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Isolation and characterization of cellulase overproducing mutants of *Trichoderma harzianum*

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ABSTRACT

Nine *Trichoderma* isolates were isolated from agricultural soil samples collected from Fayoum governorates, Egypt. Based on morphological and cultural characteristics, the *Trichoderma* isolates was identified as *Trichoderma harzianum*. All the nine *Trichoderma* isolates showed positive test for cellulose production. The percentage inhibitory effect of all the nine *Trichoderma* isolates against growth of *Rhizoctonia solani* was calculated and ranged from 57.6% to 64.9%. The isolate FUGT3 was showed the greatest antagonistic effect to the *R. solani* (64.9%) whereas isolate FUGT4 showed lowest antagonistic against *R. solani*. Cellulase producing from *Trichoderm* isolates was determined spectrophotometry and isolates FUGT3 showed highest CMCase activities (0.32 IU/ml). Molecular phylogenetic analyses indicate that the isolates FUGT3 was closely related to *T. harzianum* with accession number OL953189. The selected strain FUGT3 was subjected to mutation using UV-radiation and 12 mutants were obtained. These mutants were tested for their cellulase productivity compared to their wild type and the results showed that, the wild strain exhibited the cellulase activity 0.1 IU/ml on carboxy methyl cellulose (1.0%). Upon mutation by UV exposure the *Trichoderma* mutants produced cellulase 1 IU/ml. The 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 pattern attained by primers illustrated that all the mutant genotypes depicted entirely different patterns of DNA amplification in comparison to the wild type strain. The obtained mutant *Trichoderma* strain had high potential to overproduce the cellulase.

1. Introduction

For many years, a wide variety of microorganisms that produce different cellulolytic enzymes have been studied. *Trichoderma* is a particularly well-known genus for manufacturing cellulolytic enzymes with comparatively high enzymatic activity. According to economic assessments, the cost of producing cellulase is still a consideration. Therefore, it is essential to increase cellulase production in order to make the procedure more profitable. Thus, numerous conventional mutagenesis techniques have been used to enhance cellulase production. In comparison to other methods, this one is straightforward and frequently effective [1]. Biological control agents can be used as an alternative to chemical pesticides to control infections. A safe and effective strategy for both humans and the environment is biological control. One of the best biocontrol agents is *Trichoderma*. Many plant pathogens can be controlled by this fungus [2]. *Trichoderma* fungus was modified to increase its effectiveness as a biocontrol agent against pathogenic fungi. Following this, antagonistic properties of derived mutants and those of wild isolates were compared, and eventually some isolates were introduced that had stronger antagonistic effects than the wild isolate. By regulating specific signals and causing the production of certain enzymes or proteins that enable the pathogens to degrade, genes play a significant part in the biocontrol cycle and are therefore referred to as biocontrol genes. Increased gene expression contributes to improved biocontrol action, which supports plant growth and inhibits pathogen attack. Therefore, the biocontrol genes can be cloned and produced in large quantities for commercial applications [3]. Due to the importance shown by mutations in improving microbial products, a strong importance placed on a number of methods, including chemical mutation, UV exposure, and genetic engineering, to create improved cellulase-producing strains [4]. The success of enhanced strains' industrial applications hinges on a system that can quickly diagnose them and characterizes their genetics and physiological makeup. These mutation detection processes are based on a variety of methods, and resistant mutants can also be used for this purpose. Although random mutagenesis techniques are straightforward and simple to use, they have drawbacks like instability. One such method

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that encourages the genetic structure of the targeted species to diversify is mutagenesis. According to many investigations [5, 6], UV mutant strains of *A. niger* produce excessive amounts of cellulase. These experiments showed that the primary factors affecting the yield of cellulase production are the microorganism's proximity to the UV source and the duration of its UV exposure. The purpose of this work was to induce mutants using UV irradiation and evaluation of their antagonistic potential against the soil-borne fungal pathogens, also this work aimed to assess the genetic variability of these mutants with their wild strain using ISSR molecular marker.

2. Materials and Methods

2.1. Isolation and identification of *Trichoderma* isolates from soil samples

Trichoderma isolates were obtained from rhizosphere samples using the soil dilution plate method according to Rahman et al. [7]. The characteristics of conidiophores, such as phialides, cysts, and chlamydospores, were identified using the identification keys after the isolates had been isolated and purified [8, 9].

2.2. Qualitative evaluation of cellulase production in isolated *Trichoderma*

As stated by Wang et al. [10], *Trichoderma* isolates from various sources were qualitatively tested. The culture medium was 1% carboxymethyl cellulose (CMC) and fresh culture plugs of the *Trichoderma* isolates were positioned in the center of the plate. For 96 hours, plates were incubated at 28 °C. Separate staining preparations were also made for the replica plates. The replica plates were saturated for 20 minutes with 0.3% Congo red. After pouring out the stain, 1.0 M NaCl was used to wash the plates. Cellulase activity was determined to be present when a clear zone developed around the expanding colonies of cellulase positive cultures against dark red backgrounds. The *Trichoderma* isolates that showed good clearance outside of its growing zones were chosen for further studies.

2.3. Evaluation of *Trichoderma* species' antagonistic effects on *R. solani in vitro*

The ability of *Trichoderma* isolates to inhibit the plant pathogen *R. solani in vitro* was assessed using a dual culture methodology in accordance with the bioassay method outlined by Zhang and Wang [11] on PDA medium.

2.4. Quantitative evaluation of cellulase production in isolated *Trichoderma*

According to Shawky and Hickisch [12], the probable *Trichoderma* isolates chosen by the initial qualitative screening were subsequently assessed for enzyme production and employed for the quantitative screening of CMCase activity.

2.5. Amplification of ITS region from wild type *Trichoderma* isolate and DNA sequencing

To amplify the Internal Transcribed Spacer1 (ITS1) regions between the small and large nuclear rDNA of the chosen *Trichoderma* isolate (FUGT3), PCR was carried out 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, as described by White et al. [13]. Sequencing was done on the roughly 600 bp-long purified PCR product by lab technology services in Korea using an Applied Biosystems model 3730XL automated DNA sequencing instrument. The chosen *Trichoderma* isolate's ITS regions' nucleotide sequence was compared to ITS sequences acquired from *Trichoderma* sequences found in the GenBank database (http://www.ncbi.nlm.nih.gov/Blast). According to Tamura et al. [14], the phylogenetic tree was built.

2.6. UV radiation-induced *Trichoderma* mutants.

To avoid shielding, 1 ml of spore suspension (1x108 spores per ml) was added to a Petri plate without a lid. To treat the spores, a Philips TUV 15W lamp was put 20 cm away from the treated spores. All UV irradiations were carried out in laminar air flow under aseptic conditions for varying durations (0, 5, 10, 15, 20, 25, 30, 35, and 40 min) while stirring. To prevent any light from reaching the plates during transfer, just one plate with fresh, diluted spore suspension was put in the chamber for each time interval's needed irradiation time. After being exposed to UV light, the spores were serially diluted and then plated on PDA plates with 0.1% Triton X-100 as a colony limitation factor, which led the fungus to grow in small colonies [15]. After seven days of daily colony monitoring on the plates, the surviving spores formed small mutant colonies that were harvested, transferred to PDA plates, and incubated at 28°C for 72 hours. As a control, a plate with conidia that had not been treated was kept. Following incubation, a colony from a single spore was transplanted onto new PDA medium.

2.7. Evaluation of *Trichoderma* mutants' antagonistic effects on *R. solani in vitro*

Following each mutagenesis, the ability of each *Trichoderma* spore mutant to inhibit *R. solani in vitro* was assessed using the dual culture technique in accordance with the bioassay method outlined in the preceding section.

2.8. Qualitative and quantitative screening of *Trichoderma* mutants for cellulase production.

Following each mutation, the mutated spore suspensions were plated onto CMC screening medium and cultured at 30 °C for seven days. The quantitative cellulase activities of each spore mutant were then determined. The mutant derivatives that passed the screening process were then subjected to quantitative enzyme assays using the procedures outlined above.

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

Initially, the ISSR assay's PCR optimization was looked at, including DNA concentration, primer concentration, PCR cycle count, and annealing temperature. According to Bornet et al. [16], Table1displayed the sequence of the six ISSR primers that were employed in the current investigation to compare the genetic makeup of the *Trichoderma* wild type and its mutations.

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

No.	Primer code	Primers sequence {5'-3'}	Annealing temperature
1	ISSR1	CTACACACACACACACAC	**48**
2	ISSR 2	AGAGAGAGAGAGAGAGTA	**43**
3	ISSR 3	ACACAACAACAACAACAACAA	**48**
4	ISSR 4	AGAGAGAGAGAGAGAGACC	**48**
5	ISSR 7	CAACAACAACAACAAACG	**43**
6	ISSR 10	AGAGAGAGAGAGAGAGTA	**43**

2.10. Analysis of Data

The one-way analysis of variance (ANOVA) was used to analyze variations of the antagonistic activity according to Snedecor and Cochran [17]. Duncan's multiple range tests were also used. The amplification products ISSR were scored for the presence and absence of particular bands for each primer. The scores obtained after using all primers were pooled for constructing a single data matrix were evaluated by calculating the Jaccard‟s similarity coefficient for pair wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to cluster analysis by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and a dendrogram was generated. The computations were performed using the NTSYSpc version 2.1 program [18]. The Jaccard‟s similarity matrix was subjected to principal component analysis. The cluster analysis (dendrogram) was performed using UPGMA method and viewed through Mega 6 software, an application to obtain the confidence for certain branches of the trees.

3. Results and Discussion

3.1. Isolation and morphological identification of *Trichoderma* isolates

Utilizing different morphological traits such rapid development, colony appearance, and pigments, the *Trichoderma* isolates were identified. Nine different *Trichoderma* spp. isolates were isolated in the rhizosphere soil of two different cultivated crops, Faba bean and Wheat. *Trichoderma* isolates were conformed for species-level identification using an interactive key found at http://nt.arsgrin.gov/taxadescriptions/keys/FrameKey.cfm?gen=*Trichoderma*. According to Anees et al. [19], morphological characterization has traditionally been used to identify *Trichoderma* species and is still a viable technique for doing so. The present study demonstrated the necessity of DNA-based features for complete identification and demonstrated how the few morphological characters with limited variation may cause overlap and misidentification of the strains.

3.2. Qualitative evaluation of cellulase production in isolated *Trichoderma*

Based on the colour intensity and diameter of the yellow coloured zone encircling the colony on cellulase detection media, each of the nine *Trichoderma* isolates was screened and chosen for production of cellulytic enzyme. Results were positive cellulase producers for plates flooded with Congo red, as shown in Fig. 1and Table 2. The ability of *Trichoderma* isolates to produce cellulase is measured by measuring the area around the colony that forms as a result of their capacity to hydrolyze cellulose. After a significant amount of time has passed, clearing zones surrounding *Trichoderma* developing colonies show that they are capable of producing cellulase. This technique was utilized in numerous investigations to screen the various *Trichoderma* cellulolytic isolates [20]. Exoglucanases, endoglucanases, and beta-glucosidase must work together for cellulose to be successfully degraded during enzyme hydrolysis [11].

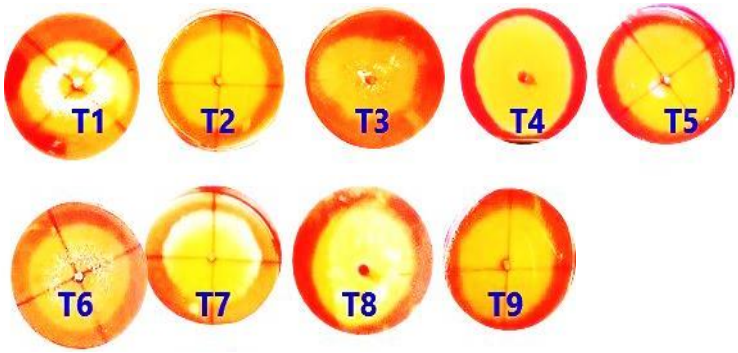

Fig.1. Screening for cellulolytic *Trichoderma* isolates by covering the petri dishes with Congo red dye. A zone of clearance surrounding the colonies is indicative of carboxymethyl cellulose (CMC) hydrolysis by secreted CMCase.

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±0.058b
FUGT2	*Trichoderma harzianum*	4.25±0.029c
FUGT3	*Trichoderma harzianum*	3.50±0.058a
FUGT4	*Trichoderma harzianum*	5. 30±0.058g
FUGT5	*Trichoderma harzianum*	5.00±0.058f
FUGT6	*Trichoderma harzianum*	4.50±0.058d
FUGT7	*Trichoderma harzianum*	4.45±0.029d
FUGT8	*Trichoderma harzianum*	4.75±0.029e
FUGT9	*Trichoderma harzianum*	4.25±0.029c

3.3. Antagonistic efficacy of *Trichoderma* isolates against *R. solani*

Trichoderma isolates were showed a significant reduction in mycelia growth of fungal colonies of *R. solani* face the *Trichoderma* spp. compared to the control. FUGT3 strain showed the highest inhibition (64.9%) of *R. solani* growth. On the other hand, FUGT4 strain showed the lowest inhibition (57.6%). The remaining isolates showed intermediate values of percentage of inhibition of *R. solani* growth (Fig.2 and Table 3). Competition, antibiosis, parasitism, and triggering host defense systems are just a few of the ways a biocontrol agent may work against pathogens [21]. The findings of the current study are in agreement with those of numerous researchers from around the world and in Egypt: a number of species in the genus *Trichoderma* are capable of parasitizing fungi that cause plant diseases like *R. solani*, producing antibiotics that are effective against soil-borne pathogens, and competing with pathogens for infection sites. *R. solani* was completely inhibited by the isolates due to an antagonist overgrowth. It has been discovered that several *Trichoderma* spp. suppress infections *in vitro* via overgrowing. According to Bastakoti et al. [22], *Trichoderma* sp. was observed to be growing on top of the colony expansion of *S. rolfsii* on the fourth day of incubation. The growth of the test fungal pathogen was discovered to be severely reduced as a result of the overgrowth of *Trichoderma* species in the plate.

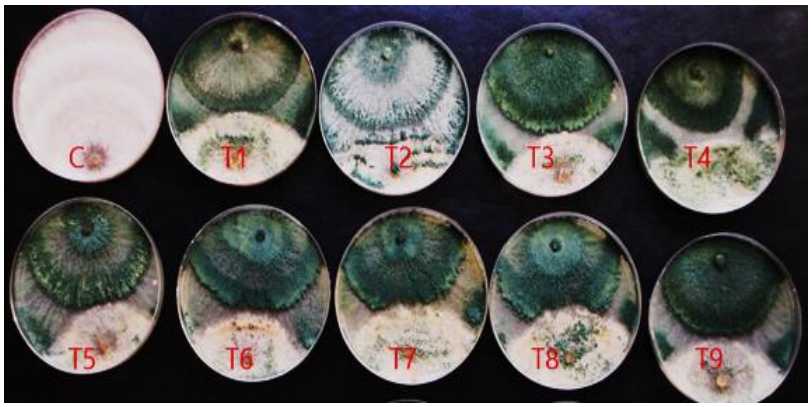

Fig.2. Inhibition of mycelial growth of *Rhizoctonia solani* by different *Trichoderma* isolates on PDA after 7 days.

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

Isolate code	*Trichoderma* species	Cellulose activity (IU/ml)	Total protein
FUGT1	*Trichoderma harzianum*	0.0406±.006a	0.3046±.057b
FUGT2	*Trichoderma harzianum*	0.0506±.006a	0.3246±.057b
FUGT3	*Trichoderma harzianum*	0.3163±.058b	0.1144±.058a
FUGT4	*Trichoderma harzianum*	0.0575±.006a	0.0989±.032a
FUGT5	*Trichoderma harzianum*	0.0474±.0057a	0.925±.032c
FUGT6	*Trichoderma harzianum*	0.0297±.0057a	0.0951±.0044a
FUGT7	*Trichoderma harzianum*	0.04647±.0057a	0.1268±.033a
FUGT8	*Trichoderma harzianum*	0.0404±.0057a	0.1327±.033a
FUGT9	*Trichoderma harzianum*	0.0569±.0057a	0.1439±.033a

*Values are means of 3 replicates, the means the having the same alphabetical letter in the column, using Duncan's multiple range test procedure at p< 0.05 level of significance.

3.4. Qualitative screening of *Trichoderma* isolates for their cellulase production

The *Trichoderma* isolates that demonstrated cellulase positive were examined for quantitative cellulase synthesis in accordance with the findings of earlier research, antagonistic activities, and qualitative screening for cellulase enzymes. The findings in Table 4 demonstrated that all of the chosen *Trichoderma* isolates were capable of producing cellulase enzyme when CMC served as the only carbon source. According to the findings, *Trichoderma* isolate FUGT3 produced the most cellulase with matching activity of 0.32 IU/mL. Florencio et al. [23] were the first to report a link between qualitative screening using the Congo red methodology and quantitative screening using the dinitrosalicylic acid reagent approach. According to Li et al. [24], *Trichoderma* spp. and *Aspergillus* spp. are believed to be cellulase producers, and the raw enzymes that these microbes manufacture are commercially available for use in agriculture.

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

Isolate code	*Trichoderma* species	*Rhizoctonia solani*	
		Mean pathogen edge (cm)	Inhibition%
FUGT1	*Trichoderma harzianum*	3.5±0.0577c	58.8±0.693b
FUGT2	*Trichoderma harzianum*	3.25±0.029b	61.7±0.529c
FUGT3	*Trichoderma harzianum*	3.00±0.058a	64.9±0.693d
FUGT4	*Trichoderma harzianum*	3.6±0.058c	57.6±0.66b
FUGT5	*Trichoderma harzianum*	3.00±0.058a	63.7±0.693d
FUGT6	*Trichoderma harzianum*	3.25±0.029b	61.7±0.529c
FUGT7	*Trichoderma harzianum*	3.5±0.033c	58.8±0.693b
FUGT8	*Trichoderma harzianum*	3.25±0.029b	61.7±0.529c
FUGT9	*Trichoderma harzianum*	3.50±0.033c	58.8±0.693b
Rhizoctonia solani		8.5±0.058d	0.00±0.00a

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).

3.5. PCR amplification of ITS region of rDNA of *Trichoderma* isolates.

Genomic DNA of the selected *Trichoderma* isolate FUGT3 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 the PCR amplification. 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 [25]. The ITS PCR has helped to detect polymorphism at ITS region of rDNA among the *Trichoderma* isolates. Sequencing of ITS-1 and ITS-2 of the rDNA gene complex was undertaken because these regions are known to be highly variable and suitable for phylogenetic studies of fungi at the interspecific and intraspecific level [26].

3.6. Sequencing of nucleotides and accession number

The strain amplified of the ITS was yielding a PCR product with 600 base pairs (bp), which was then immediately sequenced. After editing, the sequence for the *Trichoderma* isolate was uploaded to the Gene Bank, where homology searches were conducted using the Blast N and FASTA programs [National Centre for Biotechnology Information (NCBI), USA]. The closest match between each sequence and the test sample found using the Basic Local

Alignment Search Tool (BLAST) search results was utilized to identify the species of *Trichoderma* isolates. The isolate FUGT3 shared a tight relationship with *T. harzianum*, accession OL953189.

3.7. Computational analysis (Blast) and construction of phylogenetic tree.

Fig.3 shows the phylogenetic tree that was created using the ITS region of the rDNA from *T. harzianum* FUGT3 and sequences from other *Trichoderma* spp. that were obtained from sequencing databanks. They disclosed that *T. harzianum* was identified to be *Trichoderma* isolate FUGT3. The isolated *Trichoderma* strains were recognized both visually and molecularly. When compared to morphological identification, the outcomes of molecular identification were radically different. The ribosomal DNA genes (rDNA) have properties that are ideal for the species-level identification of fungal isolates. These rDNA have a mosaic of conserved and different areas within the genome and are quite stable [27]. Additionally, they can be found in tandem repeats in up to 200 copies per haploid genome, with each repetition containing the genes for the 18S small subunit (SSU), 5.8S, and 28S large subunit (LSU). Specific primers for closely related fungus species have been created using internal, transcribed spacer (ITS) regions [28]. The ITS sections of ribosomal genes were used to create primers that can be used to identify *Trichoderma* spp. In a broader sense, taxon-selective amplification of ITS regions is anticipated to become a popular strategy in molecular identification strategies.

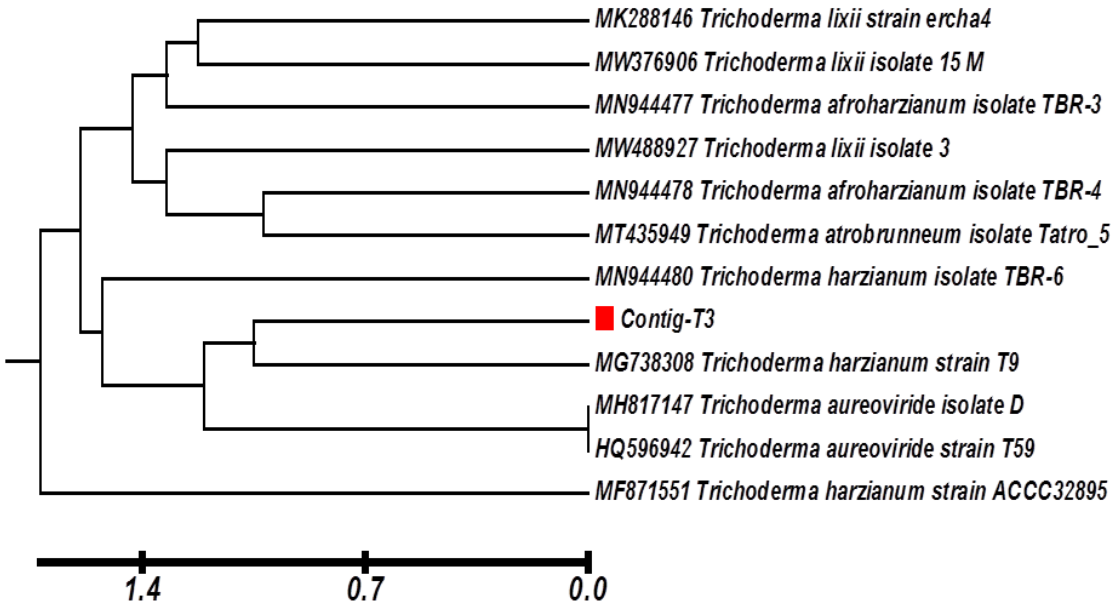

Fig.3. Phylogenetic tree showing the relationship between *Trichoderma* isolate FUGT3 (*T. harzianum*) and its homologues strains in Genbank. The tree was constructed using MEGA6 sequence alignment programs.

3.8. Induction of *Trichoderma* mutants

The findings demonstrated that 12 mutants were produced as a result of UV radiation. Several mutants that were obtained showed morphological differences in growth characteristics, including variations in colony morphology, colony colour, sporulation rate, and pigmentation. During the selection of better strains, 12 surviving colonies were created on plate-screening medium. These colonies were chosen because they produced zones of hydrolysis that were substantially greater than those produced by the matching parental strain. The percentage of conidia that survived after being exposed to UV radiation for varying lengths of time (5–40 min) was significantly decreased. After 35 minutes of UV radiation treatment, conidia died 100% of the time. When UV exposure period was steadily extended from 5 to 30 min, there was a steady rise in mutant frequency, followed by a fall. Due of its mutagenic impact on DNA structure, UV light was employed to encourage genetic diversity in wild type *Trichoderma* spp. The ability to induce genome-wide random alterations also offers an excellent option for genetic improvement, even though focused mutagenesis approaches are increasingly accessible for filamentous fungi [29]. Various UV exposures can have varied mutagenesis effects [30]. Cellular repair mechanisms such as photo reactivation, excision repair, and post-replication repair offer a likely explanation for how UV-surviving mutants were able to survive the stress brought on by DNA damage as a result of UV radiation mutagenesis [31].

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

The Twelve *Trichoderma* mutants were evaluated for cellulase production based on the antagonistic potential against *R.solani* as compared to wild strain. The results for selected mutants are presented in Fig.4 and Table 5. 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 demonstrated that, when tested against *R. solani*, all mutants induced from the wild type *Trichoderma* (FUGT3) performed significantly better than the wild type, with

the 12 mutants selected by UV treatment scoring the highest growth inhibition, ranging from 78.3% to 79.4%, compared to the wild type, which scored inhibition 64.9%. According to Li et al. [24], secretory enzyme production rose when fungi were cultured with mutagens at sub-lethal concentrations, but they also demonstrated that the dying rate was nearly 100% after UV exposure. Using a combination of microwave and UV radiation, the mutant strains M-B1, M-B2, M-B3, M-B5, and M-B7 produced significant amounts of CMCase and were stable for a lengthy time of 9 generations to create cellulase [24]. Because various strains or isolates of the same species might display varied biocontrol potential against *R. solani*, antagonism is not a characteristic of *Trichoderma* spp. According to Daguerre et al. [32], better antagonists are strains or isolates whose genes implicated in antagonist action against *R. solani* are efficiently and quickly produced.

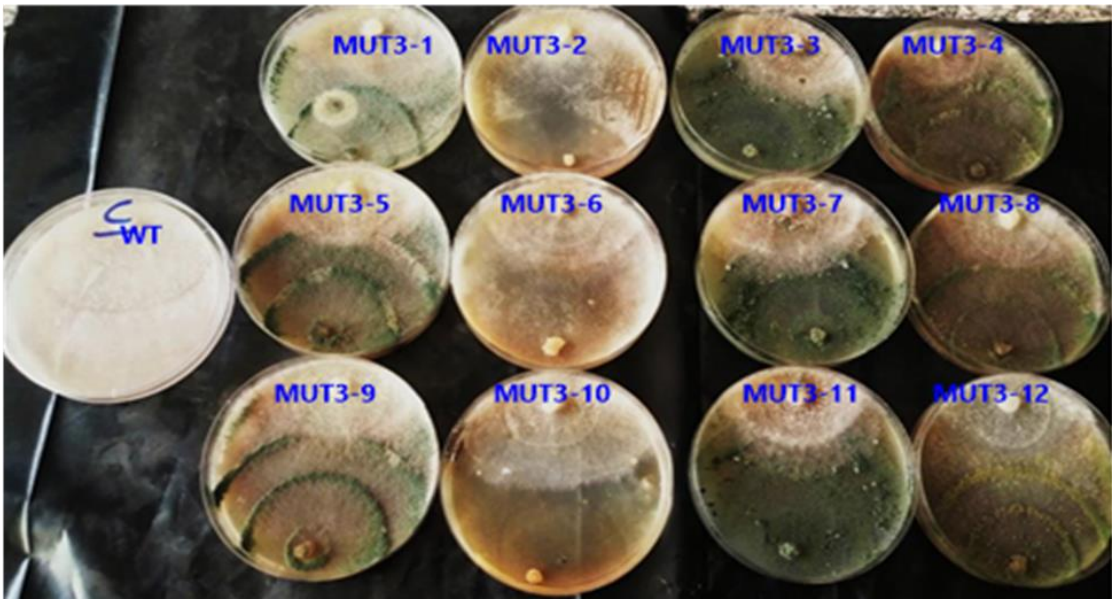

Fig.4. Inhibition of mycelial growth of *Rhizoctonia solani* by *Trichoderma* mutants induced by UV radiation after three rounds of successive sub-culture on PDA (MUT3-1 and MUT3-2) at 5 min, (MUT3-3 and MUT3-4) at 10 min., (MUT3-5 and MUT3-6) at 15min., (MUT3-7 and MUT3-8) at 20 min., (MUT3-9 and MUT3-10) at 25min., (MUT3-11 and MUT3-12) at 30 min and C(*Rhizoctonia solani*).

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±.028g	00.00±.00a
0	FUGT3 WT	3.5±.033f	61.1±.384b
5	MUT3-1 (5 min)	3.2±.058ef	64.4±.058d
	MUT3-2 (5 min)	3.25±.029ef	63.9±.058c
10	MUT3-3 (10 min)	3.0±.115de	66.7±.121e
	MUT3-4 (10 min)	2.8±.058bcd	68.9±.058g
15	MUT3-5 (15 min)	2.9±.058cde	67.8±.115f
	MUT3-6 (15 min)	2.88±.058cde	68.0±.288f
20	MUT3-7(20 min)	2.75±.029bcd	69.4±.058h
	MUT3-8(20 min)	2.8±.058bcd	68.9±.058g
25	MUT3-9(25 min)	2.5±.288bc	72.2±.058i
	MUT3-10(25 min)	2.5±.288bc	72.2±.058i
30	MUT3-11(30 min)	2.45±.288b	72.8±.115j
	MUT3-12(30 min)	2.1±.058a	76.6±.203k

3.10.

Quantitative screening of *Trichoderma* mutants for their cellulase production

The selected mutants were subjected to quantitative analysis by shake flask cultures. Twelve mutants were analyzed quantitatively in submerged fermentation for their potential cellulase enzyme activity. The results in Table 6 showed that mutant strains were variable and higher than those of the parental strain. However, an isolates picked after treated with UV for 5 and 10 min showed high produced cellulase 1IU/ml compared its wild type 0.1 IU/ml. There was a significant difference between the enzymatic activity of the wild strain and the UV-treated candidate mutants. However, all the mutants exhibited significantly higher activity of cellulase enzyme than the wild type strain. The data analysis revealed that MUT3-2 and MUT3-3 demonstrated about a 10 fold enhancement in enzyme activity. The improvement in enzyme activity may be due to the photolysis of pyrimidine's in adjacent pyrimidine's to form dimmers. They may cause error at the next replication and so result in mutation. The gene responsible for the production of cellulase may be over expressed due to mutation, as a result increase in enzyme activity [33]. The main effect of mutagenic agents is to induce a lesion in or modification of the base sequence of DNA molecule; a mutation appears if this lesion remains unrepaired [34]. The perusal of data obtained from chemical treatment revealed that EMS proved more effective as it enhanced cellulase activity to the greater extent as compared to UV. This might be due

to the fact that EMS is strong mutagenic agent and induces permanent changes in DNA structure (frame shift mutation). Similar research was conducted by Hamad et al. [35], where they reported that chemical treatment is more efficient in inducing high-level mutations as compared to UV irradiation.

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)
FUGT3 WT	0.24±.006a	0.104±.006a
MUT3-1 (5 min)	0.97±.029a	0.477±.004b
MUT3-2 (5 min)	1.20±.058a	1.000±.058e
MUT3-3 (10 min)	1.20±.058a	1.000±.058e
MUT3-4 (10 min)	0.85±.098b	0.613±.033c
MUT3-5 (15 min)	0.34±.006a	0.477±.058b
MUT3-6 (15 min)	0.26±.068a	0.474±.058b
MUT3-7(20 min)	0.35±.029a	0.811±.058d
MUT3-8(20 min)	1.00±.058a	0.467±.058b
MUT3-9(25 min)	0.15±.029a	0.481±.058b
MUT3-10(25 min)	0.21±.006a	0.503±.029bc
MUT3-11(30 min)	0.26±.067a	0.746±.018d
MUT3-12(30 min)	0.72±.015a	0.925±.021e

3.11. 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. Each of the primers produced distinct polymorphic banding patterns in all the genotypes examined (Fig.5). 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 1200 bp were attained. Within a total of 46 bands primed, 28 were polymorphic and the remaining 18 were common in all of the genotypes. All of the primers revealed a varying degree of polymorphism among the genotypes, in the range 28.5–100%. The profiles obtained with ISSR primers are shown in Fig.5. 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. Genetic similarity between the individuals was calculated from the rectangular matrix of the presence/absence of bands. The similarity matrix determined through the UPGMA method and the dendrogram generated is shown in Fig. 6. A total of 46 bands from ISSR markers were used for determination of genetic similarities and designing a phylogenetic tree for these *Trichoderma* and their mutants. According to genetic similarity, FUGT3 wild type strain and 6 mutants were grouped into two different clusters. The FUGT3WT strain and 5 mutants were grouped in the first cluster and only MUT3-12 mutant was grouped in the second cluster (Fig.6). The genetic similarity between the wild type and the mutant strain (MUT3-8) which obtained by using UV irradiation for 20 min showed low similarity with the wild type. In order to increase the efficacy of native strains, *Trichoderma* spp. have effectively undergone genetic improvement by induced mutation employing physical and chemical mutagens throughout the past few decades [36]. In *Trichoderma*, the application of UV-blue light, low pH, nutritional stress, or mechanical injury to the mycelium can all cause the change from vegetative development to the conidial phase. In comparison to their parent strains, some *Trichoderma* spp. mutants have been shown to have higher rhizosphere competence. A better approach to managing plant diseases may be to choose such advantageous variants. According to Tulipa and Roy [37], 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*. This was based on the antagonistic potential of stable mutated *Trichoderma* isolates as well as their respective parental strains against the soil-borne fungal pathogens. ISSR and ITS markers proved to be useful as genetic markers in *Trichoderma* spp. fingerprinting [38]. The advantages of the ISSRs are the requirement for small amount of DNA (5-20 ng), single short (9 to 10 bp) primers of arbitrary sequence, and the rapidity to screen for polymorphisms. In addition, no prior knowledge of sequence is required. Though ISSR has some limitations, but it is being used as one of the powerful techniques for genetic studies, for example, analysis of genetic variation in fungi and bacteria [39].

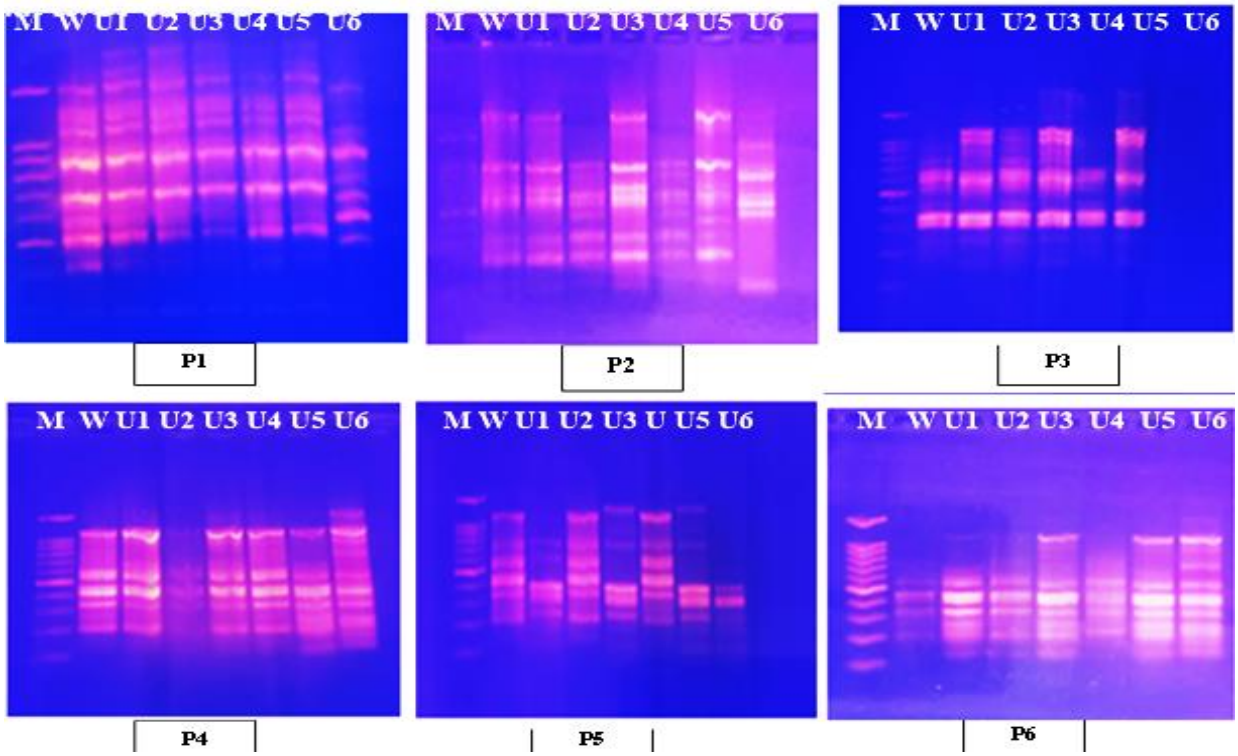

Fig.5. ISSR-PCR profile of parent *Trichoderma* strain FUGT3WT (*T. harzianum*) and their 6 corresponding mutants (lane 1 to lane 8); lane 1 (M) 100 bp DNA marker; lane 2 (W) FUGT3 wild type; lane 3 (U1) MUT3-1; lane 4 (U2) MUT3-3; lane 5 (U3) MUT3-5; lane 6 (U4) MUT3-8; lane7 (U5) MUT3-10 and lane 8 (U6) MUT3-12.

Table 7. Polymorphic bands of each ISSR primers and percentage of polymorphism in FUGT3 wild type strain and their corresponding mutants.

FUGT3					
Primer Number	**Total bands**	**Polymorphic**	**Monomorphic**		**Polymorphism %**
			Positive	Negative	
1	10	4	2	4	40
2	7	2	2	3	28.5
3	8	8	0	0	100
4	7	5	1	1	71.4
5	7	4	0	3	57.14
6	7	5	1	1	71.4
Total	46	28	6	12	

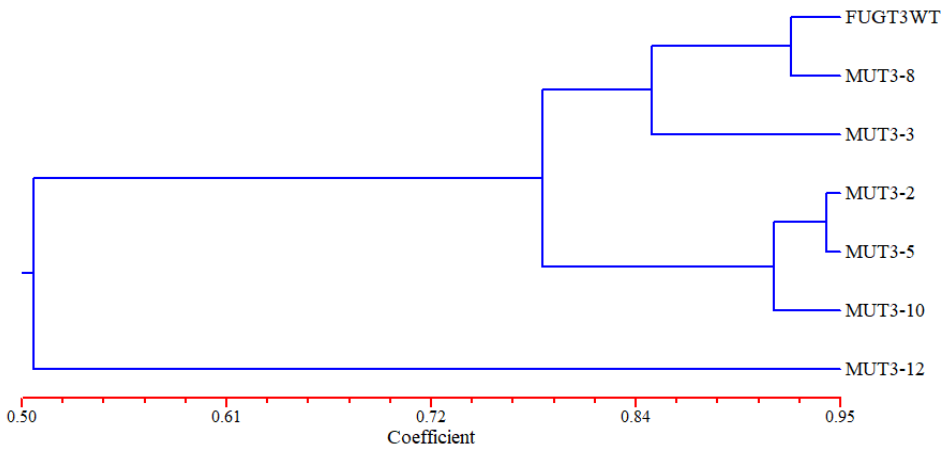

Fig.6. Phylogenetic tree showing the relationship between the *Trichoderma* wild type FUGT3WT strain and its mutants induced by UV radiation (MUT3-2, MUT3-3, MUT3-5, MUT3-8, MUT3-10and MUT3-12).

4. Conclusions

The best defense against plant diseases may be biological control, and the generation of mutants is a key strategy in strain improvement for plant pathogen suppression, which produces trustworthy strains for biocontrol. More focus should be placed on mutagenic techniques since strains developed through mutagenesis can be registered more quickly than strains developed through protoplast fusion and transformation or gene cloning for field use. UV irradiation was used to provide a straightforward, speedy, and one-step mutation approach to cause mutagenesis in *Trichoderma*. The acquired mutant strain has a significant potential for cellulase overproduction.

Author Contributions

All authors contributed to this work. E. Mahmoud and G.M. Hassan collected the samples and completed the experimental measurements. Both A. Teleb and N. Hemeda shared writing and followed the performance of the experiments. G.M. Hassan completed the paper writing, analyzing the data, and validation. G.M. Hassan followed the revision and submission of the manuscript for publication. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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