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Induction and characterization of a cellulase overproducing mutant strains of *Trichoderma harzianum*

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Abstract

Nine *Trichoderma* isolates were isolated from different agricultural soil samples. All the Nine *Trichoderma* isolates showed positive test for cellulase as revealed by the formation of a clear zone on the screening medium containing Congo red as indicator. The percentage inhibitory effect of all the Nine *Trichoderma* isolates against growth of *R. solani* was calculated and ranged from 58 % to 64.9%. The qualitative and antagonistic results showed the isolate FUGT4 had the highest efficiency in antagonistic against *R. solani*. Molecular phylogenetic analysis was performed based on nucleotide sequences. The results indicate that the isolate FUGT4 are closely related to *Trichoderma harzianum,* with accession number OL953189. The selected strain was subjected to mutation using UV-radiation and 12 mutants were obtained when exposed to different time periods of radiation.These mutants were tested for their cellulase productivity compared to their wild type and the results showed that, the mutants were produced cellulase 0.921 IU/mL cellulase activities**.** The ISSR analysis of genomic DNA was performed to detect genetic diversity of these mutants with the wild type by using 6 primers. The mutants and their wild counterparts were tested for their antagonistic potently against to *R. solani.* The results showed that all mutants induced from the wild type *Trichoderma* were significantly better than the wild type when tested against *R. solani*.

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Introduction

Many microorganisms that produce various cellulolytic enzymes have been studied for several decades. The genus of *Trichoderma* has been especially famous for producing cellulolytic enzymes with relatively high enzymatic activity. Economic analyses have indicated that the production of cellulase is still a cost factor. It is therefore imperative to improve the production of cellulase in order to make the process more economically viable. Hence many traditional mutagenesis strategies have been applied to improve the production of cellulase. This technique is simple and successful many times as compare to the complication exists (Chand *et al*., 2005).

One of the alternative methods to control pathogens is to use biological control agents instead of chemical pesticides. Biological control is a safe and suitable method for humans and the environment. *Trichoderma* is one of the most suitable biocontrol agents. This fungus has the ability to control many plant pathogens (Waghunde *et al*., 2016). Mutations were used to improve the efficiency of *Trichoderma* fungus as a biocontrol agent against pathogenic fungi. Afterwards, derived mutants were compared with wild isolates in respect to antagonistic properties; and finally some isolates have been introduced with higher antagonistic effect than the wild isolate. Genes play a major role in the biocontrol cycle by controlling certain signals and leading to the secretion of certain enzymes or proteins that enable the pathogens to degrade and hence are known as biocontrol genes. Increased gene expression helps in enhanced biocontrol activity which helps to promote plant growth and prevents the plant from attacking pathogen. For commercial applications, therefore, the biocontrol genes can be cloned and generated in large amounts (Massart and Jijakli, 2007). Several approaches including chemical mutation, UV irradiation and genetic engineering to obtain enhanced cellulase producing strains have been given a high priority in the last decade (Kotchoni and Shonukan, 2002). Industrial application success for improved strains depends on their genetics and physiological characterization with a system that allows quick diagnosis. These diagnostic procedures for mutation are based upon a number of techniques in which resistant type of mutants can also be used for this purpose. Random mutagenesis methods are simple and easy to operate, though they have limitations, such as lack of stability. Mutagenesis is one such approach that induces diversification of the genetic structure of targeted organisms. There are several reports indicating the overproduction of cellulase by UV mutant strains of *A. niger* (Kang *et al*., 2004 and Nicolás-Santiago *et al*., 2006). These studies revealed that the UV exposure time and distance of the microorganism to the UV source are the main parameters which influence the yield of cellulase production. The aim of this study was to induce mutants using chemical mutagens and UV and evaluation of their antagonistic potential against the soil-borne fungal pathogens. Resolve the genetic variability of mutant derivatives with their wild strain using ISSR markers and isolation of the cbh1 gene, encoding for the exo-cellobiohydrolas from *Trichoderma* and their mutants and confirmed by PCR using specific primers.

Materials and methods

Isolation and identification of Trichoderma isolates from collected samples

Trichoderma isolates were isolated from rhizosphere samples according to soil dilution plate method described by Rahman *et al.* (2011). After isolation and purification of the isolates, the characteristics of conidiophores, such as phialides, cysts, and chlamydospores were detected using the identification keys (Gams and Bissett, 2002 and Samuels *et al.*, 2002).

Qualitative screening of Trichoderma isolates for their cellulase production

Trichoderma isolates from different sources were screened qualitatively as described by Wang *et*

al. (2003). 1% of carboxymethyl cellulose (CMC) was added into culture medium and fresh culture plugs of the *Trichoderma* isolates were placed in the middle of the plate. Plates were incubated at 28°C for 96 h. The replica plates were also prepared separately for staining. The replica plates were flooded with 0.3% Congo red for 20 min. The stain was poured off and the plates were washed with 1.0 M NaCl. A clear zone formed around the growing colonies of cellulase positive cultures against dark red backgrounds was taken as the indication of cellulase activity. The *Trichoderma* isolates exhibited good clearance beyond the areas of its growth was then selected for further studies.

In vitro evaluation of antagonism of Trichoderma sp. against R. solani

Trichoderma isolates were evaluated for their potential to antagonize the plant pathogen *R. solani in vitro* using dual culture technique according to the bioassay method described by Zhang and Wang, (2010) on PDA medium.

Quantitative screening of Trichoderma isolates for their cellulase production

The potential *Trichoderma* isolates selected by primary qualitative screening were then evaluated for enzyme production and used for quantitative screening of CMCase activity according to Shawky and Hickisch, (1984)

PCR amplification of 18SrDNA region of Trichoderma isolates

PCR was performed using two universal primers ITS1 (5'- TCCGTAGGTGAACCTGCGG -3') for forward primer and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') for reverse primer to amplify the internal Transcribed Spacer1 (ITS) regions between the small and large nuclear rDNA of the selected *Trichoderma* isolate (FUGT4) according to White *et al*. (1990).

Sequencing of ITS region of Trichoderma isolates

The purified PCR product of approximately 600 bp was subjected to sequencing through lab technology services located in Korea and performed at Applied Biosystems model 3730XL automated DNA sequencing system. The nucleotide sequence of the ITS regions of rDNA obtained from the selected *Trichoderma* isolate was compared with ITS sequences collected from *Trichoderma* sequences available in GenBank database (http://www.ncbi.nlm.nih.gov/ Blast). The phylogenetic tree was constructed according to Tamura *et al. (*2011).

Induction of Trichoderma mutants by UV radiation

1ml of spore suspension ($1x10^8$ spores per ml) was poured in the Petri plate without the lid to prevent shielding and subjected to UV light using a Philips TUV 15W lamp, which was placed at a distance of 20 cm from treated spores. All UV irradiations were performed under aseptic condition in laminar air flow for different time intervals (0, 5, 10, 15, 20, 25, 30, 35 and 40 min) with stirring. For each time interval, only one plate with fresh diluted spore suspension was placed in the chamber for the required irradiation time; this was done to prevent any light from reaching the plates during transfer. Following the treatment, UV-treated spores were serially diluted and plated on PDA plates containing 0.1% Triton X-100 as a colony restriction factor, which caused the fungus to grow in small colonies (Kallinen, 2016). The plates were then incubated at 28ºC for 7 days with daily monitoring of colonies, surviving spores developed into small mutant colonies that were picked, transferred to PDA plates and incubated at 28°C for72 h. An untreated plate containing conidia was maintained as a control. After incubation, colony developed from single spore was transferred on fresh PDA medium.

In vitro evaluation of antagonism of Trichoderma mutants against R.solani

After each mutagenesis, for each spore mutant of *Trichoderma* was evaluated for its potential to antagonize the *R. solani in vitro* using dual culture technique according to the bioassay method described in the above section.

Qualitative and Quantitative screening of Trichoderma mutants for their cellulase production

After each mutagenesis, the mutated spore suspensions were plated onto CMC screening medium and cultured at 30^{o}C for seven days, and then quantitative cellulase activities were determined for each spore mutant. The screened out mutant derivatives were then assayed quantitatively for enzyme assayed according to the methods as described above.

Inter-simple sequence repeats (ISSR) analysis for Trichoderma wild type and mutants

ISSR assay initially optimization of PCR was examined, including concentration of DNA, primers and number of PCR cycles as well as annealing temperature. Table (1) showed the sequence of six ISSR primers were used in present study to determine the genetic similarity between the *Trichoderma* wild type and their mutants according to Bornet *et al.* (2002)

Table 1. Nucleotide sequence of primers used for ISSR analysis of *Trichoderma* wild type and their mutants.

SL	Primer code	Primers sequence {5'-3'}	Annealing temperature
1	ISSR1	CTACACACACACACAC	48
2	ISSR 2	AGAGAGAGAGAGAGTA	43
3	ISSR 3	ACACAACAACAACAACAACAA	48
4	ISSR 4	AGAGAGAGAGAGAGACC	48
5	ISSR 7	CAACAACAACAACAAACG	43
6	ISSR 10	AGAGAGAGAGAGAGTA	43

Data Analysis

The analysis of the variance of the antagonistic activity was done using one-way analysis of variance, using SPSS software var. 16. Duncan's multiple range tests, as reported by Snedecor and Cochran (1989).

Results and discussion

Isolation and morphological identification of Trichoderma isolates

The *Trichoderma* isolates were characterized by using distinctive morphological characters that includes rapid growth, colony appearance and pigments. A total of nine isolates of *Trichoderma* spp. were isolated from rhizospher soil of different cultivation crops (Wheat and Faba bean). The conformation of species-level identification of *Trichoderma* isolates was carried out according to an interactive key http://nt.arsgrin.gov/taxadescriptions/keys/FrameKey.cfm?gen=*Trichoderma*. Morphological characterization was conventionally used in the identification of *Trichoderma* species and it remains as a potential method to characterize and distinguish *Trichoderma* species (Anees *et al.,* 2010). The few morphological characters with limited variation may lead to an overlap and misidentification of the strains and showed the necessary of DNA-based characters to complete identification evident from the present study.

Qualitative screening of Trichoderma isolates for their cellulase production

Each of the 9 *Trichoderma* isolates was screened and selected for cellulytic enzyme production based upon the color intensity and diameter of the yellow colored zone surrounding the colony on cellulase detection medium. Results present in Fig. (1) and Table (2) showed positive cellulase producers for plates flooded Congo red. The cellulase production ability of fungi assessed by estimating zone around the colony formed due to ability of fungal isolates to hydrolyse cellulose. Clearing zones surrounding *Trichoderma* growing colonies after incubating for a suitable period indicating their ability for cellulase production. Kasana *et al.* (2008) discovered that Gram's iodine for plate flooding in place of hexadecyltrimethyl ammonium bromide or Congo red, gave a more rapid and highly apparent result. Clearing zones surrounding *Trichoderma* growing colonies after incubating for a suitable period indicating their ability for cellulase production. Various studies used this method in screening the different *Trichoderma* cellulolytic isolates (Castrillo *et al*., 2021). Enzymatic hydrolysis requires the cooperation of exoglucanases, endoglucanases, and beta-

glucosidase for the successful degradation of cellulose (Zhang *et al.,* 2010).

Table 2. Diameter size of yellow clearing zone surrounding the colony of *Trichoderma* on the plate screening cellulase medium.

Isolate code	Species	Mean of yellow zone (cm)
FUGT1	*Trichoderma harzianum*	4.00
FUGT2	*Trichoderma harzianum*	4.25
FUGT3	*Trichoderma harzianum*	3.50
FUGT4	*Trichoderma harzianum*	5. 30
FUGT5	*Trichoderma harzianum*	5.00
FUGT6	*Trichoderma harzianum*	4.50
FUGT7	*Trichoderma harzianum*	4.45
FUGT8	*Trichoderma harzianum*	4.75
FUGT9	*Trichoderma harzianum*	4.25

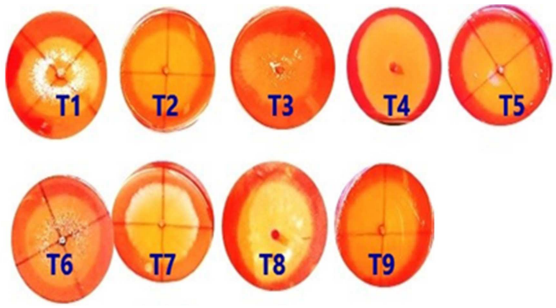

Fig. 1. Screening for cellulolytic *Trichoderma* isolates by covering the petri dishes with Congo red dye. A zone of clearance surrounding the colonies is indicative of carboxymethyl cellulose (CMC) hydrolysis by secreted CMCase.

Antagonistic efficacy of Trichoderma isolates against R. solani

The *Trichoderma* isolates were evaluated *in vitro* for their potential antagonizes the plant pathogenic fungus *R. solani*. The results of antagonism between *Trichoderma* isolates and *R. solani* showed in Fig. (2) and Table (3). *Trichoderma* isolates were showed a significant reduction in mycelia growth of fungal colonies of *R. solani* face the *Trichoderma* isolates compared to the control. *T. harzianum* (FUGT4) showed the highest inhibition (64.9%) of *R. solani* growth. On the other hand, *T. harzianum* (FUGT4) showed the lowest inhibition (57.6%). The remaniant isolates showed intermediate values of percentage of inhibition of *R. solani* growth (Table 3). The antagonism was observed with the naked eye (Fig. 2). A biocontrol agent may act against pathogens by using one or more of the following mechanisms: competition, antibiosis, and parasitism as well as activating host defense mechanisms (Papavizas and Lumsden, 1980). Results of the present study agree with the finding of many researchers all over the world as well as in Egypt: several species belonging to the genus *Trichoderma* are capable of parasitizing fungal plant pathogens such as *R. solani*, producing antibiotics effective against soil borne pathogens and competing for infection sites against pathogens. Isolates exhibited caused complete inhibition of *R. solani* due overgrowth of antagonist. Various *Trichoderma* spp. have been found to inhibit pathogens *in vitro* by over growth. According to Bastakoti *et al.* (2017) the colony growth of *S. rolfsii* on the fourth day incubation was found to be covered by the growth of *Trichoderma* sp. Due to over growth of *Trichoderma* species in the plate, the growth of test fungal pathogen was found to be highly inhibited.

Table 3. Percentage inhibition of radial growth of *Rhizoctonia solani* in dual cultures with *Trichoderma* isolates on PDA.

Isolate code	*Trichoderma species*	*Rhizoctonia solani*	
		Mean pathogen edge (cm)	Inhibition%
FUGT1	*Trichoderma harzianum*	3.5	58.8
FUGT2	*Trichoderma harzianum*	3.25	61.7
FUGT3	*Trichoderma harzianum*	3.6	57.6
FUGT4	*Trichoderma harzianum*	3.00	64.9
FUGT5	*Trichoderma harzianum*	3.00	63.7
FUGT6	*Trichoderma harzianum*	3.25	61.7
FUGT7	*Trichoderma harzianum*	3.5	58.8
FUGT8	*Trichoderma harzianum*	3.25	61.7
FUGT9	*Trichoderma harzianum*	3.50	58.8
Rhizoctonia solani		8.5	0.00

* Values are means of 3 replicates, the means the having the same alphabetical letter in

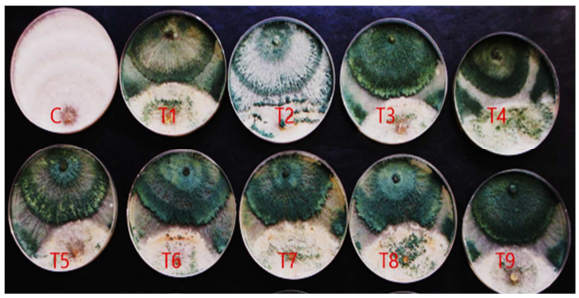

Fig. 2. Inhibition of mycelial growth of *Rhizoctonia solani* by different *Trichoderma* isolates on PDA after 7 days.

Quantitative screening for cellulase production from Trichoderma isolates

According to the results of the previous experiments, antagonistic activities and qualitative screening for cellulase enzymes, the *Trichoderma* isolates exhibiting cellulase positive were checked for quantitative cellulase production. The results presented in Table (4) showed that all the selected *Trichoderma* isolates were able to produced cellulase enzyme in the presence of CMC as the sole carbon source. The results indicate that *Trichoderma* isolates FUGT4 showed the highest cellulase production with corresponding activities 0.32 IU/mL. The correlation between qualitative screening with Congo red technique and quantitative screening with dinitrosalicylic acid reagent method was first reported by Florencio *et al.* (2012). Li *et al.* (2010) who reported that *Trichoderma* spp. and *Aspergillus* spp. are thought to be cellulase producers, and crude enzymes produced by these microorganisms are commercially available for agricultural use.

Table 4. Quantitative assay of cellulase activity for nine selected *Trichoderma* isolates.

Isolate code	*Trichoderma* species	Cellulose activity (IU/ml)	Total protein
FUGT1	*Trichoderma harzianum*	0.0406	0.3046
FUGT2	*Trichoderma harzianum*	0.0506	0.3246
FUGT3	*Trichoderma harzianum*	0.0575	0.0989
FUGT4	*Trichoderma harzianum*	0.3163	0.1144
FUGT5	*Trichoderma harzianum*	0.0474	0.925
FUGT6	*Trichoderma harzianum*	0.0297	0.0951
FUGT7	*Trichoderma harzianum*	0.04647	0.1268
FUGT8	*Trichoderma harzianum*	0.0404	0.1327
FUGT9	*Trichoderma harzianum*	0.0569	0.1439

The data are the mean of three replicates ±SE. Means having the same letter are not significantly different using Duncan's multiple range test (DMRT) ($P<0.05$)

PCR amplification of ITS region of rDNA of Trichoderma isolates

The few morphological characters with limited variations may lead to an overlap and misidentification of the strains, so the morphological and physiological identification of the isolates were confirmed by molecular identification based on internal transcripted spacers (ITS region) of rDNA gene. Genomic DNA of all four isolates of *Trichoderma* was analyzed by PCR amplification of rDNA gene including 5.8S gene and the flanking intergenic transcribed spacer ITS region of rDNA.

Amplification of the ITS with primers ITS1 and ITS4 yielded a single product estimated by gel electrophoresis of approximately 600 bp was obtained from all the PCR amplifications for biocontrol isolates of *Trichoderma* spp. Use of the ITS region is a recognized method for *Trichoderma* species identification; the *Trichoderma* species specific PCR primers, ITS1 and ITS4 provide a fast and accurate tool for identification and characterization of *Trichoderma* spp. The analysis of the sequences of ITS-1, the 5.8S rDNA and ITS-2 regions has greatly improved the identification of *Trichoderma* species (Hermosa *et al.*, 2004).

Nucleotide sequencing and accession number

The ITS regions were amplified in different strains, resulting in PCR product of 600 base pairs (bp), which was sequenced directly. The sequence for the selected *Trichoderma* isolate after editing was submitted to the Gene Bank and homology searches done against all the published *Trichoderma* sequences using Blast N and FASTA programs [National Center for Biotechnology Information (NCBI), USA].

Basic Local Alignment Search Tool (BLAST) search results of each sequence giving the closest match to the test sample was used to determine the species of *Trichoderma* isolates. The isolate FUGT4 was closely related to *T harzianum* with accession number OL953189.

Computational analysis (Blast) and construction of phylogenetic tree

The phylogenetic tree obtained by sequence analysis of ITS region of rDNA of *T. harzianum* FUGT4 and the sequences of other *Trichoderma* spp. obtained from sequence databanks is represented in Fig. (3). They revealed that *Trichoderma* isolates FUGT4 was identified as *Trichoderma harzianum*.

The *Trichoderma* isolates were identified both morphologically and on the molecular level. The results of molecular identification were completely different compared with morphological identification. The ribosomal DNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett, 1992).

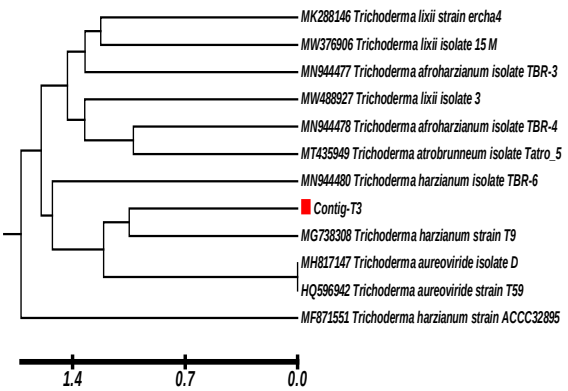

Fig. 3. Phylogenetic tree showing the relationship between *Trichoderma* isolate FUGT4 (*T. harzianum*) and its homologues strains in Genbank. The tree was constructed using MEGA6 sequence alignment programs.

They also occur in multiple copies with up to 200 copies per haploid genome arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S and the 28S large subunit (LSU) genes. Internal, transcribed spacer (ITS) regions have been used to generate specific primers capable of closely related fungal species (Bryan *et al.*, 1995).

In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies, the ITS regions of ribosomal genes were used for the construction of primers that can be used to identify *Trichoderma* spp.

Induction of Trichoderma mutants

The results showed that following UV exposure, 12 mutants were obtained. Morphological changes in growth features were evident in a number of obtained mutants and included changes in colony appearance, colony colour, sporulation rate and pigmentation.

A total of 12 surviving colonies were developed on plate-screening medium during the selection of improved strains. These colonies were selected that formed proficiently and significantly larger zones of hydrolysis than the corresponding parental strain. The exposure to UV irradiation for different time intervals (5-40 min) dramatically reduced the percentage survival of conidia.

One hundred percent mortality of conidia was recorded at 35 min of UV irradiation treatment. There was a progressive increase in mutant frequency when UV exposure time was gradually increased from 5 to 30 min and a decline thereafter. To induce diversification in the genetic material of wild type *Trichoderma* spp. UV light was used for its mutagenic effect on DNA structure.

While targeted mutagenesis techniques are becoming widely available for filamentous fungi (Nodvig *et al.*, 2015), the ability to introduce genome-wide random modifications also provides an invaluable option for genetic improvement (Bose, 2014).

This is in agreement with Ikehata and Ono, (2011) who reported that different UV treatments can induce distinct mutagenic consequences. Photo reactivation, excision repair and post-replication

repair are cellular repair systems that provide a probable explanation for why UV-surviving mutants were able to withstand the stress induced in the form of DNA damage as a result of UV light mutagenesis (Yasui *et al*., 2003).

In vitro evaluation of antagonistic potential of selected Trichoderma mutants against R. solani

To enhancement of cellulolytic activity by the *Trichoderma* strains, conidial suspension was subjected to UV irradiations. Among the 20 survivals, 12 mutants of the selected *Trichoderma* strain were selected for hyper-production of extra cellular cellulases. The selection of these cellulase producing strains was based on the antagonistic potential against *R.solani* as compared to wild strain. Competition efficiencies of *Trichoderma* mutants were examined by testing there *in vitro* antagonistic against *R. solania*.

The result for selected strain was presented in Table (5) and Figs (4). Certain mutants proved to be effective antagonists against the examined plant pathogenic fungus, they were growing as a thick culture on the surface of the colony of the phytopathogens. Colony diameters of plant pathogen *R. solani* co-cultivated with wild type and mutant *Trichoderma* spp. were measured, and growth inhibition was calculated using the aforementioned formulae. Statistical analysis of the obtained results shows significant difference between the inhibitory effect of the wild type and its mutants on *R. solani*.

The data in Table (5) and Fig. (4) which showed that all mutants induced from the wild type *Trichoderma* (FUGT4) were significantly better than the wild type when tested against *R. solani*, with the 12 selected mutants induce by UV were scoring the highest growth inhibition ranged from 78.3% to 79.4% compared with T3 wild type which scoring inhibition % 64.9%. Li *et al*., (2010) reported that when fungi were grown with mutagens at sub lethal concentrations, secretory enzyme production increased, but they showed, the killing rate was nearly 100% after UV treatment. Mutant strains M-B1, M-B2, M-B3, M-B5 and M-B7 produced high levels of CMCase, which were obtained by compound mutation of microwave and ultraviolet, and these were also stable for a long period of 9 generations to produce cellulase (Li, *et al*., 2010).

Antagonism is not a property of *Trichoderma* spp. because different strains or isolates of the same species can exhibit varying biocontrol potential against *R. solani*. The strains or isolates which genes are efficiently and rapidly expressed involved in antagonist activity against *R. solani* are infect better antagonists (Daguerre *et al*., 2014).

Table 5. Antagonistic effect of *Trichoderma* mutants against growth of *Rhizoctonia solani* after three rounds of successive sub-culture on PDA.

Exposure Time (min)	Isolate code	Mean Pathogen edge (cm)	Inhibition %
	C (*F. solani*)	9	00.00
0	T4 W.T	3.5	61.1
5	FUGT3(1)	3.2	64.4
	FUGT3(2)	3.25	63.9
10	FUGT3(1)	3.0	66.7
	FUGT3(2)	2.8	68.9
15	FUGT3(1)	2.9	67.8
	FUGT3(2)	2.88	68.0
20	FUGT3(1)	2.75	69.4
	FUGT3(2)	2.8	68.9
25	FUGT3(1)	2.5	72.2
	FUGT3(2)	2.5	72.2
30	FUGT3(1)	2.45	72.8
	FUGT3(2)	2.1	76.6

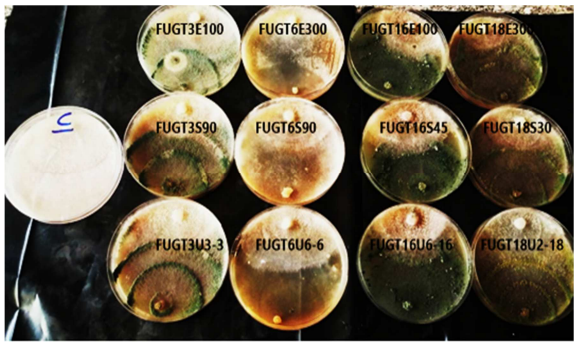

Fig. 4. Inhibition of mycelial growth of *Rhizoctonia solani* by *Trichoderma* mutants after three rounds of successive sub-culture on PDA.

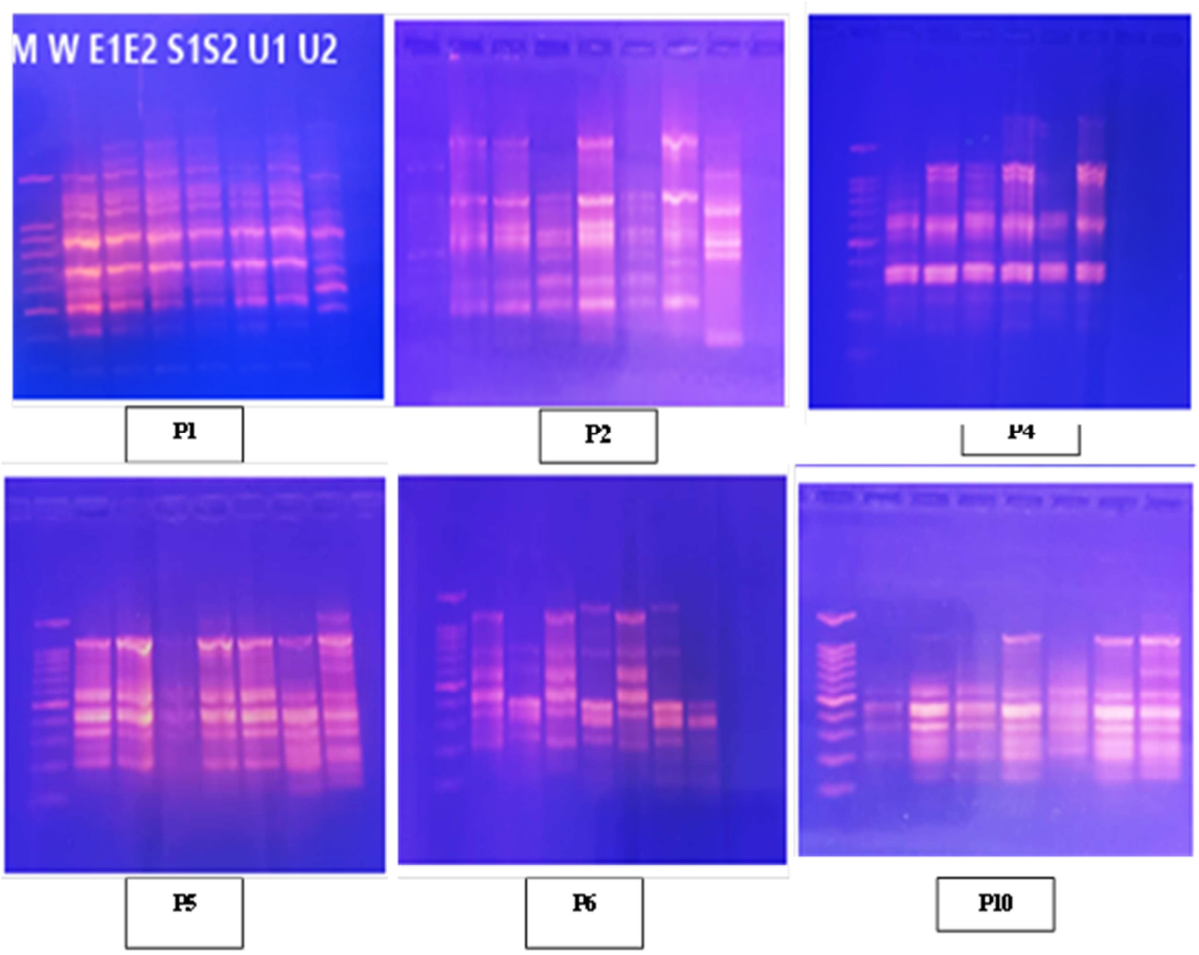

Fig. 5. Grouping of each *Trichoderma* wild type and its mutants induced by mutagen agents based on genetic similarity. Unweighted pair-group method algorithm dendrogram of relative genetic similarity among parent strain and 6 mutants (A: FUGT3, B: FUGT6M C: FUGT16 and D: FUGT18), calculated in the ISSR analysis with 6 primers and using Jaccard's coefficient.

Quantitative screening of Trichoderma mutants for their cellulase production

The selected mutants were subjected to quantitative analysis by shake flask cultures. A total of 12 mutants, isolated after UV were scored as improved putative cellulase mutant colonies on the basis of a comparatively large diameter of clear zones around the colonies on plate-screening medium as compared to the wild strain; these were analyzed quantitatively in submerged fermentation for their potential cellulase enzyme activity (Table 5) All computed activities of the mutant strains were variable and higher than those of the parental strain. However, an isolates picked after UV for 30 min showed hig produced cellulase 1, 1 and 0.92 IU/ml compared its wild type 0.08, 0.05 and 0.3 IU/ml respectively. These mutants were selected as the best isolates for hyperactivity of extra cellular cellulases and labeled MET18, MST6 and MUT16. There was a significant difference between the enzymatic activity of the wild strain. However, all the mutants exhibited significantly higher activity of cellulase enzyme than the wild type strain. The data analysis revealed that MET18 and MST6 demonstrated about a 14 fold enhancement in enzyme activity. The wilde type of *Tricchoderma* was mutagenized and genetically modified to develop a mutant strains capable of exhibiting high levels of cellulase activity because fungal strains have a unique character to pass over the environmental stress including chemical and irradiative mutagenesis and are highly susceptible to various physical mutagenic agents.

Table 6. Quanitative screening of *Trichoderma* mutants for their cellulase production after three rounds of successive sub-culture on PDA.

Mutants		Total protein	Celulase assay (U/ml)
FUGT4 WT		0.24	0.104
MUT4-1	(5 min)	0.97	0.477
MUT4-2	(5 min)	1.20	1.000
MUT4-3	(10 min)	1.20	1.000
MUT4-4	(10 min)	0.85	0.613
MUT4-5	(15 min)	0.34	0.477
MUT4-6	(15 min)	0.26	0.474
MUT4-7	(20 min)	0.35	0.811
MUT4-8	(20 min)	1.00	0.467
MUT4-9	(25 min)	0.15	0.481
MUT4-10	(25 min)	0.21	0.503
MUT4-11	(30 min)	0.26	0.746
MUT4-12	(30 min)	0.72	0.925

Genetic variability assay for Trichoderma wild type and its mutants using Inter-simple sequence repeats (ISSR)

The top mutants from UV were selected and ISSR analysis of genomic DNA was performed to detect genetic diversity of these mutants with the wild type by using 6 primers. The primer-wise details of DNA polymorphism detected in *Trichoderma* genotypes are elaborated in Table (1). Each of the primers produced distinct polymorphic banding patterns in all the genotypes examined.

The level of polymorphism was different with each primer among the genotypes and the number of bands observed for all the genotypes examined with each primer is presented in Table (7). Because of these genetic changes, these mutants might have exhibited different production profiles. Major and minor ISSR fragments ranging from 100 to 1200bp were attained. Within a total of 279 bands primed, 188 were polymorphic and the remaining 91 were common in all of the genotypes.

All of the primers revealed a varying degree of polymorphism among the genotypes, in the range 57.8–79.3%. The profiles obtained with ISSR primers are shown in Fig. (7). All genotypes exhibited significant variation in the expression profile of different genes, with all the primers in correspondence with their wild strain. The pattern attained by primers illustrated that all the mutant genotypes depicted entirely different patterns of DNA amplification in comparison to the native strain. Recorded dissimilarities in genetic make-up of the test mutants seemingly lead to the enhancement of their enzyme-activity potential.

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 (Walnuj and Jhon 2013). The transition from vegetative growth to conidial phase can be triggered in *Trichoderma* by the application of UV-blue light, low pH, nutrient stress, or mechanical injury to the mycelium.

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. Tulipa and Roy (2022) reported that, the antagonistic potential of stable mutated *Trichoderma* isolates as well their respective parental strains against the soil borne fungal pathogens indicated that treatment T9 of UBT-18 (*T. harzianum*) exhibited higher percentage inhibition of growth over control compared to the wild UBT-18 in case of pathogen *Fusarium oxysporum* f.sp. lentis.

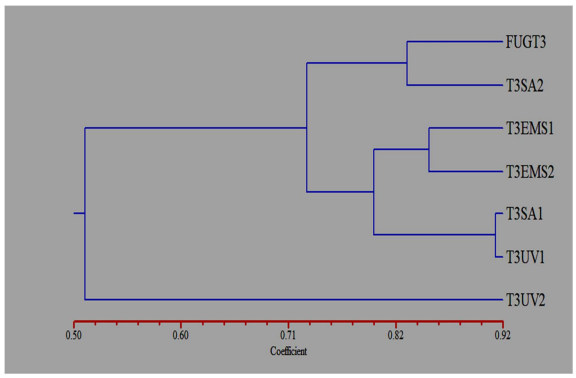

Fig. 6. Phylogenetic tree showing the relationship between *Trichoderma* isolate FUGT3 (*T. harzianum*) and its homologues strains in Genbank. The tree was constructed using MEGA6 sequence alignment programs.

Conclusion

Biological control could be the best alternative against plant pathogens, and development of mutants is an important technique in strain improvement toward plant pathogen suppression, which yields reliable strains for biocontrol. Since strains bred by mutagenesis can get registration more easily than strains produced by protoplast fusion and transformation or via gene cloning for field use, more attention should be paid to the mutagenic methods. A simple, rapid and one step mutation process was developed to induce mutagenesis in *Trichoderma* using UV irradiation. The obtained mutant strain had high potential to overproduce the cellulase.

References

Anees M, Tronsmo A, Edel-Hermann V, Hjeljord L, Heraud C, Steinberg C. 2010. Characterization of field isolates of *Trichoderma* antagonistic against *Rhizoctonia solani*. Fungal Biology **114,** 691-701.

Bastakoti S, Belbase S, Manandhar S, Arjyal C. 2017. *Trichoderma* species as biocontrol agent against soil borne fungal pathogens. Nepal Journal of Biotechnology **5,** 39-45.

Bornet B, Goraguer F, Joly G, Branchard M. 2002. Genetic diversity in European and Argentinian cultivated potatoes (*Solanum tuberosum* subsp. *tuberosum*) detected by inter-simple sequence repeats (ISSRs). Genome **45,** 481-484.

Bose JL. 2014. Chemical and UV mutagenesis. In: Bose J., editor. Vol. 1373. Humana Press; New York, NY. The Genetic Manipulation of *Staphylococci*. Methods in Molecular Biology.

Bryan G, Daniels T, Osbourn A. 1995. Comparison of fungi within the Gaeumannomyces and *Phialophora complex* by analysis of ribosomal DNA sequence. Applied and Environmental Microbiology **61,** 681-689.

Castrillo M, Bich G, Amerio N, Rodríguez M, Zapata D, Villalba L. 2021. Assessment of cellulase complex secretory capacity of *Trichoderma* strains and morphological and molecular identification of the isolate with the highest enzymatic secretion capacity. Journal Microbiology, Biotechnology and Food Sciences **10,** 1-13.

Chand P, Aruna A, Maqsood A, Rao L. 2005. Novel mutation method for increased cellulase production. Journal Applied Microbiology **98,** 318-323.

Daguerre Y, Siegel K, Edel-Hermann V, Steinberg C. 2014. Fungal proteins and genes associated with biocontrol mechanisms of soilborne pathogens: A review. Fungal Biology Reviews **28,** 97-125.

Florencio C, Couri S, Sanchez F. 2012. Correlation between agar plate screening and solid-state fermentation for the prediction of cellulase production by *Trichoderma* strains. Enzyme Research 1-8.

Gams W, Bissett J. 2002. Morphology and identification of *Trichoderma*. In: Kubicek, C. P. and Harman, G. E. (Eds.). *Trichoderma* and *Gliocladium*: Basic biology, taxonomy and genetics. Taylor & Francis Ltd 3-31.

Hermosa M, Keck E, Chamorro I, Rubio B, Sanz L, Vizcaíno J, Grondona I, Minte E. 2004. Genetic diversity shown in *Trichoderma* biocontrol isolates. Mycology Research **108,** 897-906.

Hibbett D. 1992. Ribosomal RNA and fungal systematics. Transactions of the British Mycological Society **33,** 533-556.

Ikehata H, Ono T. 2011. The mechanisms of UV mutagenesis. Journal of Radiation Research **52,** 115-125.

Kallinen K. 2016. Behavioral inhibition and activation personality traits moderate safety culture and risk behavior pp. 1-4. (Proc. Eur. Conf. Cogn. Ergon. ECCE 16).

Kang W, Park S, Lee S, Hong I, Kim W. 2004. Production of cellulases and hemi cellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. Bioresour Technol **91,** 153-156.

Kasana C, Salawan R, Dhar H, Dutt S, Gulati A. 2008. A rapid and easy method for the detection of microbial celluloses on agar plates using gram's iodine. Current Microbiology **57,** 503-507.

Kotchoni S, Shonukan O. 2002. Regulatory mutations affecting the synthesis of cellulose in *B. pumilus*. World Journal of Microbiology and Biotechnology **18,** 487-491.

Li H, Yang J, Roy B, Park Y, Jiang J, Wang D, Miao G. 2010. Enhanced cellulase production of the *Trichoderma viride* mutated by microwave and ultraviolet. Microbiological Research **165,** 190-198.

Massart S, Jijakli M. 2007. Use of molecular techniques to elucidate the mechanisms of action of biocontrol agents: A review. Journal Microbiological Methods **69,** 229-241.

Nicolas-Santiago D, Regalado-González C, García-Almendárez B, Fernández J, Téllez-Jurado A, Huerta-Ochoa S. 2006. Physiological, morphological, and mannanase production studies on *Aspergillus niger* uam-gs1 mutants. Electronic Journal of Biotechnology **9,** 50-60.

Nodvig S, Nielsen B, Kogle E, Mortensen H. 2015. A CRISPR-cas9 system for genetic engineering of filamentous fungi. PLoS One **10,** e0133085.

Papavizas C, Lumsden D. 1980. Biological control of soil borne fungal propagules. Annual Review of Phytopathology **18,** 389 - 413.

Rahman M, Rahman M, Azad A, Alam M. 2011. Inhibitory effect of different plant extracts and antifungal metabolites of *Trichoderma* strains on the conidial germination and germ tube growth of *Colletotrichum capsici* causing chili anthracnose. International Journal of Agronomy and Agricultural Research **1,** 20-28.

Samuels J, Dodd L, Gams W, Castlebury A, Petrini O. 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. Mycologia **94,** 146-170.

Shawky T, Hickisch B. 1984. Celluloytic activity of *Trichoderma* sp. Strain G, grown on various cellulose substrates. Zbl. Mikrobiol **139,** 91-96.

Snedecor W, Cochran G. 1989. Statistical methods, 8th edn. Iowa State University Press, Ames

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution **28,** 2731-2739.

Tulipa D, Roy A. 2022. Antagonistic Property of the Stable *Trichoderma* Mutants against *Fusarium oxysporum* f.sp. Lentis and *Rhizoctonia solani*. Environment and Ecology **40,** 1476-1480.

Waghunde R, Shelake M, Sabalpara N. 2016. *Trichoderma*: A significant fungus for agriculture and environment. African Journal of Agricultural Research **11,** 1952-1965.

Walnuj A, Jhon P. 2013. Role of mutation in *Trichoderma* spp. Rashtriya Krishi **8,** 68

Wang L, Yen H, Shih L, Chang C, Chang T, Wu C, Chai D. 2003. Production of xylanases from rice bran by *Streptomyces actuosus* A-151. Enzyme and Microbial Technology **33,** 917- 925.

White J, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols. A Guide to Methods and Applications **18,** 315-322.

Yasui A, Kanno S, Takao M. 2003. DNA damage and repair. Nihon Ronen Igakkai zasshi Japanese Journal of Geriatrics **58,** 577-580.

Zhang J, Zhong Y, Zhao X, Wang T. 2010. Development of the cellulolytic fungus *Trichoderma reesei* strain with enhanced β-glucosidase and filter paper activity using strong artifical cellobiohydrolase 1 promoter. Bioresource Technology **101,** 9815-9818.