7 ^{bcdefgh}	21.46(0.22) ^{bcdefg}
T31	51.3 ^{h}	42.96(0.44) ^{a}
T32	72.3 ^{bcdef}	19.59(0.20) ^{cdefg}
T33	74.4 ^{abc}	17.25(0.17) ^{fg}
T34	67.1 ^{bcdefgh}	25.43(0.26) ^{abcdefg}
T35	57.6 ^{cdefgh}	35.64(0.37) ^{abcdef}
T36	65.3 ^{bcdefgh}	27.43(0.28) ^{abcdefg}
T37	74.3 ^{abcd}	17.42(0.18) ^{fg}
T38	54.9 ^{fgh}	38.94(0.40) ^{abc}
T39	66.6 ^{bcdefgh}	25.93(0.26) ^{abcdefg}
T40	55.8 ^{efgh}	37.94(0.39) ^{abcd}
T41	59.0 ^{bcdefgh}	34.43(0.35) ^{abcdefg}
T42	61.2 ^{bcdefgh}	31.93(0.33) ^{abcdefg}
T43	62.1 ^{bcdefgh}	30.93(0.31) ^{abcdefg}
Control	90.0 ^{a}	

*Values in parentheses are values after arcsine transformation. Means with atleast one letter common are not statistically significant using TUKEY's Honest Significant Difference.

4.1.4 Selection of *Trichoderma* isolates

Trichoderma exhibits numerous mechanisms during pathogen suppression. Hence to select the most potent isolates for further study, additive effect of the isolates were considered (Table 6). On considering the additive effect of three screening methods, the isolate T38 showed maximum inhibition (213.07) followed by

T36 and T32 with scores of 203.28 and 201.9 respectively. The isolates ranked first 26 were selected for further study.

Table 6: The ranking of *Trichoderma* based on cumulative effect shown by the isolates

Isolate code	Cumulative inhibition	Rank
T38	213.07	1
T36	203.28	2
T32	201.91	3
T40	200.67	4
T6	195.36	5
T34	194.81	6
T15	190.48	7
T2	189.70	8
T42	189.55	9
T14	187.81	10
T41	187.46	11
T43	184.96	12
T35	184.62	13
T16	184.44	14
T11	183.21	15
T28	181.43	16
T18	181.28	17
T17	178.92	18
T13	177.57	19
T7	177.37	20
T19	176.80	21
T3	176.70	22
T29	175.18	23
T1	174.94	24
T20	174.40	25
T5	174.15	26

4.2 BIOCHEMICAL ASSAY

4.2.1 *N*-acetyl glucosamine (GlcNAc) standard curve

Three replicates were tested for each concentration of GlcNAc. Using the standard curve (Fig. 3) the concentration of *N*-acetyl glucosamine (in mg ml^{-1}) corresponding to a particular absorbance was determined.

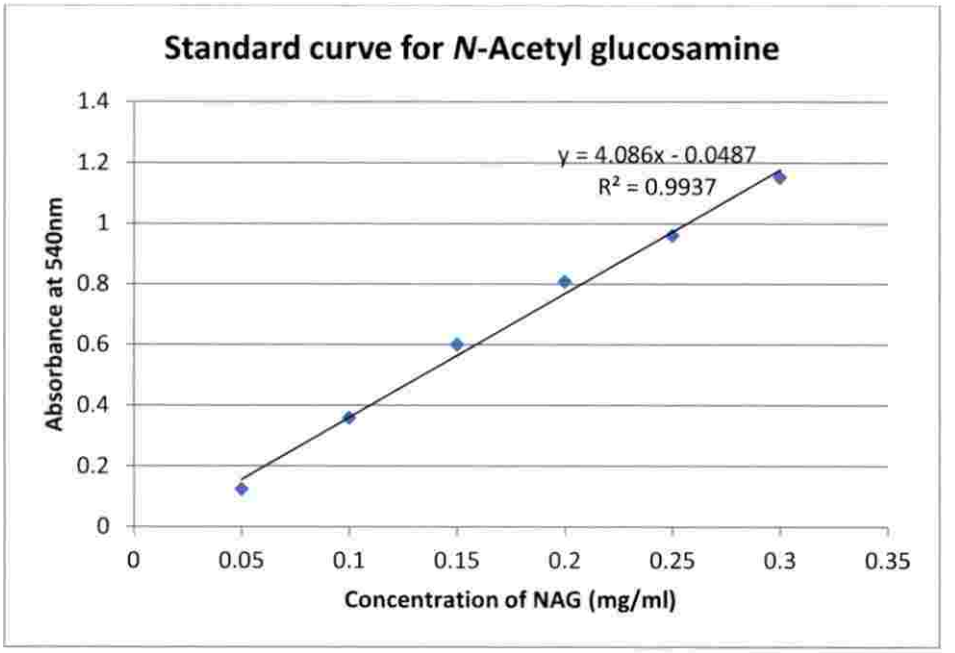


Fig. 3 Standard curve for *N*-acetyl glucosamine (GlcNAc)

4.2.2 Glucose standard curve

Three replicates were tested for each concentration of glucose. Using the standard curve (Fig. 4) the concentration of glucose (in mg ml^{-1}) corresponding to a particular absorbance value can be determined.

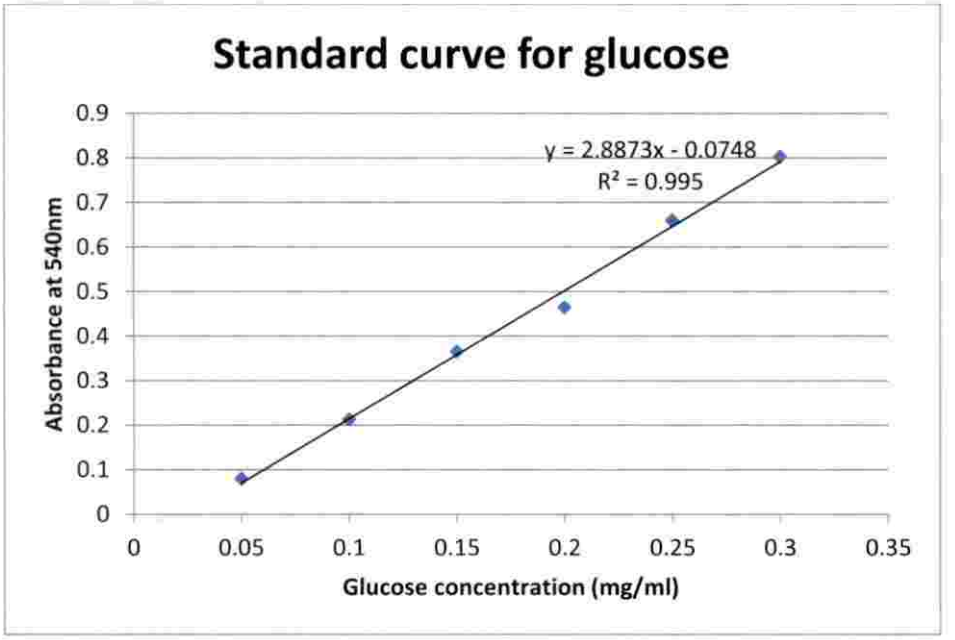


Fig.4 Standard curve for glucose

4.2.3 Chitinase and β-1,3-glucanase enzyme assay

Isolates showed significantly high variability in chitinase production (Table 7). The isolate T7 showed maximum chitinase activity (74.48 pkat ml^{-1}) and the production was significantly higher than all other 25 isolates. The least chitinase activity was shown by T38 (22.11 pkat ml^{-1}). There was no correlation observed between antagonistic potential of isolates and induction of chitinase enzyme. However, some of the isolates (T32, T34, T40, T15 and T2) showed high suppression of pathogen as well as induction of chitinase enzyme.

In general, antagonistic potential and induction of glucanase activity are positively correlated. The highest antagonistic potential as well as β-1,3-glucanase enzyme activity was given by T38 isolate. The enzyme activity ranged from 2.11nkat ml^{-1}(T38) to 0.21nkat ml^{-1}(T20). Among the isolates which ranked as top 10 isolates based on the antagonistic potential, 7 isolates produced high glucanase activity also.

Some of the isolates *viz*., T2, T15, T32, T34 and T40 showed >50 pkat ml^{-1}chitinase activity and >1nkat ml^{-1} β-1,3-glucanase enzyme activity.

Table7. Chitinase and β-1,3-glucanase enzyme activity expressed by *Trichoderma* isolates in media supplemented with *S. rolfsii* cell wall as carbon source

	Chitinase enzyme assay		**β-1,3-glucanase enzyme assay**	
Isolate code	**GlcNAc released in mg ml^{-1} per hour**	**Chitinase enzyme activity in terms of pkat ml^{-1}**	**Glucose released in mg ml^{-1} per hour**	**β-1,3-glucanase enzyme activity in terms of nkat ml^{-1}**
T1	0.02^{kl}	28.12^{kl}	0.48^{ij}	0.75^{ij}
T2	0.05^{f}	58.12^{f}	0.91^{cd}	1.40^{cd}
T3	0.04^{gh}	45.92^{gh}	0.48^{ij}	0.74^{ij}
T5	0.02^{jkl}	29.61^{jkl}	0.14^{o}	0.22^{o}
T6	0.03^{hij}	38.75^{hij}	0.19^{no}	0.29^{no}
T7	0.54^{a}	674.48^{a}	0.60^{h}	0.92^{h}
T11	0.04^{gh}	45.30^{gh}	0.47^{ij}	0.73^{ij}
T13	0.03^{hijk}	36.49^{hijk}	0.57^{hi}	0.88^{hi}
T14	0.14^{c}	175.58^{c}	0.29^{lmn}	0.45^{lmn}
T15	0.05^{f}	58.52^{f}	0.85^{de}	1.31^{de}
T16	0.07^{e}	93.84^{e}	0.15^{o}	0.24^{o}
T17	0.03^{ijkl}	32.00^{ijkl}	0.75^{ef}	1.16^{ef}
T18	0.04^{fg}	51.40^{fg}	0.46^{ij}	0.71^{ij}
T19	0.03^{ijk}	32.94^{ijk}	0.44^{j}	0.68^{j}
T20	0.03^{hij}	38.15^{hij}	0.14^{o}	0.21^{o}
T28	0.03^{hij}	38.69^{hij}	0.23^{mno}	0.36^{mno}
T29	0.05^{f}	56.69^{f}	0.24^{mno}	0.37^{mno}
Table 7 continued				

Table 7 continued				
T32	0.16^{b}	200.35^{b}	0.63^{gh}	0.98^{gh}
T34	0.07^{e}	93.60^{e}	0.72^{fg}	1.10^{fg}
T35	0.03^{hi}	40.58^{hi}	0.41^{jk}	0.64^{jk}
T36	0.02^{kl}	26.89^{kl}	1.25^{b}	1.93^{b}
T38	0.02^{l}	22.11^{l}	1.37^{a}	2.11^{a}
T40	0.11^{d}	142.14^{d}	1.00^{c}	1.55^{c}
T41	0.03^{ijk}	33.28^{ijk}	0.32^{klm}	0.50^{klm}
T42	0.03^{ijk}	34.33^{ijk}	0.39^{jkl}	0.60^{jkl}
T43	0.03^{ijk}	32.10^{ijk}	0.30^{klm}	0.47^{klm}

4.2.2 Effect of VOCs on plant growth

Mustard seeds were used to assess the effect of VOCs produced by *Trichoderma* isolates on plant growth. VOCs of isolates showed differential response to various growth parameters. In general, many of the isolates promoted plant growth in terms of fresh weight, number of leaves, root length, shoot length and number of shoot lets (Table 8 and Plate 10). Fresh weight ranged from 0.05 g (Control) to 0.16 g (T34) and five isolates *viz.*, T15, T34, T35, T36 and T43 showed significant increase in weight by the emission of VOCs. The number of leaf production did not differ significantly by VOCs. The least number was noted in control (1.70) and the maximum was with T41 (2.50). Even though the growth promotion exhibited by VOCs of the isolates was not statistically significant barring a few isolates, all the isolates could increase fresh weight as well as production of leaves (Fig. 5).

Root length of the isolates varied significantly among the isolates. Three isolates (T3, T14 and T28), negative effect was noticed. The root length varied from 2 cm (T3) to 15.68 cm (T40). Shoot length and number of shoot let also varied significantly. Shoot length varied from 6.58 cm (T3) to 12.63 cm (T5) and 2 isolates

(T3 and T14) showed growth retardation compared to control (7.35 cm). In case of number of shoot let, only 13 isolates showed increase in number compared to control. It varied from 1 (T20) to 2.75 (T16 and T41).

Table 8. The growth parameters recorded after VOCs exposure

Isolate code	Fresh weight of the shoot (g)	No. of leafs	Root length (cm)	Shoot length (cm)	No. of shoot lets
T1	0.12^{abcd}	2.00	9.63^{abc}	9.80^{abcd}	2.00^{ab}
T2	0.11^{abcd}	1.75	11.25^{abc}	12.38^{ab}	1.75^{ab}
T3	0.05^{d}	2.00	2.00^{c}	6.58^{d}	1.00^{b}
T5	0.10^{abcd}	1.75	7.00^{abc}	12.63^{a}	1.75^{ab}
T6	0.11^{abcd}	1.75	8.88^{abc}	12.33^{abc}	1.50^{ab}
T7	0.11^{abcd}	1.75	8.88^{abc}	12.03^{abc}	1.50^{ab}
T11	0.11^{abcd}	2.00	8.00^{abc}	12.58^{a}	1.25^{ab}
T13	0.07^{bcd}	1.75	11.25^{abc}	8.03^{abcd}	1.50^{ab}
T14	0.06^{cd}	2.00	3.45^{bc}	6.95^{d}	1.50^{ab}
T15	0.13^{abc}	2.00	12.00^{ab}	12.50^{a}	2.00^{ab}
T16	0.11^{abcd}	2.25	9.00^{abc}	9.38^{abcd}	2.75^{a}
T17	0.07^{bcd}	1.75	8.75^{abc}	8.03^{abcd}	1.25^{ab}
T18	0.12^{abcd}	1.75	11.25^{abc}	12.35^{ab}	1.75^{ab}
T19	0.07^{bcd}	1.75	11.25^{abc}	8.03^{abcd}	1.50^{ab}
T20	0.07^{bcd}	2.00	8.10^{abc}	7.63^{abcd}	1.00^{b}
T28	0.10^{abcd}	2.00	6.45^{abc}	9.88^{abcd}	1.75^{ab}
T29	0.10^{abcd}	1.75	11.75^{ab}	8.73^{abcd}	2.00^{ab}
T32	0.12^{abcd}	2.25	9.18^{abc}	11.50^{abcd}	2.25^{ab}
T34	0.16^{a}	2.00	9.75^{abc}	11.43^{abcd}	2.25^{ab}

Table 8 continued

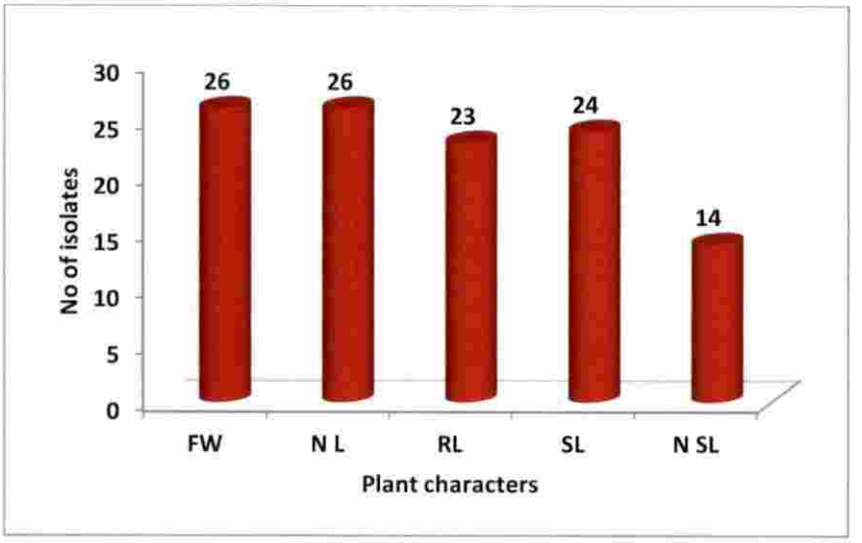


Fig. 5 Number of *Trichoderma* isolates with growth promotion activity

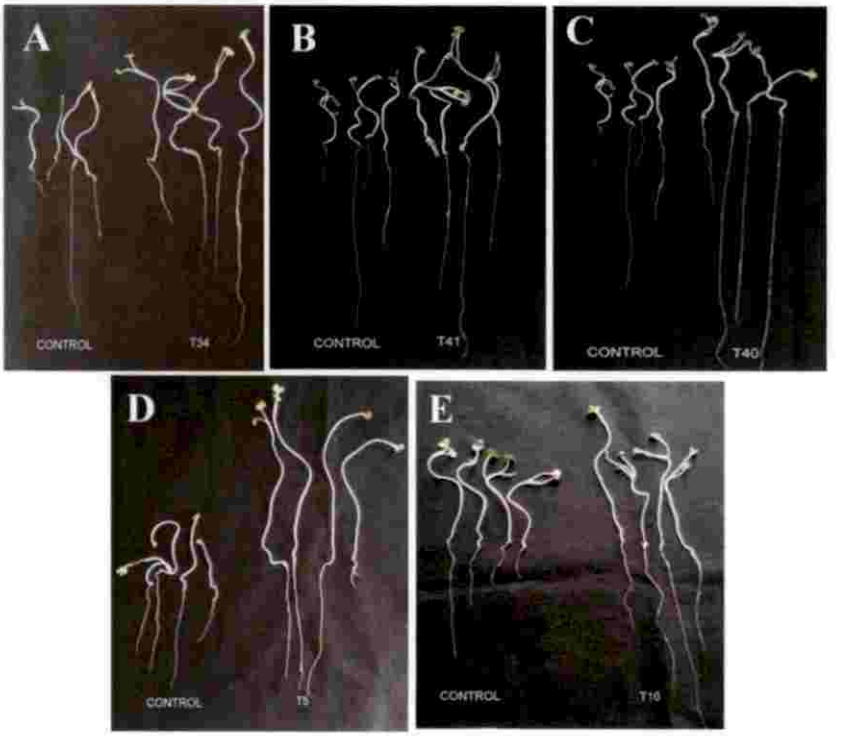


Plate 10. Growth promotion in mustard seedlings by VOCs of T34, T41, T40, T5 and T16 isolates

Table 8 continued					
T35	0.13^{abc}	2.00	10.50^{abc}	10.40^{abcd}	2.00^{ab}
T36	0.14^{ab}	2.00	10.83^{abc}	10.43^{abcd}	2.00^{ab}
T38	0.13^{abcd}	1.75	9.13^{abc}	9.88^{abcd}	2.25^{ab}
T40	0.09^{abcd}	2.00	15.68^{a}	8.15^{abcd}	2.25^{ab}
T41	0.12^{abcd}	2.50	9.88^{abc}	9.25^{abcd}	2.75^{a}
T42	0.09^{abcd}	2.00	9.58^{abc}	7.40^{bcd}	2.25^{ab}
T43	0.14^{ab}	2.50	7.38^{abc}	10.75^{abcd}	2.75^{a}
Control	0.05^{d}	1.70	6.55^{abc}	7.35^{cd}	1.90^{ab}

The results showed that in general, the VOCs from all the isolates could increase one or more growth parameters. However, some of the isolates could negatively affect root length, shoot length and production of shoot lets. The production of shoot let was very much influenced by the emission of VOCs.

4.3 MORPHOLOGICAL CHARACTERIZATION OF *Trichoderma* ISOLATES

Macro and micro morphology of *Trichoderma* isolates were studied (Table 9 and Plate 11-13). Mycelial growth rate, colony colour, reverse colony colour, presence or absence of coconut odour, shape of conidia, conidiophores branching, size of conidia and size of phialides were recorded. Growth rate was highly variable and it ranged from 17.7mm/day (T32) to 44.83mm/day (T36) followed by T38 (44.6) and T2 (42.0mm/day). Colony colour ranged from white to dark green and maximum number isolates showed dark green colony colour. The isolates showed colorless/cream/yellow/pale yellow/cream/pale greenish yellow reverse colony colour and 38.4% of the isolates showed colorless reverse colony colour. Barring eight isolates, all other isolates produced coconut odour. All the 26 isolates produced sub globose shaped conidia and in 22 isolates, the conidiophores were highly branched. The size of conidia as well as size of phialides also showed high variability among the

isolates. The conidial size ranged from 3.06µm (T18) to 5.83 µm (T28) whereas, the size of phialides varied from 7.65 µm (T14) to 13.70 µm (T28). The morphological characters studied were overlapping and it was very difficult to assign the isolates to specific species. Among the characters studied, colony morphology was most useful in characterization.

The macro morphological characters observed were compared with the characters explained in "Practical Handbook of the Biology and Molecular Diversity of *Trichoderma* Species from Tropical Regions" by Siddiquee (2017). The colony of T1, T2, T3, T5, T11, T13, T14, T15, T16, T17, T18, T19, T20, T41, T42 and T43 isolates were characterized by dense white conidial production, green colored colony, cream color on the reverse side of the colony and no color diffusion or pigment production throughout the PDA plate. The isolates were tentatively identified as *T. asperellum.* The isolates T35 and T36 were characterized with intense yellow pigment and yellowish green conidia. These isolates were tentatively identified as *T. reesei.* The colony of T38 isolate was found white in color and the reverse side of the colony was found pale greenish-yellow in color and was identified as *T. longibrachiatum.* The isolate T40 form dense green conidia on white colony and developed pustules and the isolate was tentatively identified as *T. erinaceum.* T6, T7, T28 and T29 isolates shows dull green colored colony with cream color on reverse side of the colony and it covers the entire surface of the plate in a concentrical pattern they were identified as *T. virens*. T32 and T34 isolates forms similar colony pattern of green conidia clumped on white colony with white reverse colony color. Based on the morphological characters it was found difficult to identify all isolates up to species level. T32 and T34 were not identified by morphology-based characterization. Only the tentative identification was possible using morphological characters.

Table 9. Macro and micro morphological characters of *Trichoderma* isolates

Isolate code	Macro morphological characters				Micro morphological characters			
	Growth rate (mm/ day)	Colony color	Reverse colony color	Coconut odor	Shape of conidia	Conidiophore branching	Size of conidia (µm)	Size of phialides (µm)
T1	40.0^{abcde}	Dark green	Cream	Present	Subglobose	Highly branched	4.48±1.05	8.96±0.75
T2	41.0^{abcd}	Dark green	Cream	Present	Subglobose	Highly branched	3.4±0.71	9.57±5.00
T3	42.0^{ab}	Dark green	Cream	Absent	Subglobose	Highly branched	3.55±0.41	9.33±1.10
T5	40.5^{abcd}	Dark green	Cream	Absent	Subglobose	Highly branched	3.64±0.89	11.57±2.99
T6	40.5^{abcd}	Light green	Cream	Present	Subglobose	Less branched	3.59±0.50	10.12±2.20
T7	39.8^{abcde}	Light green	Cream	Present	Subglobose	Less branched	4.74±0.77	12.00±1.20
T11	37.2^{bcdef}	Dark green	Colorless	Absent	Subglobose	Highly branched	4.06±0.26	11.12±1.18
T13	33.2^{fg}	Dark green	Colorless	Absent	Subglobose	Highly branched	3.69±0.18	13.20±0.36
T14	35.8^{defg}	Dark green	Colorless	Present	Subglobose	Highly branched	3.80±0.70	7.65±0.94
T15	34.0^{fg}	Dark green	Colorless	Absent	Subglobose	Highly branched	3.62±0.18	10.66±1.28
T16	37.7^{bcdef}	Dark green	Colorless	Present	Subglobose	Less branched	4.13±0.16	14.00±1.05
T17	41.3^{abc}	Dark green	Colorless	Present	Subglobose	Highly branched	3.69±0.05	11.20±0.87
T18	31.2^{gh}	Dark green	Colorless	Present	Subglobose	Highly branched	3.06±0.46	9.97±2.74
T19	35.0^{efg}	Dark green	Colorless	Present	Subglobose	Highly branched	4.07±0.03	12.30±1.65
T20	27.8^{hi}	Dark green	Colorless	Present	Subglobose	Highly branched	3.80±0.16	12.50±0.26
T28	26.5^{hi}	Light green	Cream	Present	Subglobose	Highly branched	5.83±0.91	13.70±3.29
T29	24.7^{i}	Light green	Cream	Present	Subglobose	Highly branched	3.77±0.26	11.73±1.68
T32	17.7^{i}	White	Colorless	Absent	Subglobose	Highly branched	3.92±0.28	8.89±0.64
T34	37.9^{bcdef}	White	Cream	Absent	Subglobose	Highly branched	3.70±0.16	13.13±0.47
T35	41.7^{abc}	Yellowish	Yellow	Present	Subglobose	Less branched	3.52±0.03	10.16±0.73
T36	44.8^{a}	Yellowish	Pale yellow	Present	Subglobose	Highly branched	3.67±0.04	9.84±0.21
T38	44.7^{a}	White	Pale greenish yellow	Present	Subglobose	Highly branched	3.33±0.12	11.53±0.76
T40	34.0^{fg}	White	Cream	Absent	Subglobose	Highly branched	3.47±0.04	10.56±0.70
T41	35.2^{efg}	Yellowish green	Cream	Present	Subglobose	Highly branched	3.37±0.12	12.30±0.26
T42	36.5^{cdef}	Yellowish green	Cream	Present	Subglobose	Highly branched	3.87±0.18	10.78±1.09
T43	35.8^{defg}	Yellowish green	Cream	Present	Subglobose	Highly branched	4.56±0.24	9.59±0.24
	5.2415							

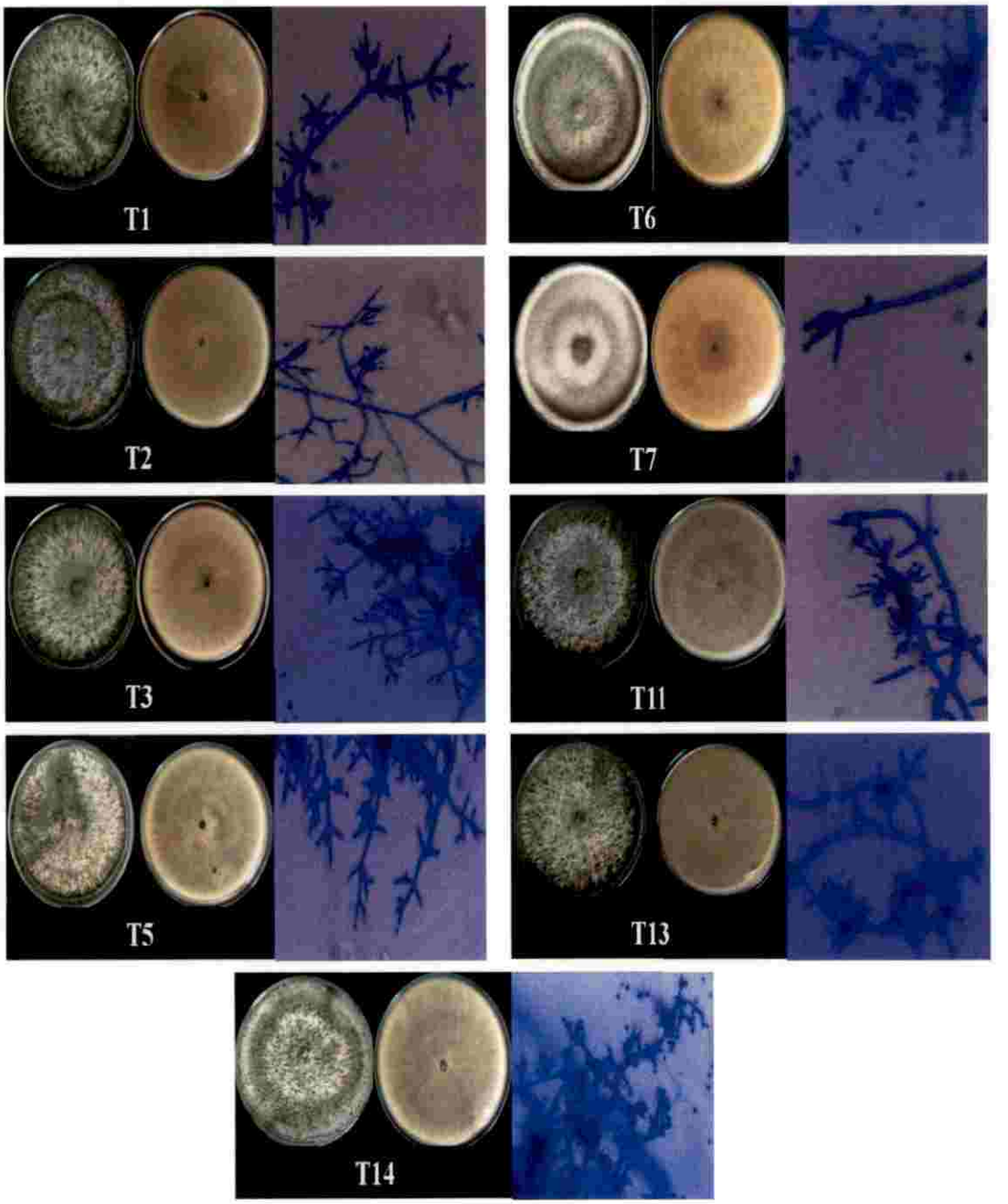


Plate 11. Colony appearance of each isolate on PDA (7DAI), reverse colony color, microscopic view of conidiophore at 40X magnification

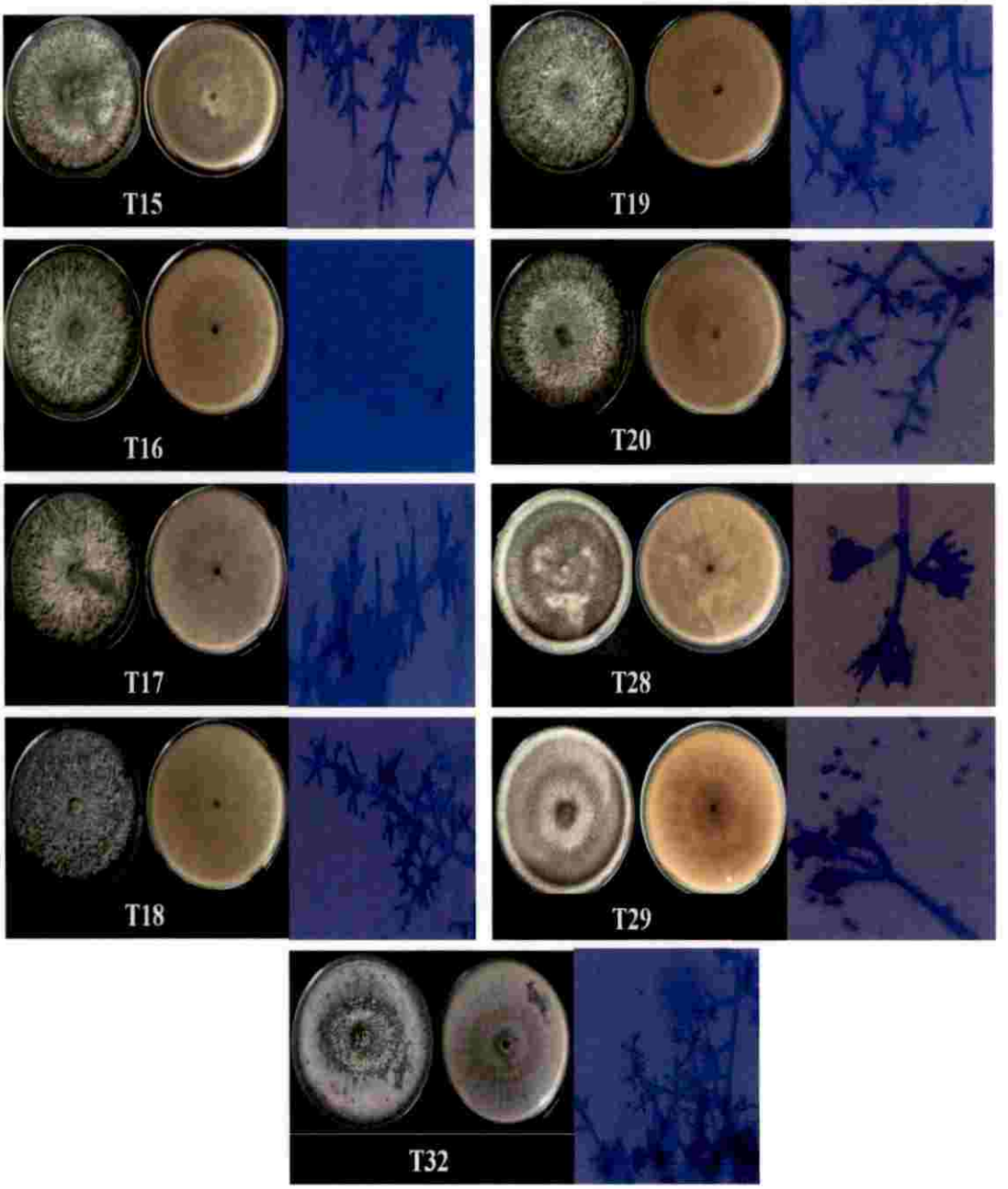


Plate 12. Colony appearance of each isolate on PDA (7DAI), reverse colony color, microscopic view of conidiophore at 40X magnification

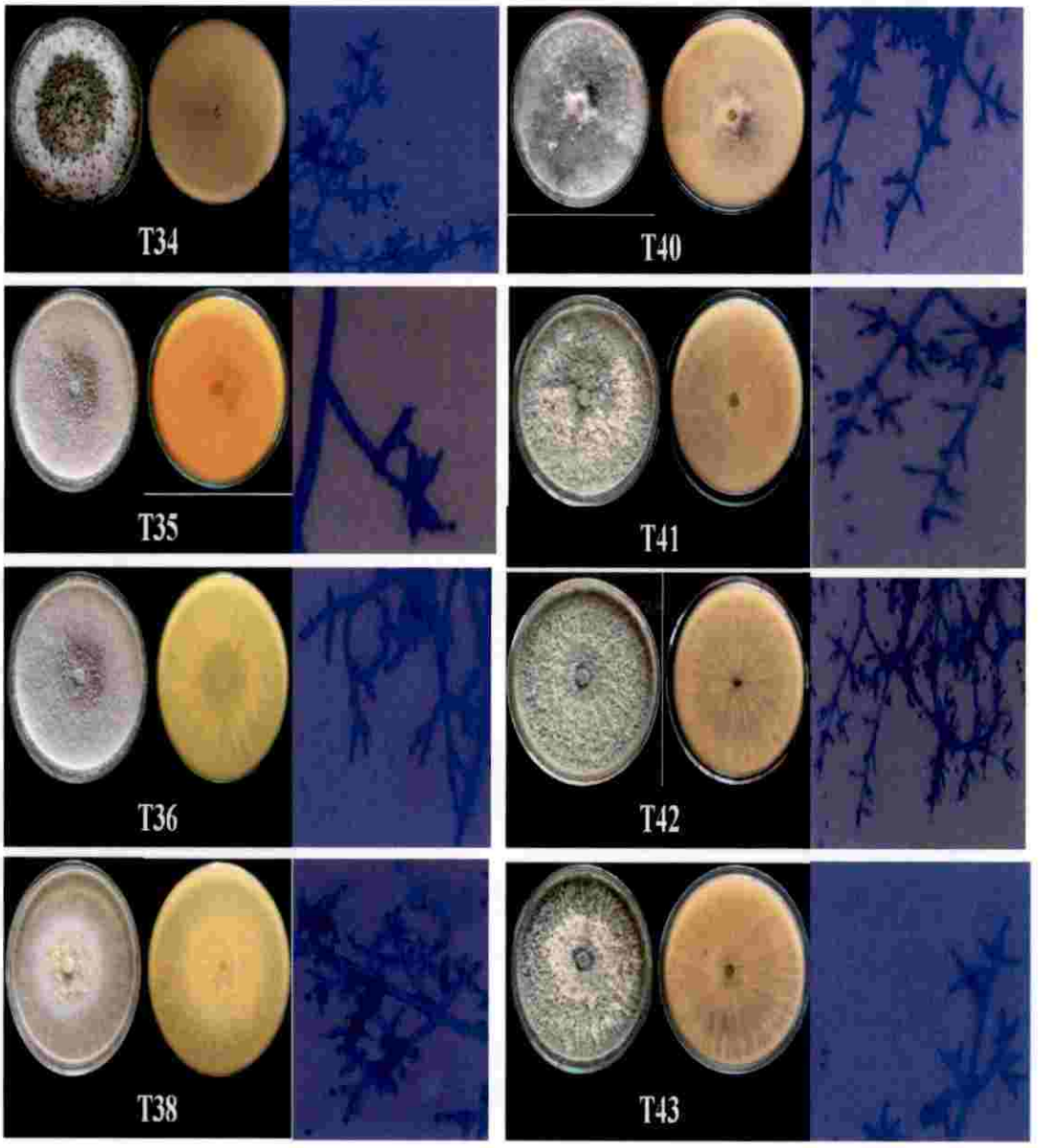


Plate 13. Colony appearance of each isolate on PDA (7DAI), reverse colony color, microscopic view of conidiophore at 40X magnification

4.4 MOLECULAR CHARACTERIZATION OF *Trichoderma* ISOLATES

4.4.1 Genomic DNA isolation

The DNA was extracted from 26 *Trichoderma* isolates using SDS method explained by George *et al.* (2018). The procedure was modified as two time wash with phenol- chloroform-iso amyl alcohol (25:24:1) and chloroform: iso amyl alcohol (24:1). The concentration of DNA and its purity were checked using Nanodrop spectrophotometer (Denovix) and are given below in the Table 10. A maximum yield upto 3409 ng μl^{-1} of DNA was obtained by this method. $A_{260/280}$ ratio ranges from 1.82 to 2.19 and $A_{260/230}$ratio ranges from 1.98 to 2.35. The quality of diluted DNA sample was also checked in 1% agarose gel (Plate 14).

Table 10. Quantity and quality of DNA isolated from 26 *Trichoderma* isolates (Measured using Nanodrop spectrophotometer)

Sample	DNA yield (ng μl^{-1})	$A_{260/280}$	$A_{260/230}$
T1	1812	1.88	2.07
T2	2391	1.98	2.05
T3	1132	2.13	2.16
T5	1710	2.03	2.17
T6	3049	1.88	2.16
T7	2388	1.99	2.18
T11	1014	2.01	2.14
T13	2146	1.83	2.15
T14	2205	1.85	2.03
T15	1752	1.82	2.17
T16	1004	1.91	2.15
T17	1539	2.00	2.00
T18	1590	2.01	2.22
T19	3409	1.87	2.35
T20	2992	1.82	2.15
T28	2153	2.14	2.21
T29	1479	1.97	2.08
T32	1171	1.85	1.98
T34	310	2.06	2.14

T35	1760	1.84	2.21
T36	2250	1.93	2.11
T38	1934	2.04	2.24
T40	2580	1.87	2.13
T41	680	2.19	2.17
T42	1634	2.06	2.31
T43	2678	1.96	2.29

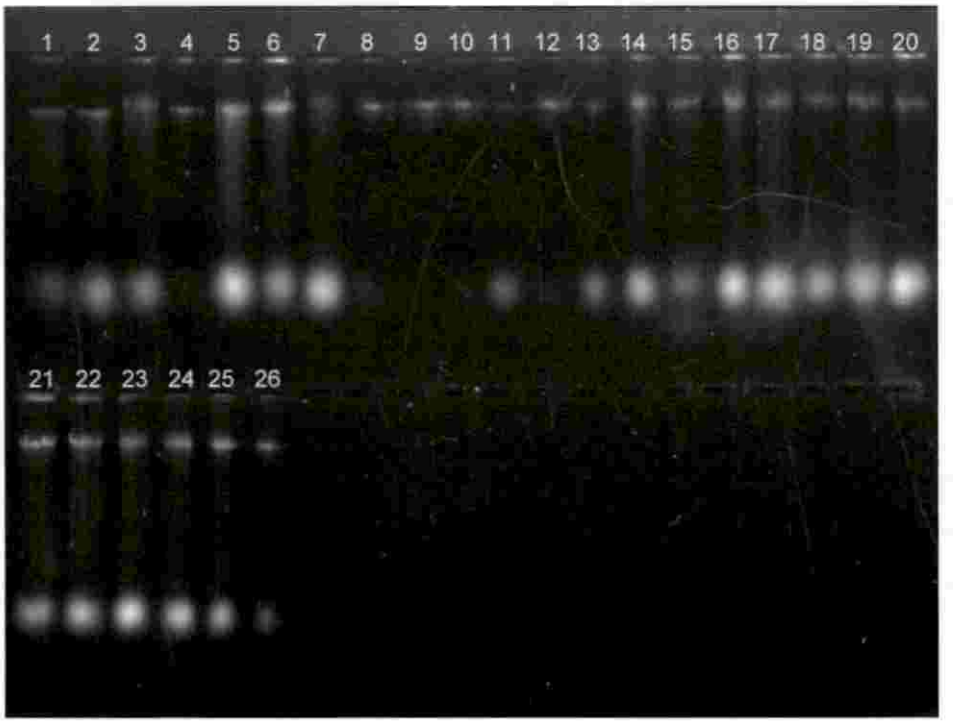


Plate 14 Gel profile of diluted DNA of 26 isolates in 1% agarose

4.4.2 Molecular identification of *Trichoderma* isolates based on ITS gene

The PCR amplification of ITS 1 and 2 genes of 26 *Trichoderma* isolates were done using the primers ITS1 and ITS4. On checking the PCR product in 1.2 % agarose gel, all isolates showed band at the 600bp region (Plate 15). The sequencing of PCR product was done at AgriGenome Labs Private Limited, Kochi, India. The obtained sequences were analyzed using NCBI BLAST and isolates were identified based on the nucleotide sequence similarity (Table 11). The percentage identity

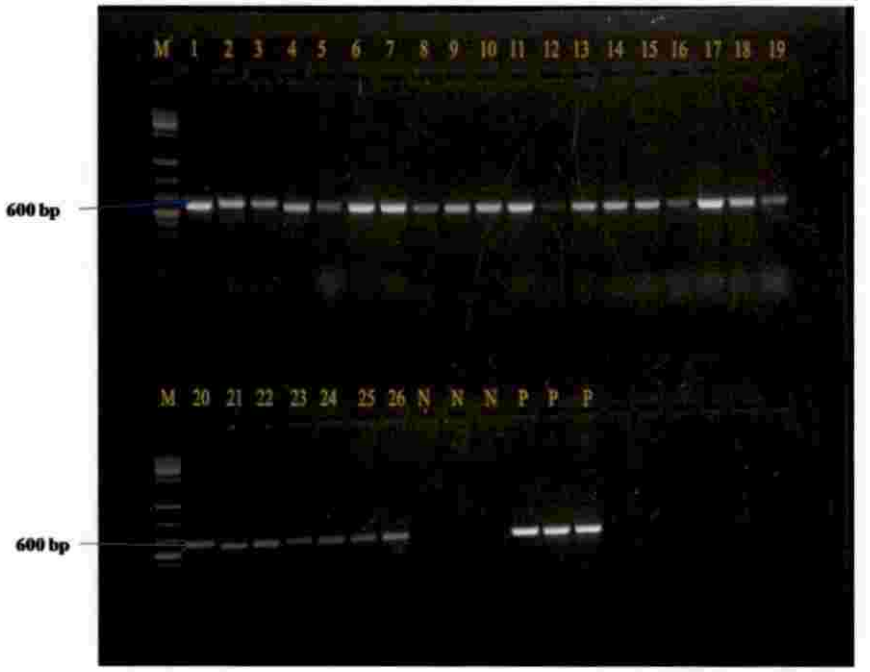


Plate 15. Gel profile of amplified ITS 1 and 2 gene

M – ladder 1kb plus

1 to 26 – ITS gene of selected isolates

N- Negative control

P- Positive control

ranged from 93.54% to 100%. Six *Trichoderma* species were identified from this 26 isolates. They are *T. asperellum*, *T. virens*, *T. hamatum*, *T. reesei*, *T. longibrachiatum* and *T. erinaceum*. Among the isolates, *T. asperellum* was the most common species. The species identification using NCBI BLAST was further checked by *Trich*OKEY 2 program available online from the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (ISTH). It also confirmed the identification with high reliability except for T14, T35, T36 and T40 isolates due to incomplete query sequence. Figures 7, 8, 9, 10, 11 and 12 shows the BLAST and *Trich*OKEY output description of T1, T28, T38, T14, T35 and T40 isolate respectively.

Table 11. Details of species identification based on ITS gene sequence

Isolate	Species identified	NCBI accession number to which the isolate shown similarity	Identity (%)	Genbank accession no.	Identification reliability in ISTH
T1	*Trichoderma asperellum*	MH908503.1	100	MN176406.1	High
T2	*T. asperellum*	KU170996.1	99.64	MN176380.1	High
T3	*T. asperellum*	MK027316.1	98.58	MN176404.1	High
T5	*T. asperellum*	MK210428.1	99.65	MN176408.1	High
T6	*T. virens*	MF952679.1	93.88	MN165549.1	High
T7	*Hypocrea virens*	FJ884748.1	99.48	MN176398.1	High
T11	*T. asperellum*	MH752042.1	99.30	MN176388.1	High
T13	*T. asperellum*	KU198280.1	98.95	MN176392.1	High
T14	*T. hamatum*	KC403936.1	99.30	MN176381.1	-
T15	*T. asperellum*	MH908503.1	99.29	MN176379.1	High
T16	*T. asperellum*	MF408314.1	98.41	MN173868.1	High
T17	*T. asperellum*	KC113288.1	99.64	MN173869.1	High
T18	*T. asperellum*	MK255053.1	94.21	MN173871.1	High
T19	*T. asperellum*	KU198280.1	99.3	MN173875.1	High
T20	*T. asperellum*	KM875460.1	98.95	MN173873.1	High
T28	*T. virens*	KT363920.1	94.12	MN165539.1	High
T29	*T. virens*	MF408299.1	97.74	MN173849.1	High

Fig. 6 A neighbor-joining phylogenetic tree constructed based on the ITS gene sequences

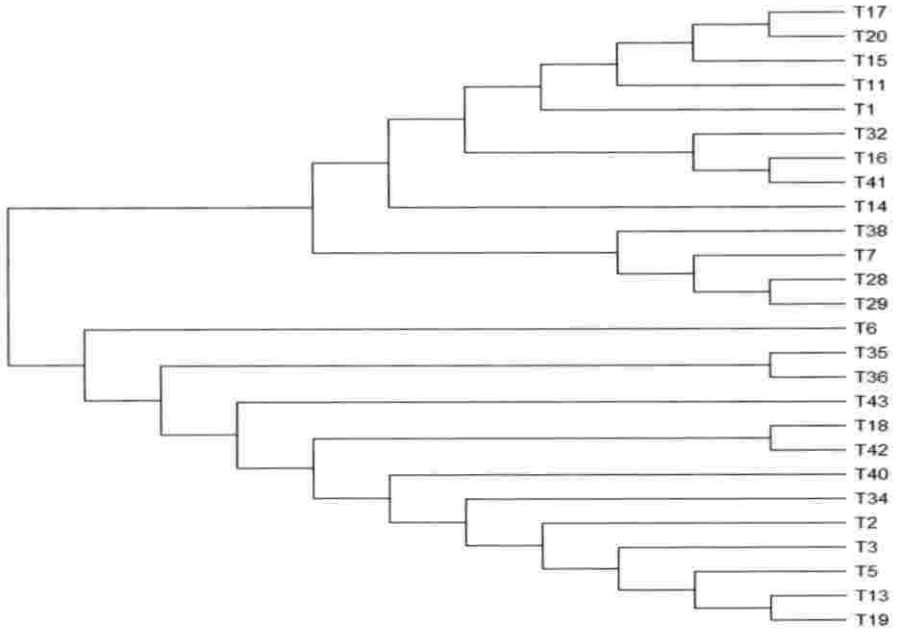


Fig. 7a BLAST output description of T1 isolate

Fig. 7b *Trichо*BLAST output description of T1 isolate

Fig. 7 BLAST and *Trichо*BLAST output descriptions of T1 isolate

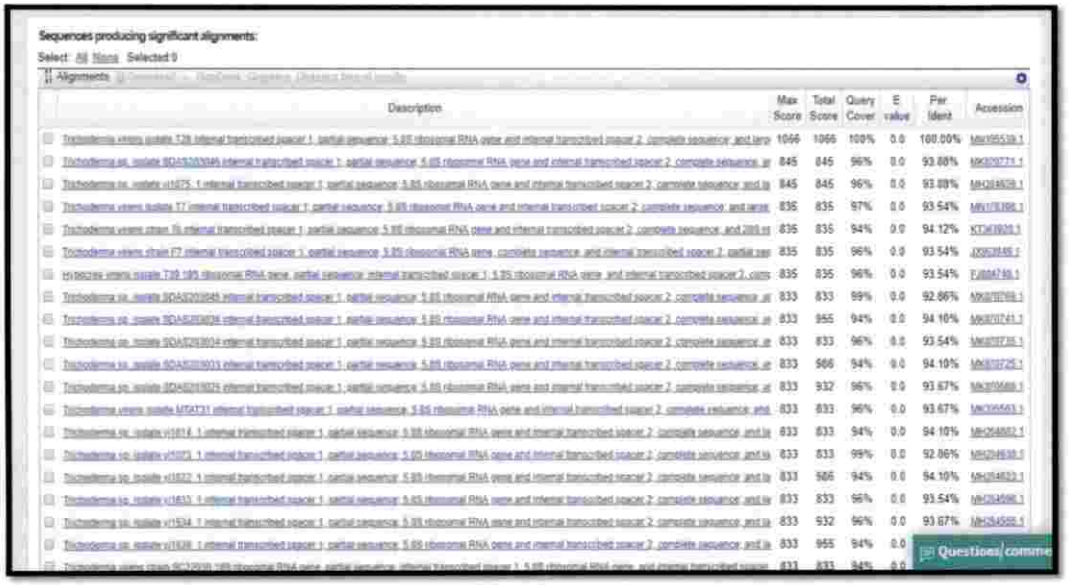


Fig. 8a BLAST output description of T28 isolate

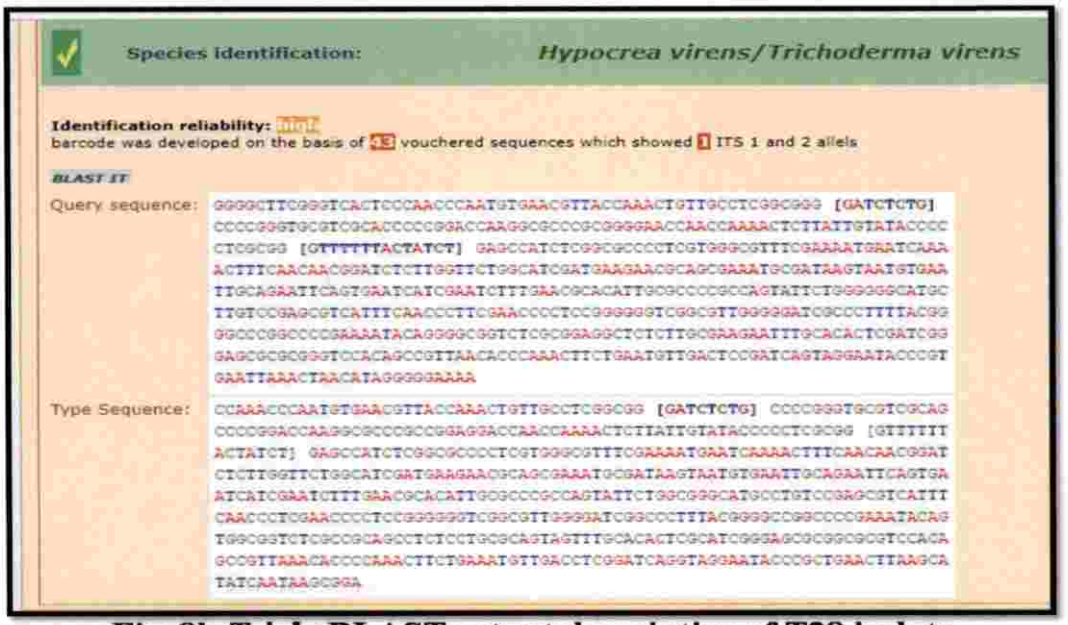


Fig. 8b *Trichо*BLAST output description of T28 isolate

Fig. 8 BLAST and *Trichо*BLAST output description of T28 isolate

Fig. 9a BLAST output description of T38 isolate

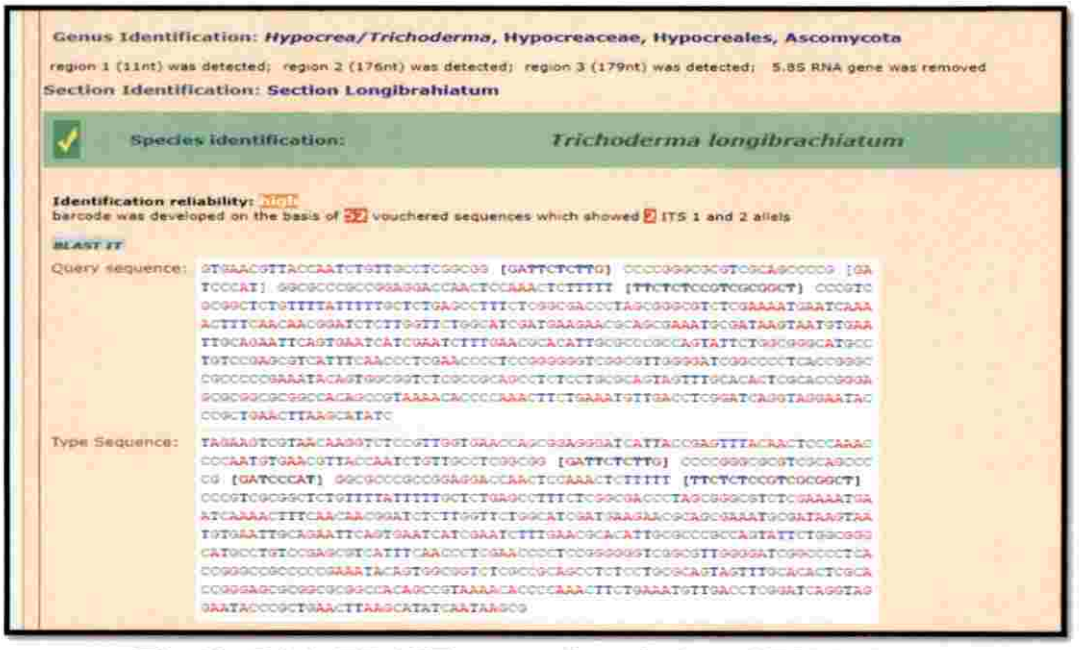
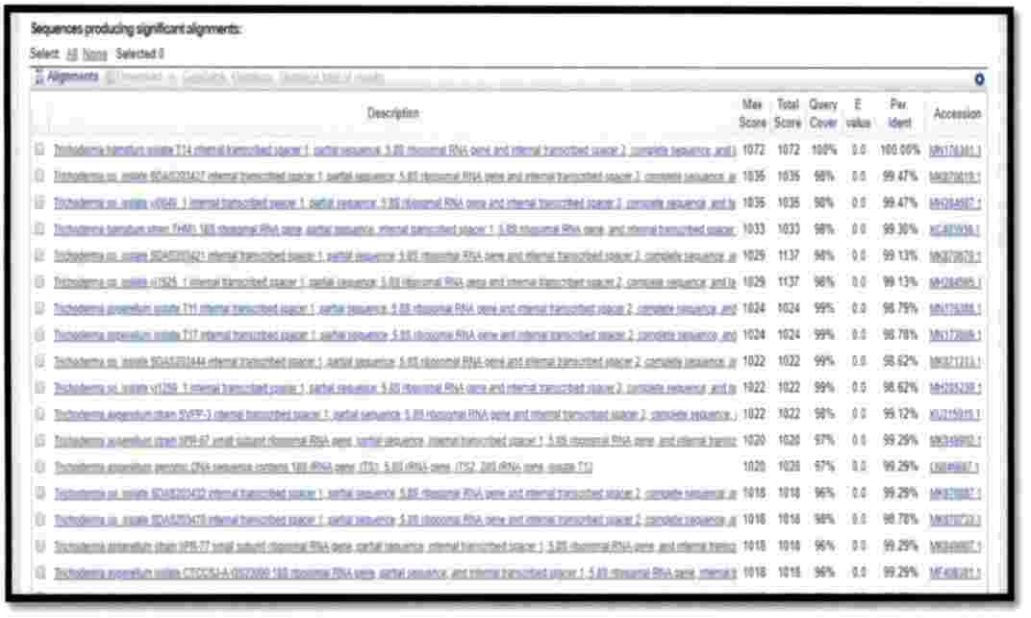


Fig. 9b *Trichо*BLAST output description of T38 isolate

Fig. 9 BLAST and *Trichо*BLAST output description of T38 isolate



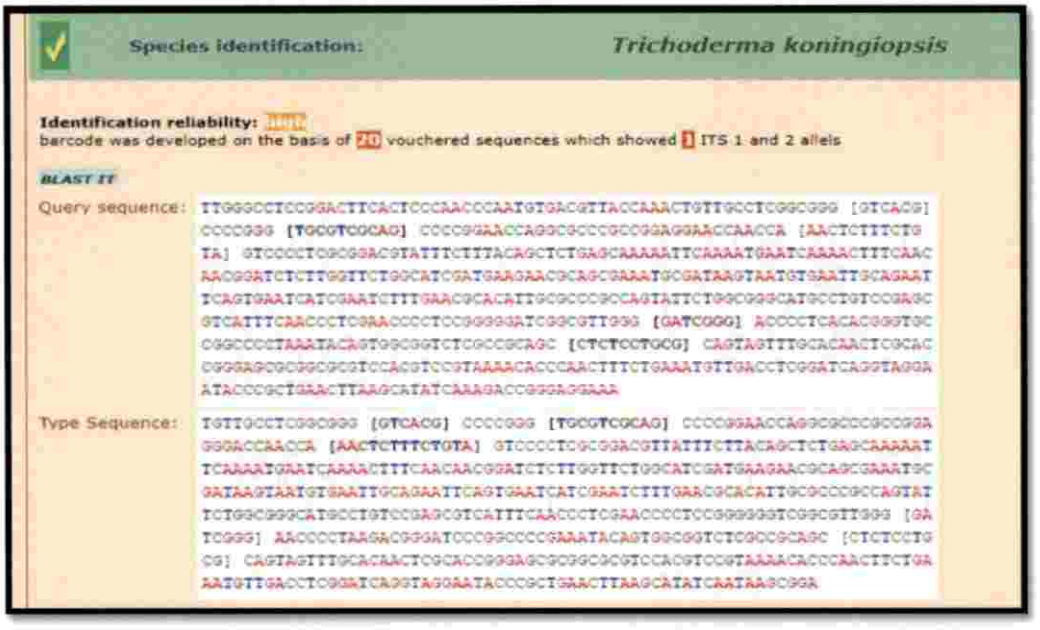
Sequences producing significant alignments:

Select: All None Selected: 0

Alignments

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Trichoderma harzianum isolate T14 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and l	1072	1072	100%	0.0	100.00%	MN176361.1
Trichoderma sp. isolate SDAS203427 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; a	1035	1035	98%	0.0	99.47%	MK879619.1
Trichoderma sp. isolate v3649_1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and la	1035	1035	98%	0.0	99.47%	MH284567.1
Trichoderma harzianum strain THW1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer	1033	1033	98%	0.0	99.30%	KC489936.1
Trichoderma sp. isolate SDAS203421 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; a	1029	1137	98%	0.0	99.13%	MK879578.1
Trichoderma sp. isolate v1525_1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and la	1029	1137	98%	0.0	99.13%	MH284565.1
Trichoderma gamsii isolate T11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and	1024	1024	99%	0.0	98.79%	MN176388.1
Trichoderma asperellum isolate T17 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and	1024	1024	99%	0.0	98.78%	MN173006.1
Trichoderma sp. isolate SDAS203444 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; a	1022	1022	99%	0.0	98.62%	MK871213.1
Trichoderma sp. isolate v11758_1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and la	1022	1022	99%	0.0	98.62%	MH285238.1
Trichoderma asperellum strain SVFF-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence;	1022	1022	98%	0.0	99.12%	KU215915.1
Trichoderma asperellum strain WR-67 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1020	1020	97%	0.0	99.29%	MK949902.1
Trichoderma asperellum genomic DNA sequence, contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, isolate T12	1020	1020	97%	0.0	99.29%	LN849887.1
Trichoderma sp. isolate SDAS203452 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; a	1018	1018	96%	0.0	99.29%	MK879587.1
Trichoderma sp. isolate SDAS203478 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; a	1018	1018	96%	0.0	98.78%	MK879723.1
Trichoderma asperellum strain WR-77 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1018	1018	96%	0.0	99.29%	MK949907.1
Trichoderma asperellum isolate CTCCSU-A-I0072000 18S ribosomal RNA gene, partial sequence; and internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal t	1018	1018	96%	0.0	99.29%	MF408281.1

Fig. 10a BLAST output description of T14 isolate



Species identification: *Trichoderma koningiopsis*

Identification reliability: HIGH

barcode was developed on the basis of 210 vouchered sequences which showed 1 ITS 1 and 2 allels

BLAST IT

Query sequence:

TTGGGCCTCCGGACTTCACTCCCAACCCAATGTGACGTTACCAAACTGTTGCCTCGGCGGG [GTCACG] CCCCGGG [TGCGTCGCAG] CCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCA [AACTCTTTCTG TA] GTCCCCTCGCGGACGTATTTCTTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAAC AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAAT TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGC GTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGG [GATCGGG] ACCCCTCACACGGGTGC CGGCCCCTAAATACAGTGGCGGTCTCGCCGCAGC [CTCTCCTGCG] CAGTAGTTTGCACAACTCGCAC CGGGAGCGCGGCGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGTAGGA ATACCCGCTGAACTTAAGCATATCAAAGACCGGGAGGAAA

Type Sequence:

TGTTGCCTCGGCGGG [GTCACG] CCCCGGG [TGCGTCGCAG] CCCCGGAACCAGGCGCCCGCCGGA GGGACCAACCA [AACTCTTTCTGTA] GTCCCCTCGCGGACGTTATTTCTTACAGCTCTGAGCAAAAAT TCAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT TCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGTCGGCGTTGGG [GA TCGGG] AACCCCTAAGACGGGATCCCGGCCCCGAAATACAGTGGCGGTCTCGCCGCAGC [CTCTCCTG CG] CAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCGTCCACGTCCGTAAAACACCCAACTTCTGA AATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGA

Fig. 10b *Trichо*BLAST output description of T14 isolate

Fig. 10 BLAST and *Trichо*BLAST output description of T14 isolate

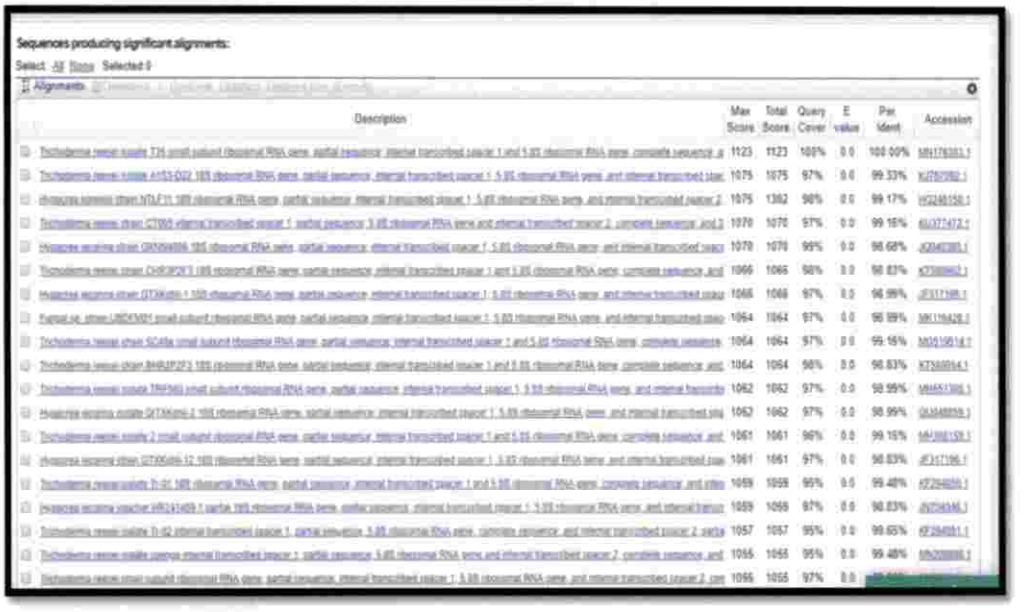


Fig. 11a BLAST output description of T35 isolate

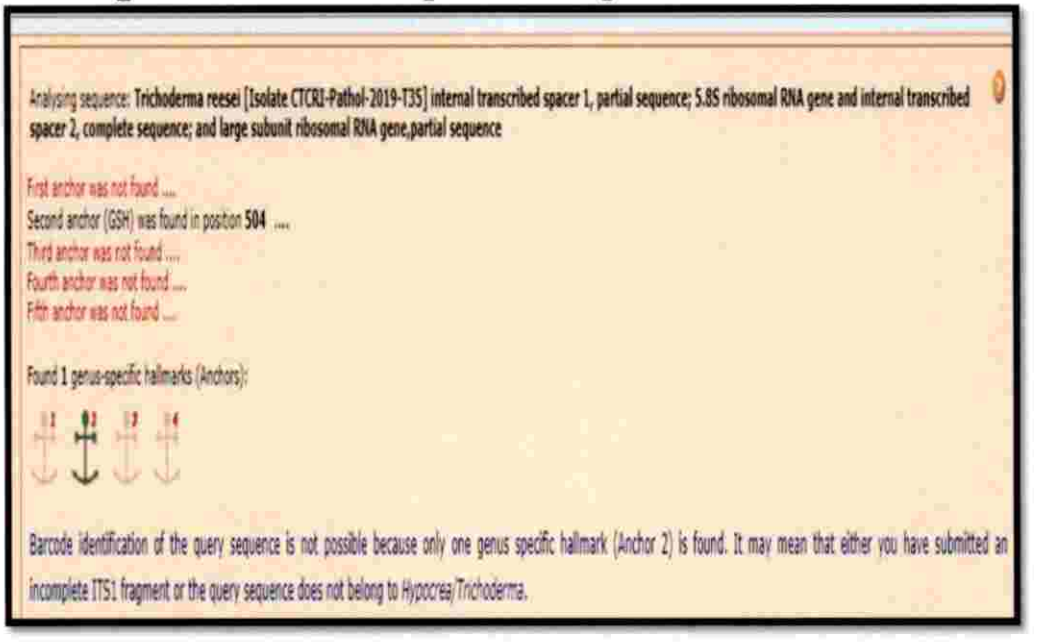


Fig. 11b *Trichо*BLAST output description of T35 isolate

Fig. 11 BLAST and *Trichо*BLAST output description of T35 isolate

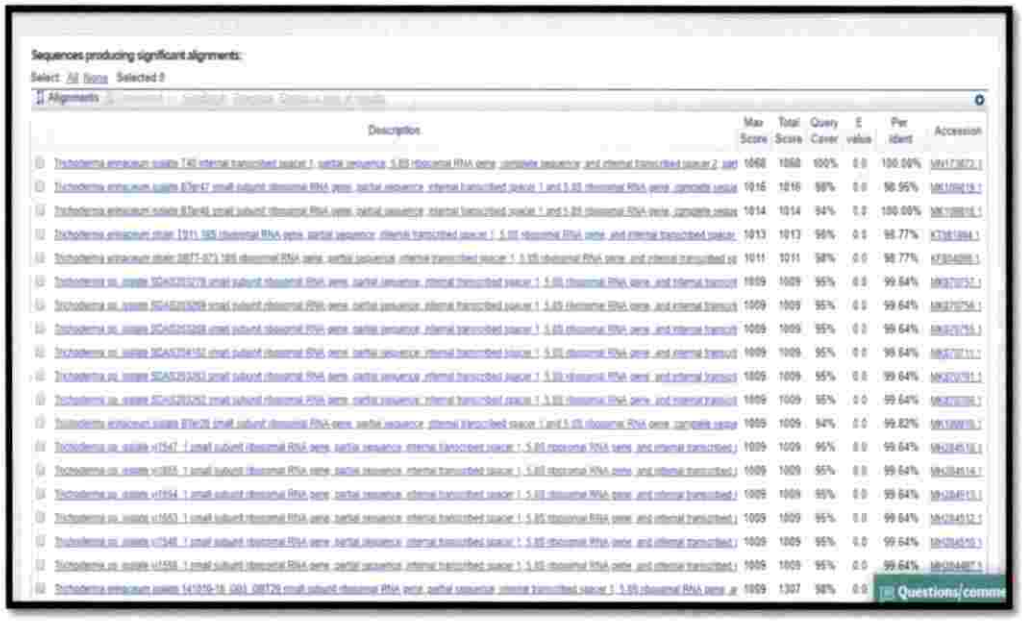


Fig. 12a BLAST output description of T40 isolate

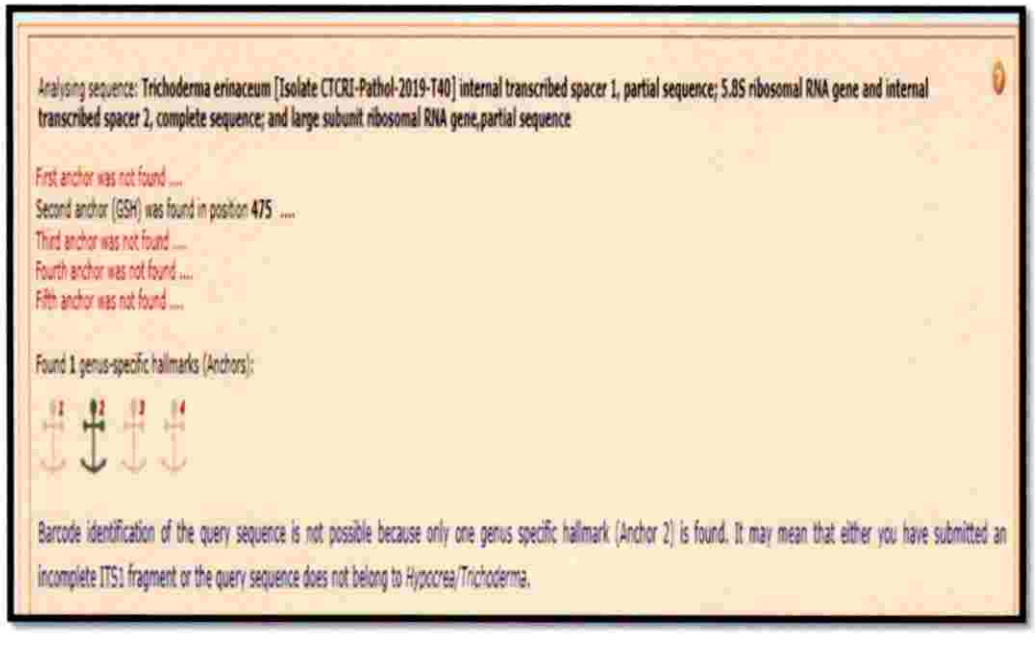


Fig. 12b *Trichо*BLAST output description of T40 isolate

Fig. 12 BLAST and *Trichо*BLAST output description of T40 isolate

T32	*T. asperellum*	MH752042.1	100	MN128228.1	High
T34	*T. asperellum*	MK611655.1	100	MN165490	High
T35	*T. reesei*	KJ767092.1	99.33	MN176383.1	-
T36	*T. reesei*	MG575492.1	99.47	MN165446.1	-
T38	*T. longibrachiatum*	MG962377.1	100	MN165477.1	High
T40	*T. erinaceum*	MK109819.1	98.95	MN173872.1	-
T41	*T. asperellum*	KC113288.1	98.58	MN173865.1	High
T42	*T. asperellum*	MK032262.1	99.22	MN173860.1	High
T43	*T. asperellum*	MF919486.1	93.54	MN173867.1	High

Phylogenetic analysis was done and the phylogram was drawn with the help of MEGA software version 7. It indicated the considerable difference in isolates. Even same species identified by sequence similarity were clustered into different groups. The phylogenetic tree got divided into two clusters. On analyzing the phylogenetic tree (Fig. 6), isolates T35 and T36 (*T. reesei*) were found to be similar. *T. virens* isolates like T7, T28 and T29 were also found to be similar. However, Isolate T6, which was also identified as *T. virens* was clustered separately and found to be different according to the phylogenetic tree.

4.4.3 Molecular identification of *Trichoderma* isolates based on *tef*1 gene

The PCR amplification of *tef*1 gene of 26 *Trichoderma* isolates was done using the primers *tef*85f (5' AG GAC AAG ACT CAC ATC AAC G 3') and *tef*954r primer sequence: (5'AGT ACC AGT GAT CAT GTT CTT G 3') (John Bissett, 2009). The PCR product was checked in 1.2 % agarose gel, all isolates shows band at the 900 bp region (Plate 16). The sequencing of the PCR product was done at AgriGenome Labs Private Limited, Kochi, India. The obtained sequences were analyzed using NCBI BLAST and isolates were identified based on the nucleotide sequence similarity (Table 12). The percentage identity ranges from 83.29% to 100%. The species identification using NCBI BLAST was further checked by *Trich*OKEY 2 program available online from the International Subcommission on *Trichoderma* and

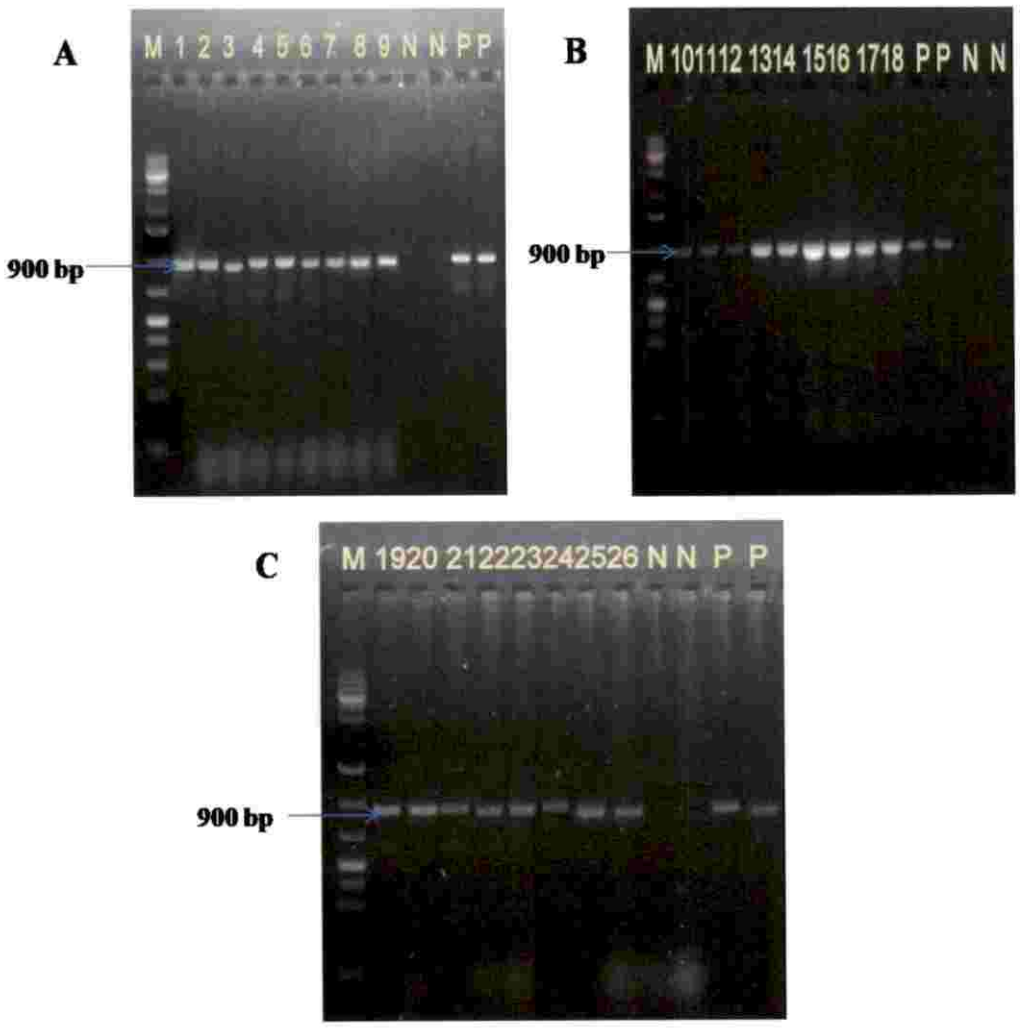


Plate 16. A, B and C gel profile of *tef*1 gene amplification of 26 *Trichoderma* isolate

M – ladder 1kb plus

1 to 26 – *tef*1 gene of selected isolates

N- Negative control

P- Positive control

Hypocrea Taxonomy (ISTH). Due to incomplete sequence details, the identification was not possible with *Trich*OKEY.

Table 12. Details of species identification based on *tef1* gene sequence

Isolate	Species identified	Per. Identity	NCBI accession number to which the isolate shown similarity
T1	*Trichoderma asperellum*	100%	KT302162.1
T2	*T. asperellum*	100%	MH017203.1
T3	*T. asperellum*	100%	KT302162.1
T5	*T. asperellum*	100%	KT302162.1
T6	*T. virens*	96.13%	KU301728.1
T7	*T. virens*	83.29%	KU301728.1
T11	*T. asperellum*	90.85%	MH017203.1
T13	*T. asperellum*	90.37%	MH017203.1
T14	*T. asperellum*	96.53%	KP696459.1
T15	*T. asperellum*	97.13%	KT302162.1
T16	*T. asperellum*	100%	KY213959.1
T17	*T. asperellum*	98.25%	KT302162.1
T18	*T.asperellum*	98.99%	MH017203.1
T19	*T. asperellum*	97.53%	KT302162.1
T20	*T. asperellum*	98.41%	KT302162.1
T28	*T. virens*	100%	KU301729.1
T29	*T. virens*	96.15%	KU301728.1
T32	*T. asperellum*	100%	KY213959.1
T34	*T. asperellum*	100%	KY213959.1
T35	*T. reesei*	90.17%	KU301742.1
T36	*T. reesei*	92.86%	KU301742.1

T38	*T. longibrachiatum*	87.50%	HG931246.1
T40	*T. erinaceum*	100%	EU280025.1
T41	*T. asperellum*	87.75%	MH017203.1
T42	*T. asperellum*	99.10%	KT302162.1
T43	*T. asperellum*	100%	KT302162.1

4.5 MOLECULAR DIVERSITY ANALYSIS OF *Trichoderma* ISOLATES

4.5.1 Diversity analysis using SSR markers

Ten SSR primers were used to study the genetic diversity of selected 26 *Trichoderma* isolates. The annealing temperature for each primer was determined by gradient PCR. The annealing temperature ranges between 45.2 °C to 57.4 °C (Table 13). The amplified products were resolved on 2% agarose gel and compared the size of amplicon with 1kb plus ladder (Plate 17-22). Ten SSR primers produced a total of 32 reproducible and scorable amplicons with an average of 3.2 amplicons per primer (Table 13). The percentage of polymorphism generated by the SSR markers was 100% among all the 26 isolates of *Trichoderma*. Maximum number of alleles was amplified by ThSSR1 primer. Minimum number of allele was amplified by TaSSR1.

4.5.2 Similarity matrix for SSR analysis

The data for each primer amplicon was noted in Microsoft excel sheet and generated a pair-wise similarity matrix using NTSYS version 2.02. The Jaccard's similarity coefficient of 10 SSR primer banding patterns was estimated for the 26 isolates of *Trichoderma* and which was used for cluster analysis to present genetic relationship in the form of dendrogram shown in Fig. 14. The result from the UPGMA dendrogram generated for *Trichoderma* isolates revealed that it was divided into two main clusters. Among 26 isolates, 16 isolates came under cluster A and 10 isolates came under cluster B. Cluster B further sub clustered into two. First sub cluster of cluster B consist of all *T. virens* isolates (T6, T29, T7 and T28). All *T. asperellum* isolates except T41, T32 and T34 were came under cluster A. The second

sub cluster of cluster A was separately for *T. erinaceum* (T40). Two *T. reesei* isolates (T35 and T36) were found in a single cluster. Jaccard's coefficient value based on SSR banding pattern for 26 isolates of *Trichoderma* are presented in Fig. 13. The similarity coefficient range varies from 0.31 to 1.00.

Table 13 Percent polymorphism observed in SSR primer

Sl. No.	Primer name	Annealing temperature (°C)	Total bands	Polymorphic bands	Monomorphic bands	% Polymorphism	Cross species amplification	Expected allele size (bp)
1	TvSSR-1	57.4	2	2	0	100	*Ta, Tha, Tr, Ter*	340
2	TaSSR-1	57.4	1	1	0	100	*Tha, Ter*	142
3	TaSSR-4	49.5	3	3	0	100	*Tvr, Tha, Tr*	397
4	ThSSR-1	45.2	6	6	0	100	*Ta, Tvr, Tr, Tl, Ter*	213
5	ThSSR-4	45.2	6	6	0	100	*Ta, Tvr, Tr, Ter*	147
6	ThSSR-5	51.3	2	2	0	100	*Ta, Tvr, Tr, Ter*	375
7	ThSSR-6	51.3	3	3	0	100	*Ta, Tvr, Tr, Tl, Ter*	313
8	TvSSR-4	55.5	2	2	0	100	–	194
9	TvSSR-5	55.5	2	2	0	100	*Ta, Tha*	199
10	TvSSR-2	46.3	5	5	0	100	*Ta, Tha, Tr, Tl, Ter*	391
	Total		32	32	0	1000		
	Average		3.2	3.2	0	100		

*(*Ta- T. asperellum, Tvr- T. virens, Tha- T. hamatum, Tr- T. reesei, Tl- T. longibrachiatum, Ter- T. erinaceum)*

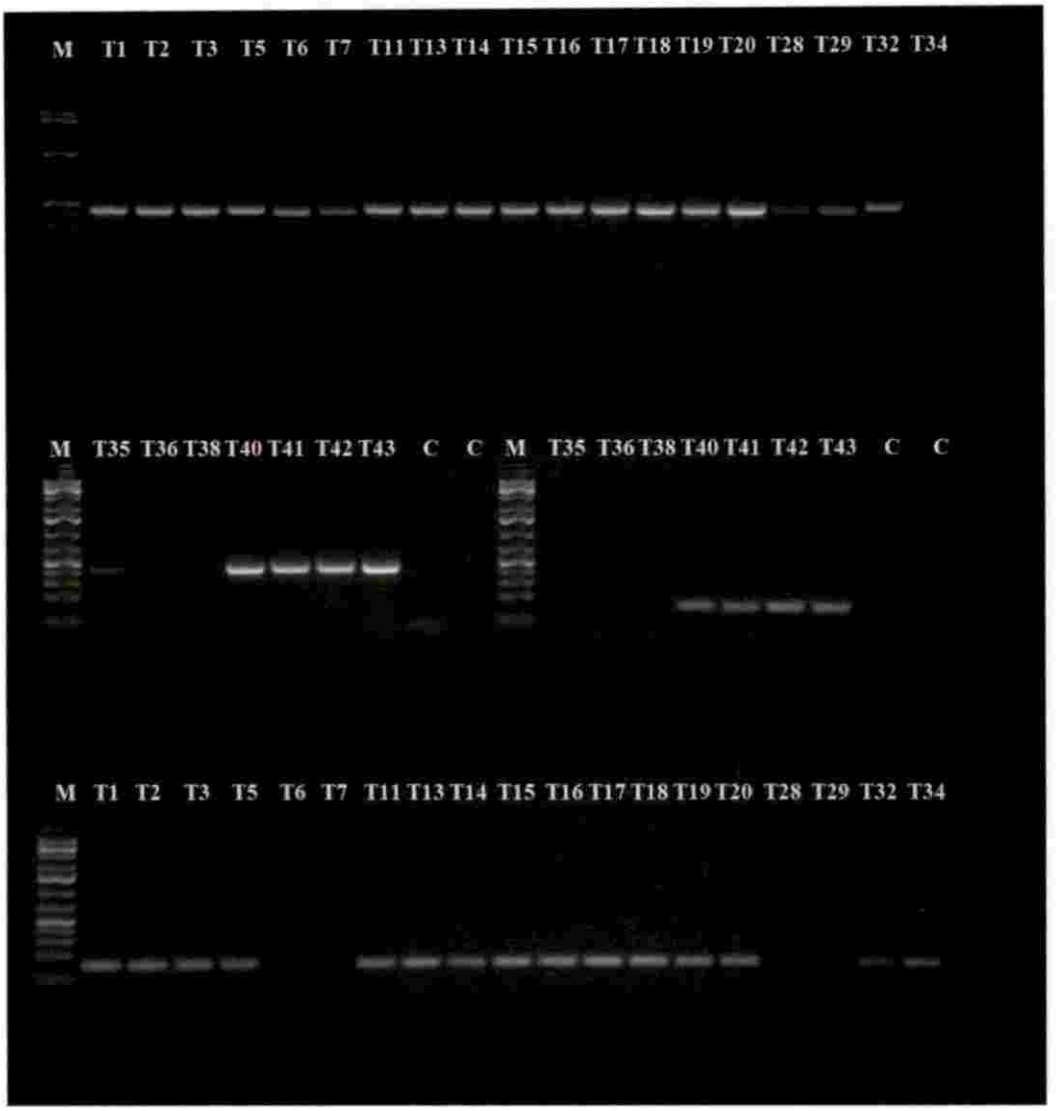


Plate 17. Gel profile of 26 isolates using the primer pair TvSSR1f/TvSSR1r and TaSSR1f/TaSSR1r

M – ladder 1kb plus

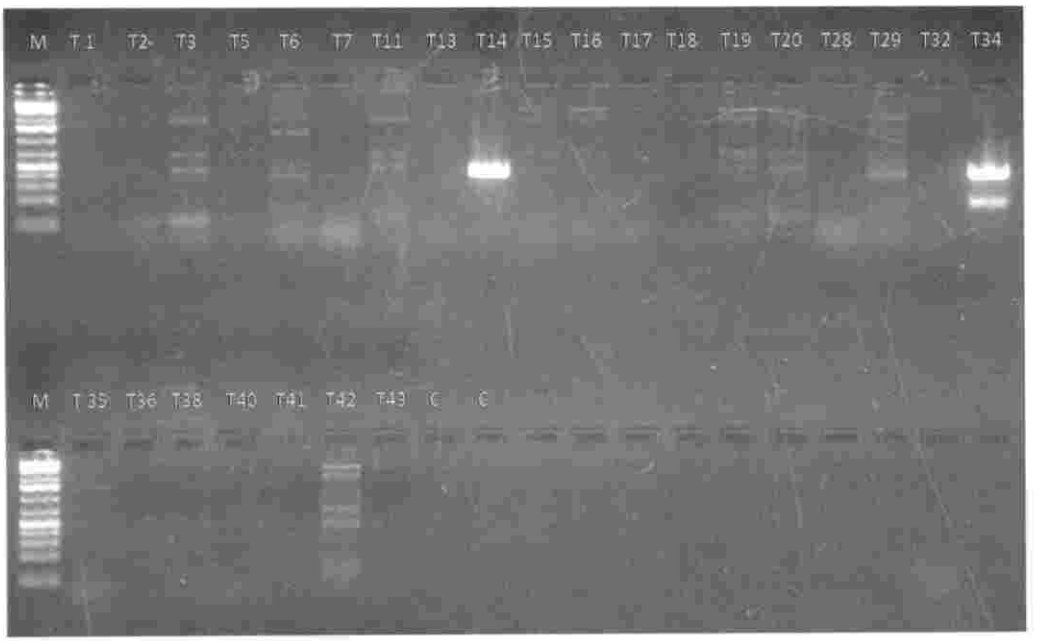


Plate 18. Gel profile of 26 isolates using the primer pair TaSSR4f/TaSSR4r

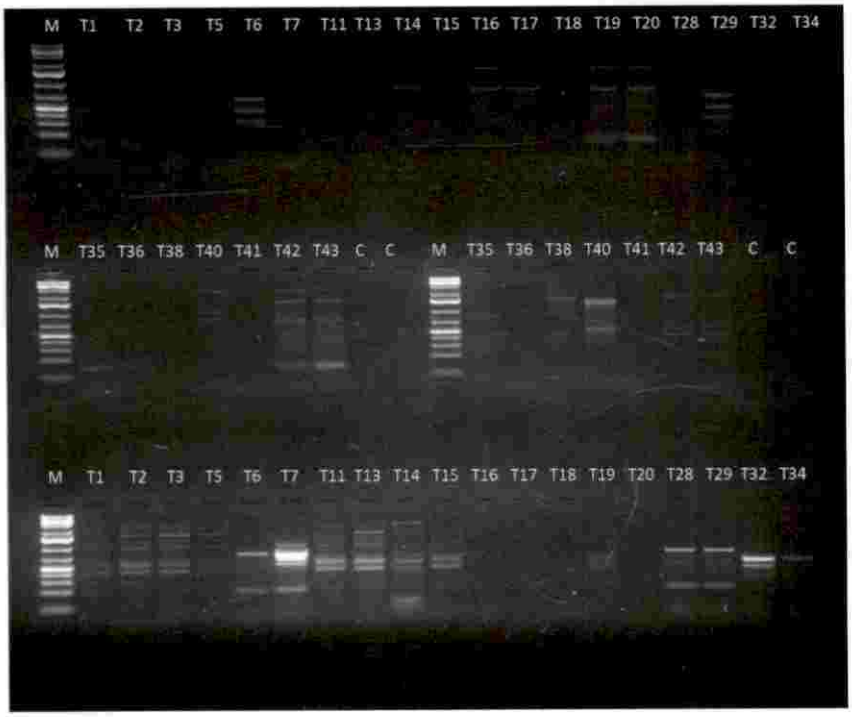


Plate 19. Gel profile of 26 isolates using the primer pair ThSSR4f/ThSSR4r and ThSSR1f/ThSSR1r

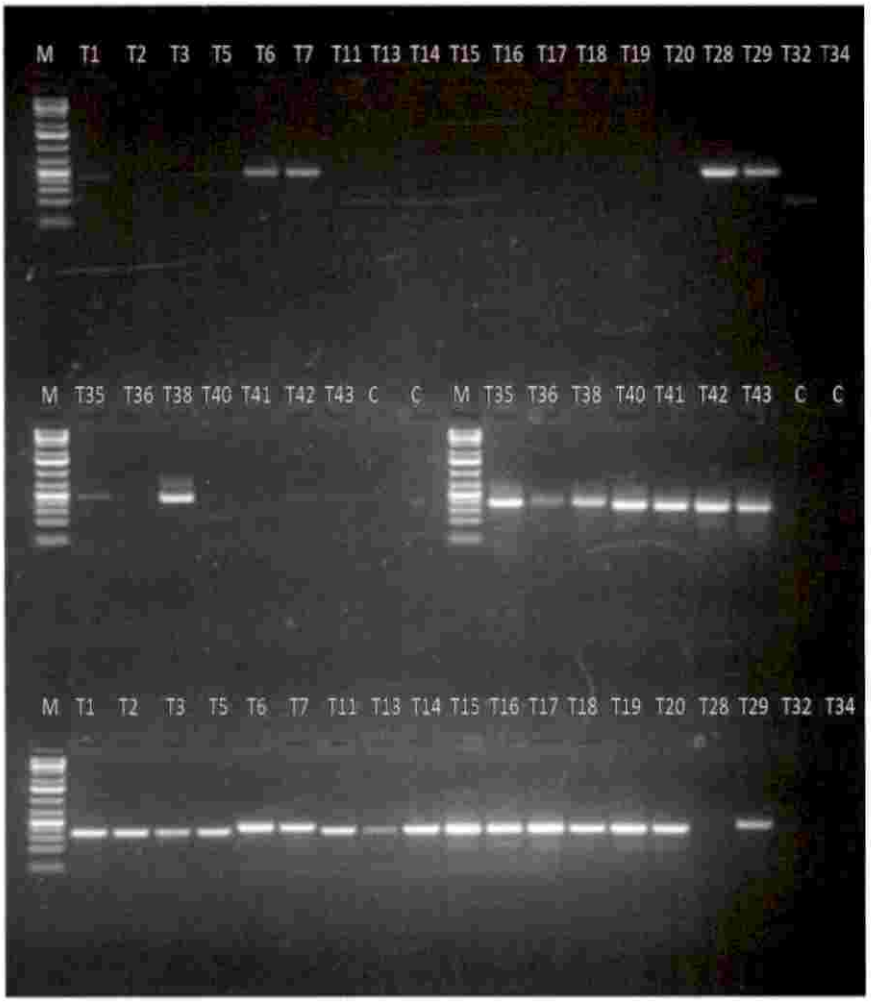


Plate 20. Gel profile of 26 isolates using the primer pair ThSSR5f/ThSSR5r and ThSSR6f/ThSSR6r

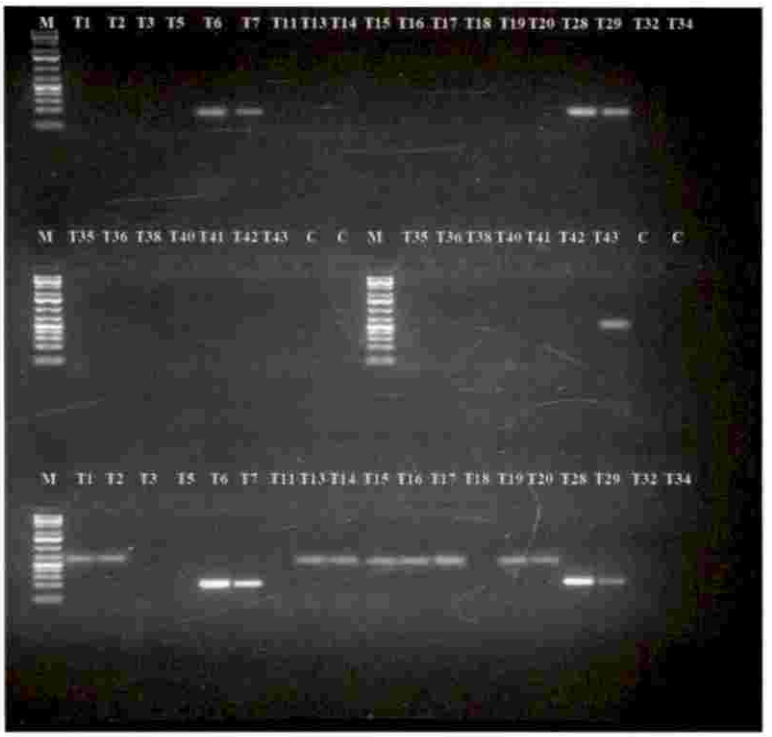


Plate 21. Gel profile of 26 isolates using the primer pair TvSSR4f/TvSSR4r and TvSSR5f/TvSSR5r

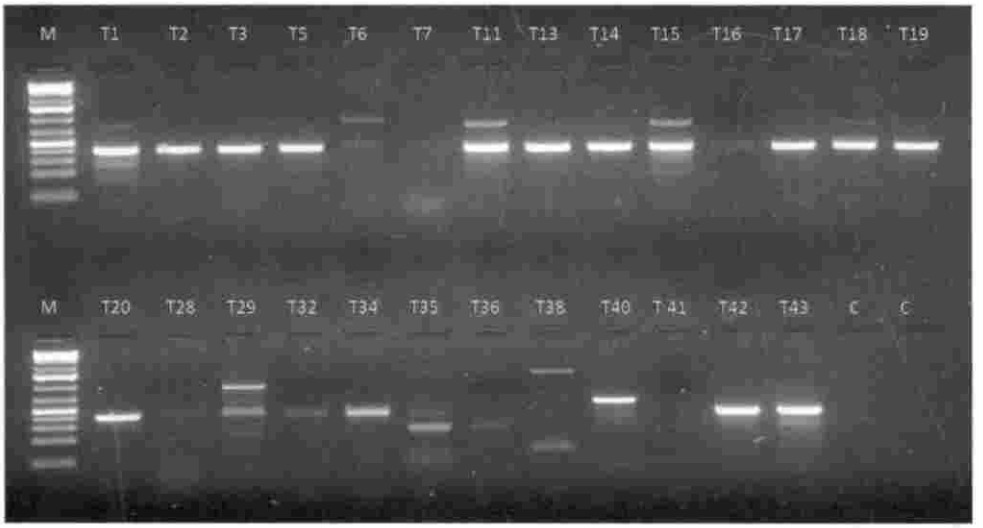
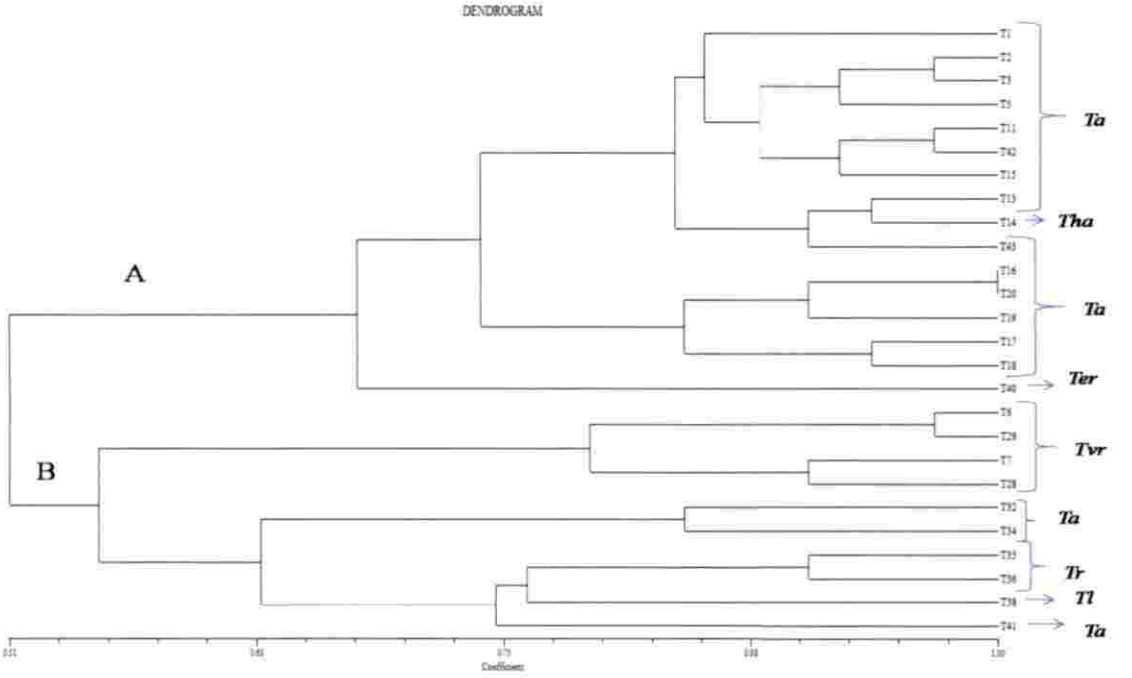


Plate 22. Gel profile of 26 isolates using the primer pair TvSSR2f/TvSSR2r

Fig. 13 The similarity coefficient for SSR analysis

	T1	T2	T3	T5	T6	T7	T11	T13	T14	T15	T16	T17	T18	T19	T20	T28	T29	T32	T34	T35	T36	T38	T40	T41	T42	T43
T1	1.00																									
T2	0.88	1.00																								
T3	0.84	0.97	1.00																							
T5	0.78	0.91	0.94	1.00																						
T6	0.47	0.47	0.50	0.44	1.00																					
T7	0.41	0.41	0.44	0.50	0.81	1.00																				
T11	0.84	0.91	0.94	0.88	0.50	0.50	1.00																			
T13	0.75	0.88	0.84	0.91	0.34	0.53	0.84	1.00																		
T14	0.81	0.88	0.84	0.84	0.34	0.47	0.84	0.94	1.00																	
T15	0.91	0.91	0.88	0.81	0.44	0.44	0.94	0.84	0.91	1.00																
T16	0.72	0.78	0.75	0.69	0.44	0.38	0.75	0.72	0.78	0.81	1.00															
T17	0.63	0.69	0.66	0.72	0.34	0.47	0.66	0.81	0.81	0.72	0.91	1.00														
T18	0.63	0.63	0.66	0.72	0.41	0.53	0.72	0.75	0.75	0.72	0.84	0.94	1.00													
T19	0.81	0.88	0.84	0.78	0.47	0.41	0.84	0.81	0.88	0.91	0.91	0.81	0.75	1.00												
T20	0.72	0.78	0.75	0.69	0.44	0.38	0.75	0.72	0.78	0.81	1.00	0.91	0.84	0.91	1.00											
T28	0.38	0.38	0.41	0.47	0.78	0.91	0.47	0.50	0.44	0.41	0.47	0.56	0.63	0.44	0.47	1.00										
T29	0.44	0.44	0.47	0.41	0.97	0.78	0.47	0.31	0.31	0.41	0.47	0.38	0.44	0.44	0.47	0.81	1.00									
T32	0.44	0.56	0.59	0.66	0.34	0.53	0.59	0.69	0.63	0.53	0.47	0.56	0.56	0.50	0.47	0.50	0.31	1.00								
T34	0.41	0.53	0.56	0.56	0.44	0.44	0.56	0.59	0.59	0.50	0.56	0.59	0.59	0.53	0.56	0.47	0.41	0.84	1.00							
T35	0.50	0.50	0.53	0.59	0.66	0.72	0.53	0.56	0.50	0.47	0.59	0.63	0.63	0.56	0.59	0.75	0.63	0.63	0.59	1.00						
T36	0.41	0.41	0.44	0.50	0.56	0.69	0.44	0.53	0.47	0.38	0.56	0.66	0.66	0.47	0.56	0.78	0.59	0.66	0.63	0.91	1.00					
T38	0.34	0.41	0.44	0.50	0.44	0.63	0.44	0.53	0.47	0.38	0.44	0.53	0.53	0.34	0.44	0.66	0.47	0.66	0.56	0.72	0.81	1.00				
T40	0.59	0.66	0.69	0.75	0.44	0.56	0.75	0.78	0.72	0.69	0.56	0.66	0.72	0.59	0.56	0.53	0.41	0.66	0.69	0.53	0.50	0.56	1.00			
T41	0.44	0.56	0.59	0.66	0.47	0.59	0.59	0.69	0.63	0.53	0.72	0.81	0.81	0.63	0.72	0.69	0.50	0.69	0.66	0.75	0.78	0.72	0.72	1.00		
T42	0.88	0.88	0.91	0.84	0.53	0.53	0.97	0.81	0.81	0.91	0.72	0.63	0.69	0.81	0.72	0.50	0.50	0.56	0.53	0.56	0.47	0.47	0.72	0.56	1.00	
T43	0.81	0.81	0.78	0.84	0.41	0.59	0.84	0.94	0.88	0.84	0.66	0.75	0.75	0.75	0.66	0.56	0.38	0.63	0.53	0.56	0.53	0.53	0.78	0.63	0.88	1.00

Fig. 14 UPGMA dendrogram obtained by SSR analysis of *Trichoderma* isolates based on Jaccard's similarity coefficient



*(*Ta- T. asperellum, Tvr- T. virens, Tha- T. hamatum, Tr- T. reesei, Tl- T. longibrachiatum, Ter- T. erinaceum*)

DISCUSSION

5. DISCUSSION

Elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) is an important tuber crop popular as a food security crop and as a remunerative cash crop (John *et al.,* 2015). Andhra Pradesh, Tamil Nadu, Kerala and West Bengal are the states were *A. paeonifolius* (Dennst.) is being commercially cultivated in India (Srinivas and Ramanathan 2005). *Amorphophallus* is susceptible to a number of diseases often causing heavy loss to the crop (Misra and Nedunchezhiyan, 2008). Collar rot caused by *Sclerotium rolfsii* is the most destructive and predominant disease among the diseases affecting elephant foot yam (Misra, 1997). *S. rolfsii* being non specialized soil borne pathogen and affects more than 500 species of economically important crops, the management of this pathogen with a single strategy including use of chemical fungicides seems to be difficult (Gogoi *et al.,* 2002). The present management strategy of collar rot disease includes application of various physical, chemical and biological control methods (Punja, 1985; Punja and Rahe, 1993). The mounting reluctance to use hazardous fungicides has led the policy makers to encourage bio-intensive management practices (El Komy *et al.*, 2015). Among the investigated biological control agents, *Trichoderma* species have attracted special position due to the particular biological characteristics since the early 1930s (You *et al.,* 2016). The précised identification and screening of *Trichoderma* isolates forms the basis of its application as a management strategy to tackle the deadly pathogen.

The present study, "Morphological, biochemical and molecular characterization of *Trichoderma* isolates from tuber crop ecosystem" aimed to identify the best *Trichoderma* isolate with maximum antagonistic potential against *S. rolfsii,* the pathogen responsible for collar rot disease in elephant foot yam and to characterize the isolates by adopting morphological, biochemical and molecular approaches. Knowledge regarding the behavior of *Trichoderma* isolates and more precise

identification is very essential for their effective use (Consolo *et al.,* 2012). Forty-three isolates of *Trichoderma* obtained from tuber crops ecosystem and maintained at Microbial repository, ICAR-CTCRI were used for the study. Various methods are being considered to determine the potency of *Trichoderma* isolates against various pathogens (Hirpara *et al.,* 2017). Three methods were adopted in present study to select the potent isolates of *Trichoderma.*

Screening results helps to clearly differentiate the isolates based on its antagonistic potential. Isolates showed high variability in their inhibitory potential. In the present study, maximum number of *Trichoderma* isolates was collected from the rhizosphere of tuber crop ecosystems in Kerala. Out of 43 isolates, 15 isolates were collected from different fields of ICAR- CTCRI, Sreekariyam. In dual culture, 73.3% of these isolates showed more than 50% inhibition which ranged from 45.67% (T4) - 74.13% (T38). In antibiosis test for production of diffusible inhibitory metabolites, 86.6% of isolates showed 100% inhibition against the test fungus. The inhibition varied from 41.85% (T21)–100%. Eleven isolates were collected from Pathanamthitta district of Kerala. In this, nine isolates were collected after 2018 flood in Kerala from the rhizosphere of tuber crops in Pathanamthitta district. In dual culture, 63.6% of the isolates showed more than 50% inhibition and which ranged from 9.44% (T26) – 58.17% (T21). Similarly, 36.3% of these isolate showed 100% inhibition against the test fungus in antibiosis test for production of diffusible inhibitory metabolites. The inhibition varied from 3.70% (T31) to 100%. In antibiosis test for production of volatile compounds by the isolates against pathogen, 53.3% and 63.6% of CTCRI isolates and of Pathanamthitta isolates respectively showed more than 30% inhibition. Thus the isolates under study showed high variability in their antagonistic potential irrespective of their place of collection or method used for evaluation. The result indicates the need for the use of suitable isolate for the management.

Competition for nutrients or space, production of lytic enzymes, inactivation of the pathogen's enzymes and parasitism are the direct mechanisms exhibited by *Trichoderma* isolates to elicit its bio-control action (Harman, 2006). Maximum

inhibition was recorded with the method, production of diffusible metabolites. It suggests the involvement of lytic enzymes and other metabolites in pathogen suppression. The efficiency of *Trichoderma* spp. to manage plant diseases is majorly because of their direct antagonistic effects on the fungal pathogen and mainly their ability to produce lytic enzymes (Benítez *et al.,* 2004; Viterbo *et al.,* 2002). Harman *et al.* (2004) suggested the key role of antibiosis or production of lytic enzymes during antagonism. The least inhibition was recorded with volatile metabolites. John *et al.* (2015) reported the inhibition of *S. rolfsii* by volatile metabolites as non significant.

Chitin and β-1,3 glucan are the main structural components of fungal cell wall. Chitinases and β -1,3 glucanases produced by *Trichoderma* isolates are the major enzymes resulting in the lysis of cell walls during mycoparasitic action (Kullnig *et al.*, 2000; Kubicek *et al.*, 2001). The efficiency of selected 26 isolates on induction of chitinase and β-1,3-glucanase enzyme against the cell wall of *S. rolfsii* was studied. The isolates varied in their ability to induce lytic enzymes. Even isolates of same species also showed variation in enzyme induction. The activity of cell wall-degrading enzymes, chitinase and β-1,3-glucanase is positively correlated with growth inhibition of the test pathogen (Gajera *et al.*, 2009; Consolo *et al.*, 2012: Hirpara *et al.*, 2017). In the present study, there was no positive correlation observed between chitinase enzyme production and antagonistic potential in terms of growth inhibition. However, some of the isolates (T32, T34, T40, T15 and T2) possess high degree of pathogen suppression as well as induction of chitinase and glucanase enzymes (Fig. 15). John *et al.* (2015) also reported strong positive correlation between antagonistic potential and induction of exochitinase compared to β-1,3-glucanase. Contradiction to this report was observed in the present study. Some studies have shown that *Trichoderma* exhibit considerable variability among strains with respect to their production of lytic enzymes (Sivan and Chet 1992). An isolate of *T. harzianum* induced high levels of chitinase and β -1-3 glucanase when grown on mycelia of *Rhizoctonia solani*, whereas it induced low levels of enzymes with *S.*

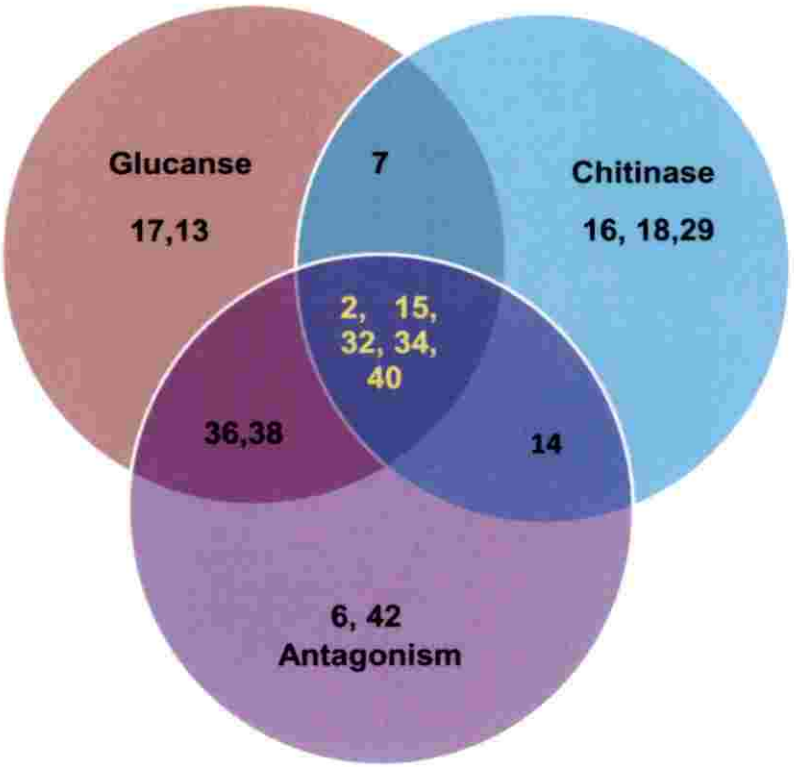


Fig. 15 Isolates (top 10) with antagonistic and induction of lytic enzymes potential

rolfsii (Elad *et al.*, 1982; Grondona *et al.*, 1997). No correlation was observed between high coiling capacity and chitinase activity with 2/5 *Trichoderma* isolates tested against *R. solani* (Almeida *et al.*, 2007). Similar mechanism could be operating between some of the *Trichoderma* isolates used in the present study and *S. rolfsii*. As suggested by Sivasithamparam and Ghisalberti (1998), other compounds with biological activity could be being produced by these isolates and causing the inhibition.

Antagonistic potential of the *Trichoderma* isolates and glucanase induction activities are positively correlated (Table 14) as reported by many earlier workers (Gajera *et al.*, 2009; Consolo *et al.*, 2012: Hirpara *et al.*, 2017). Pearson correlation coefficient indicates the strength of association between two variables. On analyzing the data obtained, it is clear that glucanase activity of the isolates is positively correlated with the antagonistic potential. Whether it is in terms of results obtained from dual culture method used for screening or the cumulative inhibition of the isolates calculated. Since the results of dual culturing and cumulative inhibition is also positively correlated, inhibition in dual culturing as well as glucanase induction potential of the isolates can be considered as yardsticks while selecting potent isolates for bio- intensive management.

Table 14. Pearson correlation coefficients

	Additive	Chitinase	Dual culture	Glucanase	Metabolite production	Volatile production
Additive						
Chitinase	-0.04315 0.8342					
Dual culture	0.78284 **<.0001**	-0.06049 0.7691				
Glucanase	0.68368 **0.0001**	0.05119 0.8038	0.51503 **0.0071**			
Metabolite production	0.05244 0.7992	0.10268 0.6177	-0.03094 0.8807	0.29669 0.1411		
Volatile production	0.50447 **0.0086**	-0.00588 0.9773	-0.07924 0.7004	0.36014 0.0707	0.06440 0.7546	

Isolate, T38 (*T. longibrachiatum*) showed maximum antagonistic potential against *S. rolfsii* and it showed maximum glucanase activity (2.11) also. Similarly, isolate T36 showed second highest pathogen suppression as well as glucanase activity (1.93). Seven isolates out of ten best performing isolates, the relationship is positive. The β-1,3-glucanase enzyme production was not only influenced by a single factor, a number of factors like type of strain, growth condition and the type of substrate used play a key role (Harman *et al.,* 2004). The ability of isolates to induce chitinase and β-1,3-glucanase production can serve as a marker to select isolates with high potential. However, environmental conditions and competitions from other organisms can restrict the performance of bio-agents under field conditions.

Genus *Trichoderma* is known to produce volatile organic compounds (VOCs) and it can act against the pathogens and can confer plant growth promoting effects as well as systemic resistance to plants (Tabarestani *et al.,* 2016). Effect of VOCs produced by the isolates on plant growth was assessed by using mustard seeds. The isolates promoted plant growth in terms of fresh weight, number of leaves, root length, shoot length and number of shoot lets. The growth promotion potential by VOCs also varied among isolates as in the case of antagonistic potential and lytic enzymes. The isolates could not uniformly promote all growth parameters in a significant manner. The differential emission of VOCs by various *Trichoderma* isolates and subsequent growth promotion in plants had been reported by many researchers (Coria *et al*., 2016; Lee *et al.,* 2016; Tabarestani *et al*., 2016; Flores *et al.,* 2017 and Perez *et al.,* 2017). The volatile-mediated growth promotion depended on species of *Trichoderma*, culture, stage of the plant, duration of the exposure (Hung *et al.*, 2013; Lee *et al.*, 2015). Similarly, Jalali *et al.* (2017) studied beneficial effects of VOCs from 13 strains of *Trichoderma* and found that *Arabidopsis* plants responded differently to VOCs of various strains and showed no change to significant growth promotion. The result of the present study confirms the findings of earlier workers.

To find out the most promising agents for growth promotion, additive effect on plant growth by the isolates was considered. T15 (*T. asperellum*) obtained from organically grown cassava ranked first followed by T32 (*T. asperellum*), T34 (*T. asperellum*), T41 (*T. asperellum*), T43 (*T. asperellum*) and T36 (*T. reesei*). These isolates, T15, T32, T34 and T36 are earlier identified with high pathogen suppression as well as high inducers of chitinase and β-1,3-glucanase production. The isolates have multipronged potential and can be utilized for the eco-friendly management of collar rot incidence and growth promotion in elephant foot yam.

Considering the diverse economical applications of *Trichoderma*, the correct species identification of *Trichoderma* is vital (Samuels, 2002). Morphological characterization was traditionally used in the identification of *Trichoderma* species, and it continues as an efficient method to identify *Trichoderma* species (Anees *et al.*, 2010).With an objective of identifying the isolates to species level, the macro morphological characters on PDA medium such as growth rate of colony, colony color, reverse colony color and odor of culture were studied. On the basis of morphological characteristics, 75% of isolates of *Trichoderma* were identified to species level (Fahmi *et al.*, 2016). Odor of culture was found to be inadequate for identifying up to species level since more than one species shows this character. Coconut odor was reported in *T. harzianum* and *T. viride* (Siddiquee, 2017). Even though many micro and macro characters of the isolates were considered for species identification in the present study, the characters were overlapping and make a clear-cut identification. Among the characters, colony color and pattern were useful in identification.*T. asperellum* is characterized by dense conidial production, white conidia towards the green centre, cream color on the reverse side of the colony and no color diffusion or pigment production throughout the PDA plate. Based on this, isolates T1, T2, T3, T5, T11, T13, T14, T15, T16, T17, T18, T19, T20, T41, T42 and T43 were tentatively identified as *T. asperellum*. Two isolates, T35 and T36 were with an intense yellow pigment and yellowish green conidia and tentatively identified

as *T. reesei.* The reverse colony color of T38 was pale greenish-yellow in color and showed more similarity to colony morphology of *T. longibrachiatum.* The isolate is tentatively identified as *T. longibrachiatum* (Siddiquee, 2017). Identification of *Trichoderma* up to species level, purely depending on morphological characters is insufficient because they have few morphological characters and less variation that cause overlapping and improper identification of the isolates (Fahmi *et al.*, 2016). Only tentative identification was possible with morphological characters.

Many *Trichoderma* species shows similar appearance under cultural conditions, although they are completely different species (Siddiquee, 2017). Hence more reliable methods for species identification like molecular methods were adopted. The internal Transcribed Spacer (ITS) region is the most widely sequenced region of fungal DNA. Identification of *Trichoderma* isolate to the species level was done by DNA sequencing of two regions, the ribosomal DNA (rDNA) - ITS region and the *tef1* gene (Nath *et al.*, 2014). On analyzing ITS region and *tef*1 sequences using NCBI BLAST, isolates were identified based on the nucleotide sequence similarity. The six species that are identified from 26 isolates are *T. asperellum*, *T. virens*, *T. hamatum*, *T. reesei*, *T. longibrachiatum* and *T. erinaceum*. Among 6 species identified in this study, *T. asperellum* was found to be the common species. Previously, Nath *et al.* (2014) also reported *T. asperellum* as widely distributed species.

In the genetic diversity study, minimum similarity of 0.31 was observed between T29 (Aranmula, Pathanamthitta) and T13 (Sreekariyam) isolates. The isolates T29 (Aranmula, Pathanamthitta) and T14 (Pathanamthitta) also showed the minimum similarity of 0.31. In many studies, minimum similarity coefficient indicates the isolates are from distant locations. But considering the isolates T29 and T13 as well as T29 and T14, the minimum similarity was due to the difference in species. The maximum similarity of 1.00 was observed between T20 (Sreekariyam) and T16 (Arunachal Pradesh) isolates. However, T20 and T16 are from distant

locations they showed maximum similarity because they came under same species. The dendrogram (Fig. 14) helped in better understanding of genetic relatedness between *Trichoderma* species. An important feature of SSR markers are its cross transferability across related species (Chandel *et al.,* 2011). Cross amplification was observed in primers except TvSSR-4 (Table 13). Ten SSR primers produced a total of 32 reproducible and scorable amplicons with an average of 3.2 amplicons per primer. The percentage of polymorphism generated by the SSR markers was 100 % among all the 26 isolates of *Trichoderma.*

Considering the antagonistic potential, high chitinase and glucanase production and plant growth promotion, isolates such as T2, T15, T32, T34 (*T. asperellum*) and T40 (*T. erinaceum*) are selected as effective bio-control agents (Fig. 15). After mass multiplication of these isolates and their field validation, these can be recommended as a management strategy to tackle the deadly pathogen *S. rolfsii.*

The present study helped in identifying the *Trichoderma* isolate with high antagonistic potential against *S. rolfsii* and the entire process ensured more precised and targeted application of *Trichoderma* isolate in field condition. The outcome of the study will be a key factor in developing appropriate management strategy to mitigate collar rot disease in elephant foot yam.

SUMMARY

6. SUMMARY

The study entitled "Morphological, biochemical and molecular characterization of *Trichoderma* isolates from tuber crop ecosystem" was carried out at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during the period 2018-2019. The objectives of this research programme was to study the differential antagonistic potential of *Trichoderma* isolates against *Sclerotium rolfsii,* the collar rot pathogen of *Amorphophallus paeonifolius* (Dennst.), to characterize the isolates using morphological, biochemical and molecular approaches and to analyse the molecular diversity.

Forty-three isolates of *Trichoderma* obtained from tuber crops ecosystem and maintained at Microbial repository, ICAR-CTCRI were used for the study. The differential antagonistic potential of *Trichoderma* isolates was studied against *S. rolfsii* by adopting three different *in vitro* screening methods like dual culture, production of diffusible metabolites and volatiles. The isolates showed differential response in checking the mycelial growth of the pathogen. *Trichoderma* uses different mechanisms to inhibit the pathogen. Considering this point, additive effect of three screening methods were considered for short listing the isolates. T38 showed maximum inhibition (213.07) followed by T36 and T32 with scores of 203.28 and 201.9 respectively. Twenty-six isolates were selected for further study based on the individual as well as additive effects.

The efficiency of 26 isolates for induction of chitinase and β-1,3-glucanase was studied using the cell wall of *S. rolfsii* as carbon source. There was no correlation between antagonistic potential of the isolates and induction of chitinase enzyme. However, some of the isolates (T32, T34, T40, T15 and T2) showed high pathogen suppressive action as well as induction of chitinase enzyme. A positive correlation was observed between antagonistic potential and induction of β-1,3-glucanase enzyme. The highest antagonistic potential as well as β-1,3-glucanase enzyme activity

was given by T38 isolate. Further, the effect of volatile organic compounds (VOCs) on plant growth was studied using mustard seeds. VOCs of isolates showed differential response to various growth parameters like fresh weight, number of leaves, root length, shoot length and number of shoot lets. Considering all factors, isolates T15, T32, T34, T41, T43 and T36 showed growth promotion of mustard seedlings.

The macro and micro morphological characters of 26 isolates were studied and compared with the characters reported in "Practical Handbook of the Biology and Molecular Diversity of *Trichoderma* Species from Tropical Regions" by Shafiquzzaman Siddiquee in 2017. Twenty-six isolates were tentatively identified into five species based on morphological, cultural and microscopic characters. The species identified are *T. asperellum* (T1, T2, T3, T5, T11, T13, T14, T15, T16, T17, T18, T19, T20, T41, T42 and T43), *T. virens* (T6, T7, T28 and T29), *T. reesei* (T35 and T36), *T. longibrachiatum* (T38) and *T. erinaceum* (T40). T32 and T34 were not identified by morphology-based characterization.

The identities were further checked by amplifying and analyzing the sequences of ITS gene 1 and 2 and *tef*1. BLAST analysis of ITS sequence showed 93.54% to 100% similarity to the corresponding sequences available at NCBI GenBank. BLAST analysis of *tef*1 sequence showed 83.29% to 100% similarity to the corresponding sequences available at NCBI GenBank. The isolates were identified as *T. asperellum*, *T. virens*, *T. hamatum*, *T. reesei*, *T. longibrachiatum* and *T. erinaceum*. Among 26 isolates, *T. asperellum* was the most common species.

Ten reported SSR markers were used to study the genetic diversity of selected 26 *Trichoderma* isolates (Rai *et al.*, 2016). The annealing temperature for each primer was determined by gradient PCR. The annealing temperature ranges between 45.2 °C to 57.4 °C. The Jaccard's similarity coefficient of 10 SSR primer banding patterns varies from 0.31 to 1.00. Significant variability was observed even within the same

species. Dendrogram was constructed based on the scoring data. The 26 isolates were clustered into two clusters and the first cluster contains maximum number of accessions.

Considering the antagonistic potential, high chitinase and glucanase production and plant growth promotion, isolates such as T2, T15, T32, T34 (*T. asperellum*) and T40 (*T. erinaceum*) are selected as effective bio-control agents. The present study helped in identifying the *Trichoderma* isolate with high antagonistic potential against *S. rolfsii* and the entire process ensured more precised and targeted application of *Trichoderma* isolate in field condition. The outcome of the study will be a key factor in developing appropriate management strategy to mitigate collar rot disease in elephant foot yam.

REFERENCES

7. REFERENCES

Agrawal, T. and Kotasthane, A. S. 2012. Chitinolytic assay of indigenous *Trichoderma* isolates collected from different geographical locations of Chhattisgarh in Central India. *Springer Plus*, 1(1): 73.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215(3): 403-410.

Anees, M., Tronsmo, A., Edel-Hermann, V., Hjeljord, L. G., Héraud, C. and Steinberg, C. 2010. Characterization of field isolates of *Trichoderma* antagonistic against *Rhizoctonia solani*. *Fungal Biol.* 114(9): 691-701.

Ashwini, N. and Srividya, S. 2013. Potentiality of *Bacillus subtilis* as biocontrol agent for management of anthracnose disease of chilli caused by *Colletotrichum gloeosporioides* OGC1. *3 Biotech.* 4(2): 127-136.

Atanasova, L., Druzhinina, I. S., Jaklitsch, W. M., Mukherjee, P., Horwitz, B. and Singh, U. 2013. Two hundred *Trichoderma* species recognized on the basis of molecular phylogeny. *Trichoderma: Boil. and Appl. CABI, Wallingford*, pp.10-42.

Behera, S. S. and Ray, R. C. 2016. Konjac glucomannan, a promising polysaccharide of *Amorphophallus* konjac K. Koch in health care. *Int. J. Biol. Macromolecules* 92: 942-956.

Benítez, T., Rincón, A. M., Limón, M. C. and Codón, A. C. 2004. Mecanismos de biocontrol de cepas de *Trichoderma*. *Int. Microbiol.* 7(4): 249-260.

Brotman, Y., Kapuganti, J. G., and Viterbo, A. 2010. *Trichoderma Curr. Biol.* 20(9): 390-391.

Calistru, C., McLean, M. and Berjak, P. 1997. *In vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species. *Mycopathologia*, 137(2): 115-124.

Chandel, G. Dubey, M. Samual, P. and Meena, R. 2011. *In silico* mining and characterization of novel SSRs and candidate genes within QTLs controlling grain protein contents using MPSS signatures and micro array analysis in rice (*Oryza sativa* L.) *Plant Omics J.* 4(3): 126-135.

Chen, C., Bauske, E. M., Musson, G., Rodriguezkabana, R. and Kloepper, J. W. 1995. Biological control of *Fusarium* wilt on cotton by use of endophytic bacteria. *Biol. control* 5(1): 83-91.

Consolo, V. F., Mónaco, C. I., Cordo, C. A. and Salerno, G. L. 2012. Characterization of novel *Trichoderma* spp. isolates as a search for effective bio-controllers of fungal diseases of economically important crops in Argentina. *World J. Microbiol. and Biotechnol.* 28(4): 1389-1398.

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Alfaro-Cuevas, R. and López-Bucio, J. 2014. *Trichoderma* spp. improve growth of *Arabidopsis* seedlings under salt stress through enhanced root development, osmolite production, and Na+ elimination through root exudates. *Mol. Plant-Microbe Interactions* 27(6): 503-514.

Cook, R. J. and Baker, K. F. 1983. The nature and practice of biological control of plant pathogens. *American Phytopathological Soc.*

Cook, R. J. 1985. Biological control of plant pathogens: theory to application. *Phytopathol.* 75(1): 25-29.

Dekker, J. and Georgopolous, S. G. 1982 Wageningen, Netherlands, Pudoc, *Fungicide Resistance in Crop Prot.*

Dennis, C. and Webster, J. 1971a. Antagonistic properties of species-groups of *Trichoderma*: I. Production of non-volatile antibiotics. *Trans. Br. Mycological Soc.* 57 (1): 25-39.

Dennis, C. and Webster, J. 1971b. Antagonistic properties of species-groups of *Trichoderma*: II. Production of volatile antibiotics. *Trans. Br. Mycological Soc.* 57 (1): 41-48.

dos Reis Almeida, F. B., Cerqueira, F. M., do Nascimento Silva, R., Ulhoa, C. J. and Lima, A. L. 2007. Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: evaluation of coiling and hydrolytic enzyme production. *Biotechnol. Letters* 29(8): 1189-1193.

Druzhinina, I. and Kubicek, C. P. 2005. Species concepts and biodiversity in *Trichoderma* and *Hypocrea:* from aggregate species to species clusters. *J. Zhejiang Univ. Sci. B* 6(2): 100.

Druzhinina, I. S., Kopchinskiy, A. G., Komoń, M., Bissett, J., Szakacs, G. and Kubicek, C. P. 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet. Biol. 42*(10): 813-828.

Druzhinina, I. S., Komoń-Zelazowska, M., Kredics, L., Hatvani, L., Antal, Z., Belayneh, T. and Kubicek, C. P. 2008. Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. *Microbiol.* 154(11): 3447-3459.

Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., Mukherjee, P. K., Zeilinger, S., Grigoriev, I. V. and Kubicek, C. P. 2011. *Trichoderma:* the genomics of opportunistic success. *Nat. Rev. Microbial.* 9(10): 749.

Edison, S., Anantharaman, M. and Srinivas, T. 2006. Status of cassava in India: An overall view, Central Tuber Crops Research Institute (Indian Council of Agricultural Research). *Tech. Bull. Ser. 46.*

Edington, L. V., Khew, K. L. and Barron, G. I. 1971. Fungitoxic spectrum of benzimidazole compounds. *Phytopathol.* 61: 42-44.

Elad, Y., Chet, I. and Henis, Y. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. *Can. J. Microbiol.* 28(7): 719-725.

El-Komy, M. H., Saleh, A. A., Eranthodi, A. and Molan, Y. Y. 2015. Characterization of novel *Trichoderma asperellum* isolates to select effective biocontrol agents against tomato *Fusarium* wilt. *The Plant Pathol. J.* 31(1): 50.

Fahmi, A. I., Ragaa A Eissa1, Khalil A El-Halfawi, Hanafy A Hamza. and Mahmoud S Helwa. 2016. Identification of *Trichoderma* spp. by DNA barcode and screening for cellulolytic Activity. *J. Microb. Biochem. Technol.* 8(3): 202-209.

Fenice, M., Selbmann, L., Di Giambattista, R. and Federici, F. 1998. Chitinolytic activity at low temperature of an Antarctic strain (A3) of *Verticillium lecanii*. *Res. Microbiol.* 149(4): 289-300.

Fróes, A., Macrae, A., Rosa, J., Franco, M., Souza, R., Soares, R. and Coelho, R. 2012. Selection of a *Streptomyces* strain able to produce cell wall degrading enzymes and active against *Sclerotinia sclerotium*. *J. Microbiol.* 50(5): 798-806.

Gajera, H. P., Bambharolia, R. M., Patel, S. V., Mandavia, M. K. and Golakiya, B. A. 2009. Significance of lytic enzymes from *Trichoderma* in the *in vitro* biocontrol of fungal plant pathogen *Fusarium oxysporum f.* sp. *Ciceri*. *Indian J. Agric. Biochem.* 22(1): 31-37.

Gajera, H. P., Bambharolia, R. P., Patel, S. V., Khatrani, T. J. and Goalkiya, B. A. 2012. Antagonism of *Trichoderma* spp. against *Macrophomina phaseolina*: evaluation of coiling and cell wall degrading enzymatic activities. *J. Plant Pathol. Microbiol.* 3(7).

Gajera, H. P., Savaliya, D. D., Patel, S. V., and Golakiya, B. A. 2015. *Trichoderma viride* induces pathogenesis related defense response against rot pathogen infection in groundnut (*Arachis hypogaea* L.). *Infection, Genet. Evol.* 34: 314-325.

Gajera, H. P. and Vakharia, D. N. 2012. Production of lytic enzymes by *Trichoderma* isolates during *in vitro* antagonism with *Aspergillus niger*, the causal agent of collar rot of peanut. *Brazilian J. Microbiol.* 43(1): 43-52.

Galindo, E., Flores, C., Larralde-Corona, P., Corkidi-Blanco, G., Rocha-Valadez, J. A. and Serrano-Carreón, L. 2004. Production of 6-pentyl-α-pyrone by *Trichoderma harzianum* cultured in unbaffled and baffled shake flasks. *Biochemical Eng. J.* 18(1): 1-8.

Gams W. and Bissett J. 1998. Morphology and identification of *Trichoderma*. In: Kubicek C. P., Harman G. E. (eds) *Trichoderma* and *Gliocladium*, Basic biology, taxonomy and genetics, vol 1. Taylor and Francis Ltd., London, pp. 3-34.

George, A., Jeeva, M. L., Nath, V. S., Sreelatha, G. L., and Sujina, M. G. 2018. Simple and efficient genomic DNA extraction protocol for molecular characterisation of *Phytophthora colocasiae* causing taro leaf blight. *Arch. Phytopathol. Plant Prot.* 1-11.

Geraldine, A. M., Lopes, F. A. C., Carvalho, D. D. C., Barbosa, E. T., Rodrigues, A. R., Brandão, R. S., Ulhoa, C. J. and Junior, M. L. 2013. Cell wall-degrading

enzymes and parasitism of sclerotia are key factors on field biocontrol of white mold by *Trichoderma* spp. *Biol. Control* 67(3): 308-316.

Gogoi, N. K., Phookan, A. K, Narzay, B. D. 2002. Management of collar rot of elephant foot yam. *Indian Phytopath.* 55(2): 238-240.

Gogoi, H. K. and Veer, V. 2015. Antagonistic potential of native *Trichoderma viride* strain against potent tea fungal pathogens in North East India. *The Plant Pathol. J.* 31(3): 278.

Goldman, G. H., Hayes, C. and Harman, G. E. 1994. Molecular and cellular biology of biocontrol by *Trichoderma* spp. *Trends in Biotechnol.* 12(12): 478-482.

Grondona, I., Hermosa, R., Tejada, M., Gomis, M. D., Mateos, P. F., Bridge, P. D., Monte, E. and Garcia-Acha, I. 1997. Physiological and biochemical characterization of *Trichoderma harzianum*, a biological control agent against soilborne fungal plant pathogens. *Appl. Environ. Microbiol.* 63(8): 3189-3198.

Gupta, V. G., Schmoll, M., Herrera-Estrella, A., Upadhyay, R. S., Druzhinina, I. and Tuohy, M. eds. 2014. *Biotechnol. and Biol. of Trichoderma.*

Harman, G. E. and Kubicek, C. P. 1998. *Trichoderma and Gliocladium, Volume 2: Enzymes, Biological Control and commercial applications*. CRC Press.

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I. and Lorito, M. 2004. *Trichoderma* species-opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2(1): 43.

Harman, G. E. 2006. Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathol.* 96(2): 190-194.

Hibbett, D. S., Ohman, A., Glotzer, D., Nuhn, M., Kirk, P. and Nilsson, R. H. 2011. Progress in molecular and morphological taxon discovery in Fungi and options

for formal classification of environmental sequences. *Fungal Biol. Rev. 25*(1): 38-47.

Hirpara, D. G., Gajera, H. P., Hirpara, H. Z. and Golakiya, B. A. 2017. Antipathy of *Trichoderma* against *Sclerotium rolfsii Sacc.*: Evaluation of cell wall-degrading enzymatic activities and molecular diversity analysis of antagonists. *J. Mol. Microbiol. and Biotechnol.* 27(1): 22-28.

Hjeljord L. and Tronsmo A. 1998. *Trichoderma* and *Gliocladium* in biocontrol: an overview. In: Kubicek CP, Harman GE (eds), *Trichoderma* and *Gliocladium*. London, UK, Taylor and Francis, pp. 135–151.

Hung, R., Lee, S. and Bennett, J. W. 2013. *Arabidopsis thaliana* as a model system for testing the effect of *Trichoderma* volatile organic compounds. *Fungal Ecol.* 6(1): 19-26.

Insam, H. and Seewald, M.S. 2010. Volatile organic compounds (VOCs) in soils. *Biol. Fertil. Soils* 46(3): 199-213.

Jalali, F., Zafari, D. and Salari, H. 2017. Volatile organic compounds of some *Trichoderma* spp. increase growth and induce salt tolerance in *Arabidopsis thaliana. Fungal Ecol.* 29: 67-75.

Jarne, P. and Lagoda, P. J. 1996. Microsatellites, from molecules to populations and back. *Trends in Ecol. Evol.* 11(10): 424-429.

John, N. S., Anjanadevi, I. P., Nath, V. S., Sankar, S. A., Jeeva, M. L., John, K. S. and Misra, R. S. 2015. Characterization of *Trichoderma* isolates against *Sclerotium rolfsii*, the collar rot pathogen of *Amorphophallus*–A polyphasic approach. *Biol. Control* 90: 164-172.

Khatri, D. K., Tiwari, D. N. and Bariya, H. S. 2017. Chitinolytic efficacy and secretion of cell wall-degrading enzymes from *Trichoderma* spp. in response to phytopathological fungi. *J. Applied Biol. Biotechnol.* 5(6): 1-8.

Kitamura, E. and Kamei, Y. 2003. Molecular cloning, sequencing and expression of the gene encoding a novel chitinase A from a marine bacterium, *Pseudomonas* sp. PE2, and its domain structure. *Applied Microbiol. Biotechnol.* 61(2): 140-149.

Kottb, M., Gigolashvili, T., Großkinsky, D. K. and Piechulla, B. 2015. *Trichoderma* volatiles effecting *Arabidopsis*: from inhibition to protection against phytopathogenic fungi. *Frontiers in Microbiol.* 6: 995.

Kubicek, C. P., Mach, R. L., Peterbauer, C. K. and Lorito, M. 2001. *Trichoderma*: from genes to biocontrol. *J. Plant Pathol.* pp.11-23.

Kullnig, C., Mach, R. L., Lorito, M. and Kubicek, C.P. 2000. Enzyme diffusion from *Trichoderma atroviride* (= *T. harzianum* P1) to *Rhizoctonia solani* is a prerequisite for triggering of *Trichoderma ech*42 gene expression before mycoparasitic contact. *Appl. Environ. Microbiol.* 66(5): 2232-2234.

Kullnig-Gradinger, C. M., Szakacs, G. and Kubicek, C. P. 2002. Phylogeny and evolution of the genus *Trichoderma*: a multigene approach. *Mycol. Res.* 106(7): 757-767.

Kumar, S., Maurya, D., Shalini, R.A.I., Kashyap, P. L. and Srivastava, A. K. 2012. Computational Mining and Genome Wide Distribution of Microsatellite in *Fusarium oxysporum f.* sp. *lycopersici*. *Notulae Scientia Biologicae* 4(4): 127-131.

Kumari R, Singh P. P., and Rai R. C. 2013. Integrated management of diseases of elephant foot yam (*Amorphophallus paeoniifolius*). In: Misra R. S.,

Neduchezhiyan M (eds) Aroids: opportunities and challenges. Allied Publishers Pvt Ltd, New Delhi, 240–245

Kredics, L., Hatvani, L., Naeimi, S., Körmöczi, P., Manczinger, L., Vágvölgyi, C. and Druzhinina, I. 2014. Biodiversity of the genus *Hypocrea/Trichoderma* in different habitats. In *Biotechnol. Biol. Trichoderma, Elsevier*, 3-24.

Lee, S., Hung, R., Yap, M. and Bennett, J. W. 2015. Age matters: the effects of volatile organic compounds emitted by *Trichoderma atroviride* on plant growth. *Arch. Microbiol.* 197(5): 723-727.

Lee, S., Yap, M., Behringer, G., Hung, R. and Bennett, J.W. 2016. Volatile organic compounds emitted by *Trichoderma* species mediate plant growth. *Fungal Biol. Biotechnol. 3*(1): 7.

Lieckfeldt, E., Samuels, G. J., Nirenberg, H. I. and Petrini, O. 1999. A morphological and molecular perspective of *Trichoderma viride*. *Appl. Environ. Microbiol.*, *65*(6): 2418-2428.

LoBuglio, K. F., Pitt, J. I. and Taylor, J. W. 1993. Phylogenetic analysis of two ribosomal DNA regions indicates multiple independent losses of a sexual *Talaromyces* state among asexual *Penicillium* species in subgenus *Biverticillium*. *Mycol. 85*(4): 592-604.

Lopes, F. A. C., Steindorff, A. S., Geraldine, A. M., Brandão, R. S., Monteiro, V. N., Júnior, M. L., Coelho, A. S. G., Ulhoa, C. J. and Silva, R. N. 2012. Biochemical and metabolic profiles of *Trichoderma* strains isolated from common bean crops in the Brazilian Cerrado, and potential antagonism against Sclerotinia sclerotiorum. *Fungal Biol.* 116(7): 815-824.

López-Coria, M., Hernández-Mendoza, J. L. and Sánchez-Nieto, S. 2016. *Trichoderma asperellum* induces maize seedling growth by activating the

plasma membrane H+-ATPase. *Mol. Plant-Microbe Interactions* 29(10): 797-806.

Mahfooz, S., Maurya, D. K., Srivastava, A. K., Kumar, S. and Arora, D. K. 2012. A comparative *in silico* analysis on frequency and distribution of microsatellites in coding regions of three formae speciales of *Fusarium oxysporum* and development of EST–SSR markers for polymorphism studies. *Fed. Eur. Microbiol. Soc. Microbiol. Lett.* 328(1): 54-60

Mathivanan, N., Prabavathy, V. R. and Vijayanandraj, V. R. 2008. The effect of fungal secondary metabolites on bacterial and fungal pathogens. In *Secondary Metabolites in Soil Ecol. Springer*, Berlin, Heidelberg. 129-140.

Mathys, J., De Cremer, K., Timmermans, P., Van Kerkhove, S., Lievens, B., Vanhaecke, M., Cammue, B. and De Coninck, B. 2012. Genome-wide characterization of ISR induced in *Arabidopsis thaliana* by *Trichoderma hamatum* T382 against *Botrytis cinerea* infection. *Frontiers in Plant Sci.* 3: 108.

Misra, R. S. 1997. *Diseases of tuber crops in Northern and Eastern India*. CTCRI Technical Series No. 22, CTCRI, Trivandrum, Kerala, India, 27p.

Misra, R.S., Sharma, K. and Mishra, A.K. 2008. *Phytophthora* leaf blight of Taro (*Colocasia esculenta*)-a review. *Asian Aust. J. Plant Sci. Biotechnol. 2*: 55-63.

Misra, R. S. and Nedunchezhiyan, M. 2008. *Amorphophallus* diseases and their integrated management. In: *Natl. Seminar on Amorphophallus: Innovative Technol.* pp. 19-20.

Mittal, N. and Dubey, A. K. 2009. Microsatellite markers in molecular genetics. *Pharmacog. Rev.* 3(6): 235.

Molano, J., Durán, A. and Cabib, E. 1977. A rapid and sensitive assay for chitinase using titrated chitin. *Anal. Biochem.* 83(2): 648-656.

Mukherjee, P. K., Horwitz, B. A. and Kenerley, C. M. 2012. Secondary metabolism in *Trichoderma*–a genomic perspective. *Microbiol.* 158(1): 35-45.

Munir, S., Ahmed, N., Abid, M., Rehman, S. U., Ashraf, M., Zhang, L. and Anees, M. 2019. Chitinolytic activity of the indigenous *Trichoderma* spp. from the North West of Pakistan against the fungal phytopathogens. *Pakistan J. Bot.* 51(2): 711-716.

Muthu, K. A. and Sharma, P. 2011. Molecular and morphological characters: An appurtenance for antagonism in *Trichoderma* spp. *Afr. J. Biotechnol.* 10(22): 4532-4543.

Nath, V. S., John, N. S., Anjanadevi, I. P., Hegde, V. M., Jeeva, M. L., Misra, R. S. and Veena, S. S. 2014. Characterization of *Trichoderma* spp. antagonistic to *Phytophthora colocasiae* associated with leaf blight of taro. *An. Microbiol.* 64(4): 1513-1522.

Ospina-Giraldo, M. D., Royse, D. J., Thon, M. R., Chen, X. and Romaine, C. P. 1998. Phylogenetic relationships of *Trichoderma harzianum* causing mushroom green mold in Europe and North America to other species of *Trichoderma* from world-wide sources. *Mycol.* 90(1): 76-81.

Pelagio-Flores, R., Esparza-Reynoso, S., Garnica-Vergara, A., López-Bucio, J. and Herrera-Estrella, A. 2017. *Trichoderma*-induced acidification is an early trigger for changes in *Arabidopsis* root growth and determines fungal phytostimulation. *Frontiers in Plant Sci. 8*: 822.

Pérez, A., Hermosa, R. and Monte, E. 2017. *Trichoderma* biocontrol activity against plant pathogenic *ascomycetes*. *Farm J. 2*(1): 85.

Punja, Z. K. 1985. The biology, ecology, and control of *Sclerotium rolfsii*. *Annu. Rev. Phytopathol.* 23(1): 97-127.

Prasetyawan, S. and Sulistyowati, L. 2018. Glucanase and chitinase from some isolates of endophytic fungus *Trichoderma* spp. In *IOP Conference Series: Materials Science and Engineering* (Vol. 299). IOP Publishing.

Rai, S., Kashyap, P. L., Kumar, S., Srivastava, A. K. and Ramteke, P. W. 2016. Comparative analysis of microsatellites in five different antagonistic *Trichoderma* species for diversity assessment. *World J. Microbiol. Biotechnol.* 32(1): 8.

Rao, S. N., Anahosur, K. H. and Kulkarni, S. 2004. Evaluation of antagonistic microorganisms against *Sclerotium rolfsii* causing wilt of potato. *J. Mycol. Plant Pathol.* 34: 298–299.

Ratan, S., Ranwah, B. R. and Singh, R. P. 2001. Tissue culture studies in Indian mustard (*Brassica juncea* L. Czern & Coss). *Indian J. Agric. Res. 35*(4): 259-262.

Reddy, B. N., Saritha, K. V. and Hindumathi, A. 2014. *In vitro* screening for antagonistic potential of seven species of *Trichoderma* against different plant pathogenic fungi. *Res. J. Biol.*2: 29–36.

Reddy, P. P., 2015. *Plant protection in tropical root and tuber crops.* New Delhi: *Springer India.*

Rifai, M. A. 1969. A revision of the genus *Trichoderma*. *Mycol. Pap.* 116: 1-56.

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Wei, H. X., Paré, P. W. and Kloepper, J. W. 2003. Bacterial volatiles promote growth in *Arabidopsis*. *Proc. Natl Acad. Sci.* 100(8): 4927-4932.

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Kloepper, J. W. and Paré, P. W. 2004. Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiol.* 134(3): 1017-1026.

Sadfi-Zouaoui, N., Cha^abani, S., Rouaissi, M., Hedi, A., Hajlaoui, M. R. and Boudabous, A. 2009. Analysis of the diversity of *Trichoderma* spp. in soil horizons using digested ITS regions. *Ann. Microbiol.* 59: 459–463.

Samuels, G. J., Chaverri, P., Farr, D. F., McCray, E. B. 2002. *Trichoderma* Online. Systematic Mycology and Microbiology Laboratory, ARS, USDA.

Samuels, G. J., Ismaiel, A., Bon, M. C., De Respinis, S. and Petrini, O. 2010. *Trichoderma asperellum* sensu lato consists of two cryptic species. *Mycologia*, 102(4): 944-966.

Saranraj, P., Behera, S. S. and Ray, R. C. 2019. Traditional foods from tropical root and tuber crops: innovations and challenges. In *Innovations in Tradit. Foods*. Woodhead Publishing. 159-191

Savitha, M. J. and Sriram, S. 2015. Morphological and molecular identification of *Trichoderma* isolates with biocontrol potential against *Phytophthora blight* in red pepper. *Pest Manag. Hortic. Ecosyst.* 21(2): 194-202.

Shahid, M., Srivastava, M., Sharma, A., Kumar, V., Pandey, S. and Singh, A. 2013. Morphological, molecular identification and SSR marker analysis of a potential strain of *Trichoderma/Hypocrea* for production of a bioformulation. *J. Plant Pathol. Microbiol.* 4(10)

Shah, S., Nasreen, S. and Sheikh, P. A., 2012. Cultural and morphological characterization of *Trichoderma* spp. associated with green mold disease of *Pleurotus* spp. in Kashmirm. *Res. J. Microbiol*, 7(2): 139-144.

Shahid, M., Srivastava, M., Kumar, V., Singh, A., Sharma, A., Pandey, S., Rastogi, S., Pathak, N. and Srivastava, A. K. 2014. Phylogenetic diversity analysis of *Trichoderma* species based on internal transcribed spacer (ITS) marker. *African J. Biotechnol.* 13(3).

Shalini Devi S., Sreenivasulu Y., Saritha S., Ram Kumar M., Praveen Kumar K. and Sudhakar P. 2012. Molecular diversity of native *Trichoderma* isolates against *Fusarium oxysporum f.* sp. L*ycopersici (Sacc.*). A casual agent of *Fusarium* wilt in tomato (*Lycopersicon esculentum* Mill.). *Arch. Phytopathol. Plant Prot.* 45: 686–698.

Sharma K, Mishra A. K. and Misra R. S. 2009. Morphological, biochemical and molecular characterization of *Trichoderma harzianum* isolates for their efficacy as biocontrol agents. *J. Phytopathol* 157:51–56

Siddiquee, S., 2017. Practical Handbook of the Biology and Molecular Diversity of *Trichoderma* Species from Tropical Regions. Springer International Publishing.

Singh, A., Mehta, S., Singh, H. B. and Nautiyal, C. S. 2003. Biocontrol of collar rot disease of betelvine (*Piper betle* L.) caused by *Sclerotium rolfsii* by using rhizosphere-competent *Pseudomonas fluorescens* NBRI-N6 and *P. fluorescens* NBRI-N. *Curr. Microbiol.* 47(2): 0153-0158.

Singh, R., Singh, P. P. and Singh, V. 2006. Integrated management of collar rot of *Amorphophallus paeoniifolius* blume caused by *Sclerotium rolfsii* Saccardo. *Veg. Sci.* 33(1): 45-49.

Singh, A., Shahid, M., Srivastava, M. and Biswas, S. K., 2013. Molecular and antagonistic variability of *Trichoderma atroviride* against legume crop pathogens in Uttar Pradesh, India. *Int. J. Bio-resource and Stress Manag.* 4(4): 582-587.

Singh, R., Kumar, S., Kashyap, P. L., Srivastava, A. K., Mishra, S. and Sharma, A.K. 2014. Identification and characterization of microsatellite from *Alternaria brassicicola* to assess cross-species transferability and utility as a diagnostic marker. *Mol. Biotechnol.* 56(11): 1049-1059.

Sivasithamparam, K. and Ghisalberti, E. L. 1998. Secondary metabolism in *Trichoderma* and *Gliocladium*. *Trichoderma and Gliocladium Basic Biol. Taxon. Genet.* 1: 139-191.

Sivan, A. and Chet, I 1992. Microbial control of plant diseases. *Environ. Microbiol.* 335-354.

Skidmore, A. M. and Dickinson, C. H. 1976. Colony interactions and hyphal interference between *Septoria nodorum* and phylloplane fungi. *Trans. Br. Mycol. Soc.* 66 (1): 57-64.

Skoneczny, D., Oskiera, M., Szczech, M. and Bartoszewski, G. 2015. Genetic diversity of *Trichoderma atroviride* strains collected in Poland and identification of loci useful in detection of within-species diversity. *Folia Microbiologica* 60(4): 297-307.

Srinivasa, N., Sriram, S., Singh, C. and Shivashankar, K. S. 2017. Secondary metabolites approach to study the bio-efficacy of *Trichoderma asperellum* isolates in India. *Int. J. Curr. Microbiol. App. Sci. 6*(5): 1105-1123.

Srinivas, T. and Ramanathan, S., 2005. A study on economic analysis of elephant foot yam production in India. *Agric. Econ. Res. Rev.* 18(347): 241-252.

Srinivas, T. and Ramanathan, S., 2005. A study on economic analysis of elephant foot yam production in India. *Agric. Econ. Res. Rev.* 18(347-2016-16681): 241-252.

Srivastava, A. K., Singh, R. N., Kumar, S., Kashyap, P. L. and Arora, D. K. 2012. Growth promotion and management of *Alternaria* leaf spot in chilli by *Trichoderma harzianum*. *IJIH*, 2: 158-163.

Tabarestani, M. S., Rahnama, K., Jahanshahi, M., Nasrollanejad, S. and Fatemi, M. H. 2016. Identification of volatile organic compounds from *Trichoderma virens* (6011) by GC-MS and separation of a bioactive compound *via* nanotechnology. *Int. J. Eng.-Trans.* 29(10): 1347-1353.

Tweddell, R. J., Jabaji-Hare, S. H. and Charest, P. M. 1994. Production of chitinases and β-1, 3-glucanases by *Stachybotrys elegans*, a mycoparasite of *Rhizoctonia solani*. *Appl. Environ. Microbiol.* 60(2): 489-495.

Vahabi K., Sharifnabi B. and Zafari D. 2009. Genetic diversity of *Trichoderma* spp. associated with button mushroom, *Agaricus bisporus*, inferred from AFLP markers and ITS sequencing. *Acta. Phytopathol. Entomol. Hung* 44: 239–253.

Verma, M., Brar, S.K., Tyagi, R.D., Surampalli, R.Y. and Valero, J.R. 2007. Antagonistic fungi, *Trichoderma* spp.: panoply of biological control. *Biochemical Eng. J. 37*(1): 1-20.

Viterbo, A., Ramot, O., Chernin, L. and Chet, I. 2002. Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. *Antonie Van Leeuwenhoek* 81(1-4): 549-556.

Vos, C. M., De Cremer, K., Cammue, B. P. and De Coninck, B. 2015. The tool box of *Trichoderma* spp. in the biocontrol of *Botrytis cinerea* disease. *Mol. Plant Pathol.* 16(4): 400-412.

Wang, M. L., Barkley, N. A. and Jenkins, T. M. 2009. Microsatellite markers in plants and insects part I: Application of Biotechnology. *Genes, Genomes and Genomics* 3: 54-67

Weller, D. M. 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathol.* 97(2): 250-256.

White, T. J., Bruns, T., Lee, S., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a Guide to Methods and Applications*. Innis, M. A., Gelfand, D. H., Sninsky J. J, White, T. J (eds). Academic, New York, pp. 315-332.

You, J., Zhang, J., Wu, M., Yang, L., Chen, W. and Li, G. 2016. Multiple criteria-based screening of *Trichoderma* isolates for biological control of *Botrytis cinerea* on tomato. *Biol. Control* 101: 31-38.

Yuzana, K. and Thein, H.M. 2018. Antagonistic Activity of *Trichoderma* strains against pathogenic fungi of *Arachis hypogaea* L. *1st Myanmar-Korea Conference*.

APPENDICES

APPENDIX I

CULTURE MEDIA- COMPOSITIONS

Potato dextrose agar medium (PDA)

Potato	250 g/l
Dextrose	20 g/l
Agar	20 g/l

Modified Czapek's broth

A. Lyophilized mycelia of pathogen	10 g
Distilled water	150 ml
B. KH_2PO_4	1 g
Distilled water	100 ml
C. KCl	0.5 g
$MgSO_4.\ 7H_2O$	0.5 g
$FeSO_4.\ 7H_2O$	0.05 g
$ZnSO_4.\ 7H_2O$	0.05 g
$NaNO_3$	1 g
Distilled water	150 ml
D. Distilled water	600 ml

APPENDIX II

REAGENTS FOR BIOCHEMICAL ASSAYS

DNSA Reagent (1%)

Solution A - Dissolve 1.00 g of DNS in 20 ml of 2M NaOH

Solution B - Add 30 g of Sodium potassium tartrate tetrahydrate in 50 ml of distilled water. Stir until complete dissolution.

Add solution A into solution B and homogenize it by heating.

Make up the volume to 100 ml with distilled water and store in an amber bottle.

APPENDIX III

REAGENTS FOR DNA EXTRACTION

SDS Extraction Buffer

Tris - HCl	1M
EDTA (pH=8)	0.5 M
NaCl	5 M
SDS	1 %
PVP	1 % (w/v) (Warm it before adding β-mercaptoethanol)
β-mercaptoethanol	0.2 % (v/v)
Distilled water	

TE Buffer (10X)

Tris – HCl (pH 8.0)	10 mM
EDTA	1 mM

TAE Buffer (50 X)

Tris-base	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA	100 ml

Dissolve in 600 ml of distilled water, adjust the pH to 8.0 and make up to 1L with distilled water.

Ethidium Bromide (10 mg ml^{-1})

Add 1 g of Ethidium bromide to 100 ml of water. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Transfer the solution to a dark bottle and store at room temperature.

Gel loading dye (6X)

Bromophenol blue (w/v) 0.25%

Xylene cyanol FF (w/v) 0.25%

Glycerol 50%

EDTA (pH 8.0) 10 mM

Dissolve these components in nuclease free water and store at -20 ^{0}C.

APPENDIX IV

NCBI SUBMISSIONS

MN176406.1 *Trichoderma asperellum* isolate T1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN176380.1 *Trichoderma asperellum* isolate T2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

MN176404.1 *Trichoderma asperellum* isolate T3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

MN176408.1 *Trichoderma asperellum* isolate T5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

MN165549.1 *Trichoderma virens* isolate T6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

MN176398.1 *Trichoderma virens* isolate T7 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN176388.1 *Trichoderma asperellum* isolate T11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN176392.1 *Trichoderma asperellum* isolate T13 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

MN176381.1 *Trichoderma hamatum* isolate T14 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN176379.1 *Trichoderma asperellum* isolate T15 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN173868.1 *Trichoderma asperellum* isolate T16 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN173869.1 *Trichoderma asperellum* isolate T17 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN173871.1 *Trichoderma asperellum* isolate T18 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

MN173875.1 *Trichoderma asperellum* isolate T19 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

MN173873.1 *Trichoderma asperellum* isolate T20 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN165539.1 *Trichoderma virens* isolate T28 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN173849.1 *Trichoderma virens* isolate T29 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN128228.1 *Trichoderma asperellum* isolate T32 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN165490.1 *Trichoderma asperellum* isolate T34 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

MN176383.1 *Trichoderma reesei* isolate T35 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

MN165446.1 *Trichoderma reesei* isolate T36 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

MN165477.1 *Trichoderma longibrachiatum* isolate T38 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN173872.1 *Trichoderma erinaceum* isolate T40 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

MN173865.1 *Trichoderma asperellum* isolate T41 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

MN173860.1 *Trichoderma asperellum* isolate T42 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

MN173867.1 *Trichoderma asperellum* isolate T43 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

147

MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *Trichoderma* ISOLATES FROM TUBER CROP ECOSYSTEM

By,

LINET K. JOSEPH

(2014-09-114)

Abstract of thesis

Submitted in partial fulfilment of the

requirement for the degree of

B. Sc. - M. Sc. (INTEGRATED) BIOTECHNOLOGY

Faculty of Agriculture

Kerala Agricultural University, Thrissur

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695 522

KERALA, INDIA

2019

9. ABSTRACT

Elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) is an important tuber crop popular as a food security crop and as a remunerative cash crop. Collar rot caused by *Sclerotium rolfsii* is the most destructive and predominant disease causing great crop loss in elephant foot yam. Application of *Trichoderma* spp is being recommended as the eco-friendly strategy to combat the crop loss. The present study, "Morphological, biochemical and molecular characterization of *Trichoderma* isolates from tuber crop ecosystem" was conducted at the Division of Crop Protection, ICAR- Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram during October 2018 – August 2019. The study was performed to evaluate 43 *Trichoderma* isolates obtained from tuber crop ecosystem for their bio-control potential against *S. rolfsii*, to characterize the isolates using morphological, biochemical and molecular approaches and to analyse the molecular diversity.

The differential antagonistic potential of the isolates were assessed by adopting three *in vitro* screening methods. The screening methods executed were dual culture/ direct confrontation method, antibiosis test for production of diffusible metabolites and volatiles. Based on the additive effect of each mode of inhibition, it was concluded that the isolates *viz*., T38, T36, T32, T40 and T6 have excellent antagonistic potential. Twenty six best isolates were selected for further study based on the ranking of additive result.

The efficiency of 26 isolates for induction of chitinase and β-1,3-glucanase was studied against the cell wall of *S. rolfsii* as carbon source. There was no direct correlation observed between antagonistic potential of isolates and induction of chitinase enzyme. Whereas, positive correlation was observed between antagonistic potential and induction of β-1,3-glucanase enzyme. The effect of volatile organic compounds (VOCs) on plant growth was studied using mustard seeds. The isolates

showed differential response to various growth parameters like fresh weight, number of leaves, root length, shoot length and number of shoot lets. For morphological characterization, the macro and micro morphological characters such as growth rate, colony color, reverse colony color, odor of culture and branching pattern of conidiophore, size of conidia and phialides of isolates were studied. Morphological identification of *Trichoderma* isolates up to species level was found difficult due to the overlapped expression of these characters.

The molecular characterization was done by amplifying and analyzing the sequences of ITS gene 1 and 2 and *tef*1 gene. The six different species identified are *T. asperellum*, *T. virens*, *T. hamatum*, *T. reesei*, *T. longibrachiatum* and *T. erinaceum*. The variability was studied using SSR markers and it was found that Jaccard's similarity coefficient of 10 SSR primer banding patterns varies from 0.31 to 1.00. Fourteen *T. asperellum* isolates (T1, T2, T3, T5, T11, T13, T15, T16, T17, T18, T19, T20, T42, T43) were clustered in cluster A and the remaining three *T. asperellum* isolates (T32, T34 and T41) were clustered into cluster B. It showed the significant variability even within the same species.

Considering the antagonistic potential, high chitinase and glucanase production and plant growth promotion, isolates T2, T15, T32, T34 (*T. asperellum*) and T40 (*T. erinaceum*) were selected as effective bio-control agents. The present study helped in identifying the *Trichoderma* isolate with high antagonistic potential against *S. rolfsii* and the entire process ensured more précised and targeted application of *Trichoderma* isolate in field condition. The outcome of the study will be a key factor in developing appropriate management strategy to mitigate collar rot disease in elephant foot yam.