Đánh giá khả năng đối kháng của nấm *Trichoderma* spp. với nấm *Colletotrichum* spp. gây bệnh thán thư trên ớt chỉ thiên trong điều kiện *in vitro*

TÓM TẮT

Colletotrichum là một chi nấm gây bệnh thực vật quan trọng, lây nhiễm cho nhiều loại cây chủ. *Colletotrichum* spp. gây ra bệnh thán thư, dẫn đến tổn thất kinh tế nghiêm trọng trong nông nghiệp. *Trichoderma* spp. là một loại nấm đất phổ biến có hoạt tính đối kháng với nhiều loại mầm bệnh thực vật, bao gồm *Colletotrichum* spp., tác nhân gây bệnh thán thư trên ớt. Để xác định *Trichoderma* spp. có hoạt tính đối kháng cao với bệnh thán thư, ba mẫu đất đã được thu thập từ các vùng trồng ớt chỉ thiên tại huyện Phù Mỹ. Dựa trên các đặc điểm hình thái, bốn chủng *Trichoderma* spp. đã được phân lập và hai chủng có hoạt tính đối kháng cao với bệnh thán thư đã được chọn. Trong số đó, Tr.1 và Tr.2 thể hiện chỉ số đối kháng cao lần lượt là 74,78% và 85,44%, trong điều kiện *in vitro*. Phân tích trình tự ITS xác định Tr.1 là *T. viride* và Tr.2 là *T. asperellum*. Hai chủng đối kháng này có tiềm năng đầy hứa hẹn trong việc kiểm soát sinh học bệnh thán thư ở ớt chỉ thiên.

Từ khóa: *Đối kháng, bệnh thán thư, tác nhân gây bệnh nấm, Colletotrichum indemuthianum, Trichoderma.*.

Evaluation of the antagonistic activity of *Trichoderma* spp. against *Colletotrichum* spp. causing anthracnose in chili pepper under *in vitro*

ABSTRACT

Colletotrichum is an important genus of plant-pathogenic fungi that infects a wide range of host plants. *Colletotrichum* spp. causes diseases such as anthracnose, leading to significant economic losses in agriculture. *Trichoderma* spp. is a common soil fungus with antagonistic activity against various plant pathogens, including *Colletotrichum* spp., the causal agent of anthracnose in chili pepper. To identify *Trichoderma* spp. with high antagonistic activity against anthracnose, three soil samples were collected from skyward pointing chili pepper growing areas in Phu My District. Based on morphological characteristics, four *Trichoderma* spp. strains were isolated, and two strains with high antagonistic activity against anthracnose were selected. Among them, Tr.1 and Tr.2, exhibited high antagonistic indices of 74,78% and 85,44%, respectively, under *in vitro* conditions. ITS sequence analysis identified Tr.1 as *T. viride* and Tr.2 as *T. asperellum*. These two antagonistic strains hold promising potential for biological control of anthracnose in skyward pointing chili pepper.

Keywords: *Antagonistics, anthracnose, fungal pathogens, Colletotrichum indemuthianum, Trichoderma.*

1. INTRODUCTION

Chili peppers (*Capsicum frutescens* L. var. fasciculatum (Sturt) Bailey), belonging to the Solanaceae family, are one of the most important and widely grown spice plants in the world.[1,2] Vietnam's chili growing areas span the country, from North to South, covering approximately 50.000 hectares.[3] Of which, Binh Dinh province (Phu My, Phu Cat, and Tay Son districts) is the largest producer. However, fungal diseases, particularly anthracnose, significantly impact yields and cause substantial economic losses.[4] Anthracnose is characterized by small, round spots initially appearing on chili pepper leaves and fruits. These spots progress, causing infected areas to become sunken and dry.[5] The disease is caused by various *Colletotrichum* species. Previous studies believed that at least five species have been associated with anthracnose in peppers, including *C. gloeosporioides*, *C. acutatum*, *C. coccoides*, *C. dematium*, and *C. truncatum*.[6]

Chemical control of chili anthracnose presents significant drawbacks.[7] Pesticide use contributes to environmental pollution, poses risks to human health, and drives the development of pesticide-resistant fungal strains. Consequently, alternative and sustainable methods such as biological control have been studied and applied for creating a safer and cleaner agricultural system. Antagonists (also known as biological control agents) are often soil microorganisms. They can suppress fungal pathogens through various mechanisms including competition, antibiosis (antibiotic production), and parasitism.[8] *Trichoderma* spp., for example, is a well-established antagonist of soilborne pathogens, including *C. truncatum*, a causative agent of chili anthracnose.[9] Trichoderma-based biocontrol agents are increasingly utilized globally as a safer and more environmentally alternative to chemical pesticides.[10]

T. viride shows the strongest potentials for inhibiting mycelial growth and spore germination of *C. lindemuthianum*.[11] *T. asperellum*, in dual-culture studies, effectively inhibited various filamentous fungal pathogens. Furthermore, *T. asperellum* exhibits plant growth-promoting properties, including IAA production, phosphate solubilization, and positive impacts on seed germination, root and leaf development.[12]

This study aims to: (1) isolate and identify *Trichoderma* spp. species from skyward pointing chili pepper soil samples in Phu My, Binh Dinh; (2) select *Trichoderma* spp. strains with antagonistic activities against skyward pointing chili peppers' anthracnose.

2. MATERIALS AND METHOD

2.1. Material

Pathogenic fungal samples on chili fruits and soil samples from chili-growing areas in My Cat Commune, Phu My District, Binh Dinh Province.

Isolation of *Trichoderma* fungi from soil samples collected in chili-growing areas in My Cat Commune, Phu My District, Binh Dinh Province.

Fungal culture medium: Potato Dextrose Agar (PDA) medium.

Essential chemicals and materials for the research: Agar, glucose, petri dishes, test tubes, sample cutting knives, forceps, alcohol lamp, measuring cylinders, filter paper, glass slides, cover slips, pots, jars, inoculating loops, drying oven, autoclave, refrigerator, incubator, biosafety cabinet and microscope.

2.2. Method

2.2.1. Sample collection method

Soil sample collection method

Soil samples were collected from the rhizosphere of healthy skyward pointing chili pepper plants in three communes (My Cat, My Tai, and My Trinh), Phu My, Binh Dinh.

Three soil samples (500g each) were collected and stored in sterile bags, and transported to the laboratory for analysis within one day (maximum one week at 2-4°C).[13]

- Collection of pathogenic fungal samples of skyward pointing chili peppers

Pathogenic fungal samples of chili fruits were collected at growing areas from My Cat commune. They were similarly stored and transported to the laboratory for analysis within one day (maximum one week at 2-4°C).

2.2.2. Isolation methods

Isolation pathogenic fungi

Diseased chili fruits were thoroughly washed under running tap water. Small tissue pieces (0,5-1 cm), excised from the border between healthy and diseased tissue using a sterile blade, were surface-sterilized in NaCl 0,9% for one minute and rinsed with sterile distilled water. Sterilized tissue pieces were inoculated onto PDA (Potato Dextrose Agar) plates and incubated at 28 ± 2°C for 48 hours. Putative *Colletotrichum* colonies on PDA were purified, and pure cultures were stored at 4°C for further analysis.[14]

- Isolation *Trichoderma*

One gram of soil from each sample was suspended in NaCl 0,9% and serially diluted to 10^6. 0,1 ml of the 10^{-4}, 10^{-5}, 10^{-6} dilutions were spread onto PDA plates and incubated at 28 ± 2°C for 48 hours. Putative *Trichoderma* colonies on PDA were purified, and pure cultures were stored at 4 ◦C for further analysis.[15]

2.2.3. Morphological identification method

Fungal colonies from the third generation of isolation were subcultured onto fresh PDA plates and incubated at 28°C ± 2°C for three days. Colony morphology was assessed daily, while spore and hyphal structures were examined using an optical microscope. The morphological characteristics were then compared with

reference descriptions from previous for identification.[16]

2.2.4. Molecular identification method

Molecular identification method of fungi: Ribosomal RNA barcode gene region was used for identification. Total DNA was extracted from purified fungal samples by CTAB method.[17] Primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3', forward) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3', reverse) were used to amplify the ITS region by PCR technique (2,5 µL PCR buffer; 0,5 µL total DNA; 0,5 µL dNTP; 1 µL each primer and 0,2 µL Taq polymerase).[18] The thermal cycling conditions included an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 5 min. The PCR product was purified using PureLink Quick Gel Extraction Kit (Invitrogen, USA) for 2-way direct sequencing (NK Biotek Laboratory, Ho Chi Minh City, Vietnam).

2.2.5. Sequence Analysis

Sequencing data were analyzed using the BLAST algorithm available in the National Center for Biotechnology Information (NCBI) nucleic acid database. Phylogenetic relationships were inferred using the Neighbor-Joining (NJ) method with the maximum composite likelihood substitution model in MEGA 7.0.[16] Tree reliability was assessed through 1000 bootstrap (BS) replicates.

2.2.6. Method for testing Trichoderma spp. antagonism against Colletotrichum spp. causing anthracnose in vitro.

- A symmetrical inoculation method was used on Petri dishes under laboratory conditions, employing two treatments:

+ Control (Ctrl): *Colletotrichum* spp. inoculation only

+ Experiment (Ex): Simultaneous inoculation of *Trichoderma* spp. and *Colletotrichum* spp.

- Fungal strains were inoculated onto PDA at 28 ± 2°C. Colony diameter and growth were monitored.[19]

$$\text{Antagonistic ratio} = \frac{(R_{ctrl} - R_o) - (R_{ex} - R_o)}{(R_{ctrl} - R_o)} X100$$

In which:

Antagonistic ratio (T)

R_{ctrl}: radius of the pathogenic fungal colony on the control plate; R_{ex}: radius of the pathogenic fungal colony on the treatment plates; R_o: radius of the initial fungal inoculum (3 mm).

Antagonistic efficiency (H):

• 75%: very high antagonism

• 60–75%: high antagonism

• 50–60%: medium antagonism

• ≤50%: low antagonism

2.2.7. Data collection and processing methods

The reported data are the average values of 3 experimental repetitions. The data were processed using Statgraphics 19 - X64. The average values were analyzed by ANOVA using the Tukey test and the value of $p < 0.05$ indicates a statistically significant difference.

3. RESULT

3.1. Isolation and morphological identification of anthracnose fungus on chili peppers.

The diseased chili samples collected from chili fields of farmers in My Cat commune, Phu My District, Binh Dinh Province, were isolated on a PDA medium. The isolation results are presented in Figure 1.

The results of morphological observations showed that the isolated samples were *Colletotrichum* spp. with the characteristics of aerial mycelium growing loosely or close to the surface of the agar.[20] The fungal colony is concentrically circular, the upper surface is white then turns gray, the lower surface is pink-orange to black. The fungus has septa and branches. The transparent spores are cylindrical in shape, with two rounded ends or one rounded end and one pointed end. On PDA, spores appear after 96 hours. Sclerotia appear after 7 days.

Based on the morphological assessment of the isolated samples, we determined that the agent was *Colletotrichum* spp., although there was still not enough evidence to determine the exact species.

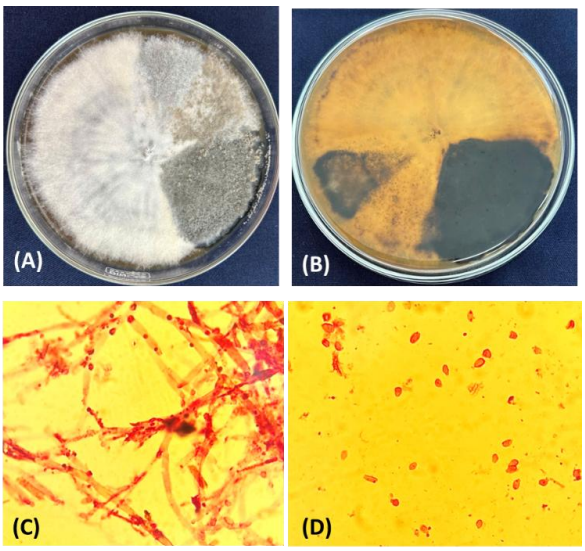

Figure 1. Morphological characteristics of fungal samples on PGA medium after 7 days, observed under an Olympus microscope with a magnification of 60X. (A): front of the mushroom disc, (B): back of the mushroom disc, (C): Mycelium, (D): Spore shape.

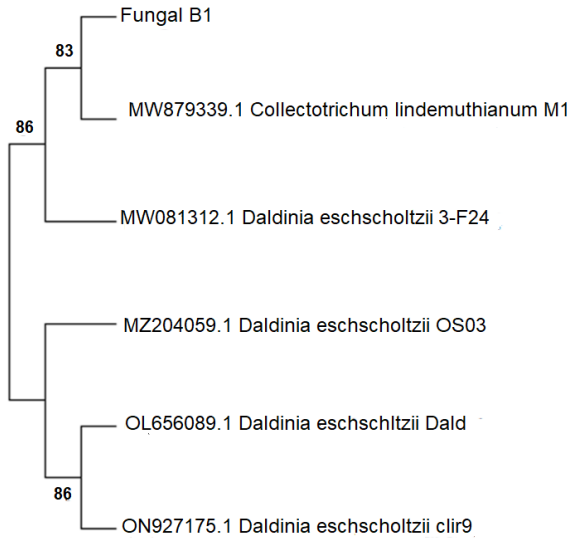

Figure 2. Genetic tree of the Fungal B1 isolate with some samples in the gene bank.

Therefore, the fungal samples were then identified by PCR technique with the primer pair ITS1 - ITS4. The results of the species clustering tree analysis showed that the Fungal B1 isolate was in the same branch as *Colletotrichum lindemuthianum* M1 with a Bootstrap value of 83% (Figure 2), the similarity level of the Fungal B1 isolate with *Colletotrichum lindemuthianum* M1 was 99.62%.

Based on the results of the identification of the agent causing anthracnose on chili plants as *Colletotrichum lindemuthianum*, this source of isolated samples was used to conduct further studies.

3.2. Isolation and identification of *Trichoderma* spp. Fungi

Soil samples from chili fields were collected from many locations with different ecological environments in Phu My district, Binh Dinh, to isolate *Trichoderma* spp. The results isolated a total of 4 fungal strains with typical morphology of *Trichoderma* spp. (white, spongy fungal colonies, forming elliptical/spherical conidia, smooth surface). After 5 days of culture, the fungal colonies gradually turned green, forming many conidia, and the flask was cylindrical in shape (Figure 3).

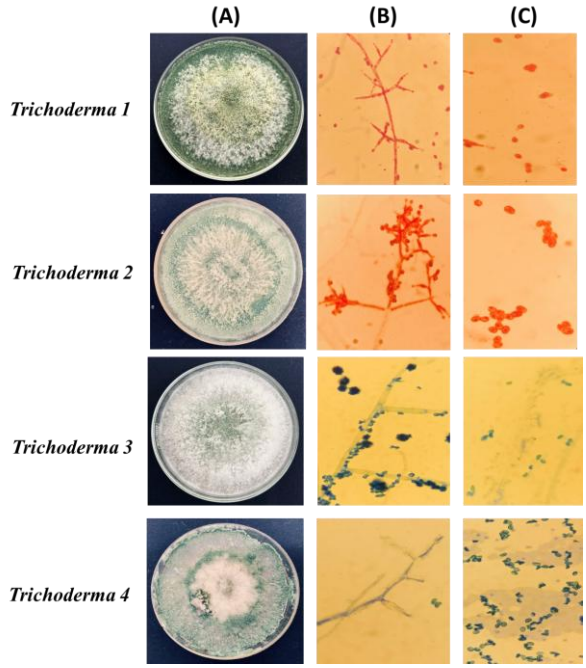

Figure 3. Morphology of colony (A), colonia (B) and Spore (C) of Trichoderma on PDA. Analysis performed at 60x.

3.3. Evaluation of antagonistic efficacy between *Trichoderma* spp. and anthracnose fungi on chili plants under *in vitro* conditions.

Next, with the aim of evaluating the antagonistic ability against *Colletotrichum lindemuthianum*, the 4 isolated *Trichoderma* spp. strains were cultured symmetrically on both sides on PDA medium (Figure 4).

The results showed that the 4 *Trichoderma* spp. strains all showed the ability to antagonize the pathogen, with an efficacy of 54.66 - 85.88% (Table 1). Of which 2 strains of *Trichoderma* spp., Tr.1 and Tr.2, have the strongest antagonistic effect against *Colletotrichum lindemuthianum* causing anthracnose, with an inhibitory effect of 74,78 – 85,88% (Table 1). In previous studies, *Trichoderma* spp. have been noted to have good inhibitory ability against anthracnose pathogens in chili. Previous studies

by other authors showed that preparations containing *Trichoderma* spp. have the ability to significantly limit anthracnose caused by *Colletotrichum* spp. on chili plants.[21,22,23] Spraying the preparation evenly on the plant combined with spreading the preparation around the base is a feasible measure to limit anthracnose. Based on this result, 2 strains of *Trichoderma* spp., Tr.1 and Tr.2, were selected for scientific identification in order to potentially use them in the future to protect the plants.

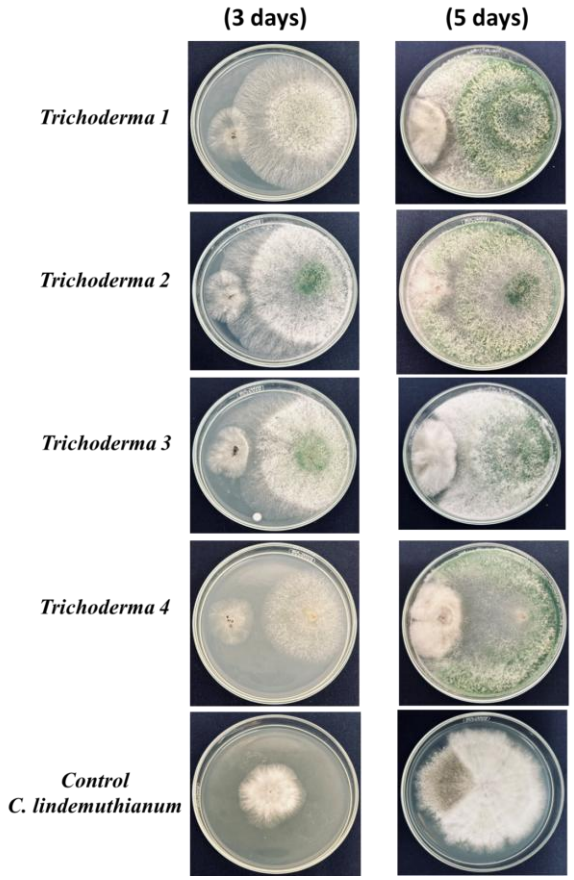

Figure 4. Antagonism of *Trichoderma* spp. against *C. lindemuthianum* on PDA, at 28ºC ±2 after 3 days and 5 days.

The result is consistent with previous studies on *Trichoderma* spp. Pham Thi Ly Thu (2021) selected *Trichoderma* spp. with high antagonistic activity against fungi causing anthracnose on mango trees in provinces of the Mekong area. The result recorded 6 strains Tr.X1, Tr.X2, Tr.X3, Tr.X4, T1, M2 with the highest antagonistic activity from 77,76-86,25% *in vitro*.[24] Nguyen Anh Dung (2016) examed the antagonistic ability of 14 *Trichoderma* strains isolated on agricultural land with the ability to antagonize the *Corticium samonicolor* fungus on rubber trees.[7] In addition, Aisha Saleh Alwadai (2022) isolated 48 *Trichoderma* spp. from the soil samples having antagonist potential against the tested plant pathogenic fungi. There were three strains with highly effective in reducing the growth of tested plant pathogenic fungi. *Trichoderma* A (1) 2.1 T was highly effective against *F. oxysporum* (82%), *Trichoderma* A (6) 2.2 T prevented the maximal growth of *H. rostratum* (77%) according to the dual culture data.[25]

Table 1. Antagonistic efficiency between *Trichoderma* spp. and *C. lindemuthianum* on PDA, at 28ºC ±2

Tr.	3 days		5 days	
	T (%)	H	T (%)	H
Tr. 1	57,22± 11,12[a]	Medium	74,78± 4,51[b]	High
Tr. 2	58,61± 7,67[a]	Medium	85,44± 5,25[c]	Very high
Tr. 3	55,04± 7,73[a]	Medium	58,40± 2,17[a]	Medium
Tr. 4	58,62± 7,16[a]	Medium	54,66± 2,34[a]	Medium

Note: Numbers in the same column followed by one or more identical letters are not significantly different by Duncan's test.

3.4. Identification of *Trichoderma* spp. fungi antagonistic to anthracnose pathogens in chili plants.

To identify the *Trichoderma* spp. molecularly, this study sequenced the ITS region of the fungal strains and constructed a phylogenetic tree with known data on *Trichoderma* species based on the GeneBank database.

The results showed that the Tr.1 strain belonged to the *Trichoderma viride T*-VT-15 species with a similarity level of ITS sequence reaching 100% and a Bootstrap value of 35%, while the Tr.2 strain belonged to the *Trichoderma asperellum* Z228 species with a similarity level of ITS sequence reaching 100% and a Bootstrap value of 100% (Figure 5).

Previous studies have shown that the antagonistic ability of *Trichoderma* spp. against pathogens is explained by the exogenous cellulase, protease and chitinase production properties to destroy the cell wall of pathogenic fungi.[26]

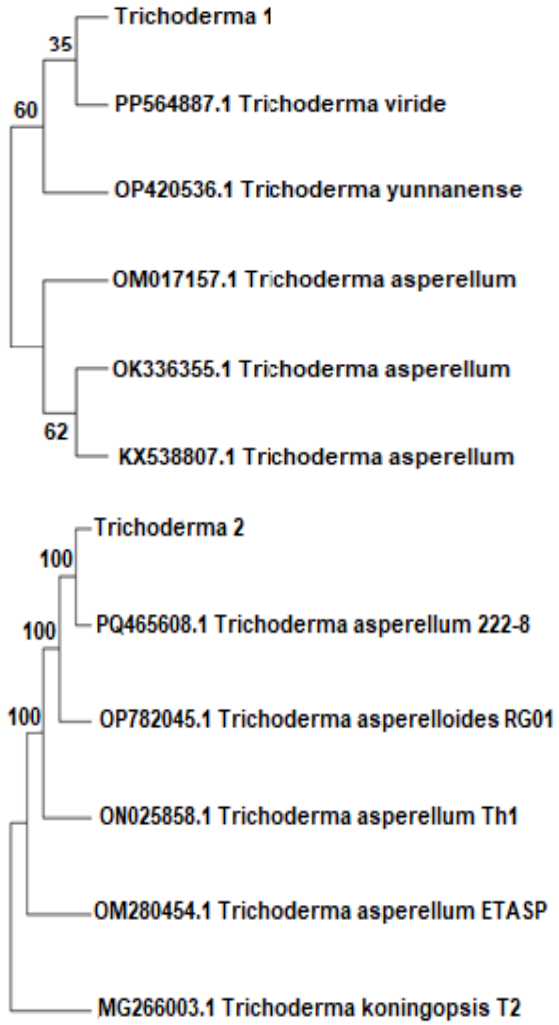

Figure 5. Genetic tree of the *Trichoderma* 1 and 2 with some samples in the gene bank.

4. CONCLUSION

From soil samples collected in chili growing areas in My Cat commune, Phu My District, Binh Dinh Province, the study isolated 4 strains of *Trichoderma* spp. Under *in vitro* conditions, 2 out of 4 strains of *Trichoderma* spp. showed a strong antagonism against *Colletotrichum lindemuthianum* causing anthracnose in chili plants with an efficacy ranging from 74,78 to 85,88%.

Based on morphological characteristics and ITS region sequencing, *Trichoderma* Tr. 1 strain was identified as belonging to the *Trichoderma viride* species and *Trichoderma* Tr. 2 to the *Trichoderma asperellum* species. The study will continue to test the effectiveness of using *Trichoderma* Tr. 1 and Tr. 2 strains to produce biological products for application in controlling anthracnose on chili plants.

Acknowledgement

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