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TRICHODERMA ATROVIRIDE (T14) DERIVED PUTATIVE MUTANTS WITH ENHANCED CHITINASES ABILITY AGAINST *RHIZOCTONIA SOLANI*

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ABSTRACT

Filamentous fungi of the genus *Trichoderma* are renowned for their opportunistic nature, exhibiting a wide array of interactions with plants, including the ability to combat plant-pathogenic fungi, promote plant growth, and enhance plant defence responses. Gamma-ray irradiation of wild-type *Trichoderma atroviride* (T14) led to the development of various mutant strains with enhanced genetic diversity, which are promising candidates for strain improvement. Through biochemical screening and in vitro confrontation assays, we identified mutants that exhibit superior chitinase production and enhanced mycoparasitic activity against *R. solani*. These findings suggest that gamma-ray-induced mutants of *T. atroviride* (T14) with increased chitinase production and antagonistic capabilities hold significant potential for use as effective biological control agents against various pathogenic fungi.
Keywords **:** *Trichoderma*, strain improvement, mutation, mycoparasitism

Introduction

Trichoderma, a genus of fungi in the Hypocreaceae family, is ubiquitous in soil and thrives in a variety of natural and artificial environments, demonstrating remarkable adaptability (Druzhinina *et al*., 2011, 2012). This genus, comprising species of Ascomycetes in the Hypocreales order, holds significant economic value due to its production of enzymes, antibiotics, plant growth promoters, and xenobiotic degraders, and is particularly noted for its role as a commercial bio-fungicide (Mukherjee, 1999). *Trichoderma* species excel as colonizers and competitors in their habitats, leveraging their enzymatic machinery to decompose diverse substrates, including lignocellulosic biomass and fungal cell wall chitin. As endophytes, they colonize crop roots effectively, enhancing plant growth through increased root density, nutrient uptake, mineral ion solubilization, and induced defence responses against various stresses (Harman, 1992; Mastouri *et al*., 2010). Mutation via gamma irradiation is a well-established genetic manipulation technique for enhancing antagonistic and plant growth-promoting activities. Gamma rays can induce genetic diversity and mutations in filamentous fungi, leading to improved metabolic activities such as the secretion of extracellular cell wall-degrading enzymes and antibiotics, and enhanced mycoparasitic abilities (Kumakura *et al*., 1984; Bailey and Tahtiharju, 2003; Jiang *et al*., 2011; Li *et al*., 2010; Zaldivar *et al*., 2001). Notably, *Trichoderma atroviride* (T14) has shown a positive correlation between plant growth promotion and metabolite production (Kotasthane *et al*., 2014). Given *T. atroviride* (T14)'s chitinase and cellulase activities, and its hormonal-like effects, we propose its genetic improvement through gamma radiation. We aim to utilize the superior putative mutants of *T. atroviride* (T14) as effective biocontrol agents against phytopathogens and explore their commercial potential.

Material and Methods

Wild type isolate *T. atroviride* (T-14): Earlier identified as potential isolate (T-14) of *T. atroviride* with ability to produce large number of secondary metabolites, mycoparasitism (antagonism of class 1 against *R. solani* and *S. rolfsii*) ability to induce plant growth promoting ability (effects similar to hormonal application measurable in terms of high chlorophyll

content, indole acetic acid production, phosphate solubilisation and siderophore production).

Gamma irradiation and single spore isolation: Sporulating cultures of wild type isolate of *T. atroviride* (T-14) (on potato dextrose agar slants) were exposed to gamma radiation at 1000 and 1250 Gray at BARC, Trombay, Mumbai. Single spore putative mutants were derived and were designated as M-# ("M" = putative mutant, "#" = no. of putative mutant) from irradiated culture and multiplied on PDA slants (Kotasthane & Agrawal, 2010).

Bipartite interactions were performed following a simple confrontation assay technique to identify prospective bio-agent. Nine putative mutants of *Trichoderma atroviride* (T 14) were evaluated for their antagonistic effect on the mycelium growth of *R. solani* under *in vitro* condition.

For dual culture 5-mm diameter mycelial block (derived from 7-days-old culture of each *Trichoderma* putative mutants and of *R. solani*) were placed on the opposite side on pre sterilized and cooled PDA surface under aseptic conditions and care was taken to avoid contamination. Petri dishes containing dual cultures were incubated at 27±1°C under continuous light and were observed at regular intervals until mycelia growth of the oppositely paired cultures merged. The experiment was conducted in two replications. Mycoparasitic activity of the respective *Trichoderma* putative mutants was recorded using the standard scale representing growth of *Trichoderma* and pathogen.

Scoring for degree of antagonism was done as per Bell *et al.,* 1980 was on a scale of classes 1-5: Class 1= *Trichoderma* completely overgrew the pathogen and covered the entire medium surface, Class 2= *Trichoderma* overgrew at least two-third of the medium surface, class 3 = *Trichoderma* and the pathogen each colonized approximately one-half of the medium surface (more than one third and less than two-third) and neither organism appeared to dominate the other, class 4 = The pathogen colonized at least two-third of the medium surface and appeared to withstand encroachment by *Trichoderma*, and class 5 = the pathogen completely overgrew the *Trichoderma* and occupied the entire medium surface.

Paired cultures were observed for a total of 9 days before being discarded.

The % growth inhibition of the test fungus was calculated using the formula:

$$\text{Per cent growth inhibition} = \frac{R_1 - R_2}{R_1} \times 200$$

Where,

R_1= Radial growth of test fungus in control plate

R_2= Radial growth of test fungus in presence of antagonist

Chitinase activity

Chitinase activity was detected following the technique developed by Agrawal and Kotasthane (2012). Chitinase detection medium consisted of a basal medium comprising 0.3 g of $MgSO_4.7H_2O$, 3.0 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, 1.0 g of citric acid monohydrate, 15 g of agar, 200 µl of Tween-80, 4.5g of colloidal chitin and 0.15 g of bromocresol purple per litre; pH 4.7. Fresh culture plugs of the isolates to be tested for chitinase activity were inoculated into the medium in petri plates and incubated at 25±2 °C. Chitinase activity was identified as formation of purple coloured zone in the inoculated medium. Observations were recorded for the colour intensity and diameter of the purple-coloured zone until 3rd day after inoculation.

Results

Confrontation assay

Bipartite interactions were performed following a simple confrontation assay technique to identify prospective bio-agent. Nine putative mutants of *Trichoderma atrovide* (T 14) were evaluated for their antagonistic effect on the mycelium growth of *R. solani* under *in vitro* condition.

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the pathogen completely overgrew the *Trichoderma* and occupied the entire medium surface.

Paired cultures were observed for a total of 9 days before being discarded. We considered an isolate of *Trichoderma* to be antagonistic to the pathogen if the mean score for a given comparison was ≤ 2, but not highly antagonistic if the number was ≥3. Selected cultures from pairings of *Trichoderma* x *R. solani* resulting in different antagonism classes were viewed microscopically to determine the approximate state of the pathogen thalli after 9 days. This system appeared to be adequate only for determining the viability of *R. solani*.

There was difference in the antagonistic abilities of *Trichoderma* isolates against *R. solani. Trichoderma atroviride* isolates M18, M23, M46, M88, M136, M144 expressed a high level of antagonism (score 2,3) whereas rest of the putative mutants (M143, M151 and M152) could not express apparent antagonism (score 4) against *R. solani* (Table: 1, Fig: 1). However, the area occupied by *Trichoderma* is appearing less in media plate but it overgrew on the colony of *R. solani* (Fig. 1).

Table 1: Bipartite interaction of putative mutants of *T. atroviride* (T14) with *Rhizoctonia solani* following confrontation assay technique

Confrontation assay			
Treatment	**Isolates**	**% inhibition**	**Score***
T1	Control	00	NA
T2	M18	61.01^{a}±0.75	2
T3	M23	45.94^{b}±0.95	3
T4	M46	41.15^{c}±0.64	3
T5	M88	45.62^{b}±0.64	3
T6	M136	44.98^{b}±1.27	3
T7	M143	35.92^{d}±0.67	4
T8	M144	43.71^{bc}±0.64	3
T9	M151	35.24^{d}±1.35	4
T10	M152	36.58^{d}±1.33	4
C.D.		2.92	
SE(m)		0.915	
SE(d)		1.294	
C.V.		3.315	

*Score as per Bell *et al.,* 1980

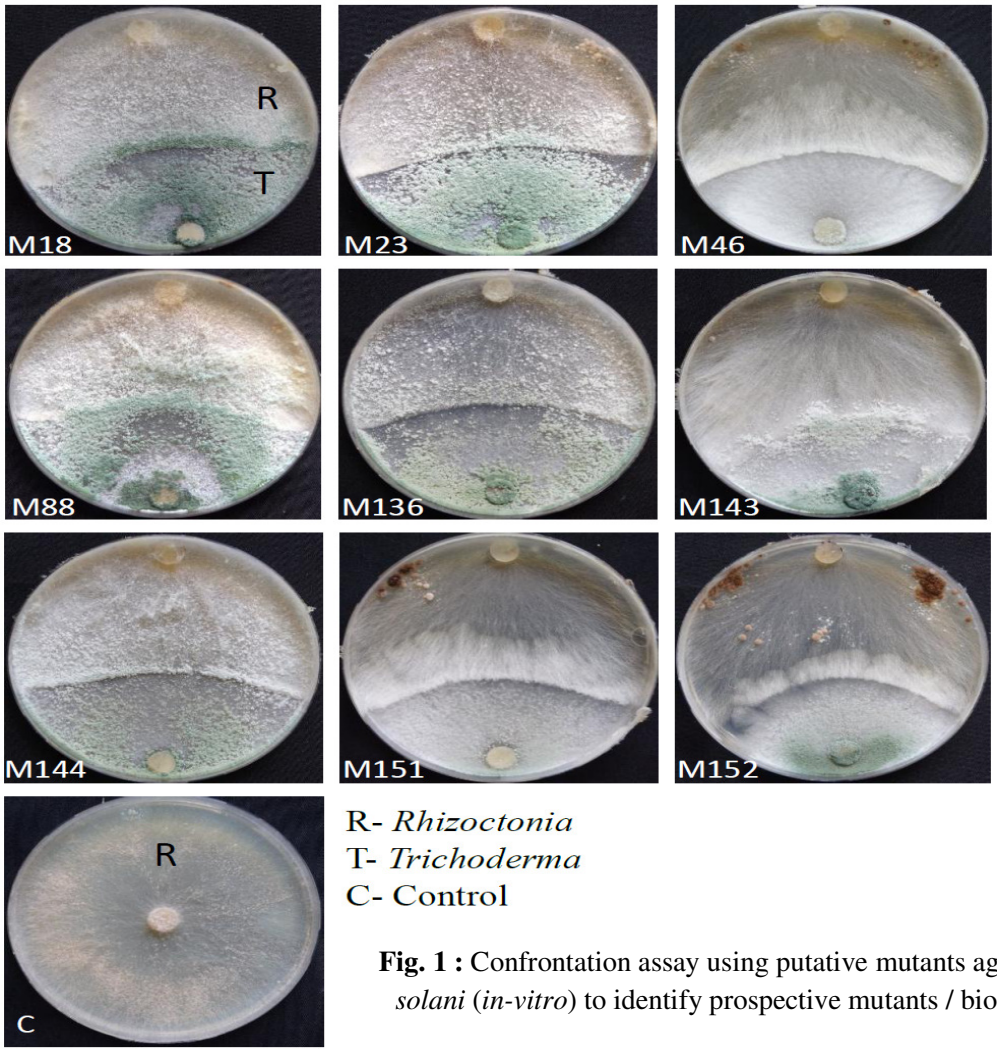

Fig. 1 : Confrontation assay using putative mutants against *R. solani* (*in-vitro*) to identify prospective mutants / bio-agent

Characterization of the isolates for chitinase activity

Using basal medium with colloidal chitin (*Rhizoctonia solani* cell wall derived Colloidal chitin and commercial chitin) as sole carbon source supplemented with Bromo cresol purple (pH indicator dye) was used to evaluate putative mutant populations of *Trichoderma* for chitinase activity. Colloidal chitin media containing bromocresol purple (pH 4.7) when inoculated with chitinolytic *Trichoderma*, resulted in breakdown of chitin into N- acetyl glucosamine causing a corresponding shift in pH towards alkalinity and change of color of pH indicator dye (BCP) from yellow to purple zone surrounding the inoculated fresh culture plugs in the region of chitin utilization. Chitinase activity exhibited by nine putative mutant isolates of *Trichoderma* was determined by the diameter of the purple colored zone after 3 days of incubation in the colloidal chitin supplemented agar medium.

Putative mutant progenies revealed wide variation in quantized amount of growth of isolates which ranged from 8.5 to 33 mm. M88 (33 mm radius) expressed significantly high amount of chitinase in media supplemented with commercial chitin derived colloidal chitin, followed by M144 (29 mm radius) (Table 2). Similarly, chitinase activity on *Rhizoctonia solani* cell wall derived colloidal chitin of the putative mutant isolate revealed wide variation in quantized amount of growth and of isolates which ranged from 7 to 32 mm. M136 (32 mm) expressed significantly high amount of chitinase in media supplemented with *Rhizoctonia solani* cell wall derived colloidal chitin followed by M143 and M144 (28 mm).

Table 2: Chitin hydrolysis activity of derived putative mutant isolates in colloidal chitin (prepared from Commercial chitin) amended media

Analysis for Chitinase (Commercial chitin)		
Treatment	**Isolates**	**diameter of the purple color zone (mm)**
T1	M18	25.25^{c}±0.5
T2	M23	28^{b}±1
T3	M46	8.5^{g}±0.5
T4	M88	11^{f}±0.5
T5	M136	33^{a}±0.5
T6	M143	20^{d}±0.5
T7	M144	29^{b}±0.5
T8	M151	13^{f}±1
T9	M152	16^{e}±1
	C.D.	2.294
	SE(m)	0.707
	SE(d)	1
	C.V.	4.898

Table 3: Chitin hydrolysis activity of derived putative mutant isolates in colloidal chitin (prepared from chitin isolated from cell wall of *Rhizoctonia*) amended media

Analysis for Chitinase (chitin isolated from cell wall of *Rhizoctonia*)		
Treatment	**Isolates**	**diameter of the purple color zone (mm)**
T1	M18	23^{c}±1
T2	M23	12^{f}±0.5
T3	M46	7^{g}±0
T4	M88	18^{de}±1
T5	M136	32^{a}±1
T6	M143	28^{b}±1
T7	M144	28^{b}±1
T8	M151	16^{e}±1
T9	M152	20^{d}±0.5
	C.D.	2.96
	SE(m)	0.894
	SE(d)	1.264
	C.V.	6.182

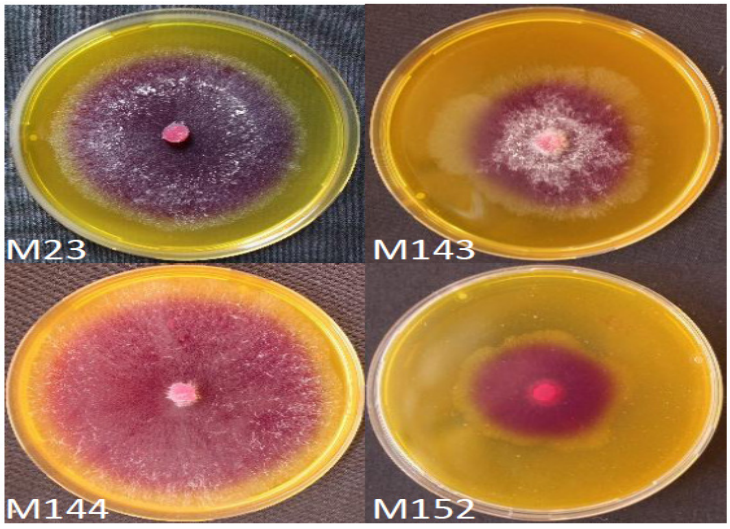

Fig. 2 : Chitin hydrolysis activity of derived putative mutant isolates in colloidal chitin (prepared from Commercial chitin) amended media

Discussion

Inducing random mutations through ionizing and non-ionizing radiation, or chemical mutagens like acridine orange and ethyl methane sulfonate, has proven to be an effective tool for strain improvement (Griffiths *et al*., 2000). In our study, we subjected the wild-type *T. atroviride* (T14) culture to gamma irradiation at doses of 1000 and 1250 Gy. This approach, alongside previous research, has shown promise in identifying potential biocontrol agents, such as fluorescent Pseudomonas and Trichoderma spp., against various plant pathogens, including soil-borne ones like *R. solani* and *S. rolfsii*, through confrontation assays in petri plates (Kotasthane *et al*., 2017; Sharma *et al*., 2012; Toppo and Tiwari, 2015; Yadav *et al*., 2018). The use of colloidal chitin as a sole carbon source, indicated by color changes in the media, helped us identify chitinase-positive superior mutants. We identified four superior chitinase producers on media supplemented with both *Rhizoctonia solani* cell wall-derived colloidal chitin and commercial chitin-derived colloidal chitin. Evidence suggests that random mutations enhance the efficiency of *Trichoderma* in producing chitinase, β-1,3-glucanase, and β-1,4-endoglucanase (Dutta and Chatterji, 2004; Patil, 2012). Various strategies, including salt stress (Mohamed and Haggag, 2006), pesticide resistance (Hatvani *et al*., 2006), and chemical mutations (Shafique *et al*., 2010), have been used to improve Trichoderma spp. efficiency. Gamma irradiation has been particularly effective in creating numerous mutants of *Trichoderma* that are efficient against soil-borne plant pathogens by increasing their extracellular cell wall-degrading enzyme content (Abomohra *et al*., 2016; Moussa and Rizk, 2003). Chitinase enzymes are of particular interest because chitinolytic strains of *Trichoderma* are among the most effective biological control agents for plant diseases (Harman *et al*., 1993; Samuels, 1996; Spiegel & Chet, 1998; Kubicek *et al*., 2001; Viterbo *et al*., 2002; Benitez *et al*., 2004; Navazio *et al*., 2007; Goswami *et al*., 2008; Vinale *et al*., 2009; Karlsson *et al*., 2010). Previous studies by Agrawal and Kotasthane (2012) indicated variable preferences for media supplemented with commercial chitin (Cc) (Himedia) and *Rhizoctonia* cell wall (RCW) derived colloidal chitin. Sivan and Chet (1989) found that the production of extracellular β-glucanases, chitinases (Schuster and Schmoll, 2010; Agrawal & Kotasthane, 2009), and proteinases significantly increases when *Trichoderma* spp. are grown on media supplemented with autoclaved mycelium or purified host fungal cell walls. Other studies have used various substrates, such as p-nitro phenyl keto oligomers, differentially purified chitins, or fungal cell walls, to assay, purify, and characterize chitinolytic enzymes from Trichoderma (Harman *et al*., 1993; Lorito *et al*., 1994; Ulhoa & Peberdy, 1993; Schickler *et al*., 1998).

Conclusion

We successfully demonstrated that gamma irradiation-induced gene mutations can produce numerous beneficial mutants. In this study, we identified superior putative mutants derived from gamma-irradiated *T. atroviride* (T14) that exhibit enhanced chitinase and mycoparasitic activities. The identification of these superior mutants suggests that gamma irradiation holds significant potential for improving the *T. atroviride* (T14) strain. These superior mutants can be utilized as plant growth promoters, incorporated into disease management strategies, and exploited for commercial production, offering valuable benefits for farmers.

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Conflicts of Interest: Authors declared no conflict of interest.

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