# ANTAGONASTIC POTENTIAL OF TRICHODERMA VIRIDE MUTANT AND THEIR COMPATABILITY WITH FUNGISIDES

**THESIS**

**Submitted to**
**Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola**
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## (PLANT PATHOLOGY)

**By**

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## DECLARATION OF STUDENT

I hereby declare that the experimental work and its interpretation of Thesis entitled **"ANTAGONASTIC POTENTIAL OF TRICHODERMA VIRIDE MUTANT AND THEIR COMPATABILITY WITH FUNGISIDES"** or part there of has neither been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis or publication of University or Scientific Organization. The sources of material used and all assistance received during the course of investigation have been duly acknowledged.

Place: Akola (Vyawahare Manoj Prakash)

Date: / /2018 Enrolment No. KK/1868

# CERTIFICATE

This is to certify that the thesis entitled **"ANTAGONASTIC POTENTIAL OF TRICHODERMA VIRIDE MUTANT AND THEIR COMPATABILITY WITH FUNGISIDES"** submitted in partial fulfillment of the requirements for the degree of "**Master of science in Agriculture (Plant Pathology)"** of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafied research work carried out by **VYAWAHARE MANOJ PRAKASH** under my guidance and supervision.

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

Place: Akola
Date: / /2018

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**Place :** Akola. **(Vyawahare Manoj Prakash)**

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# Table of Contents

## (A) List of Tables

## (B) List of Figures

## (C) List of Plates

## (D) List of Abbreviations

| | | |
|---|---|---|
| % | - | Per cent |
| @ | - | At the rate of |
| $^0$C | - | Degree celcius |
| C.D. | - | Critical difference |
| DAI | - | Days after inocubation |
| e.g. | - | Exampli gratia (For example) |
| et.al | - | Et alia (and others) |
| etc | - | Et cetra |
| EMS | - | Ethyal methyl sulphonate |
| Fig | - | Figure |
| g | - | Gram |
| h | - | Hours |
| HA | - | Hydroxyl Amine |
| M | - | Molar |
| mg | - | milligram |
| ml | - | millilitre |
| µl | - | microlitre |
| mM | - | Millimolar |
| µm | - | micrometer |
| nm | - | nanometer |
| PDA | - | Potato Dextrose Agar |
| m | - | minutes |
| FCRD | - | Factorial completely randomized design |
| Dr. PDKV | - | Dr. PanjabraoDeshmukhKrishiVidyapeeth |
| i.e. | - | That is |
| *In vitro* | - | In laboratory |
| *In vivo* | - | In field |
| J | - | Journal |
| Kg | - | Kilogram |
| Ml | - | Millilitre |
| mm | - | Millimeter |
| No. | - | Number |

| | | |
|---|---|---|
| S.E.(m)+ | - | Standard error of mean |
| Sig. | - | Significant |
| Spp./sp | - | Species |
| PGI | - | Per cent growth inhibition |
| TvME | - | *Trichoderma viride*mutants obtained by Ethyl methane sulphonate (EMS) |
| TvMH | - | *Trichoderma viride*mutants obtained by Hydroxyl amine (HA) |
| TvC | - | *Trichoderma viride* (mother culture) |

## (F) Thesis Abstract

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### ABSTRACT

The present investigations were on "Antagonistic potential of *Trichoderma viride* mutants and their compatibility with fungicides" with an objectives to Induce the mutation in *Trichoderma viride* by using chemicals and compatibility of mutants with fungicides. An experiment was conducted

at Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth Akola (M.S.) during 2017-18.

An antagonistic ability of a biocontrol agent was determined by its physiological state, so that change in physiological or genetical conditions could alter the antagonism and hence the genetic modification using chemical mutagenesis i.e. Ethyl Methane Sulphonate (EMS) and Hydroxyl Amine (HA), @ 50, 100,150 200 and 250 µl/ml and time interval at 60 minutes was carried out. Ten *Trichoderma viride* mutants were obtained after chemical mutagenesis and check their stability upto $7^{th}$ generation by observing morphological characters. Efficient mutants were then tested for their antagonistic ability *in vitro* against predominant soil borne plant pathogens viz., *Sclerotium rolfsii, Rhizoctonia bataticola, Fusarium udum* by adopting dual culture technique.

Among the mutants most of them were found efficient against *Sclerotium rolfsii, Rhizoctonia bataticola, Fusarium udum*. The mutants TvME-4 ($T_4$), TvMH-8 ($T_8$), TvME-3 ($T_3$) and TvMH-9 ($T_9$) exhibited maximum antagonistic activity against *S. rolfsii* which showed 79.06, 78.98, 78.10 and 77.90 per cent growth inhibition. In case of *R. bataticola,* the mutants TvME-4($T_4$), TvMH-8 ($T_8$), TvME-3 ($T_3$) and TvMH-10 ($T_{10}$) showed maximum growth inhibition i.e. 73.75, 73.56, 72.21 and 69.91% growth inhibition. *Fusarium udum* was also inhibited by mutants TvME-3 ($T_3$), TvME-4 ($T_4$), TvMH-9 ($T_9$) and TvMH-8 ($T_8$) showed maximum per cent growth inhibition i.e.78.88, 77.90, 76.94 and 76.54 per cent growth inhibition. The common efficient mutants which showed antagonistic activities against all three pathogens are TvME-4, TvMH-8 and TvME-3 where as mother culture showed least efficacy against tested pathogens.

The highest chitinase enzyme units/mg of protein i.e. 0.64, 0.63 and 0.62 was exerted in TvME-4 ($T_4$), TvMH-9 ($T_9$) and TvME-3 ($T_3$) respectively. However TvMH-8 ($T_8$) contains 0.61 enzyme units, it showed maximum inhibitory ability against *F. udum*. Mother culture of *T. viride* contain only 0.38 chitinase enzyme units/mg of protein. Hence, present study proved that mutagenesis by using chemicals is the efficient tool for improving bio efficiency of *Trichoderma viride.*

The mother culture of *T. viride* and its mutants showed differences in the compatability with fungicides. Carbendazim 50% WP @ 0.1 %, Tebuconazole 25% WG @ 0.05%, Mancozeb 75% WP @ 0.2 % having inhibitory effect and found not compitable but Azoxystrobin 23% SC @ 0.2 % and Chlorothalonil 75 % WP @ 0.2 % were compatible with the all mutants and mother culture of *T. viride*. Among mutants TvME-4 ($T_4$), TvMH-8 ($T_8$), TvMH-9 ($T_9$), and TvMH-10 ($T_{10}$), were found more compatible as compare to mother culture. Therefore, it is assumed that *T. viride* mutants can be safely incorporated into IDM of soil borne diseases.

# CHAPTER I

# INTRODUCTION

## 1.1 Background Information

The present day world is facing various problems regarding food security. The traditional agriculture is affected by various problems such as drought, pest and diseases, reduced availability of the land, increase in population. Among them, pests and diseases cause major losses. According to a study conducted by Bowyer (1999), the total loss as a consequence of plant diseases could be as high as 25 per cent of the yield in western countries and almost 50 % in developing countries. Of these, one third is due to fungal infection. In addition to causing yield losses, fungal infection reduces the quality of the product due to the presence of toxic metabolites (Alderman *et al*., 1996).

Plant diseases alone contribute more than 10 percent loss in global food production. Indiscriminate use of chemicals to overcome the pathogens has caused enhancement of over head costs, accumulation of toxic chemical residues in food chain and soil pollution leading to loss of soil health. Apart from this, the chemicals tend to become less efficient due to the development of resistance among the pathogens over time scale. Under these circumstances, the use of various eco-friendly biocontrol agents is increasingly being emphasized as an important component of the integrated pest management.

The concept of biocontrol embodies introduction of antagonists into cropping systems. A living multiplying biocontrol agent potentially provides a continuous, non chemical control of pathogen. Morever chemical measures may establish imbalance in the microbiological community i.e. unfavourable situation for activity of beneficial organisms. So direct application of antagonist would be safer method for introducing micro organisms into the soil for biological control of soil borne plant pathogen.

Many fungi are used as biocontrol agents but *Trichoderma* spp. is widely used as bio agent, biological control of plant diseases used

to be, more successful to date in rhizosphere than in phyllosphere. *Trichoderma* is an ubiquitous, easy to isolate and cultured, grown rapidly on many substrate, acts as a mycoparasite, competes well for food and site, produces antibiotics and capable of attacking a wide range of plant pathogens. Among their other activities, they grow tropically towards and attach to the hyphae of host fungi via coiling, hooks and appressorium like bodies and penetrate the host cell wall by secreting lytic enzymes such as chitinase and glucanase. This process (mycoparasitism) limits growth and activity of plant pathogenic fungi. Some times in conjunction with mycoparasitism, individual strain also produces antibiotics.There are about 41 species in the genus *Trichoderma.* The species and the isolates within the same species differ in their biocontrol potential (Goes *et al*. 2002; Umamaheswari and Sankaralingam, 2005; Upendra, 2006). Therefore, isolation and characterizeation of *Trichoderma* from different geographical locations is likely to provide an array of diverse isolates within desired biocontrol potential against plant diseases.

Soil borne fungal pathogen such as *Rhizoctonia bataticola, Fusarium udum, Sclerotium rolfsii, Pythium, Phytopthora* etc. cause greater reduction in crop yield. The use of fungicides for control of these pathogens has met with moderate success and their future use is a question due to increased regulatory restrictions. In addition, a number of currently used fungicides, such as mercurials have been withdrawn from the market, minimising the use of fungicides as means of managing soil borne plant pathogens. One means of overcoming these problems is to use an Integrated Disease Management (IDM) system which would include bio control strategies also. A component of such strategies might include the use of non-pathogenic soil borne fungi which secrete low molecular weight protein compound that may be toxic to the pathogen. The discovery of new biological control agents and the demonstration of their value is reducing disease incidence and severity have opened new promising avenues for practical applications in agriculture and for promoting environmental safety (Boland, 1990). Since the pioneering work of the use of *Trichoderma* strains to control damping off caused by *Rhizoctonia solan*i in citrus,

considerable attention has been focused over the past 25 years on effective as chemicals in suppressing fungal pathogens. volatile and non-volatile antibiotics produced by *T. harzianum* inhibited *Fusarium culmorum* by 42.3 per cent on agar plates (Michrina *et al*., 1995). The fungal antagonists like *T. viride*, *T. harzianum*, *T. hamatum, T. koningii, T. pseudokoningii, T. longibrachiatum* and *Gliocladium virens* were evaluated against black gram root rot fungus *M. phaseolina*. *T. harzianum* and *T. longibrachiatum* were on par in controlling *M. phaseolina* (Indra *et al*., 2003).

The relationship between mycolytic enzymes produced by *Trichoderma* and their significance in host cell degradation. The production of non-volatile (diffusible) antibiotics by *Trichoderma* spp. by an “agar layer technique”.

**1.2 Importance of Study**

Genetic transformation of existing biocontrol fungi that are well adjusted to their environment is likely to enhance their biocontrol capabilities. One attractive approach would be to select the stable and safe transgenic fungi with different genes coding for cell wall degrading enzymes (chitinase and glucanase) which express co-ordinately to combat the phytopathogen. Chitin, glucan and proteins are the main structural components of fungal cell wall. In plants, induction of chitinase, glucanase and other hydrolytic enzymes is one of the coordinated, often complex and multifaceted defence mechanism triggered in response to phytopathogen attack. But hydrolytic enzymes produced by plant itself are not sufficient to confer resistance to multiple pathogens. Fungal chitinases and glucanases appear to be more effective in their ability to inhibit pathogenic fungi than enzymes from other sources. As fungi play a major role in causing most of the plant diseases, genetic engineering of plants by transferring genes encoding chitinase and glucanase to develop plants resistant to fungal diseases is an approach of worth consideration.

The effect of volatile compounds of *T. harzianum* on the growth of *S. rolfsii* when *Trichoderma* isolates were grown for three days and *S. rolfsii* was exposed for 48 hours to vapour, the growth of *S. rolfsii*

was reduced in case of isolates 3 and 4 and stimulated by isolates 1 and 2. The vapour action of *T. harzianum* on 6th day was most inhibitory and thereafter it declined and reached zero on 15th day.

It is necessary to mutagenesis of the microorganisms including those used as biocontrol agents were applied to improve of the antifungal production and antagonistic potential over a broad spectrum of phytopathogens, survival, longevity and activity (Ximena *et al*., 2007). Several successful endeavours had been made to rectify the biocontrol potential of *Trichoderma* or *Gliocladum* species (which were bankrupted to generate new biotypes) by exposing the spores to chemical or physical mutagens. Physical mutagen like UV-ray (Melo *et al.,* 1997 and Brunner *et al*., 2005).

Haggag and Mohamed (2002) found that mutagenesis of three *Trichoderma* species by gamma irradiation exhibited high capabilities to produce efficient antibiotics, enzymes and phenols, corresponded to better on white rot disease control in overall biocontrol ability.

In the mutational studies of *Trichoderma viride* conducted by using chemical mutagenesis i.e. Polyethylene glycol (PEG),(Sreerama Kumar and palakshappa, 2005) and physical mutagens like UV irradiations.

Dinakaran and Marimuthu (1998) developed nine mutants of *T. viride* using different mutagenic agents viz. UV irradiation and gamma irradiation (150 kr). Selvakumar (2000) observed UV light and EMS induced mutants in *T. viride*, which showed stable colony characters, fungicide tolerance and antagonistic potential from a parent strain. Papavizas and Lewis (1982) evaluated wild strain WT-6 of *T. harzianum* by exposing to UV irradiation.

*Trichoderma viride* is a potential biocontrol agent against several soil and seed borne pathogens. Owing to its antagonistic effect on inimical organism, it ranks as one of the most successful agents for biological control of pathogens (Gupta, 2004). As some fungicides are effectively used as seed-dressing, it is necessary to study compatibility of these with the commonly used bioagent, *T. viride.* (Bindu *et. al*. 2011).

Study of compatibility with commonly used fungicides, insecticides, plant extracts and bio-agents on growth of native *Trichoderma* spp. is advocated to achieve effective control of plant diseases. Compatibility of these bioagents will make them highly suitable for exploitation in future within framework of integrated disease management program.

### 1.3 Objectives of the Study

Advances in molecular biology have laid the foundation for isolation of valuable genes and their transfer to target plants through novel transgenic approach. Chitinases, *ß*-1, 3- glucanases and proteases are the best studied antifungal proteins. Chitinase encoding genes are being used to improve plant defence against fungal pathogens. These enzymes are capable of degrading the linear homopolymer of ß-1, 4-N-acetyl-Dglucosamine, the main cell wall component of most phytopathogenic fungi, showing strong inhibitory activity *in vitro* on spore germination and hyphal growth (Lorito *et al*., 1994). Plants do have chitinases, but are not as effective as microbial chitinases. Therefore, cloning and characterization of genes from biocontrol microbes such as *Trichoderma* is very important. There is still possibility to make genetical changes even though not using modern genetical engineering for specific implementation. Compatatibility of *Trichoderm viride* with fungicide will make it highly suitable for explotation in futureTherefore, considering the importance of *Trichoderma* as potential biocontrol agent the present investigation on title “Antagonistic potential of *Trichoderma viride* mutants and their compatability with fungicides” was carried out with the following objectives:

1. Induction of mutation in *Trichoderma viride*
2. Compatibility of *Trichoderma viride* mutants with fungicides.

### 1.4 Scope and Limitations

*Trichoderma* has been widely studied for their biocontrol ability, but *Trichoderma* have some limitations in their use as biocontrol agents in agriculture is due to the unpredictable efficiency which is affected by biotic and abiotic factors in soil. There is also limitation to use bio-control agents along with the fungicide because of their adverse effect on

*Trichoderma*. Hence it is essential to increase their bio control efficacy against targeted pathogen and to made, it tolerant to fungicide, mutagenesis is the tool for improving genetic makeup of bio agents.

### 1.5 Hypothesis

*Trichoderma* is a promising antagonistic organism for the biological control of plant pathogenic fungi. While the use of *Trichoderma* as bio-control agent against target pathogens, it is important to consider its efficiency. Mutagenesis is one of the important mean to enhance the antagonistic properties of *Trichoderma* against target pathogen. Considering this possibilities, present study was conducted.

## CHAPTER II

# REVIEW OF LITERATURE

**2.1. History**.

The genus *Trichoderma* was introduced by Persoon (1794) for four microscopically similar fungi. The four species originally described in *Trichoderma* viz., *T. aureum, T. nigrescens, T.roseum* and *T.viride* each with different coloured conidia. As per Gilman and Abbots classification (1927). *Trichoderma* was a monolytic genus and all green spored *Trichoderma* isolated were indiscriminately identified as, *T. lingorum* (Tode) Harz, a synonym of *T. viride*

Different biological control agents can be used for the control of diseases. These include bacteria, fungi and actinomycetes. With the advent of biocontrol as one of the component in integrated pest and disease management in the area of fungi mediated plant disease control, the genus *Trichoderma* has gained considerable importance in recent times (Mrinalini and Lalithakumari, 1996).

Mycoparasitism, the direct attack of one fungus on another, is a very complex process that involves sequential event, including recognition, attack, subsequent penetration and killing of the host. The cell wall degrading enzymes (CWDEs) of *Trichoderma* such as different chitinolytic enzymes, glucanases and proteases are considered important in mycoparasitism ( Cherif and Benhamou, 1990; Harman *et al.*, 2004; Howell, 2003). Chitin and β-1, 3 glucan are the main structural components of the fungal cell wall and chitinases and β-1, 3 glucanases have been proposed as the key enzymes in the degradation of cell wall during mycoparasitism against phytopathogenic fungi.

Antibiosis occurs during interactions with other microorganisms involving low molecular weight diffusible volatile and nonvolatile toxic metabolite compounds or antibiotics like harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl 1,3-pyrone,

massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others (Vey *et al.,* 2001).

### 2.2. Role of *Tichoderma* as a biocontrol agent

Webster and Lomas (1964) reported that many fungal isolates recovered from the soil had been shown to produce gliotoxin and viridin, Trichodermin an antibiotic produced by *Trichoderma*. role of *Trichoderma* as a biocontrol agent, the broad concept that biocontrol of plant diseases involves all the interactions of plant pathogens and their antagonists. Under nutrient rich condition many *Trichoderma* isolates posses three antagonistic mechanisms; competition, antibiosis and mycoparasitism

Howell (2003) isolated and described a new antibiotic, gliovirin, from *Gliocladium* (*Trichoderma*) *virens* (GV-P) that was strongly inhibitory to *Pythium ultimum* and a *Phytopthora* species.

Elad *et al*. (1983) showed with the help of scanning electron microscopy and fluroscence microscopy that *Trichoderma* entered *R. solani* or *S. rolfsii* by dissolving and forming holes in the host hyphae. Removal of the coiled hyphae also showed partial lysis outlining the area of former contact.

Brahmabhatt *et al*. (1989) reported the parasitic and antibiotic activity of an isolate of *T. harzianum* against *Pythium aphanidermatum* from tobacco. Cell free culture of the *Trichoderma* caused 17.9 % inhibition of growth of the *Pythium*.

Dhedi *et al.* (1990) reported influence of metabolites of microorganisms on growth of *Fusarium oxysporum* f. sp. *udum* the pathogen of *Cicer aerientium in vitro*. The growth of pathogen was inhibited by metabolites from *Bacillus* spp*., Streptomyces* spp*., Penicillium* spp. And *Trichoderma* spp.

Cherif and Benhamou (1990) revealed that colonisation of the agar medium by the *Trichoderma* occurred earlier and to much higher extent than that caused by *Fusarium oxysporum radicis lycopersici*. The

mycelium of the pathogen was almost totally encircled by the fast growing colony of *Trichoderma.*

Khara and Hawdan (1990) studied the antagonism of *Trichoderma* spp. against *R. solani* the causal agent of damping off of tomato. Both the *T. pseudokoningii* and *T. koningii* showed the maximum antagonism against *R*. *solani* in glucose peptone media.

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

Melo (1991) tested 33 isolates of *Trichoderma* spp. and all isolates had shown antagonism against *Sclerotium minor* and 3 *Scerotium*. Culture filtrates of *T. viride* 2 b was toxigenic to both pathogen tested.

Belanger *et al*. (1995) reported that strain of *T. harzianum* antagonized first and foremost by antibiosis leading to cell death, followed by degradation of cell wall by means of chitinolytic enzymes an suggested that production of antibiotics is more important than that of chitinolytic enzymes in conferring superior biocontrol properties to *Trichoderma harzianum*.

Mukharjee *et al*. (1995) noted the parasitic activity of *Trichoderma harzianum and Gliocladium virens* on the hyphae and sclerotia of *S. rolfsii.*

Majumdar *et al*. *(*1996) observed the antagonistic activity of biocontrol agents *T. viride, T. harzianum* and *Bacillus subtilis* against *Macrophomina phasiolina,* the incitant of blight of moth bean. Among three antagonists *T. harzianum* recorded maximum growth inhibition of pathogens.

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

Totawar and Somani (1999) noticed mycoparasitism and lysis of mycelium of *Rhizoctonia bataticola* by *Trichoderma harzianum* and *T. viride* in dual culture.

Dubey and Patel (2000) tested biological agents *viz. Trichoderma viride, Trichoderma harzianum and Gliocladium virens* against *Rhizoctonia solani* causing web blight of groundnut *in vitro* by dual culture technique and observed that *Gliocladium virens* was most effective, which inhibited maximum mycelial growth (59.8%) followed by *Trichoderma viride* (36.3%) and *Trichoderma harzianum* (34.0%).

Metcalf and Wilson (2001) described the colonization of onion roots, infected with *Sclerotium cepivorum,* by *T. koningii* (Tr5). Hyphae of the biocontrol agent penetrated into infected epidermal and cortical tissue of the root to destroy the hyphae of the pathogen, with little or no damage to uninfected plant tissue.

Sindhan *et al*. (2002) noted antagonistic activity of *Trichoderma viride* and *Trichoderma harzianum* against *Rhizoctonia bataticola in vitro* separately using dual culture method and noted that all the antagonists inhibited mycelial growth in comparison to control. Though the fungal cell wall is made up of mainly of glucan and chitin, the β-1, 3-glucanase and chitinase are key enzymes responsible for fungal cell wall lysis and degradation. (Kucuk and Kivanc, 2004).

Chakraborty *et al*. (2004) studied the volatile and non-volatile antibiotics of *Trichoderma* on growth inhibition of the die back pathogen (*Botryodiplodia theobrome)* of Bottle brush. *T. harzianum* showed maximum growth inhibition (75.33%) if of the pathogen through mycoparasitism and the non-volatiles produced by the same agent exhibited its excellent antagonism to the growth of the pathogen (91.11%) under in vitro condition.

Pandey *et al*. (2005) stated that the *Trichoderma* spp. i.e*. T. harzianum* and *T. viride* was effective mycoparasite for controlling *Fusarium* and *Rhizoctonia.* Parasitism of *Rhizoctonia* by *Trichoderma* characteristically show the coiling tight looping followed by penetration of pegs and knobs like haustoria within hyphae.

Mohamed *et al*. (2006) obtained two stable salt tolerant mutants having great biological proficiency against *Fusarium oxysporum* the causal agent of tomato wilt disease.

Babu and Kumar (2008) observed that *Trichoderma harzianum* -3 (Th-3) inhibited mycelial growth of *Sclerotium rolfsii* by 83 per cent in dual culture among nine antagonistic rhizosphere fungal mycoflora of groundnut

Chang *et al*. (2009) evaluated eighteen strains of *Trichoderma* spp. for antagonism against isolates of *Fusarium* spp. The *Trichoderma* strains exhibited various degrees of overgrowth of *Fusarium* spp. in paired culture on PDA.

Anitha and Rabeeth (2010) studied in vitro interactions between *Streptomyces griseus* strains and some soil borne plant pathogens (*Fusarium oxysporum, Alternaria alternate, Rhizoctonia solani* and *Fusarium solani*) chitinase enzyme produced by *S. griseus* in medium inhibits growth of test pathogen.

Qaisar *et al*. (2011) evaluated three bioagents viz. *Trichoderma viride, Trichoderma harzianum* and *Gliocladium virens in vitro* against *Fusarium oxysporum* f. sp. *plini* causing root rot of kail pine (Pinus wallichhiana). All three bio agents significantly inhibited the mycelial growth of the test pathogen. In case of *Trichoderma viride* (65.33%) followed by *T. harzianum* (64.0%) and *Gliocladium virens* (52.0%).

**2.3. Mutation**

Papavizas and Lewis (1982) evaluated wild strain WT 6 of *T. harzianum* for 3 times after harvesting the surfacing conidia each time and then tolerated to high concentration of benomyl (100-500 mg/ml). The UV induced biotypes differed considerably from WT 6 in appearance, growth habbit fungitoxic metabolite production and also effectively repressed the growth of *Pythium ultimum* of pea *Rhizoctonia solani* of cotton and radish and *Sclerotium lepillorum* of onion.

Durand *et al*. (1988) adopted mutagenesis in *Trichoderma reesei* for large scale production of cellulase after screening mutants up to

six generations, each mutant of them fulfilled three criteria viz., improved productivity, high stability, ability to be further improved. The strain CL 847 found to be high cellulase productivity, resistance to catabolite repression, increased β-d-glucosidase specific activity and it was partially constitutive.

Rajappan (1996) obtained mutants of *Trichoderma viride* by UV irradiation that found superior in production of biomass, conidia and chlamydospores at higher pH levels compared to mother culture.

Mukherjee *et al*. (1997) reported that the fifteen stable benomyl tolerant mutants of *T. viride* obtained after exposing to N-methyln-nitro-N-nitrosaguanidine (100 mg/l in 0.05 M Tris buffer pH 6.0) for 1 h having significant difference in ability to produce antifungal metabolites in liquid culture.

Dinkaran and Marirmuthu (1998) developed nine mutants of *T. viride* using different mutagenic agents viz., UV irradiation 120 min at 30 cm distance from UV light, EMS + UV irradiation and gamma irradiation (150 kr).

Selvakumar (2000) observed UV light and EMS induced mutants in *T. viride*, which showed stable colony characters, fungicide tolerance and antagonistic potential from a parent strain.

Zaldivar *et al*. (2001) treated *Trichoderma aureoviride* strains with N-methyl-N-nitro-N-nitrosoguanidine. The mutant strain showed enhanced production of fungal cell wall degrading enzymes; chitinase, β-1, 3-glucanases and proteases.

Haggag (2002) carried out mutagenesis of *Trichoderma harzianum* and *T. koningii* with 50 and 75 kilo-rad doses of gamma irradiation which result four mutants of each *T. harzianum* and *T. koningii* capable of producing high level of chitinase. These mutants were stable and superior to wild type (WT) with respect to growth, sporulation and biocontrol potential against *B. cinerea.*

Sreerama kumar and Palakshappa (2005) studied the effect of Polyethylene glycol (PEG) at 0.25, 0.5 and 1% concentration on *Trichoderma* spp. and observed that there was no abnormal effect of

polymer on the *Trichoderma* spp. in respect of wet, dry mycelial weight and conidial formation grown in liquid culture but helps to minimize pellet formation in *Trichoderma*. Also there was an early initiation of conidial production in the biomass.

Sereih *et al*. (2007) treated *Trichoderma harzianum* and *Fusarium oxysporum* f sp.*sesami* by five concentration of chitosan. Increase in dose of chitosan, inhibited growth of *Fusarium*. While, *Trichoderma* was found affected genetically and enhanced the antagonistic properties of *Trichoderma* against *Fusarium.*

Balasubramanian *et al*. (2010) growned *Trichoderma harzianum* on colloidal chitin medium for adaptation to obtain chitinase rich mutants and same wild type was mutated by UV irradiation. Jaivel and Marimuthi (2010) carried out mutagenesis for strain improvement of *Aspergillus terreus* by exposing spores to UV light and Ethyl methane sulphonate at different time intervals.

Chandra *et al*. (2010) developed a mutant strain of *Trichoderma citrinoviride* by multiple exposures to EMS and Ethidium bromide.

Mohamed *et al*. (2010) applied UV treatment of mutagenesis in genetically breeding program of the bioagent *Trichoderma viride* to enhance three effective hydrolytic enzymes viz., chitinase, β-1,3—galactoronase and cellulases in their biocontrol abilities against *S. rolfsii* and *Sclerotinia sclerotiorum*. The greatest enzyme production by *T. viride* mutants was cellulases followed by β-1, 3—galactoronase then chitinase.

Patil and Kamble (2011) obtained five *T. koningii* mutants by UV treatments of different time variables. Among these five mutants *T.Koningii*-2 showed maximum antagonistic activity against the charcoal rot pathogen in vitro.

**2.6. Chitinase Study**

Since chitin is the major component of most fungal cell walls, a principle role has been attributed to chitinases in the control of a wide range of phytopathogens (Collinge *et al.*, 1993; Gokul *et al.*, 2000).

*In vitro* experiments demonstrate that there are no chitin containing phytopathogens that would be resistant to chitinases of *Trichoderma* (Lorito *et al*., 1993-1994). Introduction of a single fungal gene into a cultured plant is expected to produce a transgene with increased resistance to a broad range of pathogenic fungi (multiple introductions are required if plant genes are involved). The gene *ech*42 from *T. harzianum*, (Garcia *et al*., 1994; Hayes *et al*., 1994), codes for a chitinase with significantly higher activity against a broad range of phytopathogenic fungi than other chitinolytic enzymes.

Chitinases from other sources were also cloned and investigated from fungus such as *Trichoderma harzianum* endochitinase (Hayes *et al*., 1994) which introduced into apple (Wong *et al*., 1999; Bolar *et al*., 2000), potato (*Solanum tuberosum* L.), broccoli (Mora and Earl, 2001), tobacco (Lorito *et al*., 1998). In 1992, De La Cruz *et al.* were the first to isolate, purify, and characterize chitinases of the mycoparasitic fungus *T. harzianum*, used as a means of biocontrol.

Kapat *et al.* (1996) used the colorimetric assay to determine the chitinase activity of the *T. harzianum.* The reducing sugar was measured by the dinitrosalisylic method as described by the Miller (1959). One unit of enzyme activity was defined as the amount of enzyme that releases 1 gmol of N-acetyl-D-glucosamine from the substrate in 1 minute per ml of culture filtrate at a defined temperature.

Bolar *et al*. (2000) obtained several lines of very susceptible ‘McIntosh’ apple with varying levels of *ech*42 expression. Some of them exhibited increased tolerance to *V. inaequalis*.

Kikkert *et al.* (2000) reported that chitinases from the biocontrol fungus *Trichoderma harzianum* inhibit spore germination and hyphal elongation of the grapevine pathogens *Botrytis cinerea* (the causal agent of bunch rot) and *Uncinula necator* (the causal agent of powdery mildew) in *in vitro* assays.

Harjono and Widyastuti (2001) assayed the *T. reesei* endochitinase activity on the basis of reduction of turbidity of suspension of

colloidal chitin as described by the Tronsmo and Harman (1993); whereas colloidal chitin was prepared as described by

Vessey and Pegg (1973) from crab shell chitin. One enzyme unit was defined as the amount of enzyme required to reduce the turbidity of a chitin suspension by 5 per centat 510 nm (Tronsmo and Harman, 1993).

Omumasaba (2001) studied and described the purification and characterization of one chitinolytic enzyme from commercial prepration, Usukizyme derived from *T. viride* under cellulase inducible conditions, which is widely used in protoplast fusion studies of molds and mushrooms.

Ike *et al.* (2006) used the substrate chitosan 7B and chitosan 10B for the determination of chitinase activity. The reaction mixture contained 1 ml of enzyme in 50mM sodium acetate buffer (pH 5.5) and the substrate, incubated at 300C for appropriate time. This reaction was terminated by immersing the reaction mixture in boiling water for 10 minutes and reducing sugar released was estimated. Chitin, a β-1-(1,4)-linked polymer of N-acetyl D-glucosamine is widely distributed in nature, particularly as a structural polysaccharide in fungal cell walls in the exoskeleton of arthropods, the outer shell of crustaceans, nematodes etc. (Dahiya *et al*., 2006).

Kulkarni and Ramanujam *et al.* (2010) studied the ability of *Trichoderma* isolates to produce chitinase enzyme through polyacralamid gel electrophoresis (SDS-PAGE) method and relate it to their antagonistic ability which will help to identify the markers and it can be inserted in to the plant itself through genetic engineering to evolve resistant varieties or these markers may be inserted into *Trichoderma species* itself to promote its antagonistic ability.

K. K. Suryawanshi *et al.* (2012) obtained Twenty four *T. viride* mutants by chemical mutagenesis i.e. Ethyl methyl sulphonate (EMS) and Hydroxyl amine (HA) treatments of different doses and time variables. Among these Twenty four mutants *T.viride* TVME3c, TVME4c, TVME4a

showed maximum antagonistic activity against the *Sclerotium rolfsii, Rhizoctonia bataticola and Fusarium solani*.and also having the highest chitinase enzyme units/mg of protein i.e. 0.62, 0.63 and 0.61 enzyme units/mg of protein.

Savita ***et al.*** (2015) studied sixteen *Trichoderma viride* mutants by gamma irradiation and tested for their antagonistic activities *in vitro*. Morphological characters of efficient mutants were tested up to six generation to check their stability. Among these TVGM1 were proved as effective antagonists against *Sclerotium rolfsii, Rhizoctonia bataticola* and *Fusarium oxysporum* f.sp. *ciceri* basis of their maximum bioefficiency. The highest chitinase enzyme units/mg of protein *i.e*. 0.62 was exerted in TVGM1.

**2.7 Compatibility with fungicide**

Sharma *et al*. (1999) tested the compatibility of *Trichoderma viride* with mancozeb and found it to be compatible at lower concentrations.

Chand and Tripathi (2002) found that growth of *Trichoderma viride* was inhibited by carbendazim.

Hatvani *et. al*. (2006) Tested 16 pesticides tested, seven fungicides: copper sulfate, carbendazim, mancozeb, tebuconazole, imazalil, captan and thiram inhibited colony growth of the test strains significantly with minimal inhibitory concentrations of 300, 0.4,50, 100, 100, 100 and 50 lg/ml, respectively. Mutants resistant to carbendazim and tebuconazole were produced from both wild type strains by means of UV-mutagenesis. Carbendazim resistant mutants showed total cross-resistance to benomyl and thiabendazole at a concentration of 20 lg/ml, which may have the potential to be applied in IPM strategies.

Bhattiprolu S. L. (2007) Compatibility of *T. viride* was tested against carbendazim 0.1%, dithane-M-45 0.25%, thiram 0.3%, copperoxychloride 0.3%, thiophanate methyl 0.1%, hexaconazole 0.2% by employing poisoned food technique. The control plates were fully covered with *T. viride* in four days. The radial growth in different treatments ranged from 0.0 to 88.7 mm. Growth of *T. viride* in dithane-M-45 amended PDA

(88.3 mm) was on par with the control plates (88.7mm) followed by copperoxychloride treatment (80mm). *T. viride* did not grow in the presence of carbendazim, hexaconazole and thiophanate methyl at recommended doses but could tolerate thiram with 31.4 mm radial growth.

Madhusudhan *et. al.* (2010) evaluated among the six fungicides, Carbendazim (50% WP) Propiconazole (25% EC), Tridemorph *(BO%* EC) Chlorothalonil (75% WP) and hexaconazole (5% EC) for their compatibility with *T. viride*. Among them chlorothalonil (75% WP) was found safe up to 40ppm. Other fungicide, were found effective against *Fusarium solani* but isolation not compatable with *Trichoderma viride* even at very low concentration.

Bagwan (2010) reported *Trichoderma* was most sensitive to capton, tebuconazole, vitavax, propiconazole and chlorothalonil.

Bindu *et. al.* (2011) reported that *T. viride* showed compatibility with seed treatment chemical Imidacloprid followed by Mancozeb and Tebuconazole while being totally incompatible with systemic fungicides like Carbendazim, Hexaconazole, Tebuconazole and Propiconazole**.**

Patil *et al.* (2011) reported that Trichoderma was not compatible with carbendazim (0.1%), exhibited the complete inhibition of *Trichoderma* fungus and thiram (0.3%) showed the maximum suppression of *Trichoderma* on various substrate used during the study.

Archana *et. al.* (2012) conducted study the compatibility of azoxystrobin 23 SC with bacterial and fungal biocontrol againto show they found that azoxystrobin 23 SC was compatible bacterial bioagent even at a high concentration of 300 ppm, where as fungal biocontrol agent *Trichoderma viride* was inhibited by azoxystrobin 23 SC at a concentration above 15 ppm.

Bheemaraya *et al.* (2012) studied that mancozeb was compatible with growth of *Trichoderma spp*. while carbendazim, captan and propiconazole completely inhibited radial mycelial growth hence, where not compatible.

Nandeesha *et al*. (2013) studied the invitro efficacy of four systemic fungicide carbendazim, propiconazole and hexaconazole and tebuconazole and two systemic fungicides viz. mancozeb, and captan at various concentrations against Aspergillus niger. All systemic fungicide was highly effective and completely inhibited the mycelial growth of pathogen followed by mancozeb. Among all fungicides mancozeb was found highly compatible with *Trichoderma viride.*

Sita Ram Bana *et. al.* (2017) evaluate the efficacy of fungicides (Calcium chloride, Mancozeb, Carbendazim), bio-agents (*Trichoderma harzianum, T. viride* and *Pseudomonas fluorescens*) against root rot of fennel (*Fusarium oxysporum*). All the treatments found effective against *Fusarium oxysporum*. Maximum inhibition per cent was recorded on Carbendazim (T1-99.44%) which was statistically superior on Mancozeb (T3- 98.77%) and T3 statistically superior on Calcium chloride (T2-81.33% ) which was statistically at par with *T. harzianum* (T5-79.44%) followed by *Trichoderma viride* (T4- 76.88%) *Pseudomonas fluorescens* (T6- 72.66%) while the minimum in control (T0- 51.00%) under in vitro conditions. Among all the treatments the minimum disease incidence (%) of root rot was recorded in T1 - carbendazim (13.50%), followed by T3-mancozeb (15.00%), T2-Calcium chloride (18.65%), T5-*Trichoderma harzianum* (21.00%) T4- *Trichoderma viride* (21.65%), T6-*Pseudomonas fluorescence* (25.27%).

Nongmaithem (2015) reported that carbendazim was highly incompatible with Trichoderma viride and also concluded that fungicide is more toxic to *Trichoderma viride* as compare to bio pesticide.

## CHAPTER III

# MATERIAL AND METHODS

The present investigation on “Antagonistic potential of *Trichoderma viride* mutants and their compatability with fungicides” was carried out during 2017-2018 at Department of Plant pathology, PGI Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

### 3.1 Material

Following material was used for experimentation.

#### 3.1.1. Glass wares and Plastic wares

During the course of investigation following glass wares and plastic wares were used glass Petri plate’s (Borosil and Schott duran, Germany), test tubes (Borosil, Germany), conical flasks of 250ml, 500ml and 1000ml (Schott Duran, Germany), funnel (Schott Duran, Germany), beaker (Schott Duran, Germany), glass pippet (Schott Duran, Germany), measuring cylinder (Schott Duran, Germany), cavity slides, cover slip, glass rod, polypropylene Petri plates, and polypropylene centrifuge tube, eppendorf tubes were used.

#### 3.1.2 Equipments

Standard laboratory equipments used for different experiments were Autoclave (Equitron, India), BOD incubator (Sanco, India), laminar airflow (Klenzaids, India), student microscope (Olympus, India), stereoscopic binocular (Nikon, India), refrigerator (LG, India), hot air oven (Bio-techniques, India), haemocytometer (Improved Neubauer, China), stage and ocular micrometer, digital weighing balance (Anamed, India), centrifuge (Biofuse, Germany), table top centrifuge (Hawkins, India), Bunsen burner, digital camera (Kodak, India), double distillation unit (JSGW, India), soil sterilization tank (locally made).

#### 3.1.3 Chemicals

Chemicals used during the course of research work were Ethyl Methyl Sulphonate, Hydroxyl Amine, Ethanol, Crab shell chitin,

Sodium azide and others such as formaldehyde, streptomycin sulphate, mercuric chloride etc.

### 3.1.4 Other materials

Blotter paper, non absorbant cotton, muslin cloth, plastic pots, earthen pots, polyethylene bags, cork borer (6 mm), inoculation needle, dissection needle, forceps, paper bags, butter paper bags, pencil, permanent marker, cello tapes, Whatman filter paper (4 mm), tags, polyethylene sheets, soil, sand, FYM, test tube stand, tray, hand sprayer, wash bottle, thread, wooden sticks, potato, rubber band, mortar pestle, scissors, etc.

### 3.1.5 Collection of pure cultures of *Trichoderma viride*

Pure culture of *Trichoderma viride* culture was collected from the Department of Plant Pathology Dr. PDKV, Akola.

### 3.1.6 Collection of pure cultures of soil inhibiting plant pathogens

Pure cultures of soil born pathogen viz., *Rhizoctonia bataticola, Sclerotium rolfsii, Fusarium udum* were collected from Department of Plant Pathology, Dr. P.D.K.V, Akola.

## 3.2 Methods

### 3.2.1 Sterilization of glass-wares and culture media

The glasswares (Borosil make) viz.; Petri plates, conical flasks, beakers, pipettes, measuring cylinder and test tubes were used during the research work. Glasswares were cleaned with cleaning solution (Detergents, Potassium dichromate solution and solution of H2SO4) and repeated washings with tap water followed by distilled water. The cleaned and dried glasswares were sterilized in hot air oven at 1800C for one hour before use. Distilled water and media were sterilized in autoclave at 1.04 kg/cm2 for 15 minutes.

### 3.2.2 Preparation of Potato dextrose agar medium (PDA)

Potato dextrose agar medium (PDA) was used for the dual culture study and maintenance of pure culture of the test pathogen.

**The composition of PDA**

| | |
|---|---|
| Peeled Potato | 200g |
| Dextrose | 20g |
| Agar | 20g |
| Distilled water | 1000ml |

Healthy peeled potatoes, 200 g were cut into pieces and boiled in 500 ml sterilized distilled water in sauce pan for 30 minutes. The extract was strained through muslin cloth and quantity was measured. In remaining 500 ml water, agar (20 g) and dextrose (20 g) were dissolved by heating. Both were mixed and volume was made up to one litre. The medium was filtered through muslin cloth and poured into conical flask and test tubes, then plugged with non absorbent cotton and autoclaved at 1.04 kg/cm2 for 15 minutes. Autoclaved tubes were kept in slanting position toobtain slants for maintenance of cultures.

### 3.2.3 Preparation of Czapek's Broth

*Trichoderma* cultures were grown on Czapek's broth for estimation of Chitinase enzyme. Composition of media is given in appendix1.

### 3.2.4 Morphological study

*Trichoderma viride* mother culture and mutants were grown on PDA medium in Petri plates, with the help of cork borer six mm disc from the periphery of the colony were used for inoculation purpose. One disc of six mm was put in the centre of Petri plate containing PDA media. Entire work was carried out in aseptic conditions. Following observations were recorded.

1. Colony characters: Colour and growth

2. Morphological characters: spore shape, size and pigmentation.

Measurement of spore size was done by using micrometry with simple microscope. The graticule (line engraved on ocular micrometer) was calibrated against a stage micrometer to obtain the exact size of the spores in micron. After calibration, stage micrometer was removed and

spore dimensions was measured at high power objective lense (40X) and finally size of spores was computed.

### 3.2.5 Spore count

Haemocytometer was cleaned with alcohol and placed 0.1ml of well suspended spore suspension at the centre and covered with cover glass. The preparation was allowed to stand for 2 min before counting so that spores settle to the bottom of the square. Five squares were chosen randomly and the spores inside the square were counted. The spore load was calculated by using the following formula.

$$\text{Spore load/ml solution} = N \times 10^4 \times 400$$

Where,

N = Total no of spores counted/no of squares.

### 3.2.4 Chemical mutagenesis

Induction of Chemical mutagenesis was carried according to procedure of Chandra *et al*. (2009) and Durand *et al*. (1988): Conidiospores of 8 days old culture of *T. viride* were used for mutagenesis. Spore suspension of *T. viride* was treated with Ethyl Methyl Sulphonate (EMS) and Hydroxyl Amine (HA) @ 50 µl/ml, 100 µl/ml, 150 µl/ml, 200 µl/ml and 250µl/Mi incubate at $28^{O}$C in orbital shaker for 60 minutes. Then kept in centrifuge machine at 5000 rpm to remove the chemical traces, centrifuge it for three times and then washed with distilled water. Suspension was spread on to the surface PDA medium and incubated at $28^{O}$C for 72 hrs. 60 minutes. In each treatment maintained three replications. After incubation colonies were transferred on fresh PDA medium and grown up to six generations to check the stability of *Tichoderma viride* mutants.

### 3.2.5 Details of experiment

The experiment was conducted in the laboratory with following details.

**Design** - Completely Randomized Design (CRD)

**Treatments** - 11

**Replication** – 3

**Table1. Treatment detail of *Trichoderma viride* mutants and its mother culture**

| Treatments | Code name | Code No | Description |
|---|---|---|---|
| $T_1$ | TvME-1 | 1 | Spore suspension of *T. Viride* treated with Ethyl Methyl Sulphonate (EMS) @ 50 µl/ml for 60minutes. |
| $T_2$ | TvME-2 | 2 | Spore suspension of *T. viride* treated with Ethyl Methyl Sulphonate (EMS) @ 100 µl/ml for 60 minutes. |
| $T_3$ | TvME-3 | 3 | Spore suspension of *T. viride* treated with Ethyl Methyl Sulphonate (EMS) @ 150 µl/ml for 60 minutes |
| $T_4$ | TvME-4 | 4 | Spore suspension of *T. viride* treated with Ethyl methyl sulphonate (EMS) @ 200 µl/ml for 60 minutes. |
| $T_5$ | TvME-5 | 5 | Spore suspension of *T. Viride* treated with Ethyl Methyl Sulphonate (EMS) @ 250 µl/ml for 60 minutes. |
| $T_6$ | TvMH-6 | 6 | Spore suspension of *T. viride* treated with Hydroxyl Amine (HA) @ 50 µl/ml for 60 minutes. |
| $T_7$ | TvMH-7 | 7 | Spore suspension of *T. viride* treated with Hydroxyl Amine (HA) @ 100 µl/ml for 60 minutes. |
| $T_8$ | TvMH-8 | 8 | Spore suspension of *T. viride* treated with Hydroxyl Amine (HA) @ 150 µl/ml for 60 minutes. |
| $T_9$ | TvMH-9 | 9 | Spore suspension of *T. viride* treated with Hydroxyl amine (HA) @ 200 µl/ml for 60 minutes. |
| $T_{10}$ | TvMH-10 | 10 | Spore suspension of *T. viride* treated with Hydroxyl Amine (HA) @ 250 µl/ml for 60 minutes. |
| $T_{11}$ | TvC-11 | 11 | Untreated control i.e. *T. viride* mother culture |

**Chemical:**

A. Ethyl Methane Sulphonate @ 50, 100, 150, 200 and 250 µl/ml

B. Hydroxyl amine @ 50, 100, 150, 200 and 250 µl/ml, time interval 60 minutes

### 3.2.6 Dual culture Technique

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

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

Where,

C = Mycelial growth (mm) in control plate.

T = Mycelial growth (mm) in treatment plate.

### 3.2.7 Estimation of chitinase enzyme

Estimation of chitinase enzyme in effective mutants was done by method suggested by Kulkarni and Ramanujan *et al*. (2010). *Trichoderma viride* mutants were grown on synthetic media (Czapek's broth) along with crab shell chitin (50 ml in 250 ml flask). After inoculating with 5 x $20^6$ / ml conidia these flasks were kept on rotary shaker at 140 rpm

at 25$^{O}$ C for 4-5 days. Culture filtrate was collected after separating the biomass filtered with nylon cloth and dialyzed with 50 mm potassium phosphate buffer PH 6.7 (6: 1) at 40$^{O}$ C overnight. Sodium azide was added to keep it for further usage.

### 3.2.8 Measurement of chitinase activity

#### 3.2.8.1 Turbidity method

Endochitinase activity was measured by the reduction of turbidity of a suspension of colloidal chitin. A suspension containing 1% (w/v) or moist colloidal chitin was prepared in 50 mm potassium phosphatebuffer, pH 6.7. A mixture consisting of 0.5 ml each of chitin suspension and the enzyme solution to be tested was prepared and inculcated for 24 h at 30$^{O}$ C. Subsequently the mixture was diluted with 5 ml and the optical density was read at 510 nm. Enzyme activity was calculated as the percentage of reduction of a chitin suspension by 5 percent.

#### 3.2.8.2 Preparation of Colloidal Chitin

Colloidal chitin was prepared as per the method of Roberts and Selintrenikoff (1988).

1. 5 g of chitin powder (HiMedia Laboratories Pvt. Ltd., Mumbai) was added slowly into 60 ml of concentrated HCl (Sd. Fine Chemicals Ltd., Mumbai) and left for vigorous shaking overnight at 4$^{O}$ C.
2. The mixture was added to two liters of ice-cold 95 per cent ethanol with rapid stirring and kept overnight at room temperature (25$^{O}$C).
3. The precipitate was collected by centrifugation at 5,000g for 20 minutes at 4$^{O}$ C and then washed with sterile distilled water until the pH of the colloidal chitin turned neutral (pH 7.0).
4. Later, colloidal chitin solution (5 per cent) was prepared and stored at 4$^{O}$ C for further use.

#### 3.2.8.3 Preparation of Phosphate Buffer (pH 6.7)

- Pottasium Dihydrogen Phosphate (KH2 PO4 1 M) 136 gm in 1000 ml of distilled water.

- Pottasium hypophosphate (K2 HPO4 1 M) 174 gm in 1000 ml of distilled water. Both were mixed together and dilute up to required concentration (50mm) and pH should be maintained 6.7.

### 3.2.8.4 Preparation of standard graph

The standard graph was constructed by using dextrose ('AR' grade) as glucose source. Standard solutions of glucose (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 %) were prepared. 0.5 ml of each standard solution and chitin suspension were mixed in test tubes and incubated for 24 hr at $30^{O}$C. The absorbance at 510 nm was recorded using systronics make spectrophotometer after dilution with 5 ml distilled water.

### 3.2.9 Compatibility with fungicide

The fungicide were evaluated for their compatibility by employing poison food technique. Potato Dextrose Agar (PDA) medium was prepared, equally distributed measuring 100 ml in 250 ml conical flask and sterilized in autoclave. Requisite quantity of each of the fungicides (as per concentration) was added in sterilized melted ($45^{0}$C) PDA separately so as to obtain desired concentration. Flask containing poisoned medium was shaken well to have even and uniform distribution of fungicides. About 20 ml of melted poisoned PDA was poured in each sterilized Petri plate and allow to solidify. These Petri plates were inoculated by *Trichoderma viride* separately. Five mm disc of one week old *Trichoderma viride* culture was cut with sterilized cork borer, lifted and transferred aseptically in the centre of Petri plate containing the medium poisoned with test fungicide. The control plates were kept the culture disc grown in same condition on PDA without fungicides. Treated plates were incubated at room temperature (25± $2^{0}$C) for a period of seven days. Colony diameter was recorded in mm and per cent mycelial growth inhibition was calculated as per Vincent's formula based on the average colony diameter. The data was subjected to statistical analysis wherever necessary.

**Table2. Fungicide detail.**

| Sr. no. | Chemical name | Trade name | Company | Concentration (%) |
|---|---|---|---|---|
| **A)** | **Chemicals** | | | |
| 1 | Carbendazim 50% WP | Starbenz | Swal co-operation Ltd. | 0.1 |
| 2 | Chlorothalonil 75 % WP | Kavach | Syngenta | 0.2 |
| 3 | Mancozeb 75 % WP | Indofil M-45 | Indofil Industries Ltd. | 0.2 |
| 4 | Tebuconazole 25 % WG | Caviet | Exel group Pvt. | 0.05 |
| 5 | Azoxystrobin 23% SC | Amistar | Syngenta | 0.2 |

## CHAPTER IV

# RESULTS AND DISCUSSION

In the present investigation efforts have been made to generate efficient mutants of *T. viride* having more antagonistic potential against soil borne pathogens and enzymic activities compared to mother culture. This chapter deals with the results obtained on the basis of studies carried out and the fact along with the discussion hereunder.

### 4.1 Morphological study

#### 4.1.1 *Trichoderma viride* (mother culture).

Colony was milky white coloured at initial stage later turned dark green. subarial and dispersed growth was observed with moderate sporulation, giving yellow colour pigmentation on PDA medium. phialides were grouped opposite with globose to obvoid shaped conidia, traditionally the isolates with warted conidia were identified as *T. viride*, Lieckfeldt *et al.* (1999) observed two morphologically distinct warted conidia –Type-I and Type-II. The molecular studies (RFLP analysis of endochitinase gene, PCR finger printing and ITS analysis and rDNA) revealed that type – I corresponds to *T.viride* (Teleomorph- *H. rufa*) and Type II corresponds to *T. asperellum* and cannot be linked to teleomorph. *T. asperellum* was distinguished from *T. viride* by finer conidial ornamentation, slightly ovoidal conidia, a faster growth rate, mostly pared branches, ampulliform phialides and the consistent presence of chlamydospores (Samuels *et el*. 1999).

#### 4.1.2 *Trichoderma viride* mutants

**1) TvME-1**

Subarial and dispersed growth was observed with moderate sporulation, colony was milky white at initial stage, later turned to dark green, colour dark yellow colour pigmentation on PDA medium, concentric rings were formed, phialides branched some have simple, conidiophores irregularly paired and subglobose roughened conidiation, conidia were globose to ellipsoidal.

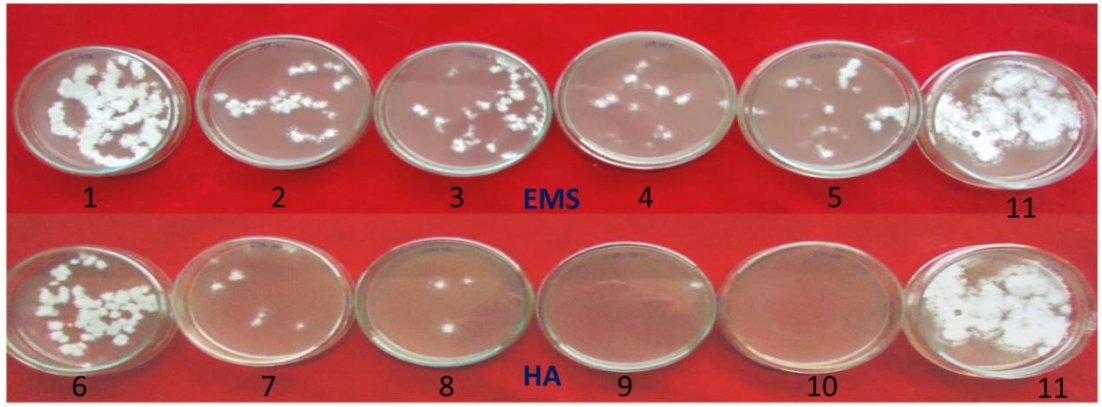


**Plate 1a.** ***Trichoderma viride*** **mutants and mother culture 1 st after treatment**

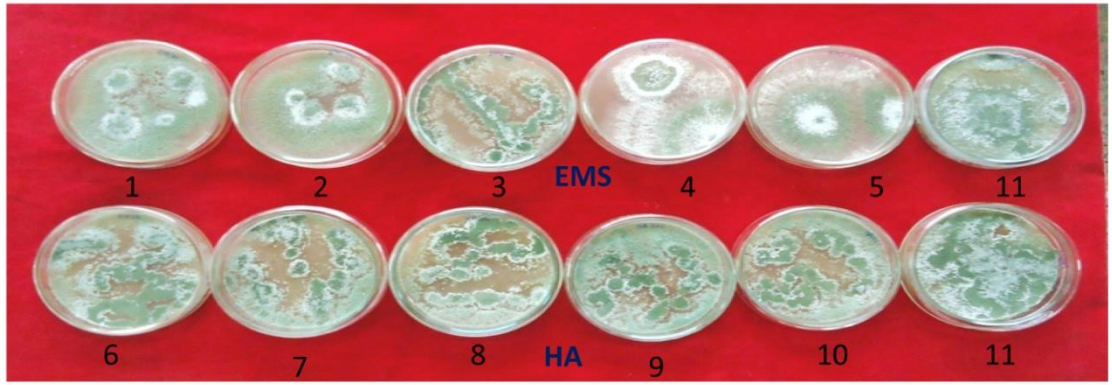


**Plate 1b.** ***Trichoderma viride*** **mutants and mother culture 7 th after treatment**

**2) TvME-2**

Colony milky white to light green mycelial growth at initial stage, later turned to light greyish green, aggregating at central region, subarial and dispersed was observed with moderate sporulation, concentric rings were produced on PDA medium, Dark yellow pigmentation was observed on PDA, phialides grouped simple, ellipsoidal shaped conidia.

**3) TvME-3**

Subarial and dispersed growth observed with maximum sporulation, colony milky white to light green mycelial growth at initial stage, later turned to dark green colour giving dark yellow colour pigmentation on PDA medium and concentric rings were formed, phialides were branched, ellipsoidal shape conidia were noticed under microscope.

**4) TvME-5**

Colony was milky white coloured at initial stage, later turned light green. flat and superficial growth observed This mutant slightly differed from other was fast growing forming concentric rings with maximum sporulation, colour of the medium changes with light yellow pigmentation on PDA, phialides were branched, conidia roughened, globose to ellipsoidal shape conidia.

**5) TvME-5**

Flat and superficial growth was observed with moderate sporulation, colony was milky white at initial stage, later turned to dark green, giving dark yellow colour pigmentation on PDA medium, phialides were grouped simple with ellipsoidal shaped conidia.

**6) TvMH-6**

Colony was milky white coloured at initial stage, later turned to light green with moderate sporulation, and giving light yellow pigmentation on PDA medium. subarial and dispersed growth was observed, phialides were grouped simple, a conidia were ellipsoidal in shape.

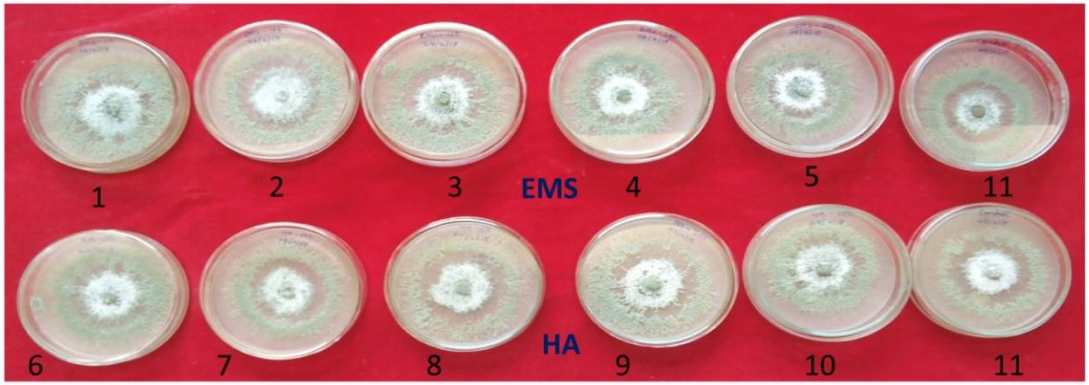


**Plate 2a. *Trichoderma viride* mutants and mother culture 2 nd Generation**

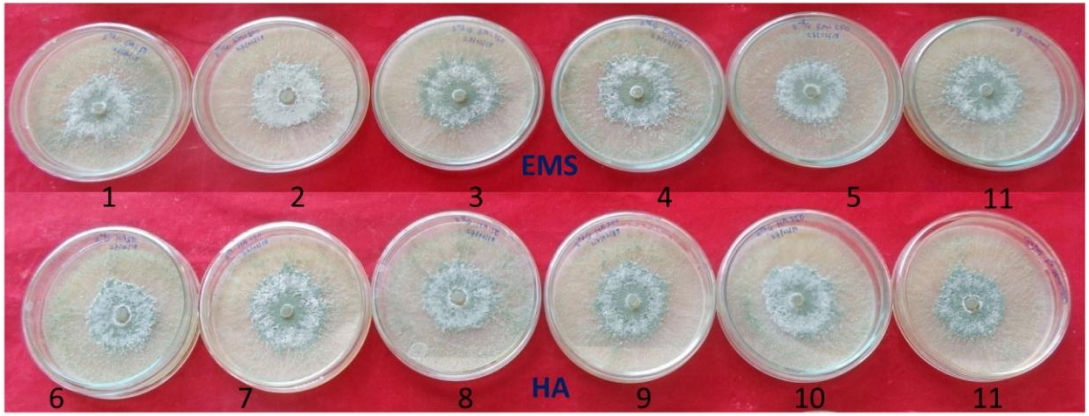


**Plate 2b. *Trichoderma viride* mutants and mother culture 3 rd generation**

**7) TvMH-7**

Colony was milky white coloured at initial stage, later turned to light green with moderate sporulation, giving light yellow colour pigmentation on PDA medium, subarial and dispersed growth was observed, phialides were branched, conidia was ellipsoidal in shape

**8) TvMH-8**

Colony was milky white coloured at initial stage later turned to dark green, concentric rings, fast growing and maximum sporulation with light yellow colour pigmentation was observed, phialides were branched, conidia globose to ellipsoidal in shape.

**9) TvMH-9**

Subarial and dispersed growth was observed with maximum sporulation, colony was dark grey at initial stage, later turned to light green, colour dark yellow colour pigmentation on PDA medium, concentric rings were formed, phialides grouped some have simple and subglobose roughened conidiation, conidia ellipsoidal shape.

**10) TvMH-10**

Colony was milky white coloured at initial stage, later turned to light green with maximum sporulation, giving dark yellow colour pigmentation on PDA medium. subarial and dispersed growth was observed, phialides were grouped simple, subglobose roughened conidiation, conidia was ellipsoidal in shape.

The genus *Trichoderma* Pers.:Fr. is defined to include anamorphs of Hypocrea, having elongated phialides and irregularly branched conidiophores Rifai (1969).

Visual appearance of *Trichoderma* on plates exhibited fast growing flat, superficial or subaerial mycelial growth, forming mostly concentric rings or discreet, disperse or some isolates producing good aerial and congregated growth at central and peripheral regions, conidiophores in *Trichoderma* is repeatedly branched at an indefinite number of levels with the primary branches producing smaller secondary

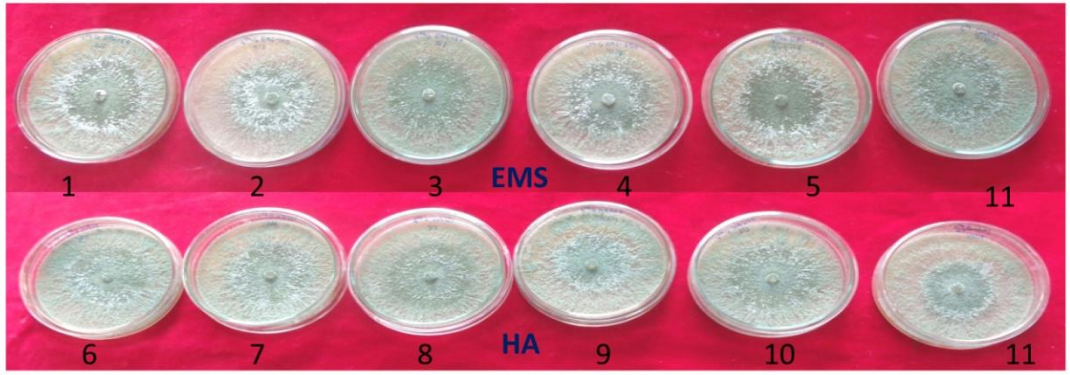


Plate 3a. *Trichoderma viride* mutants and mother culture 4 th generation

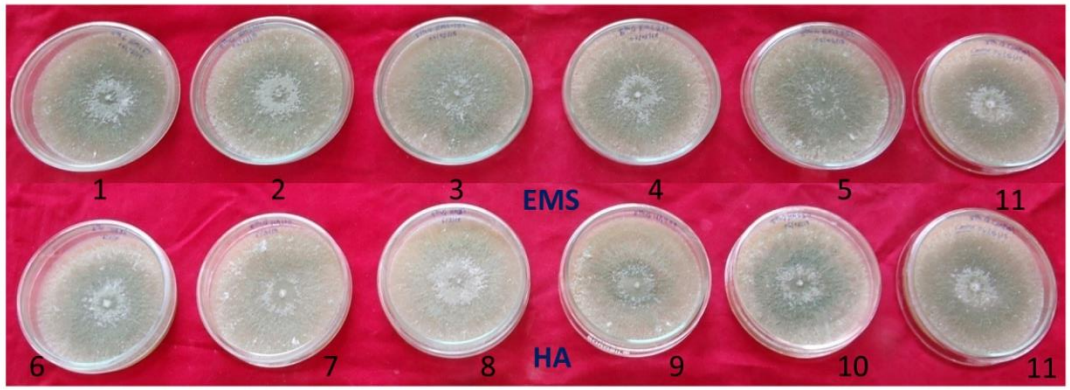


Plate 3b. *Trichoderma viride* mutants and mother culture 5 th generation

branches that also may branch and at higher levels apically and distally progressively shorter. Phialides are ampuliform to lageniform, usually constricted at the base, more or less swollen near the middle. Phialides are disposed in verticilster minally on branches of the conidiophore. Conidia are hyaline or more usually green, smooth walled in *T. harzianum* and roughened in *T. viride*. And conidia shape varies from globose, to ellipsoidal, obovoidal, or short-cylindrical, with the basal end more or less tapering and truncate. Similar type of morphological characters were also observed by Rifai (1969), Bissett (1991a, 1991b). Most isolates of *T. viride* produced characteristic dark red/or red to light, yellow pigmentation in media also corroborates the results of present studies.

Selvakumar (2000) also showed stable colony characters of *T. viride* in UV light and EMS induced mutants which differs from a parent strain.

Haggag (2002) carried out mutagenesis of *Trichoderma harzianum* and *T. koningii* with 50 and 75 kilo-rad doses of gamma which result four mutants of each *T. harzianum* and *T. koningii* capable of producing high level of chitinase. These mutants were stable and superior to wild type (WT) with respect to growth, sporulation and potential against *B. cinerea.*

Mohamed and Haggag (2002) mutagenesis of three *Trichoderma spp.* With 20 and 40 kilo-rad doses of gamma and reported four mutants of each spp. Including *T. harzianum, T. viride* and *T. koningi* were obtained by gamma irradiation to enhance their biocontrol abilities against *S. cepivorum* and their production of antifungal metabolites.

Mohamed et al. (2002) reported that mutant species of exhibited high capabilities to produce many efficient enzymes and other metabolites with better control of onion whit rot disease.

Induced mutation is one of the most widely used tools to get variants which might have improved biocontrol properties like survival ability, antagonistic and biocontrol potential and ability to colonize plant parts (Baker, 1991).

**Table3. Morphological characteristics of *Trichoderma viride* mother culture and mutants (7th generation).**

| Sr .no | *Trichoderma viride* mutants | Morphological characters | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Colony diameter/Radial mycelial growth (mm) at 7 DAI | Colony growth type | Colony colour | Pigmentation | Phialides | Conidia shape | Sporulation |
| 11 | TvC-11 | 90.00 | Subarial and dispersed | Milky white to dark green | Yellow | Grouped opposite | Globose to obvoid | ++ |
| 1 | TvME-1 | 88.50 | Subarial and dispersed | Milky white to Darkgreen | Dark Yellow | Branched | globose to ellipsoidal | ++ |
| 2 | TvME-2 | 90.00 | Subarial and dispersed | Milky white to light green | Dark Yellow | Grouped simple | Ellipsoidal | ++ |
| 3 | TvME-3 | 89.00 | Subarial and dispersed | Milky white to light green | Dark Yellow | Branched | Ellipsoidal | +++ |
| 4 | TvME-4 | 90.00 | flat and superficial | Milky white to light green | Light Yellow | Branched | globose to ellipsoidal | +++ |
| 5 | TvME-5 | 89.50 | flat and superficial | Milky white to Darkgreen | Dark Yellow | Grouped simple | Ellipsoidal | ++ |
| 6 | TvMH-6 | 89.57 | Subarial and dispersed | Milky white to light green | Light Yellow | Grouped simple | Ellipsoidal | ++ |
| 7 | TvMH-7 | 89.70 | Subarial and dispersed | Milky white to light green | Light Yellow | Branched | Ellipsoidal | ++ |
| 8 | TvMH-8 | 90.00 | Subarial and dispersed | Milky white to Darkgreen | Light Yellow | Branched | globose to ellipsoidal | +++ |
| 9 | TvMH-9 | 89.50 | Subarial and dispersed | Dark grey to light green | Dark Yellow | Grouped simple | Ellipsoidal | +++ |
| 10 | TvMH-10 | 90.00 | Subarial and dispersed | Milky white to light green | Dark Yellow | Grouped simple | Ellipsoidal | +++ |

+++: Maximum sporulation ++: Moderate sporulation +: discrete sporulation-: No sporulation

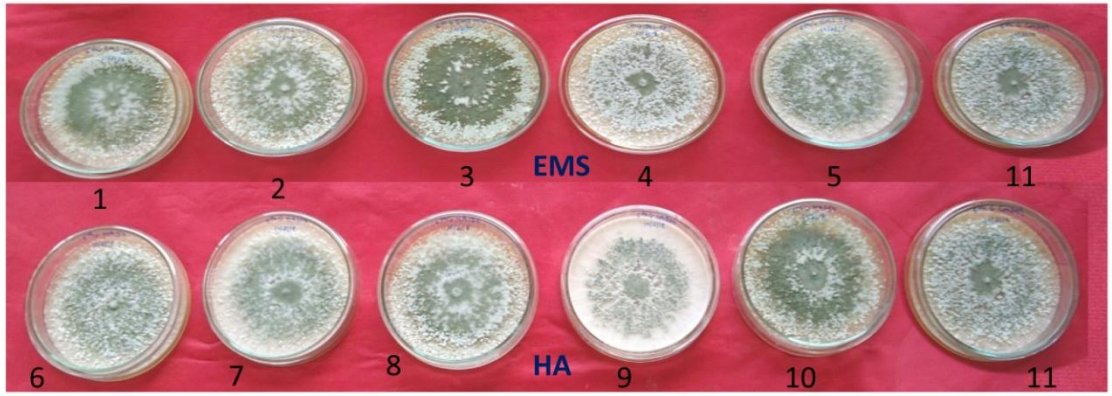


**Plate 4a. *Trichoderma viride* mutants and mother culture 6 th generation**

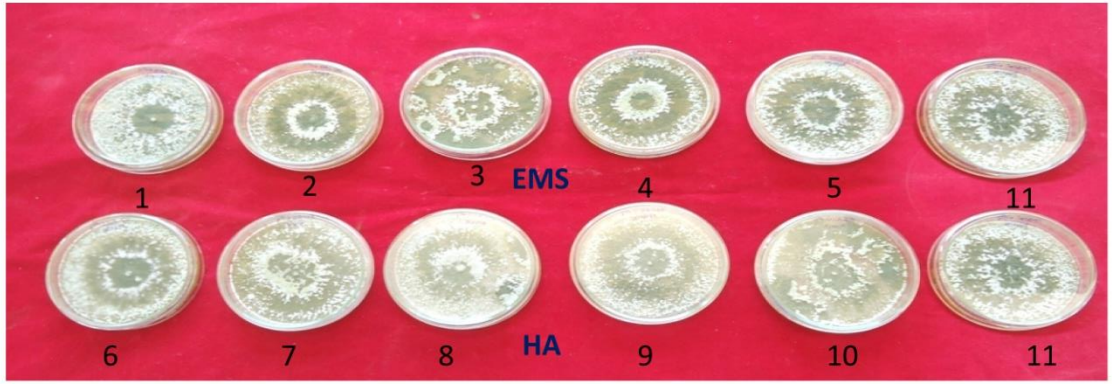


**Plate 4b. *Trichoderma viride* mutants and mother culture 7 th generation**

### 4.2 Efficacy of *Trichoderma viride* mother culture and mutants agains *Sclerotium rolfsii*.

Data presented in table 4, revealed that statistically significant differences were observed, among the mother culture *T. viride* and its mutants over control. The most effective treatment in respect of per cent growth inhibition of *S. rolfsii* was TvME-4 ($T_4$) i.e. 79.06 % which was at par with TvMH-8 ($T_8$), TvME-3 ($T_3$) and TvMH-9 ($T_9$) i.e.78.98 %, 78.10 % and 77.90 % growth inhibition respectively. The lowest per cent growth inhibition of *S. rolfsii* was exerted by TvMH-6 ($T_6$) i.e. 72.66 % and it was found at par with TvC-11($T_{11}$) mother culture. (fig. 1and plate 5).

**Table4. Efficacy of *T. viride* mother culture and mutants against *S. rolfsii* (per cent growth inhibition) at 7 DAI.**

| Treatments | Code name | Mean Radial Growth (mm) | Per cent Growth Inhibition |
|---|---|---|---|
| $T_1$ | TvME-1 | 21.67 | 74.80 |
| $T_2$ | TvME-2 | 22.50 | 73.83 |
| $T_3$ | TvME-3 | 18.83 | 78.10 |
| $T_4$ | TvME-4 | 18.00 | 79.06 |
| $T_5$ | TvME-5 | 22.67 | 73.63 |
| $T_6$ | TvMH-6 | 23.51 | 72.66 |
| $T_7$ | TvMH-7 | 21.83 | 74.61 |
| $T_8$ | TvMH-8 | 18.07 | 78.98 |
| $T_9$ | TvMH-9 | 19.00 | 77.90 |
| $T_{10}$ | TvMH-10 | 19.67 | 77.12 |
| $T_{11}$ | TvC-11 | 23.00 | 73.25 |
| | Control | 86.00 | 00.00 |
| | ‘F’ test | sig | |
| | SE(m)± | 0.80 | |
| | CD(P=0.01) | 3.17 | |

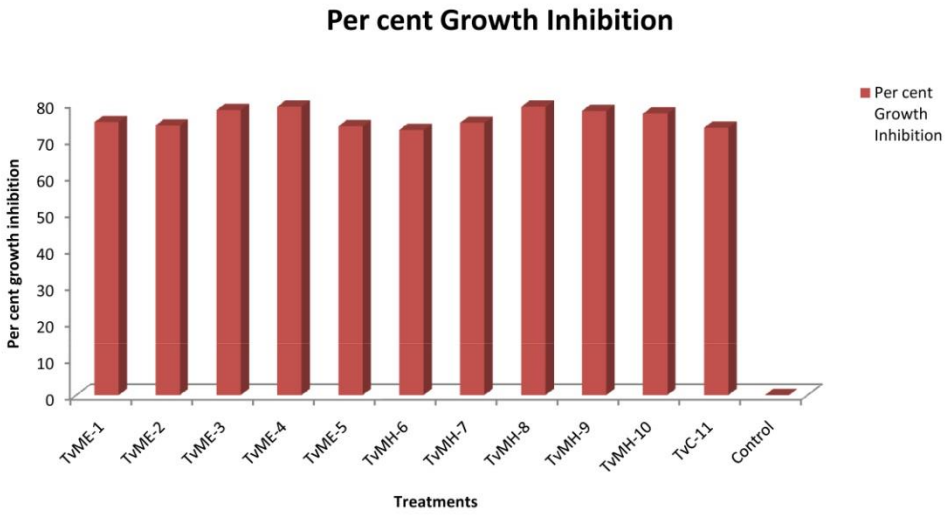


**Fig.1 Efficacy of *T. viride* mother culture and mutants against *S. rolfsii* at 7 DAI.**

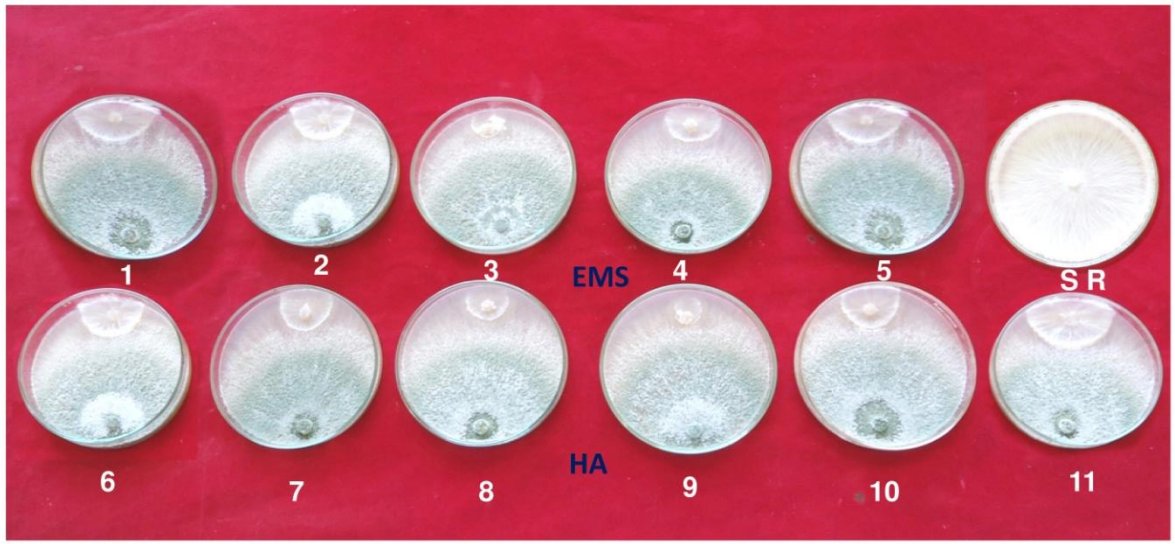


**Plate 5. Efficacy of *Trichoderma viride* mother culture and mutants against *S. rolfsii* (per cent growth inhibition) at 7 DAI**

Current findings are in agreement with Papavizas and Lewis (1982) who evaluated the UV induced biotypes of *T. viride* differed considerably from with type WT 6 in appearance, growth habit and fungitoxic metabolite production and also effectively arrested the growth of *S. lepillorum* of onion and *R. solani* of cotton and radish.

Chaudhary (2009) also evaluated biopesticides Niprot (*T. viride*) for calibration of dosage extent and suppression of collar rot caused by *S. rolfsii* and observed that Niprot was able to suppress the disease.

Mohamed et al. (2010) adopted UV treatment for mutagenesis of *T. viride* to enhance three effective hydrolytic enzymes viz., chitinase, β-1, 3- galactoronase and cellulases which having biocontrol abilities against *S. rolfsii*.

### 4.3 Efficacy of *Trichoderma viride* mother culture and mutants against *Rhizoctonia bataticola*.

Data are presented in table 5, revealed that mother culture of *T. viride* and its mutants were significant in inhibiting the radial mycelial growth of *R. Bataticola,* among them efficient treatment was TvME-4 ($T_4$) i.e. 73.75 % which was at par with TvMH-8 ($T_8$), TvME-3 ($T_3$) and TvMH-10 ($T_{10}$) i.e. 73.56, 72.21 and 69.91 per cent growth inhibition respectively. Lowest per cent growth inhibition of *R. bataticola* was found by TvME-1 ($T_1$) and TvMH-6 ($T_6$) i.e. 65.51 and 65.89 %. (fig 2 and plate 6).

Prashanthi and Vaishnav (2000) studied eight antagonistic microorganisms for their efficacy in suppressing *R. bataticola* by dual culture method. Further they found that *T. viride* completely suppressed the growth of *R. bataticola* at 7DAI.

Patil and Kamble (2011) obtained five *T. koningii mutants* by UV treatments with different time variables. Among these five mutants *T. koningii* showed maximum antagonistic activity against *R. bataticola.*

**Table5. Efficacy of *T. viride* mother culture and mutants against *R. bataticola* (per cent growth inhibition) at 7 DAI.**

| Treatments | Code name | Mean Radial Growth (mm) | Per cent Growth Inhibition |
|---|---|---|---|
| $T_1$ | TvME-1 | 30.00 | 65.51 |
| $T_2$ | TvME-2 | 29.00 | 66.66 |
| $T_3$ | TvME-3 | 24.17 | 72.21 |
| $T_4$ | TvME-4 | 22.83 | 73.75 |
| $T_5$ | TvME-5 | 28.00 | 67.81 |
| $T_6$ | TvMH-6 | 29.67 | 65.89 |
| $T_7$ | TvMH-7 | 29.00 | 66.66 |
| $T_8$ | TvMH-8 | 23.00 | 73.56 |
| $T_9$ | TvMH-9 | 26.33 | 69.73 |
| $T_{10}$ | TvMH-10 | 26.17 | 69.91 |
| $T_{11}$ | TvC-11 | 29.00 | 66.66 |
| | Control | 86.00 | 00.00 |
| | 'F' test | sig | |
| | SE(m)± | 0.61 | |
| | CD(P=0.01) | 2.45 | |

Saini and Nathawat (2016) evaluate four efficient mutants strains were obtained from the wild *T. viride* strain after UV treatment of different time period exposure ranging from 20 to 80 minutes. in dual culture studies inoculation with *T. viride* mutant TvM2-UV2/60 showed 80.37% mycelial inhibition of *R. solani volatile* compounds that released by *T. viride* mutant TvM2-UV2/60 inhibited mycelia growth of *R. solani* 82.12%. Mutants showed better sporulation and highest growth on PDA plates and covered whole plate in 4 days than their wild type strain in 7 days.

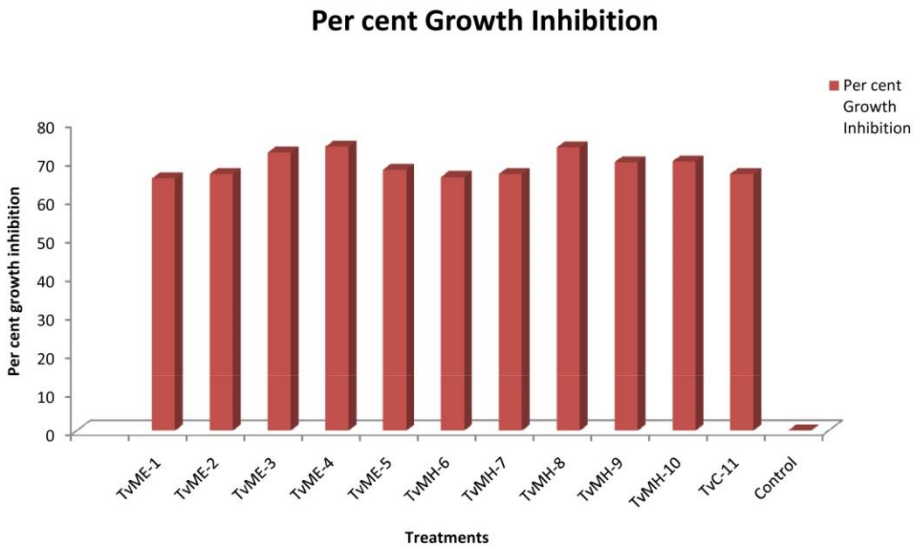


**Fig. 2 Efficacy of *T. viride* mother culture and mutants against *R. bataticola* at 7 DAI**

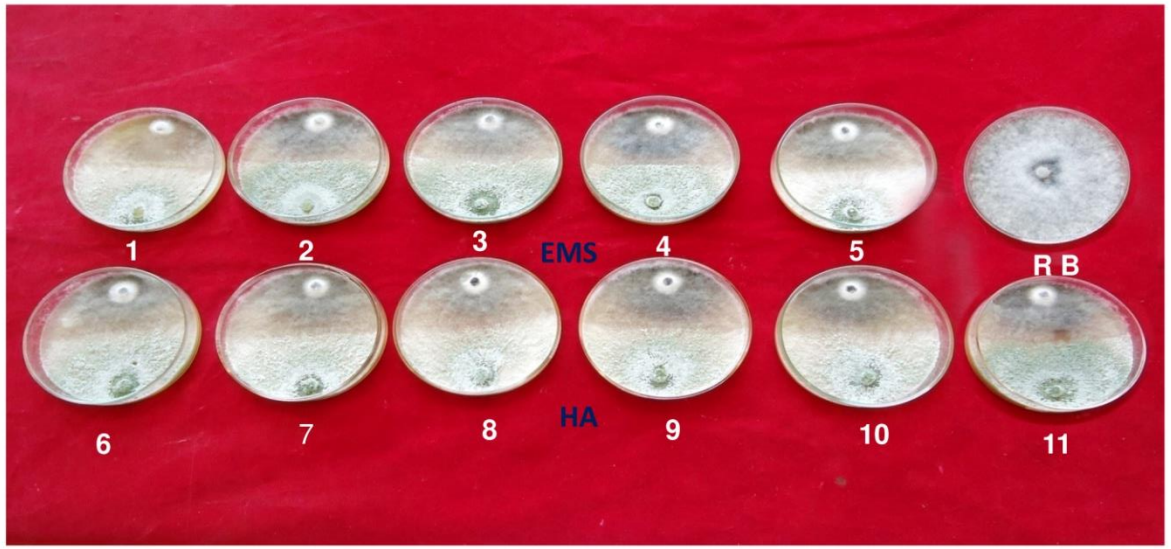


**Plate 6. Efficacy of *Trichoderma viride* mother culture and mutants against *R. bataticola* (per cent growth inhibition) at 7 DAI.**

### 4.4 Efficacy of *Trichoderma viride* mother culture and mutants against *fusarium udum* .

The data presented in table 6, showed per cent growth inhibition of *F. udum* by mother culture of *T. viride* and its mutants•

**Table6. Efficacy of *T. viride* mother culture and mutants against *F. udum* (per cent growth inhibition) at 7 DAI**

| Treatments | Code name | Mean Radial Growth (mm) | Per cent Growth Inhibition |
|---|---|---|---|
| $T_1$ | TvME-1 | 22.33 | 74.03 |
| $T_2$ | TvME-2 | 24.00 | 72.09 |
| $T_3$ | TvME-3 | 18.17 | 78.88 |
| $T_4$ | TvME-4 | 19.00 | 77.90 |
| $T_5$ | TvME-5 | 21.50 | 75.00 |
| $T_6$ | TvMH-6 | 23.00 | 73.25 |
| $T_7$ | TvMH-7 | 24.17 | 71.89 |
| $T_8$ | TvMH-8 | 20.17 | 76.54 |
| $T_9$ | TvMH-9 | 19.83 | 76.94 |
| $T_{10}$ | TvMH-10 | 21.00 | 75.58 |
| $T_{11}$ | TvC-11 | 23.00 | 73.26 |
| | Control | 89 | 00.00 |
| | 'F' test | sig | |
| | SE(m)± | 0.61 | |
| | CD(P=0.01) | 2.42 | |

It revealed that all mutants were significant in inhibiting the radial mycelial growth of the *F. udum,* among them TvME-3 ($T_3$) was most effective (78.88%) and which was at par with TvME-4 (T4), TvMH-9 ($T_9$) and TvMH-8 ($T_8$) i.e. 77.90%, 76.94% and 76.54%. The lowest per cent growth inhibition of *F. udum was* found in TvMH-7 ($T_7$) i.e. 71.89%. (fig 3 and plate 7)

Padamodaya and Reddy (1996) screened ten isolates of *Trichoderma spp*. for their efficacy in arresting the growth *of F. oxysporum*

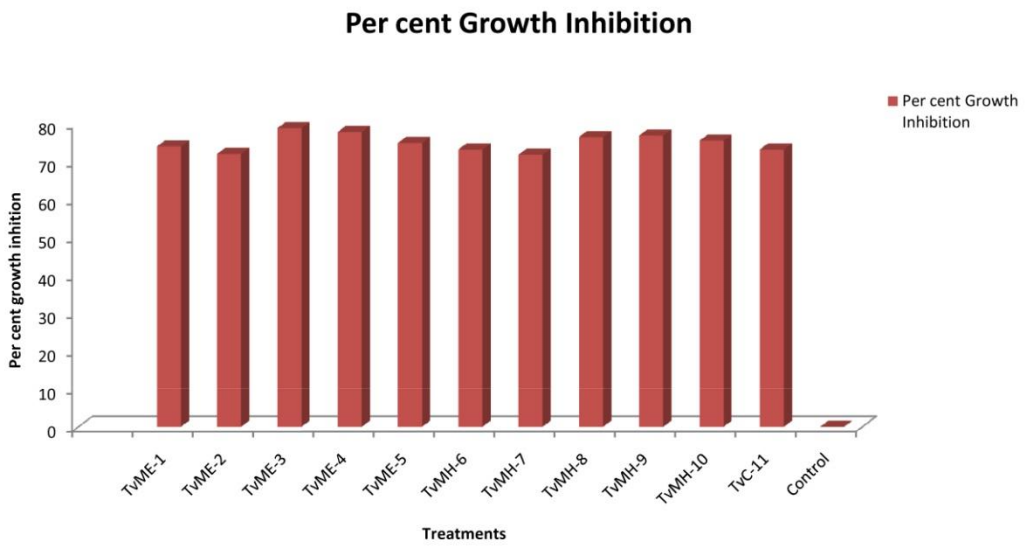


**Fig.3 Efficacy of *T. viride* mother culture and mutants against *F. udum* at 7 DAI.**

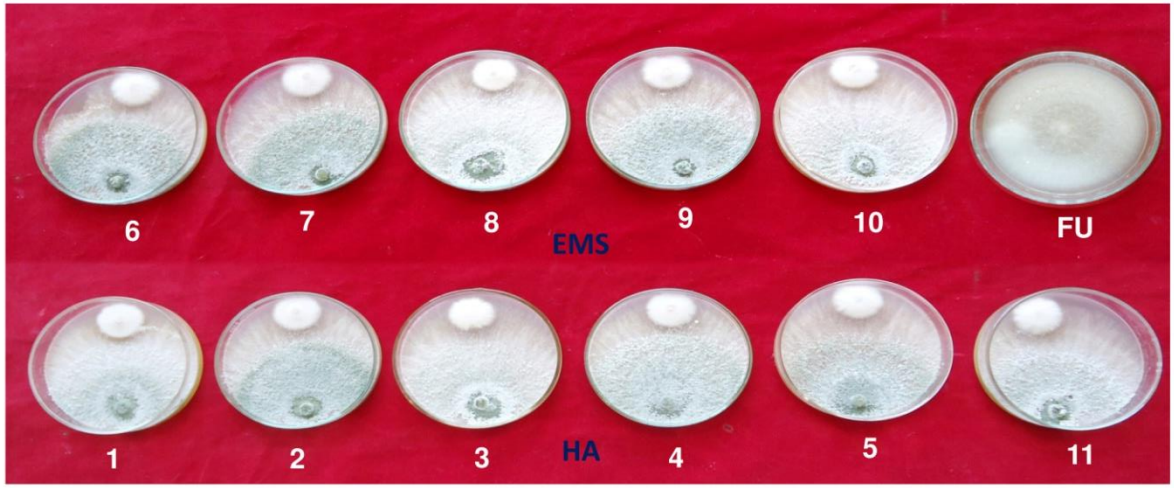


**Plate 7 . Efficacy of *Trichoderma viride* mother culture and mutants against *F. udum* (per cent growth inhibition) at 7 DAI**

*in vitro* and reported that *T. viride* was highly inhibitory in dual culture. Present investigations are in agreement with Mohamed *et al*. (2006) who obtained two stable salt tolerant mutants having great biological proficiency against *F. oxysporum* the causal agent of tomato wilt disease.

Saini and Kshirsagar (2015) observed tested selected mutants were tested for *in vitro* inhibition of predominant soil borne fungal pathogen *F. oxysporum* by using various plate culture techniques (dual culture and inverted plate technique) for their bio-efficacy. In dual culture studies inoculation with *T. viride* mutant TvM2-UV2/60 showed 90.30% mycelial inhibition of *F. oxysporum*, 89.80% which was maximum over other mutants and wild strain under study on volatile compounds that released by *T*. *viride* mutant TvM2-UV2/60 inhibited mycelia growth rate of *F. oxysporum* 83.34 per cent over other mutants and wild strain under study *in vitro.*

### 4.6 Chitinase enzyme units in *Trichoderma viride* mother culture and mutants

The data presented in table 7, showed that TvME-4 ($T_4$) content maximum i.e. 0.64 chitinase enzyme units/ mg of protein units followed by TvMH-9 ($T_9$) and only TvME-3 ($T_3$) i.e. 0.63 and 0.62. The next best mutants were (TvMH-8) ($T_8$), TvME-5 ($T_5$) and TvMH-10 ($T_{10}$) which contain 0.62, 0.60 and 0.60 chitinase enzyme units/mg of protein unit respectively. TvMH-7 ($T_7$), and TvME-2 ($T_2$) were at par with each other. *T. viride* mother culture contain 0.38 and the lowest chitinase enzyme units were estimated in TvME-1 ($T_1$) i.e. 0.37 enzyme units/mg of protein.

Haggag and mohamad (2002) carried out mutagenesis of *T. harzianum* and *T. koningii* with 50 and 75 kilo-rad doses of gamma irradiation which resulted four mutants of each *T. harzianum* and *T. koningii* capable of producing high level of chitinase. These mutants were stable and superior than wild type (WT) with respect to growth, sporulation and biocontrol potential against *B. cinerea*. The fast growing characteristics of different mutants with expression the high units of chitinase enzyme reflected in maximum inhibitory ability of test pathogens .i.e. *S.rolfsii, R.*

*bataticola, F. solani* and *F.udum*. Present studies proved the earlier reports in respect to mutants activities

Zaldivar *et. al.* (2001) treated *T. aureoviride* strains with N methyl- N-nitro-N-nitrosoguanidine. The mutant strains showed enhanced production of fungal cell wall degrading enzymes i.e. chitinase, β-1, 3-glucanases and proteases.

Kulkarni and Ramanujan *et a*l. (2010) also studied the ability of *Trichoderma* isolates to produce chitinase enzyme through polyacralamide gel electrophoresis (SDS-PAGE) method related to their antagonistic ability will help to identify the markers and it can be inserted in to the plant itself through genetic engineering to evolve resistant varieties or these markers may be inserted into *Trichoderma species* itself to promote its antagonistic ability.

**Table7. Chitinase enzyme units in *T. viride* mother culture and mutants**

| Treatments | Code name | Chitinase enzyme units/ mg of protein |
|---|---|---|
| $T_1$ | TvME-1 | 0.37 (0.61)* |
| $T_2$ | TvME-2 | 0.52 (0.72) |
| $T_3$ | TvME-3 | 0.62 (0.79) |
| $T_4$ | TvME-4 | 0.64 (0.80) |
| $T_5$ | TvME-5 | 0.60 (0.78) |
| $T_6$ | TvMH-6 | 0.41 (0.64) |
| $T_7$ | TvMH-7 | 0.57 (0.75) |
| $T_8$ | TvMH-8 | 0.61 (0.78) |
| $T_9$ | TvMH-9 | 0.63 (0.79) |
| $T_{10}$ | TvMH-10 | 0.60 (0.78) |
| $T_{11}$ | TvC-11 | 0.38 (0.62) |
| | ‘F’ test | sig |
| | SE(m)± | 0.01 |
| | CD(P=0.01) | 0.05 |

*Figure in the parentheses are square root transformed values.

**Chitinase enzyme estimate value on spectrophotometer at 510 nm wavelength

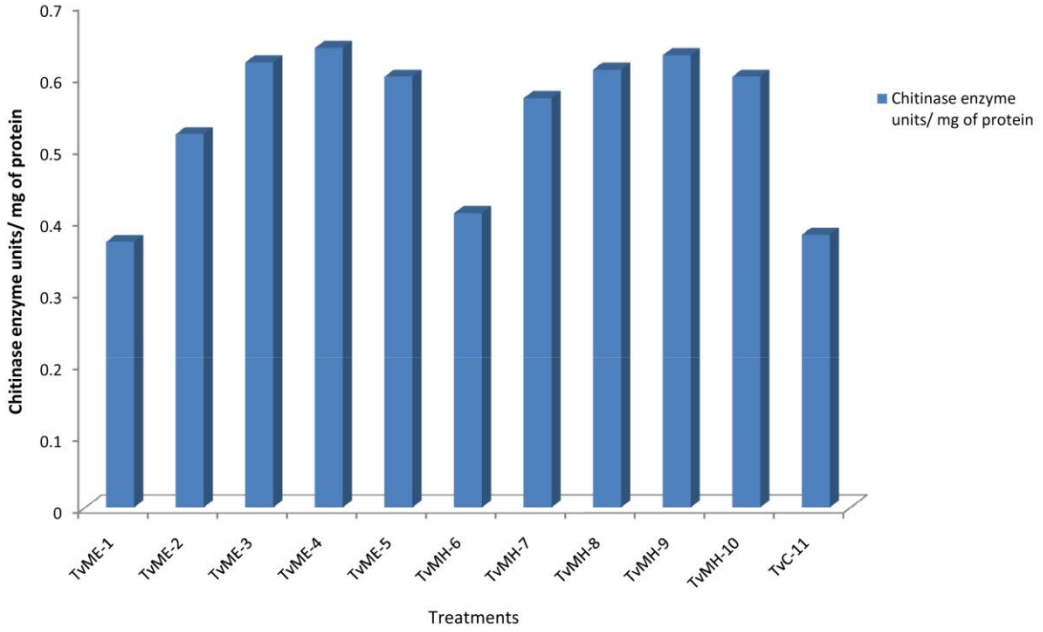


**Fig .4 Chitinase enzyme units in Trichoderma viride mother culture and mutants**

Agrawal and Kothasthani (2012) also studied the chitinase activity of *Trichoderma* on CCA medium. Chitin is second most abundant polymer in nature after cellulose and play major role in fungal cell wall.

K. K. Suryawanshi *et al.* (2012) obtained twenty four *T. viride* mutants by chemical mutagenesis i.e. Ethyl methyl sulphonate (EMS) and Hydroxyl amine (HA) treatments of different doses and time variables. Among these twenty four mutants, *T. viride* TVME3c, TVME4c, TVME4a showed maximum antagonistic activity against the *S. rolfsii, R. bataticola and F. solani*, and also having the highest chitinase enzyme units/mg of protein i.e. 0.62, 0.63 and 0.61 enzyme units/mg of protein.

Savita *et al.* (2015) studied sixteen *T. viride* mutants by gamma irradiation and tested for their antagonistic activities *in vitro*. Morphological characters of efficient mutants were tested up to six generation to check their stability. Among these TVGM1 were proved as effective antagonists against *S. rolfsii, R. bataticola* and *F. oxysporum* f.sp. *ciceri* basis of their maximum bioefficiency. The highest chitinase enzyme units/mg of protein *i.e*. 0.62 was exerted in TVGM1.

### 4.7 Compatibility of *Trichoderma viride* mother culture and its mutants with Fungicides

An experiment on compatibility of *T. viride* mutants with fungicides was conducted *in vitro*, by adopting poison food technique. The observations were on recorded at 7 DAI, the data is presented in table 8, and it showed that all fungicides were found incompatible except Azoxystrobin 23% SC @ 0.2 % and Chlorothalonil 50% WP @ 0.2 % concentration.

Carbendazim 50% WP @ 0.1 %, Tebuconazole 25% WG @0.05%, Mancozeb 75% WP @ 0.2 %, recorded complete growth inhibition of all mutants and mother culture of *T. viride.* Mycelial growth of all mutants in the range of 62 to 80 mm was recorded in Azoxystrobin 23% SC @ 0.2 %, where as Chlorothalonil 75% WP @ 0.2 % recorded only 28 to 50 mm mycelial growth. It proved that fungicides, Azoxystrobin 23% SC

**Table 8.Compatibility of *Trichoderma viride* mother culture and mutants with Fungicides**

| Treatments | Code name | Mean Radial Growth at 7 DAI. | | | | | |
|---|---|---|---|---|---|---|---|
| | | Carbendazim 50% WP | Chlorothalonil (75 WP) | Tebuconazole 25 % WG | Azoxystrobin 23% SC | Mancozeb 75 % WP | Control |
| | | 0.1 | 0.2 | 0.05 | 0.1 | 0.2 | |
| T1 | TvME-1 | 00.00 | 28.00 | 00.00 | 66.00 | 00.00 | 90.00 |
| T2 | TvME-2 | 00.00 | 23.00 | 00.00 | 68.00 | 00.00 | 90.00 |
| T3 | TvME-3 | 00.00 | 45.00 | 00.00 | 75.00 | 00.00 | 90.00 |
| T4 | TvME-4 | 00.00 | 50.00 | 00.00 | 80.00 | 00.00 | 90.00 |
| T5 | TvME-5 | 00.00 | 48.00 | 00.00 | 75.00 | 00.00 | 90.00 |
| T6 | TvMH-6 | 00.00 | 27.00 | 00.00 | 54.00 | 00.00 | 90.00 |
| T7 | TvMH-7 | 00.00 | 27.00 | 00.00 | 65.00 | 00.00 | 90.00 |
| T8 | TvMH-8 | 00.00 | 48.85 | 00.00 | 78.29 | 00.00 | 90.00 |
| T9 | TvMH-9 | 00.00 | 47.00 | 00.00 | 77.00 | 00.00 | 90.00 |
| T10 | TvMH-10 | 00.00 | 46.00 | 00.00 | 75.52 | 00.00 | 90.00 |
| T11 | TvC-11 | 00.00 | 19.00 | 00.00 | 66.00 | 00.00 | 90.00 |
| | ‘F’ test | | sig | | sig | | |
| | SE(m)± | | 0.57 | | 0.58 | | |
| | CD(P=0.01) | | 2.26 | | 2.30 | | |

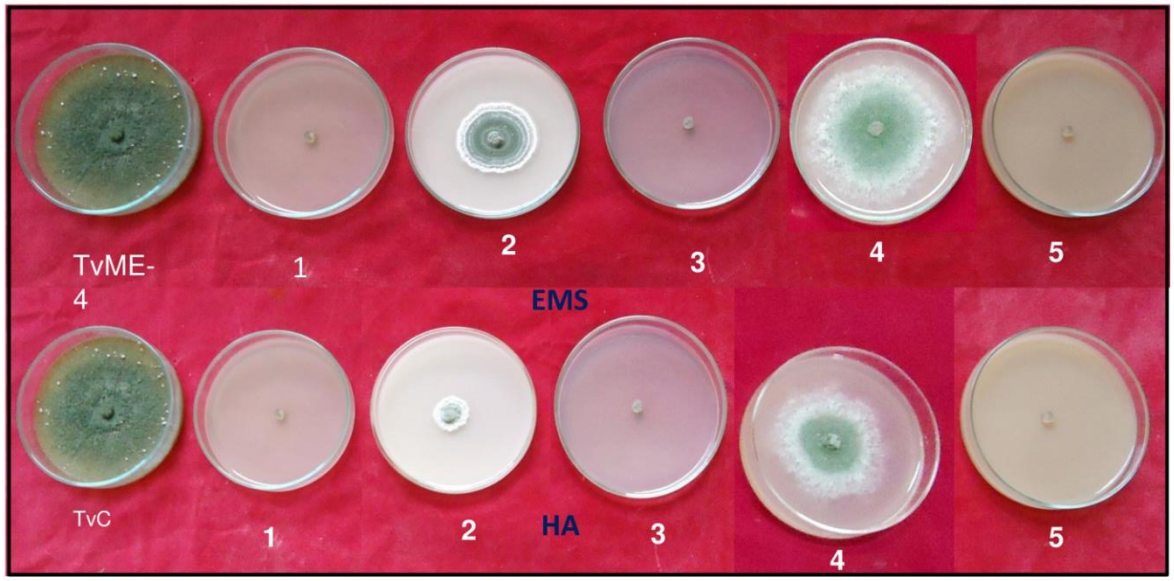


**Plate 8. Compatibility of *Trichoderma viride* mutants and mother culture with fungicides**

was more compatible with *Trichoderma* mutants including mother culture than Chlorothalonil 75% WP @ 0.2 % concentration.

Among the mutants,TvME-4 ($T_4$) was recorded 80.00 mm mycelia growth with Azoxystrobin 23% SC @ 0.2 % at 7 DAI, where as TvMH-8 ($T_8$), TvMH-9 ($T_9$), TvMH-10 ($T_{10}$) and TvME-3 ($T_3$) recorded 78.29, 77.00 75.52 and 75.00 mm growth. Chlorothalonil 75% WP @ 0.2% TvME-4 ($T_4$) also recorded maximum growth (50 mm) followed by TvMH-8 ($T_8$), TvME-5 ($T_5$), TvME-9 ($T_9$) and TvMH-10 ($T_{10}$) i. e. 48.85, 48.00, 47.00 and 46 respectively.

Madhusudhan *et. al.* (2010) evaluated six fungicides, Carbendazim (50% WP) Propiconazole (25% EC), Tridemorph (BO% EC) Chlorothalonil (75% WP) and hexaconazole (5% EC) for their compatibility with *T. viride*. Among them Chlorothalonil (75% WP) was found safe at 40 ppm. Other fungicides were not compatable with *Trichoderma viride* even at very low concentration. Similar observation was also reported by Bagwan (2010). Sharma *et al.* (1999) and Bindu *et. al.* (2011) reported that *T. viride* was compatible with Mancozeb and Tebuconazole while systemic fungicides like Carbendazim, Hexaconazole, and Propiconazole were found incompatible with *T. viride*. Nongmaithem (2015) reported that Carbendazim was highly incompatible with *Trichoderma viride*.

In the present study, Azoxystrobin 23% SC @ 0.2 % and Chlorothalonil 75 % WP @ 0.2 % was found compatible with *T. viride* mutants and mother culture. Similar observations were reported by Archana *et. al.* (2012). The slight difference observed may be due to the mutation. Based on these observation it is concluded that *T. viride* mutants can be safely incorporated into IDM on soil borne diseases caused by *Sclerotium rolfsii, Rhizoctonia bataticola, Fusarium udum.*

## CHAPTER V

# SUMMARY AND CONCLUSIONS

The present work aimed to apply the mutagenesis and irradiation techniques for genetical improvement of the bioagent *Trichoderma viride* to enhance their biocontrol abilities against predominant soil borne fungal pathogens *viz., Sclerotium rolfsii Rhizoctonia bataticola,* and *Fusarium udum*. These are important soil borne fungal pathogens affecting wide range of hosts and have a worldwide distribution on numerous field crops and vegetables.

The present investigation entitled “Antagonistic potential of *Trichoderma viride* mutants and their compatability with fungicides” was carried out during 2017-2018 at Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. The objectives of the studies were

1. Induction of mutation in *Trichoderma viride.*
2. Compatibility of *Trichoderma viride* mutants with fungicides.

*Trichoderma viride* mother culture obtained from Department of Plant Pathology, PGI, Dr. PDKV Akola and it was subjected to chemical mutagenesis i.e. Ethyl Methane Sulphonate and Hydroxyl Amine, different doses and time duration. Eleven mutants were obtained and compared for their morphology variation and biocontrol efficacy against predominant soil borne plant pathogens, viz., *Sclerotium rolfsii, Rhizoctonia bataticola, Fusarium udum.*

Morphological characters of *Trichoderma* mutants exhibited morphological variation such as growth rate, colony diameter, colony type, colony colour, pigmentation, phialides, conidial shape and size and sporulation. Mutants were differed in their biocontrol potential against soil borne pathogen as well as chitinase enzyme content.

Among the mutants most of them were found efficient against *Sclerotium rolfsii, Rhizoctonia bataticola, Fusarium udum* in dual culture technique. The mutants TvME-4 ($T_4$), TvMH-8 ($T_8$), TvME-3 ($T_3$) and TvMH-

9 ($T_9$) exhibited maximum antagonistic activity against *Sclerotium rolfsii* which showed 79.06 %, 78.98 %,78.10 % and 77.90 per cent growth inhibition respectively.

In case of *Rhizoctonia bataticola,* the mutants TvME-4($T_4$), TvMH-8 ($T_8$), TvME-3 ($T_3$) and TvMH-10 ($T_{10}$) exhibited maximum per cent growth inhibition i.e.73.75 %, 73.56 %,72.21 % and 69.91per cent growth inhibition respectively.

*Fusarium udum* was also inhibited by mutants TvME-3 ($T_3$), TvMH-4 ($T_4$), TvMH-9 ($T_9$) and TvMH-8 ($T_8$) showed maximum per cent growth inhibition i.e.78.88%, 77.90 %,76.94 % and 76.54 per cent growth inhibition respectively.

The mutants were assayed for estimation of chitinase enzyme and the mutants TvME-4 ($T_4$), TvMH-9 ($T_9$), TvME-3 ($T_3$) and TvMH-8 ($T_8$) found to possesses highest chitinase enzyme units/mg of protein i.e. 0.64, 0.63, 0.62 and 0.61 respectively. The ability of high quantum of chitinase with higher growth rate and sporulation was directly related to maximum inhibition ability of mutants.

In compatibility study all the mutants and mother cultre of *Trichoderma viride* was found compatible with the fungicide Azoxystrobin 23% SC and Chlorothalonil 50% WP. Among the mutant TvME-4 ($T_4$) showed highly compatible with fungicide as compared to other mutants and mother culture of *Trichoderma viride.*

**Conclusions**

- There was existence of some morphological variation in mutants and mother culture of *Trichoderma viride*. Mutants were differed in their biocontrol potential against tested plant pathogen as well as chitinase enzyme units.
- The mutants TvME-4 ($T_4$), TvMH-8 ($T_8$), TvME-3 ($T_3$) and TvMH-9 ($T_9$) were found effective against *Slerotium rolfsii*.
- The mutants TvME-4 ($T_4$), TvMH-8 ($T_8$), TvME-3 ($T_3$) and TvMH-10 ($T_{10}$) were found effective against *Rhizoctonia bataticola*.

- The mutants TvME-3 ($T_3$), TvMH-4 ($T_4$), TvME-9 ($T_9$) and TvMH-8 ($T_8$) were found effective against *Fusarium udum.*
- The mutants TvME-4 ($T_4$), TvMH-9 ($T_9$), TvME-3 ($T_3$) and TvMH-8 ($T_8$) contains highest chitinase enzyme units/mg of protein i.e. 0.64, 0.63, 0.62 and 0.61 respectively.
- All mutants and mother culture of *T. viride* was found compatible with fungicide Azoxystrobin 23% SC at the rate 0.2 % and Chlorothalonil 75 % WP at the rate 0.2%. Among the mutant TvME-4 ($T_4$) was more compatibility with Azoxystrobin 23% SC and Chlorothalonil 75 % WP fungicides.

## CHAPTER VI

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## Appendix-I

**Potato Dextrose Agar**

| | | |
|---|---|---|
| Peeled potato | 200 | g |
| Dextrose | 20 | g |
| Agar | 20 | g |
| Distilled water | 1000 | ml |

**Czapek's-Dox broth/medium**

| | |
|---|---|
| Sodium nitrate | 2.0 g |
| K2HPO4 | 1.0 g |
| Magnesium sulphate | 0.5 g |
| Potassium chloride | 0.5 g |
| Ferrous sulphate | 0.01 g |
| Sucrose | 30 g |
| Agar | 20 g |
| Distilled water | 1000ml |