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Antagonistic activity of *Trichoderma* fungi isolated from the soil

Otgonjargal Khureldagva[1*], Ariunjargal Jargalsaikhan[2] and Gantuya Myagmarsaikhan[1]

[1]Institute of Plant protection research, Committee 13, Bayanzurkh district, Ulaanbaatar, 13330, Mongolia
[2] Ulaanbaatar Elite International School, Seoul Street, Sukhbaatar District, Ulaanbaatar, Mongolia

*Corresponding author: otgonjargal@plantprotection.mn

https://orcid.org/0009-0001-3192-6493

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Abstract
Approximately 200 soil samples were collected from pastures, forests, and cultivated fields in Ulaanbaatar city, as well as provinces such as Tuv, Selenge, and Bulgan in 2018. These samples were diluted using Koch's serial dilution method and cultured on Potato Dextrose Agar (PDA) medium supplemented with Streptomycin (0.03 g). Pure cultures of *Trichoderma* were selected based on their morphological characteristics after 7-10 days of incubation at 25° C. The antagonistic activity of *Trichoderma* against various fungal pathogens was determined using the dual culture method. For the identification of *Trichoderma* species, DNA was extracted using CTAB buffer, and the ITS1 and ITS4 rDNA regions were PCR amplified, purified, and sequenced at Macrogenes, in South Korea. The sequences were compared to those in the NCBI GenBank database to establish phylogenetic relationships. *Trichoderma* was detected in 11 samples out of the approximately 200 soil samples. Eleven pure cultures of *Trichoderma* fungi were isolated and identified as *T. harzianum, T. citrinoviride, T. sinuosum,* and *T. virens* based on molecular biology and sequence analysis. When tested for antagonistic activity against *A. alternata, Cladosporium fulvum*, and *F. oxysporum* pathogens, all 11 pure cultures of *Trichoderma* showed more than 54% activity. On average, the *T. citrinoviride 31/4* pure culture showed 67.8% antagonistic activity, while the *T. harzianum 186/4* pure culture showed 70.5% antagonistic activity.

Keywords: Plant pathogens, biocontrol, antagonistic fungi

Introduction

The genus *Trichoderma* was described by Christiaan Hendrik Persoon in 1794. *Trichoderma harzianum* is a highly active fungus against major pathogens such as *P. notatum, R. solani, F. oxysporum, A. solani, A. alternata, and P. infestans*, which affect crops like wheat, tomatoes, potatoes, onions, rice and corn. In India several products, including Bioderma H, Bioharz Commander Fungicide, Sardar EcoGreen, Ecosom-TH Lyophilized, and Tricone V have been developed and introduced for production using *Trichoderma harzianum*. These products offer an effective alternative to synthetic chemicals, providing environmentally friendly solutions that are harmless to humans and animals [1]. This beneficial fungus is used worldwide to produce over 250 types of biopesticides for agriculture, farming, and plant protection. *Trichoderma* fungi play a key role in enhancing soil fertility by breaking down organic compounds into essential minerals. *Trichoderma* fungi contribute significantly to soil fertility by decomposing organic matter in the soil into valuable minerals. They typically grow at temperatures between 25-30 C, although some species can survive at temperatures as high as 45 C. On a PDA nutrient medium, the fungus generally forms green-colored mycelium within a week, although some species may produce mycelium with a slight yellowish variant. *Trichoderma* reproduces through asexual spores, which do not form in sporangia but instead develop on specialized reproductive hyphae. Additionally, the hyphae can fragment to form new structures. The conidiophores have a highly branched structure, originating from slightly septate hyphae, making them easy to measure [1]. *Cladosporium fulvum* (Cooke) is the fungus responsible for cladosporiosis, a disease that primarily affects tomatoes in greenhouses. In recent years, the disease has been observed spreading in greenhouse areas near hearing

facilities, beginning in August, with a spread rate of 15-20% [2].

Fusarium is characterized by its ability to infect the stem, upper plant parts, branches, and petioles, damaging the plant. The fungus attacks the main stem, leading to wilting and drying of the plant. As a result, the plant experiences a lack of water and minerals, leading to a halt in metabolism and overall drying and decaying of the plant. As a result, the plant experiences a lack of water and minerals, leading to a halt in metabolism and overall drying and decaying of the plant. *Fusarium* not only affects living plants but can also spread to stored produce under suitable conditions, damaging fruit skins and causing decay [2].

Alternaria disease significantly affects tomatoes in greenhouses. It is characterized by large brown spots with concentric rings appearing on the leaves, causing them to dry out and die. These spots can spread and merge with each other. Damaged leaves dry out and die. On the fruit, sunken spots with blackish mold appear, and spotting can also occur on the stems [2].

It is important to identify the potential of producing eco-friendly biological biofungicides effective against plant diseases by isolating antagonistic *Trichoderma* fungi.

The objectives of this study were to isolate *Trichoderma* from the soil and assess its ability to control certain plant diseases.

Materials and methods

Soil samples

A total of approximately 200 soil samples were collected from forests, pastures, and agricultural fields in the Tuv, Selenge, and Bulgan provinces, as well as around Ulaanbaatar.

Method for isolating pure fungal cultures from soil

Soil samples were diluted using the Koch method up to a dilution of 10^{-4} and cultured on PDA medium with the addition of 0.03g Streptomycin, and incubated at 25 ± 0.5°C for 7-10 days. Colonies with a morphology resembling *Trichoderma* were selected and incubated on PDA medium at 25±0.5°C for 7-10 days to isolate pure cultures. For fungal species identification, DNA was extracted using CTAB buffer, and the ITS1-5’ TCCGTFGGTGFFCCTGCGG and ITS4-5’ TCCTCCGCTTATTGATATGC regions of rDNA were amplified via PCR. The purified products were sequenced by the Macrogen company in South Korea. The sequences were then analyzed using the NCBI GenBank database to construct a phylogenetic tree and determine species relationships

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Method for determining antagonistic activity

To determine antagonistic activity against pathogens such as *A. alternata, C. fulvum, and F. oxysporum*, the dual culture method was used.

Dual culture method:

In PDA medium, a slice of the pathogen fungus and the fungus being studied (approximately 6-8 mm in diameter) were placed into the same petri dish using sterile tools. One slice of the pathogen and one slice of the fungus under study were placed in a single petri dish and incubated at 25°C for 7 days. The antagonistic activity was determined by measuring the extent of inhibition of colony growth.

Statistical Analysis

The antagonistic activity data were statistically analyzed using Excel’s Data Analysis tool with an ANOVA test ($\alpha = 0.05$). Data were considered statistically significant when $P < 0.05$.

Results

Based on the research conducted to isolate *Trichoderma* fungi from approximately 200 soil samples, 11 pure cultures with similar morphological characteristics were identified. These cultures were predominantly found in forest soil (Table 1).

Table 1.

Pure cultures of *Trichoderma* fungi isolated from soil

No.	Cultures	Species name	Soil sample collected location	Area
1	3-4	*T. citrinoviride*	Ulaanbaatar, Zaisan	Forest
2	31-4	*T. citrinoviride*	Bogd mountain	Golf course
3	75-2	*T. harzianum*	Bayanchandmani soum, Tuv province	Forest
4	93-3	*T. citrinoviride*	Bayangol, Bornuur soum, Tuv province	S-1 15-20
5	96-3	*T. citrinoviride*	Tuj Pine, Shaamar soum, Selenge Province	Forest
6	114-3	*T. citrinoviride*	Gurt bridge, Khutag-Undur soum, Bulgan province	Forest
7	115-3	*T. citrinoviride*		Forest
8	116-3	*T. sinuosum*		Forest
9	186-4	*T. harzianum*	Nuht, Ulaanbaatar	Around river
10	198-3	*T. citrinoviride*	Sharga morit	Forest
11	198-4	*T. virens*		

Some morphological characteristics of Trichoderma

Eleven pure cultures similar to the *Trichoderma* genus were cultivated in PDA nutrient medium to determine their morphological features. They formed colonies with green, light green, and yellow-green mycelium. Under a microscope at 1600 x magnification, they exhibited septate hyphae, branched conidiophores, and spherical spores.

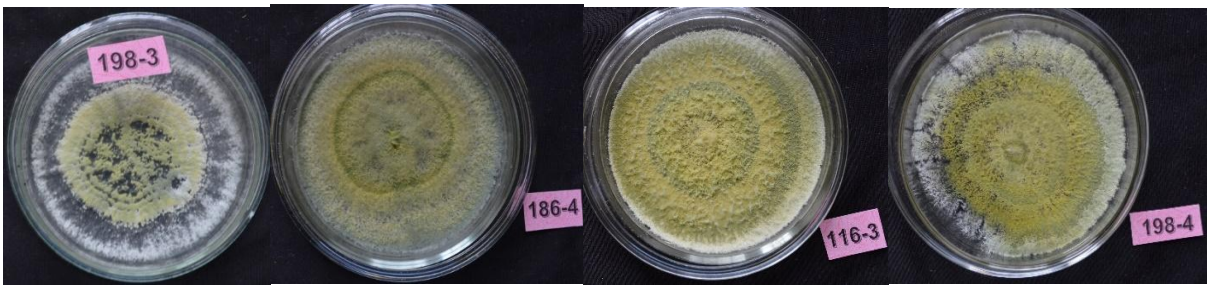

Figure 1. Mycelium of *Trichoderma* / in PDA medium /

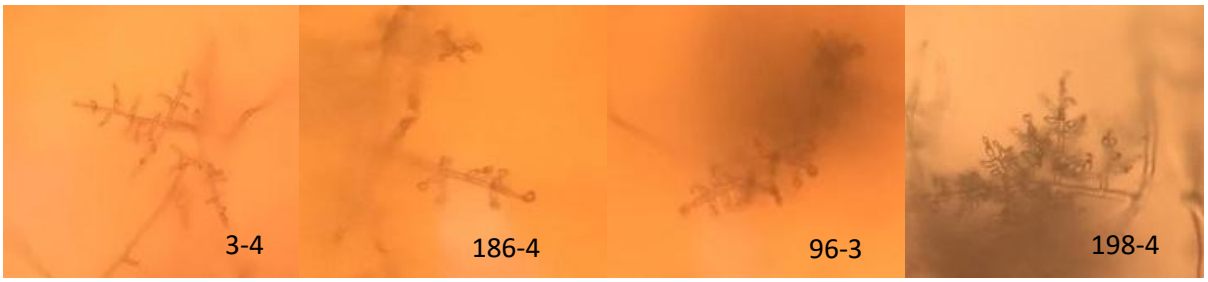

Figure 2. Phialide and spore of *Trichoderma* / magnification x 640 /

Results of sequence analysis of Trichoderma fungi

DNA was extracted from the isolated pure cultures of fungi (3-4, 31-4, 75-2, 93-3, 96-3, 114-3, 115-3, 116-3, 186-4, 198-3, 198-4), and PCR was conducted. The reaction products were purified, and genomic sequencing was performed by Macrogen company in South Korea. Upon searching the nucleotide sequences of the samples using the NCBI BLAST search system, they matched with species *T. harzanium, T. citrinoviride, T. sinuosum, and T. virens* with a similarity of 98.63% to 99% (Figure 3).

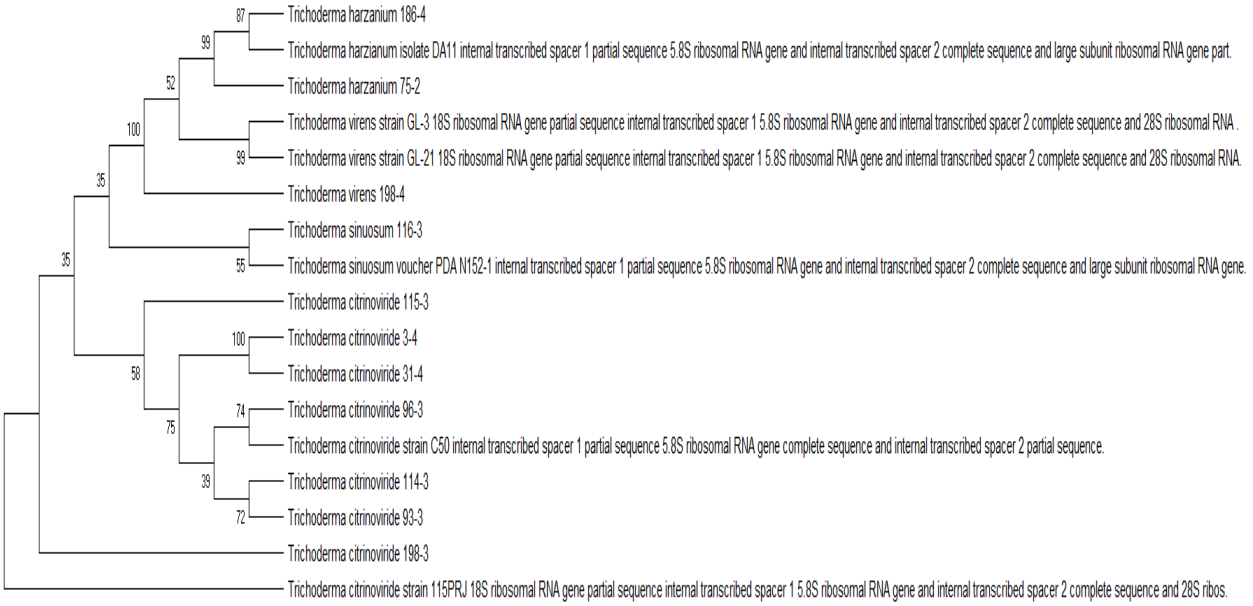

Figure 3. A phylogenetic tree constructed using the Neighbor-Joining method, with Bootstrap values calculated based on 1000 replications, incorporating sequences stored in GenBank and those identified in the study. The nucleotide sequences *of T. harzanium, T. virens, T. citrinoviride, and T. sinuosum* identified in the study were compared to reference sequences from GenBank: MF144552.1, MN396739.1, HQ654903.1, AF099008.1, AF099006.1, KU319049.1.

Antagonistic activity of Trichoderma fungi against pathogens

The study evaluated the antagonistic activity of the 11 isolated pure cultures against tomato pathogens, specifically *A. alternata, C. fulvum,* and *F. oxysporum*, using the dual culture methods. The experiment was conducted on each pathogen with 12 variants in 3 replicates over 7 days. On the 7th day, the colony radius of the pathogenic fungi was measured, and the antagonistic activity was assessed by comparing it to the control variant (Table 2, Figure 4).

Table 2.

Antagonistic activity *of Trichoderma*

Variants	*Fusarium*		*Cladosporium*		*Alternaria*		Antogonist activity /average %/
	Colony radius /mm/	Antogonist activity %	Colony radius /mm/	Antogonist activity %	Colony radius /mm/	Antogonist activity %	
Control	49		39		40		
T. citrinoviride 115-3	26	46.94	16	58.97	14	65.00	56.97
T. citrinoviride 198-3	19	61.22	16	57.69	12	70.00	62.97
T. citrinoviride 114-3	21	57.14	16.5	57.69	16	60.00	58.28
T. citrinoviride 96-3	15	69.39	15.5	61.54	13	67.50	66.14
T. harzianum 186-4	16	67.35	12	69.23	10	75.00	**70.53**
T. harzianum 75-2	26	46.94	14	64.10	17	57.50	56.18
T. citrinoviride 31-4	20	59.18	14	66.67	9	77.50	**67.78**
T. citrinoviride 3.0-4	24	51.02	15	64.10	17.5	56.25	57.12
T. virens 198-4	26	46.94	15	61.54	18	55.00	54.49
T. sinuosum 116-3	24	51.02	17	55.98	17	57.50	54.83
T. citrinoviride 93-3	23	53.06	15	61.54	12.5	68.75	61.12

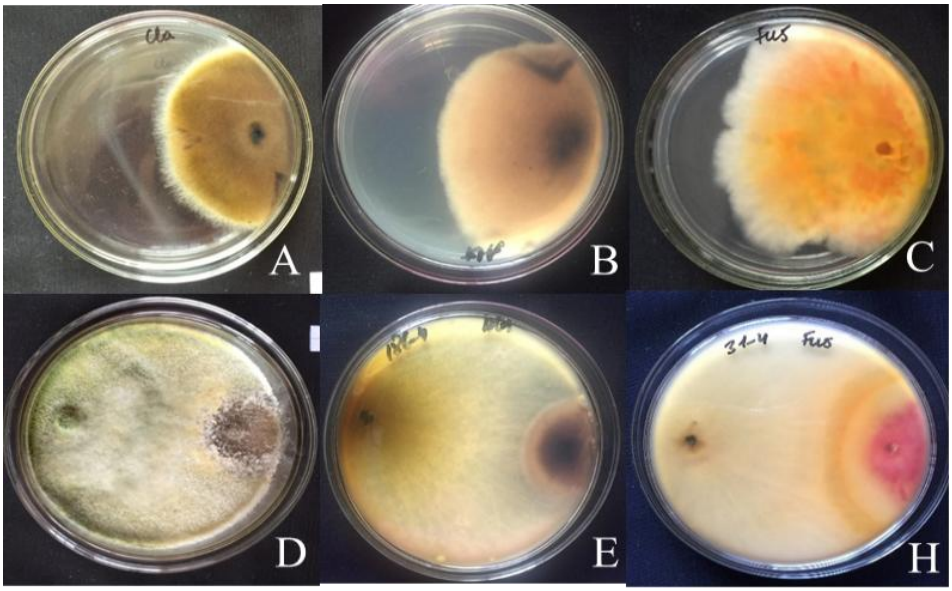

Figure 4. A-Control of *C. fulvum*, B-Control of *A. alternata*, C-Control of *F. oxysporum*, D-Culture of *T. harzianum 186/4* with *C. fulvum*, E-Culture of *T. harzianum 186/4* with *A. alternata,* H-Culture of T. *citrinoviride 31/4* with *F. oxysporum.*

The results indicated that most cultures exhibited over 50% antagonistic activity. For *A. alternata*, the most active were *T. citrinoviride 31-4, T. harzianum 186-4,* and *T. citrinoviride 198-3;* for *C. fulvum, T. citrinoviride 31-4* and *T. harzianum 186-4;* and for *F. oxysporum, T. citrinoviride 96-3 and T. harzianum 186-4* showed the highest antagonistic activity. The results demonstrated that *T. harzianum 186-4* exhibited 70.53% antagonistic activity against the three tested pathogens, while *T. citrinoviride 31-4* showed 67.78% antagonistic activity. The ANOVA test revealed significant differences between the variants ($p < 0.05$).

Discussion

The Polish researcher Lidia Błaszczyk isolated 170 cultures of *Trichoderma* from soils and decaying wood, identifying 14 species based on morphological characteristics and analysis of their ITS1 and ITS2 nucleotide sequences. The most abundant species (25%) among the isolates was *T. harzianum* [3] .

Indian researcher Pandey found that two strains of *Trichoderma* exhibited 66-67% antagonistic activity against the disease caused by *A. Alternata* [4]. Egyptian researchers Kamal A. M. Abo-Elyousr1 reported that nine pure strains of *Trichoderma* showed 70-73% antagonistic activity against the pathogen of *A. porri,* which is similar to our research work [5]. Indian researcher determined the antagonistic activity of *Trichoderma* against the pathogen *Cladosporium spherospermum* using the dual culture method, finding it to have 72% antagonistic activity [6]. Researcher Pakkala Abhiram et al. also assessed the antagonistic activity of *Trichoderma viride* against the pathogen *Fusarium oxysporum* using the same method, finding it to exhibit 71.00% antagonistic activity [7]. Fifteen *Trichoderma* antagonists were isolated from tomato rhizosphere. Under in vitro conditions, the results showed that the *Trichoderma harzianum* (ANR-1) isolate effectively inhibited the radial mycelial growth of the *Fusarium* pathogen by 53%, compared to all other isolates [8].

In the first study of its kind in Mongolia, we isolated *Trichoderma* fungi from the soil for potential use against plant diseases and investigated its antagonistic activity against pathogens such as *Fusarium, Alternaria, and Cladosporium*. The strain of *T. harzianum 186-4* showed antagonistic activities of 75% against *A. alternata*, 69.2% against *Cladosporium*, and 67.3% against *Fusarium*, which are similar to the results of the above-mentioned researchers. Future research into the production of biofungicides using *Trichoderma* cultures could lead to the development of eco-friendly biofungicides that effectively combat plant pathogens, which coluld be integrated into plant protection strategies.

Conclusion

1. Eleven pure cultures of *Trichoderma* fungi were isolated from the soil, and molecular biology and sequence analysis confirmed their identification as *T. sinuosum, T. harzanium, T. citrinoviride, and T. virens.*
2. All pure cultures exhibited antagonistic activity against the pathogens *A. alternata, C. fulvum, and F. oxysporum*, with all cultures showing over 54% activity. Notably, the pure culture *T. citrinoviride 31*/4 displayed 67.8% antagonistic activity, while *T. harzianum 186/4* showed 70.5% antagonistic activity.

Acknowledgment

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Contribution

O.Kh. designed the concept of this study. A.J., O.Kh., G.M., T.J. equally conducted laboratory work. A.J., T.J., analyzed data and validated. Writing including original draft preparation, review, and editing was performed by O.Kh. All authors read and approved the final manuscript.

Conflict of Interests

The authors declare no conflict of interests.

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