MOLECULAR CHARACTERIZATION OF *Trichoderma* spp. ISOLATED FROM SALINE SOIL

THESIS

Submitted to
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
in partial fulfilment of the requirements
for the Degree of

MASTER OF SCIENCE
IN
AGRICULTURE
(PLANT PATHOLOGY)

By
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Enrolment Number - MM/1866 **2020**

DECLARATION OF STUDENT

I hereby declare that the experimental work and its interpretation in the thesis entitled **"MOLECULAR CHARACTERIZATION OF *Trichoderma* spp. ISOLATED FROM SALINE SOIL"** or part thereof 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 source of materials used and all assistance received during the course of investigation have been duly acknowledged.

Place : Akola (Rasna Sharma)

Date : / /2020 Enrolment No. MM/1866

CERTIFICATE

This is to certify that thesis entitled "**MOLECULAR CHARACTERIZATION OF *Trichoderma* spp. ISOLATED FROM SALINE SOIL**" submitted in partial fulfilment 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 **Rasna Sharma** under my guidance and supervision.

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

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AKNOWLEDGEMENT

I feel immense pleasure in taking this opportunity of expressing my profound, humble indebtedness and deepest sense of gratitude and thanks from core of my heart towards my respected guide Dr. S. T. Ingle, Associate Professor, Department of Plant Pathology, Dr. PDKV, Akola, for his valuable guidance, constant inspiration, keep interest, kind advice and constructive criticism, right from selection of research problem up to final shaping of the thesis in the present form.

It is my privilege to record my sincere and devoted thanks to members of my advisory committee, Dr. S. S. Mane, Professor and Head, Department of Plant Pathology, Dr. P. V. Jadhav, Assistant Professor, Biotechnology Centre, Department of Agricultural Botany and Shri R. S. Ghawade, Assistant Professor of Plant Pathology, RRC Amravati, Dr. PDKV, Akola for their keen interest and valuable suggestion time to time in pursuit of present investigation.

I am extremely thankful to honourable Vice-Chancellor Dr. V. M. Bhale, Dr. M. B. Nagdeve, Director of research and Dr. D. B. Undirwade Associate Dean, PGI, Dr. PDKV, Akola, for providing all necessary facilities during the course of investigation to complete the research work.

I express my deep sense of gratitude to, Dr. G. K. Giri, Professor, Dr. R. W. Ingle, Professor Dr. S. B. Brahmankar, Associate Professor and all the staffs of Department of Plant Pathology, PGI, Dr. PDKV, Akola for their timely cooperation and valuable suggestion during the course of research work.

I am extremely thankful to Dr. Darasingh Rathod, Assistant Professor, Biotechnology Centre, Department of Agricultural Botany, PGI, DR. PDKV, Akola for providing laboratory facilities (PCR Machine) for the completion of my research work.

It is my proud privileges to record my deepest sense of gratitude and cordial thanks to affectionate all staff members of Department

of Plant Pathology, PGI, Dr. PDKV, Akola for their valuable suggestions and comments during my research investigation.

Words may be more powerful than swords, but they are the generate of great movement and revolt and revolution. Still words many times are not adequate enough to express the sentiments which bloom in the secret chamber of heart and minute modes of mind. I am trying little for thanks giving which may look bit of formal but its true and wholly from my heart. I shall ever remain grateful to them.

I think words with me are insufficient to express the feelings of hearts to acknowledge my parents, Shri. Ramji Lal Sharma and Mrs. Kamla Devi for their hard work to educate me. I am very thankful to my husband Mr. Prasoon Vyas, sisters , brother and my inlaws for their affection, enlivened my ability and evoked me to complete this work. I also extend my special and heartiest thanks to my classmates for your timely co-operation and support during the course of study and making my cheerful stay in Akola. I express my sincere thanks to all my seniors, and colleagues who supported me morally during course of investigation.

I am very much thankful and indebted to all authors and researchers whose articles had helped me in conducting my research work and utilizing their findings for interpretation of the results.

I was fortunate enough to receive the kind co-operation from almost every one way or the other, during my stay at this institute. It is extremely difficult to thank all of them individually by name, this short coming may please be pardoned.

Lastly but not the least, I want to express my grateful gratitude to one and all who helped me in one way or the other for the completion of my research work.

Place : Akola (Rasna Sharma)

Date : / /2020 Enrolment No. MM/1866

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Abbreviations		Full form
%	:	Per cent
/	:	Per
@	:	At the rate of
µg	:	Micro grams
µl	:	Micro litre (s)
°C	:	Degree Celsius
AFLP	:	Amplified Fragment Length Polymorphism
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
ISSR	:	Inter Simple Sequence Repeat
ITS	:	Internal Transcript Spacer
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
PDB	:	Potato Dextrose Broth
RFLP	:	Restriction Fragment Length Polymorphism
rpm	:	Revolution per minute
SSR	:	Simple Sequence Repeat
Taq polymerase	:	Thermus aquaticus DNA polymerase Enzyme
TBE buffer	:	Tris hydroxymethyl amino methane And boric acid ethylene diamine tetra Acetic acid
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)

E) Thesis Abstract

a)	**Title of the thesis**	**:**	**"MOLECULAR CHARACTERIZATION OF *Trichoderma* spp. ISOLATED FROM SALINE SOIL"**
b)	**Full name of student**	**:**	**RASNA SHARMA**
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d)	**Degree to be awarded**	**:**	M.Sc. (Agri.)
e)	**Year of award of degree**	**:**	2020
f)	**Major subject**	**:**	Plant Pathology
g)	**Total number of pages in the thesis**	**:**	63
h)	**Number of words in the abstract**	**:**	424
i)	**Signature of the Student**	**:**	
j)	**Signature, name and address of forwarding authority**	**:**	

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ABSTRACT

Genus Trichoderma contain many species that are of great economic importance because of ability to suppress pathogens and enhance capabilities of plants against soil borne pathogens. The present study was conducted to characterize the molecular variation in *Trichoderma* isolated from saline soil. Molecular techniques are important

analytical tool to characterized genetic variability and diagnosis of microbial population.

Molecular variability of *Trichoderma* isolates was studied by using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) techniques. The ITS universal markers was successfully used to amplify genomic DNA of *Trichoderma*. Eight isolates of Trichoderma confirmed with ITS-1and ITS-4 primers which gaves band size of 500bp to 600bp.

Nine RAPD primers of OPA series were produced 61 scorable bands among them 57 bands were polymorphic and level of polymorphism was up to 93.44%. Three primers namely OPA-13, OPA-10 and OPA-20 each showed monomorphic band. similarity coefficient value ranged from 0.923 to 0.143 across Trichoderma isolates indicating the diversity among isolates. On the basis of dendrogram the isolates were divided into three clusters. Cluster –A, includes TD-1, TT-2, TC-3, TS-4 and TJ-5. Second group is named as cluster –B which includeTM-6. Third group is named as cluster -C which includes TA-7 and TA-8. The TJ-5 was found to have higher similarity index and the TM-6 was found to have lower similarity index. The maximum and minimum value of cophenetic matrix was 0.923 and 0.255 respectively. Isolate TS-4 was found to have a higher Cophenetic distance matrix with TJ-5 **(**0.923**)** and TM-6 with all isolates **(**0.255**).** In summary statistical observations, the minimum and maximum value was 0.000 and 1.000 respectively. The maximum and minimum standard deviation was TD-1 (0.504) and TA-7(0.413).

In ISSR, 4 primer were tested and which produced 30 scorable bands among which 28 bands were polymorphic and level of polymorphism was up to 93.33%. Two clusters were observed in Dendrogram i.e. Cluster A includes four isolates i.e. TD-1, TS-4, TJ-5 and TM-6 and cluster B includes four isolates i.e.TT-2, TC-3, TA-7 and TA-8. Similarity coefficient value ranged from 0.048 to 0.824 across *Trichoderma* isolates. TS-4 was found to have a higher Cophenetic distance matrix with TJ-5 (0.824) and TM-6 with all isolates (0.158). summary statistics, on the basis of 30 total observations, the maximum and minimum value recorded

between 0.000 to 1.000. The maximum standard deviation was observed in TJ-5 (0.509) and the minimum was observed in TM-6 and TA-8 (0.479).

On the basis of RAPD and ISSR primers tested there is the existence of molecular variability among the tested isolates of *Trichoderma.*

CHAPTER I

INTRODUCTION

1.1 Background information

Fungal species belonging to the genus *Trichoderma* are worldwide in occurrence and easily isolated from soil, decaying wood, and other forms of plant organic matter (Howell, 2003). *Trichoderma* is characterised as fast growing ability to form colonies bearing tufted or postulate, repeatedly branched conidiophores with phialides and hyaline conidia borne in head and can be easily isolate from acidic soil having pH 3.5 to 4.5 (Kotasthene and Shalini, 2007). *Trichoderma* belongs to-

Kingdom	-	Fungi
Division	-	Ascomycota
Sub division	-	Pezizomycotina
Class	-	Sordariomycetes
Order	-	Hypocreales
Family	-	Hypocreaceae
Genus	-	*Trichoderma*

Trichoderma is used in different crop like rice, wheat, pulses (black gram, cowpea, and chickpea) vegetable (tomato, brinjal, and chili) against a wide range of plant pathogen. One of the popular species, *Trichoderma asperellum* is effective against all soil borne fungal pathogen like Phytophthora, Fusarium, Pythium, Rhizoctonia, Sclerotium. Trichoderma, reduce growth, survival or infection caused by pathogen by different mechanism. In addition, some species of the genus are economically important producers of industrial enzymes (*Trichoderma reesei, Hypocrea jecorina*) and antibiotics (Sivasithamparam and Ghisalberti, 1998). Due to its cosmopolitan occurrence in almost all soils usually associated with organic matter. It is among the most studied biocontrol agent in the world, mainly because it is harmless to non-target organisms and safe to the environment (Harman *et al*., 2006).

Trichoderma spp. are ubiquitous colonizers of cellulosic materials and can thus often be found wherever decaying plant material is available as well as in the rhizosphere of plants, where they can induce systemic resistance against pathogen *Trichoderma* spp. are highly successful colonizers of these habitats, which is reflected both by their efficient utilization of the substrate at hand as well as their secretion capacity for antibiotic metabolites and enzymes. Besides they have effects on growth, reproduction, and secondary metabolite biosynthesis (Schuster and Schmoll, 2010).

The status of bio pesticide showed that India has the capacity to produce 1850MT of Trichoderma formulation per annum. While, the requirement is 22038 MT with market value of 260 crores. There are around 150 registered manufacturers of *Trichoderma* in India.

The potential of *Trichoderma* spp. as bioagent was first recognized by Weindling (1932). Several reports are available on *Trichoderma* spp. as an important biocontrol agent against soil borne plant pathogens (Elad et al., 1979 and Sing et al., 2007). Antagonistic microorganism such as *Trichoderma* reduce growth, survival or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hypal interactions and enzyme secretion (Pan and Jash, 2009). Different species of *Trichoderma* well known for their biocontrol activity, rhizosphere competence and production of hydrolytic enzymes such as cellulases, chitinases, glucanases and proteases as well as many secondary metabolites (Sharma et al., 2011).

Recently molecular techniques such as DNA sequencing (Apple and Gordon, 1996), RAPD (Random Amplification of Polymorphic DNA) analysis, ITS (Internal Transcribed Sequences) of the ribosomal DNA analysis (rDNA-ITS1) and UP-PCR (Universally Primed Polymerase Chain Reaction) used for the characterization of *Trichoderma* isolates (Cumagun et.al, 1996). The another effective method to characterize genetic variations in *Trichoderma* is ISSR (Inter Simple Sequences Repeats). The developmental rate within ISSR is significantly higher than other type of

DNA, the likelihood of finding polymorphism is greater as compare to RAPD.

The molecular techniques like Random Amplified Polymorphic DNA (RAPD) developed by Williams *et al.* (1990) has been used for genetic and taxonomic studies for several fungi including *Trichoderma* spp. A high level of genetic variability in Trichoderma spp. has been reported (Chakraborty et al., 2010) that can be used to produce a wide range of products of commercial and ecological interest.

1.2 Importance of study

Trichoderma is easily identified in culture media, which produces large number of characteristics small, green or white conidia, from phialides present on the profusely or meagerly branched conidiophores. However, the identification of isolates to species level is difficult and confusing due to the complexity and closely related characters of the species*. Trichoderma* isolates were differentiated by mycelial growth rate and colony appearance, as well as microscopic morphological features, including phialides and spores. These can also be distinguished by molecular techniques like DNA sequencing, Random Amplification of Polymorphic DNA (RAPD) analysis, Restriction Fragment Length Polymorphism (RFLP) analysis, internal transcribed sequences (ITS) of the ribosomal DNA (rDNA) analysis (rDNA-ITS1) and universally primed polymerase chain reaction (UP-PCR) have been used to characterize isolates of *Trichoderma* (Cumagun et al., 1999). Inter simple sequence repeats (ISSR) have been used as another effective method to characterize genetic variability. Since the evolutionary rate within ISSR is considerably higher than other types of DNA, the likelihood of finding polymorphism is greater compared to RAPD. Since the first reports of RAPD markers by Williams et al. (1990), this method has been widely used for identification of species. This technique has been used in some cases for species identification.

1.3 Objective of the study

- To analyse the molecular variability of *Trichoderma* spp. isolated from saline soil by using RAPD and ISSR markers.

1.4 Hypothesis

Trichoderma most commonly used as biocontrol agent to control the plant pathogens by using different mechanism such as mycoparasitism, enzyme secretion, competition, mycoparasitism and antibiosis (Soesanto and Rahayuniati, 2011). Several investigations could distinguish between different *Trichoderma* strains by randomly amplified polymorphic DNA (RAPD-PCR) and sequence analysis of ribosomal DNA (Siameto *et al*., 2011).RAPD-PCR helps to determine the molecular variability in different biological control agents for determination of diversity and characterization of biocontrol agents (gherbawy *et al*., 2014). The aim of this study is to determine the molecular variability of *Trichoderma* species isolated from saline soil by using RAPD and ISSR markers.

1.5 Scope and limitation

Trichoderma are important biological control agents of several soil borne plant pathogens. *Trichoderma* species serves as a potential alternative to chemical control measure and growing pathogen resistance crop cultivars (Kushwaha and Verma. 2014). *Trichoderma* is easy to identify in culture media, because it produces large number of characteristics small, green or white conidia from phialides present on the luxuriantly or meagerly branched conidiophores. However, the identification of isolates to species level is difficult and confusing due to the complexity and closely related characters of the species. Molecular analysis of several strains revealed that classification based on morphological data has been, erroneous to great extent resulting in re-classification of several isolates and species (Samuels, 1996). Trichoderma use different mechanism for control of phytopathogens which includes mycoparasitism, out compete pathogenic fungi for nutrients, secretion for antibiotics and fungal cell wall degrading enzymes. Furthermore it is difficult to predict the degree of synergism and the behaviour of a biocontrol agent in a natural

pathosystem. Thus the present study was conducted to characterize their molecular variability of Trichoderma species associated with saline soil. Molecular techniques are important analytical tools for the characterization and diagnosis of microbial population. DNA extraction is the primary and basic step of all these techniques. Therefore, these procedures must provide DNA in sufficient quantity and purity for further analysis. Isolation of genomic DNA of fungi is laborious, expensive and time consuming.

CHAPTER II

REVIEW OF LITERATURE

2.1 History

Persoon (1794) was first described *Trichoderma* more than two hundred years ago by and was later envisaged into four genera.

Thakur and Norris (1928) In India isolated *Trichoderma* from the soils of Madras. There upon it was reported from various substrates and locations. Mostly identifications were based on the morphological characters. Various papers were published in India on the bio-control efficiency of *T. harzianum; T. koningi; T. longibrachiatum; T. virens; T. hamatum* and *T. viride.* Even-though the species names are mentioned in research papers, invalid names are still used which has given misconception regarding the identification of the species.

Bisset (1991) elevated species aggregate to special level and recognized several species within each of five sections of the genus Trichoderma.

Samuels (1996) *Trichoderma* is easily identified in culture media, which produces large number of small green or white conidia from phialides present on the profusely or meagerly branched conidiophores. However, the identification of the isolates to species level is difficult and confusing due to the complexity and closely related character of the species. Species concept within the *Trichoderma* is very wide and this has resulted in the establishment of many specific and sub-specific taxa.

Schuster and Schmoll (2010) *Trichoderma s*pecies are green spore ascomycetes present in nearly all type of temperate and tropical soils. They can often be found in decaying plant material and rhizosphere of plants.

2.2 *Trichoderma* as a Biocontrol Agent

Elad *et al.* (1979) reported *Trichoderma harzianum* is a biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani.* An isolate of *Trichoderma harzianum* capable of lysing mycelia of the wheat bran preparation of *T. harzianum* increased growth of bean

Sclerotium rolfsii and *Rhizoctonia solani* was isolated from a soil naturally plants in a noninfested soil and it controlled *S. rolfsii* more efficiently than a infested with those pathogens. In culture, *T. harzianum* grew better than S. conidial suspension of the same antagonist. An uninoculated wheat bran rolfsii and invaded its mycelium under growth conditions adverse to the preparation increased disease incidence. In naturally infested soils, wheat pathogen; eg. High pentachloro nitrobenzene concentrations, high pH bran preparations of *T.harzianum* inoculum significantly decreased levels, or low temperatures. Under greenhouse conditions, incorporation of diseases caused by *S. rolfsii* or *R. solani* in three field experiments with the wheatbran inoculum preparation of *T. harzianum* in pathogen-infested beans, cotton, or tomatoes, and they significantly increased the yield of soil significantly reduced bean diseases caused by *S. rolfsii, R. solani,* or beans. both, but its biocontrol capacity was inversely correlated with temperature.

Gaur and Sharma (1991) observed *Trichoderma harzianum* was most effective on different media against *F. udam.*

Harman *et al.* (1999) recorded in growth chamber that rice sheath blight caused by *Rhizoctonia bataticola* was reduced significantly by application of *Trichoderma harzianum* strain.

Harman *et al.* (2004) observed that many species in *Trichoderma* genus can be characterized as opportunistic avirulent plant symbionts. The genus comprises a great number of fungal strains that act as biological control agents, the antagonistic properties of which are based on the activation of multiple mechanisms (Benitez et al., 2004).

Saba *et al.* (2012) reported fungus *Trichoderma,* a low cost biocontrol agent that can establish itself in different pathosystems, has moderate effects on soil balance and does not harm beneficial organisms that contribute towards pathogen's control. Fungi of the genus *Trichoderma* are soilborne, green-spored ascomycetes that are ubiquitous in nature. Trichoderma spp. are characterized by rapid growth, mostly bright green conidia and a repetitively branched conidiophore structure. As opportunistic plant symbionts and effective mycoparasites, numerous species of this

genus have the potential to become commercial biofungicides. This biocontrol agent has no harmful effects on humans, wild life and other beneficial organisms. It is safe and effective in both natural and controlled a environment that does not accumulate in the food chain.

Xiaoxue *et al.* (2013) observed the mycoparasitic activity *Trichoderma asperellum* depends on the secretion of complex mixtures of hydrolytic enzymes like protease able to degrade the host cell wall and host.

Naher *et al.* (2014) used *Trichoderma* spp. as a biocontrol agent for sustainable management of plant diseases. The antagonistic activity of *Trichoderma* spp. showed that it is parasitic on many soil-borne and foliage pathogens. Recent discoveries show that the fungi not only act as biocontrol agents, but also stimulate plant resistance, and plant growth and development resulting in an increase in crop production. The biocontrol activity involving mycoparasitism, antibiotics and competition for nutrients, also induces defence responses or systemic resistance responses in plants. Currently, *Trichoderma* spp., is being used to control plant diseases in sustainable diseases management systems.

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. The percent growth inhibition of *S.rolfsii* by various *Trichoderma* strain was recorded at 5 days after inoculation at 28^0C 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 rolfsi*i) 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 from 71.85 - 61.11per cent. Effect of volatile compound produced by *Trichoderma* spp. against *S. rolfsii* showed inhibition ranged from 20.00 - 30.00 per cent.

Kushwaha and Verma (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.*

Muhammad *et al.* (2019) described the plant defence against fungal pathogens by antagonistic fungi with Trichoderma and focused upon *Trichoderma* mediated fungal diseases management via illustrating its taxonomy, important strains, biodiversity and mode of action.

2.3 Biodiversity of Trichoderma

Most of the *Trichoderma* species are morphologically very similar and were considered for many years as a single species: *Trichoderma viride* (Bisby 1939). Since new species were discovered, a consolidated taxonomical scheme was needed and Rifai (1969) proposed and defined nine morphological species aggregates. DNA methods brought additional valuable criteria to the taxonomy of *Trichoderma which* are being are used today for studies that includes identification (Hermosa et al., 2001; lubeck *et al*., 2000) and phylogenetic classification (Kullnig *et al*., 2002; Lieckfeldt and Seifert 2000).

2.4 Morphology

Rifai (1969) reported Conidiophores and their side branches long and slender without sterile hyphal elongation, phialids not crowded, rather slender, colonies yellowish, bright, dull to dark green, floccose or with compact conidiophores tufts. Conidia smooth walled. Conidiophores with complicated dendroid branching system, phialids regularly disposed in numbers of 3 or more. Conidia (2.8 to 3.2 × 2.5 to 2.8 µm) globose, subglobose or short obovoid, with length: width ratio of less than 1.25; colony reverse uncolored; colonies reaching >9 cm diameter in 5 days at 200C on Oat Meal Agar (OA) Media.

Grondona *et al.* (1997) monoconidial cultures of 15 isolates of *Trichoderma harzianum* were characterized on the basis of 82 morphological, physiological, and biochemical features and 99 isoenzyme bands from seven enzyme systems. The results were subjected to numerical analysis which revealed four distinct groups. Representative sequences of the internal transcribed spacer 1 (ITS 1)-ITS 2 region in the ribosomal DNA gene cluster were compared between groups confirming this distribution. The utility of the groupings generated from the morphological, physiological, and biochemical data was assessed by including an additional environmental isolate in the electrophoretic analysis. The in vitro antibiotic activity of the *T. harzianum* isolates was assayed against 10 isolates of five different soil borne fungal plant pathogens.

Soesanto and Rahayuniati (2011) done the morphological characterization of *Trichoderma* spp. 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. Result of the research showed that the antagonistic fungi of *Trichoderma* spp. and the endophytic *Fusarium* 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. *Trichoderma harzianum* could grow on Czapek and gelatin media, while endophytic *Fusarium oxysporum* and *T. pseudokoningii* could grow on Czapek, nitrit agar, and gelatin media. The temperature range for growth of *T. harzianum* and *T. pseudokongii* was 20-35ºC with the optimum at 30ºC.

Consolo *et al.* (2012) mono conidial cultures of 33 isolates of *Trichoderma* from Buenos Aires Province, Argentina were characterized on the basis of twenty eight morphological, physiological and biochemical features. All of them were screened for proteinase, endochitinase and b1,3 glucanase activity. Universally primed PCR (UP-PCR) and inter simple sequence repeat (ISSR) techniques were used to examine the genetic variability among isolates, which resulted in 127 bands for the total number of isolates. These results were subjected to numerical analysis revealing 20 haplotypes grouped in five clusters.

Srivastava *et al.,* (2012) Collected many isolates of *Trichoderma atroviride* isolates 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 color, growth, shape of conidiophore, phialides and conidia.

2.5 Molecular work on Trichoderma

2.5.1 Isolation of DNA

Hima *et al.* (2016) described a rapid, inexpensive and reproducible protocol for the isolation of DNA from the filamentous fungus, *Trichoderma* spp. The present protocol is based on the sodium dodecyl sulfate method without using β- mercaptoethanol and devoid of liquid nitrogen for the maceration. The precipitation of DNA was done using isopropanol and ethanol. The A260/280 absorbance ratio of isolated DNA was ≈1.9 indicating that DNA fraction was pure and could be used for further analysis. The result of DNA electrophoresis in 0.8% agarose gel showed sharp clear bands of DNA, indicated the good quality of DNA. Since this protocol yielded the genetic DNA in sufficient quality and quantity this can be used for the successful isolation of DNA from *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 includes the use of 20% CTAB, 1.4 M NaCl, 10% Poly Vinyl Pyrrolidone (PVP), 5% Sodium Dodecyl Sulphte (SDS) and 70% ethanol. During centrifugation chloroform : isoamyl alcohol (24:1) was used for separating and precipitating of the DNA. This method solved the problems of DNA degradation, contamination and low yield.

2.5.2 RAPD Primer

Williams *et al.* (1990) random Amplified Polymorphic DNA (RAPD) analysis is a polymerase chain reaction (PCR) technique that utilizes short, arbitrary oligonucleotides to generate amplification products that can be used as genetic markers Because amplifications are performed on total genomic DNA, RAPD analysis can be used to assess genetic variation within the entire genome rather than variation within a single genetic region (rDNA).

Fujimori and Okuda (1994) examined 74 strains of *Trichoderma* by RAPD profiles and the results were consistent with the morphological, physiological and ecological data of these strains.

Zimand *et al*. (1994) used RAPD markers obtained from 9 arbitrary primers to distinguish strains of *Trichoderma.* Ten of the strains identified as *T. harzianum* exhibited similarities, and it was possible to distinguish the isolate T-39, used commercially as bio-control agent of *Botrytis cinerea.*

Schlick *et al.* (1994) used RAPD to analyzed strains of *T. harzianum* and mutants induced by gamma radiation originated from one wild isolate, verifying that with RAPD, it was possible to differentiate all the mutants strains for at least one primer and concluding that the method was valuable for identification and fast differentiation of strains.

Turcozi *et al.* (1996) characterized biologically and molecularly some potential biocontrol strains of Trichoderma. Thirty four strains of *Trichoderma,* including *T. hamatum, T. harzianum,* and *T. viride* were compared by means of RAPD and for direct mycoparasitic activity, chitinase production, and antibiotic activity.

Gomez *et al.* (1997) analyzed the RAPD profiles of strains of *T. harzianum* and classified them in different groups according to their capacity for control of plant pathogenic fungi.

Muthumeenaksh *et al*. (1998) characterized genetically 15 strains of *T. harzianum* aggressive for edible mushrooms in the United States and England using RAPD, which were designated "*T. harzianum* group 4", that presented a high homogeneity degree. Comparison of molecular data of group 4 with group 2 (the causal agent of the epidemic green mold in industrial mushrooms in England) indicated that the isolates of *T. harzianum* group 4 were different from that of group 2.

Hermosa *et al*. (2001) applied the RAPD fingerprinting technique to analyze the genetic variation among the sixteen strains of *Trichoderma* spp. Selected as biocontrol agents and, in particular to used the RAPD patterns generated to develop a rapid method for discriminating those strains with identical ITS sequences. Thirty arbitrary decamer primers were used to generate RAPD markers patterns from the 16 strains of *Trichoderma* spp. selected as biocontrol agent.

Kullnig *et al.* (2002) reported that RAPD analysis of *Trichoderma* species from different part of Russia, Siberia and the Himalaya revealed genetic homogeneity.

Goes *et al.* (2002) examined the genetic variability among 14 isolates of Trichoderma (6 of *T. viride,* 6 of *T. harzianum*, one of *T. polysporum* and one of *T. pseudokoningii*) and their RAPD analysis revealed 197 bands for the 14 isolates. Their results showed the variability among the isolates of *Trichoderma* was very high and no relationship was found between the polymorphism showed by the isolates and their hardness, origin and substrate.

Wuczkowski *et al.* (2003) shows the occurrence and genetic diversity of *Trichoderma* in which forty-six strains were identified at the species level by analysis of morphological characters following sequence analysis in RAPD analysis.

Shalini *et al.* (2006) investigated two PCR-based techniques (RAPD and restriction analysis of the amplified ITS1-5.8 S - ITS 2 region of the nuclear ribosomal DNA) to distinguish Indian isolates of six *Trichoderma* species, namely *T. virens*, *T. pseudo koningii, T. hamatum*, *T. harzianum, T. viride* and *T. koningii*.

Sharma *et al.* (2009) showed the variability among the isolates of *Trichoderma harzianum* using RAPD markers and proved RAPD to be an efficient molecular technique in demonstrating the high intraspecific genetic variation among isolates.

Chakraborty *et al.(*2010) studied nineteen isolates of *T. viride* and *T. harzianum* from rhizosphere soil of plantation crops, forest soil and agriculture fields of North Bengal region 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 dendogram revealed that similarity coefficient ranged from 0.67 to 0.95.

Gupta *et al.* (2010) isolates of seven *Trichoderma* spp. by randomly amplified polymorphic DNA RAPD-PCR analysis and there in vitro antagonism against wilt pathogens of *Psidium guava* L. viz. *Fusarium oxysporum* 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 .From the seven isolates of *Trichoderma*, isolates two species, *T. virens* and *T. viride* were superior in inhibiting the growth of both *Fusarium* spp. *Fusarium* isolates showed intra species variability.

Sagar *et al*. (2011) collected some *Trichoderma* isolates from different locations of Bangladesh for evaluating 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.

Siemeto *et al*. (2011) characterized seven isolates using RAPD-PCR procedure to determine genetic variability. Genetic similarities generated using Jacquard's coefficient of similarity ranged from 0.231 between isolates 055E and 011E to 0.857 between isolates 010E and 015E. The technique of RAPD was efficient in demonstrating the DNA polymorphism in the isolates of *T. harzianum* tested showing intraspecific genetic variability.

Sriram *et al*. (2013) studied the most widely used fungal antagonist for plant disease management in India, *Trichoderma viride* is *Trichoderma asperellum* as confirmed by oligonucleotide barcode and morphological characters. Thirty isolates identified earlier as *T. viride* were selected and characterized using morphological characters and their identification was confirmed by oligonucleotide barcode that employed amplification of ITS and tef1 regions. All the isolates were confirmed to be

T. asperellum. Analysis using sequence polymorphism-derived markers not only confirmed their identity as *T. asperellum*, but also showed new patterns among Indian isolates and presence of cryptic species *T. asperelloides*. Analysis of sequences submitted from India to GenBank as *T. viride* or *T. asperellum* using barcode also showed that they belong to *T. asperellum* .

Gurumurthy *et al*. (2013) 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. 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. Eight RAPD primers showed the amplification which was a good sign to predict the genetic diversity and molecular variability in the *Trichoderma* spp. isolated from chickpea fields of Uttar Pradesh.

Anuradha Singh *et al.* (2013) reported antagonistic and molecular variability among eight isolates of *Trichoderma atroviride,* collected from rhizosphere soil of legumes from different places of Uttar Pradesh, India. Antagonistic variability of isolates of *T. atroviride* revealed significant suppression in the radial growth of Fusarium *oxysporum f.sp. ciceri, F. oxysporum f. sp. lentis* and *Fusarium udum.* The maximum inhibition of 47.91% of mycelial growth of F. udum was recorded in case of TAU8 isolate, which is isolated from soil sample of Azeetmal. TSI3 and TKD3 isolates of Misihinrick and Maitha block of Sitapur and Kanpur dehat, respectively was found (41.66%) least effective against *F. udum.* Similarly, maximum inhibition of mycelial growth of *F. oxysporum* f. sp. *ciceri* was recorded as 55.08% by the TH3 isolate, which was isolated from soil sample of Bilgram block of Hardoi district. Molecular variability among the isolates showed that total number of amplified bands was found 74 out of which 65 were showing polymorphic and 19 were monomorphic and the size of amplified product varied from 0.1 kb to 0.75 kb.

Saravanan *et al.* (2014) obtained 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, molecular characterization using Random Amplified Polymorphic DNA and Inter Simple Sequence Repeats-Polymerase Chain Reaction.

Shahid (2014) focused on the molecular identification and analysis of genetic variability of a specific strain of *Trichoderma* based on antagonistic and RAPD analysis in some leguminous crops (pigeonpea, lentil and chickpea) produced in U.P.

Araji (2016) used the PCR-based technique of randomly amplified polymorphic DNA (RAPD) to characterize and assess the genetic relatedness of eight *Trichoderma harzianum* isolates. Genomic DNA of each species was extracted at a final concentration of 400 - 600 µg / 2-3 g of wet mycelium and at a purity of 1.6-1.8. Each DNA sample was amplified with each of 15 primers and the products were resolved electrophoretic ally on 1.2% agarose gel, stained with ethidium bromide and photographed under UV. Six primers failed to support amplification while the remaining nine primers produced a total of 128 main bands (1120 per primer) across the eight isolates. Cluster analysis based on the genetic distances split the eight isolates into two major group genotypes.

Lakhani *et al.* (2016) studied the molecular characterization of two parent's strains (*Trichoderma harzianum* NBAII Th 1, *Trichoderma viride* NBAII Tv 23) and their Corresponding fusants, produced by protoplast fusion, was investigated by random amplified polymorphic DNA (RAPD), ISSR (inter-simple sequence repeats) and internal transcribed spacer (ITS)markers. These markers produced different fragment patterns with varied number of bands and yielded a total of 419 distinct bands. 13.3% were considered as polymorphic bands and 86.7% were considered as monomorphic bands. For RAPD marker, the OPC-04 primer has showed the highest polymorphism, 58.3%. While in ISSR marker, the UBC-835 primer has showed the highest polymorphism, 57.2%.

Shahbazi *et al.* (2017) study the effects of irradiation on genetic diversity in mutated isolates of *Trichoderma* spp. RAPD (molecular marker) was used. To induce mutation in these two Trichoderma species (*T. viride and T.harzianum*), spore suspension have been gamma radiated by 0 to450 Gry Gamma ray (with 50 Gry intervals). Optimum dose for inducing mutation have been evaluated on 250 Gary. The phenotypic data showed that, gamma irradiation effects on mycelial growth, color and colony shape and spore reproduction in *Trichoderma.* Among 10 RAPD primers which have been reported for *Trichoderma* bio-diversity diagnosis, five RAPD primers (OPA 09, OPA10, OPA11, OPA14 and OPA 16) were chosen, because, these primers amplified different pattern of bands and distinctly grouped the mutants of *T. viride* and *T.harzianum* spp. The results of RAPD marker at 84% similarity, divided *T. viride* mutant to 12, 16, 14, 20 and 19 Group respectively. Dendrograms by using UPGMA method based on Jaccard's similarity coefficient at similarity level of 84%, were divided *T. harzianum* mutated isolates into three groups.

Rani *et al*., (2017) A total of 9 *Trichoderma* isolates were obtained from 27 rhizosphere samples collected from different cropping systems i.e. groundnut, red gram and tomato. A RAPD markers 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 25bp 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.

Radhika Deshmukh *et al*. (2018) studied molecular variability of *Trichoderma* mother culture and its mutant by using RAPD marker. 20 RAPD primers of OPA series were tested, of which tested 14 primers

produced 72 scorable bands among them 52 bands were polymorphic and level of polymorphic was upto 72%.

Meshu *et al.,* (2019) studied molecular variability of *Trichoderma* species isolated from commercial products by using 19 RAPD primers of A series of which 15 primers produces 127 scorable bands, among them 121 bands were polymorphic and show 95.27% polymorphism.

Purnima Singh *et al*., (2020) studied morphological and molecular variability among different *Trichoderma* isolates by using 13 RAPD primers among which 6 primers produced consistently strong amplification products and polymorphic banding pattern. Selected 6 RAPD primers amplified a total of 26 loci. The band size of amplified fragments ranged from 100 to 220bp. Out of 26 bands, 8 were monomorphic (30.7%) and remaining 18 were polymorphic (69.2%).

2.5.3 Inter-Simple Sequence Repeats (ISSR)

Bornet *et al.,* (2002) described procedure with minor modification was used for carrying out the polymerase chain reaction (PCR) reaction for ISSR analysis. Nineteen primer were tested for amplification at different annealing temperatures of genomic DNA of the isolates. The PCR amplification was carried out with 25 ng of genomic DNA, 2.5 mM $MgCl_2$, 1 µM ISSR primer and 0.2 Mm dNTP mix. The volume was made up to 25 µl. PCR reaction were carried out in a Perkin Elmer Gene Amp 9600 thermocycler under the condition involving denaturation at 94ºC for 5 min; 30 cycles of denaturation at 94ºC for 1 min, primer annealing at temperature specific to each primer for 1 min and primer extension at 72ºC for 2 min; final extension step at 72ºC for 7 min.

Qian *et al.* (2006) used ISSR method to detect specific fragments among sample species, and conversion of the ISSR fragment into SCAR marker helped them to distinguish Sinocalycanthuschinensis, (an endangered species endemic to China, cultivated as an ornamental landscape tree in China) from its closely related species.

Kumar and Sharma (2011) used ISSR marker for characterization of twelve isolates belonging to *Trichoderma harzianum* and *Trichoderma viride*. Fifteen ISSR primers were screened and eight of them gave satisfactory amplification and band resolution which was taken for further study. The selected primers generated 70 ISSR bands and the size of the amplification products ranged from 100 to 950 bp. The per cent polymorphism ranged from 40 to 86. The mean value of the Jaccard's similarity coefficient of the ISSR marker was 0.76. The dendrogram separated the isolates into two major clusters with similarities ranging from 76 to 94 per cent. All the five *T. harzianum* isolates clustered into a single group sharing similarity around 80 per cent. The second major cluster consisted isolates of *T. viride*. However, these isolates formed two subgroups within the clusters.

Shahid *et al*. (2014) studied genetic determination of potential *Trichoderma* species Using ISSR Marker in Uttar Pradesh using seven *Trichoderma* spp. were collected from different locations of Uttar Pradesh, for evaluating their bioefficiency by determining their genetic variations. PCR-based Inter Simple Sequence Regions (ISSR) Marker employing 6 primers produced 30 scorable bands out of which 27 bands were polymorphic. The Unweighted Pair Group Method of Arithmetic Means (UPGMA) dendrogram constructed from Nei's genetic distance produced 2 main clusters. 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.

Ghutukade *et al*. (2015) Studied molecular characterization of *Trichoderma* isolates by ISSR marker they isolated twelve isolates belonging to *Trichoderma harzianum* and *Trichoderma viride* were assessed for their antagonistic effect on *Fusarium oxysporum* f.sp. *lycopearsicie* and *Xanthomonas campestris* pv. *vesicatoria. Trichoderma harzianum* isolates were more aggressive than *T. viride* isolates. The dataset generated through morphological characters and ISSR markers showed a comparable output grouping the isolates of *T. viride* in one cluster and all *T. harzianum* isolates in another cluster. It is obvious from

the present study that genetic diversity analysis had a positive correlation with the antagonistic ability of *Trichoderma* isolates.

2.5.4 Internal Transcript Spacer (ITS) Primer

Meyer *et al*. (1992) used a DNA fingerprinting technique to analyze the nine species aggregates of *Trichoderma* and recognized only five groups. The ITS 1 region of rDNA was amplified using biotinylated ITS 1 and ITS 2 primers 27 supplied by Operon.

Hermosa *et al*. (2001) Characterization of 16 biocontrol strains, previously *Trichoderma harzianum* ”Rifaias identified— and 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.

Latha *et al.,* (2002) done ITS1-5.8S-ITS2 region of the cDNA showed a band of 600bp and size variation was also observed. Restricted analysis of this region showed inter and intra specific polymorphic.

Agrawal and Kosthane (2009) amplified the Its region of 5.8 rDNA gene which yielded a fragment of 600-650 bp length in all the 29 isolates of *Trichoderma* which did not reveal inter and intra specific ITS length diversity. Cluster analysis performed on binary matrix derived following CAPS analysis of ITS1-ITS4 and ITS1-ALRO primer and pooled data showed overlapping species identification among the isolates. However, when the subpopulation was analyzed in combination it indicates association of ITS markers with morphological descriptors.

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 (2013) 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

During the course of present studies the details of material used and methods implemented are presented in this chapter. All the work on morphological and molecular characterization of *Trichoderma* isolates were carried out in the Department of Plant Pathology, Dr. PDKV, Akola.

3.1 Material

3.1.1 Glassware

Several types of glassware viz., petri plates (90mm and 100mm), conical flask (100ml, 250ml, 500ml, 1000ml), test tubes, micropipettes, beakers, slides, cover slips, glass rod, measuring cylinders (10ml, 100ml, 250ml, and 1000ml) were used.

3.1.2 Equipments

During present investigations, following laboratory equipments viz. autoclave, hot air oven, laminar air flow, BOD incubator (Remi), research microscope, stereoscopy microscope, digital weighing balance (Wensar HBT 516), centrifuge machine (Eppendorf 5810R), PCR machine (Eppendorf GR), gel electrophoresis (Genexy, Scie-Plas), gel doc machine, deep freezer-20^{0}C (Blue Star), freezer, digital camera were used.

3.1.3 Miscellaneous material

Inoculation needle, single and double distilled water, spirit lamp, spirit, scalper, forcep, cork borer (5mm), eppendorf tube, PCR tubes and tips, blotter paper, non-absorbent cotton, dissection needle, tray, rubber bands, mortar pestles, muslin cloths, polythene bags, forceps, pencil, permanent marker, Whitman filter paper, cello tapes, test tube stand, wash bottle, scissors etc. were used in present study.

3.2 Collection and maintenance of Fungal culture

Pure culture of *Trichoderma* isolates which is isolated from saline soil collected from Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. Pure culture of Trichoderma isolates were maintained on sterilized PDA slants in refrigerator for further use and

periodic transfer of the culture was carried out on sterilized PDA slants to keep the cultures in active growth.

Table 1. Pure cultures of eight *Trichoderma* isolates

Sr. No.	Trichoderma Isolated from	Districts	code
1	Daryapur	Amravati	TD-1
2	Telhara	Akola	TT-2
3	Chandur bazar	Amravati	TC-3
4	Sangrampur	Buldana	TS-4
5	Jalgaon	Buldana	TJ-5
6	Murtijapur	Akola	TM-6
7	Akola	Akola	TA-7
8	Achalpur	Amravati	TA-8

3.3 Methods

3.3.1 Sterilization of glass-wares

The glasswares (Borosil) viz; Petri plates, conical flask, beakers, pipettes, measuring cylinder and test tubes were used during research work. Glasswares were cleaned with cleaning solution (Detergents, Potassium dichromate solution) and repeated washing with tap water and followed by distilled water. The clean and dried glassware's were sterilized in hot air oven at 180°C for one hour before use. Distilled water and media were sterilized in autoclave at 15 lbs. for 15 minutes. 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.

3.3.2 Culture Media

Potato dextrose agar (PDA) and Potato dextrose broth (PDB) media were sterilized in autoclave at 121.6^0C (15lbs) for 15 min.

3.4. Preparation of media

3.4.1 Procedure for preparation of PDA media

- The composition of PDA medium is given in Table 2. 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 (121°C) for 15-20 minutes.

Table 2. The composition of Potato Dextrose Agar (PDA) 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

3.4.2 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.
- The slants were kept at half of their volume with media and kept slant.
- One cork borer of *Trichoderma* culture media was added to each test tube and kept for growth.
- After 5-6 days of growth slants were kept in free for further storage.

3.4.3 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^{0}C for 15 minutes at 15 lbs/in^2.
- These broth are inoculated with culture media wait for mycelium mat growth which is important input for extraction of DNA.

Table 3. The composition of Potato Dextrose Broth (PDB)

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

3.4.5 Molecular study

3.4.5.1 Chemicals

Different materials were used for study of molecular characterization of *Trichoderma* isolates given below

Table 4. Chemicals used for study of Molecular characterization of *Trichoderma* isolates

Sr. No.	Name of chemicals
1.	**DNA extraction**
1	β–mercaptoethanol
2	Boric acid
3	Cetyl Trimethyl 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
11	Sodium chloride
2.	**PCR Amplification**
1	10x taq buffer
2	$MgCl_2$ 25 ml
3	Taq DNA polymerase (5U/µl)
4	dNTP mix (100 Mm)
5	Primers
3.	**Agarose Gel Electrophoresis**
1	Agarose
2	Boric acid
3	EDTA Na_2
4	Ethidium bromide
5	Methanol
6	Tris buffer
7	DNA ladder 100bp and 1kb
4.	**Softwares used**
1	The UPGMA analysis
2	Alpha ease and NTSYS Pc

3.4.5.2 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.

3.4.5.3 Extraction of DNA

- Extraction buffer [2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mMTrisHCl, 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).
- 0.5 M EDTA Na2 (pH 8.0)

3.4.5.4 PCR Amplification

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

- 10 picomole/µl Oligonucleotide RAPD and ISSR primers
- 5 U/µl Taq DNA polymerase
- 10 X Taq buffer
- 25 mM $MgCl_2$
- 10mM dNTP

3.4.5.5 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)

3.4.6 Genetic variability analysis

In present investigations, *Trichoderma* isolates were screened for molecular variability using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR).

3.4.6.1 Preparation of mycelium mat

To get enough mycelium, pure culture of *Trichoderma* isolates which isolated from saline soil were mass multiplied by transferring mycelium tissue into 100 ml of PDB and kept for incubation inside BOD incubator at 27 ± 2°C for next 7 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.

3.4.6.2 Extraction of DNA

Genomic DNA was isolated from the 8 selected isolates by the Cetyl Dimethyl ethyl Ammonium Bromide (CTAB) method (Murray and Thompson, 1980) with some modifications.

3.4.6.3 Reagents required

(1) 1M Tris HCl (pH 8.0)

30.285 gram tris HCl dissolved in 150 ml of distilled water and volume 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 gm dissolved Na_2 EDTA in 150 ml of distilled water by adding NaOH pellets till the pH 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, pH 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 Na2 EDTA, pH 8.0)

2 ml of 1M Tris, pH 8.0 and 0.4 ml of 0.5 M Na2 EDTA, pH 8.0 was added to 197.6 ml of sterile distilled water.

3.4.6.4 Procedure for isolation of fungal genomic DNA

- Seven days old mycelial mat was transferred on sterilized blotter paper, air dried to remove moisture and media adhering to the mat.
- 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.
- The powder was immediately homogenized by adding pre-warmed (65^{0}C) extraction buffer and transferred to two ml eppendorf tubes.
- The content of tube was shaken vigorously for one minute for uniform mixture of suspension.
- These tubes were incubated in a water bath at 65^{0}C for one hour with gentle shaking at every 15 minutes.
- 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.
- 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.

- The content of the tubes were gently mix for five minutes and centrifuged for 15 minutes at 10000 rpm.
- The tube was mixed gently and centrifuged for five minutes as before. Aqueous phase transferred to another tube and equal volume of ice chilled Isopropanol was added. Centrifuged for 10 minutes at 12000 rpm and decanted the supernatant.
- The pellet was washed with 70 per cent ethanol twice and suspended in $T_{50}E_{10}$ buffer (50 mMTris-HCl, pH 8.0, 10 mM EDTA).
- The DNA solution was treated with RNase at 37^0C for 1 hr. 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.
- Aqueous phase was transferred to another tube and an equal volume of chloroform was added.
- The aqueous layer was separated and DNA was precipitated by adding 2.5 volume of absolute ethanol.
- The pellet (genomic DNA) was washed twice with 70 per cent ethanol and re suspended in TE buffer (10 mMTrisHCl, 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 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.

Table 5. PCR reaction mix for 1x of 12.5 µSl reaction

Sr. No.	Master mix	1x
1	10x buffer	1.25 µl
2	$MgCl_2$(25Mm)	1.25 µl
3	dNTPs (10Mm)	0.3 µl
4	Taq polymerase	0.3 µl
5	Sterilized distilled water	5.4 µl
	Total volume	**8.5 µl**

Table 6. Constituents of PCR reaction

Sr. no.	Master mix	Quantity
1	Master mix volume	8.5 µl
2	Primer (forward)	1.0 µl
3	Primer (reverse)	1.0 µl
4	Template DNA (37.5ng)	2.0 µl
	Total reaction volume	**12.5** µl

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

3.4.6.5 Internal Transcribed Spacer (ITS) amplification

Genetic variability of Trichoderma isolates which isolated from saline soil was evaluated by using ITS primers.

Table 7. List of ITS primers used with their sequences (Genaxy)

Oligo Name	Primer sequence (5'-3')	GC%
ITS 1	TCCGTAGGTGAACCTGCGG	63
ITS4	TCCTCCGCTTATTGATATGC	50

Table 8. List of RAPD primer used with their sequences (Genaxy)

Sr no	Primers	Primer sequence	Annealing temperature(^{0}C)
1	OPA-2	TGCCGAGCTG	37
2	OPA-3	AGTCAGCCAC	37
3	OPA5	AGGGGTCTTG	37
4	OPA-9	GGGTAACGCC	37
5	OPA-10	GTGATCGCAG	37
6	OPA-13	CAGCACCCAC	37
7	OPA-15	TTCCGAACCC	37
8	OPA-16	AGCCAGCGAA	37
9	OPA-20	GTTGCGATCC	37

Table 9. List of ISSR primer used with their sequences (Genaxy)

Sr. no.	Primers	Primers sequence	Annealing temperature(^{0}C)
1	ISSR811	GAGAGAGAGAGAGAGAC	57
2	GA8YC	GAGAGAGAGAGAGAGAGCC	55
3	AG8YC	AGAGAGAGAGAGAGA	55
4	AC8YT	ACACACACACACACACGCT	55

3.4.6.6 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 given in table 5 and 6.
- 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.
- 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 steps		Temperature	Time
Initial denaturation		94^{0}C	5min.
30 cycles	Denaturation	94^{0}C	1min.
	Annealing	55^{0}C	1min.
	Extension	72^{0}C	30seconds
Final extension		72^{0}C	10min.

Table 11. Steps used for PCR-RAPD reaction

Name of steps		Temperature	Time
Initial denaturation		94^0C	5min.
35 cycles	denaturation	94^0C	1min.
	annealing	37^0C	45sec.
	extension	72^0C	2min.
Final extension		72^0C	10min.
Hold		4^0C	

Table 12. Steps used for PCR- ISSR markers reaction

Name of steps		Temperature	Time
Initial denaturation		94^0C	10 min.
35 cycles	denaturation	94^0C	45sec.
	annealing	50^0C*	45sec.
	extension	72^0C	2min
Final extension		72^0C	10min.

* Annealing temperature varied from primer to primer given in table above.

3.4.6.7 Separation of RAPD-PCR, ITS and ISSR 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. 2% agarose gel solution was prepared in 1x TBE Buffer.

- Ethidium bromide was added to the luke warm liquify 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

3.4.6.8 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 (Rohlf, 2000). Dendrogram by Unweighted pair group method of arithmetic average (UPGMA) using the SAHN (Sequential Agglomerative Hierarical Nested) cluster analysis module (Rohlf, 2000).

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

CHAPTER IV

RESULTS AND DISCUSSION

The present investigation was conducted on “Molecular characterization of *Trichoderma* spp. isolated from saline soil” with an objective “to analyze the molecular variability of *Trichoderma* spp. isolated from saline soil”. This result and discussion chapter includes results of various experiments conducted during the study are presented below.

4.1 collection of pure culture of Trichoderma isolates

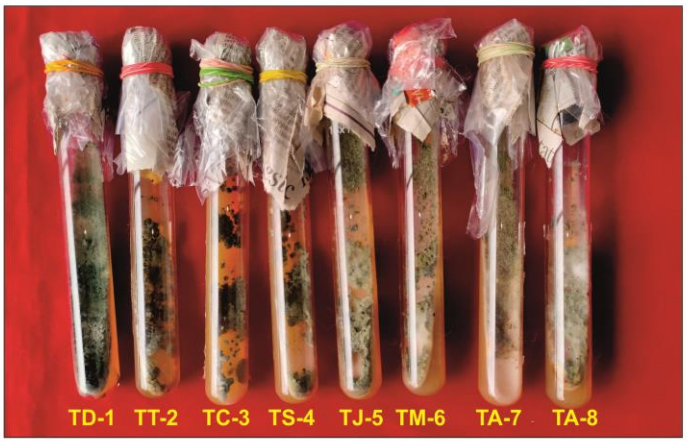
Trichoderma isolates which was isolated from saline soil tract of purna river vally located in Akola, Amravati and Buldana districts, collected from Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, for further study.

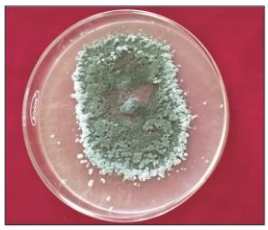
Table 13. Pure culture of Eight *Trichoderma* isolates

Sr. No.	Trichoderma Isolated from	Districts	code
1	Daryapur	Amravati	TD-1
2	Telhara	Akola	TT-2
3	Chandur bazar	Amravati	TC-3
4	Sangrampur	Buldana	TS-4
5	Jalgaon	Buldana	TJ-5
6	Murtijapur	Akola	TM-6
7	Akola	Akola	TA-7
8	Achalpur	Amravati	TA-8

4.2 Molecular study

Molecular variability of Trichoderma isolates was studied by using Randomized Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat markers (ISSR). These isolates were coded and confirmed as *Trichoderma* by using Internal Transcript Spacer (ITS) markers.





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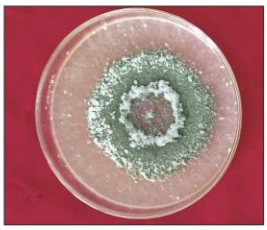

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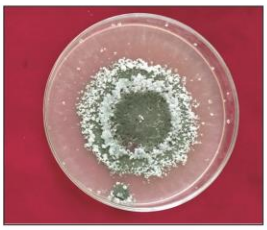

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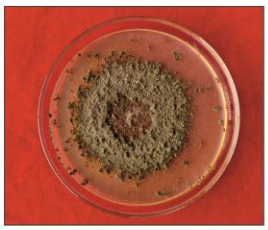
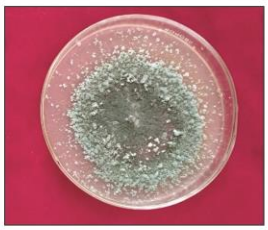


TS-4



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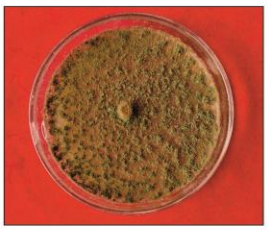
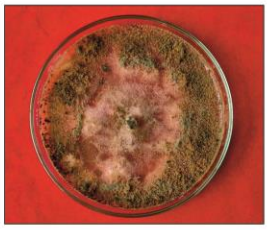
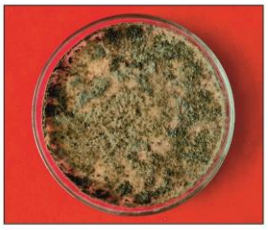


TM-6



TA-7



TA-8

Plate 1 Pure culture of *Trichoderma* isolates

Plate 2 The growth of *Trichoderma* isolates on PDA broth

4.2.1 ITS (Internal Transcript Spacer) Analysis

For the confirmation of Trichoderma isolates which isolated from saline soil was done using ITS-1 (TCCGTAGGTGAACCTGCGG) and ITS-4 (TCCTCCGCTTATTGATATGC) primer which gives band size in the range of 500bp to 600bp (Plate-3). The result are in accordance with Chakraborty (2010) who studied the identification and genetic variability of Trichoderma isolates which observed amplified DNA fragment approximately 600bp.

4.2.2 Primers selected for RAPD marker study

During present study total nine RAPD primers were randomly selected and screened of OPA series to evaluate genetic variability of isolates. PCR (Polymerase Chain Reaction) was programmed with an initial denaturation at 94^0C for 3 min. followed by 35 cycle of denaturation at 94^0C for 1 min., annealing at 37^0C for 45 sec. and extension at 72^0C for2 min. and the final extension at 72^0C for 10 min. The PCR amplified product of each primer were resolved on 1.5% agarose gel electrophoresis and the size of the amplified product was compared with 1kb DNA ladder.

Table 14. Per cent Polymorphism Observed in RAPD Primer

Sr. No.	Primers	Monomorphic amplicons	Polymorphic amplicon	Total amplicon	% polymorphism
1	OPA-2	0	6	6	100
2	OPA-3	0	7	7	100
3	OPA-5	1	8	9	88.88
4	OPA-9	0	7	7	100
5	OPA-10	2	4	6	66.66
6	OPA-13	2	5	7	71.42
7	OPA-15	0	9	9	100
8	OPA-16	0	5	5	100
9	OPA-20	1	4	5	80.00

All primers produced reproducible and scorable bands with high polymorphism. Out of these, five primers showed polymorphic bands, whereas remaining four primers namely OPA-5, OPA-13, OPA-10 and

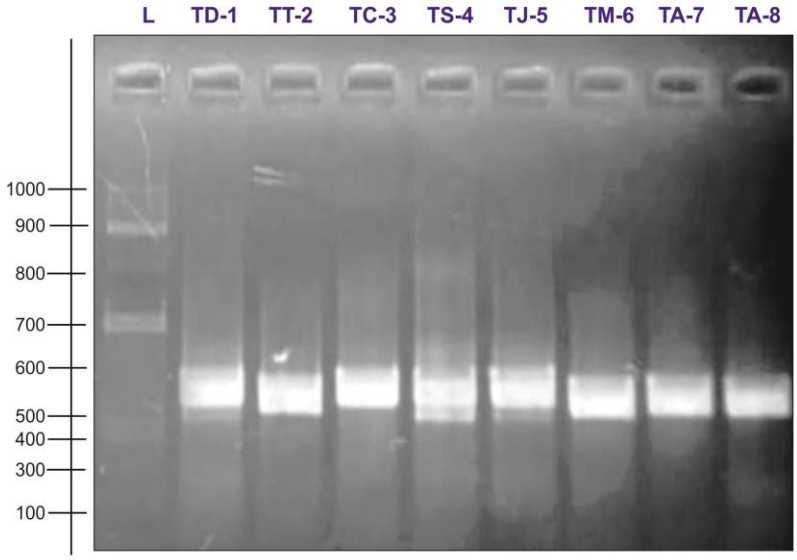


Plate 3 Confirmation of *Trichoderma* isolates by using ITS marker

OPA-20 showed monomorphic band. Total of 61 amplicons were obtained, of which, 57 amplicons were found to be polymorphic and the level of polymorphism was 93.44%. (Table14)

4.2.2.1 RAPD banding pattern

The variability observed in Trichoderma isolates and banding pattern observed in primer OPA-2 is presented in Plate 4-. The primer amplified 6 amplicons. The size of amplicons with primer OPA-2 ranged from 250bp to 1700bp. The observed polymorphism in this primer was 100%. The details of 6 RAPD band type are as follows:

Sr. No.	Amplicon Type	Specific Amplicons	Total number of specific amplicon
1	1700bp	TD-1,TC-3,TS-4,TJ-5, TM-6	5
2	1400bp	TD-1,TC-3,TS-4, TJ-5, TM-6, TA-8	6
3	1000bp	TT-2, TS-4, TJ-5, TA-7, TA-8	5
4	700bp	TM-6	1
5	600bp	TT-2	1
6	250bp	TT-2, TM-6	2

The banding pattern observed in primer OPA-3 is presented in Plate-. The primer amplified 17 amplicons. The size of amplicons with primer OPA-3 ranged from 250bp to 1500bp. The observed polymorphism in this primer was 100%. The details of 7 RAPD band type are as follows:

Sr. No.	Amplicon type	Specific amplicons	Total number of specific amplicons
1	1500bp	TD-1, TC-3, TS-4, TJ-5, TM-6	5
2	1100bp	TD-1, TC-3, TS-4, TJ-5, TM-6, TA-8	6
3	900bp	TT-2, TA-7, TA-8	3
4	750bp	TD-1, TC-3, TS-4 TJ-5, TM-6	5
5	600bp	TD-1, TC-3, TS-4 TJ-5, TM-6	5
6	500bp	TM-6, TA-7, TA-8	3
7	250bp	TD-1, TC-3, TS-4, TJ-5, TA-7, TA-8	6

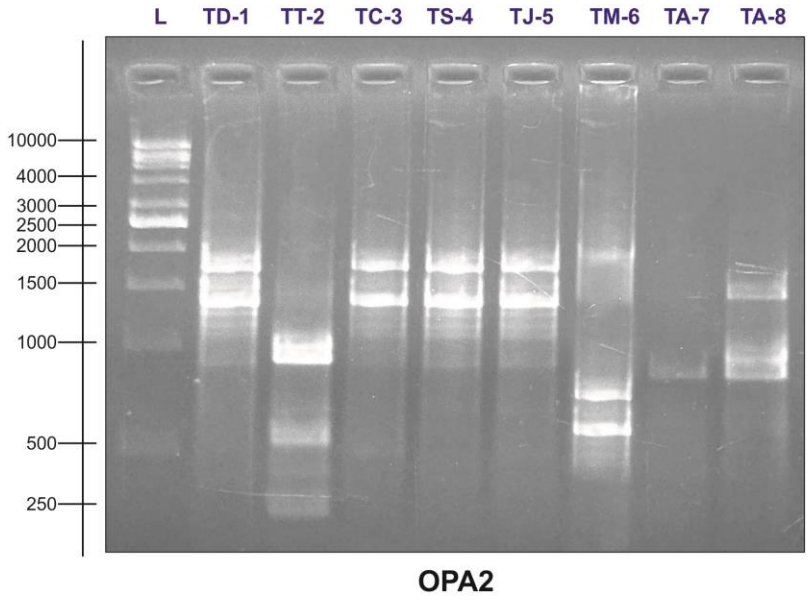


Plate 4 RAPD banding pattern of primer OPA-2 and OPA-3

The banding pattern observed in primer OPA-5 is presented in Plate-. The primer amplified 9 amplicons. The size of amplicons with primer OPA-5 ranged from 250bp to 2000bp. The observed polymorphism in this primer was 88.88%. The details of 9 RAPD band type are as follows:

Sr. No.	Amplicon type	Specific amplicons	Total number of specific amplicons
1	2000bp	TM-6	1
2	1700bp	TM-6	1
3	1300bp	TD-1, TC-3, TT-2, TS-4, TJ-5	5
4	900bp	TD-1, TT-2, TJ-5, TA-8	4
5	800bp	TC-3, TA-8	2
6	750bp	TD-1, TM-6	2
7	500bp	TD-1, TT-2, TS-4, TJ-5, TM-6	5
8	350bp	TD-1, TT-2, TS-4, TJ-5, TM-6	5
9	250bp	TM-6	1

The banding pattern observed in primer OPA-9 is presented in Plate-. The primer amplified 7 amplicons. The size of amplicons with primer OPA-9 ranged from 250bp to 2500bp. The observed polymorphism in this primer was 100%. The details of 7 RAPD band type are as follows:

Sr. No.	Amplicon type	Specific amplicons	Total number of specific amplicons
1	2500bp	TC-3, TA-8	2
2	1900bp	TD-1, TC-3, TS-4, TJ-5, TM-6	5
3	1600bp	TD-1, TC-3, TS-4, TJ-5, TM-6, TA-8	6
4	1300bp	TD-1, TC-3, TS-4, TJ-5, TA-8	5
5	700bp	TC-3, TM-6, TA-7, TA-8	4
6	500bp	TD-1, TS-4, TJ-5, TM-6	3
7	250bp	TM-6	1

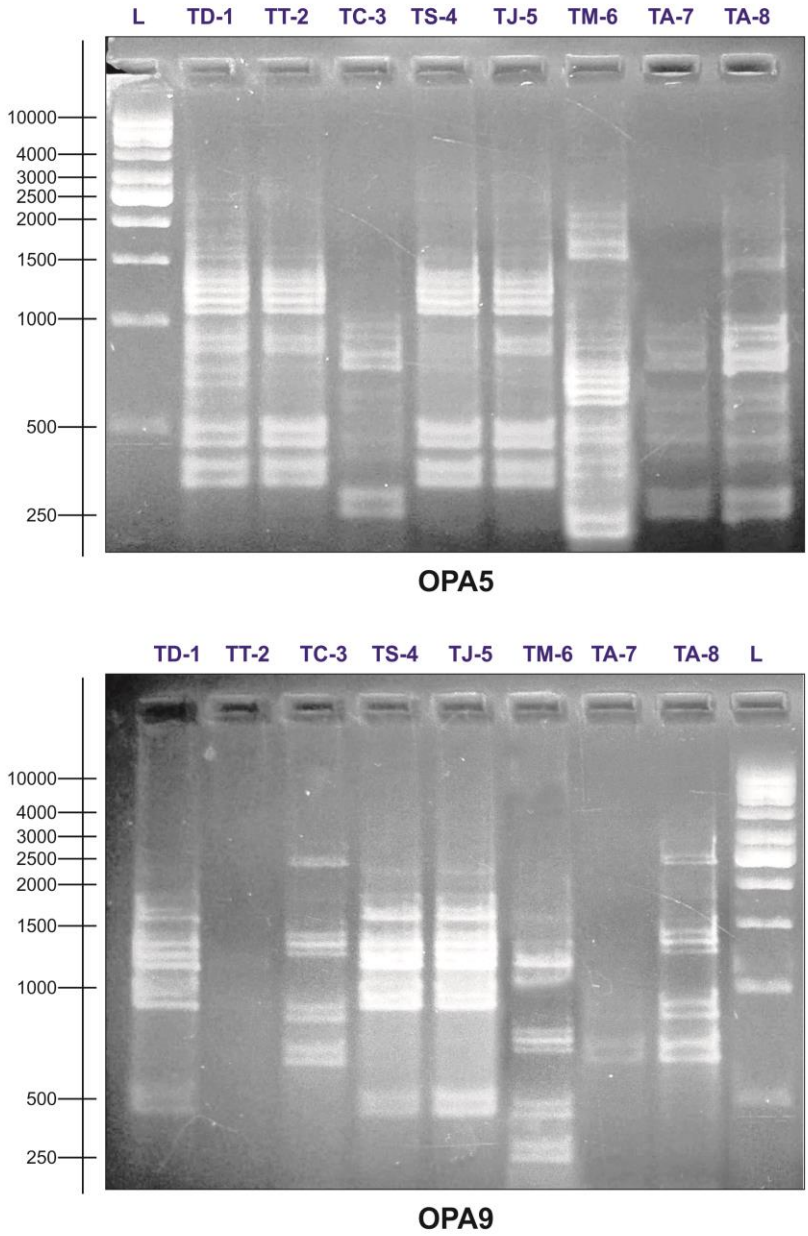


Plate 5 RAPD banding pattern of primer OPA-5 and OPA-9

The banding pattern observed in primer OPA-10 is presented in Plate-. The primer amplified 6 amplicons. The size of amplicons with primer OPA-10 ranged from 250bp to 2500bp. The observed polymorphism in this primer was 66.66%. The details of 6 RAPD band type are as follows:

Sr. No.	Amplicon type	Specific amplicons	Total number of specific amplicons
1	2500bp	TD-1, TT-2, TC-3, TS-4, TJ-5	5
2	1800bp	TD-1, TT-2, TC-3, TS-4, TJ-5, TA-8	6
3	1400bp	TD-1, TT-2, TC-3, TS-4, TJ-5, TM-6, TA-7, TA-8	8
4	1000bp	TD-1, TT-2, TC-3, TS-4, TJ-5, TM-6, TA-7, TA-8	8
5	500bp	TD-1, TT-2, TC-3, TS-4, TJ-5, TA-7, TA-8	7
6	250bp	TA-7, TA-8	2

The banding pattern observed in primer OPA-13 is presented in Plate-. The primer amplified 7 amplicons. The size of amplicons with primer OPA-13 ranged from 400bp to 3000bp. The observed polymorphism in this primer was 71.42%. The details of 7 RAPD band type are as follows:

Sr. No.	Amplicon type	Specific amplicons	Total number of specific amplicons
1	3000bp	TM-6	1
2	2500bp	TM-6	1
3	1300bp	TC-3, TM-6	2
4	900bp	TD-1, TT-2, TC-3, TS-4, TJ-5, TM-6, TA-7, TA-8	8
5	700bp	TD-1, TC-3, TS-4, TJ-5, TA-7, TA-8	6
6	600bp	TA-7, TA-8	2
7	400bp	TC-3, TM-6	2

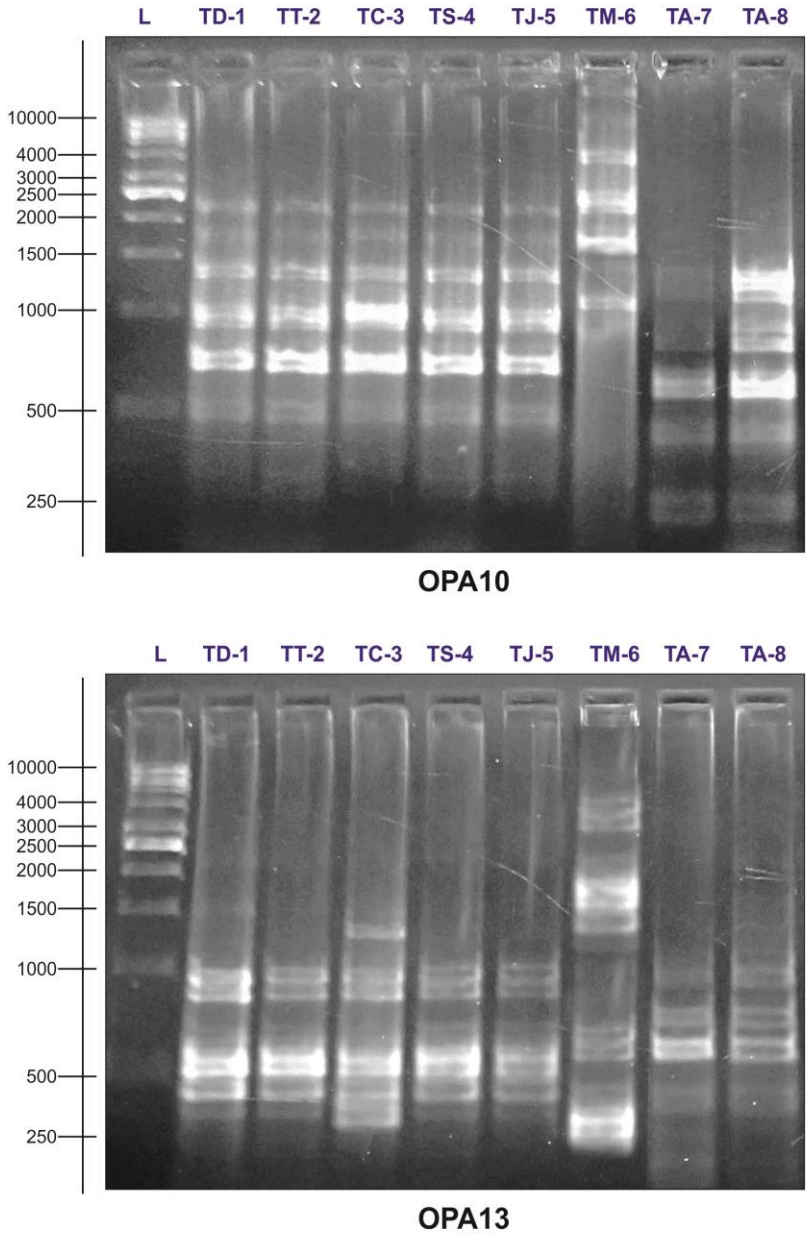


Plate 6 RAPD banding pattern of primer OPA-10 and OPA-13

The banding pattern observed in primer OPA-15 is presented in Plate-. The primer amplified 9 amplicons. The size of amplicons with primer OPA-15 ranged from 350bp to 3500bp. The observed polymorphism in this primer was 100%. The details of 7 RAPD band type are as follows:

Sr. No.	Amplicon type	Specific amplicons	Total number of specific amplicons
1	3500bp	TS-4, TJ-5, TM-6	3
2	2500bp	TD-1, TC-3, TS-4, TJ-5, TA-7, TA-8	6
3	1500bp	TC-3, TS-4, TJ-5, TA-8	4
4	1300bp	TT-2, TM-6	2
5	1000bp	TC-3, TS-4, TJ-5	3
6	900bp	TT-2, TJ-6, TA-8	3
7	750bp	TM-6	1
8	500bp	TD-1, TC-3, TJ-5	3
9	350bp	TS-4, TJ-5	2

The banding pattern observed in primer OPA-16 is presented in Plate-. The primer amplified 5 amplicons. The size of amplicons with primer OPA-16 ranged from 800bp to 4000bp. The observed polymorphism in this primer was 100%. The details of 5 RAPD band type are as follows:

Sr. No.	Amplicon type	Specific amplicons	Total number of specific amplicons
1	4000bp	TM-6	1
2	3000bp	TD-1, TS-4, TJ-5, TM-6	4
3	2500bp	TD-1, TT-2, TC-3, TS-4, TJ-5	5
4	1300bp	TD-1, TT-2, TC-3, TS-4, TJ-5, TA-8	6
5	800bp	TD-1, TC-3, TS-4, TJ-5, TA-8	5

The banding pattern observed in primer OPA-20 is presented in Plate-. The primer amplified 5 amplicons. The size of amplicons with

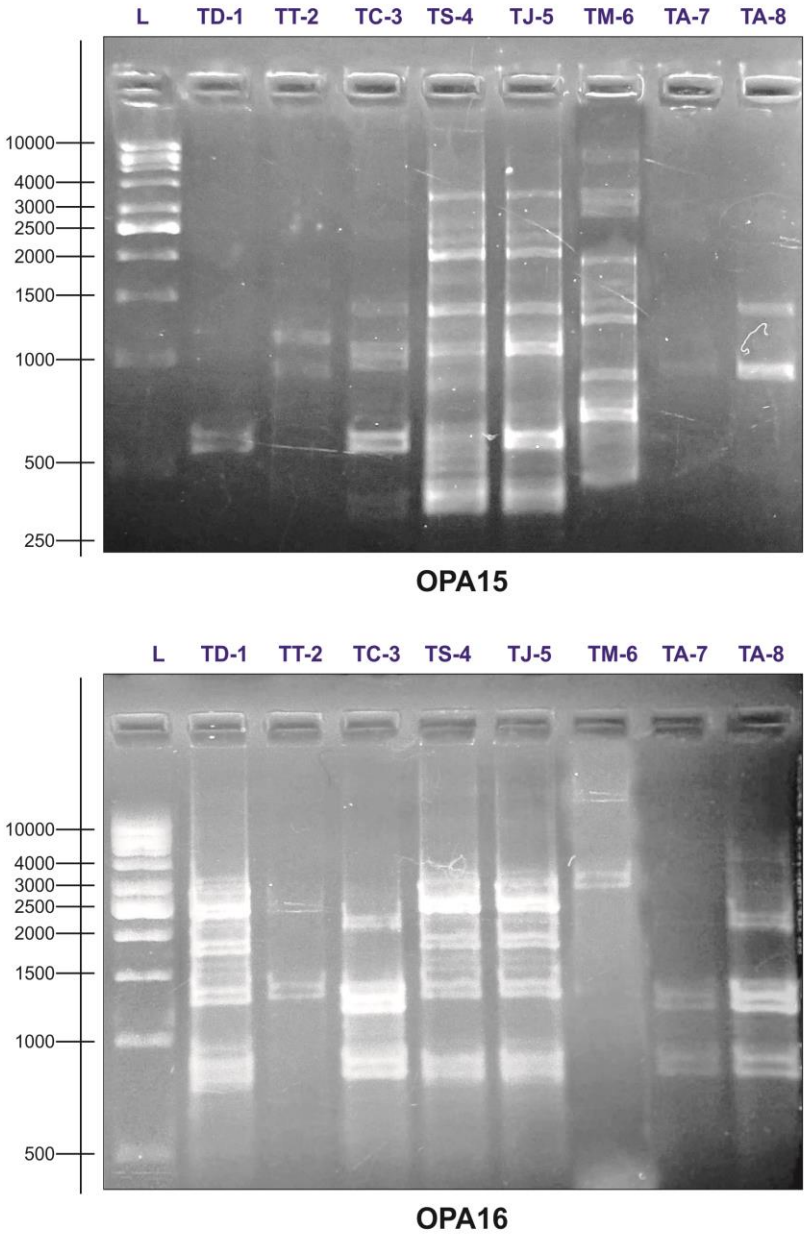


Plate 7 RAPD banding pattern of primer OPA-15 and OPA-16

primer OPA-20 ranged from 500bp to 2000bp. The observed polymorphism in this primer was 80%. The details of 6 RAPD band type are as follows:

Sr. No.	Amplicon type	Specific amplicons	Total number of specific amplicons
1	2000bp	TD-1, TS-4, TJ-5	3
2	1800bp	TD-1, TT-2, TS-4, TJ-5, TM-6	5
3	1300bp	TD-1, TT-2, TS-4, TJ-5	4
4	750bp	TD-1, TT-2, TS-4, TJ-5	4
5	500bp	TD-1, TT-2, TS-4, TJ-5, TA-7, TA-8	6

4.2.2.2 Binary similarity matrix

4.2.2.2.1. Binary similarity matrix for RAPD analysis

Binary similarity matrix of combined data from 9 RAPD primers of *Trichoderma* isolates were prepared by scoring presence or absence of band. The same molecular weight was assumed to be identical.

Table 15. Similarity coefficient for RAPD analysis

	TD-1	TT-2	TC-3	TS-4	TJ-5	TM-6	TA-7	TA-8
TD-1	1	0.447	0.538	0.744	0.775	0.314	0.184	0.357
TT-2	0.447	1	0.244	0.405	0.409	0.208	0.286	0.333
TC-3	0.538	0.244	1	0.524	0.558	0.260	0.242	0.514
TS-4	0.744	0.405	0.524	1	0.923	0.340	0.195	0.356
TJ-5	0.775	0.409	0.558	0.923	1	0.345	0.209	0.391
TM-6	0.314	0.208	0.260	0.340	0.345	1	0.143	0.176
TA-7	0.184	0.286	0.242	0.195	0.209	0.143	1	0.520
TA-8	0.357	0.333	0.514	0.356	0.391	0.176	0.520	1

On the basis of calculated similarity matrix the similarity between genotypes can be predicted. The genotypes showing similarity index “1” are presumed to be 100% similar while that of “0” are 100% genetically dissimilar. In present study the similarity coefficient value ranged from 0.923 to 0.143 across Trichoderma isolates indicating high

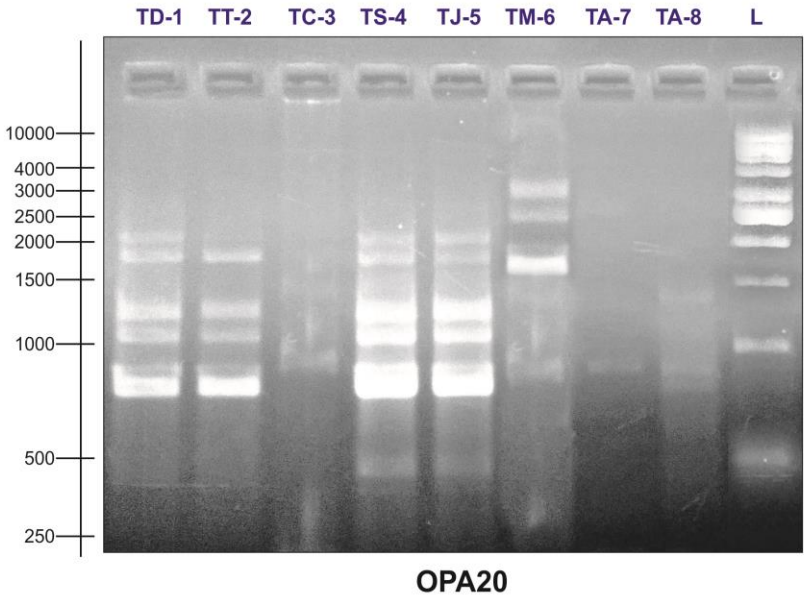


Plate 8 RAPD banding pattern of primer OPA-20

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 the form of dendrogram (Fig.1). Jaccard's coefficient value Trichoderma isolates presented in Table 15.

From the dendrogram that was analyzed, higher value of similarity coefficient 0.923 whereas 0.143 was found to have lower value of similarity coefficient. Three major clusters were obtained on the basis of analysis. First group is named as cluster –A, includes TD-1, TT-2, TC-3, TS-4 and TJ-5. Second group is named as cluster –B which includeTM-6. Third group is named as cluster -C which includes TA-7 and TA-8.

Table 16. Cophenetic distance matrix for all *Trichoderma* isolates in RAPD primer

	TD-1	TT-2	TC-3	TS-4	TJ-5	TM-6	TA-7	TA-8
TD-1	0.000	0.376	0.540	0.759	0.759	0.255	0.307	0.307
TT-2	0.376	0.000	0.376	0.376	0.376	0.255	0.307	0.307
TC-3	0.540	0.376	0.000	0.540	0.540	0.255	0.307	0.307
TS-4	0.759	0.376	0.540	0.000	0.923	0.255	0.307	0.307
TJ-5	0.759	0.376	0.540	0.923	0.000	0.255	0.307	0.307
TM-6	0.255	0.255	0.255	0.255	0.255	0.000	0.255	0.255
TA-7	0.307	0.307	0.307	0.307	0.307	0.255	0.000	0.520
TA-8	0.307	0.307	0.307	0.307	0.307	0.255	0.520	0.000

Cophenetic correlation**:** **-0.915**

From the above table the cophenetic distance matrix which is used to show how to objects or observations have to be in order to be grouped in to same cluster demonstrates the maximum and minimum value 0.923 and 0.255 respectively. According to the result TS-4 was found to have a higher Cophenetic distance matrix with TJ-5 **(0.923)** and TM-6 with all treatments **(0.255).**

Table 17. Results by classes for Jaccard's Similarity coefficient in RAPD primers

Class	1	2	3
Objects	5	1	2
Sum of weights	5	1	2
Within-class variance	9.100	0.000	6.000
Minimum distance to centroid	2.030	0.000	1.732
Average distance to centroid	2.610	0.000	1.732
Maximum distance to centroid	3.650	0.000	1.732
	TD1	TM6	TA7
	TT2 TC3 TS4 TJ5		TA8

According to the finding and as illustrated in the above table there are three classes that were grouped within three clusters which contain different isolates. From the result sum of weight is higher in cluster one (A)(5) and lower some of weight is in cluster two (B)(1). The maximum and minimum within class variance were observed in cluster one (A)(9.100) and two (B)(0.000) respectively. The maximum distance to centroid and the minimum distance to centroid were observed in cluster one (A)(3.650) and cluster two (B)(0.000) respectively.

Table 18. Summary statistics of RAPD primers for *Trichoderma* isolates

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
TD-1	61	0	61	0.000	1.000	0.525	**0.504**
TT-2	61	0	61	0.000	1.000	0.377	0.489
TC-3	61	0	61	0.000	1.000	0.459	0.502
TS-4	61	0	1	0.000	1.000	0.590	0.496
TJ-5	61	0	61	0.000	1.000	**0.639**	0.484
TM-6	61	0	61	0.000	1.000	0.574	0.499
TA-7	61	0	61	0.000	1.000	**0.213**	**0.413**
TA-8	61	0	61	0.000	1.000	0.410	0.496

In parallel study, Chakraborty *et al*. (2010) studied the genetic relatedness among eleven isolates of T. viride and eight isolates of T. harzianum 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.

Gurumurthy *et al*. (2013) 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. 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.

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.

Shahbazi *et al.* (2017) study the effects of irradiation on genetic diversity in mutated isolates of *Trichoderma* spp. RAPD (molecular marker) was used. To induce mutation in these two *Trichoderma* species (*T. viride and T.harzianum*). The phenotypic data showed that, gamma irradiation effects on mycelial growth, color and colony shape and spore reproduction in Trichoderma. . The results of RAPD marker at 84% similarity, divided T. viride mutant to 12, 16, 14, 20 and 19 Group respectively. Dendrograms by using UPGMA method based on Jaccard's

similarity coefficient at similarity level of 84%, were divided *T. harzianum* mutated isolates into three groups.

Radhika Deshmukh *et al*. (2018) studied molecular variability of *Trichoderma* mother culture and its mutant by using RAPD marker. 20 RAPD primers of OPA series were tested, of which tested 14 primers produced 72 scorable bands among them 52 bands were polymorphic and level of polymorphic was upto 72%.

Meshu *et al.,* (2019) studied molecular variability of *Trichoderma* species isolated from commercial products by using 19 RAPD primers of A series of which 15 primers produces 127 scorable bands, among them 121 bands were polymorphic and show 95.27% polymorphism.

4.2.3 Inter Simple Sequence Repeat (ISSR)

During the present investigations, five ISSR primer were screened to evaluate the genetic variability among the Trichoderma isolates. The Polymerase Chain Reaction (PCR) amplified products of each primer were resolved on 1.5 per cent agarose. The size of amplicons generated from the PCR reaction was compared with 1kb and 100bp ladder.

Table19. Percent polymorphism observed in ISSR primer

Sr. No.	Primers	Total amplicons	Polymorphic amplicons	% polymorphism
1	AG8YC	7	7	100
2	ISSR811	8	7	87.5
3	GA8YC	7	7	100
4	AC8YT	8	7	87.5
	Total	**30**	**28**	**93.33**

4.2.3.1 ISSR banding pattern

The banding pattern was observed in all 5 ISSR primers. The variability observed in *Trichoderma* isolates and the banding pattern is given below.

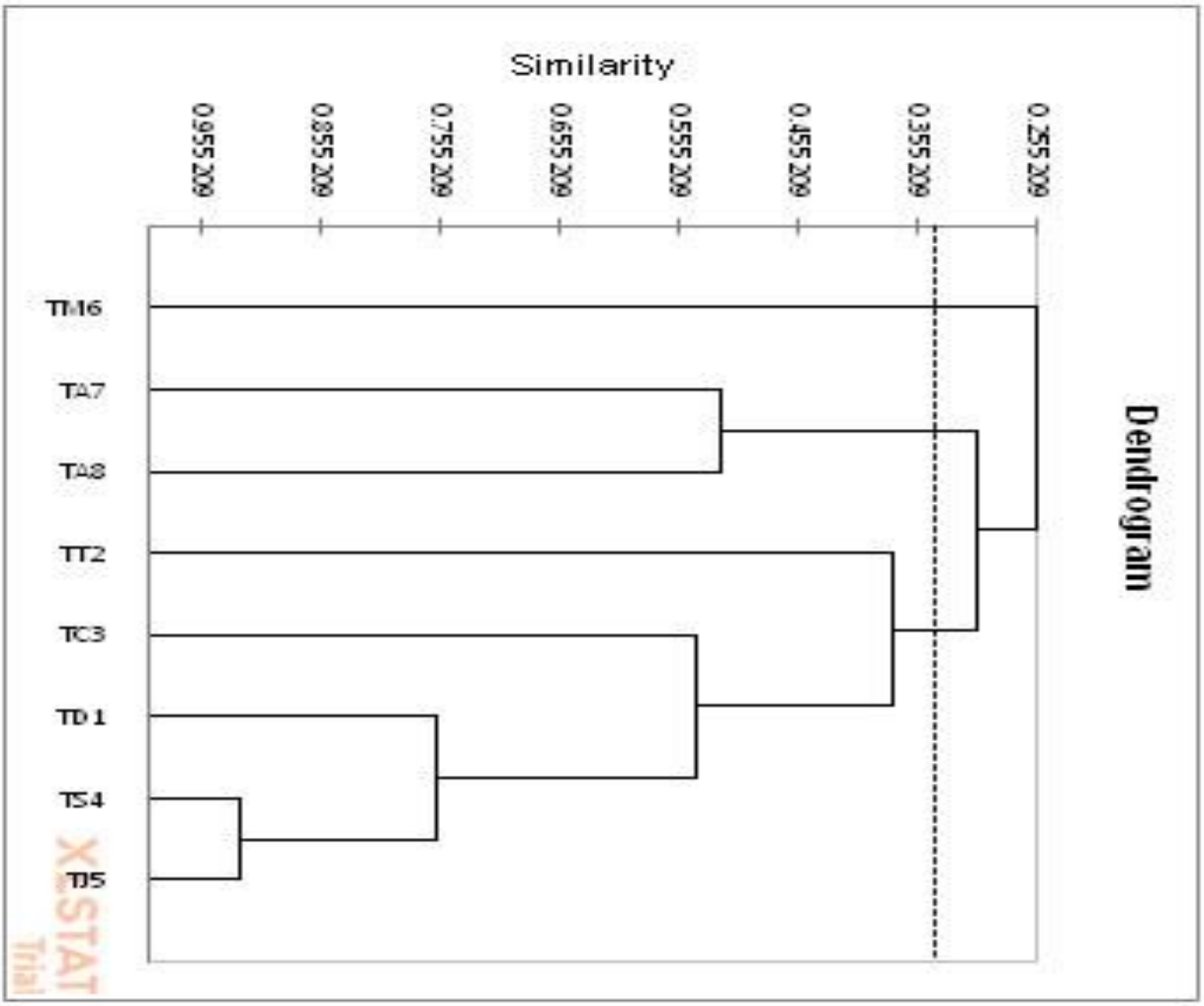


Fig. 1 RAPD UPGMA dendrogram of RAPD analysis of *Trichoderma* isolates based on Jaccard's Coefficient

The banding pattern observed in primers ISSR 811 showed in (Plate 9). The size of amplicons amplified with primer ranged from 300bp to2500bp. The polymorphism observed in this primer was 87.5%. The details of the eight bands are as follows :

Sr. No.	Amplicon type	Specific amplicons	Total number of specific amplicons
1	2500bp	TD-1, TT-2, TS-4, TJ-5	4
2	2000bp	TD-1, TS-4, TJ-5	3
3	1500bp	TT-2, TC-3, TA-7, TA-8	4
4	1200bp	TD-1, TT-2, TC-3, TS-4, TJ-5, TM-6, TA-7	7
5	800bp	TA-7	1
6	700bp	TD-1, TS-4, TJ-5, TM-6, TA-7	5
7	500bp	TT-2, TC-3, TA-7, TA-8	4
8	300bp	TM-6, TA-7	2

The banding pattern observed in primers GA8YC showed in (Plate 9). The size of amplicons amplified with primer ranged from 250bp to1000bp. The polymorphism observed in this primer was 100%. The details of the seven bands are as follows :

Sr. No.	Amplicon type	Specific amplicons	Total number of specific amplicons
1	1000bp	TD-1, TS-4, TJ-5, TM-6, TA-7	5
2	850bp	TT-2, TC-3, TA-7, TA-8	4
3	800bp	TD-1, TC-3, TS-4, TJ-5	4
4	650bp	TD-1, TS-4, TJ-5, TM-6, TA-7	5
5	500bp	TM-6	1
6	350bp	TD-1, TS-4, TJ-5	3
7	250bp	TD-1, TS-4, TJ-5, TM-6	4

The banding pattern observed in primers AG8YC showed in (Plate 10). The size of amplicons amplified with primer ranged from 250bp

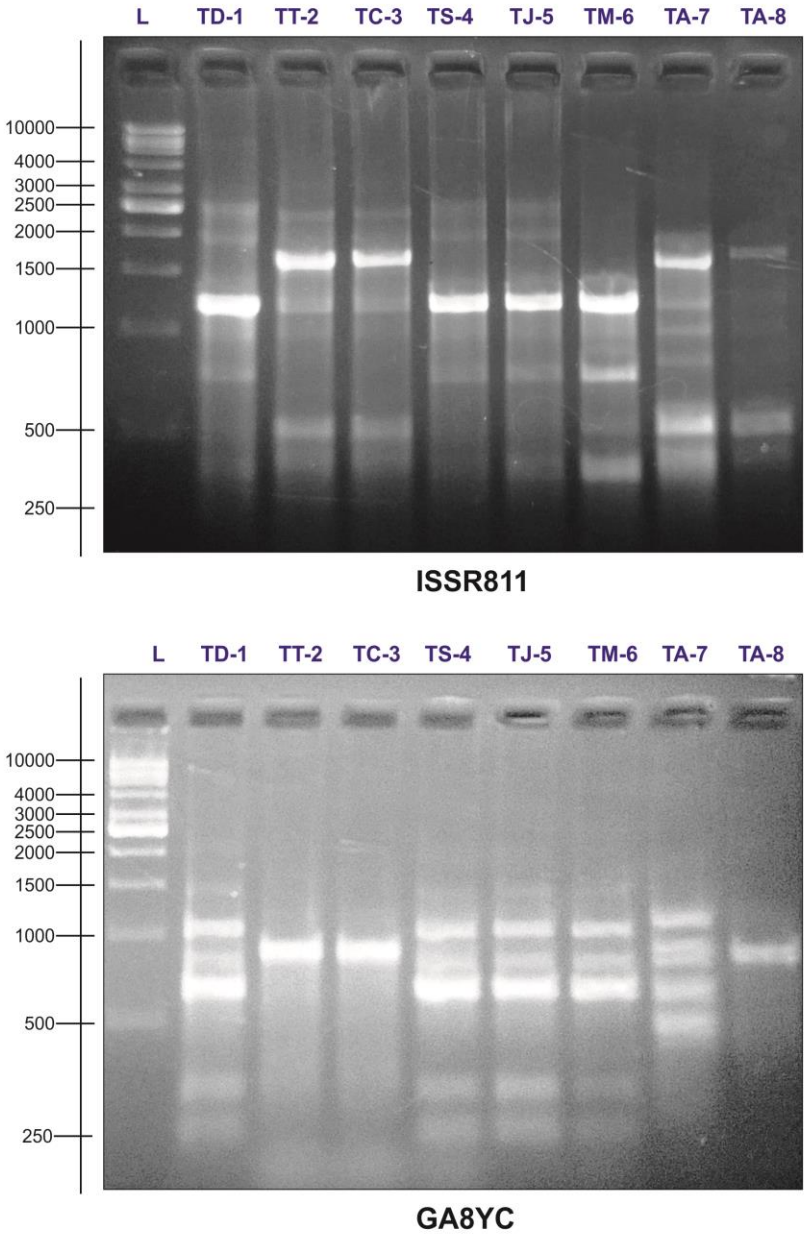


Plate 9 ISSR banding pattern of primer ISSR811 and GA8YC

to1500bp. The polymorphism observed in this primer was 100%. The details of the seven bands are as follows :

Sr. No.	Amplicon type	Specific amplicons	Total number of specific amplicons
1	1500bp	TA-7	1
2	1200bp	TT-2, TC-3 TA-8	3
3	1000bp	TD-1, TD-4, TJ-5, TM-6	4
4	900bp	TS-4, TJ-5	2
5	700bp	TT-2, TC-3, TA-7, TA-8	4
6	500bp	TS-4, TJ-5, TM-6	3
7	250bp	TT-2, TC-3, TA-7, TA-8	4

The banding pattern observed in primers AC8YT showed in (Plate 10). The size of amplicons amplified with primer ranged from 150bp to850bp. The polymorphism observed in this primer was 87.5. The details of the eight bands are as follows :

Sr. No.	Amplicon type	Specific amplicons	Total number of specific amplicons
1	2300bp	TD-1, TC-3, TS-4, TA-8	4
2	2000bp	TD-1, TC-3, TJ-5, TA-8	4
3	1400bp	TT-2, TS-4, TJ-5, TM-6, TA-8	5
4	1000bp	TT-2, TM-6	2
5	900bp	TD-1, TC-3, TS-4, TA-7	4
6	600bp	TD-1, TC-3, TS-4, TJ-5, TA-8	5
7	500bp	TT-2	1
8	250bp	TT-2	1

4.2.3.1. Binary similarity matrix and Dendrogram

On the basis of calculated similarity matrix the similarity between genotypes can be predicted. The genotypes showing similarity index “1” are presumed to be 100% similar while that of “0” are 100% genetically dissimilar. In present study the similarity coefficient value ranged from 0.048 to 0.824 across *Trichoderma* isolates indicating high degree of polymorphism in respect to genetic similarity. Genetic similarity

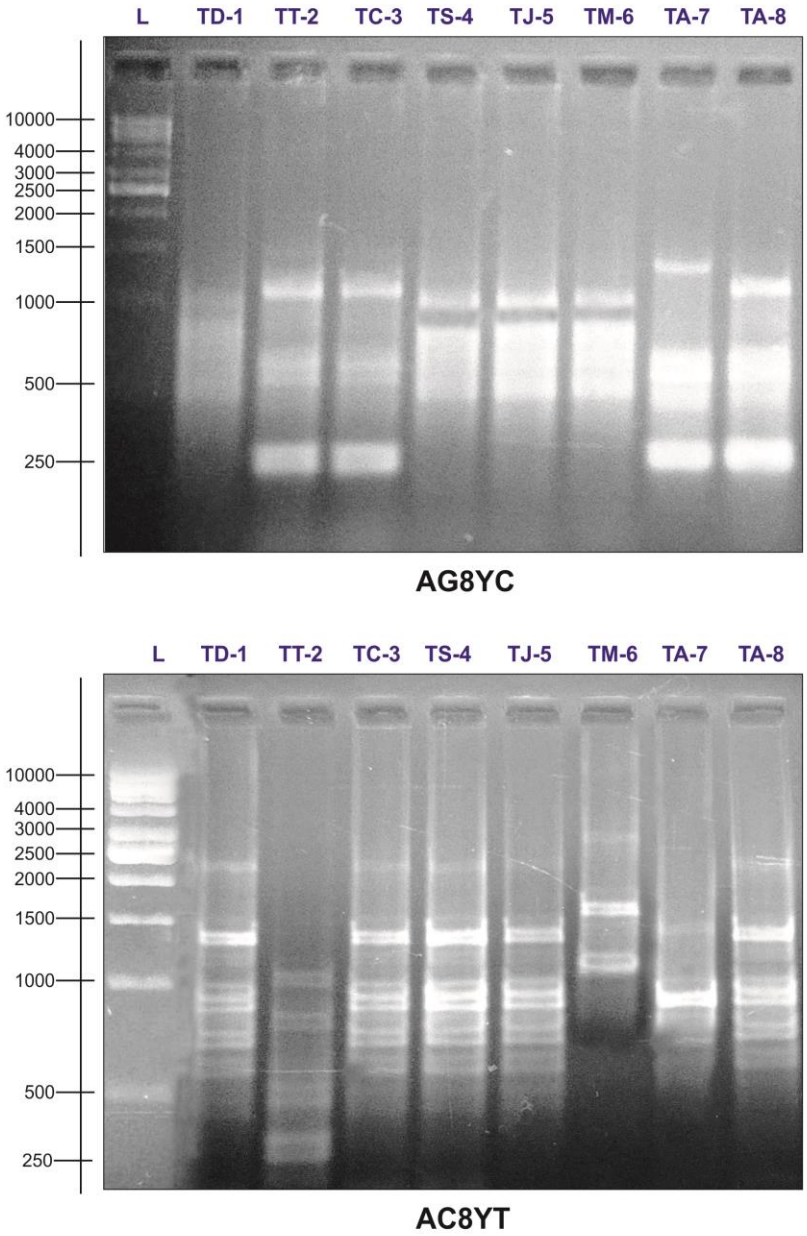


Plate 10 ISSR banding pattern of primer AG8YC and AC8YT

estimate (Jaccard's coefficient) based on ISSR banding pattern was used for cluster analysis to present genetic relationship in the form of dendrogram (Fig. 2). Jaccard's coefficient value for *Trichoderma* isolates are presented in Table -.

Table 20. Similarity coefficient for ISSR analysis

	TD-1	TT-2	TC-3	TS-4	TJ-5	TM-6	TA-7	TA-8
TD-1	1	0.083	0.300	0.765	0.706	0.333	0.217	0.143
TT-2	0.083	1	0.412	0.120	0.125	0.158	0.300	0.467
TC-3	0.300	0.412	1	0.217	0.174	0.048	0.368	0.692
TS-4	0.765	0.120	0.217	1	0.824	0.444	0.200	0.130
TJ-5	0.706	0.125	0.174	0.824	1	0.471	0.160	0.136
TM-6	0.333	0.158	0.048	0.444	0.471	1	0.263	0.053
TA-7	0.217	0.300	0.368	0.200	0.160	0.263	1	0.263
TA-8	0.143	0.467	0.692	0.130	0.136	0.053	0.263	1

In this Dendrogram was found to have higher value of similarity coefficient (0.824) whereas (0.048) was found to have lower value of similarity coefficient. Two major clusters were obtained on the basis of analysis. First group is named as cluster– A, includes TD-1, TS-4, TJ-5 and TM-6. Second group is named as cluster– B which include TT-2, TC-3, TA-7 and TA-8. The TJ-5 was found to have a higher similarity index. The TM-6 was found to have a lower similarity index.

Table 21. Cophenetic distance matrix for all Trichoderma isolates in ISSR Primers

	TD-1	TT-2	TC-3	TS-4	TJ-5	TM-6	TA-7	TA-8
TD-1	0.000	0.158	0.158	0.735	0.735	0.416	0.158	0.158
TT-2	0.158	0.000	0.439	0.158	0.158	0.158	0.311	0.439
TC-3	0.158	0.439	0.000	0.158	0.158	0.158	0.311	0.692
TS-4	0.735	0.158	0.158	0.000	0.824	0.416	0.158	0.158
TJ-5	0.735	0.158	0.158	0.824	0.000	0.416	0.158	0.158
TM-6	0.416	0.158	0.158	0.416	0.416	0.000	0.158	0.158
TA-7	0.158	0.311	0.311	0.158	0.158	0.158	0.000	0.311
TA-8	0.158	0.439	0.692	0.158	0.158	0.158	0.311	0.000

From the above table the cophenetic distance matrix which is used to show how to objects or observations have to be in order to be grouped in to same cluster demonstrates the maximum and minimum value 0.824 and 0.158 respectively. According to the result TS-4 was found to have a higher Cophenetic distance matrix with TJ-5 **(0.824)** and TM-6 with all treatments **(0.158).**

Table 22. Results by classes for Jaccard' coefficient in ISSR primers

Class	1	2
Objects	4	4
Sum of weights	4	4
Within-class variance	3.583	5.167
Minimum distance to centroid	1.250	1.620
Average distance to centroid	1.588	1.937
Maximum distance to centroid	2.250	2.475
	TD-1 TS-4 TJ-5 TM-6	TT-2 TC-3 TA-7 TA-8

According to the finding and as illustrated in the above table there are 2 classes that were grouped within two clusters which contain different isolates. The maximum and minimum within class variance were observed in cluster two (B)(5.167) and one (A) (3.583) respectively. The maximum distance to centroid and the minimum distance to centroid were observed in cluster two (B)(2.475) and cluster one (B)(1.250) respectively.

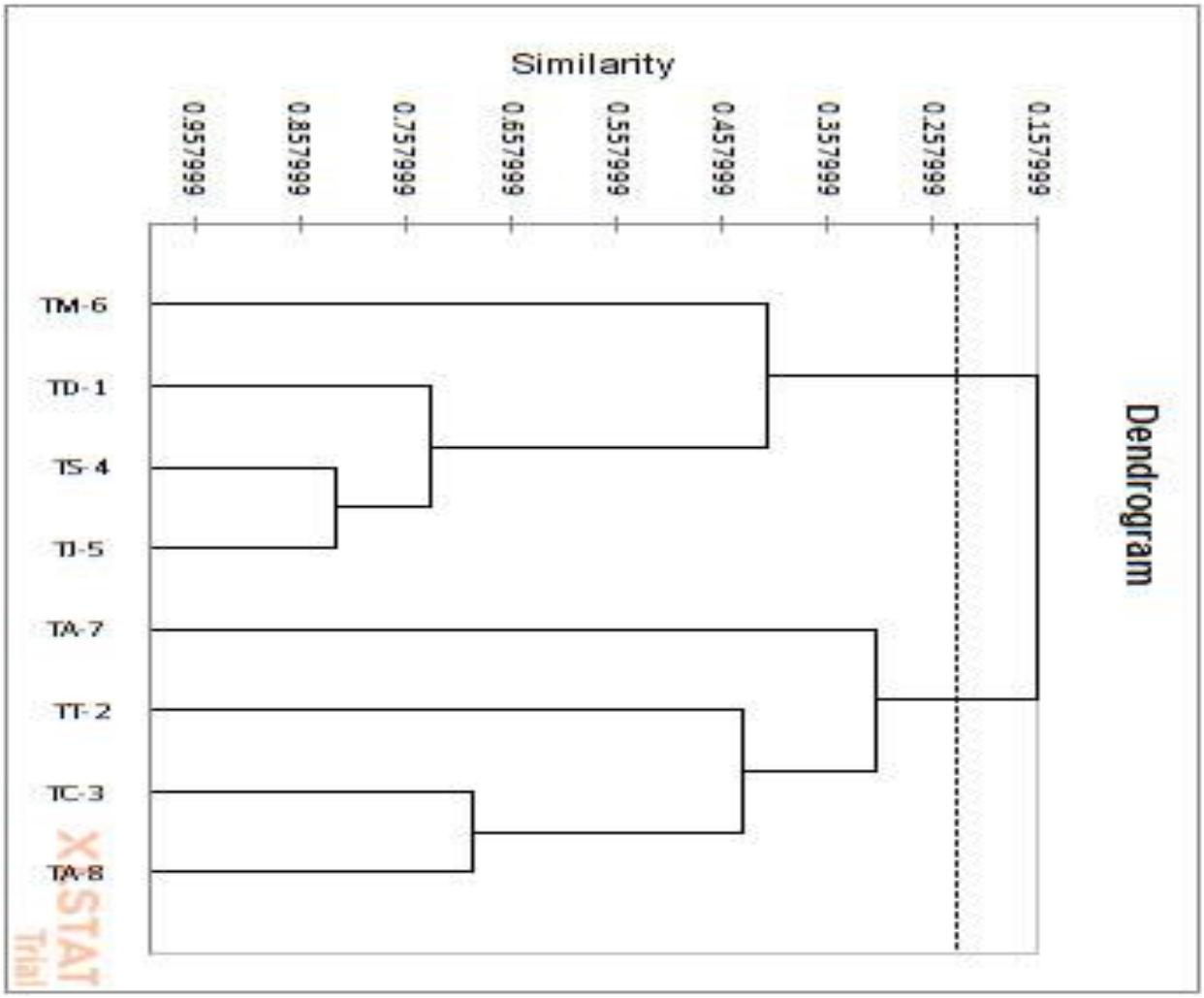


Fig. 2 UPGMA dendrogram of ISSR analysis of *Trichoderma* isolates based on Jaccard's Coefficient

Table 23. Summary statistics of ISSR primers for *Trichoderma* isolates

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
TD-1	30	0	30	0.000	1.000	0.467	0.507
TT-2	30	0	30	0.000	1.000	0.400	0.498
TC-3	30	0	30	0.000	1.000	0.400	0.498
TS-4	30	0	30	0.000	1.000	0.533	0.507
TJ-5	30	0	30	0.000	1.000	0.500	0.509
TM-6	30	0	30	0.000	1.000	0.333	0.479
TA-7	30	0	30	0.000	1.000	0.467	0.507
TA-8	30	0	30	0.000	1.000	0.333	0.479

From the above table the result showed that summary statistics of ISSR primers for *Trichoderma* isolates have 30 total observations, the minimum value of 0.000 and the maximum value of 1.000. The maximum and minimum mean values shows 0.533(TS-4) and 0.333(TM-6 & TA-8) respectively. The maximum standard deviation was observed in TJ-5 (0.509) and the minimum was observed in TM-6 and TA-8 (0.479).

Similarly, Kumar and Sharma (2011) used ISSR marker for characterization of twelve isolates belonging to Trichoderma harzianum and Trichoderma viride. Fifteen ISSR primers were screened and eight of them gave satisfactory amplification and band resolution which was taken for further study. The selected primers generated 70 ISSR bands and the size of the amplification products ranged from 100 to 950 bp. The per cent polymorphism ranged from 40 to 86. The mean value of the Jaccard's similarity coefficient of the ISSR marker was 0.76.

Shahid et al. (2014) studied genetic determination of potential Trichoderma species Using ISSR Marker in Uttar Pradesh using seven Trichoderma sp. were collected from different locations of Uttar Pradesh, for evaluating their bioefficiency by determining their genetic variations. PCR-based Inter Simple Sequence Regions (ISSR) Marker employing 6

primers produced 30 scorable bands out of which 27 bands were polymorphic.

Ghutukade et al. (2015) studied molecular characterization of Trichoderma isolates by ISSR marker they isolated twelve isolates belonging to Trichoderma harzianum and Trichoderma viride were assessed for their antagonistic effect on Fusarium oxysporum F.sp. lycopearsicie and Xanthomonas campestris. pv. vesicatoria. Trichoderma harzianum isolates were more aggressive than T. viride isolates. The dataset generated through morphological characters and ISSR markers showed a comparable output grouping the isolates of T. viride in one cluster and all T. harzianum isolates in another cluster.

CHAPTER V

SUMMARY AND CONCLUSIONS

The investigation was carried out in the Molecular Plant Pathology Laboratory of the Department of Plant Pathology and Biotechnology Center, Dr. PDKV, Akola. Eight Trichoderma isolates viz. TD-1, TT-2, TC-3, TS-4, TJ-5, TM-6, TA-7 and TA-8 selected for molecular characterization. The RAPD and ISSR marker were used to study the molecular variability. For the confirmation of fungus *Trichoderma* ITS markers ITS-1and ITS-4 were used which gives band size of 500bp to 600bp.

The molecular variability of *Trichoderma* isolates was studied by using nine RAPD primers of OPA series which produced 61 scorable bands. Among them 57 bands were polymorphic and level of polymorphism was 93.44%. The primer OPA15 and OPA 5 amplified maximum 9 bands within the size 350bp to 3500bp (OPA15) and 250bp to 2000bp (OPA5). While OPA-16 and OPA 20 amplified minimum 5 bands within the size 600 bp to 3500 bp (OPA16) and 1000 to 2500 (OPA20).

From the similarity coefficient analysis, higher and lower value of similarity coefficient is 0.923 and 0.143. *Trichoderma* isolates TJ-5 was found to have higher similarity index and TA-7 was found to have lower similarity index. On the basis of Jaccard's coefficient results and dendrogram three major clusters were obtained. First group is named as cluster –A, includes TD-1, TT-2, TC-3, TS-4 and TJ-5. Second group is named as cluster –B which includeTM-6. Third group is named as cluster - C which includes TA-7 and TA-8.

Cophenetic distance matrix which was used to show how two objects or observations have to be in order to be grouped in to same cluster, demonstrates the maximum and minimum matrix value 0.759 and 0.255 respectively. According to the result TS-4 was found to have a higher Cophenetic distance matrix with TJ-5 **(0.759)** and TM-6 with all treatments **(0.255).** The maximum and minimum within class variance were observed in cluster one (A) (9.100) and two (B)(0.000) respectively. The maximum

distance to centroid and the minimum distance to centroid were observed in cluster one (A)(3.650) and cluster two (B)(0.000) respectively.

From summary statistics of RAPD primers on the basis of 61 total observations, the maximum and minimum value recorded between 0.000 to 1.000.The maximum and minimum mean values show 0.639(TJ-5) and 0.213(TA-7) respectively. The maximum standard deviation was observed in TD-1 (0.504) and the minimum was observed in TA-7 (0.413).

In ISSR marker, 4 primers produced 30 scorable bands out of these 28 bands were polymorphic and level of polymorphism was 93.33%. From the similarity coefficient analysis ISSR marker, higher value of similarity coefficient is 0.824 whereas 0.048 was lower value of similarity coefficient. *Trichoderma* isolate TJ-5 was found to have higher similarity index and TM-6 was found to have lower similarity index. Two major clusters were obtained on the basis of analysis. First group is named as cluster– A, includes TD-1, TS-4, TJ-5 and TM-6. Second group is named as cluster– B which include TT-2, TC-3, TA-7 and TA-8. The TJ-5 was found to have a higher similarity index. The TM-6 was found to have a lower similarity index.

The cophenetic distance matrix which is used to show how two objects or observations have to be grouped in to same cluster demonstrates the maximum and minimum matrix value 0.823 and 0.158 respectively. According to the result TS-4 was found to have a higher Cophenetic distance matrix with TJ-5 (0.823) and TM-6 with all treatments (0.158).

From summary statistics of ISSR primers, on the basis of 30 total observations, the maximum and minimum value recorded between 0.000 to 1.000 The maximum and minimum mean values show 0.533(TS-4) and 0.333(TM-6 & TA-8) respectively. The maximum standard deviation was observed in TJ-5 (0.509) and the minimum was observed in TM-6 and TA-8 (0.479).

Conclusions

- *Trichoderma* isolates confirmed with ITS marker using ITS-1 and ITS-4 primers which gives band size of 500bp - 600bp.
- The molecular variability of *Trichoderma* isolates was studied by using 9 RAPD primers of OPA series which produced 61 scorable bands, 57 bands were polymorphic.
- In the RAPD markers on the basis of analysis three major clusters were obtained. First group is named as cluster –A, includes TD-1, TT-2, TC-3, TS-4 and TJ-5. Second group is named as cluster –B which includeTM-6. Third group is named as cluster -C which includes TA-7 and TA-8.
- In ISSR primer, 4 primers produced 30 scorable bands of which 24 bands were polymorphic.
- In ISSR marker two major clusters were obtained on the basis of analysis. First group is named as cluster– A, includes TD-1, TS-4, TJ-5 and TM-6. Second group is named as cluster– B which include TT-2, TC-3, TA-7 and TA-8.
- Molecular variability using RAPD and ISSR markers show that the level of polymorphism was 93.44% and 93.33%.

CHAPTER VI

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APPENDICES I

Composition of media

1) Potato Dextrose Agar (PDA) medium

Sr. No.	Ingredient	Quantity
1	Peeled and sliced potato	200gm
2	Dextrose	20gm
3	Agar-agar	20gm
4	Distilled water	1000ml

2) Potato Dextrose Broth (PDB) medium

Sr. No.	Ingredient	Quantity
1	Peeled and sliced potato	200gm
2	Dextrose	20gm
3	Distilled water	1000ml

APPENDIX II

List of bands amplified against RAPD marker

Primers	Banding type	TD1	TT2	TC3	TS4	TJ5	TM6	TA7	TA8
OPA2	1700	1	0	1	1	1	1	0	0
	1400	1	0	1	1	1	0	0	1
	1000	0	1	0	1	1	0	1	1
	700	0	0	0	0	0	1	0	0
	600	0	1	0	0	0	1	0	0
	250	0	1	0	0	0	0	0	0
OPA3	1500	1	0	1	1	1	1	0	0
	1100	1	0	1	1	1	1	0	1
	900	0	1	0	0	0	0	1	1
	750	1	0	1	1	1	1	0	0
	600	1	0	1	1	1	1	0	0
	500	0	0	0	0	0	1	1	1
	250	1	0	1	1	1	0	1	1
OPA5	2000	0	0	0	0	0	1	0	0
	1700	0	0	0	0	0	1	0	0
	1300	1	1	0	1	1	0	0	0
	900	1	1	0	0	1	0	0	1
	800	0	0	1	0	0	0	0	1
	750	1	0	0	0	0	1	0	0
	500	1	1	0	1	1	1	0	0
	350	1	1	0	1	1	1	0	0
	250	0	0	0	0	0	1	0	0
OPA9	2500	0	0	1	0	0	0	0	1
	1900	1	0	0	1	1	1	0	0
	1600	1	0	1	1	1	1	0	1
	1300	1	0	1	1	1	0	0	1
	700	0	0	1	0	1	1	1	1
	500	0	0	0	1	1	1	0	0
	250	0	0	0	0	0	1	0	0
OPA10	2500	1	1	1	1	1	0	0	0
	1800	1	1	1	1	1	0	0	1
	1400	1	1	1	1	1	1	1	1

	1000	1	1	1	1	1	1	1	1
	500	1	1	1	1	1	0	1	1
	250	0	0	0	0	0	0	1	1
OPA13	3000	0	0	0	0	0	1	0	0
	2500	0	0	0	0	0	1	0	0
	1300	0	0	1	0	0	1	0	0
	900	1	1	1	1	1	1	1	1
	700	0	0	0	0	0	0	1	1
	600	1	1	1	1	1	1	1	1
	400	0	0	1	0	0	1	0	0
OPA15	3500	0	0	0	1	1	1	0	0
	2500	0	0	0	1	1	1	0	0
	1500	0	0	1	1	1	0	0	1
	1300	0	1	0	0	0	1	0	0
	1000	0	0	1	1	1	0	0	0
	900	0	1	0	0	0	1	0	1
	750	0	0	0	0	0	1	0	0
	500	1	0	1	0	1	0	0	0
	350	0	0	0	1	1	0	0	0
OPA16	4000	0	0	0	0	0	1	0	0
	3000	1	0	0	1	1	1	0	0
	2500	1	1	1	1	1	0	0	0
	1300	1	1	1	1	1	0	0	1
	800	1	0	1	1	1	0	0	1
OPA20	2000	1	0	0	1	1	0	0	0
	1800	1	1	0	1	1	1	0	0
	1300	1	1	0	1	1	0	0	0
	750	1	1	0	1	1	0	0	0
	500	1	1	1	1	1	0	1	1

APPENDIX III

List of bands amplified against ISSR marker

Primers	Banding type	TD-1	TT-2	TC-3	TS-4	TJ-5	TM-6	TA-7	TA-8
ISSR811	2500	1	1	0	1	1	0	0	0
	2000	1	0	0	1	1	0	0	0
	1500	0	1	1	0	0	0	1	1
	1200	1	1	1	1	1	1	1	0
	800	0	0	0	0	0	0	1	0
	700	1	0	0	1	1	1	1	0
	500	0	1	1	0	0	0	1	1
	300	0	0	0	0	0	1	1	0
GA8YC	1000	1	0	0	1	1	1	1	0
	850	0	1	1	0	0	0	1	1
	800	1	0	1	1	1	0	0	0
	650	1	0	0	1	1	1	1	0
	500	0	0	0	0	0	0	1	0
	350	1	0	0	1	1	0	0	0
	250	1	0	0	1	1	1	0	0
AG8YC	1500	0	0	0	0	0	0	1	0
	1200	0	1	1	0	0	0	0	1
	1000	1	0	0	1	1	1	0	0
	900	0	0	0	1	1	0	0	0
	700	0	1	1	0	0	0	1	1
	500	0	0	0	1	1	1	0	0
	250	0	1	1	0	0	0	1	1
AC8YT	2300	1	0	1	1	0	0	0	1
	2000	1	0	1	0	1	0	0	1
	1400	0	1	0	1	1	1	0	1
	1000	0	1	0	0	0	1	0	0
	900	1	0	1	1	0	0	1	0
	600	1	0	1	1	1	0	0	1
	500	0	1	0	0	0	0	0	0
	250	0	1	0	0	0	0	0	0