MORPHOLOGIAL AND MOLECULAR CHARACTERIZATION OF *Trichoderma longibrachiatum*

THESIS

Submitted to the
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
in partial fulfilment of the requirements
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MASTER OF SCIENCE
IN
AGRICULTURE
(PLANT PATHOLOGY)

By
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Enrolment Number – KK/1175

2018

DECLARATION OF STUDENT

I hereby declare that the experimental work and its interpretation of the thesis entitled **MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Trichoderma longibrachiatum*** or part there of has neither been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis / publication of any University or Scientific Organization. The sources of material used and all assistance received during the course of investigation have been duly acknowledged.

Place : Akola. **(SHINDE PRITI BHAGWAT)**

Date : / /2018 Enrolment No. KK/1175

CERTIFICATE

This is to certify that the thesis entitled **MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Trichoderma longibrachiatum*** submitted in partial fulfillment of the requirement for the degree of "**Master of Science in Agriculture (Plant Pathology)"** of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafide research work carried out by **SHINDE PRITI BHAGWAT** under my guidance and supervision.

The subject of thesis has been approved by the Student's Advisory Committee.

Place : Akola
Date : / / 2018

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Abbreviations		Full form
%	–	Per cent
/	–	Per
@	–	At the rate of
0C	–	Degree Celsius
Mg	–	Micro grams
µl	–	Micro litre (s)
BCAs	–	Biological Control Agents
Cm	–	Centimetre(s)
CTAB	–	Cetyl Trimethyl Ammonium Bromide
dd H2O	–	Double distilled water
Deptt.	–	Department
DNA	–	Deoxyribose Nucleic Acid
dNTPs	–	Deoxyribo nucleoside triphosphate
e.g.	–	Exampli gratia (For example)
EDTA	–	Ethylene Di-amine Tetra Acetic Acid
et al.	–	Et alia (and associates)
etc.	–	Et cetera
Fig.	–	Figure
g	–	Grams
i.e.	–	That is
ITS	–	Internal Transcribed Spacer
ITCC	_	Indian Type Culture Collection
Kbp	–	Kilo base pairs
M	–	Molar
Max.	–	Maximum
mg	–	Milli gram(s)
min.	–	Minutes
ml	–	Milli litre(s)
mM	–	Milli molar
mm	–	Millimetre
ng	–	Nano gram(s)

nm	–	Nano meter
No.	–	Number
PCR	–	Polymerase Chain Reaction
PDA	_	Potato Dextrose Agar
PAB	_	Potato Dextrose Broth
RAPD	–	Random Amplified Polymorphic DNA
RFLP	–	Restriction Fragment Length Polymorphism
rDNA	–	Ribosomal Deoxyribose Nucleic Acid
Rnase	–	Ribonuclease A
rpm	–	Revolution per minute
SSR	–	Simple Sequence Repeats
Taq polymerase	–	*Thermus aquaticus* DNA polymerase Enzyme
TBE buffer	–	Tris hydroxymethyl amino methane and boric acid ethylene diamine tetra acetic acid buffer.
TE buffer	–	Tris hydroxymethyl amino methane ethylene diamine tetra acetic acid buffer
Tris	–	Tris Hydroxymethyl aminomethane
U	–	Unit
UPGMA	–	Unweighted pair group method for arithmetic mean average
UV	–	Ultra violet
viz.	–	Videlicet (namely)

F) THESIS ABSTRACT

a) Title of the thesis : **MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Trichoderma longibrachiatum***

b) Full name of student : **Shinde Priti Bhagwat**

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ABSTRACT

It is important to isolate *Trichoderma* spp. having potentially higher antagonistic efficiency by the selection of isolates. The significance of genetic variation is one of criteria for biodiversity evaluation. Genus *Trichoderma* contain many species that are of great economic importance because of ability to suppress pathogens and enhance their biocontrol

capabilities against soil borne pathogen. In order to utilize the full potential of *Trichoderma* species in specific applications, precise identification and characterization is vital. Use of molecular markers has given a boost to the analysis of the accurate variation among various isolates of bio-agents. The present study was carried out to estimate diversity among *T. longibrachiatum.*

Nine isolates of *T. longibrachiatum* were obtained from rhizosphere soil of different districts of Vidarbha region. There was variability in colony growth, colony colour, reverse pigmentation, conidial shape and size, phialides etc. Pure culture of all isolates *T. longibrachiatum* were send to Indian Type Culture Collection(ITCC), Division of Plant Pathology, IARI, New Delhi for confirmation accordingly identified as *T. longibrachitum.*

T. longibrachiatum isolates were evaluated for antagonism using dual culture technique and all isolates were found significant in controlling per cent growth inhibition of soil borne pathogens *S. rolfsii* ,*R. bataticola* and *F. udum.* Growth inhibition was also recorded due to volatile substance produced by the isolates TL-4 (Buldhana) and TL-9(Yeotmal).

Molecular techniques are important analytical tool to characterize genetic variability and diagnosis of microbial population. Molecular variability of *Trichoderma* mother culture and mutants was studied by using Random Amplified Polymorphic DNA (RAPD).The ITS-1 and ITS-4 universal primer were successfully used to amplify genomic DNA. 20 RAPD primer of OPA series were tested, of which 15 primers produced 113 scorable bands among them 110 bands were polymorphic and level of polymorphism was upto 97.34 %. Three primers namely OPA-8, OPA-9 and OPB-19 each showed one monomorphic band. Similarity coefficient was ranged from 0.21 to 0.55, indicating the significant diversity among isolates. On the basis of dendrogram the tested culture were divided into 6 cluster, The cluster A consist TL-1 (Akola), TL-2 (Amravati) and TL-3 (Bhandara). Cluster B consist only one isolate i.e. TL-4 (Buldhana). Cluster C consists of TL-5 (Chandrapur). Cluster D consist of

TL-6 (Gadchiroli). TL-7 (Gondia) and TL-8 (Washim) isolate are in cluster E and TL-9 (Yeotmal) isolate was in separate cluster F. It indicates that there are genetic diversity among the isolates of *T. longibrachitum* isolated from different districts of Vidarbha region.

CHAPTER I

INTRODUCTION

1.1 Background information

Fungi are considered as the fifth kingdom, characterized as eukaryotic heterotrophic organisms with a low level of ‘‘tissue’’ differentiation and chitin or β-glucan-containing cell walls. They form spores as a stress resistant and spreading phase of their life cycle. Approximately 120,000 species of fungi have so far been identified worldwide, but recent estimates claim about 1.6 million species in total (Zhang *et al.* 2005). Despite this high diversity, the biogeography of fungi is still a neglected topic, and molecular studies directed towards an assessment of the global biodiversity of fungi are only slowly emerging. The hymenomycete genus *Trichoderma* is characterized by species remarkable for their rapid growth, capability of utilizing diverse substrates and resistance to noxious chemicals. They are often the predominant components of the mycoflora in soils of various ecosystems, such as agricultural fields, forest, salt marshes and deserts, in all climatic zones. They make a significant contribution to the decomposition of woody and herbaceous materials and exhibit saprotrophic activities against primary wood decomposers. Some species of this genus have economic importance because of their production of enzymes and antibiotics, or use as biocontrol agents. Due to the ecological importance of *Trichoderma* and its application as a biocontrol agent in the field, it is important to understand its biodiversity and biogeography.

Trichoderma spp. are antagonistic to other fungi and have shown promise as biological control agents for several soil-borne diseases (Papavizas, 1985; Jenson and Wolffhechel, 1995). *Trichoderma spp.* have accumulated. *T. harzianum, T. viride and T. virens* are the most widely used for biological control. They are reported effective in controlling root rots /wilt complexes and foliar diseases in several crops and are reported to inhibit a number of soil borne fungi like *Rhizoctonia*, *Pythium, Sclerotinia, Sclerotium, Fusarium spp., Macrophomina* etc. and recently root knot nematode*, Meloidogyne spp*. Several potentially useful strains of

Trichoderma for the biological control are difficult to distinguish from other strains found in the field. So there is a need to find ways to monitor these strains when applied to the natural pathosystem.

Identification based on morphological characters consent a relatively simple method for classification of *Trichoderma* as genus, but the species perceptions are complex to construe and there is considerable confusion over the application of specific names. Pioneers in *Trichoderma* like (Rifai, 1969 and Bissett, 1991) observed certain cultural characters that could be used for identification and description of these species. The *Trichoderma* isolates were differentiated by mycelial growth rate and colony appearance, as well as microscopic morphological features, including philides and phialospores (Seaby, 1996).

The significance of genetic variation as one of several criteria for biodiversity evaluation is widely recognized (Humphries *et al.*, 1995), and protection of genetic diversity is incorporated into many international conventions. Some of the genera such as *Trichoderma* contain species that are of great economic importance because of their production of enzymes, abiotics or use as biocontrol agents (Harman and Kubick, 1998). Since extracellular enzymes are highly substrate-dependent and looking into the now a days growing interest in using biocontrol agents, a reliable and precise system for strain identification is important. More recently, the use of molecular markers has given a boost to the analysis of the accurate variation among various isolates of these bioagents. Latha *et al.* (2002) reported that the RAPD (random amplified polymorphic DNA) techniques can be used for distinguishing strains of bioagents. By using the RAPD procedure (Williams *et al*., 1990), which incorporates the PCR (polymerase chain reaction) technique without depending on a known DNA sequence, information can be generated on amplification patterns from only a small amount of DNA.

These can also be distinguished by Random Amplified Polymorphic DNA (RAPD)- PCR, restriction fragment length polymorphisims in mitochondrial DNA and ribosomal DNA and sequence analysis of ribosomal DNA. The Random Amplified Polymorphic DNA

(RAPD) procedure developed by Williams *et al*. (1990). Molecular characterization of potential biocontrol agents using Random Amplified Ploymorphic DNA (RAPD) and Internal Transcribe Spacer-Polymerase Chain Reaction (ITS-PCR) helps to determine the diversity and identification. The advantages of the RAPDs are, the requirement for small amount of DNA (5-20 ng), single short (9 to 10 bp) primers of arbitrary sequence, the rapidity to screen for polymorphisms, the efficiency to generate a large number of markers for genomic mapping and the potential automation of the technique. In addition, no prior knowledge of sequence is required (Sobral and Honeycutt, 1993).

1.2 Importance of study

Trichoderma spp. has received a considerable attention as a potential biocontrol agent against a wide range of soil borne pathogenic fungi (Harman et al., 2004; Verma et al 2007). In particular, soil borne pathogens cause important losses, fungi being the most aggressive. The distribution of several phytopathogenic fungi, such as *Rhizoctonia, Fusarium, Alternaria, Colletotrichum and Helminthosporium*, has spread during the last few years due to changes introduced in farming, with detrimental effects on crops of economic importance. Biological control of plant pathogens has been considered as a potential control strategy in recent years and search for these biological agents is increasing. *Trichoderma* spp. are the most commonly used fungal biological control agents and have long been known as effective antagonists against plant pathogenic fungi (Chet et al., 1981; Papavizas, 1985:). The intensified use of fungicides has resulted in accumulation of toxic compounds potentially hazardous to humans and environment (Cook and Baker, 1983) and also in the build up of resistance of the pathogens (Dekker and Georgopolous, 1982). In view of this, effective alternatives to chemical control are being investigated and the use of biological control agents seems to be one of the promising approaches (Cook, 1985).

The characterization of the antagonists plays an important role to determine its potential level to inhibit the growth of plant pathogens (Fox, 1993; Narayanasamy, 1997; Waller et al., 1998). Therefore, a

research aimed at determining morphological characteristic of potential fungal antagonists and identification of the antagonists was conducted. Further the *Trichoderma spp.* are difficult to distinguish morphologically, so molecular methods including DNA sequencing and genealogical concordance phylogenetic species recognition using several unliked genes are needed to give accurate identification of *Trichoderma spp. (*Gherbawy et al. 2014).

Proposed study is aimed to characterized 9 strains of *Trichoderma spp.* isolated from rhizospheric soil collected from different districts of Vidarbha region of Maharashtra state and asses their antagonistic ability against soil borne plant pathogen.

1.3 Obectives of the study

There is significant interest in finding more efficient mycoparasitic fungi especially within *Trichoderma spp*., which differ considerably with respect to their biocontrol effectiveness. It is important to isolate *Trichoderma spp*. having potentially higher antagonistic efficiency by the selection of isolates with high potential to secret cell wall degrading enzymes. In addition, genomic diversity and biocontrol mechanism study from less explored sites needed. Therefore, the presen investigation under title **"Morpholgical and Molecular characterization of *Trichoderma longibrachiatum*"** has been carried out to determine the species diversity of *Trichoderma* occurring in the soils of Vidarbha region of Maharashtra state and to observe the antagonistic activities of the most promising isolates of *T. longibrachiatum* against soil-borne pathogens, with following objective:

1. Morphological and colony characterization of *Trichoderma longibrachiatum*.
2. To estimate molecular diversity among *Trichoderma longibrachiatum*.

1.4 Hypothesis

Among the Bio-control agents, *Trichoderma* species are the most intensively studied species (Morgan 2011). They are the most isolated soil-borne fungi commonly found in plant root ecosystem (Vinale *et al.,* 2007). Besides that, these opportunistic, avirulent plant symbionts are antagonistic towards many phytopathogenic fungi. Depending upon the

strain, the application of *Trichoderma* is proven to improve root and plant growth, as well as to induce resistance in plants (Harman *et al.,* 2004). In order to utilize the full potential of *Trichoderma* species in specific applications, precise identification and characterization of these fungi is vital (Lieckfeldt *et al*., 1999).

Molecular characterization of the potential biocontrol agents using Random Amplified Polymorphic DNA (RAPD) and Internal Transcribe Spacer- Polymerase Chain Reaction (ITS-PCR) , helps to determine the diversity and identification. The present study was carried out to characterize morphologically and estimate diversity among *Trichoderma* isolates isolated from rhizosphere soils of Vidarbha region also to study the gentic variability in 9 isolates of *Trichoderma longibrachiatum*.

1.5. Scope and Limitation

Because of the intimate relationship between species of *Trichoderma* and human activity, there is a great need for the accurate identification of *Trichoderma* species. *Trichoderma* ecology and biological control activity are greatly influenced by soil properties and different ecological zones. *Trichoderma* have some limitations in their use as bio-control agents in agriculture is due to the unpredictable efficiency which is affected by biotic and abiotic factors in soil. Hence it is essential to know the genetic variability among the different species of the *Trichoderma.*

CHAPTER II

REVIEW OF LITRATURE

2.1 History of *Trichoderma*

Persoon (1794) described the genus *Trichoderma* for first time more than two hundred years ago in Germany. He showed macroscopically similar fungi described as appearing like mealy powder enclosed by a hairy covering. The four species were proposed by Persoon i.e. *T. viride, T. nigroscens, T. aureum and T. roseum*.

Weindling (1932) first discovered the biocontrol potential of *Trichoderma* first to demonstrate the parasitic activity against wilt of pigeon pea.

Bilai (1963) recognized four species based on colour, shape of conidia and colony appearance classification (1927) or Bisby's conclusion (1939) *Trichoderma* was a monolytic genus and all green spored *Trichoderma* isolated were indiscriminately identified as*, T. lingorum* (Tode) Harz, a synonym of *T. viride*. Mycologist in the USSR accepted the classification of *T. lingorum* and *T. koningii* as representing the genus.

Elad *et al*. (1982) observed that mycoparasitism, the direct attack of one fungus on another, is a very complex process that involves sequential event, including recognition, attack, subsequent penetration and killing of the host. The cell wall degrading enzymes (CWDEs) of *Trichoderma* such as different chitinolytic enzymes, glucanases and proteases are considered important in mycoparasitism.

Lewis and Papavizas (1991) defined biological control of plant disease is the involvement and the use of beneficial microorganisms, such as fungi and bacteria, to attack and control plant pathogens and the diseases they cause different biological control agents can be used for the control of diseases. These include bacteria, fungi and actinomycetes. With the advent of biocontrol as one of the component in integrated pest and disease management in the area of fungi mediated plant disease control, the genus *Trichoderma* has gained considerable importance in recent times.

Vey *et al*., (2001) studied that antibiosis occurs during interactions with other microorganisms involving low molecular weight diffusible volatile and nonvolatile toxic metabolite compounds or antibiotics like harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl 1,3-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others.

2.2 Isolation of Trichoderma from soil samples.

Elad *et al.* (1981) developed Trichoderma-selective agar medium (TSM) for quantitative isolation of *Trichoderma spp.* from soil. Selectivity was obtained by using chlor- amphenicol as a bacterial inhibitor, and pentachloronitrobenzene, p-dimethyl- aminobenzenediazo sodium sulfonate and rose-bengal as selective fungal inhibitors. The TSM also contains a low concentration of glucose which still allows relatively rapid growth and sporulation of *Trichoderma*, enabling convenient and rapid identification of *Trichoderma* colonies. All the 15 *Trichoderma* isolates tested formed colonies and grew well on this medium.

Papivizaz and Lumsden (1982) studied on the improved medium and used it effectively to recover the *Trichoderma spp.* from the soil by addition of alkylaryl polyether alcohol at 2.0 ml/ L alone or in combination with sodium propionate. The basal medium contain (per litre): V-8 juice, 200 ml, water 1 ml, agar 20 g, 1 gm glucose. The improved medium designated TME-SA contained the following antimicrobial agent (µg/ ml) neomycin sulphate, bactericin, penicillin G, cholroneb 100, chlorotetracycline hydrochloride 25, nystatin 20, sodium propionate 500. The alkylaryl polyether alcohol was added 2ml/ L. For benomyl- tolerant biotype of *Trichoderma spp*, the medium was supplemented with benomyl at 2µg/ ml and designated as TME- ben10 SA. Both the modified media allowed the *Trichoderma spp*. to develop on the agar and effectively suppress the rapidly growing fungi such as *Rhizopus*.

Gil *et al*. (2009) studied on soil samples collected from an assay with different crop rotations and tillage systems, and populations of *Trichoderma spp*., *Gliocladium spp*. and actinomycetes were quantified in order to select the general and selective culture media that better reflect

the changes of these microbial populations in soil. The most efficient medium for the isolation of *Trichoderma spp*. and *Gliocladium spp*. was potato dextrose agar modified by the addition of chloramphenicol, streptomycin and rose bengal, and for actinomycetes was Ku¨ster medium, with cycloheximide and sodium propionate was used.

Khang *et al*. (2013) studied on *Trichoderma spp*. with high antifungal activities against plant pathogenic fungi such as *Rhizoctonia solani*, *Fusarium moniliforme* and *Phytophthora capsic,* which are commonly present in arable soil of Mekong delta. The media used for isolation and selection were Potato Dextrose Agar (PDA) and Rose Bengal Agar (RBA). 21 *Trichoderma* strains were isolated from 24 soil samples collected at provinces as Can Tho, Hau Giang, Vinh Long, Dong Thap, Ca Mau and Dong Nai, belong to Mekong Delta.

Chennappa *et al.* (2017) *Trichoderma* species were isolated from rhizosphere soils of different cropping ecosystem from Karnataka, India using serial dilution spread plate method. *Trichoderma* colonies were appeared white in beginning, varying in culture from dark green to light green or yellowish green with advancement of age among the isolates. Apprised and flat growth was observed whereas fluffy growth was observed in few *Trichoderma* isolates.

2.3 Morphology

Soesanto *et al*. (2011) studied morphological characteristics of some antagonistic microbes. The morphological characteristic was done by observation of fungal colony growth on PDA and MEA media, on several nutrition such as nitrogen and carbon, pigmentation observation, and fungal colony growth at various temperatures. Isolates had specific morphological structure that are commonly similar for the same species and could be identified as *T. harzianum*, *T. pseudokongii*, and *F. oxysporum* but conidia size and mycelial wet or dry weight were different and gave different growth on PDA and MEA but better growth on PDA. The temperature range for growth of *T. harzianum* and *T. pseudokongii* was 20-35ºC with the optimum at 30ºC.

Srivastava *et al*. (2012) Collected many isolates of *Trichoderma atroviride* isolated from rhizospheric soils from different parts of U.P. has brought attention due to its highly antagonistic activity. These were isolated on PDA medium by serial dilution and identified based on phenotypic characters like colony colour, growth, shape of conidiophore, phialides and conidia.

Shahid *et al*. (2013) seven different strains of *Trichoderma* are isolated from wilt infected leguminous crops of an Indian state and tested for their antagonistic activity against *Fusarium* (soil borne pathogen) which is expressed as a zone of inhibition in the culture plates. The seven strains are identified as *Trichoderma viride*, *T. harzianum*, *T*. *asperellum, T. koningii, T. atroviride, T. longibrachiatum*, and *T. virens*. Upon successful identification, morphological description and sequencing of the isolated strains with the help of universal ITS primers, the sequences are submitted to NCBI and allotted with the accession numbers JX119211, KC800922, KC800921, KC800924, KC008065, JX978542 and KC800923, respectively.

Sriram *et al.* (2013) Studied thirty isolates and identified earlier as *T. viride,* isolates were characterized using morphological characters and their identification was confirmed by oligonucleotide barcode that employed amplification of ITS and tef1 regions. The conidia length ranged between 3.1 and 4.5 µm, whereas the width was between 2.1 and 3.9 µm. The phialides length ranged from 7.1 to 11.1. The phialides midpoint ranged between 1.4 and 3.5 µm, whereas the phialides base was between 1.4 and 326 µm. Radial growth on PDA at 30°C after 72 h in darkness ranged between 24 and 80 mm. In three isolates (TaCu1, TaTN1 and TaTN6C) coconut odour was recognized, whereas in others distinct odour could not be recognized.

Saravanan *et al.* (2014) Studied fifteen isolates of *Trichoderma viride*, *Trichoderma atroviride* and *Trichoderma harzianum* obtained from rhizosphere soil of plantation crops, forest soil and agricultural field of Tamilnadu region were studied using morphological characters. Done the morphological characterization based on following

characters included here viz., colony growth rate (after 7days in cm) at 28±1ºC, colony colour, reverse colony colour, colony edge, culture smell, conidiation, mycelia form, mycelia colour, conidiophores branching, phialide disposition, phialide shape, conidial shape, conidial wall etc.

Kumar and Pratibha Sharma (2016) Studied on morphology of *Trichoderma spp*. Morphological characterization was carried out for 5 isolates of *T. harzianum* and 7 isolates of *T. viride* and tested for their biocontrol efficacy. The isolates belonging to *T.harzianum* were analogous in colony colour, culture smell, mycelial colour, conidiation, conidial shape, conidial wall and conidial colour. Correspondingly the isolates of *T.viride* showed certain similarity in colony colour, colony edge, culture smell, conidiophore branching, conidial wall, conidial colour and chlamydospores. The major difference between the isolates of *T.harzianum* and *T.viride* were their conidial wall pattern, conidial shape, conidial colour, colony edge and culture smell.

Sekhar *et al*. (2017) carried out taxonomic identification of ten isolates of *Trichoderma* spp. upto species level were done based on colony colour, morphology included maximum radial growth was recorded in isolates GRT-3, GRT-4 and GRT-9 at 5th day after inoculation (90.00mm) at growth rate of 30.00mm/day. Where as least radial growth rate was observed in case of GRT-7 (73.00) with growth rate of 24.33 mm/day. Species-level identification of *Trichoderma* isolates was done based on the formation of chlamydospores, conidiophores and phialides characters, shape of conidia as the main characters to identify the species. The identified strains are *Trichoderma viride* (GRT-1, GRT-6 and GRT-9), *Trichoderma koningii* (GRT-2, GRT-5 and GRT-8*), Trichoderma* sp (GRT-3), *Trichoderma reeseii* (GRT-4), *Trichoderma harizanum* (GRT-7), *Trichoderma aureoviride* (GRT-10).

Chennappa *et al*. (2017) Studied on *Trichoderma* species isolated from rhizosphere soils of different cropping ecosystem from Karnataka, India using serial dilution spread plate method*. Trichoderma* colonies were appeared white in beginning, varying in culture from dark green to light green or yellowish green with advancement of age among the

isolates. Apprised and flat growth was observed whereas fluffy growth was observed in few *Trichoderma* isolates. Few isolates showed smooth and irregular margins whereas some isolates showed smooth and uniform margins.

2.4 Antagonism of *Trichoderma longibrachiatum* against soil borne fungal plant pathogen

Goes *et al. (2002*) collected fourteen isolates of *Trichoderma* (six of *T. viride*, six of *T. harzianum*, one *T. polysporum*, one of *T. pseudokoningii*) and one of *R. solani* (isolate registered under nº 4014 from Micoteca URM,UFPE, Recife,PE,Brazil). Fourteen isolates of *Trichoderma* and their ability to antagonize *Rhizoctonia solani* using a dual-culture assay for correlation among RAPD products and their hardness to *R. solani*. the isolates of *T. harzianum* were the most aggressive. The isolates 3302, 3601and Tm, started overlaping the colony of R. solani earlier than the other isolates. The isolate T14 (*Trichoderma pseudokoningii*) was not able of overlaping the colony of *R. solani*, but inhibited its growth.

Malathi and Sabitha Doraisamy (2003) Isolates of *Trichoderma viride*, *T*. *harzianum, T. longihrachiatum, T. hamatum, T. koningii and T. pseudokollingii* were employed at various temperatures, viz., 15, 20, 25, 30, 35, 40 and 45"C for studying Its cultural behaviour and antagonistic ability. Increased growth of the pathogen *(M*. *phaseolina*), and growth, sporulation and biomass production of the fungal antagonist (*Trichoderma spp*.) were observed between 25ºand 35ºC. Antagonistic activity of *Trichoderma spp*. against *M. phaseolina* was decreased witb increase in temperature except for *T. pseudokoningii*, which showed maximum inhibition at 35ºC. At all the temperature regimes *T. harzianum* strain Th-5 has shown higher suppression of the root-rot pathogen, better growth and survival than strains of other species.

Rao and Kulkarni (2003) studied six antagonists in vitro for their effect in suppressing the growth of *Sclerotium rolfsii* Sacco *Trichodermu hurzianum* Rifai was found more effective in suppresing the growth of *S. rolfsii* in dual culture followed by *Trichoderma viride* Pers, Fr.

Studies on production of volatile and non-volatile antibiotics revealed that *T. harzianum* and *T. viride* were highly effective in reducing the radial growth of *S*. *rolfsii.*

Shalini and A. S. Kotasthane (2007) studied seventeen *Trichoderma* strains against *R. solani* in vitro. All strains including *T. harzianum, T. viride* and *T. aureoviride* were tested inhibited the growth of *R. solani*. Light microscopic observation on dual culture assay showed that the hyphae of all *Trichoderma* isolates could grow parallel to the hypae of *R. solani*. However *Trichoderma* isolates coiled around the hyphae of *R. solani* and formed appresoria and hook-like structures.

Sharma *et al*. (2009) Collected 8 isolates of *Trichoderma harzianum*, and studied their ability to antagonize *Sclerotium rolfsii* using a dual culture assay was correlated with RAPD profiles. Using the dual culture method in antagonism experiments, the *T*. *harzianum* isolates were classified in to antagonism classes. The isolates of *T. harzianum* overlapped the colony of *S. rolfsii* in 78 h except for the isolates Th 1 and Th 8. These two isolates overlapped the colony of *S. rolfsii* earlier and were more effcient in retarding the growth of *S. rolfsii* compared to the other six isolates.

Amin *et al.* (2010) studied six isolates of *Trichoderma spp.* for their ability to produce volatile metabolites against seven fungal plant pathogens viz., *Fusarium oxysporum* (causing chilli wilt), *Rhizoctonia solani* (causing sheath blight of rice), Sclerotium *rolfsii* (causing collar rot of tomato), *Sclerotinia sclerotiorum* (causing web blight of beans), *Colletotrichum capsici* (causing anthracnose of chilli fruit), *Helminthosporium oryzae* (causing brown spot of rice), *Alternaria brassicicola* (causing Alternaria blight of cabbage). *T. viride* (Tv-2) accounted for maximum reduction in mycelial growth (30.58%) and sclerotial production (65.65%).

Gupta *et al.* (2010) studied seven *Trichoderma spp*. isolates and their in vitro antagonism against wilt pathogens of *Psidium guajava* L. viz. *Fusarium oxysporum f*. *sp. psidii* (F. o. f. sp. psidii) and

Fusarium solani. From the seven isolates of *Trichoderma*, isolates *T. harzianum*, *T. virens* and *T. viride* were evaluated for in vitro efficacy against *F. o. f. sp. psidii* and *F. solani*. Per cent inhibition was maximum by direct use of *Trichoderma spp*. in dual cultures against both pathogens, *F. o. f. sp. psidii* and *F. solani.* Two species, *T. virens* and *T. viride* were superior in inhibiting the growth of both *Fusarium spp*. Fusarium isolates showed intra species variability.

Joshi *et al*. (2010) Collected 62 isolates of *Trichoderma* species isolated from different rhizospheric soil samples from different places located in Western Himalayas region. Out of these only two species were found i.e. *Trichoderma hazianum* and *Trichoderma viride.* Studied their efficacy against soil borne plant pathogens like *Sclerotium rolfsii, Rhizoctonia solani* and *Sclerotinia sclerotiorum* revealed that only three isolates amounting to 5% of the total collected isolates of this region were found highly antagonist. Among them 5% isolates were found against *S. rolfsii*, 13% isolates against *R. solani,* 10% against *sclerotium* caused above 80% inhibition of mycelial growth respectively.

Mishra *et al*. (2011) Several isolates of *Trichoderma viride*, isolated from different region of Allahabad district were selected for antagonistic screening against fungal pathogens such as *Rhizoctonia solani, Sclerotium rolfsii, Macrophomina phaseolina, Alternaria alternata, Fusarium solani* and *Colletotrichum capsici* of Moong bean (*Vigna radiata*). In dual culture a clear zone of inhibition was observed exhibiting antibiosis between pathogen and antagonist. It was observed that Tr 8 (*T. viride*) reduced the growth of *S. rolfsii* by 68.24%.

Sreedevi *et al.* (2011) evaluated effective *Trichoderma spp*. for biocontrol of *Macrophomina phaseolina* the causative agent of root rot of groundnut. Five *Trichoderma spp.* were isolated from the rhizospheric soil evaluated for in vitro antifungal activity against *M. phaseolina* by dual culture plate technique and bioassay methods.In dual culture technique *T. viride* and *T. harzianum* reduced mycelial growth by 61.1% and 64.4% respectively. Based on the dual culture technique, *T. harzianum* (T3), *T. viride* (T1) were selected for further research. Metabolites released from *T.*

harzianum, T.viride were tested in culture medium against *M.phaseolina*. The inhibition varied depending on the *Trichoderma* species producing metabolites, *T. viride* inhibited fungal growth upto 69% and *T. harzianum* upto 72.7% in nonvolatile and 47%, 64.7% in volatile metabolites respectively.

Kamala and Indira Devi (2012) a total of 114 isolates of Trichoderma were isolated from the soil collected from diverse climatic conditions of Manipur on selective medium. Out of the total isolates, 80% shows high degree of antagonism against Fusarium *oxysporum* while 68% *Trichoderma* isolates gives strong activity against *Rhizoctonia solani*. Based on their antifungal activity in dual plate assay, 25 isolates were selected for further analysis. The interaction between the *Trichoderma* and fungal pathogens were examined microscopically.

Perveen and Bokhari (2012) Studied antagonistic potential of *Trichoderma harzianum* and *Trichoderma viride* isolated from the soil of date palm field against *F. oxysporum* in vitro. All species of *Trichoderma* showed the appreciable inhibition of mycelial growth of the pathogen. Results of dual culture interaction test showed that TvDPs (66.3%) was best antagonist in inhibiting the growth of pathogen followed by TDPs (57.4%) and T1s (56.43%) respectively. Volatile metabolites produced by T. viride (TvDPs) exhibited highest growth inhibition (40.91%) followed by T1s (25.97%) and TDPs (7.57%) respectively. The culture filtrate of all antagonists incubated at 25°C showed maximum reduction in radial growth of pathogen. The cell free culture filtrate of *T. viride* incubated at various temperatures inhibited the radial growth of *F. oxysporum* at varying degree. Whereas, culture filtrates of *T. harzianum* isolates incubated at 40oC were unable to control the growth of the pathogen. Present study clearly indicates an excellent antagonistic activity of *T. viride* (TvDPS) against *F. oxysporum* in vitro condition.

Prameela Devi *et al.* (2012) Studied molecular and morphological characters with antagonistic ability of *Trichoderma* species. On the basis of morphological and cultural characteristics, the *Trichoderma* isolates were identified as *T. virens* (11 isolates), *T. asperellum* (15), *T.*

harzianum (14) and *T. longibrachiatum* (32). *T. virens* (Vn) and *T. harzianum* (Th) were assessed for their mycoparasitic effect on soil borne plant pathogens*, Rhizoctonia solani*, *Pythium aphanidermatum*, ***Fusarium udum****, F. solani*, *Sclerotium rolfsii* and *Macrophomina phaseolina*. The percent inhibitory effect among *T. harzianum* isolates was between 70 to 90% and *T. virens* isolates ranged from 50 to 80%. However, Vn09 and Th-12 were distinguishable in exhibiting higher degree of antagonism.

Shrivastava *et al.* (2012) Studied antagonistic variability of the isolates of *T. atroviride* revealed significant suppression in the radial growth of *Fusarium oxysporum f. sp*. *ciceri, F. oxysporum f. sp. lentis* and *F. oxysporum f. sp. udum*. Maximum inhibition (60.26%) of mycelial growth was recorded in case of TH1 isolate against *Fusarium oxysporum f. sp. lentis* (F.o.l.) which was isolated from the soil sample of Bilgram (Hardoi), followed by TE8 (50%) isolated from Bharthana (Etawah). TS5 (39.76%) isolate from Lainbua (Sultanpur) was found to be least effective against F.o.l. Similarly, in case of *Fusarium oxysporum f. sp. ciceri* (F.o.c), the maximum inhibition (55.08%) of mycelial growth was shown by the isolate TH3, which was isolated from the soil sample of Bilgram (Hardoi). The next effective isolate was TS6 (53.65%) isolated from Kadipur (Sultanpur). Isolate TSi4 (37.69%) of Misrikh (Sitapur) was found least effective against F.o.c. Maximum inhibition in case of *Fusarium oxysporum f. sp. udum* (F.o.u) was recorded with isolate TAU8 (47.91%) from Azeetmal (Auraiya). Next effective isolate was TBa1 (47.25%) isolated from Ballaha (Bahraich). The least effective isolates were TSi3 (41.66%) and TKD3 (41.66%) isolated from Misrikh (Sitapur) and Maitha (Kanpur Dehat).

Javaid *et al.* (2014) Studied invitro antagonistic behavior of seven species of *Trichoderma* namely *T. pseudokoningii, T. harzianum*, *T. reesei*, *T. koningii*, *T. hanatus*, *T. viridi* and *T. aureoviridi*, against two highly problematic soil-borne plant pathogenic fungi viz*. Fusarium oxysporum f. sp. lycopersici* (Sacc.) Snyder & Hansn and *Macrophomina phaseolina* (Tassi) Goid. All the *Trichoderma* species exhibited pronounced antagonistic behavior against the target fungal pathogens in dual cultures resulting in 45–65% and 59–74% reduction in radial growth of *M*.

phaseolina and *F*. *oxysporum f. sp. lycopersici,* respectively. *T. harzianum* was found to be the most effective biocontrol agent against both the fungal pathogens followed by *T. aureoviridi* and *T. hanatus.*

Parmar *et al*. (2015) studied ability to inhibit soil born pathogen of groundnut mainly *Sclerotium rolfsii.* Six *Trichoderma* strains (collected from IARI, New Delhi and MTCC, Chandigarh) were tested for their Morphological observations of *Trichoderma* strains as well as phytopathogenic fungi *S*. *rolfsii* were made from culture grown at 28 0C for about one week on PDA media. In vitro percent growth inhibition of *S.rolfsii* by various *Trichoderma* strain was recorded at 5 days after inoculation at 28 oC in the 90 cm petriplates. Results obtained from the antagonism study indicated that *Trichoderma viride* (NBAII Tv 23) inhibited 61% growth of phytopathogenic fungi *S. rolfsii* followed by *T. harzianum* (NBAII Th1) (55% growth inhibition of pathogen). This suggested that among different *Trichoderma* strains*, T. viride* was the best bio-control agent to inhibit in vitro growth of phytopathogen *S.rolfsii* which otherwise cause stem rot disease in groundnut.

Pacheco *et al.* (2016) studied efficacy of *Trichoderma* isolates to control sclerotium wilt (*Sclerotium rolfsii*) of common-bean (*Phaseolus vulgaris*). Used two isolates of *S. rolfsii* UB 193 and UB 228. Sixty-five *Trichoderma spp*. isolates were tested and the following ones were selected in vitro for the in vivo tests: 5, 11, 12, 15, 102, 103, 127, 136, 137, 1525 (*T. longibrachiatum*), 1637 (*T. reesei*), 1642, 1643 (*T. harzianum*), 1649 (*T*. *harzianum*), 1700 (*T. asperellum*) and EST 5. These selected isolates 1649 (*T*. *harzianum*), 1525 (*T. longibrachiatum*) and 1637 (*T. reesei*) were tested for evaluation of sclerotial germination inhibition under laboratory conditions, and to evaluate the effects of these on disease of bean plants under greenhouse conditions. The *Trichoderma* isolates 1649, 1525 and 1637 were more efficient in reducing sclerotial germination. In addition to 1649, 1525 and 1637, the isolates 5, 12, 102 and 1525 (*T*. *longibrachiatum*) significantly.

Chennappa *et al*. (2017) evaluated many *Trichoderma* isolates against major soil borne pathogens viz., *Rhizoctonia solani* Kuhn,

Sclerotium rolfsii Sacc, *Fusarium, Alternaria* and *Aspergillus* tested by dual culture method. Dual culture assay against *R. solani* revealed that per cent inhibition of growth of the pathogen ranged from 61.10- 89.01 per cent. The results indicated that among all the *Trichoderma* isolates tested, Tri-9 was significantly superior over the other isolates and showed 89.01 per cent inhibition of growth of R. solani. The other isolates Tri-24, Tri-29, Tri-10 and Tri-28 showed 88.9, 88.60, 86.7 and 86.6 per cent inhibition respectively.

Jana and Mandal (2017) studied antagonist effect of *Trichoderma* isolates on *Sclerotium rolfsii.* study was carried out with an aim to screen potential *Trichoderma* strains to control *Sclerotium rolfsii*. Among the eleven isolates, Seven (T1, T3, T4, T5, T7, T9 and T11) were preliminarily identified as *T. harzianum* while the rest four (T2, T6, T8 and T10) were identified as *T. viride.* Further, results of study suggested that isolate T3, T4, T11, T2 and T10 were effective against *S. rolfsii*. In dual culture plate technique, *T. harzianum* isolate T3 gave highest inhibition of 71.67%, while *T. viride* isolate T10 stood second with an inhibition of 67.23%. Reduction in radial growth of S. rolfsii by T. harzianum and T. viride differed significantly.

Nagamani *et al.* (2017) studied on twenty *Trichoderma* isolates from chickpea rhizospheric soil and screened for their efficacy against soil borne plant pathogens namely *R. bataticola, F. oxysporum ciceri* and *S. rolfsii*. In case of *R*. *bataticola, T. asperellum* (KNO 2) inhibited the mycelial growth of test pathogen by 82.5% per cent followed by *T. asperellum* (ATPU 1and KNPG 3) with 80.6 per cent inhibition over control and least recorded in *T. viride* (KJ 12) with 64.7%.

Rashmi *et al.* (2017) studied on seven isolates of two different *Trichoderma spp*. isolated from the rhizosphere of groundnut from different locations of Manipur. In vitro study on the effect of *Trichoderma* isolates on the growth of *Sclerotium rolfsii* ranges from71.85 - 61.11per cent. Effect of volatile compound produced by *Trichoderma spp*. against *S. rolfsii* showed inhibition ranged from20.00 - 30.00 per cent.

Kushwaha *et al*. (2018) Three biocontrol agents viz., *Trichoderma viride, T. virens* and *T. harzianum* were evaluated to test the

antagonism against *Sclerotium rolfsii* under in vitro conditions. The rate of inhibition was fastest in *T. harzianum* (63.60%) followed by *T virens* (51.5 %). Least inhibition was recorded in *T. viride* (50.85%) after 72 hours of incubation. However, *T. viride* showed the highest (91.31%) reduction in sclerotia formation followed by *T. harzianum* (84.92%) and *T. virens* (84.29%) after 15 days of incubation. The volatile compounds from *Trichoderma viride* were found most effective in suppressing the mycelial growth (51.11%) and sclerotia production (95.90%) of the target pathogen. The culture filtrate from both *T. harzianum* and *T. viride* (15% concentration) was found very effective in inhibiting the radial growth (57.46 and 49.62%) and sclerotia formation (98.20 and 99.83%) of *Sclerotium rolfsii*.

2.5 DNA isolation of *Trichoderma spp.*

Raihan *et al.* (2016) Studied modified procedure based on the cetyl trimethylammonium bromide (CTAB) method to isolate DNA from *Trichoderma spp*. The modified DNA extraction protocol include the use of 20% CTAB,1.4 M NaCl, 10% polyvinylpyrrolidone (PVP), 5% sodium dodecyl sulphate (SDS) and 70% ethanol. During centrifugation chloroform:isoamyl alchohol (24:1) was used for separating and precipitating of the DNA. This method solved the problems of DNA degradation, contamination, and low yield.

2.6 Random Amplified Polymorphic DNA (RAPD)

Kuhls *et al.* (1995) observed the use of PCR-fingerprinting with primers (GACA), (GTG), M13 (core sequence of phage M13), and OPB-05 was to compare ex type strains of various species of *Trichoderma*. Ex type strains could be discriminated from a number of other strains belonging to the same species, since every strain was characterized by its individual PCR-fingerprint. PCR-fingerprinting is recommended as a basic tool for proving the identity of strains, especially with regard to comprehensive culture collections. Investigations of Trichoderma todica, a species of undefined systematic position within the genus *Trichoderma*, suggest a close relationship to *Trichoderma parceramosum*. Morphological

and molecular data indicate that Trichoderma todica and *Trichoderma parceramosum* are conspecific.

Goes *et al*. (2002) Random Amplified Polymorphic DNA (RAPD) procedure was used to examine the genetic variability among fourteen isolates of *Trichoderma* and their ability to antagonize *Rhizoctonia solani* using a dual-culture assay for correlation among RAPD products and their hardness to *R. solani.* Seven oligodeoxynucleotide primers were selected for the RAPD assays which resulted in 197 bands for 14 isolates of *Trichoderma*. The data were entered into a binary matrix and a similarity matrix was constructed using DICE similarity (SD) index. A UPGMA cluster based on SD values was generated using NTSYS (Numerical Taxonomy System, Applied Biostatistics) computer program. A mean coefficient of similarity obtained for pairwise comparisons among the most antagonistic isolates was around 40%. Results showed that the variability among the isolates of *Trichoderma* was very high. No relationship was found between the polymorphism showed by the isolates and their hardness, origin and substrata.

Sharma *et al*. (2009) Studied the genetic variability among 8 isolates of *Trichoderma harzianum*, and their ability to antagonize *Sclerotium rolfsii* using a dual culture assay was correlated with RAPD profiles. Eight oligodeoxynucleotide primers were selected for the RAPD assays, which resulted in 86 bands for 8 isolates of *T. harzianum*. An unweighted pair grouping mathematical averaging (UPGMA) cluster based on SD values was generated using the NTSYS computer program. RAPD was efficient in demonstrating the high intraspecific genetic variation among isolates. A mean coefficient of similarity obtained for pairwise comparisons was c. 30% and it showed that the variability among the isolates of *T. harzianum* was very high. The dendrogram did not show the grouping of isolates by their level of antagonism. Relationship among polymorphism existent, the aggressiveness and the origin of isolates were not found.

Chakraborty *et al.* (2010) Collected nineteen isolates of Trichoderma viride and Trichoderma harzianum obtained from rhizosphere

soil of plantation crops, forest soil and agricultural fields of North Bengal region were studied using RAPD and ITS-PCR. The genetic relatedness among eleven isolates of T. viride and eight isolates of T. harzianum were analyzed with six random primers. RAPD profiles showed genetic diversity among the isolates with the formation of eight clusters. Analysis of dendrogram revealed that similarity coefficient ranged from 0.67 to 0.95. ITS-PCR of rDNA region with ITS1 and ITS4 primers produced 600bp products in all isolates. This result indicated the identification patterns of *Trichoderma* isolates.

Gupta *et al.* (2010) Studied RAPD-PCR analysis of seven *Trichoderma* species isolates and their in vitro antagonism against wilt pathogens of *Psidium guajava* L. viz. *Fusarium oxysporum f. sp. psidii* (F. o. f. sp. psidii) and *Fusarium solani*. . Out of 10 RAPD oligonucleotides (OPA 1–OPA 10) tested, seven markers OPA 1, 3, 5, 7, 8, 9 and 10 efficiently differentiated the isolates of *Trichoderma* and showed reproducible banding patterns. A total of 248 bands were obtained from these markers along with a 61.84% per cent similarity among the seven isolates of *Trichoderma*.

Sagar *et al.* (2011) evaluated some *Trichoderma* isolates from different locations of Bangladesh for their bioefficiency by determining their genetic variations. PCR-based Random Amplified Polymorphic DNA (RAPD) Marker employing 3 decamer primers produced 29 scorable bands of which all (100%) were polymorphic. The co-efficient of gene differentiation (Gst) was 1.0000 reflecting the existence of high level of genetic diversity among the isolates. The result indicating their genetic diversity has opened new possibility of using the most efficient and more isolates of *Trichoderma* in the preparation of effective biopesticide.

Kumar *et al*. (2011) carried out randomaly amplified polymorphic DNA (RAPD) in combination with dilution plating on semi selective medium for detection and identification of *Trichoderma* a potential bio-control agent utilized in compost amended mixes. Distinct and reproducible fingerprints were obtained upon amplification of purified genomic DNA of *T. longibrachiatum* with random primers of Operon (OPA)

series. The amount of genetic variation was evaluated with a set of 20 RAPD primers. More than 50% of the amplified fragments in each case were polymorphic. It is concluded that there was good genetic variability among the isolates collected from pigeonpea fields of Uttar Pradesh.

Kumar and Pratibha Sharma (2011) Observed twelve isolates belonging to *Trichoderma harzianum* and *Trichoderma viride* were assessed for their mycoparasitic effect on phytopathogens *Pythium aphanidermatum* and *Sclerotinia sclerotiorum*. Though *T. harzianum* isolates were more aggressive than *T. viride* isolates, the percent inhibitory effect among *T. harzianum* isolates did not vary much (80 to 86%). The inhibitory effect of *T.viride* isolates ranged from 50 to 80%; however, TvChen, Tv4, and TvNir were distinguishable from other *T. viride* isolates in exhibiting higher degree of antagonism. The dataset generated through morphological characters and molecular markers (RAPD and ISSR) showed a comparable output grouping the isolates Tv4, TvChen and TvNir in one cluster and all *T. harzianum* isolates in another cluster.

Srivastava *et al*. (2012) carried out study to corroborate the positive relatedness of molecular and morphological characters with antagonistic ability. Among many isolates of *Trichoderma atroviride* isolated from rhizospheric soils from different parts of U.P. has brought attention due to its highly antagonistic activity. Molecular variability among the isolates showed 74 amplified bands out of which 65 were polymorphic and 19 were monomorphic. The size of amplified product varied from 0.1kb to 0.75kb.

Gurumurthy *et al.* (2013) Studied molecular characterization of the promising bio-control agents adopting Random Amplified Polymorphic DNA (RAPD) analysis helps to determine the diversity and identification. DNA (RAPD) analysis were employed in aggregation with dilution plating on semi selective medium for distinguishing and identification of *Trichoderma*, a potential bio-control agent utilized in compost amended mixes. Distinct and reproducible fingerprints were attained upon amplification of purified genomic DNA of *Trichoderma spp*. with random primers of Operon (OPH) series. The amount of genetic

variation was figured out with a set of 20 RAPD primers. In most cases, the amplified fragments showed more than 50% polymorphism. It is evidenced that there was good genetic variability among the isolates collected from chickpea fields of Uttar Pradesh.

Saravanan *et al.* (2014) Collected fifteen isolates of *Trichoderma viride, Trichoderma atroviride* and *Trichoderma harzianum* obtained from rhizosphere soil of plantation crops, forest soil and agricultural field of Tamilnadu region. Studied molecular characterization using Random Amplified Polymorphic DNA and Inter Simple Sequence Repeats-Polymerase Chain Reaction. The isolates were grouped into three distinct clusters of *T.harzianum, T.viride and T.atroviride*. The similarity ranged from 50 to 91%. The maximum genetic variation in the *T. viride* group was observed between the isolates T4 and T7 (90%). In *T. harzianum* cluster variation ranged between T8 and T12 (88%). Followed by T3 and T5 (79%) in the *T. atroviride* group. On the other hand maximum genetic variation of 51 % was observed between the cluster I and followed by 55% between cluster II and cluster III.

Komy *et al.* (2015) Evaluated the antagonistic activity of 30 isolates of *T. asperellum* against 4 different isolates of FOL. The production of extracellular cell wall degrading enzymes of the antagonistic isolates was also measured. The random amplified polymorphic DNA (RAPD) method was applied to assess the genetic variability among the *T. asperellum* isolates. All of the *T. asperellum* isolates significantly reduced the mycelial growth of FOL isolates but the amount of growth reduction varied significantly as well. RAPD analysis showed a high level of genetic variation among *T. asperellum* isolates. The UPGMA dendrogram revealed that *T. asperellum* isolates could not be grouped by their anta- gonistic behavior or lytic enzymes production. Six isolates of *T. asperellum* were highly antagonistic towards FOL and potentially could be used in commercial agriculture to control tomato wilt.

Korat *et al*. (2016) studied on ten isolates of *Trichoderma sp*. viz., *T. viride* (five isolate), *Trichoderma sp.* (two isolates) and *T. harzianum* (three isolates) obtained from the rhizosphere soil of tomato fields of south

Gujarat region were studied using RAPD. The genetic diversity among ten isolates of *Trichoderma* sp. were analyzed with five random RAPD primers. The percentage of polymorphism ranged from 60.00% to 83.33%. Analysis of dendrogram revealed that similarity coefficient ranged from 0.51 to 0.89. RAPD profiles showed genetic diversity among the isolates with the formation of two clusters. First cluster involved five isolates of *T. viride* and two isolates of *Trichoderma sp.* while second cluster involves three isolates of *T. harzianum*. This result indicated the identification patterns of *Trichoderma* isolates.

Mohsen (2016) studied on PCR-based technique of randomly amplified polymorphic DNA (RAPD) to characterize and assess the genetic relatedness of eight *Trichoderma harzianum* isolates. Six primers failed to support amplification while the remaining nine primers produced a total of 128 main bands (11-20 per primer) across the eight isolates. Of these bands, 120 (9-19 per primer) were polymorphic. The least efficient primer was OPY-19 (8.59), while the most efficient one was OP-B14 (15.63%). Primer OP-Y19 had the lowest (7.5%) discriminatory power while primer OP-B14 had the highest (15.8%). RAPD analysis fingerprinted six of the eight isolates through unique bands with one or more of the 9 primers. Cluster analysis based on the genetic distances split the eight isolates into two major group genotypes.

Rani *et al*. (2017) A total of nine *Trichoderma* isolates were obtained from 27 rhizosphere samples collected from different cropping systems i.e. groundnut, redgram and tomato. A random amplified polymorphic DNA (RAPD) marker was used to estimate the genetic variation among 9 isolates of *Trichoderma*. These isolates were characterized using 15 random primers of the OPA and OPM series. Out of which 9 primers gave reproducible and scorable band with high percentage of polymorphism. Fifteen selected primers gave total of 207 amplification products, out of which 196 were polymorphic. The maximum polymorphism (100%) was observed in PCR reaction with OPA-01, OPA-03, OPA-05, OPA-09, OPA-10, OPM-04 and OPM-20 with size ranging from 250bp to 2500 bp. The genetic distance between each isolate was calculated, and

cluster analysis was used to generate a dendrogram showing the relationship among them.

2.7 Internal Transcribed Spacer (ITS)marker

Hermosa *et al*. (2000) Characterization of 16 biocontrol strains, previously identified as "*Trichoderma harzianum*" Rifai and one biocontrol strain recognized as *T. viride*, was carried out using several molecular techniques. A certain degree of polymorphism was detected in hybridizations using a probe of mitochondrial DNA. Sequencing of internal transcribed spacers 1 and 2 (ITS1 and ITS2) revealed three different ITS lengths and four different sequence types. Phylogenetic analysis based on ITS1 sequences, including type strains of different species, clustered the 17 biocontrol strains into four groups: *T. harzianum-T. inhamatum* complex, *T. longibrachiatum*, *T. asperellum*, and *T.* atroviride, *T. koningii* complex. ITS2 sequences were also useful for locating the biocontrol strains in *T. atroviride* within the complex *T. atroviride-T. koningii*. None of the biocontrol strains studied corresponded to biotypes Th2 or Th4 of T. harzianum, which cause mushroom green mold.

Gherbway *et al*. (2014) Collected ninety isolates of *Trichoderma* (Teleomorph: Hypocrea) species and one isolate of *Gliocladium viride* (Tel. Hypocrea lutea) were isolated from soil samples from different locations in Taif city. Two soil samples cultivated with *Purica granatum* showed high incidences of isolates (13 and 12 isolates, respectively). Regions of nuclear rDNA, containing 18S ribosomal RNA gene (partial sequence); internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (complete sequence); and 28S ribosomal RNA gene (partial sequence) were amplified to identify the collected isolates. The sequencing results indicated that 78 isolates of the population were identified as *Trichoderma harzianum* (Tel. Hypocrea lixii). Also, two isolates were Identified as *T. longibrachiatum* (Tel. H. orientalis) and one isolate as *Gliocladium viride* (Tel. H. lutea)

Savitha and Shriram (2015) Identified ten species of *Trichoderma* antagonistic to *Phytophthora capsici* in red pepper which were characterised based on morphological features and molecular tools. Molecular characterization was done by amplifying and analysing the sequences of Internal Transcribed spacer gene 1 and 2 (ITS) and translation elongation factor 1-alpha encoding gene (tef1). The phylogenetic analyses of the *Trichoderma* isolates were carried out based on the ITS and tef1 sequences. Among 10 *Trichoderma* isolates Th9, Th10, Th19, Tv10 and Tv115 were identified as *T. harzianum.* Isolates Th16, Tv30 and Tvs7 were identified as *T. asperellum* and isolates Tvs5 and Tvs8 were identified as *T. virens.*

CHAPTER III

MATERIAL AND METHODS

All the work on the Title “Morphological and Molecular characterization of *Trichoderma longibrachiatum*” was carried out in the Department of Plant Pathology PGI, Dr. PDKV, Akola during 2017-18. The material used and methods followed are described in this chapter.

3.1 Material

3.1.1. Glass wares and Plastic wares

During the present investigation several types of glasswares viz., Glass petriplates, conical flasks (250ml, 500ml and 1000ml), test tubes, beakers, glass rod, coverslips, slides, micropipetts, polypropylene centrifuge tube, measuring cylinder (10ml, 100ml, 250ml, 500ml and 1000ml), Ocular and stage micrometer were used.

3.1.2 Equipments

Standard laboratory equipments used for different experiments were Autoclave, Hot Air Oven, Laminar Air Flow, Digital weighing balance (Wensar HBT 516), BOD incubator, Research microscope, Centrifuge (Eppendorf 5418), PCR (Eppendorf), Gel electrophoresis unit(Genexy, Scie-Plas), Microwave oven, and Gel Documentation unit, haemocytometer (Improved Neubauer, China), double distillation unit, Deep freezer -20ºC (Blue star), Digital camera etc.

3.1.3 Miscelaneous

Non-absorbent cotton, muslin cloth, polyethylene bags, cork borer (5 mm), inoculation needle, dissection needle, forceps, paper bags, butter paper bags, pencil, permanent marker, cello tapes, Whatman filter paper no. 01 (4 mm), soil, test tube stand, tray, wash bottle, wooden sticks, potato, rubber band, mortar pestle, scissors, cavity slides, glass rod, polypropylene Petri plates, and polypropylene centrifuge tube, eppendorf tubes etc were used.

3.2 Soil sampling

3.2.1 Collection of soil samples

Soil samples were collected from different districts of the Vidarbha region of Maharashtra State from rhizosphere soils of important crops. For rhizospheric soil, plant was gently and carefully uprooted, soil tightly adhering the root was collected, randomly selected, mixed and one forth part was used as composite rhizospheric soil sample of the region. The composite soil samples collected from particular field are kept in brown paper bags labeled separately. The samples were brought to laboratory and stored until used.

Table 1. Soil samples collected from different districts of Vidarbha region

Sr. No.	Location	Code name
1.	Akola	TL-1
2.	Amravati	TL-2
3.	Buldhana	TL-3
4.	Bhanadara	TL-4
5.	Chandrapur	TL-5
6.	Gadchiroli	TL-6
7.	Gondia	TL-7
8.	Washim	TL-8
9.	Yeotmal	TL-9

3.3 Isolation of *Trichoderma* from collected soil samples

3.3.1. Serial Dilution Procedure

Serial dilution technique (Johnson and Curl, 1972) was used to isolate *Trichoderma* spp. from rhizospheric soil stored earlier. Antagonistic microflora were isolated on *trichoderma* selective Medium

(TSM). One ml of soil suspension from dilutions (10-3 and 10-4) was aseptically poured on to sterilized petriplates and then medium was poured at lukewarm stage. Plates were rotated gently to get uniform distribution of soil suspension in the medium. The plates were incubated at 28±1ºC and observed at frequent intervals for the development of colonies.

3.3.2. Isolation of Trichoderma spp. on Trichderma specific medium

The TSM (Trichoderma specific medium) was used for isolation was prepared according to Elad and Chet, 1982. List of ingredients for TSM are given in Table 1.

Table 2. Ingredients of Trichoderma Selective Medium (TSM)

Sr. No.	Chemicals	Quantity
1	Glucose	3g
2	Magnesium sulphate	0.2g
3	Dipotassium hydrogen sulphate	0.9g
4	Ammonium nitrate	1.0g
5	Potassium Chloride	0.5g
6	Rose Bengal	0.033g
7	Metalaxyl	0.3g
8	Penta Chloro Nitro Benzene	0.2g
9	Chloramphenicol	0.25g
10	Agar-agar	15g
11	Distilled water	1000ml

All the chemicals except Rose Bengal, Metalaxyl, PCNB and Chloramphenicol were dissolved in distilled water. The mixture was heated slowly while stirring until it started boiling. Later on Rose Bengal and Metalaxyl were added and sterilized after it was equally distributed into 250 ml conical flasks. Chloramphenicol was added into the cooled and molten medium before being poured into the petriplates.

3.3.3 Pure cultures of Trichoderma

Three days old colonies of Trichoderma isolates were picked up and purified by single hyphal tip method (Lelliott and Stead 1987). transferred to Potato Dextrose agar media plates with help of sterilized inoculating needle and the pure cultures so obtained were stored in a incubator at 35ºC for further use.

3.3.4. Maintenance of fungal cultures

Isolated fungal cultures were maintained on sterilized PDA slants in the refrigerator at about 8º to 10º C. Periodic transfer of the cultures were carried out on sterilized PDA slants to keep the cultures in active growth.

3.3.5 Broth culture preparation

Broth culture for *Trichoderma spp*. were made using Potato Dextrose Broth (PDB) medium (PH-5.6) by transferring a loop full culture from the mother culture which were grown at 26ºC - 27ºC in BOD incubator. A fungal mat of approximately 3-4 g was grown. Further the mat was air dried for the fungal sample DNA isolation from *Trichoderma spp*.

3.4. Methods

3.4.1. Sterilization of Glass wares. Media, Water Blotter Paper and Other Material

Petri plates, test tubes and conical flasks of different capacities i.e., 1000 ml, 500 ml, 50 ml etc. of 'Borosil' make were used. The cleaning and sterilization of glasswares is necessary for their re-use. The glass wares were sterilized in hot air oven at 180°C for one hour whereas the media, distilled water and blotter paper were sterilized in autoclave at 121.6ºC at 15 lbps pressure for 15 mins. The other required material like needles, inoculating needle, forceps, scalpel were directly sterilized on flame of burner by direct heating, generally it is heated till it gets red hot.

3.4.2 Culture Media

Trichoderma selective media (TSM), Potato dextrose agar (PDA) and Potato dextrose broth (PDB) media were used during laboratory studies of fungus.

3.4.3 Morphological Study

Morphological identification was done based on cultural (colony and growth rate) characterization and microscopic observation. *Trichoderma longibrachiatum* isolates were sub-cultured from slants to PDA plates and incubated at 28ºC for 24-48 hr. After 2 days when the colonies were visibly growing, but before conidial production, a 5 mm-diameter mycelia disc were cut from actively growing edge of the colony and inoculated at the center of all freshly prepared potato dextrose agar (PDA), PDA plates were incubated at 30ºC with intermittent light. Three replicates were maintained for each isolate. Entire work was carried out in aseptic conditions. The characteristics like colony appearance, growth rate and sporulation patterns were recorded (Samuels, 2004). In order to assess the growth rate, colony diameter of each isolate growing was recorded from third to seventh day. Following observations were recorded:

1. Colony character: shape, size, growth rate, colour
2. Microscopic characters: philides, spore shape, spore size, spore count.

3.4.3.1 Measurment of spore size

The spore size was measured by using ocular micrometer and stage micro meter. The ocular index was calculated with the help of stage micrometer. The ocular micrometer was placed in eye piece of microscope and stage micrometer at the stage of microscope after replacing the ordinary glass slide which was placed for adjustment of focus of material to be placed over the surface of glass slide. Measurement of different conidia was carried out for 100 times to find the particular range of conidia. The number of division of stage micrometer coinciding with the division of ocular micrometer were noted, the ocular index (μ) was calculated by formula:

$$\text{Ocular index } (\mu) = \frac{\text{No. of division of stage micrometer coinciding with ocular division}}{\text{No. of division of ocular micrometer coinciding with stage division}} \times 10\ \mu$$

4.3.3.2 Spore count

For taking fungal spore count we used the Haemocytometer. Place a drop of conidial suspension made from liquid culture on the engraved grid and let the preparation stand for 1-2 min to allow the conidia to settle at bottom. Put a cover slip over the grid carefully so that no air bubbles enters between the slide and cover glass.Slide the cave glass backwards and forwards until coloured rings are visible or two surfaces of caves glass and slide came in contact. Count the spore of fungus in the middle square (E) which consist of 25 groups of 36 small squares each grid of 0.2mm.

The spore count was calculated by using the following formula:

Spore /ml solution= $N \times 10^4$

Where,

N = No of spores counted on the middle square of the grid

3.4.4. Preparation of media

3.4.4.1 Procedure for preparation of PDA media

The composition of PDA medium is given in Table 3. Potatoes were peeled and cut into small slices and gently washed. 200 g of sliced potato added into 500 ml of water and allowed to boil till it gets soft. These boiled potatoes slices passed through muslin cloth, filtrated was collected into 2000 ml capacity beaker and final volume was made to 1000 ml by adding distilled water. Transferred the filtrate into another pot and allowed to boil, gently added 20 g of dextrose followed by 20 g of agar-agar with intermediate shaking for 3-5 minutes. Poured 250 ml of liquefied PDA into 500 ml flask and plugged by nonabsorbent cotton and wrapped with paper over it. The PDA medium was autoclaved at for 15 lbs/in^2(121°C) for 20 minutes.

Table 3. The composition of potato dextrose agar medium

Sr. No.	Ingredient	Quantity
1	Peeled and sliced potato	200 g
2	Dextrose ($C_6H_{12}O_6$)	20 g
3	Agar-agar	20 g
4	Distilled Water	1000 ml

3.4.4.2. Preparation of PDA plates and slants

The autoclaved Petri plates were transferred to laminar air flow chamber and kept under UV light for 20 minutes for maintaining aseptic condition. The PDA medium prepared in the flask was liquified by keeping it into microwave oven for three minutes. Streptomycin @ 2.5 mg/l was added in lukewarm medium and gently shakes for uniform mixing. 15-20 ml of lukewarm PDA was poured immediately into each Petri plate. These plates were kept for 24 hour in incubator to confirm free from contamination. Similarly warm liquified PDA containing streptomycin was poured into the slants.

3.4.4.3. Preparation of Potato Dextrose Broth (PDB)

The peeled potatoes slices were boiled in 800 ml of distilled water and the extract was collected by filtering through a muslin cloth. The filtrated potato extract was transferred into another jar and 20 gram of dextrose was added to the mixture. The volume was made up to 1000 ml with distilled water. 100 ml broth was added into 250 ml flasks and autoclaved at 121.6^{o}C for 15 minutes at 15 lbs/in^2. The detail composition of PDB is given in Table

Table 4. The composition of potato dextrose broth

Sr. No.	Ingredient	Quantity
1	Peeled and sliced potato	200 g
2	Dextrose ($C_6H_{12}O_6$)	20 g
3	Distilled Water	1000 ml

3.4.4.4. Dual culture Technique

Antagonistic activity of *Trichoderma longibrachiatum* were assayed against *Rhizoctonia bataticola, Sclerotium rolfsii* and *Fusarium udam* by using dual culture inoculation technique described by Vincent, (1927), Mandal *et al*., (1999) in Petri plates. Five mm disc from the periphery of actively growing pathogen on PDA was placed in centre of 90 mm diameter Petri plates containing PDA. Three discs of each actively growing isolates of *T. longibrachiatum* were placed at equidistance on all four side 30 mm apart from centre disc of pathogenic fungus. The plates were incubated at ambient condition under alternate dark and light cycle up to 7 days. Simultaneously the pathogenic fungus disc (5mm) was incubated on PDA Petri plates alone and incubated under similar condition for same period. Plates were observed every day for nothing the behaviour at the point of intermating of two cultures under stereoscopic microscope. On seventh days after incubation, the growth of pathogenic test fungus was measured and per cent growth inhibition was calculated using the following formula.

$$\text{Per cent Growth inhibition} = \frac{C - T}{C} \times 100$$

Where,

C = Mycelial growth (mm) in control plate.

T = Mycelial growth (mm) in treatment plate.

3.4.5. Collection of pure cultures of soil inhibiting plant pathogens

Pure cultures of different soil borne plant pathogens viz. *F. udam, R. bataticola, S. rolfsii* for testing efficacy of *T. longibrachiatum* were collected from Department of Plant Pathology, Dr. P.D.K.V, Akola,

3.4.6. Antagonistic activity of *T. longibrachiatum* against soil borne plant pathogens

Experiment was conducted in the laboratory with following details.

Design – Completely Randomized Design (CRD)

Treatment - 10

Replication - 3

3.4.7. Molecular characterization

3.4.7.1 Chemicals

Different materials were used for the study of molecular variability of *Trichoderma longibrachiatum* isolates are given below

Table 5. Chemicals used for study of molecular variability of *Trichoderma longibrachiatum*

Sr. no.	Name of chemicals
1. DNA extraction	
1	β–mercaptoethanol
2	Boric acid
3	Cetyltrimethyl ammonium bromide (CTAB)
4	Chloroform
5	Ethyline Diamine Tetra Acetic Acid Disodium Salt ($EDTANa_2$)
6	Ethanol
7	Isoamyl alcohol
8	Isopropanol
9	Phenol
10	Ribonuclease A (RNAase A)
11	Sodium chloride
2. PCR Amplification	
12	10X *Taq* buffer
13	$MgCl_2$ 25 mM
14	*Taq* DNA polymerase (5 U/µl)

15	dNTP Mix (10mM)		
16	Primers		
3. Agarose gel electrophoresis			
17	Agarose	21	Methanol
18	Boric acid	22	Ethidium bromide
19	EDTA Na_2	23	Tris buffer
20	Ethidium bromide	24	DNA ladder 1 kb
4. Software's used			
25	Alpha Ease		
26	NTSYS Pc		

3.4.7.2 Different solutions, buffers and reagents used for study

Different solutions, buffers and reagents used to perform present study are listed below along with their composition.

3.4.7.3. Extraction of DNA

- Extraction buffer [2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mMTris-HCl, 0.4% β–mercaptoethanol (added at the time of use)]. (The extraction buffer was autoclaved before addition of β–mercaptoethanol)
- Chloroform : Isoamyl alcohol (24:1)
- 100 % Isopropanol (Ice-cold)
- Wash buffer : 70 % ethanol
- TE buffer (10 mMTris and 1 mM EDTA, pH 8.0) Autoclaved before use.
- 5 M NaCl (solution was sterilized by autoclaving).
- 1 M Tris HCl (pH 8.0).
- 5 M EDTA Na_2 (pH 8.0)

3.5.7.4 PCR Amplification

Various reagents used for the ITS, RAPD and amplification are listed below:

- 10 picomole/µl Oligonucleotide RAPD and primers

- 5 U/µl *Taq* DNA polymerase
- 10 X *Taq* buffer
- 25 mM $MgCl_2$
- 10mM dNTP

3.5.7.5 Agarose gel electrophoresis

- 10 x TBE (0.9 M Tris base, 0.9 M Boric acid, 0.10 M EDTA)
- 6X Gel loading dye (10 mMTris-Cl pH 7.6, 0.03 % bromophenol blue, 0.03 % xylene cyanol FF, 60 % glycerol in water).
- Ethidium bromide (0.5 mg/ml).

Table 6. Primers selected for PCR with their sequence code

Sr. No.	Primers	Primer code
1	OPA 1	CAGGCCCTTC
2	OPA 2	TGCCGAGCTG
3	OPA 3	AGTCAGCCAC
4	OPA 4	AATCGGGCTG
5	OPA 5	AGGGGTCTTG
6	OPA 6	GGTCCCTGAC
7	OPA 7	GAAACGGGTG
8	OPA 8	GTGACGTAGG
9	OPA 9	GGGTAACGCC
10	OPA10	GTGATCGCAG
11	OPA11	GGACTGGAGT
12	OPA 12	TCTGTGCTGG
13	OPA 13	CAGCACCCAC
14	OPA15	TTCCGAACCC
15	OPA18	AGGTGACCGT
16	OPA19	CAAACGTCGG
17	OPA20	GTTGCGATCC
18	OPB 5	TGGGCCCTTC
19	OPB-17	AGGGAACGAG
20	OPB-20	ACTTCGCCAC

3.5.8. Molecular variability analysis

In present investigations, 9 isolates of *Trichoderma longibrachiatum* were screened for molecular variability using Random Amplified Polymorphic DNA (RAPD).

3.5.8.1. Preparation of mycelium mat

To get enough mycelium, pure culture of six isolats of *Trichoderma viride* were mass multiplied by transferring mycelium tissue into 100 ml of PDB and kept for incubation inside BOD incubator at 28 ± 2°C for next seven days without disturbing the flasks. The mycelial mat from broth was transferred to blotting paper and allowed to air dry for 20 min at room temperature. This mycelial mat was used for isolation of genomic DNA.

3.5.8.2. Extraction of DNA

Genomic DNA was isolated from the six selected isolates by the cetyldimethylethyl ammonium bromide (CTAB) method with some modifications.

3.5.8.3. Reagents required

1) TrisHCl (pH 8.0)

30.285 gram tris HCl dissolved in 150 ml of distilled water and volume 1M was made up to 250 ml with distilled water. The buffer was autoclaved and stored at room temperature.

2) 0.5M Na_2 EDTA

37.224 g dissolved Na_2 EDTA in 150 ml of distilled water by adding NaOH pellets till the P^H of the buffer came to 8.0 and the total volume of buffer was made up to 200 ml with distilled water. The buffer was autoclaved and stored at room temperature.

3) Extraction buffer

TrisHCl 50 mM (pH 8.0), NaCl 500 mM, Na_2 EDTA 50 mM, P^H 8.0 and 1 per cent (v/v) β-Mercaptoethanol (Added immediately before use).

4) Chloroform: Isoamyl alcohol (24: 1 v/v)

480 ml chloroform and 20 ml of iso-amyl alcohol was mixed together.

5) Isopropanol

6) 70 per cent Ethanol

7) TE10:1, pH 8.0 (10 mMTris; 1 mM Na_2 EDTA, pH 8.0)

2 ml of 1M Tris, pH 8.0 and 0.4 ml of 0.5 M Na_2 EDTA, pH 8.0 was added to 197.6 ml of sterile distilled water.

3.5.8.4. Procedure for isolation of fungal genomic DNA

1. Seven days old mycelial mat was transferred on sterilized blotter paper, air dried to remove moisture and media adhering to the mat.
2. This dried mycelium mat was used for DNA isolation. Approximately, one gram of air dried fungal mat was quickly frozen in liquid nitrogen (-196^{0}C) and crushed into powder form with the help of sterilized mortar and pestle.
3. The powder was immediately homogenized by adding pre-warmed (65^{0}C) extraction buffer and transferred to two ml eppendorf tubes.
4. The content of tube was shaken vigorously for one minute for uniform mixture of suspension.
5. These tubes were incubated in a water bath at 65^{0}C for one hour with gentle shaking at every 15 minutes.
6. The tubes removed from hot water bath and an equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed gently to denature proteins and centrifuged at 12000 rpm at room temperature for 20 minutes.
7. The upper aqueous phase was transferred into a new 2 ml eppendorf tubes with a wide bore pipette and equal volume of chloroform:isoamyl alcohol (24:1) was added.
8. The content of the tubes were gently mix for five minutes and centrifuged for 15 minutes at 10000 rpm.

9. The tube was mixed gently and centrifuged for five minutes as before. Aqueous phas transferred to another tube and equal volume of ice chilled Isopropanol was added. 10. Centrifuged for 10 minutes at 12000 rpm and decanted the supernatant. 10. The pellet was washed with 70 per cent ethanol twice and suspended in $T_{50}E_{10}$ buffer (50mMTris-HCl, pH 8.0, 10 mM EDTA).
10. An equal volume of phenol: chloroform (1:1) was added to the solution, mixed well for five minutes and centrifuged at 12000 rpm in a micro centrifuge.
11. Aqueous phase was transferred to another tube and an equal volume of chloroform was added.
12. The aqueous layer was separated and DNA was precipitated by adding 2.5 volume of absolute ethanol.
13. Pellet (genomic DNA) was washed twice with 70 per cent ethanol and re suspended in TE buffer (10 mMTris-HCl, pH 8.0, 1 mM EDTA) and stored at -20°C until needed.

The PCR was carried out in small reaction tubes, containing a reaction volume typically of 12.5 µl (Table 4 and 5) that was inserted into a thermal cycler (Eppendorf) that heated and cooled the reaction tubes within it to the precise temperature required for each step of the reaction. PCRs were run on the programmable thermal cycler given in Table

Table 7. PCR reaction mix for 1x of 12.5 µl reaction

Sr. No.	**Master Mix**	**1x**
1	10x *Taq* buffer	1.25 µl
2	$MgCl_2$ (25 mM)	1.25 µl
3	dNTPs (10 mM)	0.3 µl
4	*Taq* polymerase (5 U/µl)	0.3 µl
5	Sterile distilled water	5.4 µl
	Total Volume	**8.5 µl**

Table 8. Constituents of PCR reaction

Sr. No.	PCR Reaction	Quantity
1	Master Mix vol.	8.5 µl
2	Primer (Forward)	1.0 µl
3	Primer (Reverse)	1.0 µl
4	Template DNA (37.5 ng)	2.0 µl
	Total Reaction Volume	12.5 µl

After completion of the cycles keep the samples at 4^0C till electrophoresis.

3.5.9. Internal Transcribed Spacer (ITS) amplification

Genetic variability of *Trichoderma longibrachiatum* isolates was evaluated by using ITS primers.

Table 9. List of ITS primers used with their sequences (Genaxy)

Oligo Name	Primer Sequence (5'-3')	GC %
ITS 1	TCCGTAGGTGAACCTGCGG	63
ITS 4	TCCTCCGCTTATTGATATGC	50

3.5.10. Procedure for PCR reaction

Sterile PCR tubes were numbered and placed on PCR tube stand. At first 2 µl of DNA was added to each PCR tube followed by master mix. The samples were mixed by brief centrifugation to bring down the content of tube. PCRs were run on the programmable thermal cycler given in table 9 and 10. PCR products were separated by electrophoresis in 2 per cent agarose gels run in 1X TBE, stained with ethidium bromide and visualized with a UV transilluminator.

Table 10. Steps used for PCR-ITS reaction

Name of step	Temperature	Time
Initial Denaturation	94°C	5 min.
30 cycles - Denaturation	94°C	1 min.
30 cycles - Annealing	55°C	1 min.
30 cycles - Extension	72°C	30 sec.
Final Extension	72°C	10 min.

Table 11. Steps used for RAPD reaction

Name of step	Temperature	Time
Initial Denaturation	94°C	5 min.
35 cycles - Denaturation	94°C	1 min.
35 cycles - Annealing	36.5°C	1 min.
35 cycles - Extension	72°C	3 min.
Final Extension	72°C	10 min.
Hold	4°C	

3.5.10.1. Separation of RAPD-PCR and ITS amplified products by agarose gel electrophoresis

Electrophoresis of RAPD-PCR amplified analysis on agarose gel was carried out in 1x TBE buffer in horizontal gel electrophoresis. Cleaned and dried electrophoresis assembly was used for gel electrophoresis. The gel tray was wiped and cleaned with methanol. It was set to prepare the gel. 1.2% agarose gel solution was prepared in 1x TBE Buffer. Ethidium bromide was added to the luke warm liquefy gel solution.

as staining agent. PCR products were loaded in the wells on the gel along with the 6 µl ladder as marker in the first well. 2 µl 6x loading dye was loaded along with each PCR product in the wells. Then the gel was run for two hours at 80 volts. After completion of 10 cm run, the gel was observed

and captured under UV light with the help of Bio-Rad Gel documentation system

3.5.10.2 Data analysis

The gel image was captured and visualized under light in gel documentation system.Data were scored as the presence (1) or absence (0) of individual band for each isolate. This binary data was used to compute the similarity coefficient using the Jaccards similarity coefficient with the help of Numerical Taxonomy System Version 2.2 (NTSYSpc). The similarity matrix was used to construct the dendrogram by Unweighted pair group method of arithmetic average (UPGMA) using the SAHN (Sequential Agglomerative Hierarical Nested) cluster analysis module dendrogram by Unweighted pair group method of arithmetic average (UPGMA) using the SAHN (Sequential Agglomerative Hierarical Nested) cluster analysis module (Rohlf,1998). Per cent polymorphism was calculated by using the formula.

$$\text{Per cent polymorphism} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

CHAPTER IV

RESULTS AND DISCUSSION

The present investigation was conducted on “Morphological and molecular variability of *Trichoderma longibrachiatum* ” with an objectives, to study the morphology and molecular variability of *Trichoderma* isolates using RAPD marker. This result and discussion chapter includes results of various experiments conducted during the study are presented below.

4.1 Collection of soil samples

Rhizospheric soil samples collected from agricultural fields with soil pH ranging from 6.6 to 8.3 located in 9 districts of Vidarbha region of Maharashtra state viz. Akola, Amravati, Bhandara, Buldhana, Chandrapur, Gadchiroli, Gondia, Washim, Yeotmal,. Trichoderma selective medium was used for the isolation.

Table 12. Soil samples collected from different Districts of Vidarbha region.

Sr. No.	Location	Code
1.	Akola	TL-1
2.	Amravati	TL-2
3.	Buldhana	TL-3
4.	Bhanadara	TL-4
5.	Chandrapur	TL-5
6.	Gadchiroli	TL-6
7.	Gondia	TL-7
8.	Washim	TL-8
9.	Yeotmal	TL-9

4.2 Isolation of *T. longibrachiatum* from soil samples

Nine *T. longibrachiatum* isolates were isolated by following serial dilution spread plate method (Johnson and Curl, 1972). The medium used for isolation was trichoderma selective medium (Elad *et al*. 1982). Serially diluted soil samples were spread on to the media plates. After earliear whitish to latter green colonies growth appeared on TSM was

separated and purified by using single hyphal tip method and maintained on PDA medium for further study.

Similarly, Chennappa *et al.* (2017) isolated different *Trichoderma* isolates rhizosphere soils of different cropping ecosystem from Karnataka using serial dilution technique and studied further.

4.3 Morphological Characters of *Trichoderma* isolates

The Trichoderma isolates maintained on the PDA medium showed variation (plate1). The morphological studies was based on different parameters like colony growth, colony colour, pigmentation. Colony characters of Trichoderma isolates were studied using 7 days old cultures that were incubated at 28°C ± 35°C. At 28°C, all *Trichoderma longibrachiatum* isolates grew well and formed conidia within 4 days. The radial growth(mm) of all isolates was measured at 7^{th} days after inoculation. Also the spore size, spore shape, spore count and phialides were recorded. (Plate no. 1 and 2).

4.3.1 Identifiaction

Pure cultures 11 isolates of Trichoderma were send to Indian Type Culture Collection(ITCC), Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi for confirmation and identification upto species level. As per the letter No. PP/66 date 07 th April, 2018 (Appendix III). Nine isolates coaded as TL-1 to TL-9 identified as *Trichoderma longibrachiatum* and one was *Trichoderma viride* and one was not identified as it was contaminated during transit with other fungi.

4.3.1 TL-1 isolate (Akola)

The culture was initially whitish then it turns to the light green, with smooth colony edges. The reverse pigmentation was whitish to creamy white in colour. The Fungus produced light greenish oval conidia with size 1.96-2.87 x 0.95-1.50 µm. phialides were more elongeated and cylindrical.. The sporulation was Good and Compact.

Table 13. Morphological characterization of *Trichoderma longibrachiatum* isolates

Sr.no.	Isolate name	Colony Growth (mm)at 7th DAI	Colony colour	Pigmentation	Phialides	Conidia		Sporulation
						Shape	Size(L × B)	
1.	TL-1 (Akola)	88.30	Whitish to light green	Creamy white	Elongeated and cylindrical	Oval	1.96-2.87 x 0.95-1.50 µm.	++++
2.	TL-2 (Amravati)	88.50	Light to dark green	Pale yellow	Lageniform or bottle shaped	Sub globuse to oval	1.93-2.04 x 1.03-1.23 µm	++
3.	TL-3 (Bhandara)	88.33	Light olive green with yellow tinge	Colourless to yellowish	Long cylindrical	Sub globuse	1.95- 2.03 x 0.95-1.11 µm	+
4.	TL-4 (Buldhana)	80.30	cottony white	Colourless to creamy white	Cylindrical or slightly inflated	Globuse	2.02 - 2.42 x 2.01 - 2.32 µm.	+++
5.	TL-5 (Chandrapur)	**90.00**	Light green to dark green	Pale yellowish green	Ampuliform	Globuse to slightly oval	2.09-2.99 x 2.08-2.29 µm	++++
6.	TL-6 (Gadchiroli)	80.00	Whitish to light green	Dark yellow to dirty yellow	Cylindrical shape	Roundish	1.75- 2.20 x1.74-2.20 µm,	++++
7.	TL-7 (Gondia)	**90.00**	light olive green	Whitish to pale green	lageniform	Sub-cylindrical	2.29 - 3.06 x 1.83 – 2.05 µm	+++
8.	TL-8 (Washim)	86.35	whitish to pale greenish	Creamy whitish	Cylindrical or slightly inflated	Oval	1.90 – 2.86 x 1.35 – 2.02 µm	++++
9.	TL-9 (Yeotmal)	**90.00**	cottony white	Pale yellowish	Lageniniform	Oval	2.91- 3.10 x 1.86-2.59 µm	+++

L= Length, B= Breadth

+++ : Maximum sporulation

++: Moderate sporulation

+ : Discrete sporulation

++++ : Good and Compact sporulation

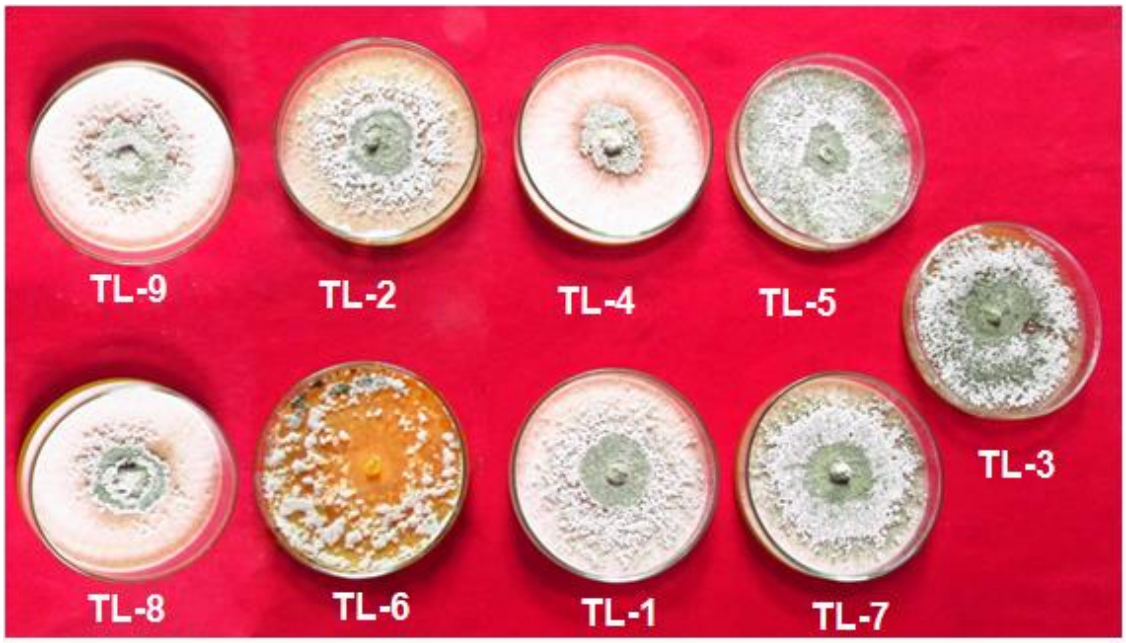


Plate 1. Morphological colonies of *Trichoderma longibrachiatum*

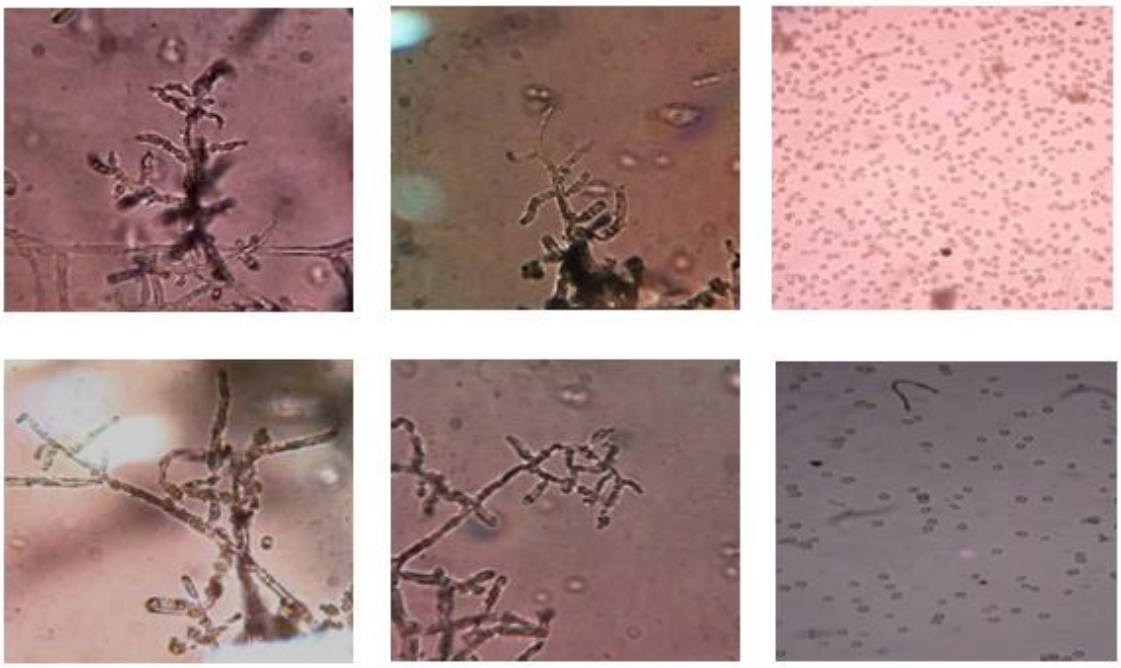


Plate 2. Microscopic photographs of *Trichoderma longibrachiatum*.

4.3.2. TL-2 isolate (Amravati)

The colony colour was light to dark green with pale yellow reverse pigmentation.The shape of phialides observed were directly along the long main branches, lageniform or bottle shaped. The fungus produce moderate sporulation with spore size 1.93-2.04 x 1.03-1.23 µm and sub globuse to oval. There was moderate sporulation.

4.3.3 TL-3 isolate (Bhandara)

Light olive green with yellow tinge colony colour with cottony growth on PDA. The reverse growth were colourless to yellowish. The phialides branches were spread and size were long cylindrical and thin. The conidial measurement were 1.95- 2.03 x 0.95-1.11 µm in size. The sporulation was very discrete.

4.3.4 TL-4 isolate (Buldhana)

Colony growth was cottony white and the pigmentation was colourless to creamy white. The phialides was cylindrical or slightly inflated. The conidial production was slow to moderate with globuse conidia shape, the size of the conidia were 2.02-2.42 x 2.01 -2.32 µm. There was maximum sporulation.

4.3.5. TL-5 isolate (Chandrapur)

Light green initially later turn greenish on 7th day on PDA medium. The Mature spore was powdery-like and reverse pigmentation were Pale yellowish green. Phialides was ampuliform with terminal phialides more elongeated. The conidial shape was globuse to slightly oval with conidial size 2.09-2.99 x 2.08-2.29 µm. The good and compact sporulation.

4.3.6 TL-6 isolate (Gadchiroli)

Superficial and dispersed growth was observed which was initially whitish but slowly turn light green in colour with irregular crystal like conidial growth. The pigmentation were dark yellow to dirty yellow. The phialides was with 2-3 whorl with cylindrical shape and sharply constricted at the tips. The conidial measurement was 1.75- 2.20 x1.74- 2.20 µm, the conidial shape was roundish. Good and compact sporulation.

4.3.7 TL-7 isolate (Gondia)

The colony colour was light olive green with flat and ring like growth on the medium. The reverse colony colour was whitish to pale green, the phialides were directly along the long main branches, lageniform .The conidia shape was Sub-cylindrical with pale green colour, the size of conidia was 2.29 - 3.06 x 1.83 – 2.05 µm.

4.3.8. TL-8 isolate (Washim)

The growth colour was whitish to pale greenish with cottony growth. The reverse pigmentation was not much changed, the pigmentation was creamy whitish. The phialides was cylindrical or slightly inflated. The conidial size measurement was 1.90 –2.86 x 1.35 – 2.02 µm, the shape of conidia was oval. The sporulation was good and compact.

4.3.9. TL-9 isolate (Yeotmal)

The cottony white growth with greenish crystal like conidial powdery growth. The reverse growth pigmentation was pale yellowish. The phialides shape was laginiform along long main branches. The conidial size was 2.91- 3.10 x 1.86-2.59 µm with oval conidial shape.

Variation in morphological characterization was reported by Sekhar *et al* (2017) while studying 10 Trichoderma isolates based on colony colour, morphology , phialides characters, shape of conidia etc. Among the ten isolates of Trichoderma spp. considerable variations were observed regarding total growth and growth rate. The maximum radial growth was recorded in isolates GRT-3, GRT-4 and GRT-9 at 5th day after inoculation (90.00mm) at rate of 30.00mm/day.Among all the isolates the least total growth rate was observed in case of GRT-7 (73.00) with growth rate of 24.33mm/day.

Shahid *et al*. (2013) studied seven isolates of *Trichoderma* species isolated from the soil of pulse fields of various districts of Uttar Pradesh, India. These include *Trichoderma viride, T. harzianum, T. asperellum, T. koningii, T. atroviride, T. longibrachiatum and T. virens. Trichoderma longibrachiatum* showed Colony Growth rate (cm/day) 8-9cm in 4 days. The colony colour and reverse pigmentation was white to green

and colourless. Colony edges were effuse. Mycelial form and mycelial colour was Floccose to Arachnoid and watery white respectively. Conidia recorded by Shahid *et al.* was smooth Green.

According to Rifai (1969) *Trichoderma longibrachiatum* is with the conidiophores complicated and progressively longer, often paired, secondary branches and phialides arising directly from secondary branches, typically not in whorls.

Table 14. Antagonism of *Trichoderma* isolates against soil borne fungal pathogen (percent growth inhibition) at 7 DAI

Sr. No.	Isolates	Mean Radial Growth (mm) of *Sclerotium Rolfsii*	PGI (%)	Mean Radial Growth (mm) of *Rhizoctonia bataticola*	PGI (%)	Mean Radial Growth (mm) of *Fusarium udam*	PGI (%)
1.	**TL-1 (Akola)**	35.18	60.91	35.28	60.80	32.02	62.42
2.	**TL-2 (Amravati)**	**12.40**	**86.22**	31.66	64.82	**39.62**	87.45
3.	**TL-3 (Bhanadara)**	37.18	58.68	29.13	67.63	11.41	87.32
4.	**TL-4 (Buldhana)**	21.67	75.92	42.61	**51.54**	25.89	71.23
5.	**TL-5 (Chandrapur)**	33.00	63.33	33.00	63.33	23.23	74.18
6.	**TL-6 (Gadchiroli)**	22.78	74.68	**43.27**	51.92	**11.00**	**87.77**
7.	**TL-7 (Gondia)**	**38.90**	**56.77**	37.17	58.70	12.45	86.66
8.	**TL-8 (Washim)**	34.65	61.50	**28.23**	**68.63**	34.32	87.72
9.	**TL-9 (Yeotmal)**	29.99	66.67	32.53	63.85	30.67	65.92
	Control	90	-	90	-	90.00	-
	F test	Sig	-	Sig	-	Sig	-
	S.E (M)±	0.49	-	0.36	-	0.19	-
	C.D. at (p= 0.01)	1.45	-	1.07	-	0.56	-

PGI- Percent growth inhibition.

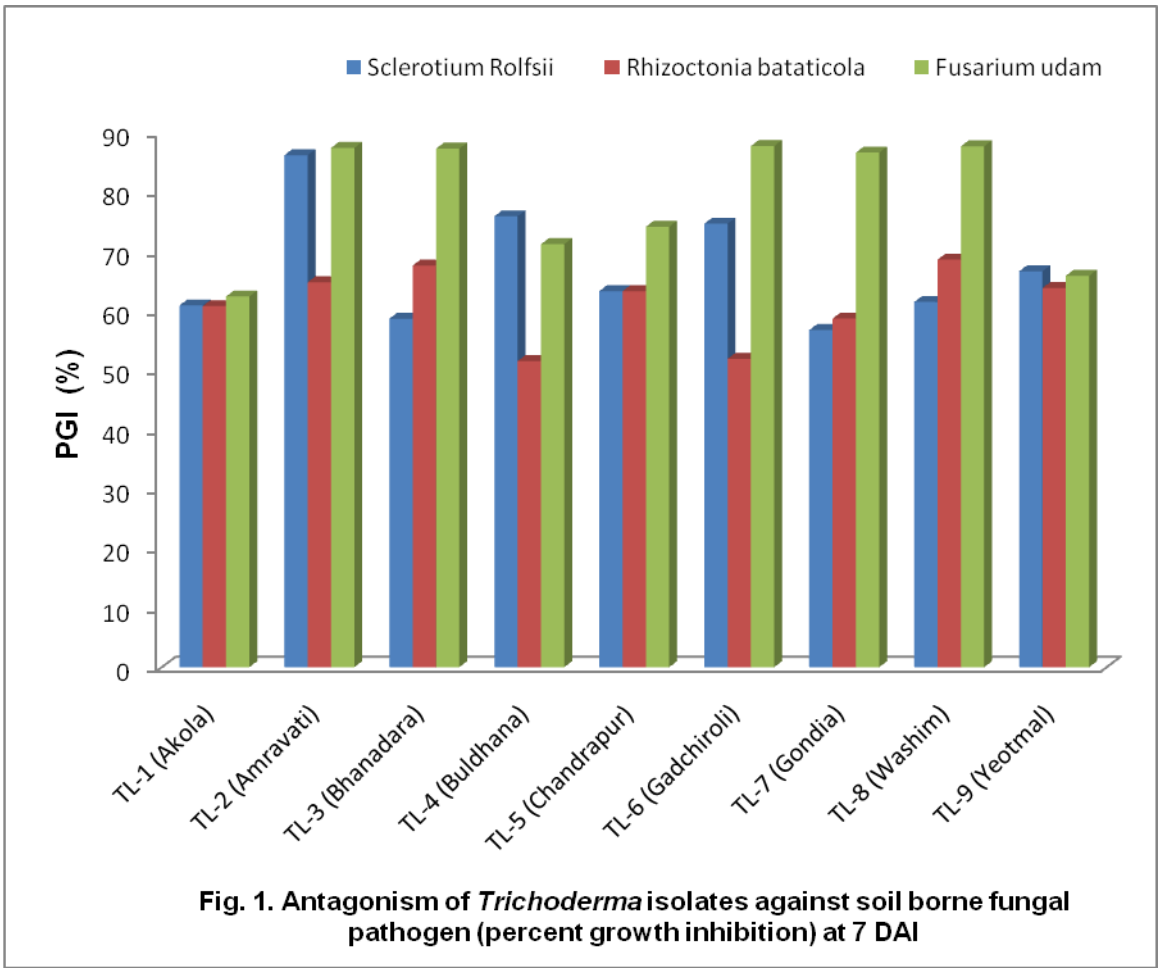


Fig. 1. Antagonism of *Trichoderma* isolates against soil borne fungal pathogen (percent growth inhibition) at 7 DAI

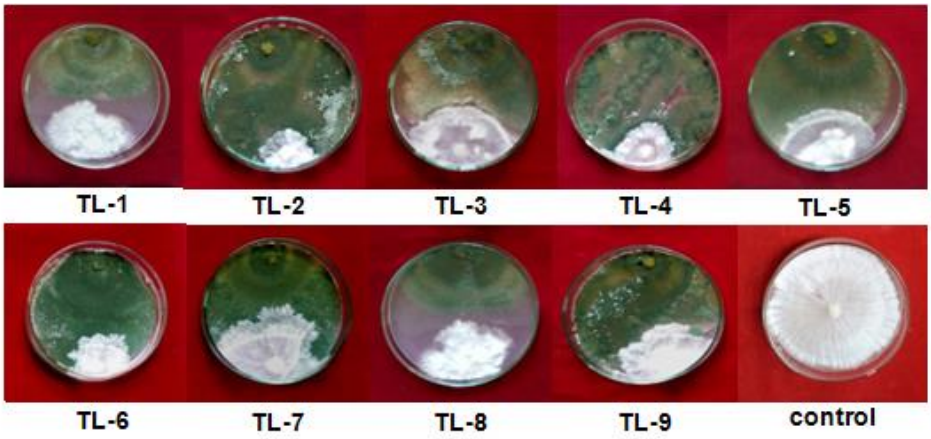


Plate 3. Antagonism of *Trichoderma longibrachiatum* against *Scelerotium rolfsii*

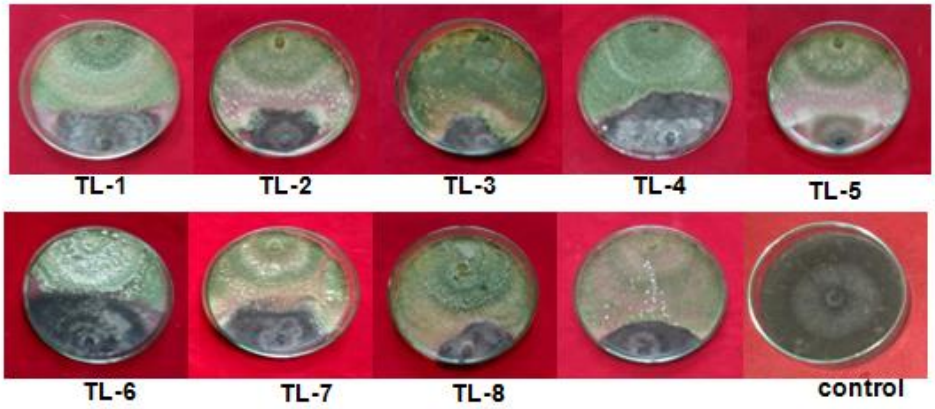


Plate 4. Antagonism of *Trichoderma longibrachiatum* against *Rhizoctonia bataticola*

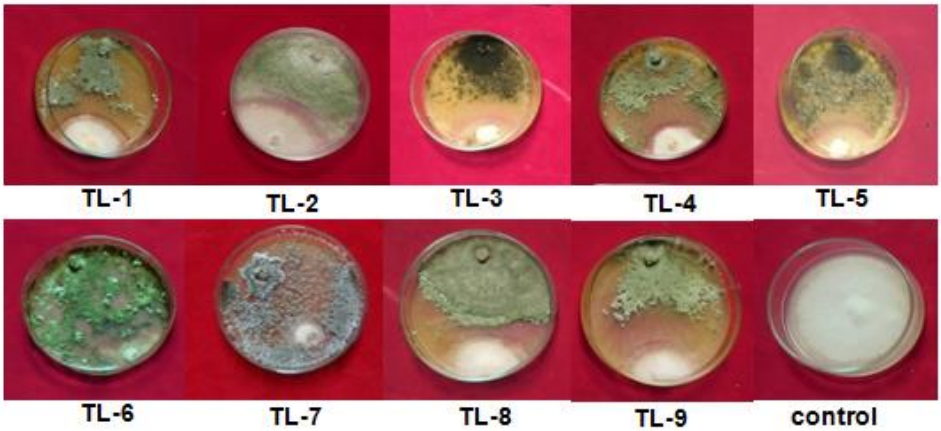


Plate 5. Antagonism of *Trichoderma longibrachiatum* against *Fusarium udam*

4.4 Efficacy of *T. longibrachiatum* against *Sclerotium rolfsii, Rhizoctonia bataticola and Fusarium udam* (per cent growth inhibition) at 7 DAI

The data presented in table 14 revealed that the maximum per cent growth inhibition (86.22%) of *Sclerotium rolfsii* was observed by the isolate TL-2(Amravati) The next best isolate was TL-4(Buldhana) which showed 75.92 % inhibition. The least per cent inhibition was observed in TL-7(Gondia) i.e. 56.77. The per cent growth inhibition of *Rhizoctonia bataticola* was maximum i.e. 68.63% by TL-8 (washim) isolate and least inhibition 51.54 % was recorded in TL-4(Buldhana) isolate. In case of F*usarium udam* the maximum inhibition was recorded by TL-6 (Gadchiroli) i.e 87.77 % where as 62.42 % growth inhibition was observed by Tl-1(Akola) isolate. (Plate no. 3,4,and 5)

Prameela Devi *et al* (2012) assessed *T. virens* (Vn) and *T. harzianum* (Th) for their mycoparasitic effect on soil borne plant pathogens, *Rhizoctonia solani, Fusarium udum, F. solani, Sclerotium rolfsii and Macrophomina phaseolina*. The present results obtained are in the conformity with Prameela Devi (2012). Shimla isolate of T. harzianum (Th12) inhibited maximum (91%) mycelial growth of *F. solani* and Hyderabad isolate of *T. virens* (Vn09) inhibited 88% of the mycelial growth of *S. rolfsii*. Also the percent inhibition in *Macrophomina phaseolina* was in the range of 51 to 83 %.

Javaid *et al.* (2014) carried out in vitro bioassays to evaluate antagonistic behavior of seven species of Trichoderma against two highly problematic soil-borne plant pathogenic fungi viz*. Fusarium oxysporum f. sp. lycopersici* and *Macrophomina phaseolina*. T. harzianum showed the highest inhibition (65%) in growth of M. phaseolina followed by T. aureoviridi (60%).

These findings showed similarity with the observations made by kumar and Sharma (2016) evaluation of *Trichderma spp*. against *S.sclerotiorum* where Th3 exhibited 90% inhibition followed by Th10 and Th30. The isolate TvNir had maximum impact of 85% against the target fungi, followed by Tv2, TvChen and Tv4.

Jana and Mandal (2017) tested eleven isolates of *Trichoderma spp.* to find out their antagonistic potential and nature of colony interaction against test pathogen, *S. rolfsii. T. harzianum* isolate T3 produced highest inhibition of 71.67%, while *T. viride* isolate T10 showed an inhibition of 67.23

Table 15. Efficacy of volatile compounds produced by *Trichoderma longibrachiatum* against soilborne fungal pathogen (percent growth inhibition) 12 DAI

Sr. No.	Isolates	Mean Radial Growth (mm) of *sclerotium rolfsii*	PGI (%)	Mean Radial Growth (mm) of *Rhizoctonia bataticola*	PGI (%)	Mean Radial Growth (mm) of *Fusarium udam*	PGI (%)
1.	**TL-1 (Akola)**	87.19	3.12	84.19	6.45	80.13	10.96
2.	**TL-2 (Amravati)**	90.00	0.00	55.67	38.14	70.85	21.27
3.	**TL-3 (Bhandara)**	83.13	7.63	89.85	0.02	88.83	1.30
4.	**TL-4 (Buldhana)**	59.97	**33.36**	89.98	0.02	48.79	**45.78**
5.	**TL-5 (Chandrapur)**	90.00	0.00	90.00	0.00	89.00	1.11
6.	**TL-6 (Gadchiroli)**	90.00	0.00	89.67	0.36	74.16	17.6
7.	**TL-7 (Gondia)**	90.00	0.00	71.09	21.01	88.59	1.56
8.	**TL-8 (Washim)**	90.00	0.00	89.99	0.01	78.44	12.84
9.	**TL-9 (Yeotmal)**	90.00	0.00	36.10	**59.88**	90.00	0.00
	control	90.00	_	90.00	_	90.00	_
	F test	Sig	-	Sig	-	Sig	-
	S.E (M)±	0.074	-	0.12	-	0.20	-
	C.D. at (p= 0.01)	0.21	-	0.37	-	0.60	-

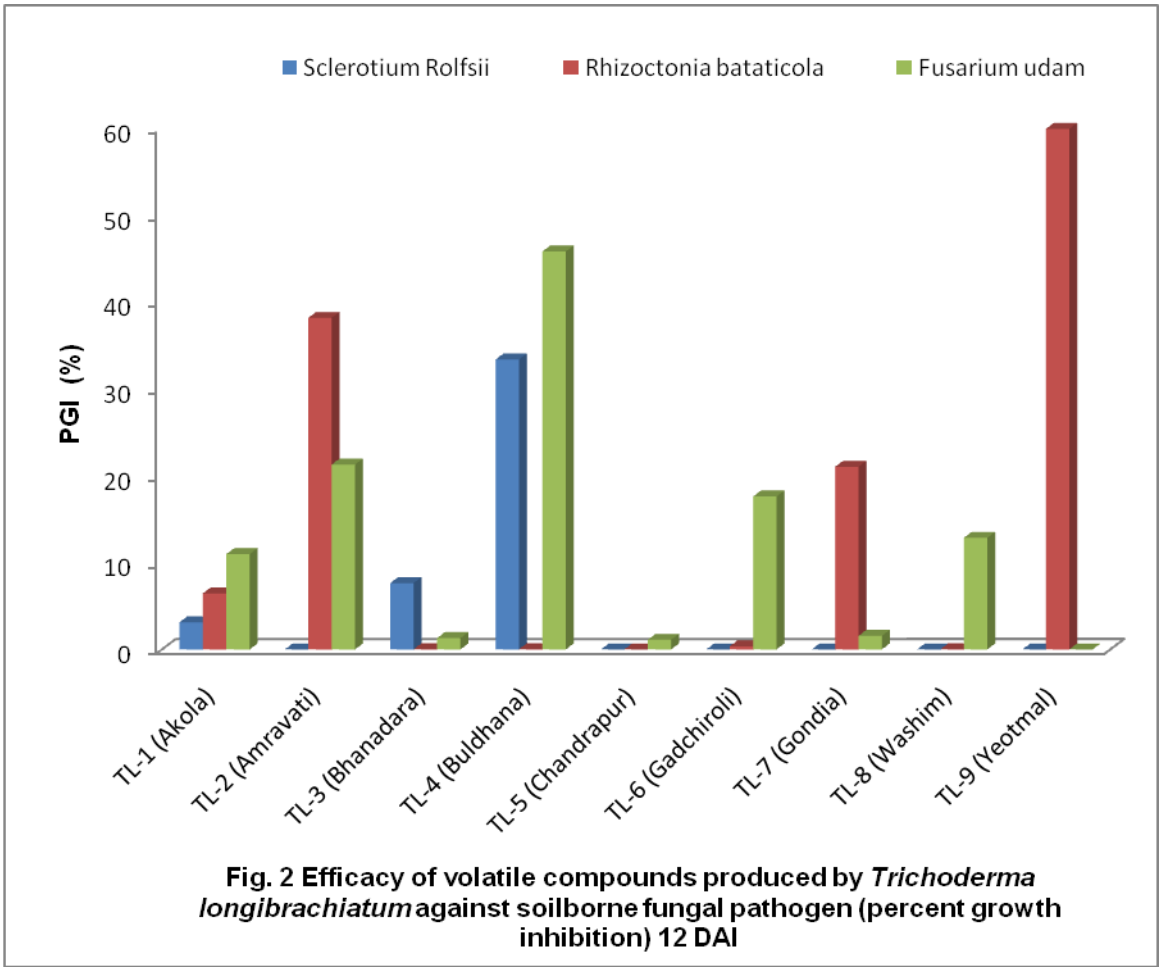


Fig. 2 Efficacy of volatile compounds produced by *Trichoderma longibrachiatum* against soilborne fungal pathogen (percent growth inhibition) 12 DAI

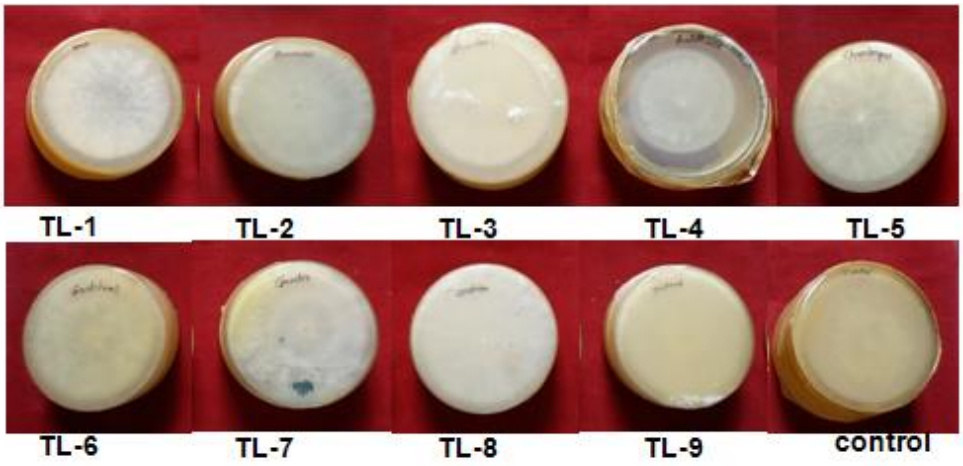


Plate 6. Efficacy volatile substance of *Trichoderma longibrachiatum* against *Scelerotium rolfsii*

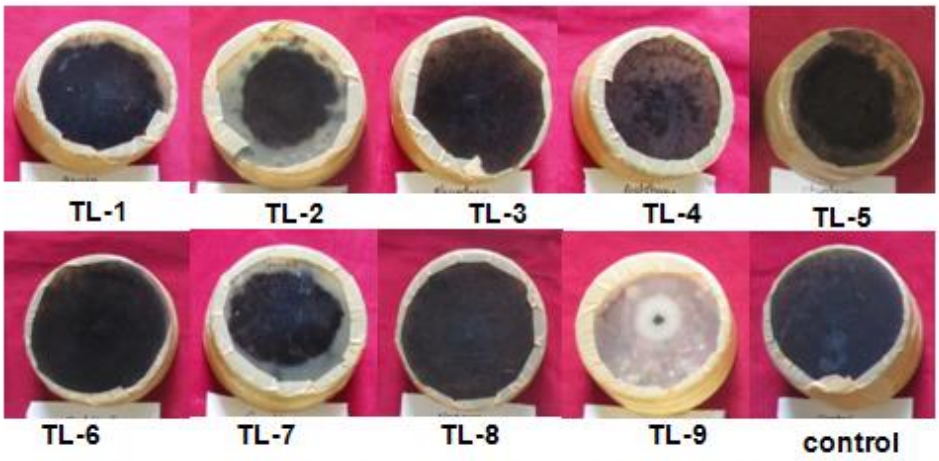


Plate 7. Efficacy volatile substance of *Trichoderma longibrachiatum* against *Rhizoctonia bataticola*

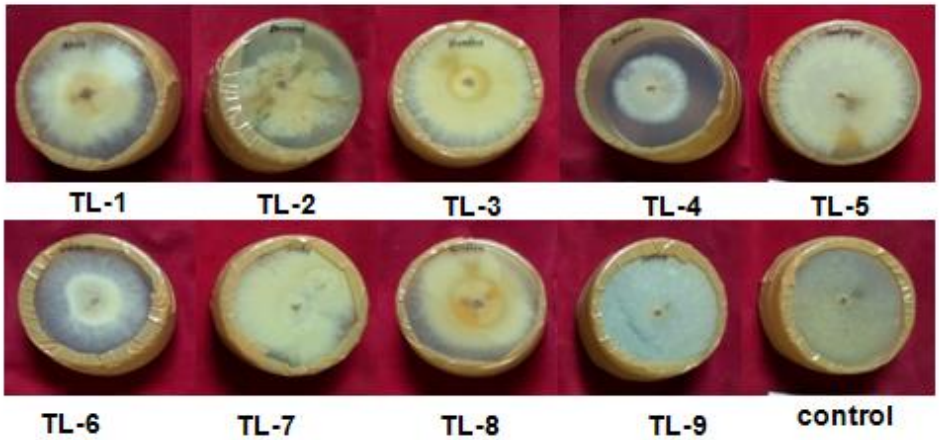


Plate 8. Efficacy volatile substance of *Trichoderma longibrachiatum* against *Rhizoctonia bataticola*

4.5 Efficacy of volatile compounds produced by *T.longibrachiatum* against *Sclerotium rolfsii, Rhizoctonia bataticola and Fusarium udam*

An experiment was conducted to evaluate the efficacy of volatile compound produced by *T. longibrachiatum* against the soil borne pathogens i.e. *Sclerotium rolfsii, Rhizoctonia bataticola, Fusarium udam* by Inverted plate technique as suggested by Dennis and Webster (1971) .

The data are presented in table 15, per cent growth inhibition of tested pathogens was recorded at 12 DAI due to volatile substance produced by *Trichoderma longibrachiatum*. TL-4 (Buldhana), isolate recorded maximum 33.36% and 45.78% growth inhibition of *Sclerotium rolfsii* and F.udum respectively where as TL-9(Yeotmal) inhibited *Rhizoctonia bataticola up to* 59.88%. (Plate no. 6, 7and 8) Amin *et al*. (2010) tested six isolates of *Trichoderma spp*. for their ability to produce volatile metabolites against several fungal plant pathogens viz., *Fusarium oxysporum* (causing chilli wilt), *Rhizoctonia solani* (causing sheath blight of rice), *Sclerotium rolfsii* (causing collar rot of tomato). Studies indicated that *T. viride* (Tv-1) was most effective in reducing the mycelial growth of *F*. *oxysporum* (41.88%), whereas, in case of *R*. *solani T. viride* (Tv-2) accounted for maximum reduction in mycelial growth (30.58%). Against *Sclerotium rolfsii*, *T. viride* (Tv-1) was most effective antagonist producing volatile metabolites, thereby inhibiting the mycelial growth by 40.68. The present results are in conformity with the findings of Amin *et al*.

Denis and Webster (1971a) suggested the possibility of production of chloroform soluble non volatile antibiotics like trichodermin and Vey *et al*., (2001) reported that there are large varieties of volatile secondary metabolites produced by Trichoderma such as ethylene, hydrogen cyanide, aldehydes and ketones which play an important role in controlling the plant pathogens peptide antibiotics.

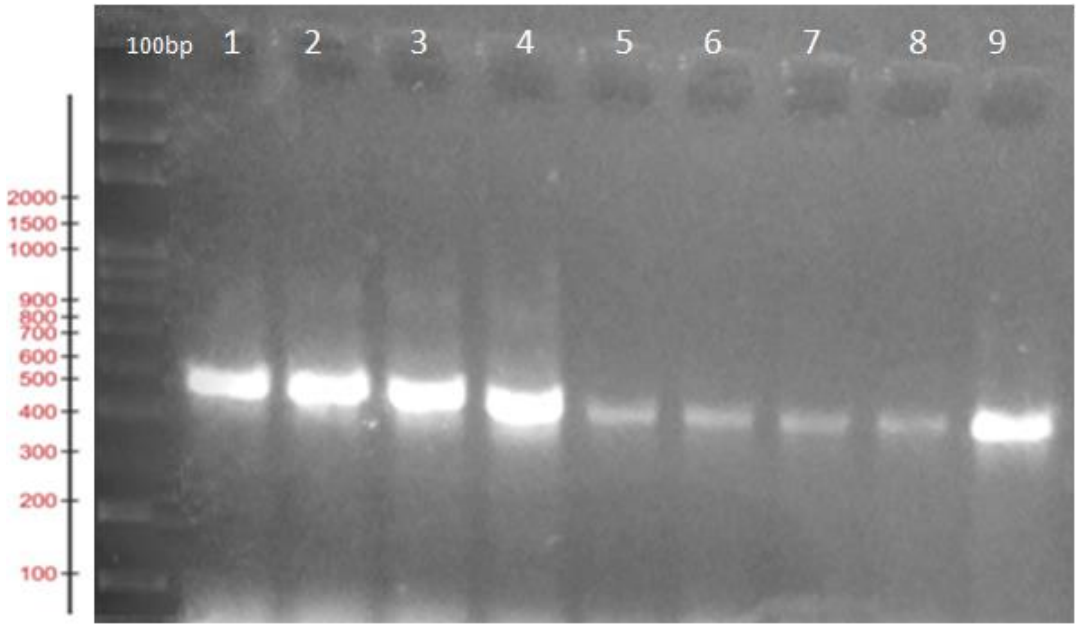


Plate 9. Identification and confirmation of *Trichoderma longibrachiatum* by ITS primer

4.5 Internal Transcript Spacer(ITS) analysis

Nine Trichoderma isolates with good antifungal activity which belonged to *Trichoderma longibrachiatum* (morphologically identified upto species level at Indian Type Culture Collection, IARI New Delhi.) was used for confirmation by sequencing of ITS region. ITS region were amplified in different isolates and gave amplicons size 500bp to 600bp. The *Trichoderma* specific ITS primers pair ITS-1 (TCCGTAGGTGAACCTGCGG) and ITS-4 (TCCTCCGCTTATTGATATGC) were used for molecular confirmation of the isolates.

The results are in accordance with Chakraborty (2010) who studied the identification and genetic variability of Trichoderma isolates which observed amplified DNA fragment approximately 600bp. (Plate no. 9)

4.6 Primer selected for RAPD Marker Study

During present study the total 20 RAPD primers were randomly selected and screened of OPA and OPB series to evaluate genetic variability of *Trichoderma longibrachiatum*. PCR was programmed with an initial denaturation at 94°C for 5 min. followed by 35cycles of denaturation at 94°C for 1 min, annealing at 36.5°C for 1 min and extension at 70°C for 1min and the final extension at 72°C for 7 min. The PCR (Polymerase Chain Reaction) amplified product of each primer were resolved on 1.2 % agarose gel electrophoresis and the size of the amplified product was compared with 1kb DNA ladder

Among 20 primers screened 15 primers produced reproducible and scorable bands with high degree of polymorphism. Out of these 15 primers, 12 primers showed all polymorphic bands, whereas remaining 3 primers namely OPA-8, OPA-9 and OPA-19 each showed one monomorphic band. A total 113 amplicons were obtained with the 15 primers. Out of 113 bands, 110 were found to be polymorphic and the level of polymorphism was 97.34 per cent (Table 16).

Table 16. Per cent polymorphism shown by RAPD primers

Sr. No.	Primers	Total amplicons	Polymorphic amplicons	Monomorphic amplicons	% Polymorphism
1	OPA-1	05	05	0	100
2	OPA-2	19	09	0	100
3	OPA-3	10	10	0	100
4	OPA-4	12	12	0	100
5	OPA-5	06	06	0	100
6	OPA-8	08	07	1	87.5
7	OPA-9	07	06	1	85.71
8	OPA-10	03	03	0	100
9	OPA-12	07	07	0	100
10	OPA-15	08	08	0	100
11	OPA-18	10	10	0	100
12	OPB-19	03	02	1	66.66
13	OPB-5	15	15	0	100
14	OPB-17	04	04	0	100
15	OPB-20	06	06	0	100
Total		**113**	**110**	**3**	**97.34**

4.7 Banding pattern of RAPD primers

Total 20 RAPD primers were screened, while the banding pattern was observed in 15 primers as given below.

The banding pattern observed in primer OPA-1 was in plate no. 10 The primer amplified five amplicons of *Trichoderma longibarchiatum* The size of amplicons amplified with primer OPA-1 ranged from 500bp to 4000bp. The polymorphism observed in this primer was 100 %. The details of the 5 RAPD band types are explained below:

Band type 1 (4000bp) : This type of band was observed in TL-9

Band type 2 (2500bp) : This type of band was observed in TL-9

Band type 3 (1000bp) : This type of band was observed in TL-2,TL-3, TL-4, TL-5, TL-6, TL-7.

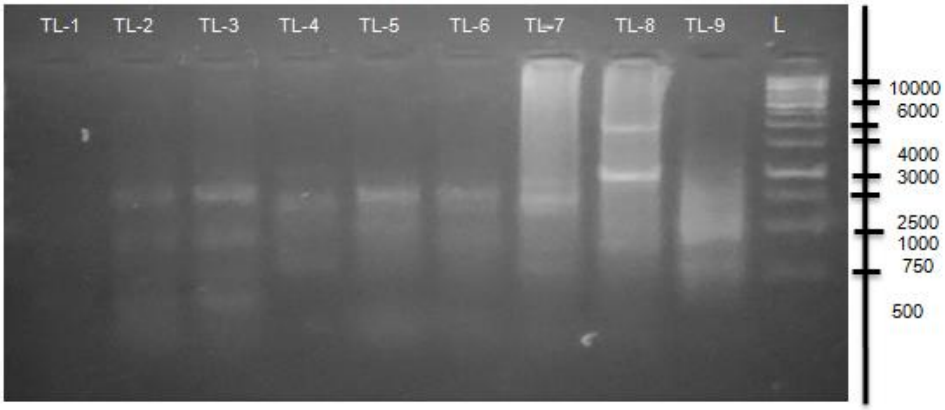
Band type 4 (700bp) : This type of band was observed in TL-2,TL-3,

Band type 5 (510bp) : This type of band was observed in TL-4, TL-7 and TL-9.

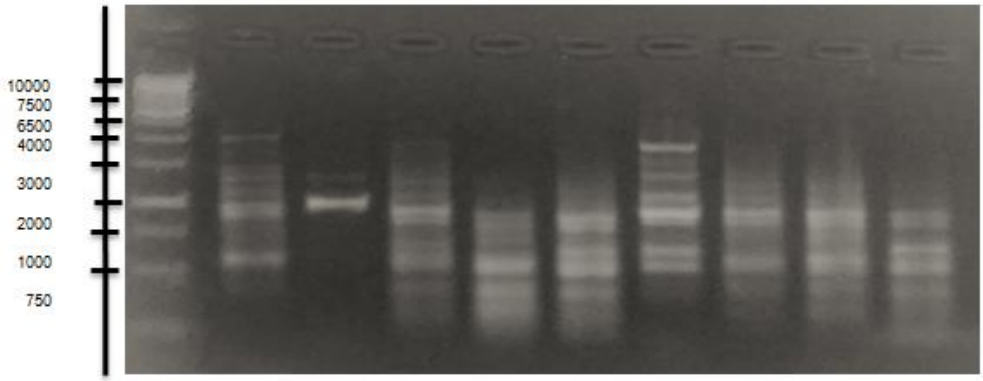
The banding pattern observed in primer OPA-2 in plate no.10. The primer amplified nine amplicons. The size of amplicons amplified ranged from 450 bp to 4000bp. The polymorphism observed in this primer was 100 %. The details of the 9 RAPD band types are as follows.

Band type 1 (4000bp)	:	This type of band was observed in isolate TL-1
Band type 2 (3400bp)	:	This type of band was observed in isolate TL-6
Band type 3 (2500bp)	:	This type of band was observed in isolates TL-1,TL-2, TL-3, TL-6.
Band type 4 (2200bp)	:	This type of band was observed in isolate TL-3
Band type 5 (2000bp)	:	This type of band was observed in isolates TL-2, TL-6
Band type 6 (1500bp)	:	This type of band was observed in isolates TL-1,TL-3,TL-4,TL-5,TL-6,TL-7, TL-8 and TL-9.
Band type 7 (850bp)	:	This type of band was observed in isolatesTL-5, TL-6,TL-9
Band type 8 (750bp)	:	This type of band was observed in isolates TL-1,TL-3,TL-4,TL-5,TL-6,TL-7,TL-8.
Band type 9 (450bp)	:	This type of band was observed in isolates TL-3,TL-4, TL-5, TL-7,TL-8,TL-9.

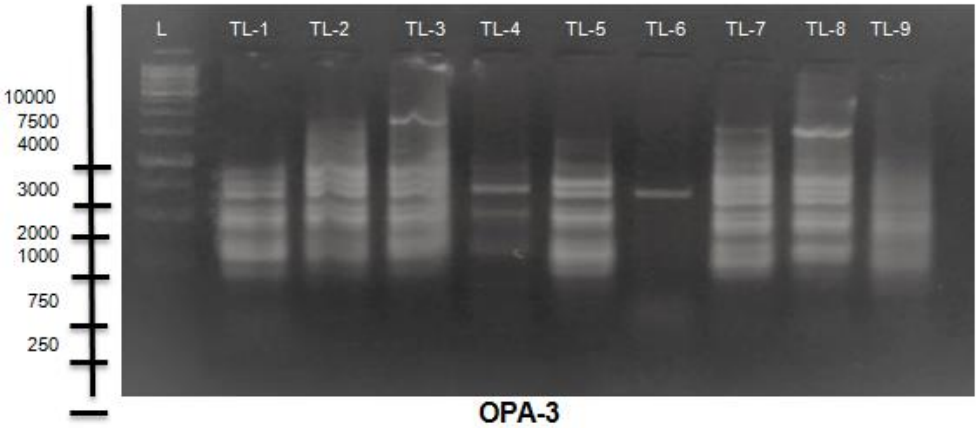
The banding pattern observed in primer OPA-3 was presented in plate no.10 . The primer amplified ten amplicons. The size of amplicons amplified ranged from 3500bp to 250bp. The polymorphism observed in this primer was 100 %. The details of the 10 RAPD band types are as follows.



OPA-1



OPA-2



OPA-3

Plate 10 . RAPD banding pattern of primer OPA-1, OPA-2, OPA-3

Band type 1 (3500bp)	:	This type of band was observed in isolates TL-2 and TL-3.
Band type 2 (3000bp)	:	This type of band was observed in isolate TL-7 and TL-8
Band type 3 (2800bp)	:	This type of band was observed in isolate TL-5
Band type 4 (2000bp)	:	This type of band was observed in isolate TL-5, TL-7 and TL-8
Band type 5 (1500bp)	:	This type of band was observed in isolates TL-1,TL-2,TL-3,TL-5 and TL-7.
Band type 6 (1000bp)	:	This type of band was observed in isolates TL-1,TL-2,TL-3,TL-4, TL-5, TL-7 and TL-8
Band type 7 (850bp)	:	This type of band was observed in isolates TL-1,TL-2,TL-3,TL-5, TL-7 and TL-8.
Band type 8 (750bp)	:	This type of band was observed in isolates TL-1,TL-2,TL-3,TL-4,TL-5, TL-7, TL-8 and TL-9.
Band type 9 (450bp)	:	This type of band was observed in isolate TL-5, TL-7 and TL-8.
Band type10 (250bp)	:	This type of band was observed in isolate TL-5 and TL-7.

The banding pattern observed in primer OPA-4 in plate no.11. The primer amplified twelve amplicons. The size of amplicons amplified ranged from 220bp to 4000bp. The polymorphism observed in this primer was 100 %. The details of the twelve RAPD band types are as follows.

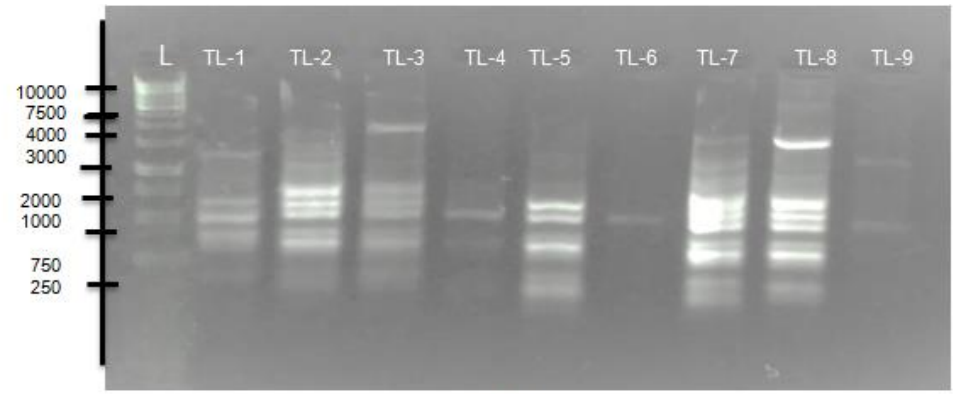
Band type 1 (4000bp)	:	This type of band was observed in isolate TL-3
Band type 2 (3000bp)	:	This type of band was observed in isolates TL-7 and TL-8
Band type 3 (2400bp)	:	This type of band was observed in isolate TL-1
Band type 4 (2000bp)	:	This type of band was observed in isolates TL-2, TL-7 and TL-9
Band type 5 (1500bp)	:	This type of band was observed in isolate TL-8
Band type 6 (1000bp)	:	This type of band was observed in isolates TL-2 and TL-3
Band type 7 (870bp)	:	This type of band was observed in isolates TL-1,TL-2,TL-3, TL-5,TL-7 and TL-8
Band type 8 (750bp)	:	This type of band was observed in isolates TL-1,TL-2,TL-3, TL-4, TL-5,TL-6, TL-7 and TL-8
Band type 9 (650bp)	:	This type of band was observed in isolates TL-7,TL-8 and TL-9
Band type10 (450bp)	:	This type of band was observed in isolates TL-1,TL-2,TL-3, TL-5,TL-7 and TL-8
Band type11(260bp)	:	This type of band was observed in isolates TL-7 and TL-8
Band type12(220bp)	:	This type of band was observed in isolates TL-7 and TL-8

The banding pattern observed in primer OPA-5 in plate no.11 . The primer amplified six amplicons. The size of amplicons amplified ranged from 820bp to 4000bp. The polymorphism observed in this primer was 100 %. The details of the 6 RAPD band types are as follows.

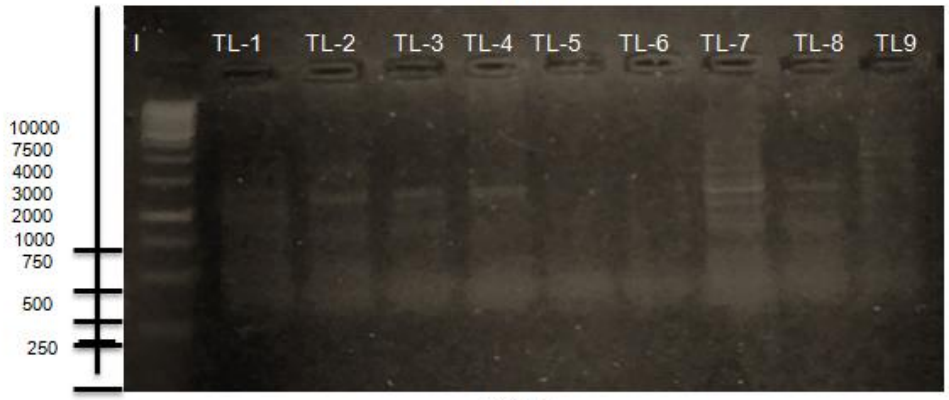
Band type 1 (4000bp)	:	This type of band was observed in isolate TL-9
Band type 2 (3000bp)	:	This type of band was observed in isolate TL-9
Band type 3 (2300bp)	:	This type of band was observed in isolates TL-7 and TL-9
Band type 4 (1300bp)	:	This type of band was observed in isolates TL-1,TL-2,TL-3,TL-4,TL-7 and TL-8
Band type 5 (1000bp)	:	This type of band was observed in isolate TL-7
Band type 6 (820bp)	:	This type of band was observed in TL-7 and TL-8

The banding pattern observed in primer OPA-8 in plate no.11. The primer amplified seven amplicons. The size of amplicons amplified ranged from 550bp to 5800bp. The polymorphism observed in this primer was 87.5 %. The details of the 7 RAPD band types are as follows.

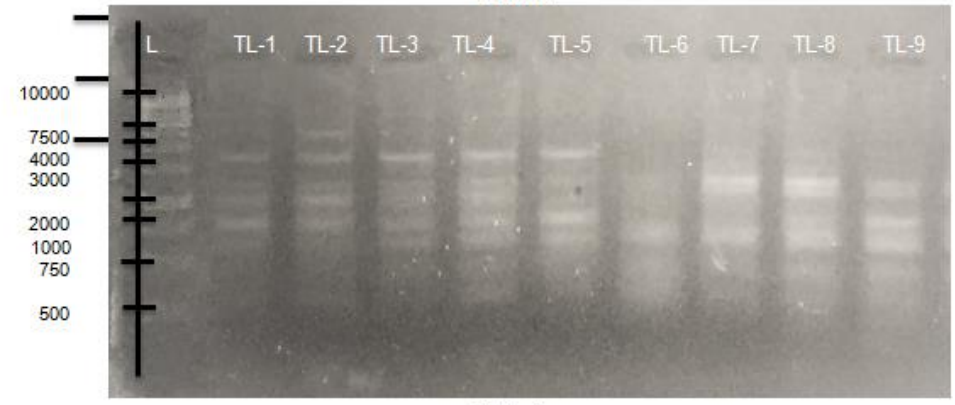
Band type 1 (5800bp)	:	This type of band was observed in isolate TL-2
Band type 2 (3500bp)	:	This type of band was observed in isolates TL-1, TL-3, TL-3,TL-4 and TL-5
Band type 3 (2700bp)	:	This type of band was observed in isolates TL-1, TL-2, TL-3,TL-4, TL-5, TL-6,TL-7, TL-8 and TL-9
Band type 4 (2000bp)	:	This type of band was observed in isolates TL-1, TL-2and TL-4
Band type 5 (1000bp)	:	This type of band was observed in isolates TL-1, TL-2, TL-3,TL-4, TL-5, TL-8 and TL-9
Band type 6 (800bp)	:	This type of band was observed in isolates TL-3,TL-4, TL-5,TL-6, TL-7, TL-8 and TL-9
Band type 7 (550bp)	:	This type of band was observed in isolate TL-4



OPA-4



OPA-5



OPA-8

Plate 11. RAPD banding pattern of primer OPA- 4, OPA-5, OPA-8

The banding pattern observed in primer OPA-9 in plate no. 12 The primer amplified seven amplicons. The size of amplicons amplified ranged from 250bp to 4000bp. The polymorphism observed in this primer was 85.71%. The details of the 7 RAPD band types are as follows.

Band type 1 (4000bp)	:	This type of band was observed in isolate TL-2
Band type 2 (3200bp)	:	This type of band was observed in isolates TL-2,TL-6 and TL-8
Band type 3 (2500bp)	:	This type of band was observed in isolates TL-1, TL-2, TL-3,TL-4, TL-5, TL-6,TL-7, TL-8 and TL-9
Band type 4 (1200bp)	:	This type of band was observed in isolates TL-1, TL-3, TL-5, TL-6,TL-7 and TL-9
Band type 5 (1000bp)	:	This type of band was observed in isolates TL-3, TL-8, TL-9
Band type 6 (650bp)	:	This type of band was observed in isolates TL-3, TL-8 and TL-9
Band type 7 (2500bp)	:	This type of band was observed in isolate TL-2, TL-6 and TL-8

The banding pattern observed in primer OPA-10 in plate no.12. The primer amplified 3 amplicons. The size of amplicons amplified ranged from 740bp to 2000bp. The polymorphism observed in this primer was 100%. The details of the 3 RAPD band types are as follows.

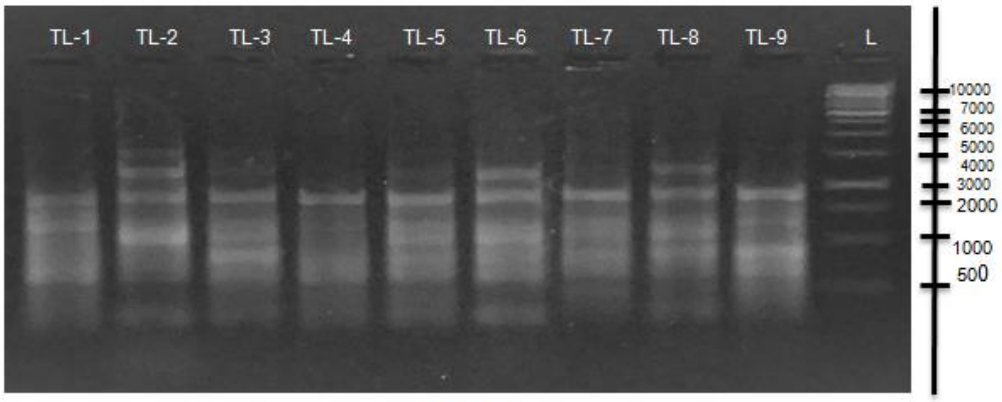
Band type 1 (2000bp)	:	This type of band was observed in isolate TL-9
Band type 2 (800bp)	:	This type of band was observed in isolates TL-1,TL-2,TL-5,TL-7, TL-8 and TL-9
Band type 3 (740bp)	:	This type of band was observed in isolates TL-1,TL-2,TL-4,TL-5,TL-7, TL-8 and TL-9

The banding pattern observed in primer OPA-12 in plate no. 12. The primer amplified 7 amplicons. The size of amplicons amplified ranged from 700bp to 5000bp. The polymorphism observed in this primer was 100%. The details of the 7 RAPD band types are as follows

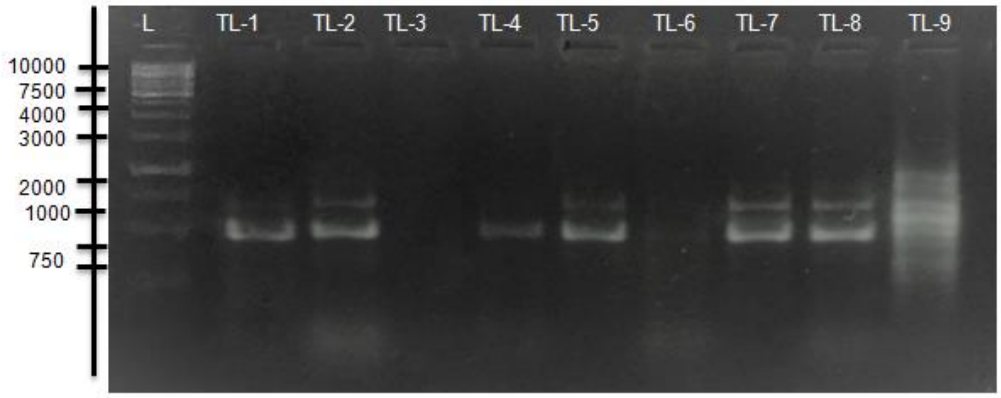
Band type 1 (5000bp)	:	This type of band was observed in isolate TL-2
Band type 2 (3300bp)	:	This type of band was observed in isolates TL-4, TL-5, TL-7 and TL-8
Band type 3 (3000bp)	:	This type of band was observed in isolate TL-5
Band type 4 (2500bp)	:	This type of band was observed in isolates TL-1 and TL-5
Band type 5 (2000bp)	:	This type of band was observed in isolates TL-1, TL-5, TL-7 and TL-8
Band type 6 (900bp)	:	This type of band was observed in isolates TL-1, TL-4 and TL-5
Band type 7 (700bp)	:	This type of band was observed in isolate TL-1, TL-2, TL-4, TL-5, TL-6 and TL-7

The banding pattern observed in primer OPA-15 was in plate no.13. The primer amplified 8 amplicons. The size of amplicons amplified ranged from 300bp to 4000bp. The polymorphism observed in this primer was 100%. The details of the 8 RAPD band types are as follows

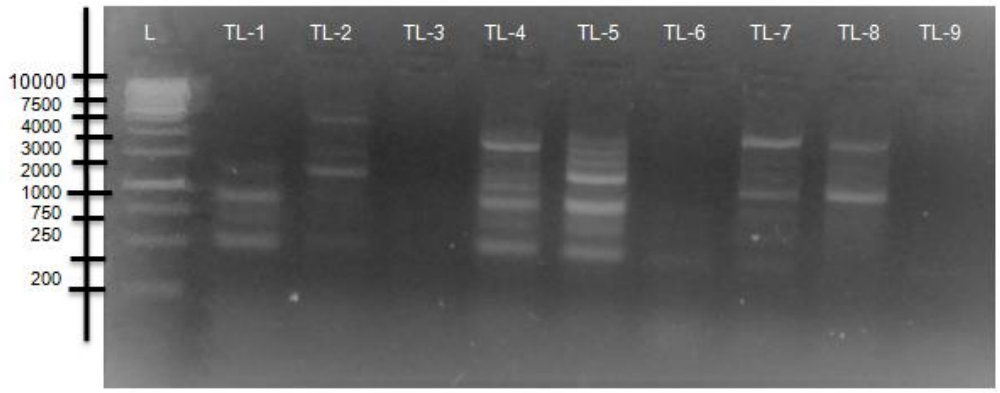
Band type 1 (4000bp)	:	This type of band was observed in isolateTL-1
Band type 2 (2000bp)	:	This type of band was observed in isolate TL-7
Band type 3 (1200bp)	:	This type of band was observed in isolates TL-2,TL-4,TL-7 and TL-8
Band type 4 (800bp)	:	This type of band was observed in isolates TL-2,TL-3,TL-5,TL-7,TL-8 and TL-9



OPA-9



OPA-10



OPA-12

Plate 12 . RAPD banding pattern of primer OPA-9, OPA-10, OPA-12

Band type 5 (750bp)	:	This type of band was observed in isolates TL-7 and TL-8
Band type 6 (700bp)	:	This type of band was observed in isolates TL-1, TL-2, TL-7 and TL-9
Band type 7 (520bp)	:	This type of band was observed in isolates TL-1, TL-2, TL-5, TL-7 and TL-8
Band type 8 (300bp)	:	This type of band was observed in isolate TL-5

The banding pattern observed in primer OPA-18 in plate no. 13 The primer amplified 10 amplicons. The size of amplicons amplified ranged from 500bp to 4000bp. The polymorphism observed in this primer was 100%. The details of the 10 RAPD band types are as follows

Band type 1 (4000bp)	:	This type of band was observed in isolates TL-2 and TL-7
Band type 2 (3200bp)	:	This type of band was observed in isolate TL-2
Band type 3 (3000bp)	:	This type of band was observed in isolates TL-2 and TL-8
Band type 4 (2300bp)	:	This type of band was observed in isolate TL-8
Band type 5 (2050bp)	:	This type of band was observed in isolates TL-1,TL-2,TL-3, TL-6 and TL-8
Band type 6 (1600bp)	:	This type of band was observed in isolates TL-2, TL-7 and TL-8
Band type 7 (1000bp)	:	This type of band was observed in isolates TL-1, TL-2 and TL-8
Band type 8 (750bp)	:	This type of band was observed in isolates TL-1 and TL-2
Band type 9 (620bp)	:	This type of band was observed in isolate TL-5
Band type10 (500bp)	:	This type of band was observed in isolate TL-8

The banding pattern observed in primer OPA-19 plate no. 13. The primer amplified 3 amplicons. The size of amplicons amplified ranged from 1000bp to 3800bp. The polymorphism observed in this primer was 66.66%. The details of the 8 RAPD band types are as follows

Band type 1 (3800bp)	:	This type of band was observed in isolates TL-7 and TL-9
Band type 2 (2500bp)	:	This type of band was observed in isolate TL-3
Band type 3 (1000bp)	:	This type of band was observed in isolatesTL-1,TL-2,TL-3, TL-4,TL-5,TL-6, TL-7, TL-8 and TL-9

The banding pattern observed in primer OPB-5 in plate no.14 The primer amplified 15 amplicons. The size of amplicons amplified ranged from 200bp to 6500bp The polymorphism observed in this primer was 100%. The details of the 15 RAPD band types are as follows

Band type 1 (6500bp)	:	This type of band was observed in isolates TL-2,TL-3 and TL-5
Band type 2 (5000bp)	:	This type of band was observed in isolate TL-7 and TL-8
Band type 3 (3500bp)	:	This type of band was observed in isolates TL-2,TL-3 and TL-5
Band type 4 (3000bp)	:	This type of band was observed in isolates TL-7 and TL-8
Band type 5 (2700bp)	:	This type of band was observed in isolates TL-2, TL-3, TL-5 and TL-6
Band type 6 (2300bp)	:	This type of band was observed in isolate TL-1, TL-2, TL-3, TL-5, TL-7, TL-8 and TL-9
Band type 7 (2000bp)	:	This type of band was observed in isolates TL-8 and TL-9
Band type 8 (1900bp)	:	This type of band was observed in isolates TL-5and TL-6

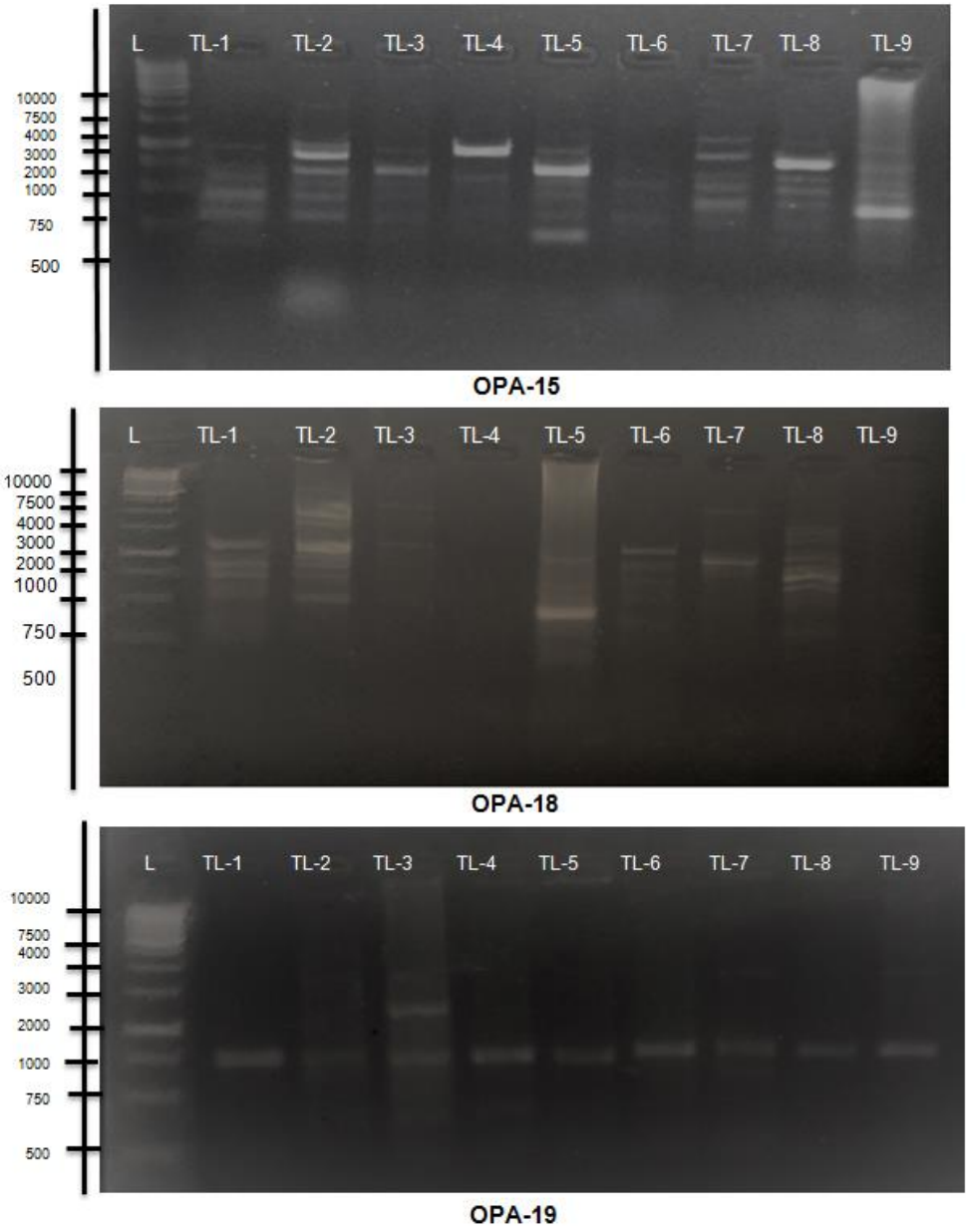


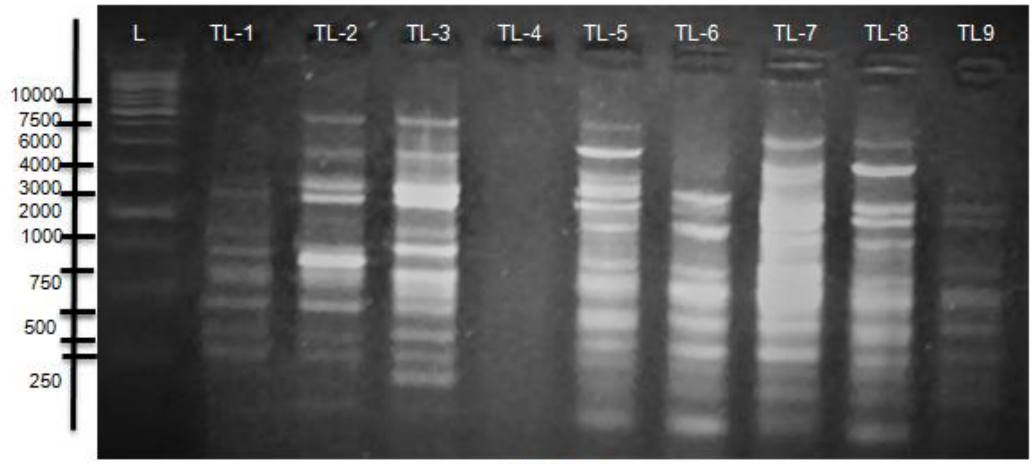
Plate 13. RAPD banding pattern of primer OPA-15, OPA-18 and OPA-19

Band type 9 (1200bp)	:	This type of band was observed in isolates TL-1, TL-3 and TL-8
4Band type10 (1000bp)	:	This type of band was observed in isolates TL-1, TL-2 and TL-3
Band type11(800bp)	:	This type of band was observed in isolates TL-1, TL-2, TL-3, TL-5, TL-7, TL-8 and TL-9
Band type12(700bp)	:	This type of band was observed in isolates TL-1, TL-2, TL-3, TL-5, TL-7, TL-8 and TL-9
Band type13(550bp)	:	This type of band was observed in isolates TL-1, TL-2, TL-3, TL-5, TL-7, TL-8 and TL-9
Band type14 (500bp)	:	This type of band was observed in isolates TL-1, TL-2, TL-3, TL-5, TL-7, TL-8 and TL-9 s
Band type15 (200bp)	:	This type of band was observed in isolates TL-5, TL-6, TL-7 and TL-8

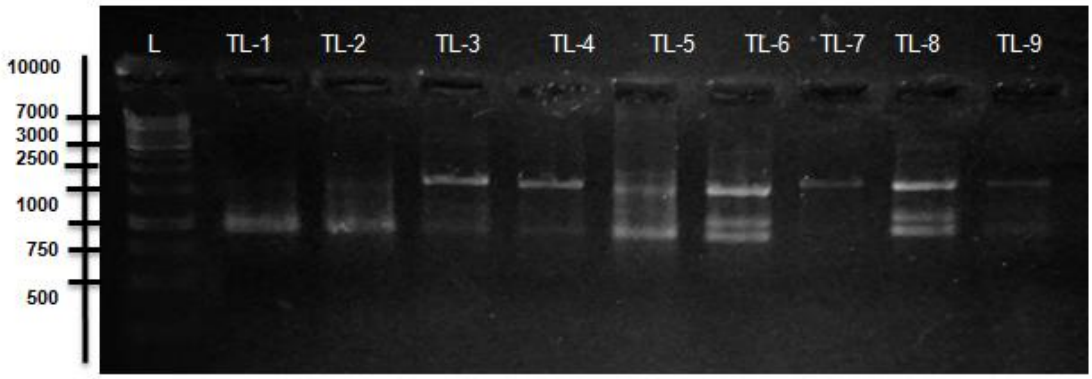
The banding pattern observed in primer OPB-17 in plate no. 14. The primer amplified 4 amplicons. The size of amplicons amplified ranged from 1000bp to 2700bp. The polymorphism observed in this primer was 100%. The details of the 4 RAPD band types are as follows

Band type 1 (2700bp)	:	This type of band was observed in isolates TL-3, TL-4, TL-7, TL-8 and TL-9
Band type 2 (2500bp)	:	This type of band was observed in isolates TL-5 and TL-6
Band type 3 (1100bp)	:	This type of band was observed in isolates TL-6 and TL-7
Band type 4 (1000bp)	:	This type of band was observed in isolates TL-1, TL-2, TL-3, TL-4, TL-5, TL-6, TL-8 and TL-9

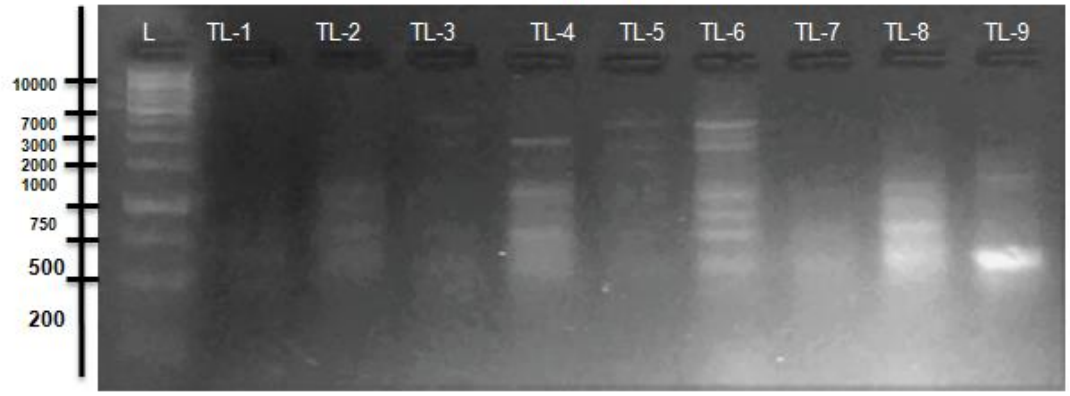
The banding pattern observed in primer OPB-20 in plate no.14. The primer amplified 6 amplicons. The size of amplicons amplified ranged from 600bp to 4000bp. The polymorphism observed in this primer was 100%. The details of the 6 RAPD band types are as follows



OPB-5



OPB-17



OPB-20

Plate 14 . RAPD banding pattern of primerOPB-5, OPB-17 andOPB-20

Band type 1 (4000bp)	:	This type of band was observed in isolates TL-3, TL-5 and TL-6
Band type 2 (3000bp)	:	This type of band was observed in isolates TL-4, TL-5 and TL-6
Band type 3 (1200bp)	:	This type of band was observed in isolates TL-4, TL-6, TL-8 and TL-9
Band type 4 (900bp)	:	This type of band was observed in isolate TL-8
Band type 5 (750bp)	:	This type of band was observed in isolates TL-6 and TL-8
Band type 6 (600bp)	:	This type of band was observed in isolates TL-6 and TL-9

4.8 Binary similarity matrix for RAPD analysis

A binary similarity matrix of combined data of 15 primers for the 9 isolates of *Trichoderma longibrachiatum* was prepared by scoring bands for presence or absence. DNA bands of same mobility (molecular weight) were assumed to be identical.

On the basis of calculated similarity matrix the similarity between isolates can be predicted. The isolates showing similarity index “1” are presumed to be 100% genetically similar and those showing similarity index “0” were considered as 100% genetically dissimilar. In the present study, the similarity coefficient value ranged from 0.21 to 0.55 across the 9 isolates. The size of the randomly amplified DNA fragments ranged from 150bp to 3100bp indicating high degree of polymorphism in respect to genetic similarity. Genetic similarity estimate (Jaccard’s coefficient) based on RAPD banding pattern was used for cluster analysis to present genetic relationship in form of dendrogram (fig.3). Jaccrd’s coefficient value of *Trichoderma longibrachiatum*, presented in Table.17

Table 17. Binary similarity matrix for RAPD analysis

	Akola	Amravati	Bhandara	Buldhana	Chandrapur	Gadchiroli	Gondia	Washim	Yeotmal
Akola	1								
Amravati	0.500	1							
Bhandara	0.464	0.492	1						
Buldhana	0.320	**0.219**	0.273	1					
Chandrapur	0.419	0.370	0.460	0.310	1				
Gadchiroli	0.293	0.279	0.317	0.235	0.393	1			
Gondia	0.368	0.346	0.347	0.306	0.451	0.250	1		
Washim	0.362	0.342	0.361	0.302	0.387	0.282	**0.557**	1	
Yeotmal	0.288	0.222	0.311	0.255	0.303	0.281	0.358	0.353	1

Results on differentiation of isolates revealed that, out of 20 primers used 15 primers showed good amplification. The highest genetic similarity to an extent of 0.55 was recorded between TL-7 (Gondia) and TL-8 (Washim) isolates followed by 0.50 similarity between TL-1(Akola) and TL-2 (Amravati) isolates and Least genetic similarity was observed in between TL-2 (Amravati) and TL-4 (Buldhana).

In parallel study Sagar *et a*l. (2011) studied *Trichoderma* isolates from different locations of Bangladesh for evaluating their bioefficiency by determining their genetic variations. genetic distance between 35 *Trichoderma* isolates were computed from combined data sets for the three primers ranging from 0.0034 to 1.4214. Comparatively higher genetic distance (1.4214) was found between T14 and T (2, 17). The lowest genetic distance (0.0034) was revealed between T24 and T27, also in T28 and T29.

4.9 Dendrogram and cluster analysis

Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) , indicated segregation of the 9 isolates of *Trichoderma longibrachiatum* into 6 clusters. The cluster A consist Akola (TL-1), Amravati (TL-2) and Bhandara (Tl-3). Cluster B consist only 1 isolate i.e. Buldhana (TL-4). Cluster C consists of Chandrapur isolate (TL-5). Cluster D consist of Gadchiroli isolate (TL-6). The Gondia (TL-7) and Washim (TL-8) isolate are in cluster E and Yeotmal (TL-9) isolate is in cluster F. The similarity ranges from 21 to 55 %. (Fig.)

Similar studies were also done by Gurumurthy *et al.* (2013) studied Molecular characterization of the promising bio-control agents adopting Random Amplified Polymorphic DNA (RAPD) analysis helps to determine the diversity and identification. DNA (RAPD) analysis. Distinct and reproducible fingerprints were attained upon amplification of purified genomic DNA of *Trichoderma spp*. with random primers of Operon (OPH) series. The amount of genetic variation was figured out with a set of 20 RAPD primers. In most cases, the amplified fragments showed more than 50% polymorphism.

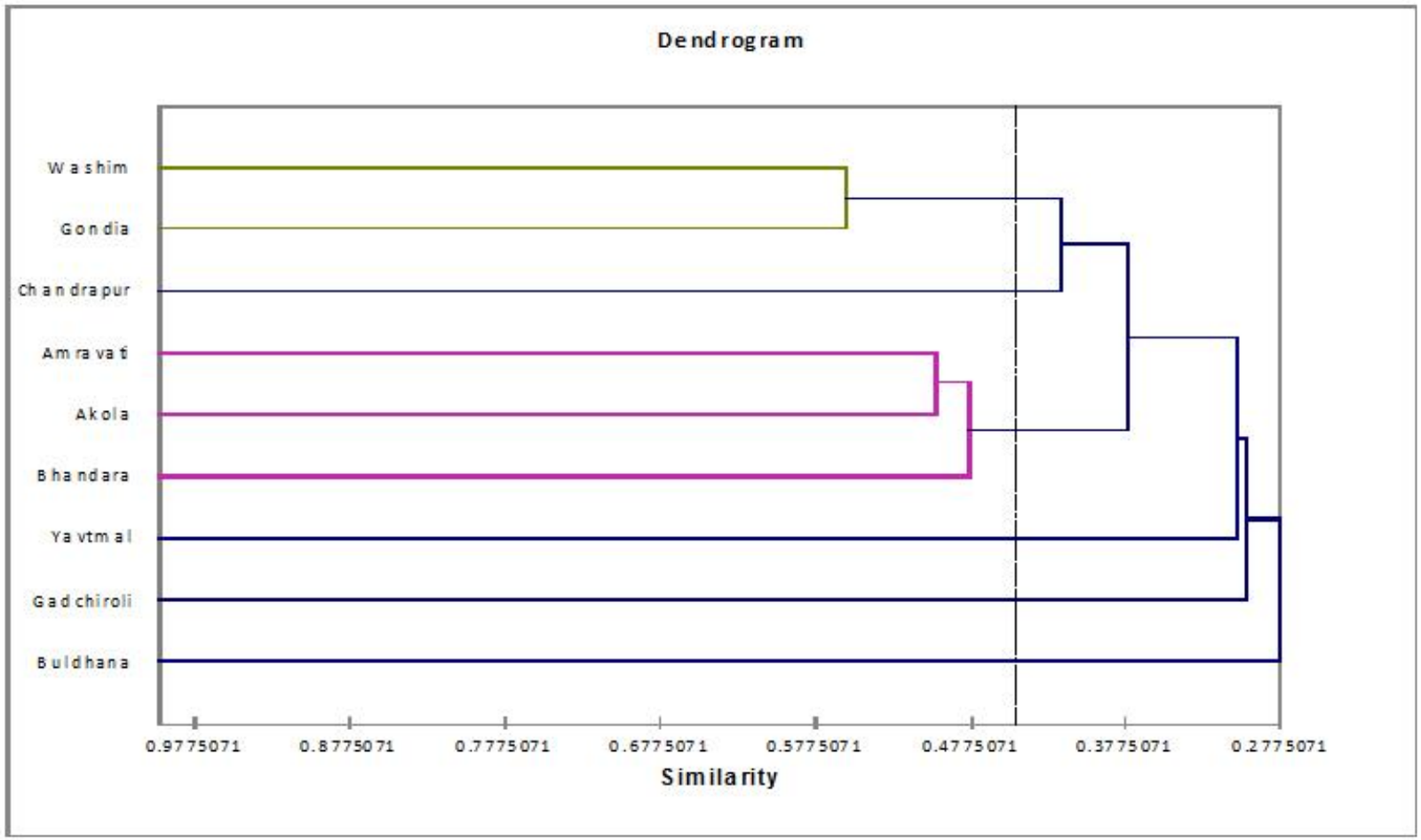


Fig. 3. RAPD UPGMA dendogram of six isolates of *Trichoderma viride* on Jaccard's similarity

Saravanan *et al.* (2014) studied 15 isolates using morphological characters, molecular characterization using Random Amplified Polymorphic DNA and Inter Simple Sequence Repeats-Polymerase Chain Reaction. The similarity ranged from 50 to 91%. The maximum genetic variation in the T. viride group was observed between the isolates T4 and T7 (90%). In *T*. *harzianum* cluster variation ranged between T8 and T12 (88%). Followed by T3 and T5 (79%) in the T. atroviride group. On the other hand maximum genetic variation of 51 % was observed between the cluster I and followed by 55% between cluster II and cluster III.

Recently Rani *et al*. (2017) studied total nine *Trichoderma* isolates were obtained from 27 rhizosphere samples collected from different cropping systems i.e. groundnut, redgram and tomato. A random amplified polymorphic DNA (RAPD) marker was used to estimate the genetic variation among 9 isolates of Trichoderma. These isolates were characterized using 15 random primers of the OPA and OPM series. Out of which 9 primers gave reproducible and scorable band with high percentage of polymorphism.

CHAPTER V

SUMMARY AND CONCLUSIONS

Several species of *Trichoderma* have been extensively studied for their ability to control different fungal plant pathogens. In the present study, an attempt was made to study morphological and molecular diversity among *Trichoderma* isolates using RAPD markers and to confirm potent *Trichoderma longibrachiatum* isolates at species level using ITS region. The results obtained in the present study are summarized.

Trichoderma isolates were isolated from rhizospheric soil samples collected from Vidarbha region of Maharashtra state viz.TL-1(Akola), TL-2 (Amravati), TL-3 (Bhandara), TL-4(Buldhana), TL-5 (Chandrapur), TL-6 (Gadchiroli), TL-7 (Gondia), TL-8 (Washim) and TL-9 (Yeotmal) by following serial dilution spread plate method on TSM

For the confirmation of the isolates Trichoderma culture was send to Indian Type Culture Collection(ITCC), Division of Plant Pathology, Indian agricultural Research Institute, New Delhi and as per the report 9 isolates were identified as *Trichoderma longibrachitum.*

Morphological observation were recorded on colony colour, colony growth, pigmentation, conidial shape and size and phialides shape etc. *Trichoderma longibrachiatum* isolates grew well and formed conidia within 4 days. The radial growth (mm) was recorded in isolate 7^{th} DAI. The maximum radial growth 90.00 mm was recorded only in isolates TL-3 (Chandrapur), TL-5 (Gondia), TL-9 (Yeotmal) . The colony colour was from whitish green to olive green, creamy to dirty yellow pigmentation was observed. Conidia globuse to sub globuse, size of conidia was in the range of 1.75 to 2.91µm length and 0.95 to 2.59 breadth. phialides shape was lageniform to cylindrical. The spore count was least to moderate. Morphological variation within the *T. longibrachiatum* isolates collected from different places.

Nine *T. longibrachiatum* isolates were evaluated for antagonistic efficacy against three pathogens and the maximum per cent growth inhibition (86.22%) of *Sclerotium rolfsii* was observed by the isolate TL-2(Amravati) and the least per cent inhibition was observed in TL-7(Gondia) i.e. 56.77. The per cent growth inhibition of *Rhizoctonia bataticola* was maximum i.e. 68.63% by TL-8 (washim) isolate and least inhibition 51.54 % was recorded in TL-4(Buldhana) isolate. In case of F*usarium udam* the maximum inhibition was recorded by TL-6 (Gadchiroli) i.e 87.77 % where as 62.42 % growth inhibition was observed by Tl-1(Akola) isolate.

Growth inhibition of *Sclerotium rolfsii, Rhizoctonia bataticola* and *F.udum* were recorded at 12 DAI due to volatile substance produced by *T. longibrachiatum*. TL-4 (Buldhana), isolate recorded maximum 33.36% and 45.78% growth inhibition of *Sclerotium rolfsii* and *F.udum* respectively where as TL-9(Yeotmal) inhibited *Rhizoctonia bataticola* up to 59.88%.

RAPD marker was used for analyzing molecular differentiation of isolates , out of 20 primers used 15 primers showed good amplification. The highest genetic similarity to an extent of 0.55 was recorded between TL-7 (Gondia) and TL-8 (Washim) followed by 0.50 similarity between TL-1 (Akola) and TL-2 (Amravati) isolates. Least genetic similarity was observed that is 0.21 between TL-2 (Amravati) and TL-4 (Buldhana) **.**

The cluster A consist Akola (TL-1), Amravati (TL-2) and Bhandara (Tl-3). Cluster B consist only 1 isolate i.e. Buldhana (TL-4). Cluster C consists of Chandrapur isolate (TL-5). Cluster D consist of Gadchiroli isolate (TL-6). The Gondia (TL-7) and Washim (TL-8) isolate are in cluster E and Yeotmal (TL-9) isolate is in cluster F. The similarity ranges from 21 to 55 %.

ITS marker was done which gives band size of 500bp to 600 bp.

Coclusion:

- Soil samples collected from the nine districts of Vidarbha region yielded nine different isolates(TL-1to TL-9) of *Trichoderma* spp . Isolates were identified as *Trichoderma longibrachiatum* by Indian Type Culture Collection(ITCC), Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi.

  Morphological variation like growth, colony colour, pigmentation, conidial size and shape and phialides shape were recored among the nine isolates of T. longibrachiatum

- Antagonistic efficacy of *Trichoderma longibrachiatum* was proved against soil borne pathogen i.e *Sclerotium rolfsii, Rhizoctonia bataicola* and *Fusarium udam.* Volatile compounds was also produced and able to arrest the growth of tested pathogens.

- Among 20 primers screened 15 primers produced reproducible and scorable bands with high degree of polymorphism. Out of these 15 primers, 12 primers showed all polymorphic bands, whereas remaining 3 primers namely OPA-8, OPA-9 and OPA-19 each showed one monomorphic band.

- A total 113 amplicons were obtained with the 15 primers. Out of 113 bands, 110 were found to be polymorphic and the level of polymorphism was 97.34 per cent.

- Twenty RAPD primers were used for the molecular diversity analysis among nine *Trichoderma longibrachiatum.* Similarity coefficient for RAPD marker ranged from 0.21 to 0.55, indicating the significant diversity among isolates.

- The maximum base pair band was 6500bp and the minimum base pair band produced was 200bp.

CHAPTER VI

LITRATURE CITED

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Place : Akola

Date : / / 2018

(Priti Bhagwat Shinde)
Signature of student

Appendix I

Composition of media

1) Potato dextrose agar (PDA) medium

Sr. No.	Ingredient	Quantity
1	Peeled and sliced potato	200 gm
2	Dextrose ($C_6H_{12}O_6$)	20 gm
3	Agar-agar	20 gm
4	Distilled Water	1000 ml

(For preparation of the broth medium no agar was added)

2) *Trichoderma* Selective Medium (TSM)

Sr. No.	Chemicals	Quantity
1	Glucose	3g
2	Magnesium sulphate	0.2g
3	Dipotassium hydrogen sulphate	0.9g
4	Ammonium nitrate	1.0g
5	Potassium Chloride	0.5g
6	Rose Bengal	0.033g
7	Metalaxyl	0.3g
8	PentaChloro Nitro Benzene	0.2g
9	Chloramphenicol	0.25g
10	Agar-agar	15g
11	Distilled water	1000ml

Appendix II

Indian Type Culture Collection
Identification / Culture Supply Services
Division of Plant Pathology
Indian Agricultural Research Institute
New Delhi- 110 012

IDENTIFICATION REPORT

Ref. No. PP/66 Date:07/04/2018

To
Dr. S.S.Mane
Dept. of Plant Pathology
Dr. Panjabrao DeshkuhKrishi Vidyapeeth, Akola
Krishinagar
Akola- **444 104**
Maharashtra

I.D. No.	Ref. No.	Source	Fungus	Identified By
10.781.18	1	Rhizospheric soil	*Trichoderma asperellum*	Dr. T. Prameela Devi
10.782.18	2	-DO-	*Trichoderma longibrachiatum*	Dr. Deeba Kamil
10.783.18	3	-DO-	*Trichoderma longibrachiatum*	
10.784.18	4	-DO-	*Trichoderma longibrachiatum*	
10.785.18	5	-DO-	*Trichoderma longibrachiatum*	
10.786.18	6	-DO-	*Trichoderma longibrachiatum*	
10.787.18	7	-DO-	*Trichoderma longibrachiatum*	
10.788.18	8	-DO-	Contaminated	
10.789.18	9	-DO-	*Trichoderma longibrachiatum*	
10.790.18	10	-DO-	*Trichoderma longibrachiatum*	
10.791.18	11	-DO-	*Trichoderma longibrachiatum*	

Note: Isolate No.8 is found contaminated please send fresh and sporulated culture tubes in duplicate.

Remarks: Recpt. No. CIC/201-18/RF/134,dt. 23/03/2018 ₹ 11,000/- sent on 02/04/2018

7/4/18
(T. Prameela Devi)
Principal Scientist (ITCC)

- See ITCC catalogue on **www. iari.res.in/files/ITCC_Catalogue_ 1936-2016-16092016.pdf**
- Kindly acknowledge ITCC in every research correspondence related to these fungi.
- The minimum time required for the identification of
- **Oomycetes, Ascomycetes and Coelomycetes- 4-6 weeks**
- **Zygomycetes and Hyphomycetes- 3-4 weeks**

Appendix III

List of bands amplified against RAPD marker

Primer	Banding type	TL-1	TL-2	TL-3	TL-4	TL-5	TL-6	TL-7	TL-8	TL-9
OPA-1	4000bp	0	0	0	0	0	0	0	0	1
	2500bp	0	0	0	0	0	0	0	0	1
	1000bp	0	1	1	1	1	1	1	0	0
	700bp	0	1	1	0	0	0	0	0	0
	510bp	0	0	0	1	0	0	1	0	1
OPA-2	4000bp	1	0	0	0	0	0	0	0	0
	3400bp	0	0	0	0	0	1	0	0	0
	2500bp	1	1	1	0	0	1	0	0	0
	2200bp	0	0	1	0	0	0	0	0	0
	2000bp	0	1	0	0	0	1	0	0	0
	1500bp	1	0	1	1	1	1	1	1	1
	850bp	0	0	0	0	1	1	0	1	1
	750bp	1	0	1	1	1	1	1	1	1
	450bp	0	0	1	1	1	0	1	1	1
OPA-3	3500bp	0	1	1	0	0	0	0	0	0
	3000bp	0	0	0	0	0	0	1	1	0
	2800bp	0	0	0	0	1	0	0	0	0
	2000bp	0	0	0	0	1	0	1	1	0
	1500bp	1	1	1	0	1	0	1	0	0
	1000bp	1	1	1	1	1	0	1	1	0
	850bp	1	1	1	0	1	1	1	1	0
	750bp	1	1	1	1	1	0	1	1	1
	450bp	0	0	0	0	1	0	1	1	0
	250bp	0	0	0	0	1	0	1	0	0
OPA-4	4000bp	0	0	1	0	0	0	0	0	0
	3000bp	0	0	0	0	0	0	1	1	0
	2400bp	1	0	0	0	0	0	0	0	0
	2000bp	0	1	0	0	0	0	1	0	1
	1500bp	0	0	0	0	0	0	0	1	0
	1000bp	0	1	1	0	0	0	0	0	0
	870bp	1	1	1	0	1	0	1	1	0
	750bp	1	1	1	1	1	1	1	1	0
	650bp	0	0	0	0	0	0	1	1	1
	450bp	1	1	1	0	1	0	1	1	0
	260bp	0	0	0	0	0	0	1	1	0
	220bp	0	0	0	0	1	0	1	1	0
OPA-5	4000bp	0	0	0	0	0	0	0	0	1
	3000bp	0	0	0	0	0	0	0	0	1
	2300bp	0	0	0	0	0	0	1	0	1
	1300bp	1	1	1	1	0	0	1	1	0
	1000bp	0	0	0	0	0	0	1	0	0
	820bp	0	0	0	0	0	0	1	1	0
OPA-8	5800bp	0	1	0	0	0	0	0	0	0
	3500bp	1	1	1	1	1	0	0	0	0

	2700bp	1	1	1	1	1	1	1	1	1
	2000bp	1	1	0	1	0	0	0	0	0
	1000bp	1	1	1	1	1	0	0	1	1
	800bp	0	0	1	1	1	1	1	1	1
	550bp	0	0	0	0	0	1	0	0	1
OPA-9	4000bp	0	1	0	0	0	0	0	0	0
	3200bp	0	1	0	0	0	1	0	1	0
	2500bp	1	1	1	1	1	1	1	1	1
	1200bp	1	0	1	0	1	1	1	0	1
	1000bp	0	1	0	0	0	0	0	0	0
	650bp	0	0	1	0	0	0	0	1	1
	250bp	0	1	0	0	0	1	0	1	0
OPA-10	2000bp	0	0	0	0	0	0	0	0	1
	800bp	1	1	0	0	1	0	1	1	1
	740bp	1	1	0	1	1	0	1	1	1
OPA-12	5000bp	0	1	0	0	0	0	0	0	0
	3300bp	0	0	0	1	1	0	1	1	0
	3000bp	0	0	0	0	1	0	0	0	0
	2500bp	0	0	0	0	1	0	0	0	0
	2000bp	1	0	0	1	0	0	1	1	0
	900bp	1	0	0	1	1	0	0	0	0
	700bp	1	1	0	1	1	1	1	0	0
OPA-15	4000bp	1	0	0	0	0	0	0	0	0
	2000bp	0	0	0	0	0	0	1	0	0
	1200bp	0	1	0	1	0	0	1	1	0
	800bp	0	1	1	0	1	0	1	1	1
	750bp	0	0	0	0	0	0	1	1	0
	700bp	1	1	0	0	0	0	1	0	1
	520bp	1	1	0	0	1	1	1	0	0
	300bp	0	0	0	0	1	0	0	0	0
OPA-18	4000bp	0	1	1	0	0	0	1	0	0
	3200bp	0	1	0	0	0	0	0	0	0
	3000bp	0	1	0	0	0	0	0	1	0
	2300bp	0	0	0	0	0	0	0	1	0
	2050bp	1	1	1	0	0	1	0	1	0
	1600bp	0	1	0	0	0	0	1	1	0
	1000bp	1	1	0	0	0	0	0	1	0
	750bp	1	1	0	0	0	0	0	0	0
	620bp	0	0	0	0	1	0	0	0	0
	500bp	0	0	0	0	0	0	0	1	0
OPA-19	3800bp	0	0	0	0	0	0	1	0	1
	2500bp	0	0	1	0	0	0	0	0	0
	1000bp	1	1	1	1	1	1	1	1	1
OPB-5	6500bp	0	1	1	0	1	0	0	0	0
	5000bp	0	0	0	0	0	0	1	1	0
	3500bp	0	1	1	0	1	0	0	0	0
	3000bp	0	0	0	0	0	0	1	1	0
	2700bp	0	1	1	0	1	1	0	0	0
	2300bp	1	1	1	0	1	0	1	1	1
	2000bp	0	0	0	0	0	0	0	1	1
	1900bp	0	0	0	0	1	1	0	0	0
	1200bp	1	0	1	0	0	0	0	1	0
	1000bp	1	1	1	0	0	0	0	0	0
	800bp	1	1	1	0	1	1	1	1	1

	700bp	1	1	1	0	1	1	1	1	1
	550bp	1	1	1	0	1	1	1	1	1
	500bp	1	1	1	0	1	1	1	1	1
	200bp	0	0	0	0	1	1	1	1	0
OPB-17	2700bp	0	0	1	1	0	0	1	1	1
	2500bp	0	0	0	0	1	1	0	0	0
	1100bp	0	0	0	0	0	1	1	0	0
	1000bp	1	1	1	0	1	1	0	1	1
OPB-20	4000bp	0	0	1	0	1	1	0	0	0
	3000bp	0	0	0	1	1	1	0	0	0
	1200bp	0	0	0	1	0	1	0	1	1
	900bp	0	0	0	0	0	1	0	0	0
	750bp	0	0	0	1	0	1	0	1	0
	600bp	0	0	0	0	0	1	0	0	1