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Exposure of *Trichoderma* isolates to different doses of UV radiation for development of mutants and their stability in subsequent generations

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Abstract
Mutagenesis is one such technique that induces diversification of genotypes of targeted organisms. Mutagen induced changes could either be random or targeted based upon the suitable mutagenesis technique. Biocontrol-associated characters of mutants obtained by successful screening and analysis can be significantly improved. Genetic improvement of *Trichoderma* spp. By induced mutation using physical and chemical mutagens have been attempted successfully to ameliorate the efficacy of native strains. Such mutation brings about changes in various morphological features and different physical and biochemical attributes as well as potential increase in antifungal metabolites of the selected mutants as compared to their wild parents has also been observed. Certain mutants of *Trichoderma* spp. have been to have better rhizosphere competence compared to their parent strains. Selection of such beneficial mutants may be a better avenue for the management of plant pathogens.
In the present investigations, two best strains of *Trichoderma* i.e. UBT-18 and T21 were isolated from soil of Cooch Behar, West Bengal and placed under UV light with different duration of exposures. The colonies from individual mutated spores of *Trichoderma* strains were repeatedly sub-cultured in test tubes up to 10th generation to obtain stable mutants. Some *Trichoderma* strains died out before reaching last generation growth due to segregation effect after random mutation.

Keywords: Trichoderma, strains, isolates, mutation

Introduction
The worldwide emphasis on use of *Trichoderma* as biological control agent is because of its universal existence, ability of rhizosphere modification, tolerance to unfavourable weather conditions, high regeneration capacity, effective nutrient mobilization, efficient in plant growth regulations and havoc aggressiveness against phytopathogenic fungi. Due to major impact of Trichoderma on human welfare, recent genome sequencing has targeted seven species mainly *T. atroviride*, *T. asperellum*, *T. citrinoviride*, *T. harzianum*, *T. longibrachiatum*, *T. reesei* and *T. virens* (Grigoriev *et al*., 2012) [5].
Mutagenesis is one such technique that induces diversification of genotypes of targeted organisms. Mutagen induced changes could either be random or targeted based upon the suitable mutagenesis technique. Biocontrol-associated characters of mutants obtained by successful screening and analysis can be significantly improved.
During the past few decades, genetic improvement of *Trichoderma* spp. by induced mutation using physical and chemical mutagens have been attempted successfully to ameliorate the efficacy of native strains (Mukherjee and Mukhopadhyay, 1993, Hazra, 2003, Walnuj and Jhon, 2013) [12, 7, 22]. Such mutation brings about changes in various morphological features and different physical and biochemical attributes as well as potential increase in antifungal metabolites of the selected mutants as compared to their wild parents has also been observed. Certain mutants of *Trichoderma* spp. have been to have better rhizosphere competence compared to their parent strains. Selection of such beneficial mutants may be a better avenue for the management of plant pathogens. *Trichoderma* spp. mutated utilizing UV light, produces two new antifungal compounds along with antibiotic produce by the parental strain (Graeme-Cook *et al*., 1991) [4]. Mutated forms of *T. harzianum* are more rhizosphere competent with exorbitant cellulose activity than non-competent ones (Baker, 1988) [1] and mutants with velvet protein (Vel 1) deleted, generate more chlamydospores under nutrient stress condition in contrast with the wild type (Mukherjee *et al*., 2013) [13]. Mutation is employed in *Trichoderma* to improve various properties like survivability, fungicide tolerance, antagonistic potentiality, genetic variability, colonizing ability and competitive saprophytic

ability (CSA) (Pecchia and Anne, 1989; Mukherjee and Mukhopadhyay, 1993; Migheli *et al*., 1998, Mukherjee *et al*., 1999; Haggag *et al*., 2010) [15, 12, 10, 11] and strain improvement. Strain improvement of wild type *Trichoderma* by mutation is a successful method to enhance the antagonistic activity, rhizosphere competence, antibiotic production, production of cell wall degrading enzymes like chitinases, glucanases, cellulases in addition to growth promotion and induction of resistance in plants against various stresses (Hazra, 2003, Roy, 2003, Villena and Guttierezz-Correa, 2006) [7, 18, 21].

Materials and Methods

The experiments were conducted at Department of Plant Pathology, Uttar Banga Krishi Viswavidyalaya during 2019-2020. The details of experimental procedures adopted, materials used and techniques followed during the course of the present investigation are described below.

Media used

Potato Dextrose Agar (PDA, Riker and Riker, 1936) [17]

It is a semi-synthetic growth media for routine culture of the test biocontrol agent *Trichoderma* spp.

Compositions	**g/L**
Potatoes, infusion from	200.0
Dextrose anhydrous	20.0
Agar agar	20.0
Distilled water	1L
Final pH (at 25 ºC) 5.6±0.2	

***Trichoderma* specific medium (TSM, Elad *et al*., 1981; modified by Saha and Pan, 1997) [2, 19]**

Due to the low glucose level of this medium, rapid growth and sporulation of fungus takes place. The medium was used for selective isolation of *Trichoderma* from soil. In present investigation the medium was also used for isolation of mutated *Trichoderma* isolates after exposure to different doses of UV radiation. The viability of mutated isolates was also examined on this medium and then studied through generation study.

Components	**g/L**
Glucose	3.0
KCl	0.15
MgSO4	0.25
NH4NO3	1.0
K2HPO4	0.9
Agar Agar	15
Distilled water	1L
Rose Bengal	0.033
Captan	0.1
Methyl Orange	0.3
Chloramphenicol	0.25
pH	5.0-5.5

Native *Trichoderma* strains used

Two best strains of *Trichoderma* i.e. UBT-18 and T21 were used. These strains were isolated from soil of Cooch Behar (vegetable plots, tea plantation).

Pure culture of *Trichoderma*

Single spore of *Trichoderma* strains is taken to get pure culture from the previous available culture. The spore is placed on fresh PDA medium on sterile Petri plate. The Petri plates are properly wrapped and incubated in BOD at 28±1 ºC for 5-7 days. The process can be repeated after 15-20 days to maintain the culture for future use.

Transfer on test tubes

From pure culture petri plates, *Trichoderma* was transferred on PDA slants that were then kept in BOD at 28±1 ºC for 5-7 days and later maintained at 4 ºC as pure culture.

UV Mutagenesis

The mutagenesis of both the *Trichoderma* strains was done in UV2 sterilizing PCR Work station Cabinet having 2UV sources of 254 nm short wave UV tubes. Timer with 10-15 min was used first to decontaminate the chamber before use. The exterior dimension of the model (W×D×H) is 737 × 610 × 729 mm and interior dimensions (W×D) of 500 × 544 mm and the distance between the Petri plates to the UV light source was 700mm.

Petri plates containing *Trichoderma* strains were placed under UV light with different duration of exposures i.e., for strain UBT-18 starting from 15 min to 2 hr 30 min at 15 min interval forming 10 different treatments. For strain T21, 5 different treatments starting from duration of 1 hr. 30 min to 2 hr 30 min keeping 15 min interval between each (Table 3.1). The treated petri plates were immediately transferred in BOD at 28±1 ºC for 18-19 hr approximately.

Table 1: Duration of exposure of fungal strains to UV-light

Fungal strain/mutant UBT-18 Treatment	Duration	Fungal strain/mutant T21 Treatment	Duration
T_1	15 min	T_1	1hr 30 min
T_2	30 min	T_2	1 hr 45 min
T_3	45 min	T_3	2hr
T_4	1hr	T_4	2hr 15 min
T_5	1hr 15 min	T_5	2hr 30 min
T_6	1 hr 30 min		
T_7	1hr 45 min		
T_8	2hr		
T_9	2hr 15 min		
T_{10}	2hr 30min		

Serial dilution of UV treated *Trichoderma* strains

After UV treatment and BOD placement, the cultures from UV treated plates were serial diluted and plated on Petri plates containing TSM. Conical flask of 50 ml was taken and filled with 45 ml distilled water and sterilized. In a single flask a single treatment of the *Trichoderma* strain was used. From

treated *Trichoderma* plates a portion of culture using cork borer is taken and dipped into the conical flask and shaken properly. Aliquot of 5 ml from the conical flask is taken using pipette and transferred into another conical flask containing only 45 ml distilled sterilized water. From the second conical flask 1 ml of the solution is taken using micropipette and placed on a sterilized Petri Plate Containing TSM.

Sufficient replication was maintained for a single treatment of a *Trichoderma* strain and for other treatments also the same method was carried out. The plates were then kept in BOD at 28±1 °C until single colony of viable *Trichoderma* spores grew into small mutated colony which subsequently was picked before merging with one another based on difference in colony appearance of native strains and transferred to test tube containing PDA slants and incubated in BOD for growth.

Generation growth of mutated *Trichoderma*

The colonies from individual mutated spores of *Trichoderma* strains were repeatedly sub-cultured in test tubes up to 10th generation. For this purpose, spores from the previous test tubes are taken and inoculated onto fresh PDA slant tubes for growth. Some *Trichoderma* colonies under some treatments even died out before reaching last generation growth due to segregation effect. During each generation of treatments, the growth pattern and visual color changes if any was observed carefully.

Results and Discussion

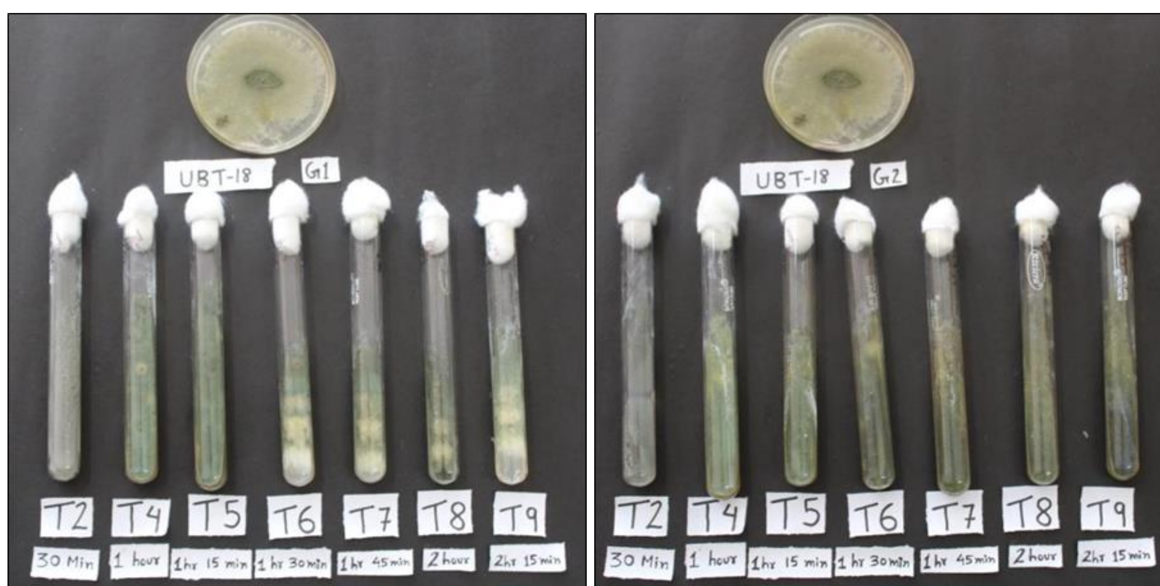
Mutagenic Treatment

The two *Trichoderma* pp. were taken which showed different visual appearances. *T. harzianum* (UBT-18) was a light yellowish green in colour with low sporulation rate. On the other hand, *T. asperellum* (T 21) exhibited dark green spores, high growth rate, granular and marginal mycelia growth. Both were exposed to UV rays following the method described in section 3.5. After incubation of 18-19hrs upon exposure the mutated conidia were plated for isolation. Based on differences in visual appearance of single colony of various exposures, following numbers of mutated isolates were transferred to PDA slants for generation studies.

Table 2: Mutated isolates cultured through generation

***T. harzianum* (UBT-18)**		***T. asperellum* (T 21)**	
Treatment	**No. of single mutated colony isolated**	**Treatment**	**No. of single mutated colony isolated**
T_1	No colonies isolated due to absence of variation in colony appearance from native culture	T_1	3
T_2	3	T_2	4
T_3	Same as T1	T_3	2
T_4	4	T_4	3
T_5	5	T_5	4
T_6	5		
T_7	4		
T_8	3		
T_9	3		
T_{10}	No colony appeared		

The mutated isolates were repeatedly sub-cultured up to 10th generation to observe the effect of segregation after random mutation. The purpose was also to get the stable mutants. During the study, a few mutants lost their viability and were excluded from the experiment. The various generations from 1st to 10th showed visual differences in the pigmentation of the spores which have been presented in plate (1 and 2). Among the stable mutated isolates obtained after 10th generation, one isolate from each treatment having maximum growth rate was selected to study *in-vitro* antagonistic potential against fungal pathogens.



UBT-18 (*T. harzianum*)-1stGeneration UBT-18 (*T. harzianum*)-2nd Generation

UBT-18 (*T. harzianum*)-3rd Generation

UBT-18 (*T. harzianum*)-4th Generation

UBT-18 (*T. harzianum*)-5th Generation

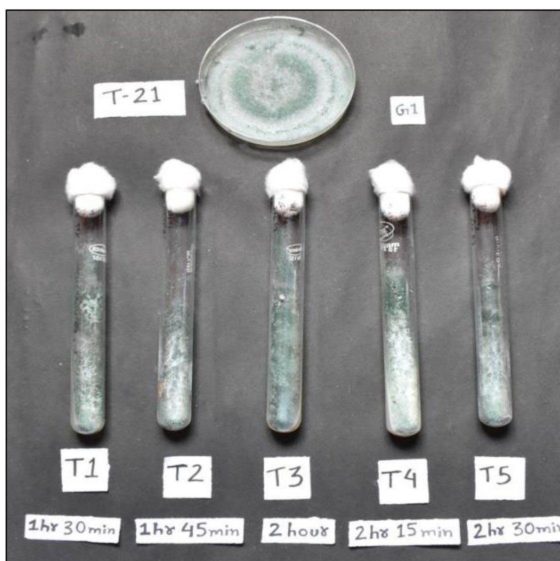
UBT-18 (*T. harzianum*)-6th Generation

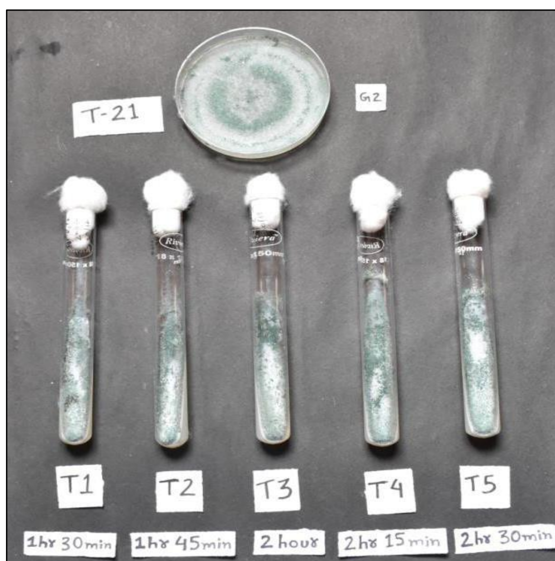
UBT-18 (*T. harzianum*)-7thGeneration

UBT-18 (*T. harzianum*)-8thGeneration

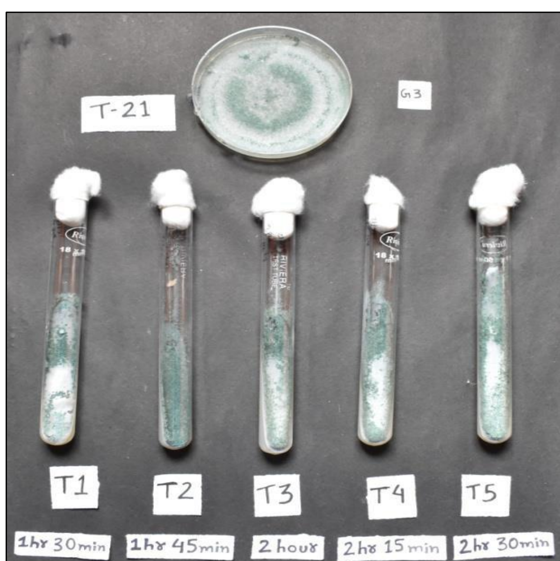
UBT-18 (*T. harzianum*)-9th Generation UBT-18 (*T. harzianum*)-10th Generation

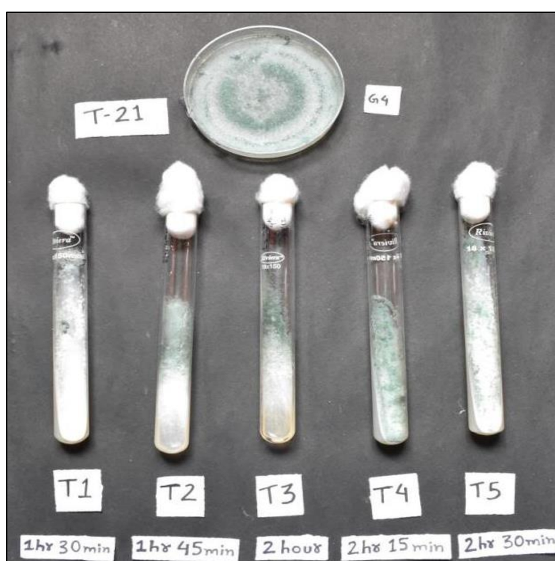
Plate 1: Generation growth of mutated UBT-18 *Trichoderma* strains



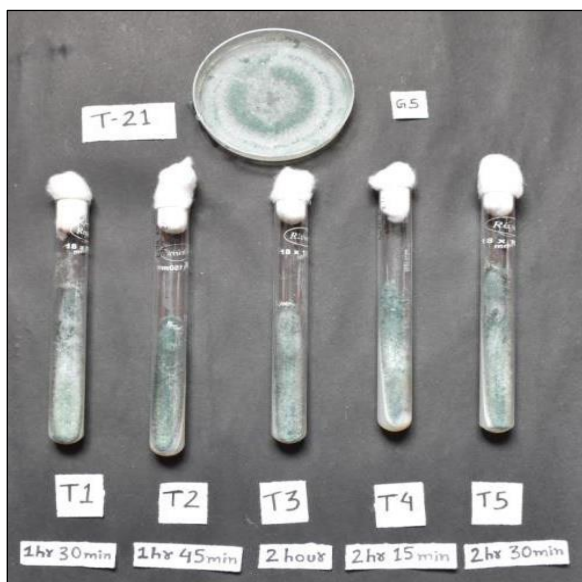


T21 (*T. asperellum*)-1st Generation T21(*T.asperellum*)-2nd Generation

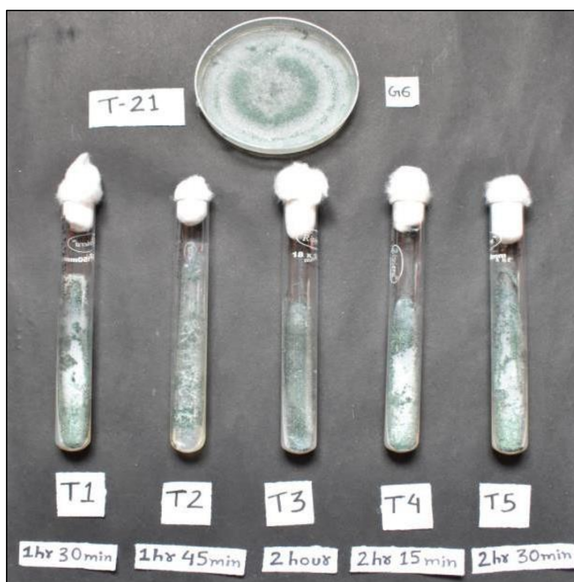




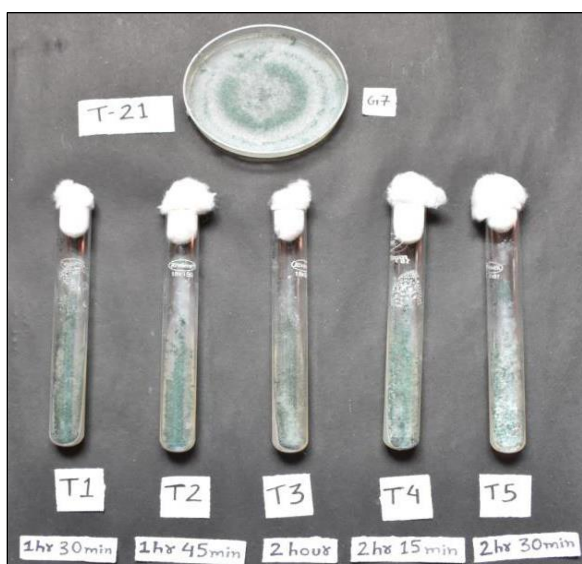
T21 (*T. asperellum*)-3rd Generation T21 (*T. asperellum*) 4th Generation



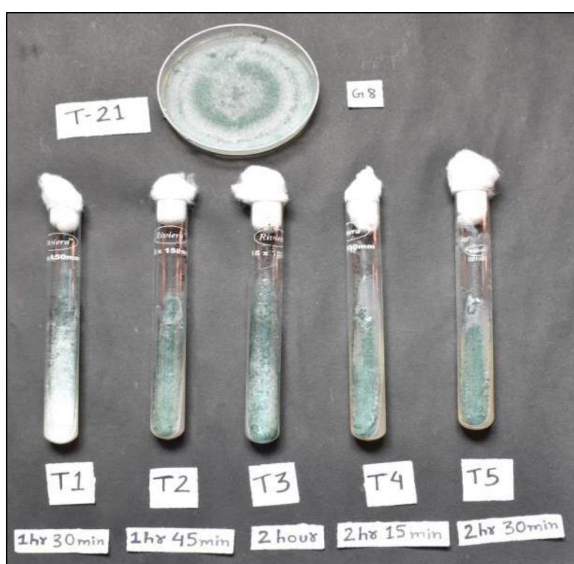
T21(*T. asperellum*)-5th Generation



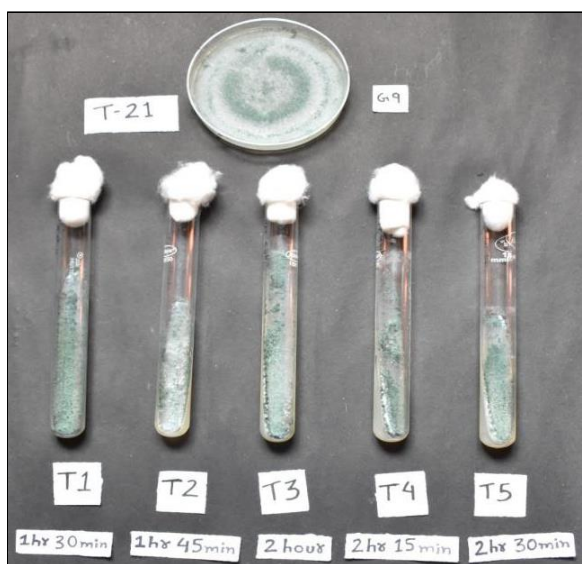
T21(*T. asperellum*)-6thGeneration



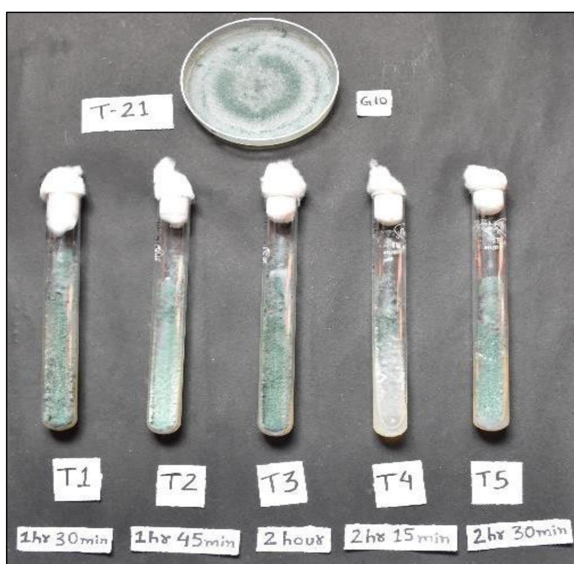
T21(*T. asperellum*)-7th Generation



T21(*T. asperellum*)-8th Generation



T21(*T. asperellum*)-9th Generation



T21(*T. asperellum*)-10th Generation

Plate 2: Generation Growth of mutated T21 *Trichoderma* strains

The mutagenic effect of UV light on DNA structure results in variations in cultures of *Trichoderma* spp. Nodvig *et al.* (2015) [14] gave the mutagenesis techniques for filamentous fungi. Certain morphological changes observed due to mutagenic consequences include the change in colony appearance, colony colour, pigmentation and sporulation rate (Ikehata and Ono, 2011; Pfeifer *et al*., 2005) [8, 16]. The mutagenic consequence seems to change with change in UV treatments. UV-surviving mutants can induce stress due to photo-reactivation, post-replication repair, excision repair and cellular repair system (Ganesan, 1974; Seeberg *et al*., 1995; Yasui *et al*., 2003) [3, 20, 23]. But cell death can occur due to excessive damage in the form of numerous DNA cross-linkages, multiple breaks in strand and cyclobutane pyrimidine dimmers (Lin and Wang, 2001) [12].

Conclusions

Two best strains of *Trichoderma* (UBT-18 and T21) isolated from soil of Cooch Behar were used. Both strains showed different visual appearance i.e., UBT-18 was light yellowish green in colour with low sporulation rate and on the other hand T21 exhibited dark green spores, high growth rate and marginal mycelial growth.

Both the *Trichoderma* spp. were exposed to UV light for different durations, immediately transferred to BOD after treatment and were later serial diluted and plated on Petri plates containing TSM. After a few days of growth on BOD, mutated colonies of *Trichoderma* spores were picked on PDA slants before the colonies merged with each other.

The colonies from individual mutated spores of *Trichoderma* strains were repeatedly sub-cultured in PDA slants up to 10th generation to obtain stable mutants. Some *Trichoderma* strains died out before reaching last generation growth due to segregation effect after and on mutation. Such viability losing mutants was excluded from the experiment. During each generation of treatments, the differences in growth pattern and visual colour if any were observed carefully. Knowledge about the stable mutant strains of *Trichoderma* will help in their efficient selection and use in various fields.

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