ISOLATION AND MASS PRODUCTION OF *TRICHODERMA*

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Abstract: *Three Trichoderma isolates (THDU-1, THDU-2, and THDU-3) were isolated from the root zone area of healthy bananas on the farm in Ba Thuoc district, Thanh Hoa province. All of these isolates showed high inhibitory activity against soil borne diseases Rhizoctonia solani and Sclerotium rolfsii. Our study proposed a protocol of Trichoderma mass multiplication using rice media based on solid substrate fermentation (SSF) to produce commercial product of Trichoderma.*

Keywords: *Trichoderma, isolation, conidia, conidial propagation, Rhizotonia solani, Sclerotium rolfsii.*

1. Introduction

Trichoderma spp. are free-living fungi and common in soil and root ecosystems. They are versatile, ubiquitous filamentous fungi, colonizing dead organic matter, and in beneficial endophytic associations with plant species. Their capability to synthesize antagonistic compounds (proteins, enzymes, and antibiotics) and micro- nutrients (vitamins, hormones, and minerals) enhance their biocontrol activity. Therefore, *Trichoderma* species are the most commonly used biological control agent against several soil borne fungal pathogens (fungi, bacteria, and nematodes) [2, 9, 13]. Effective biocontrol is achieved through a combination of mechanisms including mycoparasitism, competition for nutrients and/ or space, antibiosis, and induction of systemic resistance [1, 4, 10, 11, 15, 16]. Moreover, *Trichoderma* species also possess ability to promote plant growth and soil remediation activity through production of solubilizing enzymes, and phytohormones [3].

Trichoderma spp. produces three kinds of propagules: hyphae, chlamydospores, and conidia [13]. Chlamydospores and condia have been commonly used as the active ingredients in most *Trichoderma* spp. based products [5, 6, 8] due to reproduce well in culture. *Trichoderma* sp is multiplied by solid and liquid fermentation methods [14]. However, solid substrate fermentation is the most common method for *Trichoderma* mass-scale production for commercial use because of low cost of bedding materials with high yielding and environmental safe. The success of the biocontrol agent depends much on the establishment of the product, the formulation and delivery system.

The current research aimes at isolating the indigenous *Trichoderma* spp. and designing of solid substrate fermentation to optimize inoculum production using easily available and low cost agricultural residues combination.

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2. Materials and Methods

2.1. Isolation of native antagonistic Trichoderma spp. from plant roots

Root samples were collected from the root zone area of healthy bananas on the farm in Ba Thuoc district, Thanh Hoa province, Vietnam. The root samples were washed under tap water to remove bulk soil and cut into pieces of approximately 1.5 cm in lenghs with a sterilized knife. *Trichoderma* spp. was isolated from roots pieces using the potato dextrose agar (PDA) amended with streptomycin (1 g/L). The cultures were incubated at room temperature (26^{o}C) for 7 days, at which time colonies can be subcultured onto new plates to obtain pure cultures.

2.2. Isolation of native antagonistic Trichoderma spp. from rhizophere soil samples

15g of rhizophere soil samples around the roots soil were collected from rhizosphere of healthy plants in Ba Thuoc district, Thanh Hoa province, Vietnam. The samples were stored at 4-8^{o}C until ready for processing. Add 15 g soil sample to 9 mL sterilized distilled water (SDW) in universal bottles. The samples were shook for 10 min at maximum speed and then leave to stand for 10 min. Dilute 100, 1000, and 10 000 fold and plate 1 mL onto PDA plates amended with streptomycin (1 g/L) for each dilution. Petri plates were sealed and incubated at room temperature (26^{o}C) for 7 days, at that time colonies can be subcultured onto new plates to obtain pure cultures.

2.3. Antagonistic activity of Trichoderma isolates

Isolates of *Trichoderma* were tested for their inhibitory activity against soil born pathogen *Sclerotium rolfsii* and *Rhizoctonia solanin* by using the dual culture technique described by Morton and Stroube (1955). Each petri-dish (9 cm) containing PDA was inoculated with two 5 mm diameter mycelial discs at the same time. Plates were incubated at room temperature (25^{o}C ± 2) for 7 days. The experiment was replicated three times and percentage of growth inhibition was calculated by the following formula:

Inhibition % = (C-T)/C x 100. Where,

C: growth of the colony (*S. rolfsii*, *R. solani*) in control plates (mm)

T: growth of the colony (*S. rolfsii*, *R. solani*) in treated plates (mm)

The experimental design was used a completely randomized with four petri dishes for each isolate. This experiment was carried out at least twice.

2.4. Mass production of Trichoderma inoculum on rice (a solid state fermentation)

1. Soak brown rice overnight in water (16 hours), wash then rinse the rice with tap water and drain well. Weight 800 g of the rice and place in a 25 x 35 cm autoclave bag, add 100 mL tap water and mix thoroughly. Roll up the bag loosely, leaving enough space for evaporation of water during autoclaving.

2. Place the bags of rice in an autoclave, sterilize at 121^{o}C, 1.2 atm for 25 min. The bags are cooled to 40-45^{o}C.

3. Inoculate the sterilized bags of rice with *Trichoderma* cultured on PDA medium for 4-5 days, leaving a slightly opening.

4. Incubate at room temperature (25 - 30°C) close to a window for exposure to nature lighting for 7 days and mix vigorously every day to avoid clumping.

5. Dispense the inoculated bags into plastic containers covered with sterile paper and incubates at room temperature (25 - 30°C) until profuse condiation occurs (about 2 - 3 days).

6. Place the plastic containers into a 40°C incubator for overnight drying (16 - 24 h).

7. The dried substrates are ground to a fine powder.

8. Conidia are recovered with sterile distilled water (SDW), mixing by vortex at low rpm and three times dilution in tube, then counted with a hemocytometer in an optic microspore (40X).

9. The powder was mixed with rice bran and talc powder in 3:1 in order to adjust the number of conidia production after incubation to 1 x 10^9 condia/g.

10. The finally processed products were placed in a zip-lock plastic bag and sealed.

3. Results and discussion

3.1. Morphological characterization

Based on the observation of the colony, conidia, phialides, colony texture, chalmydospore, conidiophore morphology the isolates were confirmed to be *Trichoderma*. The morphological characters were described in Table 1.

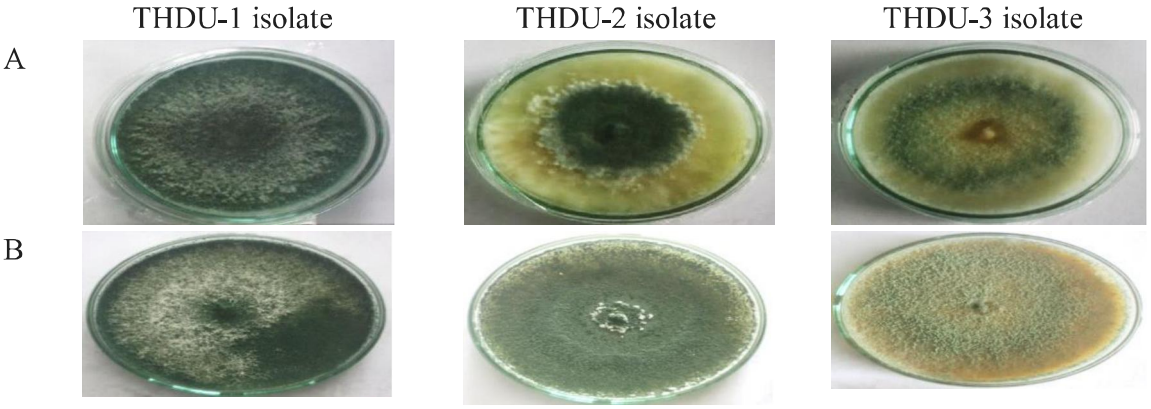

Figure 1. *Colony growth of different isolates of Trichoderma on PDA medium at 4th (A) and 7th (B) day after inoculation.*

Table 1. *Morphological descriptions of Trichoderma isolates (Figure 1)*

Isolates	**THDU-1**	**THDU-2**	**THDU-3**
Colonies grown on PDA at 28 °C ± 2 for 5 day	Form one concentric rings near the inoculum zone with a dense conidial production, with white aerial mycelium toward the green center.	Grow rapidly produce an intense diffusing yellow pigment and green conidia as the tend to form on the center of the plate.	Form one concentric ring with green conidial production in mature colonies. The mycelium is initially smooth, watery white color and sparse, until floccose aerial

		Two concentric rings, one near the margin and the other around the inoculum point.	mycelium has produced.
Colony reverse	Creamy in color	Pale yellowish	Dull yellowish
Pigment on PDA plate	Not observed	Pale yellowish	Pale yellowish-green
Aerial mycelium	Yes	Not forming	Yes
Odor	Slightly sweet coconut odor	No distinctive odor	Indistinct sweet coconut odor produced

3.2. Antagonistic activities of Trichoderma isolates

Table 2. *Antagonistic potential of Trichoderma isolates against S. rolfsii and R. solani*

Isolate name	Mycelial growth inhibition (%) at 3 dpi	
	S. rolfsii	*R. solani*
THDU-1	91.5	95.5
THDU-2	45.3	71.4
THDU-3	95.8	96.7

The antagonistic capabilities of *Trichoderma* isolates were assessed by the inhibition of *S. rolfsii* and *R. solani* growth using the dual culture test. The results showed that all *Trichoderma* isolates caused significant reduction in the mycelial growth of both *S. rolfsii* and *R. solani* (Figure 2). The highest inhibitory activity obtained from isolates THDU-1 and THDU-3. The isolate THDU-2 showed the lowest inhibition effect (45.3%) against *S. rolfsii* and the moderate inhibition effect against *R. solani* (71.4%) compared to control treatment (Table 2).

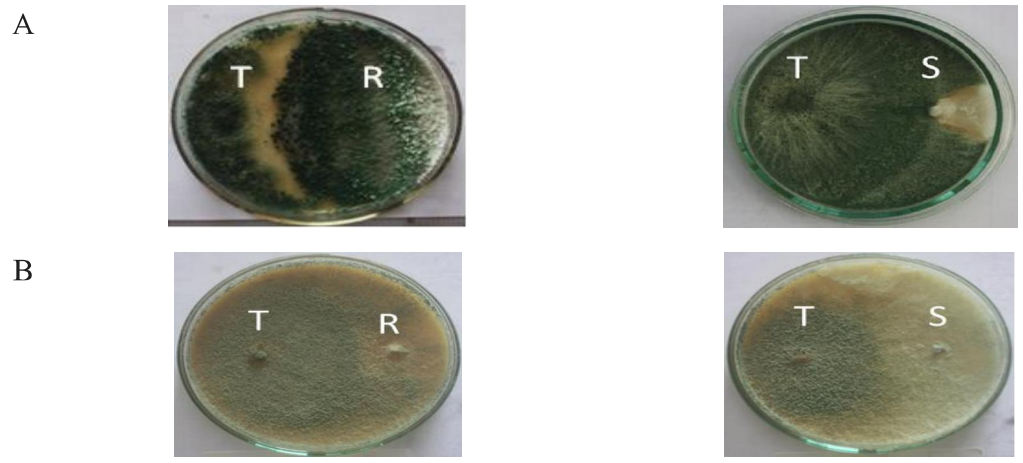

Figure 2. *Antagonistic activity (dual culture assay) of Trichoderma isolates (T) against Sclerotium rolfsii (S) and Rhizoctonia solani (R) at 6th day incubated at 26^{o}C.*
A: THDU-1 isolate; B: THDU-3 isolate

3.3. Mass production of Trichoderma isolates on rice substrate

Table 3. *Number of conidia from Trichoderma isolates incubated on rice substrate*

Isolates	Number of survived conidia during incubation times (days)			
	3	5	9	11
THDU-1	-	2.3×10^4	4.3×10^8	5.7×10^9
THDU-2	-	3.2×10^4	3.8×10^8	5.3×10^9
THDU-3	-	2.5×10^3	1.5×10^8	3.6×10^9

Note: None of conidia observed

Rice was used as substrate for mass multiplication of *Trichoderma* isolates. The number of viable conidia in per gram after 11 days incubation was obtained higher than 3×10^9 conidia/gram, indicating the substrate was potential media for the large-scale production of the fungi (Table 3 and Figure 3).

Figure 3. *Mass production of THDU-1 isolate on rice media*

4. Conclusion

Our study shows that all *Trichoderma* isolates exhibited growth inhibition of plant pathogen *R. sonali* as well as *S. rolfsii*, indicating that *Trichoderma* isolates could be potential biological control agents against soil-borne diseases. We also proposed a protocol of *Trichoderma* mass production using rice substrate which has generated high conidia yield ($\geq 3 \times 10^9$ conidia/g).

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