Study on *Trichoderma Citrinoviride* 31/4 – Antagonistic Activity and Jasmonic Acid

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Abstract — The genus *Trichoderma* was first identified by the German scientist Persoon in 1974, and there are currently 89 species in the genus. *Trichoderma* fungi often break down organic compounds in the soil into minerals and plays an important role in soil fertility formation.

Trichoderma citrinoviride 31/4 isolated from the soil forms a colony with light yellow-green mycelium on the surface of the PDA medium, with spherical spore, septate hyphae, branched conidophores.

Trichoderma citrinoviride 31/4 had antagonistic activity of 66.7% against *Cladosporium fulvum*, 77.5% against *Alternaria alternata*, and 59.1% against *Fusarium oxysporum* after 7 days.

Experimental results showed that the progress of tomato alternariosis treated with *Trichoderma citrinoviride* 31/4 was neutralized from the 7th day, and the biological activity was averagely 74% on the 21st day.

Trichoderma citrinoviride 31/4 contains 0.0135 mg / kg of jasmonic acid in plants treated with 10^9 cell / ml and 0.0076 mg/kg in plants treated with 10^8 cell / ml.

Index Terms — fungi, *Alternaria alternata*, biological activity, tomato.

I. INTRODUCTION

Trichoderma is a common fungus in the soil of the genus Hypocreaceae. *Trichoderma* fungal species differ from each other in their characteristics such as growth, conic growth, conidia and conidophores, and branching. The genus *Trichoderma* was first identified by German scientist Persoon in 1974, and there are currently 89 species in the genus. *Trichoderma* fungi often break down organic compounds in the soil into minerals and plays an important role in soil fertility formation. It grows at 25–30 °C, but for some species it grows at temperatures up to 45 °C. The PDA medium usually produces green micelles within a week, and some species produce slightly yellowish mycelium. *Trichoderma* produces the enzymes chitinase and glucanase and breaks down the cell walls of pathogenic fungi [15].

Trichoderma species are cosmopolitan fungi frequently present in all types of soil, manure, and decaying plant tissue [5].

The fungus *Trichoderma citrinoviride*, is a widespread soil fungus of the longibrachiatum clade in the genus *Trichoderma*. *T. citrinoviride* is one of the most common species of *Trichoderma* communities isolated from soil [16].

Trichoderma spp. are also used as BCAs against plant pathogenic fungi such as *B. cinerea*, *Fusarium* spp., *Pythium* spp., and *Rhizoctonia* spp [16].

Endophyte fungus *T. citrinoviride* is a highly active biocontrol agent against ginseng disease [12].

Trichoderma is effective in breaking down soil compounds and improving plant growth and soil nutrients. *Trichoderma* fungal spores are used for watering, spraying the leaves during transplanting, and before and after treatment. *Trichoderma*-based products are marketed worldwide and are used to control soil pathogens such as *Pythium* and *Rhizoctonia* in farms, nurseries and gardens [6].

Using this beneficial fungus, it is possible to develop and introduce a technology for the production of biofungicide for the control of fungal pathogens in greenhouse crops.

II. MATERIALS AND METHODOLOGY

A. Soil Samples

Taken from 0–20 cm deep soil in golf course, Bogd mountain, Ulaanbaatar city.

B. Method for Isolating Pure Fungal Cultures from Soil

After diluting the soil sample with up to 10–3 saline solution by Koch reduction dilution method and sterilizing in potato glucose agar (PDA) culture medium, pure culture was isolated by adding 0.03 gr Streptomycin and incubating at 25 °C for 7 to 10 days, transferring from green and yellow-green single-cell colonies to potato glucose agar (PDA) medium and incubating at 25 °C for 7–10 days.

C. ITS Sequencing and Phylogenetic Analysis

Fungal DNA was isolated using the CTAB buffer method. The polymerase chain reaction amplified the DNA control portion of the genome. ITS1–5'TCCGTFGGTGFFCCTGCGG and ITS4–5'TCCTCCGCTTATTGATATGC primers were used in the gDNA control zone to identify the fungal species. The total PCR reagent is calculated at 50 µl, 5 µl of 10x Dream buffer, 1 µl of dNTP, 1 µl of each primer, 3 µl of sample, 1 µl of polymerase (Dream taq polymerase), was dissolved in 39 µl of ultra-sterile water (Thermofischer Oltrapure), predenation of the reaction conditions at 94 °C for 5 min, 35 cycles: 30 sec at 94 °C, 30 sec at 58 °C, 30 sec at 72 °C, and multiplied

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by configuring for 7 min at 72 °C for the final lengthening step (My Genie™ 32 Thermal Block, Bioneer). The PCR generated product was tested by 1.5% agarose gel electrophoresis. PCR results were calculated revealing 1,500 xH gel and further study on PCR gene sequence identification study was conducted at Macrogen in the Republic of Korea, compared with a sequence registered with Genbank (NSBI), identified genetic relationship and determined the species.

D. Determination of Antagonistic Activity

a) Culturing pathogen: Three pathogens, such as *Alternaria alternata*, *Cladosporium fulvum*, and *Fusarium oxysporium*, were incubated in potato glucose agar (PDA) medium at 25 °C for 7 to 10 days.

b) Culturing antagonistic fungal: *Trichoderma* fungal culture was inoculated on potato glucose agar medium at 7 °C for 7–10 days.

c) Determining antagonistic activity: Potato glucose agar (PDA) is drilled into an agar slice (approximately 6–8 mm in diameter) in a culture medium with sterile instruments from pathogenic fungi and *Trichoderma* cultures. Placed one slice of the pathogen and 1 slice of *Trichoderma* in a Petri dish, incubated at 25 °C for 7–14 days, and the antagonistic activity was determined by dominating colony by the following formula:

$$\frac{R1 - R2}{R1} \times 100 = \%,$$

where

R1 – The diameter of the fungus grown in the control dish.

R2 – The diameter of the fungus grown in Petri dish with *Trichoderma*.

E. Determining Biological Activity against Tomato alternariosis

a) Culturing *Alternaria alternata*

The plant pathogen *Alternaria alternata* was incubated in PDA medium at 25 °C for 10 days, washed with a 0.1% solution of Twin-80 and 10⁵ spores suspension was prepared.

b) Infecting tomato with *Alternaria alternata*

When the tomatoes have 5-6 leaves, it was infected by spraying 30-40 ml spore suspension /10⁵/ per plant. To make the infection more effective, it is covered with a plastic bag for 24-48 hours after infection, and the symptoms appeared within a week.

c) Determining biological activity

When planting tomato seedlings in pots, cultures of antagonistic active fungi around the roots were irrigated with 10⁸, 10⁹ spores / ml to 50 ml. The antagonist culture was tested with 2 doses of 10⁸, 10⁹ spores/ml and 3 repetitions when the degree of disease of artificially infected tomato leaves was 1 point. There should be at least 5 tomatoes per repetition, and 30-40 ml of culture was sprayed per plant twice at 7-day intervals. Biological activity was determined by assessing disease progress and degree at 7, 14, and 21 days and comparing with control plants.

d) Methodology for plant disease detection

Detection of plant disease degree is completed by determining the percentage of diseased part (spot, stain etc.) of the plant on the total surface area the plant. The severity of

the disease is expressed in points. Usually a 5-point classification is used. A score of 1-2 indicates low morbidity, a score of 3 indicates an increase in morbidity, and a score close to 4 indicates epiphytotic. 0 point means no disease symptom on plant.

The degree of plant disease is determined by a score of 0-5, and the progress or index of plant disease is determined by the average degree of plant disease.

$$Px = \frac{\sum(a \times b)}{n \times k} \times 100\%$$

where

Px – progress of plant disease, %.

a – quantity of diseased plant.

b – disease/sickness rate, score.

n – quantity of plants taken for calculation.

k – the highest score of plant disease rate.

Biological activity of biopreparation has been calculated by using Abbott formula.

$$\vartheta = \frac{(k - 0) \times 100}{k}$$

where

ϑ – biological activity, %.

k – disease progress of plant under the control, %.

0 – disease progress in version processed by biological preparation, %.

F. Jasmonic Acid Research

After the above biological activity determination test, 2 g sample was taken from each plant treated with 10⁹ and 10⁸ cells/ml of *Trichoderma citrinoviride* 31/4 culture, diseased control, healthy control, ground it, put in a tube with 10 ml solution (95% methanol: 5% ethyl acetate) and mixed with the vortex for 15 seconds. After that centrifuged at 13,000 rpm for 10 minutes, the supernatant was separated, filtered through a 0.45 µm nylon membrane, and placed in a glass jar.

1. GC-MS Conditions

CLARUS SQ 8 GC / MS equipment is adjusted as follows using RxiR-5ms type 30 m long, 0.25 mm diameter I.D. Using the x 0.25 µm column. Detector temperature is 280°C, Source temperature is 240°C, Injector temperature is 250°C, Carrier gas helium is 1ml/min, Split total flow is 20ml/min, Injection volume is 0,2µl.

2. Mass Spectrometer Adjustment

Detector mass range – 45–480 g.

Gas Chromatograph Adjustment.

Keep the gas chromatograph oven temperature at 40 °C for 3 min. Then increase the temperature to 150 °C at 20 °C/min speed. Then increase the temperature to 280 °C at 10 °C /min speed and finish the analysis by keeping the temperature at 280 °C for 6.5 min.

III. RESULTS

A. Isolation of Pure *Trichoderma* Cultures from Soil

The soil sample was diluted and cultured in PDA culture to isolate the fungus, and pure cultures similar with *Trichoderma* in terms of morphology were isolated.

Trichoderma citrinoviride 31/4 forms a colony with light yellow-green mycelium on the surface of the PDA medium, with spherical spore, septate hyphae, branched conidophores.

Fig. 1. Pure culture of *Trichoderma citrinoviride* 31/4 /under PDA medium/.

Fig. 2. A phylogenetic tree that is stored in the General Bank and includes sequences identified by research.

The development history was determined by using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.05975663 is shown. Next to the branches, there is the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates). The evolutionary distances were computed by using the Tamura-Nei method and are in the units of the number of base substitutions per area. This analysis covered 5 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were total of 1358 positions in the final dataset. Development analyses were conducted in MEGA X.

C. Antagonist Activity

To determine antagonist activity of *Trichoderma citrinoviride* 31/4 isolated from soil, it was tested on plant pathogens such as *Alternaria alternata*, *Cladosporium fulvum*, and *Fusarium oxysporum* by double culture method. The pathogen and the studied actinomycetes were incubated in potato glucose agar medium with 4 repetitions at 25 °C for 7 days and the diameter of the pathogenic fungal colony was measured and compared with the diameter of the pathogen grown in the control dish.

B. ITS Sequencing and Phylogenetic Analysis

The DNA of the culture was isolated by the CTAB buffer method, and the sequence was determined using ITS1–5'TCCGTFGGTGGFCCTGCGG and ITS4–5'TCCTCCGCTTATTGATATGC primers, and the multiplying DNA.

TABLE 1: ANTAGONIST ACTIVITY OF *TRICHODERMA CITRINOVIRIDE* 31/4

Variants	Antagonist activity (%)			Ave- rage
	<i>Alternaria alternata</i>	<i>Cladosporium fulvum</i>	<i>Fusarium oxysporum</i>	
<i>Trichoderma citrinoviride</i> 31/4	77.5	66.7	59.1	67.8
Pathogen control				-

According to the above study, *Trichoderma citrinoviride* 31/4 had 66.7% antagonist activity against *Cladosporium fulvum*, 77.5% against *Alternaria alternata*, and 59.1% against *Fusarium oxysporum* after 7 days.

D. Biological Activity of *Trichoderma citrinoviride* 31/4 against Tomato alternariosis

After growing tomatoes in a greenhouse and artificially infecting with the pathogen *Alternaria alternata*, 10⁹, 10⁸

spores suspension of *Trichoderma citrinoviride* 31/4 were sprayed twice at 10-day intervals and biological activity was determined after 21 days.

Tomatoes began to get sick a week after being infected with the pathogen alternariosis.

The results of the study showed that the development of tomato disease treated with 1.2×10^9 spores/ml suspension of

Trichoderma citrinoviride 31/4 was reduced by 49-52% in 21 days compared with the control variant.

Experimental results showed that the course of tomato alternariosis treated with *Trichoderma citrinoviride* 31/4 was neutralized from the 7th day, and the biological activity was averagely 74% on the 21st day.

TABLE 2: BIOLOGICAL ACTIVITY OF *TRICHODERMA CITRINOVIRIDE* 31/4 / *ALTERNARIA ALTERNATA*/

№	Antagonist strain	Variant	Repeat	2019				2020				Biological activity /average %/
				Disease progress %			Biological activity %	Disease progress %			Biological activity %	
				7 th day	14 th day	21 th day		7 th day	14 th day	21 th day		
1.	<i>Trichoderma citrinoviride</i> 31-4	Хяналт	3	24.6	30	64.6	-	49.3	60	72	-	-
2.		1*10 ⁸	3	18.6	20	22.6	65.0	22.7	26.6	22.6	68.6	66.8
3.		1.2*10 ⁹	3	12	13.3	15.3	76.3	18.7	21.3	20	71.7	74

E. Results Determined Jasmonic Acid

After the above tomato experiment, 2 g sample was taken the plant and tested for jasmonic acid. Jasmonic acid is a compound that represents the plant disease resistance.

When the sample was read on a GC/MS instrument, the peak of the standard substance was observed at 13.22 minutes, and when the peak of the standard substance was compared with that of the sample, peak was observed at 13.03 and 13.01 minutes on plant samples treated with *Trichoderma citrinoviride* 31/4 culture, proving existence of jasmonic acid. Jasmonic acid was not found in diseased and healthy control plants, and plant treated with 10^9 spores/ml *Trichoderma citrinoviride* 31/4 culture contained 0.0135 mg/kg jasmonic acid, plant treated with 10^8 spores/ml contained 0.0076 mg/kg

jasmonic acid. Experiments have shown that treatment with *Trichoderma citrinoviride* 31/4 cultures forms the resistance of tomatoes to alternariosis.

TABLE 3: SAMPLING OF THE JASMONIC ACID CONTENT IN THE GC/MS DEVICE

GC/MS Sample number	Sample name	Jasmonic acid mg/kg
Ja 4	Plant treated with 10^9 cell/ml <i>Trichoderma citrinoviride</i> 31/4	0.0135
Ja 3	Plant treated with 10^8 cell/ml <i>Trichoderma citrinoviride</i> 31/4	0.0076
Ja 15	Diseased control	0
Ja 14	Healthy control	0
Ja	Jasmonic acid standart	100

Fig 3. A. Jasmonic acid standard, B. Plant treated with 10^8 cell/ml *T.citrinoviride* 31/4 culture, C. Plant treated with 10^9 cell/ml *T.citrinoviride* 31/4 culture, D., J- Healthy control, H- Diseased control.

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