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Improvement of bio-control potential of *Trichoderma asperellum* through mutagenesis

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Abstract
This study aimed to use chemical mutagenesis and irradiation to improve the genetics of *Trichoderma asperellum* in order to improve its biocontrol efficiency. *T. asperellum* mother culture was chemically mutated with ethyl methyl sulphonate (EMS) and hydroxyl amine (HA) at 200 µl/ml and physically mutated with gamma radiation (cobalt 60) at 250 gry and ultraviolet (UV) rays at 254 nm wavelength. The duration of all treatments was 30, 45, 60 and 75 min. After mutagenesis, 16 mutants were tested for their stability up to seven generations. All mutants and parent cultures of *T. asperellum* were distinguished by cultural and morphological features. The mutants showed strong antagonistic activities against the tested soil pathogens. The highest rate of growth inhibition of *Sclerotium rolfsii* was recorded in the TaH2 (T_2) mutant, i.e., 88.89%. While *Fusarium oxysporum* f.sp. *ciceri* and *Rhizoctonia bataticola*, the highest growth inhibition rates were recorded in the TaU3 (T_{11}) and TaG1 (T_{13}) mutants, 62.82 and 75.38%, respectively. All mutants recorded higher chitinase enzyme compared with culture samples. The TaH2 (T_2) mutant recorded the highest level with 0.98 units/mg protein of chitinase enzyme activity, while the parent culture TaMc (T_{17}) contained only 0.62 units/mg protein of chitinase enzyme activity.

Keywords: Bio-control potential, *Trichoderma asperellum*, mutagenesis

Introduction
Climate change and food security are two of the world's most pressing issues today. Drought, pests, diseases, reduced land availability, and population growth all have an impact on traditional agriculture. Pests and diseases are among the most damaging. Every year, yield losses of crores of money is in vain due to the damage caused by pests and diseases. The pesticides industry in India is expanding rapidly with a compounded growth rate of approximately 15% per annum. Plant diseases alone account for more than 10% of global food production losses. Plant pathogens causes huge losses to growers by causing devastating effects on crops, thereby effecting their yield upto 50%. Fungal pathogens were responsible for 70-80% of these losses. Fungal pathogens cause infection which lowers product quality due to the presence of toxic metabolites (Alderman *et al*., 1996) [1]. Fungi of the genus *Trichoderma* can be found in all climate zones. These fungi are mostly found in soil and rotting wood (Samuels *et al*., 1996; Druzhinina *et al*., 2012) [27, 4]). *Trichoderma* can be observed on sclerotia and other fungi that propagate in the soil. It colonises plant grain, leaves, and roots (Harman *et al*. 2004) [8].

Trichoderma is distinguished by its fast growth, copious spore production, and ability to produce sclerotia. Various pigments like green, yellow to a reddish tinge, grows quickly on a variety of substrates, acts as a mycoparasite, competes for food and site, produces antibiotics, and can attack a wide range of plant pathogens. *Trichoderma* shows antagonism by penetrating the host fungal cell wall through secreting lytic enzymes such as chitinase and glucanase. While other bioagents work by attacking the host fungal hyphae through hooks, coiling, and other structures like appressorium. The genus *Trichoderma* contains approximately 41 species. Diversity is seen across species and within species which reflects their variation in biocontrol potential as well (Goes *et al*. 2002) [6]. Likely wild strains of *Trichoderma* have established a significant bio-control of plant pathogens. Continuous efforts have paved the way to develop and release of more effective *Trichoderma* strains by tapping the potential of mutagenesis (Kredics *et al*., 2000; Papavizas *et al*., 1982; Mandal *et al*., 1999; Zaldivar *et al*., 2001) [9, 20, 13, 30].

Massive mutational programmes have been undertaken to boost productivity. New variants formed were subjected to screening and selection to detect the increased biocontrol efficacy.

A typical enhancement of strain is achieved by subjecting the microorganisms to mutation that produces the enzyme using techniques such as classical mutagenesis, which involves exposing the microbes to nitro-glycerine and physical mutagens such as X-rays, -rays, UV-rays. Induction of mutations in fungi were performed to improve their metabolite production, biocontrol activities, and inactivation of airborne fungal spores (Zaldivar *et al*., 2001) [30].
Therefore, this study was performed with an aim to produce stable improved biocontrol strains of *Trichoderma asperellum.*

Materials and Methods

Physical mutagenesis

Gamma irradiation induced mutagenesis

Haggag *et al*. (2002) [7] method was followed for mutagenesis by gamma irradiation. Irradiation facility was used RTM Nagpur University. *T. asperellum* mother cultures were grown in PDA Petri dishes for 7 days and the cultures sporulated from 7th day. These cultures with sporulation of *T. asperellum* were subjected to irradiation with ^{60}Co dose of 250 gry for 30, 45, 60, and 75 min and incubated for one week at 25 °C. The irradiated and non-irradiated (control) petri dishes were added with 10 ml of sterile saline solution @ 0.85% NaCl. The conidia were harvested and separated using needle which were then aseptically followed single spore isolation on PDA. Three days later these single spore colonies were sub cultured onto PDA.

UV Radiation induced mutagenesis

The conidial suspension was made using a week days old mother culture of *T. asperellum* in 10 ml of autoclaved distilled water. Centrifugation was done twice to separate the mycelium from spores at 10,000×g for five min. A pellet was formed in the suspension which was washed twice with 0.067 M phosphate buffer (pH 7.0). The spore density was adjusted to 10^5 conidia per ml with sterile water using a haemocytometer. 1 ml of conidial suspension transferred to a sterile 2 ml Eppendorf tube. The Eppendorf tube was agitated with a magnetic stirrer for 5 min to break conidial chains. 1 ml of this conidial suspension was pipetted onto sterile petri dishes with PDA. Treatment of UV light of 15 W, 25 nm was done to lidless plates for 30, 45, 60, and 75 min with a distance of 30 cm gap between fungal surface and UV lamp. Untreated plates containing conidia were kept as controls. Both treated and untreated plates were put in incubation at 28 °C for three days. The single spore colonies developed were transferred to PDA.

Chemical Mutagenesis

Chemical mutagenesis was performed by treating the spore suspension of 1×10^5 conidia per ml of *T. asperellum* with hydroxylamine (HA) and ethyl methyl sulfonate (EMS) @ 200 µL/mL for 30, 45, 60, and 75 min separately on an orbital shaker (Durand *et al*., 1988); Chandra *et al*., 2010) [5, 2]. The control was the treatment with sterile distilled water. Treated and untreated spore suspensions were washed thrice with autoclaved distilled water by centrifugation at 5000×g for 10 min. to remove trace chemicals. 1 ml of this conidial suspension was pipetted onto sterile petri dishes with PDA and put for incubation at 28 °C for three days. The single spore colonies formed were transferred to PDA.

Stability confirmation

The growth of *T. asperellum* mother strain and mutants was stable up to the 7th generation and hence mutation stability was confirmed (Mech *et al*., 2006) [14].

Antagonism

The antagonism trait of *T. asperellum* mother cultures and mutants was exploited using dual culture inoculation method against *S. rolfsii*, *R. bataticola* and *F. oxysporum* f. sp. *ciceri* (Mandal *et al*., 1999) [13]. A mycelial disk of 5 mm from the actively growing margins of the pathogen was transferred aseptically to the centre of a 90 mm Petri dish containing the PDA. In Petri dishes, on the same day, 5 mm diameter hyphal discs were placed on the opposite edge of the plate equidistant from the perimeter from the edge of week days old cultures of *Trichoderma* and soil-borne pathogens with 3 replicates for each treatment. Plates were incubated at ambient conditions with alternating light-dark cycles for 7 days. At the same time, only pathogenic fungal discs (5 mm) were incubated on PDA petri plates and incubated under similar conditions for the same duration. Observing the plates daily, under a stereomicroscope recorded the-

$$\% \text{ Growth Inhibition} = \frac{C-T}{C} \times 100$$

where,
C = mycelial growth (mm) on control plate
T = mycelial growth in treated plates (mm)

-behaviour of mixing the two cultures. The growth of the test pathogen was measured on the 7th day after culturing, and the growth inhibition rate was calculated by the following formula.

Chitinase enzyme estimation

Kulkarni and Ramanujan (2010) [10] method was followed for Chitinase enzyme estimation in efficient mutants. *T. asperellum* mutants were cultivated in crab shell chitin (50 mL in a 250 mL flask) containing Czapek broth. Inoculation was done with a conidial suspension of 5 x 10^6/ml followed by shaking the flasks at 140 rpm, 25 °C for 5 days in a rotary shaker. Later, biomass was separated using a nylon cloth and obtained culture filtrate was dialyzed with the 50 mM buffer (potassium phosphate) in 6:1 ratio at 4 °C. overnight. Added sodium azide and stored.

Chitinase activity determination

Turbidity method

Endo-chitinase enzyme activity was determined by reducing the turbidity of colloidal chitin suspensions. Suspensions with colloidal chitin 1% (w/v) were made in potassium phosphate 50 mM buffer with a pH of 6.7. The combination of 0.5 ml chitin suspension and 0.5 ml of enzyme solution was prepared and incubated at 30 °C for one day. 5ml of this diluting mixture was fed to the OD readings at 510 nm. Activity of Enzyme was The measure of percentage of the 5% reduction in chitin suspension gives the enzyme activity.

Colloidal Chitin preparation

Roberts and Selintrenikoff (1988) [25] method was followed for colloidal chitin preparation. The slow addition of 5 grams

of chitin powder to 60 ml of concentrated HCl was performed. Later, vigorously overnight shaken at 4 °C, followed by stirring rapidly in two litres of ice-cold 95% ethanol at room temperature (25 °C) overnight. Collection of precipitate was done with 20 min of 5,000×g centrifugation at 4 °C followed by washing with autoclaved distilled water to get pH 7.0 for the colloidal chitin. Preparation of 5% colloidal chitin solution was done and kept in 4 °C for storage.

Phosphate buffer preparation (pH 6.7)

One litre of distilled water was used as a solvent to dissolve the 136 grams of potassium dihydrogen phosphate (1M of KH_2PO_4) was dissolved in. 174 g of potassium hypophosphate (1M of K_2HPO_4) was dissolved in one litre of distilled water. Mixing of both solutions together to get diluted concentration of 50 mM and also a pH of 6.7.

Standard Graphs preparation

Glucose source of dextrose grade 'AR' was used to make the standard graph. Solutions of Glucose standard with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0% were prepared. Mixture of 0.5 ml of chitin suspension to each solution of standard in a test tube was done and incubated at 30 °C for two hours. After dilution with 5 ml of distilled water, OD reading at 510 nm was recorded using a Systronics spectrophotometer.

Results and Discussion

Development of *T. asperellum* mutants by mutagenesis for improving the strain in terms of effectiveness and reliability for biological control (Table 1; Fig 1; Fig 2). The Microscopic view of *T. asperellum* mutants was taken at 40X magnification (Fig 3). Efficacy of mutants was tested using dual culture technique against suppressing the soil-borne fungal plant pathogens.

Table 1: Mutagenesis of *T. asperellum* induced by different methods and their description

Mutants of *T. T. aperellum*	Method of Mutagenesis	Dosage	Treatment duration
TaH_1	Hydroxyl Amine (HA)	200 µl/ml	30 min
TaH_2	Hydroxyl Amine (HA)	200 µl/ml	45 min
TaH_3	Hydroxyl Amine (HA)	200 µl/ml	60 min
TaH_4	Hydroxyl Amine (HA)	200 µl/ml	75 min
TaE_1	Ethyl Methyl Sulphonate (EMS)	200 µl/ml	30 min
TaE_2	Ethyl Methyl Sulphonate (EMS)	200 µl/ml	45 min
TaE_3	Ethyl Methyl Sulphonate (EMS)	200 µl/ml	60 min
TaE_4	Ethyl Methyl Sulphonate (EMS)	200 µl/ml	75 min
TaU_1	15W ultraviolet light	254 nm	30 min
TaU_2	15W ultraviolet light	254 nm	45 min
TaU_3	15W ultraviolet light	254 nm	60 min
TaU_4	15W ultraviolet light	254 nm	75 min
TaG_1	Cobalt-60 (^{60}Co)	250 gry	30 min
TaG_2	Cobalt-60 (^{60}Co)	250 gry	45 min
TaG_3	Cobalt-60 (^{60}Co)	250 gry	60 min
TaG_4	Cobalt-60 (^{60}Co)	250 gry	75 min
TaMc	Mother culture of *T. asperellum*		

Fig 1.1: Chemical mutagenesis of *T. asperellum* with hydroxyl amine (HA)

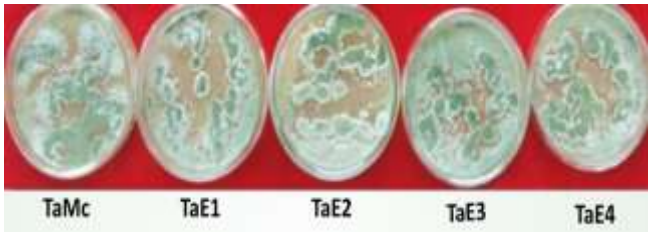

Fig 1.2: Chemical mutagenesis of *T. asperellum* with ethyl methyl sulphonate (EMS)

Fig 1.3: Physical mutagenesis of *T. asperellum* with U.V. radiation

Fig 1.4: Physical mutagenesis of *T. asperellum* with gamma radiation

Fig 1 Different mutagenesis treatments of *T. asperellum*

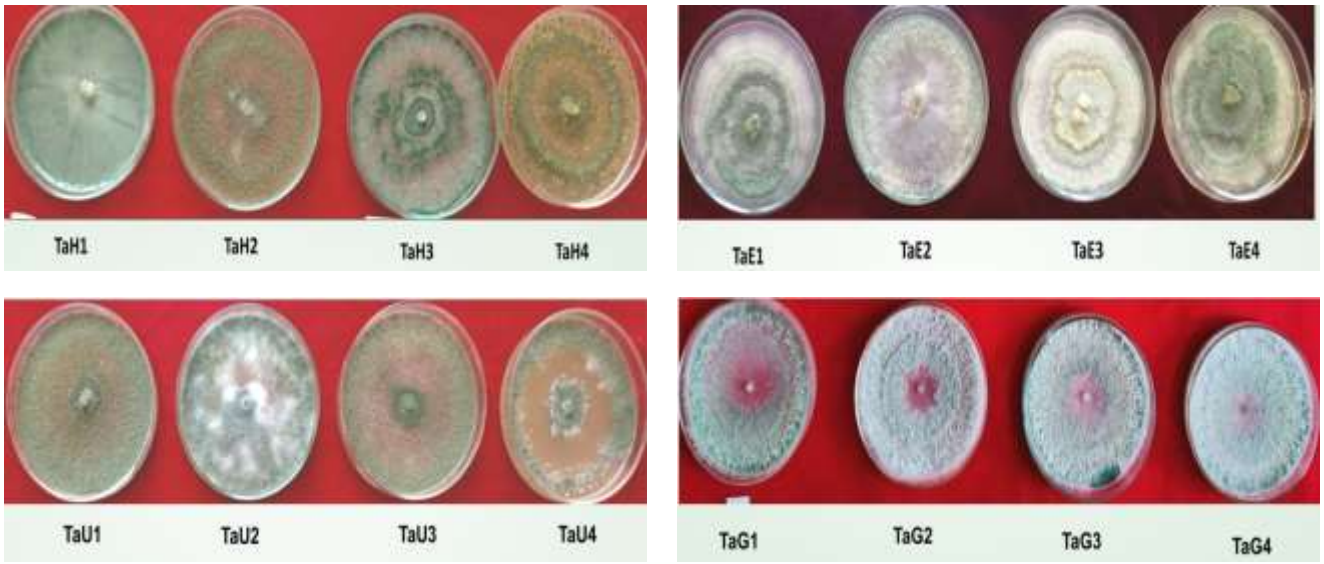

Fig 2: *T. asperellum* mutants on PDA medium (7th generation)

All treatments were significant in inhibiting *S. rolfsii* radial mycelial growth (Table 2). The mutant TaH2 (T_2) showed the greatest inhibition of mycelium growth, at 88.89% (Fig 4). TaG2 (T_{13}), TaH4 (T_4), and TaG4 (T_{16}) were the next most effective mutants, with 83.33%, 77.78%, and 75% growth inhibition, respectively. Mother culture TaMc (T_{17}) had the lowest growth inhibition of *S. rolfsii*, at 33.33%. All treatments were effective in inhibiting *R. bataticola* radial mycelial growth (Fig 5). TaU3 (T_{11}) inhibited mycelium growth the most, at 62.82%. TaE1 (T_5), TaU2 (T_{10}), and TaG2 (T_{14}) were the next most effective mutants, with 61.11%, 59.44%, and 58.89%, respectively. Mother culture TaMc (T_{17}) had the lowest growth inhibition of *R. bataticola*, at 42.1%. *T. asperellum* mutants and mother culture significantly inhibited the mycelium growth of *F. oxysporum f.sp. ciceri* (Fig 6). Maximum mycelium growth inhibition of *F. oxysporum* f.sp. *ciceri* was recorded as 75.38 % with TaG1 (T_{13}). TaG4 (T_{16}), TaH1 (T_1), and TaH4 (T_4) were the next most effective mutants, with 73.98%, 73.05%, and 71.29%, respectively. The mutant TaE2 (T_6) recorded only 55.08% growth inhibition, while the mother culture TaMc (T_{17}) recorded the lowest, 47.15% growth inhibition.

70–90% *in vitro* hyphal inhibition of *S. rolfsii* was reported with *T. viride* mutants (Papavizas, 1985; Rajappan *et al.*, 1997; Mohammed and Haggag, 2006) [21, 24, 17]. Biocontrol agents *viz*., *T. viride*, *T. virens*, and *T. harzianum* showed the potential to parasitize *S. rolfsii* and inhibit growth under *in vitro* conditions (Kushwaha *et al*., 2018) [11]. UV treatment was used for the purpose of mutagenesis of *T. viride* to improve its biocontrol ability against *S. rolfsii* (Mohammed *et al*., 2010) [16]. Different time variables assisted UV treatment was useful in developing one mutant of *T. koningii* that exhibited the most antagonistic activity *in vitro* against *R. bataticola* that causes charcoal rot (Patil and Kamble, 2011) [23]. Dual culture technique was used to test the anti-fungal activity of mutants of *Trichoderma* species (Nakkeeran *et al*., 2005; Divya *et al*., 2015) [18, 3].

4 out of 30 mutants of *T. viride* produced from diverse sources of mutagens were recorded anti-fungal activity against *R. solani* (Nakkeeran *et al*., 2005) [18]. *Trichoderma* isolates displayed the greatest ability to limit the soil-borne pathogens growth *viz*., *Macrophomina phaseolina*, *R. bataticola*, and *S. rolfsii* (Divya *et al*., 2015) [3]. *In vitro* inoculation of the *T. viride* mutant showed the greatest mycelial inhibition of *F. oxysporum*, up to 90.3% (Saini *et al*., 2015) [26]. Antifungal volatile chemicals produced by *Trichoderma* species were recorded with the *in vitro* mycelial inhibition studies resulted in the sensitivity of *F. oxysporum*. *T. viride* was extremely suppressing *F. oxysporum* during *in vitro* dual culture hampering the latter's mycelium development (Padamodaya and Reddy, 1996) [19]. Two stable mutants were highly biologically effective against *F. oxysporum*, the causative agent of tomato wilt disease (Mohammed and Haggag, 2005) [15].

Table 2: Effectiveness of *T. asperellum* mutants and mother culture at 7 DAI against *S. rolfsii*, *R. bataticola*, *and F. oxysporum* f.sp. *ciceri*

Trichoderma asperellum mutants	Mean Radial Growth of *S. rolfsii.* (mm)	Percent Growth Inhibition (%)	Mean Radial Growth of *R. bataticola* (mm)	Percent Growth Inhibition (%)	Mean Radial Growth of *F. oxysporum f.sp. ciceri* (mm)	Percent Growth Inhibition (%)
TaH_1	43.47	51.70	38.07	57.70	23.00	**73.05**
TaH_2	10.00	**88.89**	40.00	55.56	30.00	64.84
TaH_3	42.33	52.96	45.00	50.00	36.50	57.22
TaH_4	20.00	**77.78**	42.00	53.33	24.50	**71.29**
TaE_1	25.00	72.22	35.00	**61.11**	25.03	70.66
TaE_2	40.00	55.56	47.00	47.78	38.33	55.08
TaE_3	32.50	63.89	44.53	50.52	27.17	68.16
TaE_4	39.00	56.67	38.00	57.78	35.00	58.98
TaU_1	37.50	58.33	37.47	58.37	25.33	70.31
TaU_2	30.00	66.67	36.50	59.44	25.00	70.70
TaU_3	32.33	64.07	33.46	**62.82**	26.10	69.41
TaU_4	27.60	69.33	36.00	**60.00**	30.08	64.75
TaG_1	25.33	71.85	41.47	53.92	21.01	**75.38**
TaG_2	15.00	**83.33**	37.00	**58.89**	27.11	68.23
TaG_3	35.00	61.11	45.03	49.97	25.20	70.47
TaG_4	22.50	**75.00**	42.50	52.78	22.20	**73.98**
TaMc	60.00	33.33	52.03	42.19	45.10	47.15
Control	90.00	0.00	90.00	-	85.33	-
SE(m)±	0.86	-	0.68	-	1.09	-
CD (P=0.01)	2.35	-	1.87	-	2.97	-

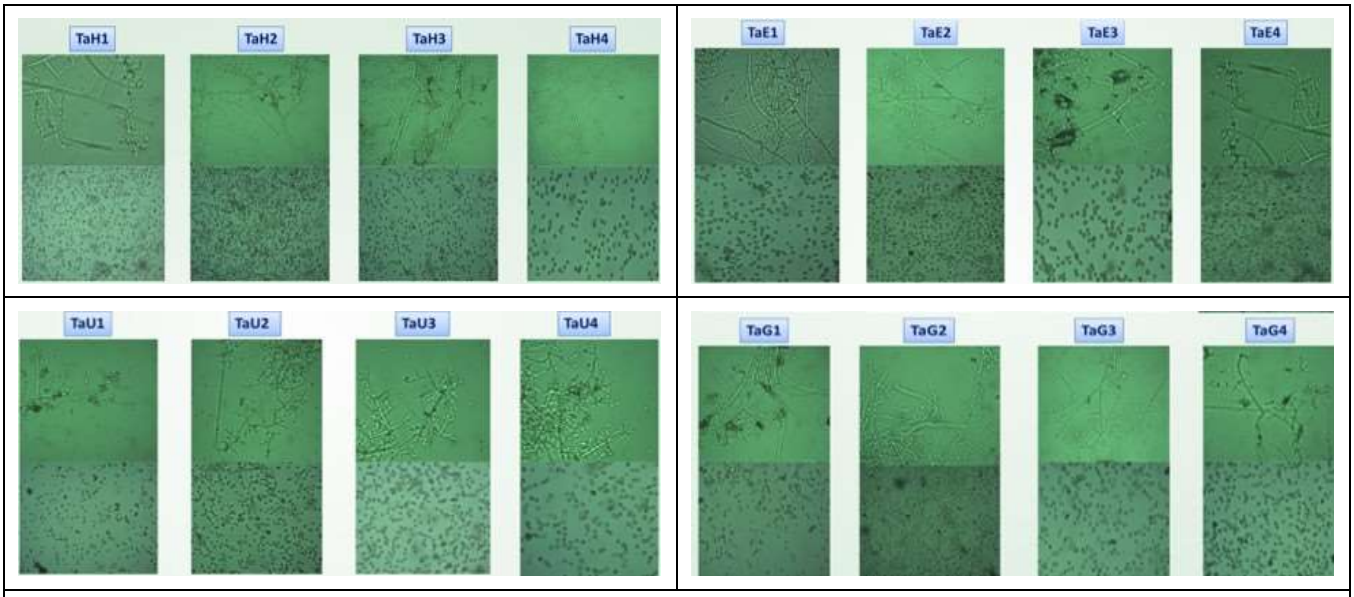

Fig 3: Microscopic view of *T. asperellum* mutants (40X magnification)

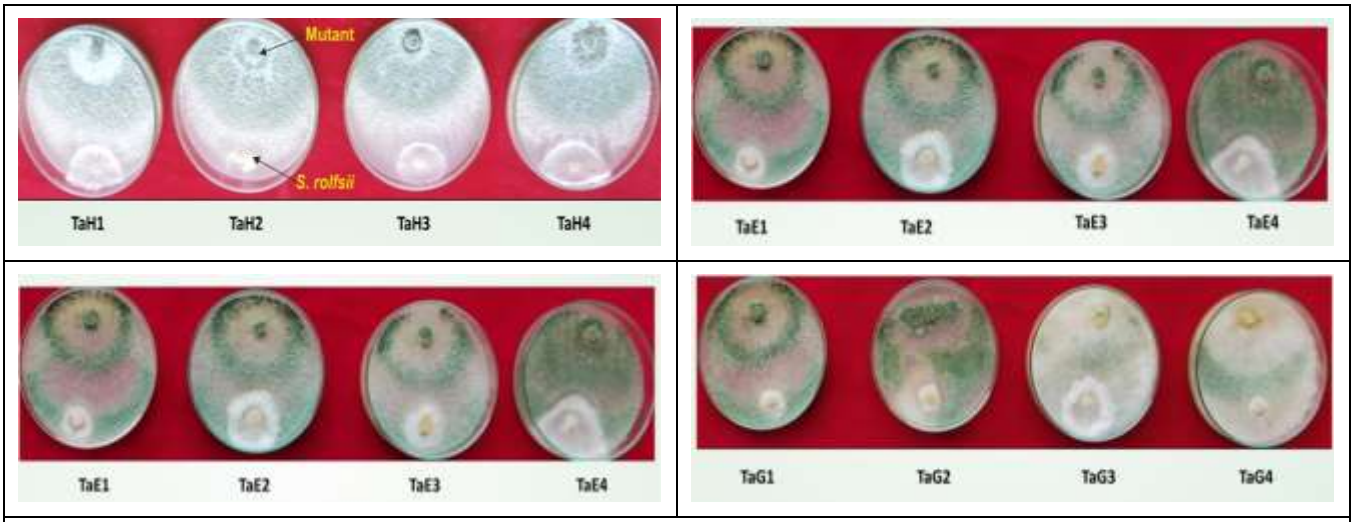

Fig 4: Antagonistic activity of *T. asperellum* mutants against *Sclerotium rolfsii* at 7DAI

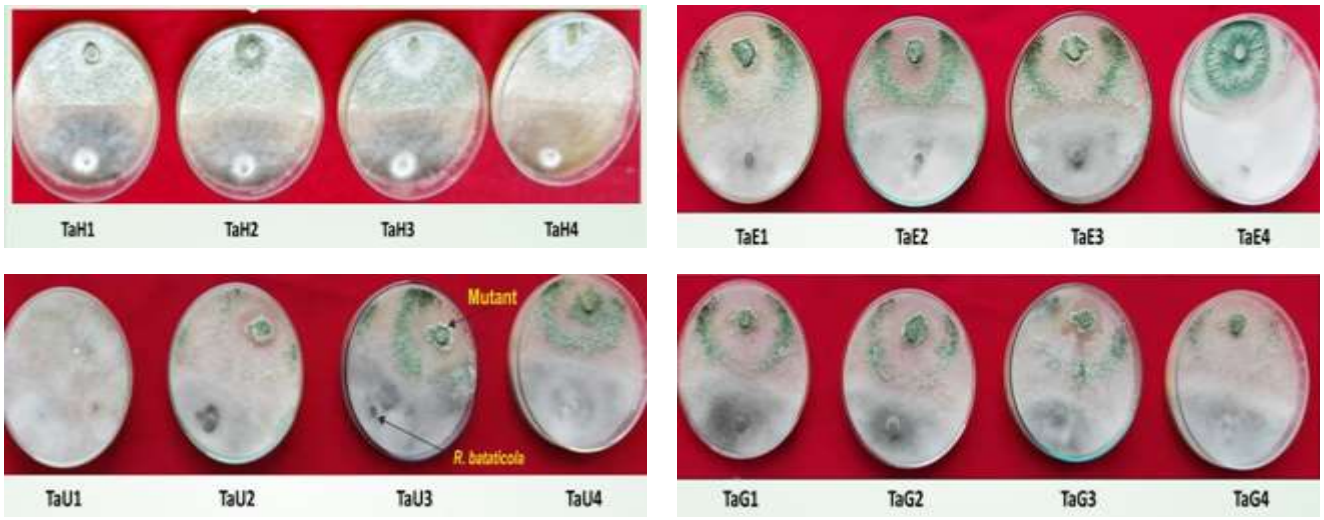

Fig 5: Antagonistic activity of *T. asperellum* mutants against *Rhizoctonia bataticola* at 7DAI

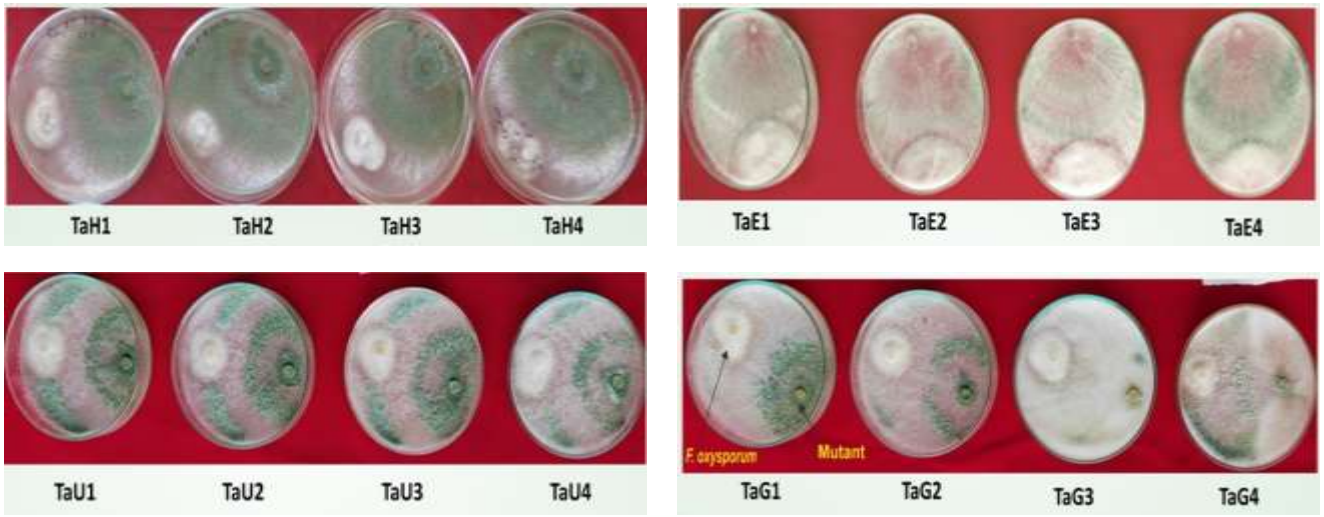

Fig 6: Antagonistic activity of *T. asperellum* mutants against *Fusarium oxysporum f.sp. ciceri* at 7DAI

All *T. asperellum* mutations outperformed mother culture (Table 3). The maximum chitinase enzyme units/mg of protein was recorded in mutant (T_2) TaH2 at 0.98 and TaG2 (T_{14}) at 0.90. The next-best mutants for chitinase enzyme unit content were recorded at 0.88 and 0.84 for TaG4 (T_{16}) and TaU2 (T_{10}) respectively. The lowest measure of 0.62 was recorded in the mother culture (T_{17}).

Trichoderma spp. have a greater capacity to produce enzymes like chitinase and antibiotics as a result of gamma irradiation (Mohammed *et al*., 2010) [16]. The mutants of *T. asperellum* produced 0.70, 0.67, 0.66, and 0.66 chitinase enzyme units/mg of protein (Shete *et al*., 2019) [28]. The mutants of *T. harzianum* obtained through gamma irradiation produce a variety of active metabolites like extracellular chitinases, cellulases, β-galactosidases, and few antibiotics like trichodermin, gliotoxin, and gliovirin. *T. harzianum* transformants with increased antifungal activity as a result of overexpression of 33-kDa chitinase (Limon *et al*., 1999) [12]. The chemical mutagenesis treatment using ethyl methane sulfonate (EMS) and hydroxyl amine (HA) produced mutants of *T. viride* isolates. These mutants produced 0.57-0.63 enzyme units more than the mother culture (Suryawanshi *et al*., 2013) [29]. Varied UV treatments lasting 2 min, 4 min, 6 min, and 8 min, isolated four mutant strains from the natural *T. harzianum* (Patil and Lunge, 2012) [22].

Table 3: Chitinase enzyme units/mg of proteins in mother culture and mutants of *T. asperellum*

S.N.	Treatment	*Trichoderma asperellum* mutants	Chitinase enzyme units/ mg of protein			Mean chitinase enzyme units/ mg of protein
			R I	R II	R III	
1	T_1	TaH_1	0.71 (0.84)	0.72 (0.85)	0.7 (0.84)	0.71 (0.84)
2	T_2	TaH_2	0.99 (0.99)	0.98 (0.99)	0.97 (0.98)	0.98 (0.99)
3	T_3	TaH_3	0.66 (0.81)	0.7 (0.84)	0.74 (0.86)	0.70 (0.84)
4	T_4	TaH_4	0.7 (0.84)	0.69 (0.83)	0.72 (0.85)	0.70 (0.84)
5	T_5	TaE_1	0.73 (0.85)	0.74 (0.86)	0.72 (0.85)	0.73 (0.85)
6	T_6	TaE_2	0.74 (0.86)	0.73 (0.85)	0.76 (0.87)	0.74 (0.86)
7	T_7	TaE_3	0.69 (0.83)	0.72 (0.85)	0.71 (0.84)	0.71 (0.84)
8	T_8	TaE_4	0.68 (0.82)	0.67 (0.82)	0.66 (0.81)	0.67 (0.82)
9	T_9	TaU_1	0.69 (0.83)	0.75 (0.87)	0.74 (0.86)	0.73 (0.85)
10	T_{10}	TaU_2	0.84 (0.92)	0.83 (0.91)	0.84 (0.92)	0.84 (0.92)
11	T_{11}	TaU_3	0.77 (0.88)	0.79 (0.89)	0.78 (0.88)	0.78 (0.88)
12	T_{12}	TaU_4	0.81 (0.90)	0.82 (0.91)	0.8 (0.89)	0.81 (0.90)
13	T_{13}	TaG_1	0.66 (0.81)	0.68 (0.82)	0.69 (0.83)	0.68 (0.82)
14	T_{14}	TaG_2	0.9 (0.95)	0.89 (0.94)	0.91 (0.95)	0.90 (0.95)
15	T_{15}	TaG_3	0.65 (0.81)	0.67 (0.82)	0.68 (0.82)	0.67 (0.82)
16	T_{16}	TaG_4	0.88 (0.94)	0.87 (0.93)	0.89 (0.94)	0.88 (0.94)
17	T_{17}	TaMc	0.61 (0.78)	0.64 (0.80)	0.6 (0.77)	0.62 (0.79)
	SE(m)±					0.01
	CD (P=0.01)					0.03

Conclusion

Mutagenesis produced positive mutation in *T. asperellum*. A significant per cent growth inhibition was recorded with the mutants against *S. rolfsii viz*., TaH_2 (88.89%), TaG_2 (83.33%), TaH_4 (77.78%), and TaG_4 (75%) over mother culture TaMc (33.33%). A significant per cent growth inhibition was recorded with the mutants against *R. bataticola viz*., TaU_3 (62.82%), TaE_1 (61.11%), TaU_4 (60.00%), and TaG_2 (58.89%) over mother culture TaMc (42.19%). A significant per cent growth inhibition was recorded with the mutants against *F. oxysporum* f.sp. *ciceri viz*., TaG_1 (75.38%), TaG_4 (73.98%), TaG_4 (73.98%), and TaH_4 (71.29%) over mother culture TaMc (47.15%). The chitinase enzyme units/mg of proteins was significantly increased in mutants *viz*., TaH_2 (0.98), TaG_2 (0.90), TaG_4 (0.88), and TaU_2 (0.84). Therefore, enhanced antagonistic potential and chitinase enzyme production of *T. asperellum* was achieved through both chemical and physical mutagenesis.

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