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Isolation and Selection of some Antagonizing Fungal Strains of Trichoderma And Chaetonium that can Inhibit the Growth of Pathogenic Fungi on Orange Trees

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ABSTRACT

Decline disease has been discovered for a long time but infection is more extensively increasing. It is difficult to detect because the disease is originated from roots. Among the causes, soil fungi have been widely determined. Using fungicides is not an effective way to control the disease. In this case, biocontrol with suitable microbial strains is a potential approach. This study aims to investigate in vitro the possibility of using Trichoderma and Chaetonium to control the causing fungi. Two Chaetonium strains and 1 Trichoderma asperellum strain were isolated from diseased-root samples. Six strains of Trichoderma (isolated strains T1, T2, T3, T4, T5, T6) and three strains of Chaetonium (isolated strains C1, C2, C3) showed a reasonably antagonistic ability to Phytophthora, Fusarium, Rhizoctonia. Base on the PCR identification method, 6 strains of Trichoderma was isolated belonging to Trichoderma asperellum species, and 3 strains of Chaetonium belonging to 2 species Chaetomium globosum and Chaetomium cichlids.

KEYWORDS: Trichoderma, Chaetonium, decline disease on citrus, antagonistic, pathogenic fungi.

1. INTRODUCTION

Orange tree (Citrus sinensis) belongs to the family *Rutaceae*, subfamily *Aurantioideae*, genus Citrus, native to tropical and subtropical Southeast Asia, distributed from 35 latitudes South and Northern hemisphere, sometimes up to 40 latitudes of the Southern and Northern Hemisphere. Orange is one of the fruit trees with high economic and nutritional value. Currently, orange trees are being expanded in the area and with the application of science and technology, the output is quite high, contributing to the development of the economy. However, the development and increase of the area of orange trees are facing difficult problems, of which the most difficult is the prevention and treatment of diseases for trees. Among the common diseases on oranges in Vietnam, the stem cracking disease caused by *Phytophthora* sp. and yellow leaf, root rot caused by *Rhizoctonia* sp., *Fusarium* sp. are common diseases, especially in Nghe An area.

For the treatment of stem cracking and pus discharge caused by *Phytophthora* sp. and yellow leaf, root rot caused by *Rhizoctonia* sp., *Fusarium* sp., chemical drugs such as Treppach Bull 607SL, Mexyl 80WP or Alpine 80WG in combination with Naturfos fertilizer or Pylacol 700WP proved to be effective. However, using chemical drugs to treat diseases of orange trees during the business period is not easy and costly, depending on the planting area. Moreover, the long-term use of chemical drugs will affect the antagonistic microflora and other beneficial insects, thereby affecting the ecological environment. Unlike chemical drugs, probiotics have advantages such as not having negative effects on human health, not polluting the environment, being able to decompose and transform organic substances, wastes, etc. contribute to clean the environment but also kill harmful fungi, so scientists in the world in general and in Vietnam, in particular, are focusing on research. For that reason, we conducted the research project "***Isolation and selection of some antagonizing fungal strains of Trichoderma and Chaetonium that can inhibit the growth of pathogenic fungi on orange trees***".

2. MATERIAL AND METHODS

2.1. Material

- Pathogenic fungi: *Fusarium* sp., *Phytophthora* sp., *Rhizoctonia* sp.

- Antagonist fungi: *Trichoderma, Chaetonium*.

- PDA media: potato 30g, dextrose 20g, distilled water 0.77l, agar 15g.

2.2. Methods

2.2.1. Isolation of antagonistic fungal strains

- *Trichoderma* strains: The sample was collected in Nghe An province (Nghia Dan and Quy Hop district. The samples were collected in sterile boxes and transferred to the laboratory.

The sample was prepared by diluting 1 g soil into 50 ml distilled water, filtered by filter paper, and collected solution. Then, the sample solution was serially diluted in a 10 ml tube separately, like 10^{-1} to 10^{-3}. Take 5 µl of each concentration by pipet was cultured on Petri plates added PDA media and incubated at 30°C for 48 to 72 hours & colonies were observed. Characteristics of Trichoderma fungal strain based on the classification key of Bui Xuan Dong (Bui Xuan Dong, 2000).

- *Chaetonium* strains: The sample was collected in Nghe An province (Nghia Dan and Quy Hop district. The samples were collected in sterile boxes and transferred to the laboratory. *Chaetonium* strains were isolated using the paper trap technique. Soil samples were dried and crushed, then placed in Petri dishes with a volume of about two-thirds of the dishes. The soil samples are moistened with sterile distilled water. The sample was filtered by filter paper, sterilized, and placed on the surface of the petri dish. The soil plates were incubated at 28°C and examined for fruiting on paper traps. When fruiting bodies appear, switch to WA medium supplemented with ampicillin (100mg/l) and streptomycin (100mg/l), then continue to inoculate on PDA medium for purification. *Chaetonium* fungal strains characteristics based on classification key of Soytong et al. (1989).

2.2.2. Determination and evaluation of antifungal activities against pathogenic fungi

The antagonistic fungal strains were tested in vitro for their antagonistic activity against *Fusarium* sp., *Phytophthora* sp., and *Rhizoctonia* sp. according to the method of Dickinson and Skidmore (1976). The confrontation assays allowed assessing the capacity of the antagonistic fungal strains to inhibit the mycelia growth of the above-mentioned phytopathogenic fungi. Malt extract agar (MEA) discs of 6 mm diameter, cut from the edge of an actively growing colony of each antagonist and pathogen, were placed at opposite sides (4.5 cm from each other) on fresh MEA medium plates (Figure 1a). The radii of the developing pathogen's mycelium were measured in the direction of the antagonist's colony (R1) (Figure 1b) three times a day, until contact. Each antagonist/pathogen combination was set up in triplicate and the inoculated plates were incubated at 30±2°C with a photoperiod of 12 hr/12 hr darkness/light. The inhibition of mycelial growth in percent PRIG% (Percentage of Radial Inhibition Growth) was calculated after 14 days using the equation (1) ascribed by Skidmore and Dickinson (1976).

PIRG (percent Inhibition of Radical Growth) = (R1-R2)/ R1 x 100 (1).

Where R1: Radius of mycelia growth of pathogenic fungus in control plates (without Trichoderma) in mm. R2: Radius of mycelia growth of pathogenic fungus in the presence of Trichoderma in mm.

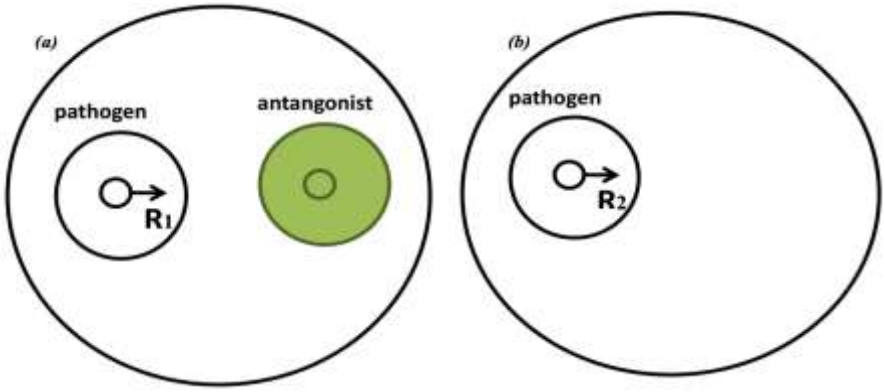


Figure 1: Dual plate culture (a) dual plates where antagonistic fungal isolate confront pathogen (b) control plates where the pathogen is growing without antagonistic fungal disc is positioning of pathogen plug and mycelium and the green disc is positioning of antagonistic fungal plug and mycelium.

2.2.3. Identification of antagonistic fungal strains

The genomic DNA of antagonistic fungal strains was extracted according to the fast DNA extraction method of fungi (El Khoury, 2011; Schmoll, 2004).

3. RESULTS AND DISCUSSION

3.1. Isolation of antagonistic fungal strains

From the sample of soil and orange root, we have isolated six *Trichoderma* and three *Chaetonium* fungal strains on PDA media. Six Trichoderma fungal isolated are named

T1, T2, T3, T4, T5, T6, and three Chaetonium fungal isolated are named C1, C2, C3.

The antagonistic fungal isolates and their mycelium, spores on PDA media were shown in Figure 2 and Figure 3.

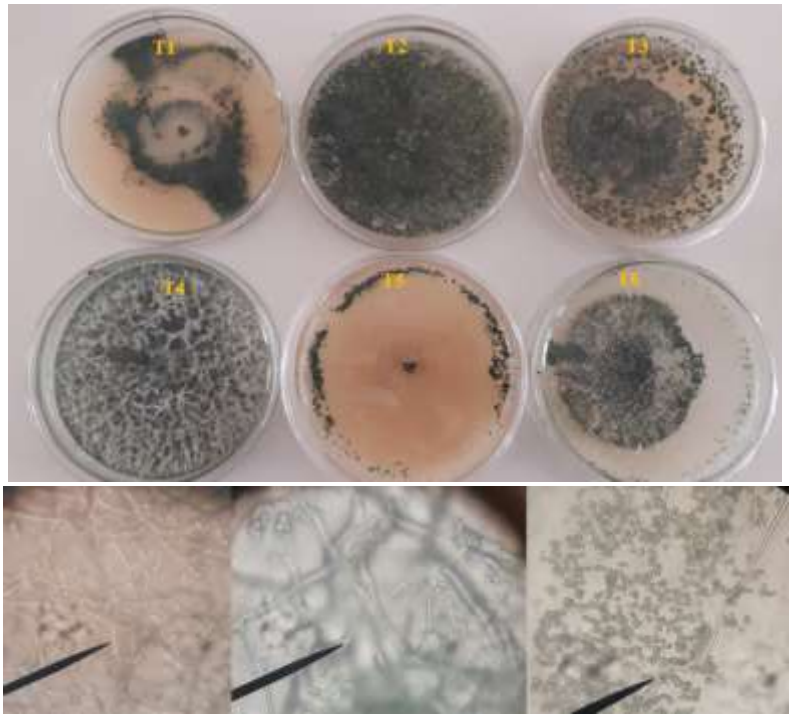


Figure 2: Trichoderma isolates and their mycelium, spores

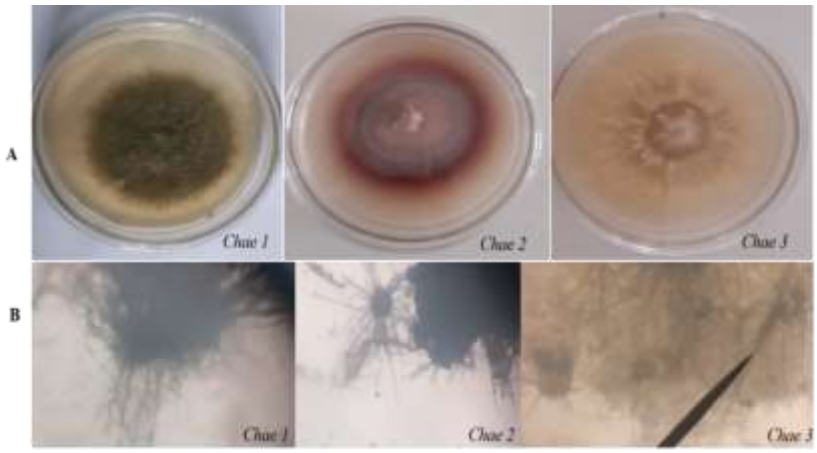


Figure 3: Chaetonium isolates (A) and their spores (B)

3.2. Determination and evaluation of antagonistic activities against pathogenic fungi

The dual plate culture method showed considerable antagonistic activity against the three different pathogens when confronting antagonistic fungal isolates and results of PRIG% means are presented in Table 1.

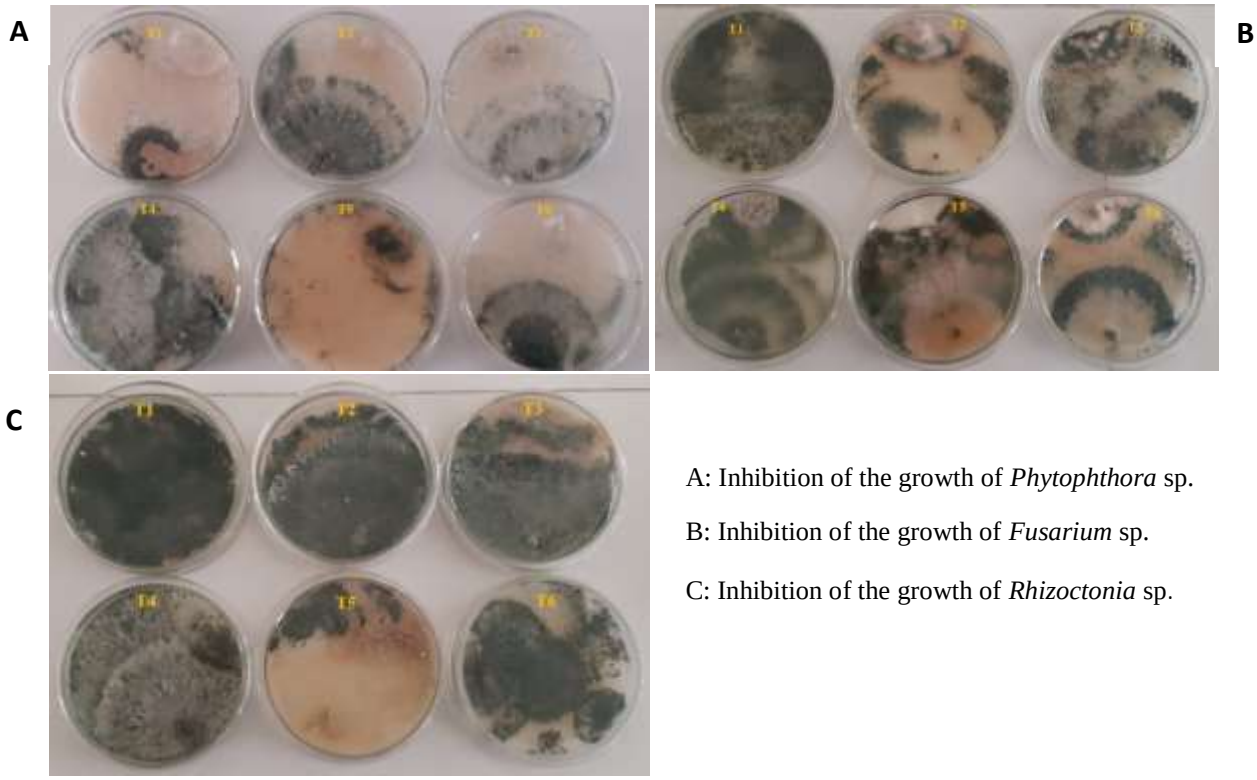
The antagonistic activity against value (Table 1) obtained is from 42.2 to 99.8 percent. We found that the antagonistic activity against the average value of six isolated *Trichoderma* is 97.67 percent, in which the isolate T3 with 99.2% as the highest PRIG% mean against three pathogens. For the *Chaetonium* isolates, the antagonistic activity against the average value of three isolated is 56.45 percent, in which the isolate C3 with 73.25% has the highest PRIG% mean against two pathogens.

Korsten (1995) developed a growth inhibition categories on a scale ranking antagonistic in vitro screening by dual plate culture where 0 scale = no inhibition growth, 1 scale = 1 to 25% inhibition, 2 scale = 25% to 50%, 3 scale = 50% to 75% and 4 scale = 75% to 100%. Therefore, we proposed to scale our antagonistic fungal isolates using the Korsten antagonism scale in Table 1. All Trichoderma isolates T1, T2, T3, T4, T5, and T6 showed the highest inhibition percentage with antagonist performance against the three pathogens on a 4 scale. Exclusively for *Chaetonium* isolates showed only C3 isolate showed the highest inhibition

percentage with antagonist performance against the two pathogens on 4 scales.

Table 1: Inhibition of the growth of *Fusarium* sp., *Phytophthora* sp. and *Rhizoctonia* sp. by antagonistic fungal isolates and their antagonistic activity against those pathogens in the dual culture test

Antagonistic fungal isolates	***Rhizoctonia* sp.**		***Phytophthora* sp.**		***Fusarium* sp.**	
	PRIG% means	**Antagonistic activity (on 1-4 scale)**	**PRIG% means**	**Antagonistic activity (on 1-4 scale)**	**PRIG% means**	**Antagonistic activity (on 1-4 scale)**
Trichoderma						
T1	99.4	4	93.6	4	99.4	4
T2	98.3	4	99.2	4	92.2	4
T3	98.8	4	99.7	4	99.1	4
T4	98.6	4	99.5	4	95.5	4
T5	98.9	4	99.8	4	93.3	4
T6	99.1	4	99.3	4	94.4	4
Chaetonium						
C1			52.2	3	51.1	3
C2			42.2	2	46.7	2
C3			78.8	4	67.7	3



A: Inhibition of the growth of *Phytophthora* sp.

B: Inhibition of the growth of *Fusarium* sp.

C: Inhibition of the growth of *Rhizoctonia* sp.

Figure 4: Inhibition of the growth of Fusarium sp., Phytophthora sp. and Rhizoctonia sp. by Six Trichoderma isolates on PDA agar after 7 days of culture

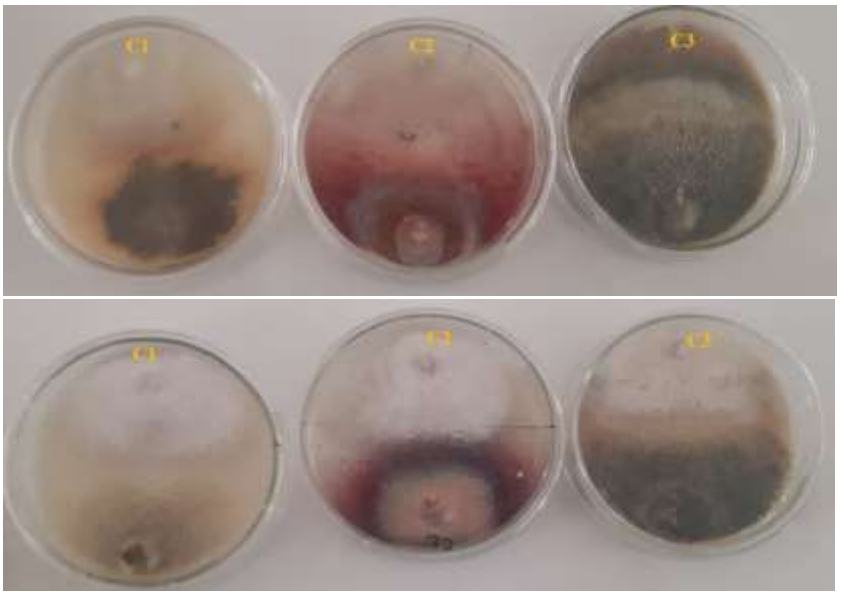


Figure 5: Chaetonium strains inhibiting the growth of Phytophthora sp. (A) and Fusarium sp. (B) on PDA agar after 9 days of culture

3.3. Identification of antagonistic fungal strains by PCR

To accurately identify 6 strains of Trichoderma and 3 isolates of Chaetonium, samples were sent to the Institute of Biotechnology for identification by 28S rRNA gene sequencing. Comparing the sequencing results with the rRNA sequence KF732005.1 on Genbank, the following conclusions can be drawn:

- The isolates T1, T2, T3, T4, T5, and T6 strains belong to Trichoderma asperellum species with 99% similarity.
- The isolated strain C1 belongs to *Chaetomium globosum* species with a 99% similarity rate.
- The isolates C2, C3 strains belong to *Chaetomium cochliodes* species with a 99% similarity rate.

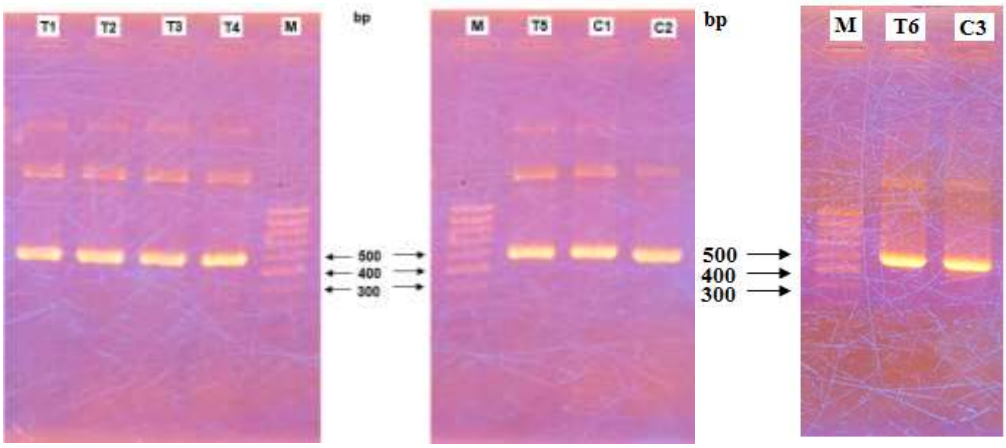


Figure 6: Amplification of 5.8S rRNA genes of antagonistic fungal strains by PCR

4. CONCLUSION

- Six strains of *Trichoderma* have been isolated with the ability to completely inhibit *Phytophthora* sp., *Fusarium* sp., *Rhizoctonia* sp. fungi.
- Three strains of *Chaetonium* have been isolated with high inhibitory ability against *Phytophthora* sp. and *Fusarium* sp. fungi.
- The identification results showed that 6 isolates of *Trichoderma* belong to *Trichoderma asperellum* species and 3 strains of *Chaetonium* belong to 2 species *Chaetomium globosum* and *Chaetomium cochliodes*.

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