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Enhanced chitinase production ability and morphological variation of *Trichoderma asperellum* Mutants

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Abstract
Genetic modification using mutagenesis leads to enhance the potential for producing improved bioprotection is likely to trigger biocontrol by *Trichoderma asperellum*. Cobalt- 60 gamma radiation were used to induce mutation in *Trichoderma asperellum.* Chemical Mutagenesis was carried out by application of different concentrations of Ethyl methane sulfonate (EMS). In order to check the stability, mutants were grown up to seven generations on PDA and Variation in the morphological and cultural character were found in mutants as compared to mother culture. These mutants were tested for their chitinase productivity. TaMG 3 (T3), TaMG 4 (T4), TaME 4 (T15) and TaMG 7 (T7) reported to be the highest producer of chitinase enzyme, since it produced 0.70, 0.67, 0.66 and 0.66 chitinase enzyme units/ mg of protein respectively. Mother culture TaMC contained only 0.39 66 chitinase enzyme units/ mg of protein.

Keywords: *Trichoderma asperellum*, mutation, gamma rays, ethyl methane sulphonate (EMS)

Introduction
Trichoderma spp. are the mycoparasites of plant pathogens used for biocontrol of diseases. Genetic modification using mutagenesis offers the potential for producing improved bioprotection is likely to enhance their biocontrol capabilities against soil borne pathogen. Advances in molecular biology have laid the foundation for isolation of valuable genes and their transfer to target plants through novel transgenic. Genetic transformation of existing biocontrol fungi that are well adjusted to their environment is likely to enhance their biocontrol capabilities. Chitin, a β- 1,4-linked polymer of N-acetylglucosamine, is a major component of the cell wall of many fungi (Austin *et al*., 1981; Peberdy, 1990) [2, 13]. Chitinases are produced by *Trichoderma* when it is grown in the presence of chitin or isolated fungal cell walls (Elad *et al*., 1983; Tokimoto, 1982) [6, 18]; 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- D glucosamine, the main cell wall component of most phytopathogenic fungi, showing strong inhibitory activity in vitro on spore germination and hyphal growth.

Material and Methods
Pure culture of *Trichoderma asperellum*, mother culture were received from the Department of Plant Pathology, Dr. P.D.K.V. Akola during 2018.

Mutation induced by Gamma Radiation.
Induction of mutation by gamma radiation was carried according to the procedure of Gadgil *et al.* (1995) [7], Migheli *et al.* (1998) [11] and Rey *et al.* (2000) [14] at Nuclear Chemistry Department, Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur (India). The 10 days sporulated culture of *Trichoderma asperellum* was irradiated with cobalt – 60 gamma radiation @ 41.6 gray/min. The applied doses level were were 5 k-rad, 7.5 k-rad, 10 k-rad, 12.5 k-rad, 15 k-rad, 17.5 k-rad, 20 k-rad, 22.5 k-rad, 25 k-rad, 27.5 k-rad and 30 k-rad. After irradiation culture were transfered on fresh PDA medium and grown up to six generation to check the stability of *Trichoderma asperellum* mutants.

Chemical mutagenesis

Chemical mutagenesis was done according to procedure suggested by Chandra *et al.* (2010) [4] and Durand *et al.* (1988) [5]. Conidiospores of 8 days old culture of *Trichoderma asperellum* were used for mutagenesis. Spore suspension of *T. asperellum* was treated with Ethyl Methane Sulphonate (EMS) @ 150 µl/ml, 175 µl/ml, 200 µl/ml, 225 µl/ml and 250µl/ml incubate at 28 ^{0}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 ^{0}C for 72 hour.

Experimental details

Treatment	Code	Description
T1	TaMG 1	*T. asperellum* treated with cobalt 60 @ 5 k-rad for 1.20 min.
T2	TaMG 2	*T. asperellum* treated with cobalt 60 @ 7.5 k-rad for 2.20 min.
T3	TaMG 3	*T. asperellum* treated with cobalt 60 @ 10 k-rad for 2.40 min.
T4	TaMG 4	*T. asperellum* treated with cobalt 60 @ 12.5 k-rad for 3.00min.
T5	TaMG 5	*T. asperellum* treated with cobalt 60 @ 15 k-rad for 4.01 min.
T6	TaMG 6	*T. asperellum* treated with cobalt 60 @ 17.5 k-rad for 4.20 min.
T7	TaMG 7	*T. asperellum* treated with cobalt 60 @ 20 k-rad for 4.49 min.
T8	TaMG 8	*T. asperellum* treated with cobalt 60 @ 22.5 k-rad for 5.40 min.
T9	TaMG 9	*T. asperellum* treated with cobalt 60 @ 25 k-rad for 6.00 min.
T10	TaMG 10	*T. asperellum* treated with cobalt 60 @ 27.5 k-rad for 7.01 min.
T11	TaMG 11	*T. asperellum* treated with cobalt 60 @ 30 k-rad for 7.11min.
T12	TaME 1	Conidial suspension of *T. asperellum* treated with Ethyl methane Sulphonate (EMS) @ 150 µl/ml for 60 minutes.
T13	TaME 2	Conidial suspension of *T. asperellum* treated with Ethyl methane Sulphonate (EMS) @ 175 µl/ml for 60 minutes.
T14	TaME 3	Conidial suspension of *T. asperellum* treated with Ethyl methane Sulphonate (EMS) @ 200 µl/ml for 60 minutes.
T15	TaME 4	Conidial suspension of *T. asperellum* treated with Ethyl methane Sulphonate (EMS) @ 225 µl/ml for 60 minutes.
T16	TaME 5	Conidial suspension of *T. asperellum* treated with Ethyl methane Sulphonate (EMS) @ 250 µl/ml for 60 minutes.
T17	Mother Culture	Untreated control ie. *T. asperellum* mother culture.

Preparation of Colloidal Chitin

Measurement of chitinase

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 inculcated for 24 h at 30 ^{0}C. Subsequently the mixture was diluted with 5 ml distilled water 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.

Preparation of Colloidal Chitin

Colloidal chitin was prepared as per the method of Roberts and Selintrenikoff (1988). 10 g of chitin powder (HiMedia Laboratories Pvt. Ltd., Mumbai) was added slowly into 120 ml of concentrated HCl (Sd. Fine Chemicals Ltd., Mumbai) and left for vigorous shaking overnight at 4 ^{0}C. The mixture was added to 2 liters of ice-cold 95 per cent ethanol with rapid stirring and kept overnight at room temperature (25 ^{0}C). The precipitate was collected by centrifugation at 5,000 rpm for 2 minutes at 4 ^{0}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 ^{0}C for further use.

Preparation of Phosphate Buffer (pH 6.7)

Potassium Dihydrogen Phosphate (KH_2PO_4) 1 M, 136 gm in 1000 ml of distilled water. Potassium hypophosphate (K_2HPO_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.

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.

Estimation of Protein

To estimate the protein concentration Lowry's method was followed (Lowry et. al. 1951) *Trichoderma* mutants and mother culture were mass-cultivated on potato dextrose broth for 7- 10 days at 28 ± 2 °C. Towards the end of the incubation period, mycelia were harvested, washed in SDW and blot-dried. The mycelial mat was crushed in sterilized, pre-chilled pestle and mortar into a fine powder using liquid nitrogen. Protein Quantity was estimated from mycelia extract. 1 ml of aliquot was taken in centrifuge tube to which 1 ml of 10% Trichloroacetic acid was added to precipitate the protein. This mixture was allowed to stand and then centrifuged. Supernatant was discarded and the procedure is repeated twice. This sample is used for protein estimation. Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube as given in the table. The final volume in each of the test tubes is 1 ml. The BSA range is 0.2 to 1 mg/ ml. The test tube with 1 ml distilled water serve as blank. Add 4.5 ml of alkaline copper sulphate reagent (analytical reagent). Mix the solutions well. This solution is incubated at room temperature for 10 mins. Then add 0.5 ml of reagent Folin Ciocalteau solution (reagent solutions) to each tube and incubate for 30 min. Take the optical density (measure the absorbance) at 660 nm. Plot the absorbance against protein concentration to get a standard calibration curve. Check the absorbance of unknown sample and Estimate the amount of protein present in the given sample from the standard graph

Results and Discussion

Morphological variations of *Trichoderma viride* mother culture and its mutants

***Trichoderma asperellum* (mother culture)**

Trichoderma asperellum colony was milky white coloured at initial stage later turned dark green. Subaerial and dispersed

growth was observed with moderate sporulation, giving yellow colour pigmentation on PDA medium. Phialides were straight, ampuliform, only slightly enlarged in the middle. Conidia shape was sub-globose or sub-globose to ovoid and conidia ornamentation was finely warted. Conidiophore were regularly branched and branches were typically paired. Chitinase enzyme (0.39) units/mg of protein was estimated in chitinase assay the data presented in table 1.

***Trichoderma asperellum* mutants**

1) TaMG1

Colony was milky white at initial stage, later turned to greenish yellow colour with yellow colour pigmentation on PDA medium. Subaerial and disperse growth was observed. Concentric rings were formed. Phialides branched some have simple, conidiophores irregularly paired and subglobose roughened conidiation and conidia shape was globose to ovoid.

2) TaMG 2

Flat and superficial growth was observed with moderate sporulation. Colony with light green to milky white mycelial growth at initial stage, later turned to light greyish green, concentric rings were produced on PDA medium. Yellow pigmentation was observed on PDA. Phialides were frequently paired. Globose to ellipsoidal shaped conidia were observed under microscope observations.

3) TaMG 3

Subaerial and raised growth was observed. This mutant slightly differed from other mutant, as it was fast growing, colony was milky white at initial stage, later turned to greenish in colour giving yellow colour pigmentation on PDA medium with concentric ring. Phialides were grouped simple, globose to ellipsoidal shape conidia, were noticed under microscope.

4) TaMG 4

Colony was light grey like milky white colour at initial stage, later turned light greenish was noticed on PDA. Subaerial and disperse growth was observed. Dark yellow colour pigmentation having concentric rings was noticed. Colony with aggregated growth at peripheral region of concentric ring. Phialides were branched with ellipsoidal shaped conidia.

5) TaMG 5

Flat and superficial growth was observed. Colony was milky white coloured at initial stage, later turned light green with milky white growth at periphery. Concentric rings were formed. Colour of the medium changes with yellow pigmentation on PDA. Phialides frequently paired conidia roughened, oblong to ellipsoid shaped.

6) TaMG 6

Subaerial and dispersed growth was observed. Colony was light green at initial stage, later turned to greenish yellow with white milky growth at periphery of the colony, giving yellow colour pigmentation on PDA medium. Phialides were branched. Globose to ovoid shaped conidia were noticed.

7) TaMG 7

Colony was milky white to greenishin colour, growth was disperse and Subaerial, dirty amber coloured pigmentation on the PDA medium. Peripheral region showed milky white growth. Phialides were branched with oblong to ellipsoidal shaped conidia.

8) TaMG 8

Flat and superficial growth was observed. Colony was slight green coloured at initial stage later turned dark greenish and milky white concentric rings was observed at peripheral region with amber colour pigmentation on PDA media. Phialides were branched; conidia were oblong to ellipsoidal shaped.

9) TaMG 9

Colony was milky white to dark green in colour with flat and disperse growth on PDA media. Colony turned to dark green, maximum sporulation was noticed on PDA, giving amber colour pigmentation. Phialides were grouped and simple whereas ellipsoidal shaped conidia.

10) TaMG 10

Colony was milky white coloured to greenish yellow, with disperse and Subaerial growth on PDA media. Colony was turned to light Green colour, fast growing, colony was in the form of concentric ring, yellow colour pigmentation was noticed 7-8 days after incubation. Phialides were branched, ellipsoidal shaped conidia were observed and chitinase.

11) TaMG 11

Colony was greenish to yellowish green on PDA media. Yellow pigmentation was noticed. Phialides were grouped simple, ellipsoidal shaped conidia was observed under microscopic observations.

12) TaME 1

Colony was milky white coloured at initial stage, later turned to greenish, having Subaerial and superficial growth. It showed yellow coloured pigmentation. Phialides were branched and ellipsoidal shaped conidia were observed.

13) TaME 2

Colony was milky white coloured at initial stage with Subaerial and disperses growth on PDA media. Colony turned to greenish, and amber coloured pigmentation was noticed. Phialides were branched, ellipsoidal shaped conidia were observed.

14) TaME 3

Colony turns to green with white milky growth at periphery of the colony with Subaerial and disperse colony growth. Yellow colour pigmentation was noticed after 7-8 days after incubation. Phialides were grouped simple and subglobose roughened conidiation and were globose to ellipsoidal in shape.

15) TaME 4

Colony was light green in colour with white milky growth at periphery, colony was in the form of concentric ring giving yellow colour pigmentation after 7-8 days of incubation. Subaerial and dispersed growth was observed. Phialides were branched, ellipsoidal shaped conidia were observed.

16) TaME 5

Colony was light greenish having milky white growth at periphery of the colony, colony was in the form of concentric ring giving yellow colour pigmentation after 7-8 days after

incubation. Flat and Superficial growth was observed. Phialides were grouped, ellipsoidal shaped conidia were observed.

Trichoderma colonies were fast growing, it shows flat, superficial or subaerial mycelial growth at first white and downy, later developing yellowish-green to deep green compact tufts, often only in small areas or in concentric ring-like zones on the plate surface. Conidiophores are repeatedly branched, irregularly verticillate, bearing clusters of divergent, Phialides were ampuliform to lageniform, usually constricted at the base, more or less swollen near the middle. Phialides were disposed on branches of the conidiophore. Conidia of *T. aperellum* and *T. viride* ultimately become dark green. Conidia of both are globose to subglobose, but in *T. asperellum* they were slightly more ovoidal than in *T. viride.* Conidial 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 (1984). Most isolates of *T. asperellum* exhibited fast growth, produced finely warted conidia and characteristic yellow pigmentation in media also corroborates the results of present studies.

Vyawahare *et al.* (2018) obtained nine *Trichoderma viride* mutants through chemical mutation using Ethyl methane sulfonate (EMS) and Hydroxyl amine (HA) and grown up to six generations to check the stability of *Trichoderma viride* mutants and reported morphological variation in mutants due to mutagenesis. Abbasi *et al.* (2016) carried out mutation of *Trichoderma* spp. through gamma irradiation and 24 mutants were selected. He reported that the gamma radiation caused differences in morphological properties of *Trichoderma* spp. such as color, colony appearance and sporulation.

Moradi (2015) showed Gamma irradiation could change the morphological characteristics such as colony shape and colour, sporulation and mycelia growth rate. The dual culture test exhibit that the mutated isolates have statistically higher antagonistic capability against soil borne plant pathogen than their parent strain.

Table 1: Morphological characteristics of *Trichoderma asperellum* mother culture and mutants (7th generation)

Sr. no	*Trichoderma asperellum* mutants	Morphological characters					
		Colony diameter/ Radial mycelial growth (mm) at 7 DAI	Colony growth type	Colony colour	Pigmentation	Phialides	Conidia shape
1.	TaMG-1	88.50	Subaerial and disperse	Greenish yellow	Yellow colour	Branched	Globose to Ovoid
2.	TaMG-2	89.50	Flat and Superficial	Light green to milky white	Yellow colour	Frequently paired	Globose to Ellipsoid
3.	TaMG-3	90.00	Subaerial and raised	Milky white to Greenish	Yellow colour	Group simple	Globose to Ellipsoid
4.	TaMG-4	90.00	Subaerial and disperse	Milky white to light Green	Dark yellow colour	Branched	Ellipsoidal
5.	TaMG-5	87.90	Flat and Superficial	Milky white to light Green	Yellow colour	Frequently paired	Oblong to Ellipsoidal
6	TaMG-6	89.50	Subaerial and disperse	Light green to light yellow	Yellow colour	Branched	Globose to Ovoid
7.	TaMG-7	89.50	Subaerial and disperse	Milky white to Greenish	Amber colour	Branched	Oblong to Ellipsoidal
8.	TaMG -8	90.00	Flat and Superficial	Greenish to light yellow	Amber colour	Branched	Oblong to Ellipsoidal
9.	TaMG-9	88.50	Flat and disperse	milky white to Light green	Amber colour	Group simple	Ellipsoidal
10.	TaMG-10	90.00	Subaerial and disperse	Milky white to Greenish yellow	Yellow colour	Branched	Ellipsoidal
11.	TaMG-11	89.50	Subaerial and disperse	Greenish to yellowish green	Yellow colour	Group simple	Ellipsoidal
12.	TaME-1	88.50	Subaerial and Superficial	Milky white green to light Greenish	Yellow colour	Branched	Globose to Ellipsoidal
13.	TaME-2	89.00	Subaerial and disperse	Milky white to Greenish yellow	Amber colour	Branched	Ellipsoidal
14.	TaME-3	90.00	Subaerial and disperse	Light milky white to dark green	Yellow colour	Group simple	Oblong to Ellipsoidal
15.	TaME-4	90.00	Subaerial and disperse	Greenish white	Yellow colour	Branched	Ellipsoidal
16.	TaME-5	88.00	Flat and Superficial	Milky white to light green	Dark yellow colour	Group simple	Ellipsoidal
17.	TaMC (Mother culture)	88.00	Subaerial and disperse	Milky white to Greenish	Yellow colour	Group simple	Globose to ovoid

Chitinase enzyme units in Trichoderma asperellum mutants and mother culture

The data presented in table 2, showed that TaMG 3 (T_3) contain highest i.e. 0.70 chitinase enzyme units/ mg of protein, which was followed by TaMG 4 (T4) i.e. 0.67 chitinase enzyme units/ mg of protein. The next best mutant in terms of containing chitinase enzyme unit were TaME 4 (T15) and TaMG 7 (T7) i.e. 0.66 chitinase enzyme units/ mg of protein units respectively. Mother culture TaMC contained only 0.39 chitinase enzyme units/ mg of protein. Lowest chitinase enzyme units i.e. 0.34 chitinase enzyme units/ mg of protein was observed in TaMG 11 (T11).

Table 2. Chitinase enzyme units in *Trichoderma asperellum* mutants and mother culture

S.N.	Treatment	Code	Chitinase enzyme units/ mg of protein			Mean Chitinase enzyme units/ mg of protein
			R1	R2	R3	
1	T1	TaMG 1	0.45(0.97)*	0.40(0.95)*	0.42(0.96)*	0.42 (0.96)*
2	T2	TaMG 2	0.44 (0.97)	0.38 (0.94)	0.39 (0.94)	0.40 (0.95)
3	T3	TaMG 3	0.66 (1.07)	0.70 (1.10)	0.74 (1.11)	0.70 (1.09)
4	T4	TaMG 4	0.70 (1.09)	0.66 (1.08)	0.64 (1.07)	0.67 (1.08)
5	T5	TaMG 5	0.57 (1.03)	0.57 (1.03)	0.59 (1.04)	0.58 (1.04)
6	T6	TaMG 6	0.42 (0.96)	0.42 (0.96)	0.40 (0.95)	0.41 (0.96)
7	T7	TaMG 7	0.66 (1.08)	0.66 (1.08)	0.65 (1.07)	0.66 (1.08)
8	T8	TaMG 8	0.41 (0.95)	0.40 (0.95)	0.43 (0.96)	0.41 (0.96)
9	T9	TaMG 9	0.59 (1.04)	0.59 (1.04)	0.57 (1.03)	0.58 (1.04)
10	T10	TaMG 10	0.46 (0.98)	0.46 (0.98)	0.45 (0.97)	0.46 (0.98)
11	T11	TaMG 11	0.34 (0.92)	0.36 (0.93)	0.33 (0.91)	0.34 (0.92)
12	T12	TaME 1	0.37 (0.93)	0.38 (0.94)	0.37 (0.93)	0.37 (0.93)
13	T13	TaME 2	0.60 (1.05)	0.57 (1.03)	0.62 (1.06)	0.60 (1.05)
14	T14	TaME 3	0.48 (0.99)	0.48 (0.99)	0.47 (0.98)	0.48 (0.99)
15	T15	TaME 4	0.66 (1.08)	0.67 (1.09)	0.66 (1.08)	0.66 (1.08)
16	T16	TaME 5	0.57 (1.04)	0.58 (1.05)	0.58 (1.05)	0.58 (1.04)
17	T17	Mother Culture	0.39 (0.94)	0.39 (0.94)	0.40 (0.95)	0.39 (0.95)
	'F' test					Sig.
	SE(m)±					0.0053
	CD (P=0.01)					0.0204

*Figure in the parentheses are square root transformed values

Similar results were recorded by Vyawahare *et al*. (2019) [19] who obtained ten Trichoderma viride mutants by chemical mutation among them the highest chitinase enzyme units/mg of protein i.e. 0.64 was in TvME 4. The results obtained in present study are also in agreement with the findings of scientists *viz*. Haggag (2002), Kulkarni and Ramanujam *et al.* (2010), Suryavanshi *et al.* (2012), and Wagh *et al.* (2015) who reported the higher chitinase enzyme activity of *Trichoderma* mutants.

Present finding on the *T. viride* mutants enhanced the production of chitinase enzyme as compaired to mother culture of *T. viride* these results was supported by the earliar worker Vyawahare *et al.* (2019) [19] and Suryawanshi *et al*. (2013) [17].

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