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Article

Genotypic Characterization and Mutagenesis of *Trichoderma S*trains to Enhance Cellulolytic Production

Ayaat A. Teleb*, Esraa A. Mahmoud, Nada F. Hemeda, and Gamal M. Hassan

Genetics Department, Faculty of Agriculture, Fayoum University, Fayoum 63514, Egypt

*Correspondence: aat01@fayoum.edu.eg

Abstract: Eight strains of Trichoderma were isolated from the agricultural soil of various crops, such as faba beans and wheat. Every one of the eight Trichoderma strains tested positive for the production of cellulase. All Trichoderma strains had a percentage inhibitory impact that ranged from 52.9% to 67.6% on Rhizoctonia solani growth. The highest carboxymethyl cellulose (CMCase) activity (0.42 IU/ml) was displayed by Trichoderma aureoviride (FUGT18). Based on nucleotide sequences of ITS, molecular phylogenetic analysis was carried out; the isolate FUGT18 is closely related to T. aureoviride with accession number OL953192. After treatment by mutagens using UV light and ethyl methanosulfate (EMS) on carboxymethyl cellulose (1.0%), the wild-type strains exhibited significant cellulase activities (0.42 IU/ml). Cellulase levels of 0.921 and 1 IU/ml were produced by Trichoderma mutants by using UV and EMS, respectively. Every mutant derived from the wild-type Trichoderma (FUGT18) outperformed the wild-type by a large margin when tested against R. solani. Through UV and EMS mutagenesis, as well as ISSR analysis of genomic DNA, the genetic variety between those mutations and the wild-type was revealed using different primers. The primer-derived pattern revealed that each mutant genotype had entirely distinct DNA amplification patterns compared to the strain of the wild-type.

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1. Introduction

One of mycoparasites of plant pathogens is *Trichoderma* spp that used for biocontrol of diseases. Genetic modification by mutagenesis has the potential to produce greater bioprotection, to increase their capacity to biocontrol soil-borne infections. The basis for separating useful genes and transferring them to target plants via innovative transgenics has been established by developments in molecular biology. Existing biocontrol fungi that have adapted well to their surroundings may be genetically modified to improve their biocontrol potential (Rahul *et al*., 2020). The genus *Trichoderma* has been demonstrated to mitigate plant diseases, stimulate vegetative growth, ehance the efficiency of nutrient assimilation, bolster plant resilience, and remediate agrochemical contamination (Tilocca *et al*., 2020; Fontana *et al*., 2021; Sánchez-Montesinos *et al*., 2021; Al-Surhanee, 2022).

Standard methods estimate the number of *Trichoderma* colonies to the genus or level of species using both microscopic (such as mycelium, conidiophore, phialides, conidia, and chlamydospores), and macroscopic (such as color, odor, physiological characteristics, and growth rate) factors. This genus of fungi may produce a wide range of enzymes that are utilized in agriculture for plant-growth-promoting-fungi (PGPF), and other fields as biocontrol-agents (BCA) to manage nematodes or fungal infestations (Michal *et al*., 2015).

Given the economic potential of cellulases, obtaining highly active cellulases at a reasonable cost is a crucial component of cellulose research. Cellulases are used in the manufacturing of pharmaceuticals, resins, fragrances, starch, waste management, and baking. Thus, the fundamental objective of research continues to focus on boosting the particular function of the enzymes in the cellulase complexes in order to the expense of cellulases working on pretreated biomass that is used to make bio-ethanol (Shazia *et al*., 2011).

ISSR was a rapid and easy method for analyzing variations between the mutant strains of the same species and determining the distinctions among closely associated species (Jones, 2000). To determine the genetic variability among *Trichoderma* mutants in comparison to

the wild type, the ISSR analysis of genomic deoxyribonucleic acid (DNA) was performed. The amplification patterns produced showed that all mutant genotypes displayed entirely different DNA amplification profiles when compared to the wild type strain. (Esraa *et al*., 2023). ISSR markers, which are different-sized DNA segments amplified from the same locus, are rarely identified.

Mutation techniques such as ultraviolet (UV) irradiation and chemical mutagens like ethyl methanesulfonate (EMS) have been used to induce genetic variability, often resulting in strains with improved enzymatic activity and pathogen inhibition (Rahul *et al*., 2020; Shazia *et al*., 2011).

The study's objectives are to fully identify *Trichoderma* isolates using both traditional and molecular methods, produce mutants using EMS and UV irradiation, and assess their ability to be hostile to the soil-borne fungal *Rhizoctonia solani*. Additionally, use the ISSR molecular marker to compare the genetic diversity of these mutants to their natural strain.

2. Materials and Methods

2.1. Sample collection

Specimens were collected from the root zone soil of different crops, such as faba bean and wheat, which were cultivated in fields spread around the Fayoum Governorate of Egypt. Soil particles firmly attached to the root surface were removed from each individual crop, and placed in sterile polyethylene bags. After that, these bags were brought to the lab and stored there at 4°C until they were required.

2.2. Trichoderma strain isolation from specimens collected

Trichoderma isolates were generated from root zone samples using the soil dilution plate technique as outlined (Rahman *et al*., 2011). Following daily inspections of the culture plates, each colony were separated, purified, and then moved to fresh-potato-dextrose-Agar-medium (PDA). Following the identification of different morphological features, the plates were kept at 4°C for upcoming tests.

2.3. Morphological identification

Following isolation and purification, the isolates were identified by first examining the color and shape of the colony's upper and lower surfaces, growth rates within the dextrose-agar-medium, and morphological features, such as the conidia manufacturing technique. Next, the identification-keys were used to identify conidiophore traits, including phialides, and cysts, as well as, chlamydospores (Gams *et al*., 2002; Samuels *et al.*, 2002).

2.4. Screening for cellulase production

Trichoderma isolates were qualitatively evaluated for cellulase production using culture media supplemented with 1% carboxymethyl cellulose (CMC) (Wang *et al*., 2003). As outlined in (Shawky and Hickisch 1984) the likely *Trichoderma* strains found following the initial qualitative screening were further subjected to enzyme biosynthesis analysis and utilized for quantitative CMCase activities screen. CMCase units are defined as the quantity of enzyme which releases one µmol for lowering sugar per minute (Azadian *et al*., 2017).

2.5. Induction of mutants

The UV irradiation and EMS treatments were employed to induce mutants from the *T. aureoviride* (FUGT18) strain for high cellulolytic output. For each trial, one ml of spores' suspension (1x108 spores/ ml) was put into a Petri dish without a lid to stop shielding and subjected to UV radiation for 30 minutes at 5-minute intervals while standing at a constant 20cm distance from a UV lamp had a wavelength at 254 nm and 220V at 50Hz (Hamad *et al*., 2001). To undertake chemical mutations, a suspended conidial of the same level was tested with suitable dilutions of 50, 100, 150, 200, and 250 mg/mL of EMS solution for 30 minutes at 37ºC in a water bath shaker. With this dose, a mortality rate of up to 90% was achieved (Morikawa *et al*., 1985). Following exposure or treatment, 200 µL of the suspended conidial was added to Petri plates including PDA along with 0.1% Triton X-100 and L-sorbose as colonies restrictors. At 30 ºC, the plates were incubated.

2.6. Qualitative and quantitative screening

Following each mutation, the changed spore suspensions have been placed into CMC screening media. Each spore mutant's qualitative cellulase activity has been evaluated after seven days of culture at 30°C. The mutant derivatives that had been eliminated through the aforementioned processes were then subjected to a quantitative analysis for enzymes. The reaction mix had been incubated at 50ºC for 15 minutes using a shaking-water-bath. Considering glucose as the reference, the 3,5-dinitrosalicylic acid (DNS) technique of Miller (Miller, 1959) was used to identify the converting sugars in cultures broth.

2.7. Fungicide tolerance test

Fungicide tolerance was evaluated using benomyl (NASR Zim 50% WP) at a concentration of 50 µg/mL in PDA. Mycelial discs from wild-type and mutant strains were inocu-

lated into poisoned media. Growth inhibition was calculated using Vincent's formula, and results were compared with non-treated controls (Miller, 1959).

2.8. ISSR analysis of genetic variation

Genomic DNA was extracted using the CTAB method (Crowhurst *et al*., 1995). Six ISSR primers (Table 1) were used for PCR amplification. PCR reactions (25 µL) were performed in a thermal cycler under the following conditions: initial denaturation at 94°C for 10 min; 35 cycles at 94°C for 1 min, primer-specific annealing at 43–48°C for 1.5 min, and extension at 72°C for 2.5 min; followed by a final extension at 72°C for 7 min (Crowhurst *et al*., 1995; Bornet *et al*., 2002). PCR products were separated on 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light. Banding patterns were scored, and genetic similarities were assessed using Jaccard's coefficient. Cluster analysis and dendrogram construction were performed using UPGMA in NTSYS-PC v2.1 (Rohlf, 1997).

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	-5′ CTACACACACACACAC 3′-	48
2	ISSR 2	-5′ AgAgAgAgAgAgAgAgTA 3′-	43
3	ISSR 3	-5′ ACACAACAACAACAACAACAA 3′-	48
4	ISSR 4	-5′ gAgAgAgAgAgAgAgAACC 3′-	48
5	ISSR 7	-5′ CAACAACAACAACAAACG3′-	43
6	ISSR 10	-5′ AgAgAgAgAgAgAgAgTA 3′	43

3. Results

3.1. Isolation and identification morphologically of isolated Trichoderma

Eight *Trichoderma* isolates were successfully recovered from the rhizospheres of faba bean and wheat plants. Initial species-level identification relied on morphological traits following established taxonomic keys, which remain relevant for preliminary classification despite the growing shift toward molecular diagnostics.

Morphological evaluation on PDA revealed distinct characteristics. The colonies of *T. aureoviride* were slow-growing and exhibited compact, dull green pustules, with a granular texture and a reverse pigmentation of brownish-yellow (Fig. 1A). Microscopy further confirmed the presence of typical conidiophores, conidia, chlamydospores, and spore structures (Fig. 1B). All isolates were ultimately identified as *T. aureoviride*, indicating its ecological prevalence in the sampled agroecosystems. Although morphological characterization provides a foundation for identification, this study underscores the necessity of integrating molecular tools due to potential phenotypic overlap among *Trichoderma* species (Larkin *et al*., 2007; Roberts *et al*., 2010 and Tamura *et al*., 2011).

(A) **(B)**

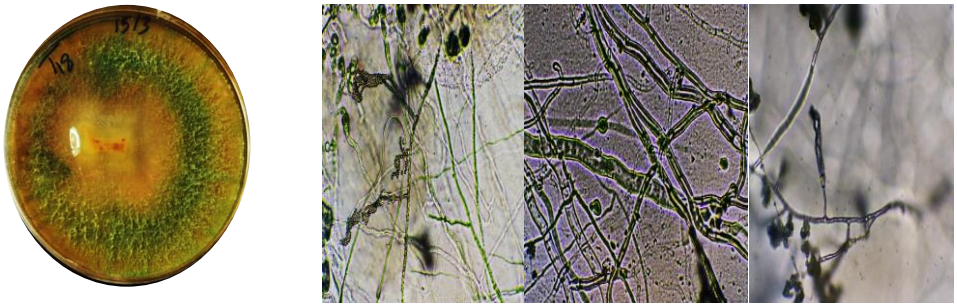

Fig. 1. (A) Colony appearances of *Trichoderma* isolate (FUGT18). (B) Microscopic features of the *Trichoderma* isolate. Conidiophore, conidia, spores and chlamydo spores of *Trichoderma* isolates cultivated on a PDA plate for seven days at 28°C.

3.2. Qualitative screening for cellulase production

Screening for cellulolytic activity was conducted using Congo red staining on CMC agar. All eight isolates displayed varying degrees of cellulase activity, indicated by the yellow clearance zones around colonies (Fig. 2). The diameter of these zones, measured in centimeters, served as a qualitative proxy for cellulase production (Table 2).

Among the isolates, FUGT18 exhibited the most prominent halo (5.00 ± 0.00 cm), indicating superior CMCase activity. Plate-based assays are convenient for high-throughput screening, their semi-quantitative nature limits precision. Numerous investigations utilizing

the Congo red methodology to evaluate the diverse cellulolytic isolates of Trichoderma have been conducted (Castrillo *et al*., 2017; Suirta *et al*., 2021; El-Sobky *et al*., 2024). Future assessments should employ fluorogenic substrates like 4-methylumbelliferone derivatives for more accurate enzyme kinetics (Kasana *et al*., 2008). This aligns with previous findings emphasizing the variability of cellulolytic potential due to genotypic and environmental factors (Shahriarnour *et al*., 2011; Fia and Giovani, 2005; Syed *et al*., 2013; Castrillo *et al*., 2021).

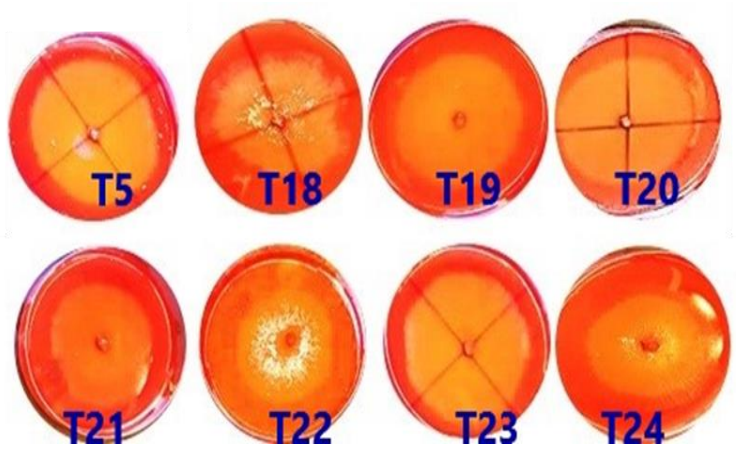

Fig. 2. Screening for isolates of cellulolytic *Trichoderma* by using Congo red dye for covering the petri dishes. A zone of clearing encircling the colonies represents the hydrolysis of carboxymethyl cellulose by secreted CMCase.

Using the clearing zone assay, a quick assessment of the tested isolates' cellulolytic activity was conducted. Clearing zones of varying sizes were present in all isolates, demonstrating the significance of this assay in the screening and pre-selection of isolates that are cellulase-positive. This technique was employed in a number of investigations to screen the various *Trichoderma* cellulolytic isolates (Suirta *et al*., 2021; Sir Lakshmi *et al*., 2012)
The variations in the cellulolytic activity of the different *Trichoderma* isolates may be caused by the isolates' genetic composition, the isolates' place of origin, and the amount of cellulase enzymes the fungus generates.

Table 2. The diameter of the yellow clearing zone around *Trichoderma* colonies on cellulase screening medium, as an indicator of CMCase activity

Isolate code	Mean of yellow zone (cm) ±S.E
FUGT5	$4.25\pm.09^{c}$
FUGT18	$5.00\pm.00^{e}$
FUGT19	$4.25\pm.09^{c}$
FUGT20	$3.50\pm.06^{bc}$
FUGT21	$2.00\pm.28^{a}$
FUGT22	$2.75\pm.14^{b}$
FUGT23	$4.75\pm.06^{d}$
FUGT24	$3.00\pm.12^{b}$

3.3. Quantitative cellulase assay

To complement qualitative findings, cellulase activity was quantified using CMC as the sole carbon source. Enzyme activities varied significantly among isolates, with FUGT18

again displaying the highest CMCase activity (0.420 IU/ml) and protein content (0.254 mg/ml), followed by FUGT24 and FUGT21 (Table 3).

Table 3. Quantitative cellulase assay of selected *Trichoderma* isolates

Isolate code	Cellulose activity (IU/ml) ± S.E	Total protein
FUGT5	$0.1213 \pm .01063^{ef}$	0.1085 ± 0.00006^{e}
FUGT18	$0.42015 \pm .0100^{cde}$	0.2543 ± 0.00006^{k}
FUGT19	$0.0406 \pm .00^{a}$	0.3046 ± 0.00058^{l}
FUGT20	0.0506 ± 0.00537^{ab}	0.3246 ± 0.00577^{m}
FUGT21	0.3163 ± 0.00867^{de}	0.1144 ± 0.00035^{f}
FUGT22	0.04113 ± 0.00593^{a}	0.1080 ± 0.00006^{e}
FUGT23	$0.1213 \pm .01063^{ef}$	0.1085 ± 0.00006^{e}
FUGT24	$0.3372 \pm .01694^{ab}$	0.1379 ± 0.00006^{i}

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) ($P<0.05$)

These results corroborate earlier findings that *Trichoderma* spp., particularly *T. harzianum*, exhibit potent cellulase and xylanase activity. The high activity of FUGT18 positions it as a promising candidate for biotechnological applications, particularly in lignocellulosic biomass degradation. Similar results were obtained in studies using *T. harzianum* and *T. viride* on different carbon sources (Wang *et al*., 2003; Pandey *et al*., 2014).

3.4. Antagonistic activity against Rhizoctonia solani

All isolates suppressed the growth of *R. solani* in dual culture assays (Fig. 3), with inhibition ranging from 52.9% to 64.7% (Table 4). FUGT18 demonstrated the highest inhibition (64.7%), supporting its potential as a biocontrol agent. These interactions are mediated via mycoparasitism, competition, and production of antifungal metabolites (Li *et al*., 2010). This antagonistic capacity is attributed to mycoparasitism, antibiosis, and competitive exclusion (Bastakoti *et al*., 2017).

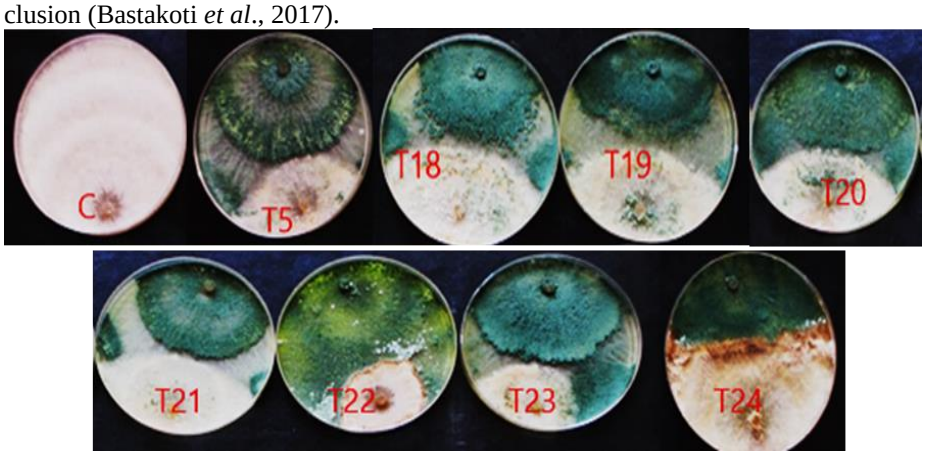

Fig. 3. *Rhizoctonia solani* colony inhibits growth by strains of *Trichoderma* on PDA after seven days.

Trichoderma spp is known to suppress soil borne pathogens through production of hydrolytic enzymes, secondary metabolites, and induction of plant defenses (Athira *et al.*, 2017; Deguerre *et al*., 2014). FUGT18's strong antagonistic activity is consistent with reports identifying *Trichderma* as an effective biocontrol agent in multiple crops (Rahman *et al*., 2011; Roberts *et al*., 2010 and Bastakoti *et al*., 2017).

Table 4. Percentage suppression of radial growth of *Rhizoctonia solani* in dual cultures involving *Trichoderma* isolates on PDA

Isolate code	*Rhizoctonia solani*	
	Mean pathogen edge (cm) ± S. E	Inhibition %
FUGT5	3±0.06^{b}	61.8±0.34^{e}
FUGT18	3±0.00^{b}	64.7±0.00^{f}
FUGT19	3.5±0.00^{d}	58.8±0.00^{d}
FUGT20	3±0.00^{b}	64.0±0.00^{f}
FUGT21	3.25±0.00^{c}	61.8±0.00^{e}
FUGT22	3.75±0.14^{e}	55.25±1.70^{c}
FUGT23	3.25±0.03^{c}	61.8±0.35^{e}
FUGT24	4±0.00^{f}	52.9±0.00^{b}
C	8.5^{g}	0.00^{a}

The main values are the averages of three replicates at the $p<0.05$ level of significance that have the same alphabetical letter in the column, according to Duncan's multiple range test protocol.

3.5. Mutagenesis and selection of high-performing mutants

Mutagenesis using EMS (ethyl methanesulfonate) and UV irradiation yielded several mutants with altered morphology and enhanced cellulase production (Table 5). EMS at 250 mg/ml produced mutants, such as MET18, with cellulase activity up to 1.0 IU/ml—a ~10-fold increase over wild-type. This confirms previous reports of EMS as an efficient mutagen for enhancing enzymatic yield in filamentous fungi (Devehand and Gwynne, 1991; Hamad *et al*., 2001; Ikehata and Ono, 2011 and Nodvig *et al*., 2015).

Table 5. Quantitative assay of cellulase production for *Trichoderma* T18 mutants (U/mL)

Wild types and Mutants *Trichoderma* strains	Total protein	Enzyme assay (U/ml)
WT18	0.1863±0.01394^{ab}	0.0882±0.00367^{a}
Ethyle methno sulfate (EMS)		
MET18 (250 µg/ml)	1.2000±0.05774^{g}	1.0000±0.24835^{bcd}
MET18 (250 µg/ml)	0.5163±0.02996^{e}	0.5140±0.17916^{bcd}
MET18 (250 µg/ml)	0.3775±0.04555^{cde}	0.4841±0.08903^{cd}
UV irradiation (at 20 cm distance)		
MUT 18 (10 min)	0.1521±0.07513^{a}	0.4814±0.02398^{b}
MUT 18 (25 min)	0.4196±0.05415^{de}	0.5271±0.02315^{bcd}
MUT 18 (30 min)	0.2335±0.03238^{abc}	0.5261±0.02315^{bcd}

The measurements represent the mean of three replicates and were analyzed using Duncan's multiple range tests. Values sharing the same letter in a column are not significantly different at the $p < 0.05$ level.

3.6. Mutant performance in antagonism and genetic stability

Mutants were re-evaluated after three sub-culture generations for consistency in antagonism and cellulase production. All retained stable enzyme activity and inhibition potential (Tables 6 and 7, and Fig. 4), confirming the heritable nature of the induced mutations. Mutants exhibiting both biocontrol and enzymatic enhancement are valuable for integrated

disease management and organic waste bioconversion. Stability across generations is a key requirement for commercial deployment (Mohsin *et al*., 2006).

Table 6. Antagonist effect of *Trichoderma* mutants against of *R. solani* after 3 rounds of successive sub-culture on PDA.

Mutants	Pathogen edge (cm)	Inhibition%
FUGT18E300(3)	1.9 ± 0.09^{ab}	78.9 ± 0.12^{d}
FUGT18S30(2)	2.3 ± 0.1^{cd}	74.4 ± 0.1^{c}
FUGT18U2-18(2)	2.5 ± 0.09^{c}	72.2 ± 0.09^{b}

The main values are the averages of three replicates at the $p<0.05$ level of significance that have the same alphabetical letter in the column, according to Duncan's multiple range test protocol.

Table 7. Quantitative assay of cellulase production of *Trichoderma* mutants after 3 rounds of successive sub-culture on PDA.

Mutants	Total protein	Cellulase assay (U/ml)
MET18 (300 µg/ml)	1.2000 ± 0.05774^{g}	1.0000 ± 0.24835^{bcd}
MST18 (30 min)	0.3459 ± 0.05439^{cde}	0.4770 ± 0.05987^{d}
MUT18 (10 min)	0.1521 ± 0.07513^{a}	0.4814 ± 0.02398^{b}
MUT18 (15 min)	0.2162 ± 0.03731^{ab}	0.5030 ± 0.04864^{bcdef}

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.

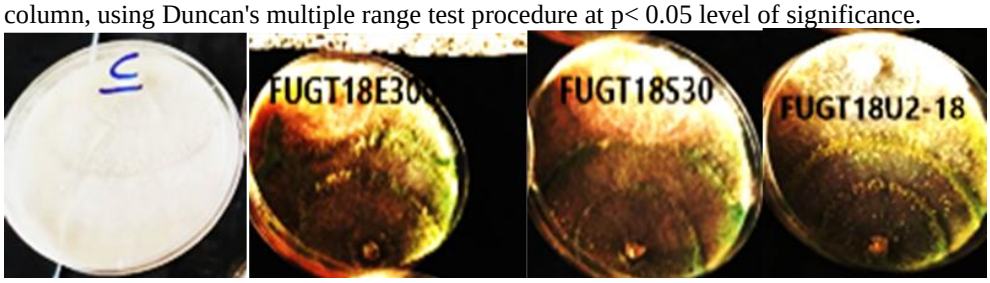

Fig. 4. *Rhizoctonia solani* colony inhibits growth by mutations of *Trichoderma* after 3 rounds of successive sub-culture on PDA.

The use of genetically modified or mutant microbial strains can lead to significantly enhanced cellulase activity. This improvement is often the result of upregulated expression of cellulase-encoding genes, leading to increased enzyme synthesis. Additionally, modifications in regulatory pathways may enhance the efficiency of enzyme secretion systems, reduce feedback inhibition, or enable the strain to better utilize substrates. Random mutagenesis, adaptive evolution, or targeted genetic engineering (e.g., CRISPR, overexpression of transcription factors) is common strategies used to develop high yield cellulase producing strains (Singh *et al*., 2024; Sveholm *et al*., 2024; Kim *et al*., 2025).

3.7. Genetic diversity via ISSR profiling

ISSR markers confirmed genetic polymorphism among wild-type and mutant strains. Six primers generated 279 bands in total, with 188 being polymorphic (67.4% average polymorphism). The highest polymorphism was recorded with primers 3 and 10 (87.5–87.6%) (Table 8 and Fig. 5).

Phylogenetic analysis clustered mutants into distinct clades, reflecting the mutagen-specific divergence (Fig. 6). This polymorphism is consistent with other reports using ISSR for fungal genotyping (Bornet *et al*., 2002; Roberts *et al*., 2010). Genomic diversity among mutants likely contributes to phenotypic variability in enzyme production and biocontrol efficacy (Azin *et al*., 2001). The ISSR analysis of genomic DNA was conducted to ascertain the genetic variability among *Trichoderma* mutants relative to the wild type, which the amplification patterns generated demonstrated that all mutant genotypes exhibited completely disparate DNA amplification profiles when juxtaposed with the wild type strain (Esraa *et al*., 2023).

Table 8. Polymorphic bands of each ISSR primers and percentage of polymorphism in T18 strains and their corresponding mutants

Primer Number	Total bands	Polymorphic	Monomorphic		Polymorphism %
			Positive	Negative	
1	11	7	2	2	63.6
2	11	7	2	2	63.6
3	8	7	1	0	87.5
4	7	6	1	0	85.5
7	8	6	1	1	75
10	8	7	0	1	87.6

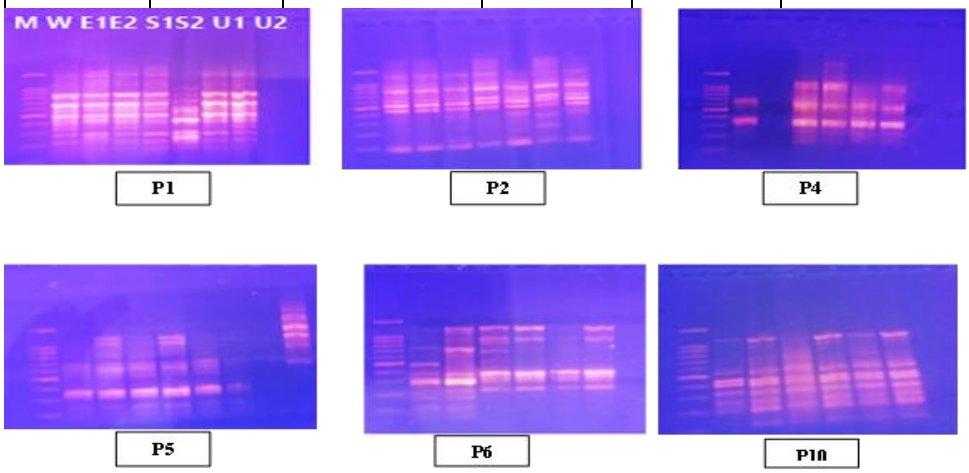

Fig. 5. ISSR-PCR profile of four parent's *Trichoderma* strains T18 (*T. aureovirde*) and their 6 corresponding mutants (lane 1 to lane 8).; M: 100 bp DNA marker; (W) wild type; (E1and E2) mutants by EMS; (S1 and S2) mutants using sodium azide and (U1 and U2) mutants using UV radiation.

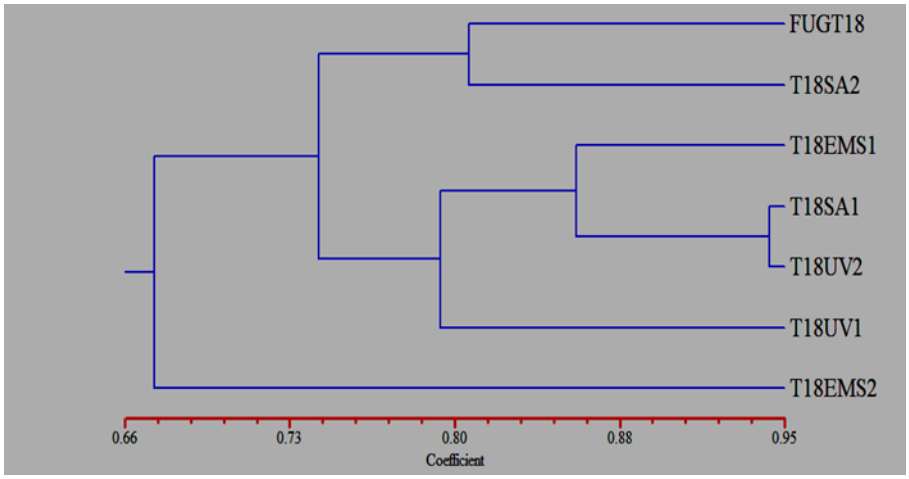

Fig. 6. Phylogenetic tree of *Trichoderma* FUGT18 wild type (parent strain) and its mutants (6 mutants) by mutagen agents based on genetic similarity. Genetic similarity calculated by NTSYS-pc 2.1, in the ISSR analysis with 6 primers.

4. Conclusions

This study successfully isolated and identified *Trichoderma aureoviride* strains from rhizosphere soils and evaluated their cellulolytic and antagonistic capabilities. Among the isolates, FUGT18 consistently exhibited the highest cellulase activity and strongest antagonism against *Rhizoctonia solani*, highlighting its potential as a biocontrol agent and industrial enzyme producer. To further enhance performance, mutagenesis via UV irradiation and EMS treatment was employed, leading to the development of mutant strains with significantly increased cellulase activity up to 10-fold higher than the wild type as well as enhanced and stable biocontrol efficacy over successive generations. ISSR profiling confirmed the genetic diversity induced by mutagenesis, supporting the observed phenotypic improvements. Such mutants hold great promise for sustainable agricultural applications and biomass degradation, combining strong biocontrol potential with elevated enzymatic production in a genetically stable framework. Enhanced Trichoderma strains exhibiting superior cellulase activity possess significant commercialization potential owing to their economical enzyme production and scalability for industrial applications, including biofuels, textiles, and the paper industry. Genetically modified Trichoderma variants facilitate more effective conversion of lignocellulosic biomass, thereby establishing them as essential components in the advancement of sustainable and economically feasible biorefineries. The elevated yield and stability of cellulases derived from optimized Trichoderma strains contribute to the reduction of downstream processing expenses, rendering them appealing for extensive enzyme markets.

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