Functional analysis of three genes downregulated in a non-conidiating mutant of *Trichoderma virens*

By

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

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List of Publications arising from the thesis

Publications in Peer Reviewed Journals

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Ravindra Bansal, Gagan Deep Gupta, Prasun K Mukherjee, 2020, Translationally Controlled Tumor Protein (TCTP) is involved in growth and antagonistic behavior of *Trichoderma virens*, Physiological and Molecular Plant Pathology **(Under review).**

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2. **Ravindra Bansal** and Prasun K Mukherjee, 2019, Ecm33, a GPI-anchored cell wall protein, is involved in conidiation and mycoparasitism in *Trichoderma virens*, Proceedings of DAE Life Science Symposium (LSS-2018) on Frontier in Sustainable Agriculture, 26-28 April 2018, Trombay, Mumbai, India. (Abstract)
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Dedicated to My

Parents, My Wife and

My Stars Shriyansh and

Sanskar

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TABLE OF CONTENTS

Table of Contents…….....1-4

Summary 5-6

List of Figures 7-9

List of Tables 10

List of Abbreviations 11

CHAPTER-1:

INTRODUCTION

1.0 Introduction…….……12-14
1.1 The classification of *Trichoderma*…….....14-14
1.2 Application of *Trichoderma*……15-18
1.3 Mechanisms of Biocontrol
1.3.1 Competition…….……..….18-19
1.3.2 Mycoparasitism…….…19-20
1.3.3 Antibiosis……...20-20
1.3.4 Induced systemic resistance……...21-22
1.3.5 Mitigation of abiotic stress by *Trichoderma*…….…....22-25
1.4 Molecular mechanisms
1.4.1 Molecular mechanisms of mycoparasitism……..,……...……..25-27
1.4.2 Molecular mechanisms of antibiosis ……...……..27-29
1.4.3 Molecular mechanisms of induced systemic resistance (ISR).……..…....29-30
1.5 Factors influencing the bioefficacy of *Trichoderma*…….…….…...30-34
1.6 Morphogenesis in filamentous fungi
1.6.1 *Neurospora crassa*- model saprophyte…….…….……34-37
1.6.2 *Magnaporthe oryzae* (*Pyricularia oryzae*)- model plant pathogen…….…….….37-38
1.6.3 *Aspergillus fumigatus*- model human pathogen…….……..…..39-40
1.6.4 Morphogenesis in *Trichoderma*……..…...40-44
1.7 Biological roles of Tcp1, Pgy1 and Ecm33 proteins in plants, microbes, and plant-microbe interactions
1.7.1 Translationally controlled tumour proteins…….…….…..45-49

1.7.2 Proline–glycine-tyrosine rich proteins …….…….....49-50

1.7.2 Ecm33 proteins…….……51-56

CHAPTER-2:

MATERIALS AND METHODS

2.1 Strains, media and growth conditions……..…….……..58-58

2.2 Reagents, media and kits……..……,……58-65

2.3 Equipments……..….65-66

2.4 Phylogenetic analysis of Tcp1, Pgy1 and Ecm33 proteins……..….66-66

2.5 Primer designing and synthesis…….……66-70

2.6 Plasmids used in the study…….……...71-71

2.7 General strategy for construction and detection of knock out mutants ……..…..72-78

2.8 Bacterial transformation……..……...……..78-81

2.9 PCR amplification for making gene deletion construct ……...81-81

2.10 Isolation and transformation of protoplasts of *Trichoderma virens*……...81-84

2.11 Isolation of RNA from transformants of *T. virens* …….…….....84-86

2.12 Determination of copy number ……..86-87

2.13 Assay for growth, dry weight, conidiation and chlamydospores production……..…87-88

2.14 Estimation of the IC_{50} concentration…….…..88-88

2.15 Cell-wall integrity assay……88-89

2.16 Transmission electron microscopy……..89-89

2.17 Test for hydrophobicity……..……89-89

2.18 Confrontation assay…….....89-90

2.19 Antibiosis assay and HPLC analysis…….…..90-90

2.20 GC-MS analysis for volatile compounds……....90-91

2.21 Root growth assay……...…….....91-92

CHAPTER-3:

***IN SILICO* ANALYSIS AND CONSTRUCTION OF KNOCKOUT MUTANTS**

3.1 Introduction……94-94

3.2 Materials and methods……...94-95

3.3 Results

3.3.1 Bioinformatics and phylogenetic analysis……...96-101
3.3.2 Conserved motif analysis in Pgy proteins……..102-102
3.3.3 Multiple sequence alignment of Pgy proteins……103-104
3.3.4 Construction of knockout mutant using split PCR technique
3.3.4.1 Protoplast transformation of PCR products……105-108
3.3.4.2 Screening and purification of knockout mutants……....109-112
3.3.4.3 Morphology of mutants……...113-113
3.3.4.4 Detection of copy number in mutants…….114-116
3.4 Discussion…….117-118
CHAPTER- 4:
GROWTH AND MORPHOLOGY
4.1 Introduction……..…120-120
4.2 Materials and methods……...121-121
4.3 Results
4.3.1 Growth and morphology ……...121-124
4.3.2 Conidiation (spore formation)…….....125-132
4.3.3 Growth on agar…….…133-134
4.3.4 Formation of chlamydospores…….....135-135
4.3.5 Transmission electron microscopy……..…136-137
4.4 Discussion…….138-141
CHAPTER- 5:
STRESS RESPONSE, HYDROPHOBICITY AND CELL WALL INTEGRITY
5.1 Introduction……..…143-144
5.2 Materials and methods……...144-144
5.3 Results
5.3.1. Estimation of IC_{50}……..…145-149
5.3.2 Role of *tcp1, pgy1* and *ecm33* gene in stress response…….…….150-154
5.3.3 Effect on menadione sodium bisulfite induced pigmentation……...155-155
5.3.4. Cell wall integrity…….156-158
5.3.5 Hydrophobicity……...…….…..159-160

5.4 Discussion ……..……..161-165

CHAPTER-6:

BIOCONTROL PROPERTIES: MYCOPARASITISM, ANTIBIOSIS AND PLANT-INTERACTIONS

6.1 Introduction…….……167-168

6.2 Materials and methods…….…..168-168

6.3 Results

6.3.1 Confrontation assay against the plant pathogens…….…. 169-173

6.3.2 Antibiosis assay and viridin biosynthesis……..174-175

6.3.3 Biosynthesis of volatile secondary metabolites…….176-177

6.3.4 *Trichoderma*-plant Interaction- Effect on fresh weight of maize roots……..…...178-179

6.4 Discussion…….180-182

CHAPTER 7:

GENERAL DISCUSSION, SUMMARY AND CONCLUSION……...…….....184-191

REFERENCES…….…….192-237

Reprint of published article

SUMMARY

In an attempt to identify the genes involved in conidiation, an SSH (suppression subtractive hybridization) library was earlier constructed in our laboratory where cDNA ofa non-conidiating mutant strain was subtracted from the cDNA of the conidiating wild type strain. Further expression analysis of the genes from the library led to identification of three genes that were down-regulated in the mutant. A homology search revealed that these three proteins are orthologs of translationally controlled tumour protein (Tcp1), Proline-, glycine-, tyrosine-rich protein (Pgy1) and Ecm33. The functions of none of these genes are known in *Trichoderma*, and hence, the present study was undertaken to unravel the role of these three genes in *Trichoderma* biology.

For functional characterization of the genes, independent knockout mutants (Δ*tcp1*, Δ*pgy1* and Δ*ecm33*) were generated by split marker PCR technique using homologous recombination approach. The role of these genes in conidiation, mycoparasitism, antibiosis, secondary metabolite biosynthesis, cell wall integrity, hydrophobicity and stress responses have been studied. All these independent mutants showed slower radial growth rate on PDA compared to wild type (WT) but among these mutants, highest reduction in radial growth was recorded in Δ*tcp1* mutant. Δ*tcp1* mutants also showed significant decrease in dry weight compared to WT. Δ*pgy1* and Δ*ecm33* mutants showed significant decrease in conidiation in lab made PDA, HiMedia PDA and VMG media while these mutants had higher number of chlamydospores in shake culture compared to WT and Δ*tcp1* mutant.

The role of these genes in stress tolerance was elucidated by comparing the change in radial growth under stress conditions. The estimated IC_{50} value of WT strain of *T. virens* was 600 µg/ml for Congo red, 1.25 mM for menadione sodium bisulfite, 2.5% for NaCl

and 150 µg/ml for SDS. On the basis of changes in radial growth of mutants, Δ*tcp1* mutant was found to be sensitive to salt while highest sensitivity to SDS and Congo red was noticed in Δ*ecm33* mutant. Treatment of mycelia with cell wall lysing enzyme cocktail confirmed that the Δ*ecm33* and Δ*tcp1* mutants had significant alteration in cell wall integrity. Δ*ecm33* mutant also showed higher sensitivity towards cell wall destabilizing agent Congo red and SDS, compared to WT. TEM images of mycelia of all the mutants showed changes in cell wall structure compared to WT.

Hydrophobicity test on Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants and WT confirmed that Δ*ecm33* mutant lost the hydrophobicity while WT and other mutants retained the hydrophobicity. Confrontation assay confirmed that, Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants lost the mycoparasitic activity against the plant pathogens *Rhizoctonia solani* and *Sclerotium rolfsii*. While Δ*pgy1* and Δ*ecm33* mutants also lost the mycoparasitic activity against the plant pathogen *Pythium aphanidermatum* but Δ*tcp1* mutant retained it. Culture filtrate of Δ*pgy1* and Δ*ecm33* mutants also lost the antibiosis activity against the plant pathogen *P. aphanidermatum*. HPLC analysis of culture filtrate showed reduced viridin biosynthesis in Δ*pgy1* and Δ*ecm33* mutants. In GC-MS analysis, Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants showed significant changes in volatile compound biosynthesis compared to WT. The effect of *Trichoderma* on maize root growth was assessed in tubes. Maize seedlings inoculated with chlamydospores from Δ*pgy*1 and Δ*ecm*33 did not show any significant change in fresh weight of roots compared to WT but maize seedlings treated with Δ*tcp1* showed significant reduction in fresh weight of roots compared to WT.

Chapter-6

Biocontrol Properties: Mycoparasitism, Antibiosis and Plant-Interactions

6.0 INTRODUCTION:

Biocontrol mechanisms of the mycoparasitic fungus *Trichoderma* consist of mycoparasitic activity, antibiosis and induced resistance. Competition for the space and nutrition is one of the best strategies to control pathogens. *Trichoderma* species and pathogens compete for the space and nutrition and *Trichoderma* grow very fast towards the pathogens (58). *Trichoderma* spp. have higher ability to mobilize soil nutrients as compared to other fungal pathogens which leads to malnourishment and consequently, death of pathogens (22). The sequence of events involved in mycoparasitism includes host recognition, penetration and killing using enzymes like β-1,3-glucanase, chitinases and proteases, which degrade the fungal cell wall (60, 61). The transcription factor Ste12, seven transmembrane protein Sfp2, and methyltransferase Lae1 are involved in mycoparasitic activity in *T. atroviride* (119, 120, 121). Adenylate-cyclase-encoding gene *tac1* in *T. virens* is involved in mycoparasitic activity (130).

Trichoderma spp. inhibit the growth of the plant pathogenic fungi by producing the antimicrobial compounds called antibiotics and the process is called antibiosis. These low molecular weight compounds are mainly terpenes, peptaibols, polyketides, alkyl pyrones, isonitriles, and sterols (68). O-Methyltranserase gene (*omtB)* and cytochrome P450 (*tri13*) are involved in demethoxy-viridin and viridin biosynthesis, while NRPS gene (*glv21*) has key role in biosynthesis of gliovirin in *T. virens* (134, 135). The induced resistance works at a location away from the application site and is effective against bacterial, fungal and even viral pathogens (28). Induced resistance in plant hosts is governed by three different pathways. Out of these three pathways, two pathways come into play using the expression and assembly of PR proteins, and the third pathway is based on rhizobacteria-induced systemic resistance (77). Chromatin remodeling protein, histone deacetylase (Hda-2) of *T.*

atroviride is involved in plant defense against foliar pathogens *Botrytis cinerea* and *Pseudomonas syringae* (19). Not only proteins but some secondary metabolites are also reported to be involved in induced resistance. The trichovorin-type 18-residue peptaibiotics of *T. virens* are involved in induced resistance in cucumber against a bacterial pathogen (83). Mukherjee et al. identified a PKS/NRPS hybrid enzyme which is involved in induced resistance in maize (84).

6.2 Materials and Methods:

Confrontation assays (fresh mycelial discs of *T. virens* strains and the plant pathogen *P. aphanidermatum* were seeded opposite to each other on PDA plate and allowed to grow towards each other) of *T. virens* WT and mutants were performed against plant pathogenic fungus *R. solani, P. aphanidermatum* and *S. rolfsii.* Viability of pathogens *P. aphanidermatum* and *S. rolfsii* from confrontation assay plates was observed on PDA amended with 10 ppm benomyl fungicide, a selective inhibitor of *Trichoderma*. Antibiosis assay was performed with the culture filtrate extracts of WT and mutants. Three mycelial plugs of *P. aphanidermatum* were seeded towards the periphery and *Trichoderma* culture filtrate extract was applied at the centre of the plate in a well. Quantitation of viridin biosynthesis by WT and mutants was done by HPLC and comparison of volatile compounds produced by WT and mutants was done by the GC-MS analysis. Maize root growth assay was performed with chlamydospores of WT and mutants.

6.3 RESULTS:

6.3.1 Confrontation assay against the plant pathogens:

Confrontation assays (a standard method to follow antagonism between fungal isolates) were used to test the ability of the mutants to suppress and overgrow on plant pathogens. The pathogen and *Trichoderma* colonies were placed simultaneously on the same culture plate but at opposite ends.

6.3.1.1 Confrontation assay against the plant pathogen *Sclerotium rolfsii*:

To know the role of *tcp1, pgy1* and *ecm33* genes in mycoparasitic activity against the plant pathogen *Sclerotium rolfsii*, confrontation assays were performed with plant pathogen *S. rolfsii* and *T. virens* strains. On opposite ends of the PDA plate, one fresh mycelial disc from *Trichoderma* strains (WT, Δ*tcp1*, Δ*pgy1* and Δ*ecm33*) and one fresh mycelial disc of plant pathogen *Sclerotium rolfsii* was inoculated and allowed to grow towards each other. After seven days of inoculation, test pathogen *Sclerotium rolfsii* grew over the Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants while at this time, wild type strain of *Trichoderma* overgrew and cover most of the pathogen **(Fig 6.1)**. Viability of the test pathogen from the confrontation assay plates was checked on PDA plates having 10 ppm benomyl, a selective inhibitor of *Trichoderma*. The pathogen taken from the confrontation assay plate with *Trichoderma* wild type strain was not viable on benomyl plate but the pathogen taken from the confrontation assay plates with either of three mutants *(*Δ*tcp1*, Δ*pgy1* and Δ*ecm33*) grew on benomyl amended PDA plates as the pathogen was resistant to benomyl **(Fig. 6.2)**, These results indicated that, mutants *(*Δ*tcp1*, Δ*pgy1* and Δ*ecm33*) lost the mycoparasitic ability against the plant pathogen *Sclerotium rolfsii*.

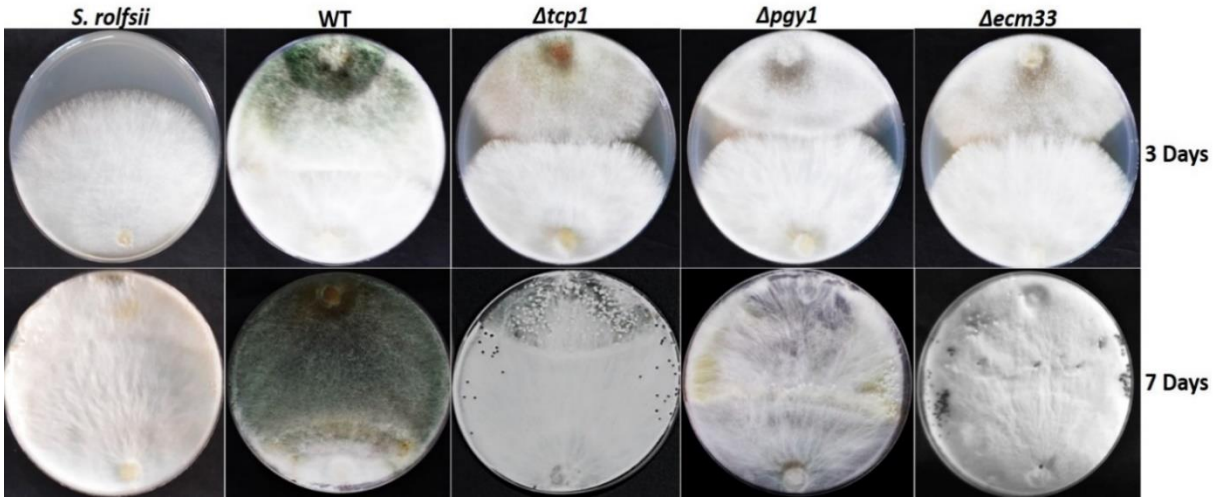

Fig. 6.1 Confrontation assay of *T. virens* WT and mutants against plant pathogenic fungus *S. rolfsii.* Fresh mycelial discs of *T. virens* strains and plant pathogen *S. rolfsii* were seeded opposite to each other on PDA plate and allowed to grow towards each other.

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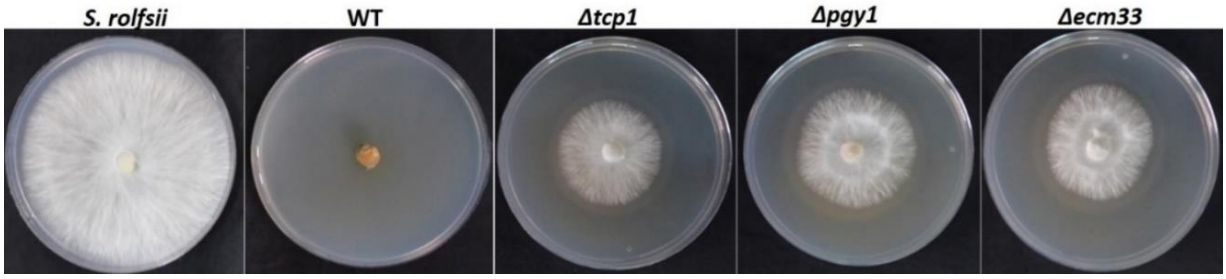


Fig. 6.2 Viability of *S. rolfsii* taken from confrontation assay plate. One 5 mm disc of *S. rolfsii* from *S. rolfsii* plate and one each from the dual culture plates (*S. rolfsii* with WT, *S. rolfsii* with Δ*tcp1, S. rolfsii* with Δ*pgy1* and *S. rolfsii* with Δ*ecm33*) were seeded at centres of PDA plates amended with 10 ppm benomyl.

6.3.1.2 Confrontation assay against the plant pathogen *Pythium aphanidermatum*:

The mycoparasitic activity of mutant strains were also compared with wild type strain of *T. virens* against the plant pathogen *Pythium aphanidermatum* using confrontation assay. After seven days of inoculation of the pathogen and *T. virens* strains at opposite ends of a plate, WT and all the three mutants (Δ*tcp1*, Δ*pgy1* and Δ*ecm33*) grew over the pathogen **(Fig. 6.3)**. The viability of the pathogen was checked on PDA plate amended with 10 ppm benomyl. The pathogen from the confrontation assay with WT or Δ*tcp1* mutant was unable to grow on benomyl plate but the pathogen from the confrontation assay with Δ*pgy1* or Δ*ecm33* grew on benomyl pate **(Fig. 6.4)**. The experiment confirmed that, Δ*tcp1* retained the mycoparasitic activity against *P. aphanidermatum* but Δ*pgy1* and Δ*ecm33* lost their mycoparasitic ability.

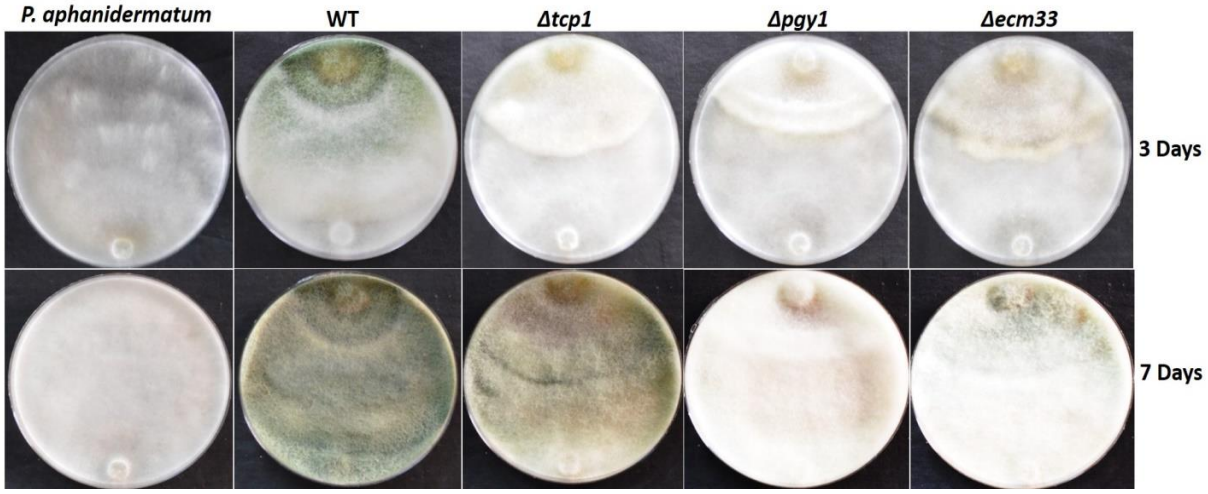


Fig. 6.3 Confrontation assay of *T. virens* WT and mutants against plant pathogen *P. aphanidermatum*. Fresh mycelial discs of *T. virens* strains and plant pathogen *P. aphanidermatum* were seeded opposite to each other on a PDA plate and allowed to grow towards each other.

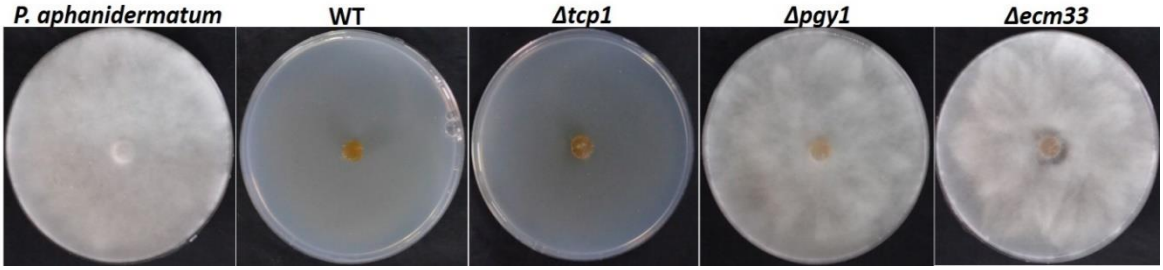


Fig. 6.4 Viability of *P. aphanidermatum* taken from confrontation assay plate. One 5 mm disc of plant pathogen *P. aphanidermatum* from only *P. aphanidermatum* plate and one each from the dual culture plates (*P. aphanidermatum* with WT*, P. aphanidermatum* with Δ*tcp1, P. aphanidermatum* with Δ*pgy1 and P. aphanidermatum* with Δ*ecm33)* were seeded at centres of PDA plates amended with 10 ppm benomyl.

6.3.1.3 Confrontation assay against the plant pathogen *Rhizoctonia solani*:

The confrontation assay was also performed with the plant pathogen *Rhizoctonia solani*. After 6 days of incubation, *Trichoderma* wild type strain grew over the pathogen. In confrontation assay with pathogen, Δ*tcp1* strain inhibited the growth of pathogen. Even after 10 days, Δ*tcp1* and the plant pathogen were not able to over-grow each other. In confrontation assay between Δ*pgy1* mutant and plant pathogen *R. solani*, pathogen overgrew on the Δ*pgy1* mutant strain of *T. virens*, but Δ*ecm33* mutant was growing even till 10 days of incubation because of very slow growth over the pathogen **(Fig. 6.5)**.

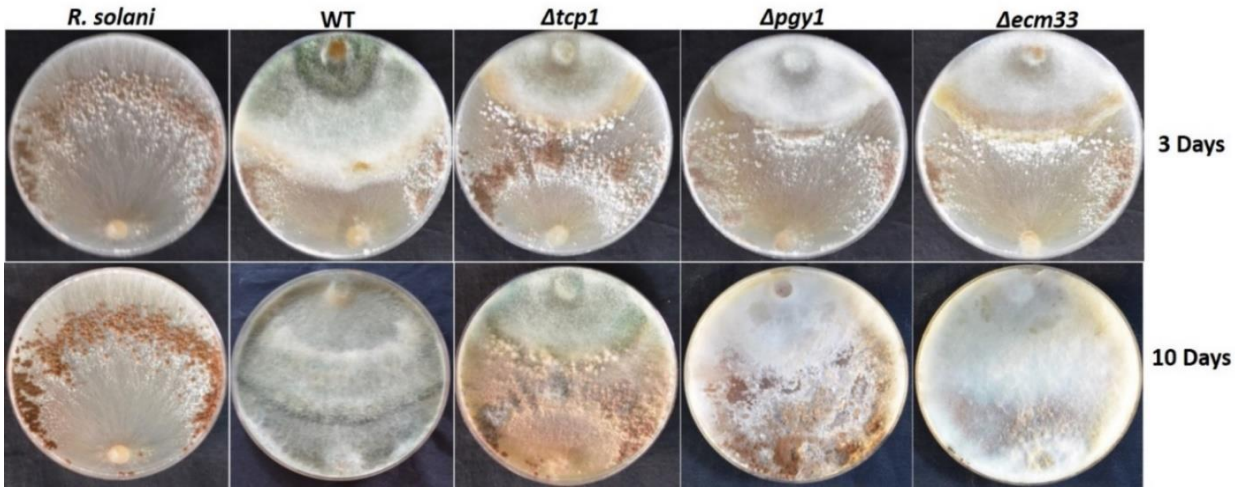


Fig. 6.5 Confrontation assay of *T. virens* WT and mutants against plant pathogenic fungus *R. solani.* Fresh mycelial discs of *T. virens* strains and plant pathogen *R. solani* were seeded opposite to each other on a PDA plate and allowed to grow towards each other.

6.3.2 Antibiosis assay against the plant pathogen *P. aphanidermatum* and biosynthesis of antimicrobial secondary metabolite viridin:

Fifty µl culture filtrate extracts from WT (10 times concentrated) were placed in a well at the centre of a PDA plate and the plates were inoculated with the test pathogen *P. aphanidermatum*. A distinct zone of inhibition was observed. The zone of inhibition was also observed with the culture filtrate of Δ*tcp1* knockout mutant. Filtrates obtained from Δ*pgy1* and Δ*ecm33* mutants, in contrast, did not produce any zone of inhibition, indicating attenuation of antibiotic properties **(Fig. 6.6)**. Twenty µl of extract was used for high pressure liquid chromatography (HPLC) analysis using acetonitrile: aqueous 0.1% phosphoric acid (72:28) as running solvent and peaks were detected at 254 nm. HPLC analysis revealed that, there was no significant difference between the production of antimicrobial secondary metabolite viridin by WT and Δ*tcp1* mutants, but Δ*pgy1* (22.28% of WT) and Δ*ecm33* (11.33% of WT) mutants produced significantly less amount of viridin **(Fig. 6.7 A, B)**.

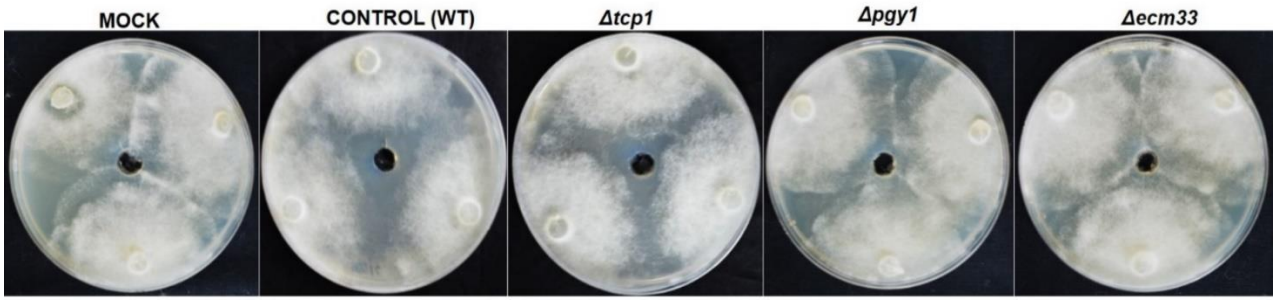
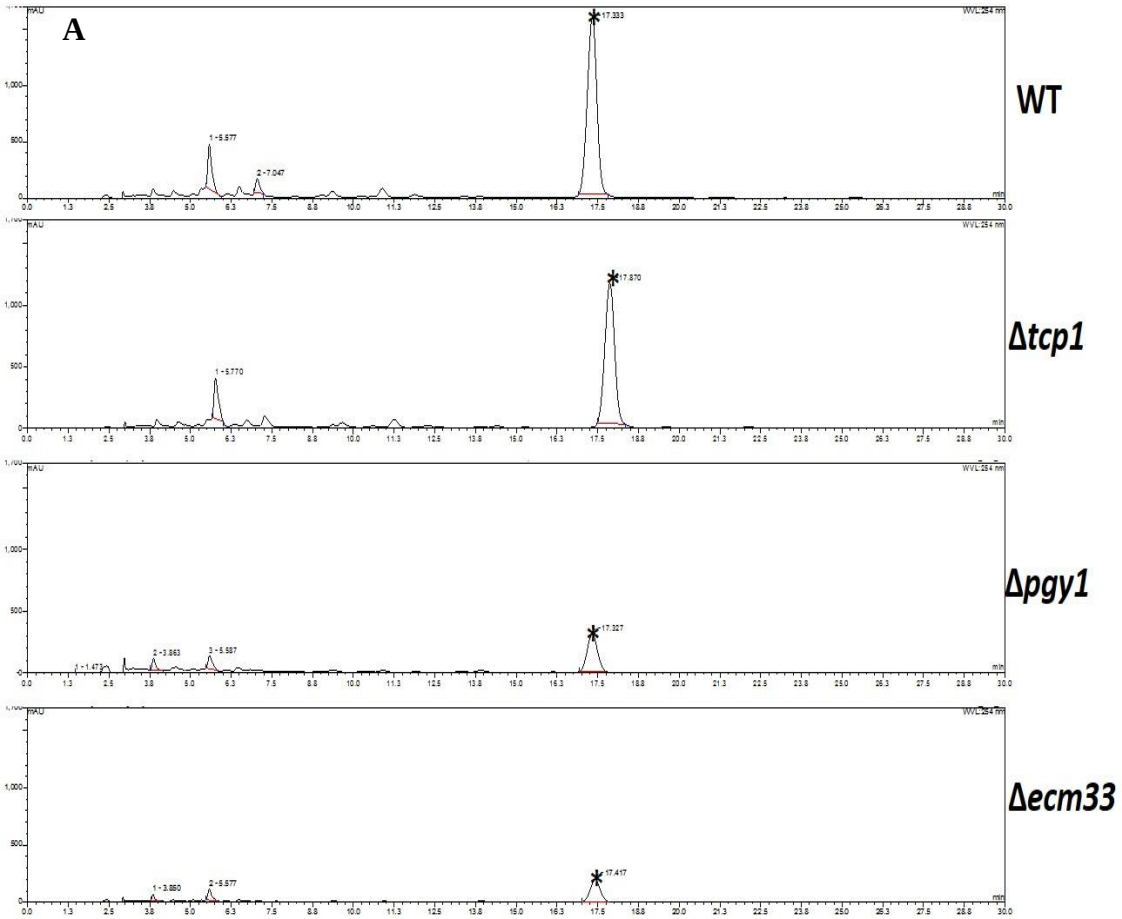


Fig. 6.6 Antibiosis assay. Inhibition of *Pythium aphanidermatum* by culture filtrate extracts of WT, Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants after 24 hours of incubation. Three mycelial plugs were seeded towards the periphery, with *Trichoderma* filtrate applied at the centre of the plate. Note the absence of inhibition by culture filtrates of the Δ*pgy1* and Δ*ecm33* mutants.



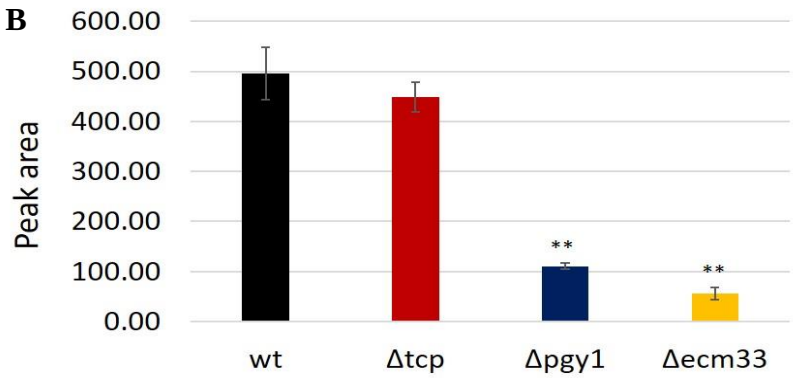


Fig. 6.7 HPLC profiles of culture filtrate extracts of *T. virens* WT and mutant strains Δ*tcp1,* Δ*pgy1* and Δ*ecm33* (A) and quantification of viridins synthesized by *T. virens* WT and mutant strains Δ*tcp1,* Δ*pgy1* and Δ*ecm33* (B) *denotes viridin. **: P< 0.01.

6.3.3 Biosynthesis of volatile secondary metabolites:

The headspace-solid phase micro-extraction (HS-SPME) and gas chromatography–mass spectrometry (GC-MS) techniques were used for detection of volatile compounds in wild type and mutants. The chromatogram of WT showed 16 peaks for volatile compounds, while Δ*tcp1* mutant had only seven peaks (including one peak representing a volatile compound which was not present in WT). Δ*tcp1* mutant also showed one big peak in GC-MS chromatogram which was neither present in WT nor had similarity with the compounds in library. GC-MS analysis of Δ*pgy1* and Δ*ecm33* showed only two peaks but theses peaks were uncommon among Δ*pgy1* and Δ*ecm33* mutants **(Fig. 6.8, 6.9)**. The details of the volatile compounds are given in **Table 6.1**.

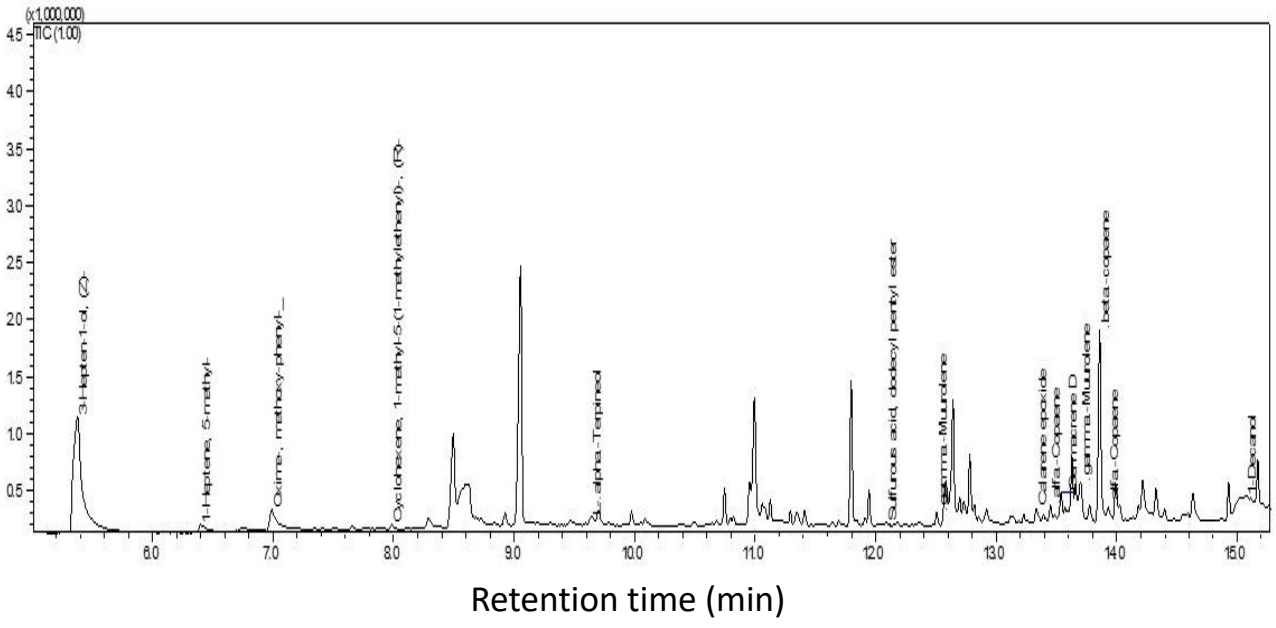


Fig. 6.8 GC-MS analysis of WT strain of *Trichoderma virens*

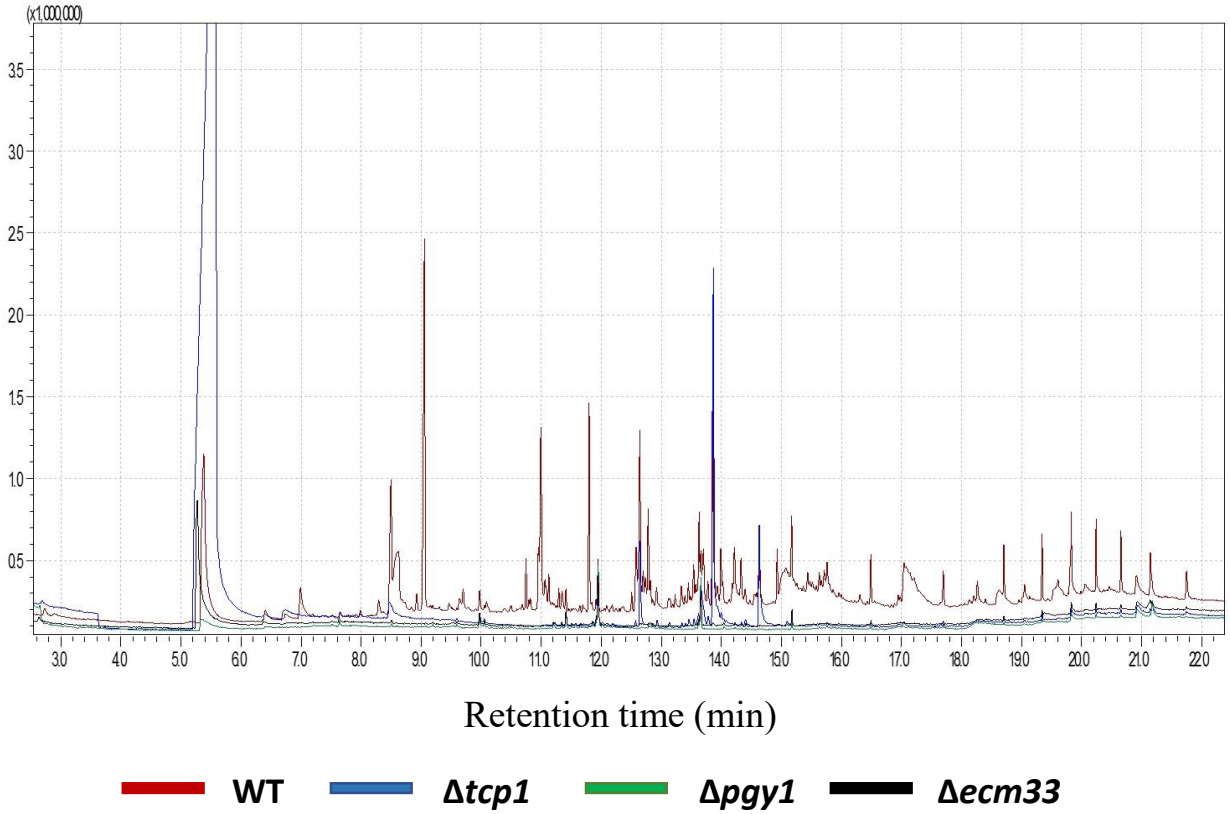


Fig. 6.9 GC-MS analysis of WT and mutant strains (Δ*tcp1*, Δ*pgy1* and Δ*ecm33*) of *T. virens*

	WT	Δtcp	Δpgy	Δecm
3-Hepten-1-ol, (Z)-	149340	0	9016	0
1-Heptene, 5-methyl-	11588	4240	0	0
Oxalic acid, 6-ethyloct-3-yl isohexyl ester	0	874	2829	0
Sulfurous acid, dodecyl pentyl ester	9003	0	0	0
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	0	0	0	438
gamma.-Muurolene	13717	1286	0	0
alfa.-Copaene	13823	4382	0	0
Germacrene D	23488	4496	0	0
beta.-copaene	165723	215406	0	1394
Oxime-, methoxy-phenyl-_	66400	0	0	0
Cyclohexene, 1-methyl-5-(1-methylethenyl)-, (R)-	6698	0	0	0
L-.alpha.-Terpineol	12882	0	0	0
alfa.-Copaene	8641	0	0	0
1-Decanol	22473	0	0	0
gamma.-Muurolene	24806	0	0	0
Calarene epoxide	8505	0	0	0

Table 6.1 - List of volatile compounds detected in GC-MS analysis of WT and mutant strains Δ*tcp1*, Δ*pgy1* and Δ*ecm33* of *T. virens*

6.3.4 *Trichoderma*-plant interaction:

6.3.4.1 Effect on fresh weight of maize roots:

The interaction effect of *Trichoderma* and maize plants on fresh weight of roots was recorded using root growth assay. Seeds were inoculated in potting-mix having chlamydospores of WT and mutants, and one set of tubes with potting-mix devoid of chlamydospores were used as a control. Maize seedlings grown in soil infested with chlamydospores of Δ*tcp1* mutant showed significant reduction in fresh weight of roots as compared to fresh weight of roots of maize seedlings inoculated with the chlamydospores of WT **(Fig. 6.10 and 6.11)**. Maize seedlings grown in soil inoculated with chlamydospores of Δ*pgy*1 and Δ*ecm*33 did not show any significant changes in fresh weight of roots compared to WT.

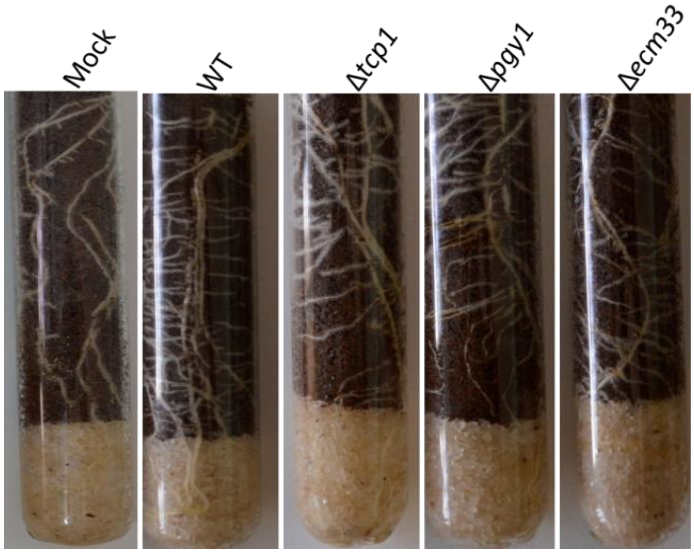


Fig. 6.10 Root growth assay. To assess the change in fresh weight of maize roots, the experiment was performed in glass tubes. Each glass tube was filled with 20 g autoclaved white sand (bottom) and 30 g potting-mix. The potting-mix was mixed with chlamydospores of WT and mutants at a rate of 1.0 g of chlamydospores per 500 g potting mix.

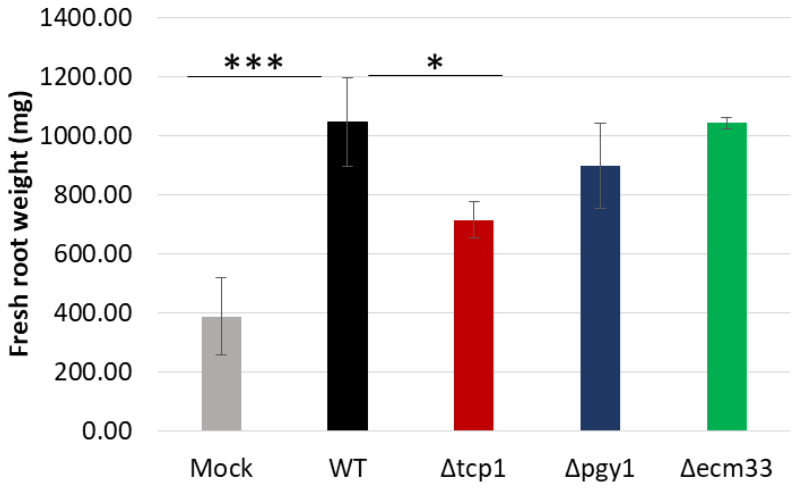


Fig. 6.11 Quantitative estimation of fresh weight of maize roots. Seeds of maize were inoculated with chlamydospores of WT and mutants. Mock was taken as negative control with no inoculation of chlamydospores ***: P< 0.001, *: P< 0.05.

6.4 DISCUSSION:

The process of mycoparasitism starts from the recognition and adhesion of *Trichoderma* to host pathogens, which is later followed by hydrolysis of host hyphae using lytic enzymes, and concludes with uptake of host cellular contents (367). *T. virens* has mycoparasitic activity against the plant pathogens *Sclerotium rolfsii, Pythium aphanidermatum* and *Rhizoctonia solani*. To know the roles of *tcp1*, *pgy1* and *ecm33* genes in mycoparasitic activity against the plant pathogens, confrontation assays were performed with wild type and mutant strains of *T. virens* against the plant pathogens. Δ*tcp1*, Δ*pgy* and Δ*ecm33* mutants lost the ability to overgrow the pathogen *Sclerotium rolfsii*, instead, the pathogen grew over the mutant strains of *Trichoderma* **(Fig 6.1)** and pathogen from these dual culture plates grew on PDA plate amended with 10 ppm benomyl **(Fig. 6.2)**.

The mycoparasitic activity of mutants was also compared with wild type against the plant pathoge*n Pythium aphanidermatum.* WT, Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants grew over the pathogen and fully covered the pathogen (Fig. 6.3). The pathogen from confrontation assays with WT or Δ*tcp1* did not grow on PDA amended with 10 ppm benomyl. The pathogen from confrontation assays with Δ*pgy1* or Δ*ecm33* grew on PDA amended with 10 ppm benomyl **(Fig. 6.4)**. The experiment concluded that, Δ*tcp1* retained the mycoparasitic activity against *P. aphanidermatum*, but Δ*pgy1* and Δ*ecm33* mutants lost their ability of mycoparasitism against this pathogen. Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants lost their mycoparasitic activity against the plant pathogen *Rhizoctonia solani.* In dual culture assay, *Rhizoctonia solani* grew over the Δ*pgy1* and Δ*ecm33* mutants, but in dual culture with Δ*tcp1* mutant, neither the mutant nor the pathogen grew over each other even after 10 days of inoculation at opposite ends of PDA plate **(Fig. 6.5)**. *T. virens* synthesized the antimicrobial secondary metabolite viridin which has antibiosis activity against the plant pathogen. Culture

filtrate of Δ*tcp1* mutant retained antibiosis activity but culture filtrates of Δ*pgy1* and Δ*ecm33* mutants lost their activity against the phytopathogen *P. aphanidermatum* **(Fig. 6.6)**. This result was further confirmed by quantitation of antimicrobial antibiotic viridin, by HPLC analysis. Culture filtrate extracts of Δ*pgy1* and Δ*ecm33* mutants showed reduced viridin biosynthesis but no significant change was observed in culture filtrate extract of Δ*tcp1* mutants as compared to WT **(Fig. 6.7 A, B)**. The mutants also showed changes in the profile of volatile compounds as compared to wild type. The gas chromatograms of WT, Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants showed 16, 7, 2 and 2 volatile compounds corresponding peaks, respectively **(Fig. 6.8, 6.9, Table 6.1)**.

Many genes are involved directly or indirectly in mycoparasitic activity of *Trichoderma* spp. In *Trichoderma harzianum,* the overexpression of the *nox1* gene showed negative effect on its mycoparasitic activity against *Pythium ultimum* and upregulation of synthesis of lytic enzyme (368). Chitinase, glucanase and protease activities are involved in host cell wall degradation (24). Integration of multiple copies of cell wall degrading enzymes like chitinase, β-glucosidase in *Trichoderma* genome increased biocontrol activity against the pathogen (369). *Trichoderma* strains overexpressing endochitinase gene (*ech42*) or protease gene (*prb1*) showed better biocontrol activities against the pathogen than the wild type strain of *Trichoderma* (370, 371). *T. atroviride* Tal6 effector protein is involved in mycoparasitism and plant association (372). Mazrou et al., highlighted the possibility of *T. harzianum* in integrated pest management as an effective biological agent (373). Recent report also confirmed that, light also have an important effect on the mycoparasitic activity of *T. atroviride* (374).

The Δ*lae1* mutants abolished sporulation triggered by mechanical injury, increased sensitivity to oxidative stress, loss of mycoparasitism and under-expression of several genes

that get upregulated during mycoparasitic interaction like proteases, glucanases and polyketide synthases (120). The Δ*epl1* mutants showed changes in mycoparasitic coiling process and expression pattern of plant defense genes compared to the wild type (122).

The transcription factor THCTF1 in *T. harzianum* has important role in biosynthesis of volatile secondary metabolite 6-pentyl-pyrone (136). Pachauri et al. confirmed the role of glyceraldehyde-3-phosphate dehydrogenase in biosynthesis of volatile sesquiterpenes in *T. virens* (137). In filamentous fungi, the intracellular siderophores have important roles in iron homeostasis which control iron related metabolic processes (375). A nonribosomal peptide synthase gene, *tex10* was predicted to be involved in synthesis of ferricrocin, an intracellular siderophore. The Δ*tex10* mutants showed enhanced growth rate, reduced conidiation, hypersensitivity to oxidative stress, reduced synthesis of gliotoxin, and enhanced ability to colonize maize seedling roots as compared to wild type (59). Novel metabolite TM2 from the *T. atroviride* has potential therapeutic activity against prostate cancer (376). Δ*tri10* mutants showed increase in production of ergosterol and polyketide compound aspinoloids, which is due to increase in intracellular levels of farnesyl diphosphate resulting from loss of trichothecene production (377).

Root system is a critical medium for the interaction between *Trichoderma* spp. and plants. *Trichoderma* treatments enhance the root biomass production and root hair development in plants (378, 379). The treatment of tomato plants with *T. harzianum* based formulations enhanced the shoot weight, root weight, and dry weight of tomato plants even when inoculated with plant pathogen *Rhizocotnia solani* (166). *Trichoderma virens* treatment promoted lateral root growth and enhanced biomass production through an auxin-dependent mechanism in *Arabidopsis* (380).

Chapter-7

General Discussion, Summary and Conclusions

An SSH (suppression subtractive hybridization) library was constructed in our laboratory, where a non-conidiating mutant strain (M7) was subtracted from the conidiating wild type strain. Three clones, C5, F3 and F6, which were under-expressed in the mutant when grown on agar as compared to shaking culture, were selected for detailed study in the present thesis. A homology search revealed that C5, F3 and F6 are orthologs of translationally controlled tumour protein (Tcp1), proline-, glycine-, tyrosine-rich protein (Pgy1) and Ecm33. These proteins are highly conserved across *Trichoderma* spp. as well as other fungi. The translationally controlled tumour proteins are highly conserved in eukaryotes and are involved in varied cellular and disease processes, while Pgy1 is a novel fungal protein (270). *T. virens* Pgy1 protein have XYPPX-like motifs at the N-terminus and a cysteine-rich CYSTM-like motif at the C-terminus. PGYRP proteins from organisms other than *Trichoderma* genus either have XYPPX domain at N-terminus or cysteine-rich transmembrane domain at C-terminus. Ecm33 is a glycosylphosphatidyl inositol anchored protein (GPI anchored) and is mainly involved in cell wall integrity (305).

Independent knockout mutants for *tcp1*, *pgy1* and *ecm33* genes were generated using split marker PCR through homologous recombination approach. All the knockout mutants were purified through single spore method and purity was confirmed by PCR and RT-PCR. All the knockout mutants have single copy integration of hygromycin resistance cassette in the genome as confirmed by real time PCR.

The morphology of the mutants had visible differences compared to WT on all the three media used in this study. Mutants of all the three genes showed slow radial growth on PDA compared to WT and highest reduction in radial growth was recorded in Δ*tcp1* mutant. The radial growth of Δ*pgy1* and Δ*ecm33* mutants was significantly less as compared to WT. Significant decrease in dry weight was observed in Δ*tcp1* mutant but there were no significant differences in dry weight of Δ*pgy1* and Δ*ecm33* mutants. Significant decrease in

conidia count was recorded in 3, 5 and 7-days old cultures of Δ*tcp1,* Δ*pgy1* and Δ*ecm33* knockout mutants on lab made PDA, HiMedia made PDA and VMG media. Microscopic analysis of cultures of WT and mutants on lab made PDA confirmed the delayed conidiation in Δ*pgy1* and Δ*ecm33* mutants. Three-days old cultures of WT and mutants showed conidiophores however, there were no conidia on the conidiophores of Δ*pgy1* and Δ*ecm33* mutants. Conidiation is a tightly regulated developmental process in many fungi, including *Trichoderma* spp., and information about these genes in *Trichoderma* would be very useful for the production of commercial biofungicide formulations.

Not only primary metabolites related genes but also the genes involved in secondary metabolites synthesis are involved in conidiation in filamentous fungi. Recent reports highlighted that histone deacetylase (*hda2*) and non-ribosomal peptide synthetase (*tex10*) genes are involved in conidiation in *T. atroviride* and *T. virens*, respectively (59, 259). Transcription factors KpeA and MrSwi6 are involved in conidiation in *Aspergillus oryzae* and *Metarhizium rileyi*, respectively (344, 345). Transcription factors Ada-6 and Chc regulate the conidiation in model fungus *N. crassa* (346). Effect of gene deletion was also seen on the growth and morphology of mutants under nutrient starvation condition imposed by putting fresh mycelial disc of WT and mutant cultures on the centre of 1% agar plate. From day three onwards, the area around the disc became green due to presence of conidia around the disc in WT but no such green growths were present around the disc of Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants. Fungi of *Trichoderma* genus produce abundant conidia under exposure to nutrient starvation (381). At day 7, more aerial hyphae were present in periphery of the Δ*ecm33* mutant as compared to the WT, Δ*tcp1* and Δ*pgy1* mutants.

Our findings also confirmed that, Δ*ecm33* and Δ*pgy1* mutants make higher number of chlamydospores in shake culture compared to WT and Δ*tcp1* mutant. Velvet protein of *T.*

virens (Vel1) is also involved in chlamydospores formation. *T. virens* Δ*vel1* mutant produced higher number of chlamydospores but lacked conidia when these mutants were grown on VMS broth and agar, respectively (349). In the present study, Δ*ecm33* and Δ*pgy1* mutant also showed fewer conidiation but higher chlamydospores formation compared to the WT. Tcp protein contains a Plk interaction domain and has key role in cell cycle progression in fungus in *A. nidulans*. Tcp deletion mutants (Δ*tcpA*) exhibited abnormal branching during vegetative growth in *A. nidulans*. The *tcpA* deletion enhanced asexual development via induction of *brlA* expression but inhibited sexual development (271). In *Arabidopsis thaliana*, Tcp is involved in the regulation of duration of the cell cycle (273). Two independent *tcp* gene deletion mutants (M3 and M35) were developed and phenotypically analysed in *Magnaporthe oryzae*. M3 and M35 showed 96.2 and 94.2% reduction in conidia count compared to WT. Conidial germination rate of M3 and M35 mutants at 2 hpi (hours post incubation) was 80.6 and 83.2% respectively, compared to the WT (272).

There is no study till now about the role of PGY protein in morphology and conidiation in any fungus. To the best of our knowledge, this is the first report on the role of Pgy1 protein in *T. virens* morphology and conidiation. The Ecm33 is a family of glycosylphosphatidylinositol-anchored (GPI-anchored) proteins and plays an important role in maintaining fungal cell wall integrity (310). Ecm33 protein is involved in conidiation in *Candida albicans, Beuveria bassiana* and *Metarhizium robertsii* (311, 323). Ecm33 deletion mutants of *A. fumigatus* showed defect in conidial separation and an increase in the conidial diameter of the mutant (327).

To find out the role of these genes in stress tolerance, we estimated the IC_{50} for different stress-causing chemicals on the basis of reduction in radial growth. The estimated IC_{50} value of WT strain of *T. virens* was 600 µg/ml for Congo red, 1.25 mM for menadione

sodium bisulfite, 2.5% for NaCl and 150 µg/ml for SDS. On the basis of change in radial growth of mutants, Δ*tcp1* mutant was found to be sensitive to salt while highest sensitivity to SDS and Congo red was noticed in Δ*ecm33* mutant. RNAi silencing of *tcp* gene in cabbage plants showed reduced vegetative growth and decreased tolerance to cold, high temperature, and salt stresses (356). Ecm33 deletion mutants showed higher sensitivity to cell wall destabilizing agent, Congo red, in *Candia albicans* due to abnormal electron-dense outer mannoproteins layer and an aberrant surface localization of the adhesion proteins (312, 320). Ecm33 protein in *Candida albicans* is essential for the proper functioning of the classical secretion pathway during host interaction (357). These studies suggested the role of *ecm33* gene in cell wall integrity in filamentous fungi. The role of *tcp1*, *pgy1* and *ecm33* genes in oxidative stress response was assessed by measuring changes in radial growth of WT and mutants on PDA plate amended with 1.25 mM menadione sodium bisulfite. Significant reduction was reported in radial growth of all three independent mutants compared to the WT.

Trichoderma treatment reduces the impact of stresses on plants by alteration in synthesis of antioxidant enzymes like peroxidase, polyphenol oxidase, catalase, glutathione reductase, phenylalanine ammonia lyase and ascorbate peroxidase (108). Salt stress reduces the plant growth and root development and this is due to blocking of auxin signaling (360). *Trichoderma* treatment increased plant tolerance against such stresses due to an improvement in plant root growth and nutrient uptake (107). Kelch domain protein of *T. harzianum*, Thkel1 conferred tolerance to salt stress when expressed in *Arabidopsis* plants and overexpressing Thkel1 facilitated root colonization which led to increase in plant productivity (85).

Trichoderma treated seeds of *Theobroma cacao* plants have a better adaptation to salt, heat and osmotic stress due to an improvement in plant root growth and water holding

capacity (100, 101). Limited molecular research has been carried out on stress tolerance mechanism in *Trichoderma*. The histone deacetylase gene (*hda2*) is involved in oxidative stress response in *T. atroviride* by regulating the expression and acetylation on the promoters of ROS-related genes (259). Salt stress response study confirmed that *tcp1* gene negatively regulates the radial growth under saline condition. Tcp1 deletion mutant also showed negative impact on salt stress response and positive impact on oxidative stress (menadione) response. Limited information is available about genes involved in saline stress response in fungi. AbSte7 negatively regulate salt tolerance and positively regulate oxidative stress tolerance in *Alternaria brassicicola* (362).

Treatment of mycelia of Δ*ecm33* and Δ*tcp1* mutants with cell wall lysing enzyme cocktail released significantly higher number of protoplast compared to WT. This observation confirmed the defect in cell wall integrity of Δ*ecm33* and Δ*tcp1* mutants. Δ*ecm33* mutant also showed higher sensitivity towards cell wall destabilizing agent Congo red and SDS, as compared to WT. TEM images of mycelia showed many changes in the cell wall structure of mutants as compared to the WT. Ecm33 is a GPI anchored protein and GPI anchor is conserved in sequence. GPI anchor proteins have important roles in post-translational modification in eukaryotes, by which many cell surface proteins such as cell surface enzymes, receptors and adhesion molecules are anchored to the cell membrane (224, 319). On the basis of localization, GPI proteins are involved in two functions. GPI proteins that are linked to cell wall are involved in filamentation, mating and adhesion to external matrix, while GPI proteins linked to plasma membrane have enzymatic activity (224, 320).

Hydrophobicity is conferred by the proteins called hydrophobins. These proteins are small secreted proteins, produced only by filamentous fungi and characterized by the presence of eight conserved cysteine residues. Hydrophobins reduce the surface tension of

the medium and are involved in a variety of biological functions in the life cycle of filamentous fungi. Hydrophobins are involve in the formation of a protective layer surrounding the sexual structures and hyphae, development of aerial hyphae, sporulation and spore dispersal, and fruiting body formation (366). Hydrophobicity test of Δ*tcp1*, Δ*pgy1* and Δ*ecm33* compared to WT confirmed that Δ*ecm33* mutant lost the hydrophobicity as compared to the WT, while the mutants of the other two genes retained their hydrophobicity. Ecm33 is a GPI anchored protein and GPI anchoring signals are composed of a C-terminal hydrophobic domain. The cells lost the hydrophobicity when glycosylphosphatidylinositol (GPI) anchor was cleaved by GPI specific phospholipase (365). These results indicate the importance of GPI anchored proteins like Ecm33 in maintaining hydrophobicity. The expression of hydrophobin genes was reduced in all the mutants as compared to WT but major reduction in expression of hydrophobin genes was recorded in Δ*ecm33*.

Trichoderma virens is a mycoparasite on the plant pathogens *Sclerotium rolfsii, Pythium aphanidermatum* and *Rhizoctonia solani*. Confrontation assay confirmed that Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants lost the mycoparasitic activity against the plant pathogens *Rhizoctonia solani* and *Sclerotium rolfsii*. Δ*pgy1* and Δ*ecm33* mutants also lost the mycoparasitic activity against the plant pathogen *Pythium aphanidermatum* but Δ*tcp1* mutant retained it. Culture filtrate of Δ*pgy1* and Δ*ecm33* mutants also lost the antibiosis activity against the plant pathogen *P. aphanidermatum*. *Trichoderma* strains overexpressing an endochitinase (*ech42*) or protease gene (*prb1*) showed better biocontrol activities against the pathogen than the wild type (370, 371). Knockout study confirmed the role of 4-phosphopantetheinyl transferase (*ppt1*) and *tal6* genes in mycoparasitism (372, 382).

HPLC analysis confirmed that Δ*pgy1* and Δ*ecm33* mutants synthesized lesser amount of viridin compared to WT but no significant change was observed in viridin quantity in

culture filtrate extract of Δ*tcp1* mutant. Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants also showed significant change in volatile compound biosynthesis as compared to the WT. Only a few genes involved in volatile compounds synthesis in *T. virens* are characterized till now and these genes are present in cluster. Terpene cyclase gene (*vir4*) is responsible for the biosynthesis of volatile terpene compounds in *Trichoderma virens* (335). Recent study of Pachauri et al., also confirmed the role of the "vir" cluster associated glyceraldehyde-3-phosphate dehydrogenase in the biosynthesis of volatile sesquiterpenes in *Trichoderma virens* (137). Our study confirmed the role of *tcp1* in biosynthesis of volatile compounds, while *pgy1* and *ecm33* are involved in the biosynthesis of both volatile and non-volatile compounds in *T. virens*.

The effect of *Trichoderma* application to maize roots on root growth was assessed. Maize seedlings inoculated with chlamydospores of Δ*pgy*1 and Δ*ecm*33 mutants did not show any significant change in fresh weight of roots as compared to those inoculated with WT. However, maize seedlings treated with Δ*tcp1* chlamydospores showed significant reduction in fresh weight of roots as compared to those inoculated with WT. Root system is a critical medium for the interaction between *Trichoderma* spp. and plants. *Trichoderma* treatment has been reported to enhance root biomass production and root hair development (378, 379). Study of *Trichoderma*-Arabidopsis interaction confirmed that auxin-dependent mechanism is involved in lateral root growth and enhanced biomass production (380). The treatment of tomato plants with *T. harzianum*-based formulation enhanced the shoot weight, root weight and dry weight of tomato plants even in the presence of the phytopathogen *Rhizoctonia solani* (166). The mycoparasitism activity of *Trichoderma* is influenced by many genes, yet there are no reports on the role of *tcp1, pgy1* and *ecm33* genes in mycoparasitism against the plant pathogens. Many genes of diverse nature like Ras-GTPase like protein *Tbrg-1*, hydrophobin

Tvhydii1 and cytochrome P450 *TvCyt2* are involved in mycoparasitism in *T. virens* (123, 125).

Phenotypic characterization of these deletion mutants gave the information whicwould help us to understand the mechanism behind the conidiation and mycoparasitism against plant pathogens. Information about the role of Ecm33 protein in hydrophobicity would help us to understand the involvement of GPI anchored protein in hydrophobicity in fungs. In continuity of this work, we will analyze the global gene expression in these mutants by RNAseq analysis, which would be very useful to identify the genes with significant changes in expression in mutants as compared to the WT. Transcriptome data will indicate the change in expression pattern of genes influenced by deletion of a particular gene, which would help us to understand the gene networks regulating conidiation, mycoparasitism and hydrophobicity in *Trichoderma*.

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Chapter-7

General Discussion, Summary and Conclusions

An SSH (suppression subtractive hybridization) library was constructed in our laboratory, where a non-conidiating mutant strain (M7) was subtracted from the conidiating wild type strain. Three clones, C5, F3 and F6, which were under-expressed in the mutant when grown on agar as compared to shaking culture, were selected for detailed study in the present thesis. A homology search revealed that C5, F3 and F6 are orthologs of translationally controlled tumour protein (Tcp1), proline-, glycine-, tyrosine-rich protein (Pgy1) and Ecm33. These proteins are highly conserved across *Trichoderma* spp. as well as other fungi. The translationally controlled tumour proteins are highly conserved in eukaryotes and are involved in varied cellular and disease processes, while Pgy1 is a novel fungal protein (270). *T. virens* Pgy1 protein have XYPPX-like motifs at the N-terminus and a cysteine-rich CYSTM-like motif at the C-terminus. PGYRP proteins from organisms other than *Trichoderma* genus either have XYPPX domain at N-terminus or cysteine-rich transmembrane domain at C-terminus. Ecm33 is a glycosylphosphatidyl inositol anchored protein (GPI anchored) and is mainly involved in cell wall integrity (305).

Independent knockout mutants for *tcp1*, *pgy1* and *ecm33* genes were generated using split marker PCR through homologous recombination approach. All the knockout mutants were purified through single spore method and purity was confirmed by PCR and RT-PCR. All the knockout mutants have single copy integration of hygromycin resistance cassette in the genome as confirmed by real time PCR.

The morphology of the mutants had visible differences compared to WT on all the three media used in this study. Mutants of all the three genes showed slow radial growth on PDA compared to WT and highest reduction in radial growth was recorded in Δ*tcp1* mutant. The radial growth of Δ*pgy1* and Δ*ecm33* mutants was significantly less as compared to WT. Significant decrease in dry weight was observed in Δ*tcp1* mutant but there were no significant differences in dry weight of Δ*pgy1* and Δ*ecm33* mutants. Significant decrease in

conidia count was recorded in 3, 5 and 7-days old cultures of Δ*tcp1,* Δ*pgy1* and Δ*ecm33* knockout mutants on lab made PDA, HiMedia made PDA and VMG media. Microscopic analysis of cultures of WT and mutants on lab made PDA confirmed the delayed conidiation in Δ*pgy1* and Δ*ecm33* mutants. Three-days old cultures of WT and mutants showed conidiophores however, there were no conidia on the conidiophores of Δ*pgy1* and Δ*ecm33* mutants. Conidiation is a tightly regulated developmental process in many fungi, including *Trichoderma* spp., and information about these genes in *Trichoderma* would be very useful for the production of commercial biofungicide formulations.

Not only primary metabolites related genes but also the genes involved in secondary metabolites synthesis are involved in conidiation in filamentous fungi. Recent reports highlighted that histone deacetylase (*hda2*) and non-ribosomal peptide synthetase (*tex10*) genes are involved in conidiation in *T. atroviride* and *T. virens*, respectively (59, 259). Transcription factors KpeA and MrSwi6 are involved in conidiation in *Aspergillus oryzae* and *Metarhizium rileyi*, respectively (344, 345). Transcription factors Ada-6 and Chc regulate the conidiation in model fungus *N. crassa* (346). Effect of gene deletion was also seen on the growth and morphology of mutants under nutrient starvation condition imposed by putting fresh mycelial disc of WT and mutant cultures on the centre of 1% agar plate. From day three onwards, the area around the disc became green due to presence of conidia around the disc in WT but no such green growths were present around the disc of Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants. Fungi of *Trichoderma* genus produce abundant conidia under exposure to nutrient starvation (381). At day 7, more aerial hyphae were present in periphery of the Δ*ecm33* mutant as compared to the WT, Δ*tcp1* and Δ*pgy1* mutants.

Our findings also confirmed that, Δ*ecm33* and Δ*pgy1* mutants make higher number of chlamydospores in shake culture compared to WT and Δ*tcp1* mutant. Velvet protein of *T.*

virens (Vel1) is also involved in chlamydospores formation. *T. virens* Δ*vel1* mutant produced higher number of chlamydospores but lacked conidia when these mutants were grown on VMS broth and agar, respectively (349). In the present study, Δ*ecm33* and Δ*pgy1* mutant also showed fewer conidiation but higher chlamydospores formation compared to the WT. Tcp protein contains a Plk interaction domain and has key role in cell cycle progression in fungus in *A. nidulans*. Tcp deletion mutants (Δ*tcpA*) exhibited abnormal branching during vegetative growth in *A. nidulans*. The *tcpA* deletion enhanced asexual development via induction of *brlA* expression but inhibited sexual development (271). In *Arabidopsis thaliana*, Tcp is involved in the regulation of duration of the cell cycle (273). Two independent *tcp* gene deletion mutants (M3 and M35) were developed and phenotypically analysed in *Magnaporthe oryzae*. M3 and M35 showed 96.2 and 94.2% reduction in conidia count compared to WT. Conidial germination rate of M3 and M35 mutants at 2 hpi (hours post incubation) was 80.6 and 83.2% respectively, compared to the WT (272).

There is no study till now about the role of PGY protein in morphology and conidiation in any fungus. To the best of our knowledge, this is the first report on the role of Pgy1 protein in *T. virens* morphology and conidiation. The Ecm33 is a family of glycosylphosphatidylinositol-anchored (GPI-anchored) proteins and plays an important role in maintaining fungal cell wall integrity (310). Ecm33 protein is involved in conidiation in *Candida albicans, Beuveria bassiana* and *Metarhizium robertsii* (311, 323). Ecm33 deletion mutants of *A. fumigatus* showed defect in conidial separation and an increase in the conidial diameter of the mutant (327).

To find out the role of these genes in stress tolerance, we estimated the IC_{50} for different stress-causing chemicals on the basis of reduction in radial growth. The estimated IC_{50} value of WT strain of *T. virens* was 600 µg/ml for Congo red, 1.25 mM for menadione

sodium bisulfite, 2.5% for NaCl and 150 µg/ml for SDS. On the basis of change in radial growth of mutants, Δ*tcp1* mutant was found to be sensitive to salt while highest sensitivity to SDS and Congo red was noticed in Δ*ecm33* mutant. RNAi silencing of *tcp* gene in cabbage plants showed reduced vegetative growth and decreased tolerance to cold, high temperature, and salt stresses (356). Ecm33 deletion mutants showed higher sensitivity to cell wall destabilizing agent, Congo red, in *Candia albicans* due to abnormal electron-dense outer mannoproteins layer and an aberrant surface localization of the adhesion proteins (312, 320). Ecm33 protein in *Candida albicans* is essential for the proper functioning of the classical secretion pathway during host interaction (357). These studies suggested the role of *ecm33* gene in cell wall integrity in filamentous fungi. The role of *tcp1*, *pgy1* and *ecm33* genes in oxidative stress response was assessed by measuring changes in radial growth of WT and mutants on PDA plate amended with 1.25 mM menadione sodium bisulfite. Significant reduction was reported in radial growth of all three independent mutants compared to the WT.

Trichoderma treatment reduces the impact of stresses on plants by alteration in synthesis of antioxidant enzymes like peroxidase, polyphenol oxidase, catalase, glutathione reductase, phenylalanine ammonia lyase and ascorbate peroxidase (108). Salt stress reduces the plant growth and root development and this is due to blocking of auxin signaling (360). *Trichoderma* treatment increased plant tolerance against such stresses due to an improvement in plant root growth and nutrient uptake (107). Kelch domain protein of *T. harzianum*, Thkel1 conferred tolerance to salt stress when expressed in *Arabidopsis* plants and overexpressing Thkel1 facilitated root colonization which led to increase in plant productivity (85).

Trichoderma treated seeds of *Theobroma cacao* plants have a better adaptation to salt, heat and osmotic stress due to an improvement in plant root growth and water holding

capacity (100, 101). Limited molecular research has been carried out on stress tolerance mechanism in *Trichoderma*. The histone deacetylase gene (*hda2*) is involved in oxidative stress response in *T. atroviride* by regulating the expression and acetylation on the promoters of ROS-related genes (259). Salt stress response study confirmed that *tcp1* gene negatively regulates the radial growth under saline condition. Tcp1 deletion mutant also showed negative impact on salt stress response and positive impact on oxidative stress (menadione) response. Limited information is available about genes involved in saline stress response in fungi. AbSte7 negatively regulate salt tolerance and positively regulate oxidative stress tolerance in *Alternaria brassicicola* (362).

Treatment of mycelia of Δ*ecm33* and Δ*tcp1* mutants with cell wall lysing enzyme cocktail released significantly higher number of protoplast compared to WT. This observation confirmed the defect in cell wall integrity of Δ*ecm33* and Δ*tcp1* mutants. Δ*ecm33* mutant also showed higher sensitivity towards cell wall destabilizing agent Congo red and SDS, as compared to WT. TEM images of mycelia showed many changes in the cell wall structure of mutants as compared to the WT. Ecm33 is a GPI anchored protein and GPI anchor is conserved in sequence. GPI anchor proteins have important roles in post-translational modification in eukaryotes, by which many cell surface proteins such as cell surface enzymes, receptors and adhesion molecules are anchored to the cell membrane (224, 319). On the basis of localization, GPI proteins are involved in two functions. GPI proteins that are linked to cell wall are involved in filamentation, mating and adhesion to external matrix, while GPI proteins linked to plasma membrane have enzymatic activity (224, 320).

Hydrophobicity is conferred by the proteins called hydrophobins. These proteins are small secreted proteins, produced only by filamentous fungi and characterized by the presence of eight conserved cysteine residues. Hydrophobins reduce the surface tension of

the medium and are involved in a variety of biological functions in the life cycle of filamentous fungi. Hydrophobins are involve in the formation of a protective layer surrounding the sexual structures and hyphae, development of aerial hyphae, sporulation and spore dispersal, and fruiting body formation (366). Hydrophobicity test of Δ*tcp1*, Δ*pgy1* and Δ*ecm33* compared to WT confirmed that Δ*ecm33* mutant lost the hydrophobicity as compared to the WT, while the mutants of the other two genes retained their hydrophobicity. Ecm33 is a GPI anchored protein and GPI anchoring signals are composed of a C-terminal hydrophobic domain. The cells lost the hydrophobicity when glycosylphosphatidylinositol (GPI) anchor was cleaved by GPI specific phospholipase (365). These results indicate the importance of GPI anchored proteins like Ecm33 in maintaining hydrophobicity. The expression of hydrophobin genes was reduced in all the mutants as compared to WT but major reduction in expression of hydrophobin genes was recorded in Δ*ecm33*.

Trichoderma virens is a mycoparasite on the plant pathogens *Sclerotium rolfsii, Pythium aphanidermatum* and *Rhizoctonia solani*. Confrontation assay confirmed that Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants lost the mycoparasitic activity against the plant pathogens *Rhizoctonia solani* and *Sclerotium rolfsii*. Δ*pgy1* and Δ*ecm33* mutants also lost the mycoparasitic activity against the plant pathogen *Pythium aphanidermatum* but Δ*tcp1* mutant retained it. Culture filtrate of Δ*pgy1* and Δ*ecm33* mutants also lost the antibiosis activity against the plant pathogen *P. aphanidermatum*. *Trichoderma* strains overexpressing an endochitinase (*ech42*) or protease gene (*prb1*) showed better biocontrol activities against the pathogen than the wild type (370, 371). Knockout study confirmed the role of 4-phosphopantetheinyl transferase (*ppt1*) and *tal6* genes in mycoparasitism (372, 382).

HPLC analysis confirmed that Δ*pgy1* and Δ*ecm33* mutants synthesized lesser amount of viridin compared to WT but no significant change was observed in viridin quantity in

culture filtrate extract of Δ*tcp1* mutant. Δ*tcp1*, Δ*pgy1* and Δ*ecm33* mutants also showed significant change in volatile compound biosynthesis as compared to the WT. Only a few genes involved in volatile compounds synthesis in *T. virens* are characterized till now and these genes are present in cluster. Terpene cyclase gene (*vir4*) is responsible for the biosynthesis of volatile terpene compounds in *Trichoderma virens* (335). Recent study of Pachauri et al., also confirmed the role of the "vir" cluster associated glyceraldehyde-3-phosphate dehydrogenase in the biosynthesis of volatile sesquiterpenes in *Trichoderma virens* (137). Our study confirmed the role of *tcp1* in biosynthesis of volatile compounds, while *pgy1* and *ecm33* are involved in the biosynthesis of both volatile and non-volatile compounds in *T. virens*.

The effect of *Trichoderma* application to maize roots on root growth was assessed. Maize seedlings inoculated with chlamydospores of Δ*pgy*1 and Δ*ecm*33 mutants did not show any significant change in fresh weight of roots as compared to those inoculated with WT. However, maize seedlings treated with Δ*tcp1* chlamydospores showed significant reduction in fresh weight of roots as compared to those inoculated with WT. Root system is a critical medium for the interaction between *Trichoderma* spp. and plants. *Trichoderma* treatment has been reported to enhance root biomass production and root hair development (378, 379). Study of *Trichoderma*-Arabidopsis interaction confirmed that auxin-dependent mechanism is involved in lateral root growth and enhanced biomass production (380). The treatment of tomato plants with *T. harzianum*-based formulation enhanced the shoot weight, root weight and dry weight of tomato plants even in the presence of the phytopathogen *Rhizoctonia solani* (166). The mycoparasitism activity of *Trichoderma* is influenced by many genes, yet there are no reports on the role of *tcp1, pgy1* and *ecm33* genes in mycoparasitism against the plant pathogens. Many genes of diverse nature like Ras-GTPase like protein *Tbrg-1*, hydrophobin

Tvhydii1 and cytochrome P450 *TvCyt2* are involved in mycoparasitism in *T. virens* (123, 125).

Phenotypic characterization of these deletion mutants gave the information whicwould help us to understand the mechanism behind the conidiation and mycoparasitism against plant pathogens. Information about the role of Ecm33 protein in hydrophobicity would help us to understand the involvement of GPI anchored protein in hydrophobicity in fungs. In continuity of this work, we will analyze the global gene expression in these mutants by RNAseq analysis, which would be very useful to identify the genes with significant changes in expression in mutants as compared to the WT. Transcriptome data will indicate the change in expression pattern of genes influenced by deletion of a particular gene, which would help us to understand the gene networks regulating conidiation, mycoparasitism and hydrophobicity in *Trichoderma*.

LIST OF FIGURES

Fig. 2.1	Schematic representation of pATBS plasmid used in construction of vectors for generation of knockout mutants	**71**
Fig. 2.2	Generation of the flanking regions along with part of the hygromycin resistance cassette	**73**
Fig. 2.3	Triple homologous recombination to generate gene deletion mutants	**74**
Fig. 2.4	RNA isolation from WT and mutants	**85**
Fig. 3.1	Phylogenetic analysis of fungal Tcp1 proteins	**96**
Fig. 3.2	Phylogenetic analysis of fungal Pgy1 protein	**98**
Fig. 3.3	Phylogenetic analysis of fungal Ecm33 proteins	**100**
Fig. 3.4	Conserved motifs analysis of Pgy1 proteins	**102**
Fig. 3.5	Alignment of fungal Pgy1 protein sequences and identification of conserved motifs	**103 & 104**
Fig. 3.6	PCR amplification of left and right flank of *tcp1*, *pgy1* and *ecm33* gene	**106**
Fig. 3.7	PCR amplification of left flank and right flank of *tcp1*, *pgy1* and *ecm33* ORF's with desired part of hygromycin marker gene cassettes	**108**
Fig. 3.8	PCR and RT-PCR confirmation of *tcp1* gene deletion	**110**
Fig. 3.9	PCR and RT-PCR confirmation of *pgy*1 gene disruption	**111**
Fig. 3.10	PCR-based confirmation of *ecm33* gene disruption	**112**
Fig. 3.11	Morphology of WT and Δ*tcp1,* Δ*pgy1* and Δ*ecm33* mutants on PDA after 7 days of incubation	**113**
Fig. 3.12	Copy number determination of integrated hygromycin resistance cassette in the knockout mutant's genome	**115**
Fig. 4.1	Colony morphology and radial growth of the WT and Δ*tcp*1, Δ*pgy1* and Δ*ecm33* mutants on PDA	**123**
Fig. 4.2	Biomass and dry weight of WT, Δ*tcp*1, Δ*pgy1* and Δ*ecm33* mutants	**124**
Fig. 4.3	Colony morphology (A) and spore count (B) of WT and mutant strains (Δ*tcp*1, Δ*pgy*1 and Δ*ecm33*) of *T. virens* on lab made PDA at 28℃	**127**
Fig. 4.4	Microscopic analysis of 3 day-old culture of WT and mutant strains (Δ*tcp*1, Δ*pgy*1 and Δ*ecm33*) of *T. virens* on potato dextrose agar plate using light microscope	**128**
Fig. 4.5	Colony morphology (A) and quantitative presentation of spore count (B)	**130**

	of WT and mutant strains (Δ*tcp*1, Δ*pgy*1 and Δ*ecm33*) of *T. virens* on HiMedia PDA at 28℃	
Fig. 4.6	Colony morphology (A) and spore count (B) of WT and mutant strains (Δ*tcp*1, Δ*pgy*1 and Δ*ecm33*) of *T. virens* on Vogel's minimal media at 28℃	**132**
Fig. 4.7	Morphology of WT and mutant strains of *T. virens* on 1% agar	**133**
Fig. 4.8	Colony morphology of WT and mutant strains (Δ*tcp*1, Δ*pgy*1 and Δ*ecm33*) of *T. virens* at the centre of 7 days old culture, 1% agar plate	**134**
Fig. 4.9	Morphology of WT and mutant strains (Δ*tcp*1, Δ*pgy*1 and Δ*ecm33*) of *T. virens* at periphery of 7 days old culture on 1% agar plate	**134**
Fig. 4.10	Chlaymydospores formation by WT and mutant strains (Δ*tcp*1, Δ*pgy*1 and Δ*ecm33*) of *T. virens*	**135**
Fig. 4.11	Transmission electron microscopy of mycelia of WT, Δ*tcp1*, Δ*pgy1* and Δ*ecm33* strains of *T. virens*	**136**
Fig. 4.12	Transmission electron microscopy of conidia of WT, Δ*tcp1*, Δ*pgy1* and Δ*ecm33* strains of *T. virens*	**137**
Fig. 5.1	Morphology and Radial growth of WT strain of *T. virens* on PDA as control media and PDA amended with sodium dodecyl sulphate (SDS)	**146**
Fig. 5.2	Morphology and radial growth of WT strain of *T. virens* on PDA as control media and PDA amended with NaCl	**147**
Fig. 5.3	Morphology and radial growth of WT strain of *T. virens* on PDA as control media and PDA amended with menadione sodium bisulfite	**148**
Fig. 5.4	Morphology and radial growth WT strain of *T. virens* on PDA as control media and PDA amended with Congo red	**149**
Fig. 5.5	Morphology of WT, Δ*tcp*1, Δ*pgy*1 and Δ*ecm33* mutants under different stress conditions compared to control media (only PDA) after 24 hr of incubation	**151**
Fig. 5.6	Morphology of WT, Δ*tcp*1, Δ*pgy*1 and Δ*ecm33* mutants under different stress conditions compared to control media (only PDA) after 48 hr of incubation	**153**
Fig. 5.7	Radial growth of WT, Δ*tcp*1, Δ*pgy*1 and Δ*ecm33* mutants under different stress conditions compared to control after 24 hr and 48 hr after incubation	**154**

Fig. 5.8	Morphology of WT, Δ*tcp*1, Δ*pgy*1 and Δ*ecm33* on PDA amended with menadione sodium bisulfite front view of plate and back view of plate after 7 days of incubation	**155**
Fig. 5.9	Release of protoplast from the mycelia of WT and mutants after treatment with cell wall lysing enzyme cocktail	**156**
Fig. 5.10	Transmission electron microscopy of mycelia of WT, Δ*tcp1*, Δ*pgy1* and Δ*ecm33* strains of *T. virens* showed alteration in cell wall integrity	**157**
Fig. 5.11	Transmission electron microscopy of conidia of WT, Δ*tcp1*, Δ*pgy1* and Δ*ecm33* strains of *T. virens* showed alteration in cell wall composition	**158**
Fig. 5.12	Hydrophobicity test of WT, Δ*tcp1,* Δ*pgy1* and Δ*ecm33* mutants and expression of hydrophobin genes in WT and mutants of *T. virens* after 7 days of incubation on PDA plates	**160**
Fig. 6.1	Confrontation assay of *T. virens* WT and mutants against plant pathogenic fungus *S. rolfsii*	**170**
Fig. 6.2	Viability of *S. rolfsii* taken from confrontation assay plate	**170**
Fig. 6.3	Confrontation assay of *T. virens* WT and mutants against plant pathogen *P. aphanidermatum*	**172**
Fig. 6.4	Viability of *P. aphanidermatum* taken from confrontation assay plate	**172**
Fig. 6.5	Confrontation assay of *T. virens* WT and mutants against plant pathogenic fungus *R. solani*	**173**
Fig. 6.6	Antibiosis assay	**174**
Fig. 6.7	HPLC profile and quantification of viridin synthesized by *T. virens* WT and mutants	**175**
Fig. 6.8	GC-MS analysis of WT strain of *Trichoderma virens*	**176**
Fig. 6.9	GC-MS analysis of WT and mutant strains (Δ*tcp1,* Δ*pgy1* and Δ*ecm33*) of *T. virens*	**177**
Fig. 6.10	Root growth assay	**179**
Fig. 6.11	Quantitative estimation of fresh weight of maize roots	**179**

LIST OF TABLES

Table 1.1	Genes/Proteins involved in morphology in *Trichoderma* spp	**43-44**
Table 1.2	Biological roles of Tcp proteins in plants, microbes, and plant microbe interactions	**47-49**
Table 1.3	Biological roles of Pgy proteins in plants, microbes, and plant microbe interactions	**50**
Table 1.4	Biological roles of Ecm33 proteins in plants, microbes, and plant microbe interactions	**54-56**
Table 2.1	Primers used in this study with purpose	**67-70**
Table 3.1	Estimation of copy no. of mutants using ΔΔCt method	**116**
Table 6.1	List of volatile compounds detected in GC-MS analysis of WT and mutant strains of *T. virens*	**177**

Thesis Highlight

Name of the Student: Ravindra Bansal
Name of the CI/OCC: BARC, Trombay **Enrolment No.:** LIFE01201504001
Thesis Title: "Functional analysis of three genes downregulated in a non-conidiating mutant of *Trichoderma virens*"
Discipline: Life Sciences **Sub-Area of Discipline:** Fungal genetics
Date of viva voce: 22-12-2020

In an attempt to identify the genes involved in conidiation, an SSH (suppression subtractive hybridization) library was earlier constructed in our laboratory where a non-conidiating mutant strain was subtracted from the conidiating wild type strain. Further expression analysis of the genes from the library led to identification of three genes that are down-regulated in the mutant. A homology search revealed that these three proteins are orthologs of translationally controlled tumour protein (Tcp1), Proline-, glycine-, tyrosine-rich protein (Pgy1) and Ecm33. The functions of none of these genes are known in *Trichoderma*, and hence, the present study was undertaken to unravel the role of these three genes in *Trichoderma* biology.

For functional characterization of the genes, independent knockout mutants (*Δtcp1, Δpgy1* and *Δecm33*) were generated by split marker PCR technique and role of these genes in conidiation, mycoparasitism, antibiosis, secondary metabolite biosynthesis, cell wall integrity, hydrophobicity and stress responses have been studied. All these independent mutants showed slower radial growth rate on PDA compared to wild type (WT) and Δ*tcp1* mutants also showed significant decrease in dry weight compared to WT. Stress tolerance response analysis of WT and mutants confirmed that, Δ*tcp1* mutant had sensitivity to salt while highest sensitivity to SDS and Congo red was noticed in Δ*ecm33* mutant.

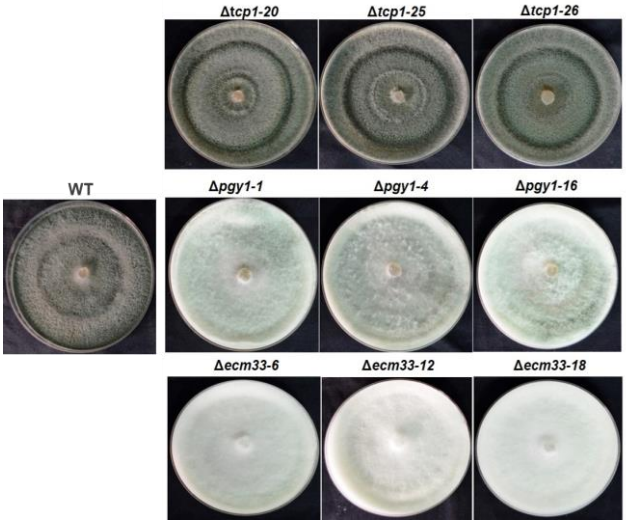

Figure 1. Morphology of WT, Δtcp1, Δpgy1 and Δecm33 mutants on PDA after 7 days of incubation.

Study of Δ*ecm33* mutant also confirmed the role of *ecm33* gene in cell wall integrity and hydrophobicity. Confrontation assay confirmed the roles of *pgy1* and *ecm33* genes in the mycoparasitic activity against the plant pathogens *Rhizoctonia solani*, *Sclerotium rolfsii* and *P. aphanidermatum* while *Δtcp1* mutants retained the mycoparasitic activity against *P. aphanidermatum*. The *pgy1* and *ecm33* genes are also involved in viridin biosynthesis while all the three genes are involved in volatile compounds biosynthesis. Maize seedlings treated with Δ*tcp1* showed significant reduction in fresh weight of roots compared to WT but no significant change was observed with chlamydospores of Δ*pgy1* and Δ*ecm33* mutants.