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Gamma Radiation Induced Mutagenesis in *Trichoderma reesei* to Enhance Chitinase Enzyme Activity

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A B S T R A C T

Trichoderma reesei is a widely distributed soil fungus that antagonizes numerous fungal phytopathogens. Random induced mutation by gamma radiation with five treatments TrME 1 (50 Kr), TrME 2 (100 Kr), TrME 3 (150 Kr), TrME 4 (200 Kr), TrME 5 (untreated) were studied for morphological study and chitinase activity. The result indicates that TrME 1 contain maximum i.e. 0.3201 chitinase enzyme units/mg of protein followed by TrME 4 recording 0.3108 chitinase enzyme units/mg of protein. This indicates that treatments TrME 1 and TrME 4 are the best mutants for use as effective mycoparasite in the biocontrol of plant pathogenic fungi.

Introduction

Trichoderma spp. has been exploited in many industries including paper, textile, biofuel and agriculture due to its prolific secretion of degrading enzymes and biocontrol activities. They produce many antifungal enzymes including chitin-degrading enzymes which used economically as a basis of these kind of proteins (Dinesh *et al*, 2017). Chitinase is a glycosyl hydrolase that catalyzes the hydrolytic degradation of chitin and is found in a wide variety of organisms including bacteria, fungi, invertebrates, plants and animals. Chitinase producing fungi especially *Trichoderma* spp. can be effective as biocontrol agents against some plant pathogenic fungi. An important part of the mechanism involved in the antagonism of *Trichoderma* spp. and fungal pathogens appears to be production of fungal cell wall lytic enzymes including chitinases. Chitin, β-1,4-linked polymer of N-acetyl glucosamine, is a major component of the cell wall of many fungi and also is an abundant biopolymer whose degradation has a significant impact on the balance of natural ecosystems. Many prokaryotic and eukaryotic microorganisms

degrade chitin by using enzyme systems such as endochitinases (EC3.2.1.14), chitobiosidases and β-N-acetylhexosamidase (N-acetyl-β-D-glucosaminidases) (EC3.2.1.30). Members of the fungal genus *Trichoderma* are known to produce chitinolytic enzymes that can degrade the cell wall of Ascomycetes and Basidiomycetes. The chitinases of mycoparasitic species, e.g., *Trichoderma harzianum*, are also involved in the antagonistic ability of these fungi against plant pathogens and in biocontrol. Although a plethora of chitinolytic enzymes have been detected and purified from various *Trichoderma* spp. only a limited number of chitinolytic genes have been cloned (ech42, chit33 and nag1) (Pebprdy & Ulhoa 1991).

Materials and Methods

Pure culture of *Trichoderma reesei* was collected from the Department of Plant pathology. Indian Type Culture Collection Centre (ITCC). New Delhi (IARI).

Mutation induced by gamma radiation

Induction of mutation by gamma radiation was carried according to the procedure of Gadgil *et al.,* (1995), Migheli *et al.,* (1998) and Rey *et al.,* (2000) at Nuclear Chemistry Department, Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur. The 10 days sporulated culture of *reesei* was irradiated with cobalt. The applied doses level were 50,100,150 and 200 Kr. After irradiation culture were transferred on fresh PDA medium and grown up to Four generation to check the stability of *reesei* mutants.

Estimation of chitinase enzyme

Estimation of chitinase enzyme in effective mutants was done by method suggested by Kulkarni and Ramanujam (2015). The *Trichoderma reesei* mutants were grown on synthetic media (Czapek's broth) along with crab shell chitin (50 ml in 250 ml flask). After inoculating with $1x10^7$ conidia / ml, these flasks were kept on rotary shaker at 140 rpm at 25^0C 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.

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^oC. 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 phosphate buffer (pH 6.7)

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

Di Potassium hydrphosphate (K_2HPO_4) 1 M, 174 gm was dissolved in 1000 ml of distilled water. Both were mixed together and diluted up to required concentration (50 mM) and the pH must be maintained at 6.7.

Estimation of protein

To estimate the protein concentration Lowry's method was followed (Lowry and Bessey, 1951).
The *Trichoderma* isolate was 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 sterilized distilled water and blot-dried. The mycelia mat was crushed in sterilized, pre-chilled pestle and mortar into a fine powder using liquid nitrogen, estimated protein quantity was estimated from mycelia extract. One ml of aliquot was taken in centrifuge tube to which 1 ml of 10% Tricholoro acetic 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 was used for protein estimation.

Reagents for protein estimation

A. 2% Sodium carbonate (Na_2CO_3) in 0.1 N NaOH

B. 1% Sodium Potassium Tartrate in H_2O

C. 0.5% $CuSO_4.5\ H_2O$ in H_2O (Copper sulphate solution)

D. Reagent I: 48 ml of A + 1 ml of B + 1 ml C (Alkaline Copper Sulphate Reagent)

E. Reagent II- 1 part Folin-Phenol [2 N]: 1 part water

BSA Standard - 1 mg/ml

Procedure for protein estimation

1. Different dilutions of Bovem serum albumin solutions are prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube. The final volume in each of the test tubes is 1 ml. The BSA range is between 0.2 to 1 mg/ ml.

2. The test tube with 1 ml distilled water serve as blank.
3. Add 4.5 ml of alkaline copper sulphate reagent (analytical reagent). Mix the solutions well. This solution is incubated at room temperature for 10 min.

4. Then add 0.5 ml of reagent Folin Ciocalteau solution (reagent solutions) to each tube and incubate for 30 min.

5. Take the optical density (measure the absorbance) at 660 nm. Plot the absorbance against protein concentration to get a standard calibration curve.

6. Check the absorbance of unknown sample and Estimate the amount of protein present in the given sample from the standard graph.

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 by using spectrophotometer after dilution with 5 ml distilled water.

Results and Discussion

Morphological study of *Trichoderma reesei* mutants

TrME-1

The Morphological characters of mutant TrME-1 was observed and found that the growth pattern of mutants TrME-1 was subarial and dispersed on PDA medium and formed a concentric rings.

The colony colour at initial stage was light green and later it turn to Dark green with the dark yellow colour pigmentation. The

phialides were found grouped opposite. Whereas conidiophores were irregularly paired and subglobose roughend conidiation. The shape of conidia were globose to ovoid but moderate sporulation with 2.15 × 10^8 conidia/ml solution was observed (Table 1).

TrME-2

From the data presented in Table 1, it is observed that the growth pattern of mutantsTrME-2 was also subarial and dispersed and formed a concentric rings on PDA medium. The colony colour also found initially at light green colour and later it turn to Dark green, with the dark yellow pigmentation. The phialids were found different as having simple branched. The conidia were found in globose to ellipsoidal shape with the moderate to heavy sporulation as 3.15 × 10^8 conidia /ml solution.

TrME-3

The colony growth type of mutant TrME 3 was subarial to circular with the formation of concentric rings on the PDA medium. The colony colour of this mutants was found milky white initially and later it turns to light green with dark yellow colour pigmentation. The phialides were found grouped and simple structure where as the conidia were ellipsoidal in shape with the moderate sporulation i.e.2.5 × 10^8 conidia/ml of solution (Table 1).

TrME-4

The mutants TrME-4 was also observed and the data present in Table 1 is revealied that the growth of mutant on PDA media is subarial dispersed along with the formation of concentric rings. Initially milky white colour colony observed which later turns to light green in colour with dark yellow colour pigmentation. The philids were found branched and the conidia were found ellisodal in shape. The sporulation of this mutant was also found High i.e 4.46 × 10^8 conidia /ml solution.

TrME-5

The morphological charecters of mother culture TrME-5 was also observed and found that the growth type of culture was totally different from all the mutants, flat and superficial growth of *T. reesei* mother culture was observed the colony colour of mother culture was initially milky white in colour which was later turn to light green colour with dark yellow colour pigmentation.

The phialides were found branched in mother culture where as the difference in phiallids was observed in different mutations. Similarly the different mutants were observed in different shape and sporulation as compared to mother culture.The conidia of mother culture were globose to ellipsoidal with high moderation was observed the sporulation was counted 4.64 × 10^8 conidia/ml of solution of mother culture of *T. reesei* (Table 1).

The *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).

Selvakumar *et al.,* (2000) also showed stable colony characters of *T. reesei* in UV light and EMS induced mutants which differs from a parent strain by method suggested by Kulakarni and Ramanujam (2015) and the data was presented in Table 2 from the data it was clearly stated that the mutant TrME-1 Contain maximum ie 0.3201 Chitinase enzyme units/mg of protein which was followed by the mutants TrME-4 recorded 0.3108 chitinase enzyme units/mg of protein.

Table.1 Morphological characteristic of *Trichoderma reesei*

Sr. no	*Trichoderma reesei* mutants	Morphological characters							
		Colony diameter/ Radial mycelial growth (mm) at 7 DAI	Colony growth type	Colony colour	Pigmentation	Phialides	Conidia shape	Sporulation	Spore count (1 ml) of solution
1	TrME -1	90.00	Subarial and dispersed	Light green to dark green	Dark Yellow	Grouped opposite	Globose to obvoid	++	2.15 × 10^8 conidia
2	TrME-2	88.50	Subarial and dispersed	Light green to Darkgreen	Dark Yellow	Branched	globose to ellipsoidal	++	3.15 × 10^8 conidia
3	TrME-3	90.00	Subarial and dispersed	Milky white to light green	Dark Yellow	Grouped simple	Ellipsoidal	++	2.5 × 10^8 conidia
4	TrME-4	89.00	Subarial and dispersed	Milky white to light green	Dark Yellow	Branched	Ellipsoidal	+++	4.46 × 10^8 conidia
5	TrME-5	90.00	flat and superficial	Milky white to light green	Dark Yellow	Branched	globose to ellipsoidal	+++	4.64 × 10^8 conidia

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

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

Sr.No.	Treatment	Code	Gamma radiation dose (Kr)	Mean Chitinase enzyme units/ mg of protein
1	T1	TrME 1	50	0.3201
2	T2	TrME2	100	0.2659
3	T3	TrME3	150	0.2908
4	T4	TrME4	200	0.3108
5	T5	TrME5	Control	0.2898
	'F' test			Sig.
	SE(m)±			0.0076
	CD (P=0.01)			0.0122

*Chitinase enzyme estimate value on spectrophotometer at 510 nm wavelength

Plate 2a 50Kr final generation (Fourth)

Plate 2b 100 Kr final generation (Fourth)

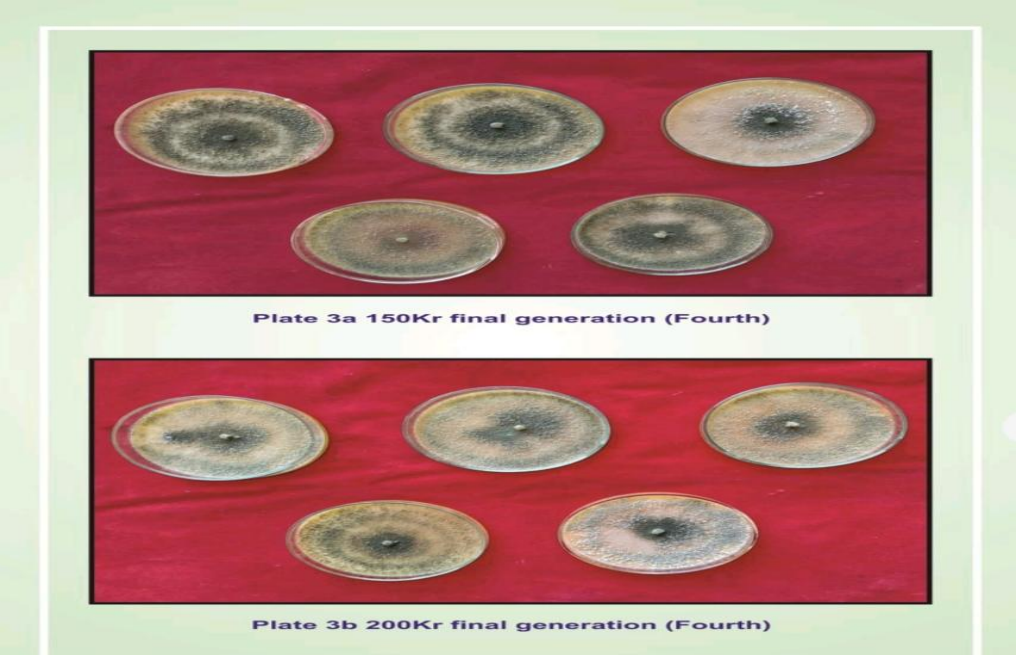

Plate 3a 150Kr final generation (Fourth)

Plate 3b 200Kr final generation (Fourth)

These Two mutant (TrME-1 & TrME-4) were found significantly superior to produced maximum Chitinase enzyme (0.3201 and 0.3108 units/mg of protein) over control (TrME-5) means the motherculture of *Trichoderma reesei* (0.2898 chitinase enzyme units/mg of protein). The minimum chitinase enzyme units mg of protein was estimated in the mutant TrME-2 i.e**,** 0.2659 followed by TrME-5 i.e, 0.2898 and TrME-3 i.e, 0.2908 chitinase enzyme units/mg of protein. This indicate that the mutants TrME-1 and TrME-4 are the best mutants for the use effectively as a mycoparasite in the biocontrol management

of plant pathogenic fungi as compare to the mother culture of *T. reesei* (TrME-5).This indicates that the treatmentsTrME-1and TrME-4 are the best mutants for use as effective mycoparasite in the biocontrol of plant pathogenic fungi.

Haggaget and Mohemad (2002) carried out mutagenesis of *Trichoderma harzianum* and *T. koningii* with 50 and 75 kr doses of gamma which result four mutants of each *T. harzianum* and *T. reesei* 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 *et al.*, (2002) mutagenesis of three *Trichoderma* spp. With 20 and 40 kr doses of gamma and reported four mutants of each spp. Including *T. harzianum, T. viride* and *T. reesei* were obtained by gamma irradiation to enhance their biocontrol abilities against *Sclerotium cepivorum* and their production of antifungal metabolites. Mutant species of exhibited high capabilities to produce many efficient enzymes and other metabolites with better control of onion white rot disease.

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