

## Original Research Article

<https://doi.org/10.20546/ijcmas.2019.801.047>

## Morphological Variation and Chitinase Production Ability of *Trichoderma viride* Mutants

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### ABSTRACT

#### Keywords

Morphological  
variation, Chitinase  
production,  
*Trichoderma viride*,  
Mutants

#### Article Info

##### Accepted:

04 December 2018

##### Available Online:

10 January 2019

In an attempt to develop superior *Trichoderma viride* isolates for improvement in chitinase Enzyme productivity, induction of mutants was applied. After application of different concentrations of Ethyl methane sulfonate (EMS) and Hydroxyl amine (HA) treatments on *T.viride* mutants were obtained. *Trichoderma viride* was determined by its physiological state so that change in genetical conditions could alter the antagonism. Hence, the genetic modification using mutagenesis offers the potential for producing improved bioprotection is likely to enhance their biocontrol. *Trichoderma viride* mutants grown up to six generations to check the stability on PDA and it was found variation in mutants compared to mother culture. These mutants were tested for their chitinase productivity. TvME-4 (T<sub>4</sub>), TvMH-9 (T<sub>9</sub>) and TvME-3 (T<sub>3</sub>) mutants proved to be the highest producer of chitinase enzyme, since it produced 0.64, 0.63 and 0.62 enzyme units respectively, greater than the mother culture.

### Introduction

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.

Chitinases,  $\beta$ -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  $\beta$ -1, 4-N-acetyl- D glucosamine, 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).

## Materials and Methods

Pure culture of *Trichoderma viride*, mother culture was received from the Department of Plant Pathology, Dr. P.D.K.V. Akola during 2018.

## Chemical mutagenesis

Induction of Chemical mutagenesis was carried according to procedure suggested by Chandra *et al.*, (2010) 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 $\mu$ l/ml, 100  $\mu$ l/ml, 150  $\mu$ l/ml, 200  $\mu$ l/ml and 250 $\mu$ l/ml and incubated at 28<sup>0</sup>C in orbital shaker for 30 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<sup>0</sup>C for 72 hour. Procedure was done for 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 *Trichoderma viride* mutants.

## Experimental details

The experiment was conducted in the laboratory bio-control efficacy of *T.viride* mutants under completely randomized design

(CRD) with treatment as T<sub>1</sub> (TvME-1) - Spore suspension of *T.viride* treated with Ethyl methyl Sulphonate (EMS) @ 50  $\mu$ l/ml for 60 minutes; T<sub>2</sub> (TvME-2) - Spore suspension of *T.viride* treated with Ethyl methyl Sulphonate (EMS) @ 100  $\mu$ l/ml for 100 minutes; T<sub>3</sub> (TvME-3) - Spore suspension of *T.viride* treated with Ethyl methyl Sulphonate (EMS) @ 150  $\mu$ l/ml for 60 minutes; T<sub>4</sub> (TvME-4) - Spore suspension of *T.viride* treated with Ethyl methyl Sulphonate (EMS) @ 200  $\mu$ l/ml for 60 minutes, T<sub>5</sub>(TvME-5) - Spore suspension of *T.viride* treated with Ethyl methyl Sulphonate (EMS) @ 250  $\mu$ l/ml for 60 minutes; T<sub>6</sub> (TvMH-6) - Spore suspension of *T.viride* treated with Hydroxyl Amine (HA) @ 50  $\mu$ l/ml for 60 minutes; T<sub>7</sub> (TvMH-7) - Spore suspension of *T.viride* treated with Hydroxyl Amine (HA) @ 100  $\mu$ l/ml for 60 minutes; T<sub>8</sub> (TvMH-8) - Spore suspension of *T.viride* treated with Hydroxyl Amine (HA) @ 150  $\mu$ l/ml for 60 minutes; T<sub>9</sub> (TvMH-9) - Spore suspension of *T.viride* treated with Hydroxyl Amine (HA) @ 200  $\mu$ l/ml for 60 minutes; T<sub>10</sub> (TvMH-10) - Spore suspension of *T.viride* treated with Hydroxyl Amine (HA) @ 250  $\mu$ l/ml for 60 minutes; T<sub>11</sub> (TvC-11) - Untreated control i.e. *T. viride* mother.

## Preparation of colloidal chitin

Colloidal chitin was prepared as per the method of Roberts and Selintrenikoff (1988).5 g of chitin powder (Hi-Media) was added slowly into 60 ml of concentrated HCl (Sd. Fine Chemicals Ltd., Mumbai) and left for vigorous shaking overnight at 40<sup>0</sup>C. The mixture was added to two liters of ice-cold 95 per cent ethanol with rapid stirring and kept overnight at room temperature (25<sup>0</sup>C). The precipitate was collected by centrifugation at 5,000g for 20 minutes at 4<sup>0</sup>C and then washed with sterile distilled water until the pH of the colloidal chitin turned neutral (pH 7.0). Later, colloidal chitin solution (5 per cent) was prepared and stored at 4<sup>0</sup>C for further use.

### **Preparation of phosphate Buffer (pH 6.7)**

Potassium Dihydrogen Phosphate ( $\text{KH}_2\text{PO}_4$  1 M) 136 gm in 1000 ml of distilled water. Potassium hypophosphate ( $\text{K}_2\text{HPO}_4$  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.

### **Measurement of chitinase activity (Turbidity method)**

Endochitinase activity was measured by the reduction of turbidity of a suspension of colloidal chitin as per the method suggested by Kulkarni *et al.*, (2010). A suspension containing 1% (w/v) or moist colloidal chitin was prepared in 50 mm potassium phosphate buffer, pH 6.7. A mixture consisting of 0.5 ml each of chitin suspension and the enzyme solution to be tested was prepared and inoculated for 24 h at 30<sup>0</sup>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 per cent. One min of enzyme activity (CU) is defined as release of 1 mMol n-glucose acetyl amine/ ml culture filtrate / min / mg protein. 1 cu = release of 1 m mol N- acetyl glucosamine/ ml of culture filtrate / mm/mg/ protein.

### **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<sup>0</sup>C. The absorbance at 510 nm was recorded using systronics make spectrophotometer after dilution with 5 ml distilled water.

### **Morphological study**

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

Colony was milky white coloured at initial stage later turned dark green. subaral 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 al.*, 1999) (Table 1).

### **Results and Discussion**

#### ***Trichoderma viride* mutants**

##### **TvME-1**

Subaral 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.

### **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.

### **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.

### **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.

### **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.

### **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.

### **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

### **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.

### **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.

### **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.

**Table.1** Morphological characteristics of *Trichoderma viride* mother culture and mutants (7<sup>th</sup> generation)

Sr.no	<i>Trichoderma viride</i> 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

**Table.2** Chitinase enzyme units in *T. viride* mother culture and mutants

Treatments	Code name	Chitinase enzyme units/ mg of protein
T <sub>1</sub>	TvME-1	0.37 (0.61)*
T <sub>2</sub>	TvME-2	0.52 (0.72)
T <sub>3</sub>	TvME-3	0.62 (0.79)
T <sub>4</sub>	TvME-4	0.64 (0.80)
T <sub>5</sub>	TvME-5	0.60 (0.78)
T <sub>6</sub>	TvMH-6	0.41 (0.64)
T <sub>7</sub>	TvMH-7	0.57 (0.75)
T <sub>8</sub>	TvMH-8	0.61 (0.78)
T <sub>9</sub>	TvMH-9	0.63 (0.79)
T <sub>10</sub>	TvMH-10	0.60 (0.78)
T <sub>11</sub>	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

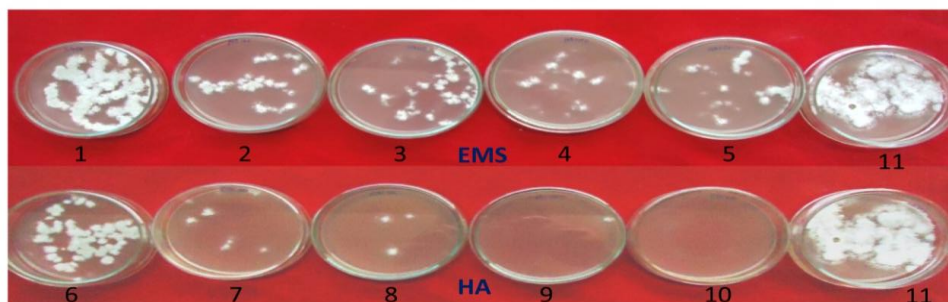


Plate 1a. *Trichoderma viride* mutants and mother culture 1 st after treatment

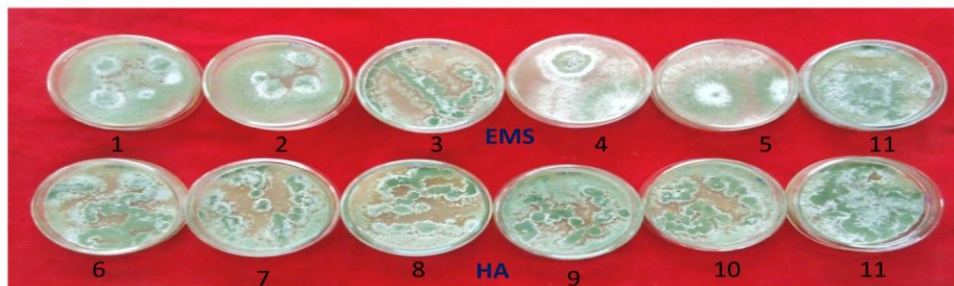


Plate 1b. *Trichoderma viride* mutants and mother culture 7 th after treatment

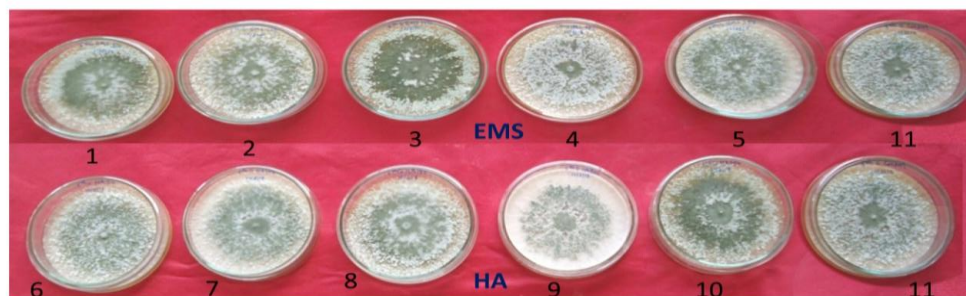


Plate 4a. *Trichoderma viride* mutants and mother culture 6 th generation

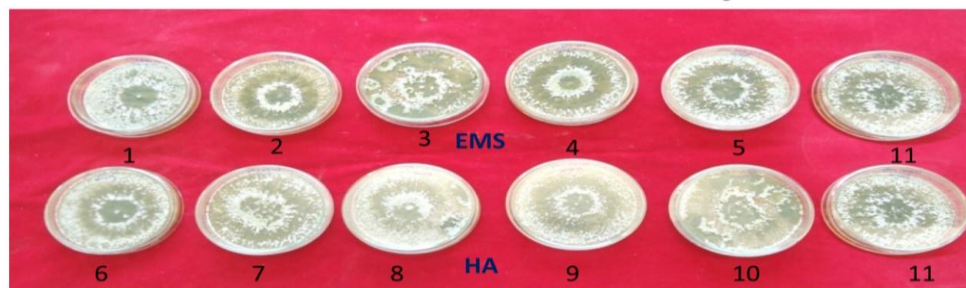


Plate 4b. *Trichoderma viride* mutants and mother culture 7 th generation

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 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 verticillasterminally 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 was 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 differ 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).

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

The data presented in table 2, showed that TvME-4 (T<sub>4</sub>) content maximum i.e. 0.64 chitinase enzyme units/ mg of protein units followed by TvMH-9 (T<sub>9</sub>) and only TvME-3 (T<sub>3</sub>) i.e. 0.63 and 0.62. The next best mutants were (TvMH-8) (T<sub>8</sub>), TvME-5 (T<sub>5</sub>) and TvMH-10 (T<sub>10</sub>) which contain 0.62, 0.60 and 0.60 chitinase enzyme units/mg of protein unit respectively. TvMH-7 (T<sub>7</sub>), and TvME-2 (T<sub>2</sub>) 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<sub>1</sub>) 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 to 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. rolfisii*, *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,  $\beta$ -1, 3-glucanases and proteases.

Kulkarni and Ramanujan *et al.*, (2010) also studied the ability of *Trichoderma* isolates to produce chitinase enzyme through polyacrilamide 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.

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**How to cite this article:**

Vyawahare, M.P., S.T. Ingle and Gathe, A.G. 2019. Morphological Variation and Chitinase Production Ability of *Trichoderma viride* Mutants. *Int.J.Curr.Microbiol.App.Sci.* 8(01): 447-455. doi: <https://doi.org/10.20546/ijcmas.2019.801.047>