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Isolation, Identification and Characterization of *Trichoderma* Species as a Potential Biocontrol Agent against *Ceratocystis paradoxa*

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ABSTRACT

Ceratocystis paradoxa is a plant pathogen causing diseases in several plants including coconut (Cocos nusifera). Infected coconut plants manifest "Stem bleeding" which is very common in Hambantota district in Sri Lanka. Coir and coir pith obtained from coconut husk provide substrates for the growth of the pathogen. The use of biological control agents (BCAs) has gained more attention in controlling plant pathogens because it minimizes the application of synthetic pesticides. In many researches, various strains of Trichoderma species have been studied to be used as BCAs against many plant pathogens. The present investigation was carried out in an attempt to isolate various strains of Trichoderma spp. from soil, litter and coir samples collected from different locations in Lunuwila area in the North Western Province of Sri Lanka, and to evaluate their potential as BCAs in controlling C. paradoxa. Several Trichoderma spp. were isolated into PDA plates. Colony morphology and morphological features of sporulating structures and spores were compared among the Trichoderma isolates. Genomic variability of isolates was determined by RFLP analysis of the PCR amplified DNA for the ITS region of the isolates using AluI enzyme. Potential ability of Trichoderma isolates in controlling the pathogen was observed using dual culture technique. Accordingly, ten different Trichoderma isolates were identified from soil but none from coir. The isolates were identified as T. viridae, T. polysporum, and T. harzianum. As BCAs, almost all Trichoderma isolates exhibited more than 60% growth inhibition of C. paradoxa on the seventh day of incubation in a dual culture.

Keywords: *Trichoderma*, *Ceratocystis paradoxa*, *Biological control, Growth inhibition, DNA*

INTRODUCTION

Ceratocystis paradoxa is a soil borne pathogen which causes diseases in several economically important plants such as sugarcane, pineapple, banana, cocoa, coconut and oil palm. Due to its infection, plant trunk either collapses on itself or the canopy suddenly falls off, both without warning. 'Stem bleeding' is a common symptom observed on *Cococs nucifera* (coconut) (Paulin-Mahady *et al*., 2002). It was reported by Petch for the first time in Sri Lanka during 1906 (Warwick and Passos, 2009). In 1995 this disease has been discovered in Hambantota district including areas such as Ambalantota, Beliatta, Netolpitiya, Tissamaharamaya and Weeraketiya. Two adjoining districts of Matara and Rathnapura have been reported to have affected palms especially in Dickwella and in Embilipitiya areas where coconut trees are grown in home lands. Although, the area was free from serious disease epidemics in the past, an epidemic of stem bleeding was reported to be spreading during 1995 (Wijesekara *et al*., 1998).

Chemicals such as methyl bromide and a fungicide named Bavistin are being frequently used to control the pathogen and to protect fields from the pathogen attack. However, use of chemicals is expensive and the heavy usage of chemicals is hazardous to the environment

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(Talukder *et al*., 2007). Biological control of plant pathogens using microorganisms has been considered as more natural and an environmentally acceptable alternative to the chemical controlling methods.

Trichoderma spp. have been found as an effective BCA against many soil borne pathogens (Eziashi *et al*., 2006). *Trichoderma* controls pathogens in an indirect way by producing several groups of antibiotics that inhibit the growth of the pathogen. Apart from that, there are direct methods showing antagonism against the pathogen which is called mycoparasitism. *Trichoderma* species can also inhibit or reduce the growth of plant pathogens especially fungi, through competition for space, enzyme substrates, nutrients, and or oxygen (Sanchez *et al*., 2006).

Therefore, *Trichoderma* species have been used as BCAs for phytopathogenic fungi to control plant diseases. The strains like *T. viridae*, *T. polysporum*, *T. hamatum*, *T. aureoviridae*, *T. harzianum* have the ability to kill plant pathogens. Remarkable results of control have been observed with strains of *T. virens* against *Pythium ultimum* infecting cotton and *Rhizoctonia solani* infecting tobacco and *T. harzianum* against *Verticillium dahliae* infecting potato (Benitez *et al*., 2004). It has been reported of the effective use of *T. hamatum* for the control of *Pythium* seed rot and *Rhizoctonia* root rot in pea (Eziashi *et al*., 2006).

Moreover, *T. viride* has a very good potential in controlling *C. paradoxa* successfully. It was also reported that *T. polysporum*, *T. hamatum*, and *T. aureoviride* have the ability of controlling *C. paradoxa* successfully (Eziashi *et al*., 2010).

The present study was undertaken in an attempt to isolate *Trichoderma* spp. from the sites near Lunuwila area in the North Western Province of Sri Lanka, where coir processing is being done and also to identify *Trichoderma* spp. using morphological and molecular techniques, and to investigate the potential in different *Trichoderma* isolates to control *C. paradoxa* that has been isolated from the same sites. Apart from that it was hypothesized that most of *Trichoderma* isolates can inhibit the growth of *C. paradoxa* according to their parallel evolution in the same location.

Random amplified polymorphic DNA (RAPD) which includes amplified essentially unknown segments of DNA and internal transcribe spacer-polymerase chain reaction (ITS-PCR) are used to carryout molecular characterization of BCAs (Chakraborty *et al*., 2010, Mbwana *et al*., 2006). In this study, amplification of 5.8S rRNA gene using primer pair ITS1 and ITS4 has been done. The two spacers, together with the 5.8S gene, are normally referred to as the ITS region (Schoh *et al*., 2012). This is a very good tool of species identification because rRNA genes are universally conserved, while the ITS region is highly variable. The ITS region evolves very fast and even within a genus it may vary among species. Therefore, the sequences of these regions can be used for identification of closely related species (White *et al*., 1990). Restriction digestion of the amplified DNA using *Alu*I enzyme was performed to differentiate the *Trichoderma* isolates depending on their genomic variability. At present, RFLP is recognized as a very good technique to differentiate species, because cleavage of DNA molecules at specific sites (Beckmann and Soller, 1983). So that, the molecular characterization was also applied during the present study in order to differentiate the isolated *Trichoderma* species.

MATERIALS AND METHODS

Sample Collection

Both fresh and decaying coir samples from old and new heaps were collected into clean polythene bags from Coir Research and Development Institute, St. Antony's Mill and Keerti Mill in Lunuwila area in the North

Western Province of Sri Lanka. The soil samples along with the litter materials were randomly collected from the same locations and stored at 4 °C until used.

Isolation of Trichoderma

Trichoderma spp. were isolated from coir and soil into PDA plates using spread plate technique. Litter materials were cultured in PDA plates for the isolation of the fungi. All of the *Trichoderma* spp. growing on PDA were then isolated into pure cultures.

Identification of Trichoderma Isolates

Colony morphology of the each *Trichoderma* isolate was recorded. For microscopic observations specimens were prepared according to the sticky tape method (Flegel, 1980). Using graticular calibrated phase contrast microscope, spore shape, size and mycelial width of each isolate were measured.

Testing the Biological Control Ability of Trichoderma Isolates

Potential ability of each *Trichoderma* isolate in controlling *C. paradoxa* was studied using dual culture technique (Imtiaj and Lee, 2008).

The percentage of inhibition (I%) on the mycelial growth of *C. paradoxa* was calculated using the following formula (Imtiaj and Lee, 2008).

$I\ \% = (r1-r2)/r1 \times 100$

(r1 is the radial growth of *C. paradoxa* in control, while r2 is the radial growth of *C. paradoxa* in dual culture plate)

Hyphal interactions between *Trichoderma* and *C. paradoxa* were observed using slide culture technique.

Molecular Characterization of Trichoderma Isolates

For DNA extraction hyphal tips of *Trichoderma* isolates were inoculated to 1.5 ml of eppendorf tube filled with 1 ml of Potato dextrose broth and allowed to grow for 72 hours at 28±2 °C incubation. The mycelial mat produced after the inoculation, was pelleted by centrifugation for 5 minutes at 13000 rpm in a microcentrifuge. The pellet was washed with 500 µl of TE (Tris-Cl, EDTA) buffer for pelleting again. The TE buffer was decanted and 300 µl of extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, and 25 mM EDTA, 0.5% SDS) was added to the pellet. The mycelium was crushed with a sterile toothpick. Subsequently, 150 µl of 3M Sodium acetate, pH 5.2 was added and tubes were placed at -20 ° C for 30 minutes. Tubes were centrifuged 10000 rpm for 10 minutes. The supernatant was transferred to another sterile eppendorf tube. Equal volume of isopropanol was added and kept for 10 minutes at room temperature. DNA was precipitated by centrifugation at 12000 rpm for 10 minutes. After a wash with 70% ethanol, the pellet was dried for 5 minutes and re-suspended in 50 µl of TE to make DNA suspension.

ITS-PCR was done for each sample in a 25 µl reaction mixture. Each reaction mixture was contained with 0.2X PCR buffer, 0.16 mM $MgCl_2$, 0.01 µM ITS1 (forward primer), 0.01 µM ITS4 (reverse primer), 0.144 mM dNTP, 0.5 mU/µl Taq polymerase, 2.00 µl of template DNA and 14.95 µl of PCR water. The Thermal cycler was programmed for 30 cycles of initial denaturation at 95 °C for 5 minutes followed by denaturation at 95 °C for one minute. Primer annealing at 55 °C for 1 minute, elongation at 72 °C for 2 minutes, final extension at 72 °C for 10 minutes and final hold 4 °C.

RFLP for each PCR product was studied with *Alu*I enzyme. Restriction digestion mixture was prepared in 30 µl volume containing 0.66X Buffer for restriction enzyme, 1 U/µl *Alu*I restriction enzyme, 15 µl of PCR product and 11.5 µl of water with 1-hour incubation at 37 °C and enzyme inactivation at 70 °C for 5 minutes.

DNA was visualized by agarose gel electrophoresis. The gel was allowed to run at 75V for 120 minutes and observed under UV trans-illuminator at 254 nm wave length.

RESULTS AND DISCUSSION

Isolation and Identification of Trichoderma

Ten different *Trichoderma* isolates have been identified from the collected samples. The identification was carried out according to the morphological and microscopic characteristics followed by the online identification key provided by Samuels and his coworkers (http://nt.arsgrin.gov/taxadescriptions/keys/FrameKey.cfm? gen=*Trichoderma*) and the Compendium of soil fungi (Domsch *et al*., 1980).

Use of morphological characteristics is one of the conventional methods to identify *Trichoderma* species and it remains as a potential method to identify *Trichoderma* up to genus level (Samuels *et al*., 2002).

Table 01 indicates the colony morphology and microscopic characteristics such as length and width (µm) of spore, phialides and mycelia of 10 *Trichoderma* isolates. Pure *Trichoderma* colonies grown in the PDA plates at room temperature showed five different growth patterns with different colony characteristics. The color of the colonies varied from light green to dark green. Each *Trichoderma* isolate having similar colony morphology, may be, most probably, belong to the same species. But colony characteristics are not sufficient to identify them into species level. Therefore, the characteristics of sporulating structures and spores were used. Morphology of the spores and sporulating structures of the isolates were more or less similar, but significantly different in length and width.

Different terms have been used in describing the shapes of the conidia because no systematic rule was established in defining the shapes of the conidia. Also the description of the shapes of conidia may be subjective. Therefore, the descriptions of the shapes of conidia were not good criteria in identifying most of the isolates. The size of spore and phialide sizes were really useful in the identification of possible *Trichoderma* species.

Identified *Trichoderma* species are indicated in Table 02 and most of the isolated species from different sites were more or less similar. Isolate 5 was easily identified as *T. polysporum*. The ellipsoidal conidia, phialides of 7.167µm length and yellow pustules were the key to distinguish those isolates as *T. polysporum*. With the spore and phialide sizes, and other microscopic and macroscopic characters such as presence of sterile hyphal elongations, absence of pustules, isolates 2 and 9 were able to be identified as *T. viridae*. Similarly isolates 4 and 7 were proposed as *T. viridae* especially using spore and phialide sizes as key characteristics. The reason for having different colony morphology may be due to different strains of *T. viridae*. Small spore size (2.6 µm) was the key character of isolates 1 and 8, which were used to predict them as *T. harzianum*. Similarly isolates 3, 6 and 10 contained small spore size (3.3 µm) which was the key feature to identify them also as *T. harzianum*. The difference that was detected in isolates 3, 6 and 10, was the presence of pustules in the culture media whereas isolates 1 and 8 have no pustules. Pustules were not a key character to differentiate them, because *T. harzianum* may or may not contain pustules in the culture. Therefore, those isolates may be different strains of the same species identified as *T. harzianum*. *T. harzianum* can be divided in to three, four, or five sub specific groups, depending on the strains (Grondona *et al*., 1997). Isolate 3, 6 and 10 produced yellow coloured water soluble pigments in PDA plates. The production of a diffusible orange pigment in the medium with gelatin by *T. harzianum* was reported by Rifai (1969) (Grondona *et al*., 1997).

Table 01: Morphological and microscopic characteristics of *Trichoderma* isolates.

Isolate	Colony on PDA		Spores	Phialides	Mycelia
1	Dark green 2 rings per plate	Width (µm)	2.60±0.22	2.083±0.25	5.20±0.10
		Length (µm)	2.60±0.19	8.167±0.34	
2	Dark green 3-4 rings per plate	Width (µm)	4.60±0.15	3.416±0.28	5.20±0.08
		Length (µm)	4.60±18	7.916±0.47	
3	Dark green Rings absent	Width (µm)	3.30±0.11	3.667±0.33	3.70±0.27
	Irregular margin Yellow coloured pigment	Length (µm)	3.30±0.09	5.916±0.51	
4	Bright green	Width (µm)	5.10±0.12	3.500±0.45	5.20±0.11
	Rings at early stage, over the plate later	Length (µm)	5.10±0.15	9.667±0.65	
5	Yellow green Rings absent	Width (µm)	2.87±0.40	3.750±0.44	4.90±0.23
	Yellow pustules over the plate	Length (µm)	3.50±0.27	7.167±0.58	
6	Dark green	Width (µm)	3.30±0.16	3.583±0.35	3.80±0.30
	Rings absent Irregular margin	Length (µm)	3.30±0.18	6.083±0.50	
7	Bright green Rings at early days	Width (µm)	5.10±0.16	3.500±0.36	5.20±0.24
	Colony over the plate later	Length (µm)	5.10±0.14	9.416±0.73	
8	Dark green 2 rings per plate	Width (µm)	2.50±0.23	2.083±0.29	5.20±0.21
		Length (µm)	2.50±0.20	8.250±0.54	
9	Dark green 3-4 rings per plate	Width (µm)	3.40±0.17	3.333±0.28	5.20±0.19
		Length (µm)	3.40±0.18	8.000±0.50	
10	Dark green	Width (µm)	3.30±0.15	3.583±0.44	3.70±0.26
	Rings absent Irregular margin	Length (µm)	3.30±0.13	6.000±0.65	

Almost all *Trichoderma* isolates grown in the PDA plates showed confluent growth within 4 days because of their higher growth rates (Table 03). But there were slight variations among some of the isolates. Among them, isolates 1, 8 (*T. harzianum*) and 7, 8 (*T. viridae*) significantly expressed the highest growth. However, the growth rate of isolate 5 (*T. polysporum*) was comparatively higher than that of isolates 2 and 9 (*T. viridae*). Meanwhile, the slowest growth rates were expressed by isolates 3, 6 and 10 (*T. harzianum*).

Restriction Digestion of ITS Region with AluI Enzyme

However, information from morphological study is not really specific to identify precisely a *Trichoderma* species because *Trichoderma* species have relatively few morphological characters and limited variations (Shahid *et al*., 2013) that may cause overlapping and misidentification of the isolates. Therefore, there is a necessity to use molecular techniques to compensate for the limitations of morphological characterization.

Table 02: *Trichoderma* spp. isolated from different sites

Isolate number	Site of collection	Trichoderma spp.
1	Coir Research and Development Institute	*T. harzianum*
2	Coir Research and Development Institute	*T. viridae*
3	Coir Research and Development Institute	*T. harzianum*
4	Coir Research and Development Institute	*T. viridae*
5	St. Antony's Mill	*T. polysporum*
6	St. Antony's Mill	*T. harzianum*
7	St. Antony's Mill	*T. viridae*
8	Keerthi Mill	*T. harzianum*
9	Keerthi Mill	*T. viridae*
10	Keerthi Mill	*T. harzianum*

Table 03: The average colony diameters of each *Trichoderma* isolates grown in PDA plates

Isolate	Species	Average colony diameter (cm)			
		Day 1	Day 2	Day 3	Day 4
1	*T. harzianum*	2.90 ±0.15	7.40 ±0.07	9.00	9.00
2	*T. viridae*	2.50 ±0.03	5.60 ±0.08	6.90 ±0.08	9.00
3	*T. harzianum*	3.30 ±0.03	4.10 ±0.27	6.40 ±0.15	9.00
4	*T. viridae*	4.10 ±0.07	8.00 ±0.22	9.00	9.00
5	*T. polysporum*	2.00 ±0.10	6.10 ±0.10	7.50 ±0.08	9.00
6	*T. harzianum*	3.30 ±0.10	3.90 ±0.2	6.30 ±0.12	9.00
7	*T. viridae*	3.90 ±0.26	8.30 ±0.12	9.00	9.00
8	*T. harzianum*	3.00 ±0.13	7.40 ±0.08	9.00	9.00
9	*T. viridae*	2.60 ±0.05	4.90 ±0.1	6.70 ±0.20	9.00
10	*T. harzianum*	3.20 ±0.05	3.90 ±0.12	6.40 ±0.07	9.00

According to the gel picture (Figure 01), the PCR amplified 5.8S rDNA flanking ITS1 and ITS4, was around 500bp length. Restriction digestion of the ITS region by *Alu*I enzyme gave 2 DNA bands around 400bp and 100bp sizes, except with isolate 9, suggesting that there is one *Alu*I site in the ITS region. According to the gel picture, five different banding patterns of *Alu*I digested DNA can be seen. There were similar fragments in lane 13 and 16 of the isolates 4 and 7 which were identified as *T. viridae* and they belong to the same strain. Isolate 2 in the lane 11 which gave another banding pattern, was also identified as *T. viridae*, confirming that there are three different *T. viridae* strains. Among the rest of banding patterns that were given by *Alu*I digestion, isolate 1 (lane 10) and isolate 3 (lane 12), which have been identified

as *T. harzianum* gave two slightly different patterns. This confirms that these two were two *T. harzianum* strains and it was also confirmed by the presence (isolate 3) and absence (isolate 1) of pustules in the PDA plate.

Biological Control Abilities of Trichoderma Species

The growth of *C. paradoxa* is significantly reduced when both *Trichoderma* (BCA) (*T. viridae*, *T. harzianum*, *T. polysporum*) and *C. paradoxa* (pathogen) are cultured in dual culture plates. The growth of *C. paradoxa* in the dual culture plate (Figure 02) is significantly lower than the growth in the single culture (Figure 03) which is incubated under similar conditions and same durations.

In almost all the dual culture plates, the contact zone appeared as a curve, with concavity oriented towards *C. paradoxa* (Figure 04). The radial growth of the colony of biocontrol agent and the colony of the pathogenic fungi in the same PDA plate depend on the growth rate of each colony. In this study, all *Trichoderma* isolates showed faster growth rate than *C. paradoxa* and it also serves as one of the characteristics of a good BCA.

The biological control potential of all *Trichoderma* isolates was determined through the average percentage inhibition of mycelial growth of pathogenic fungus: *C. paradoxa*. This approach is frequently used and shown to be a useful way in expressing the potential biological control ability of the biological control agent (Grondona *et al*., 1997).

Higher inhibition (>40%) indicates that the particular *Trichoderma* isolate as a better biological control agent. According to the results obtained (Table 04) all *Trichoderma* isolates showed significantly higher percentage inhibitions which were increased with time. Among them, isolates 3, 6 and 10 (*T. harzianum*) initially exhibited the highest percentage inhibition while on the later days, they showed the lowest percentage inhibition, confirming that the isolates have low rate of inhibition of the pathogen (Figure 06). It suggests that these isolates are not very good BCA because after some time they reduce their biological control ability. However, those *T. harzianum* isolates exhibited inhibition to the mycelial growth of *C. paradoxa* prior to mycelial contact (Figure 05). This could be due to the production of diffusible antimicrobial compounds, such as lytic enzymes or water-soluble metabolites, by *Trichoderma* isolates (Grondona *et al*., 1997).

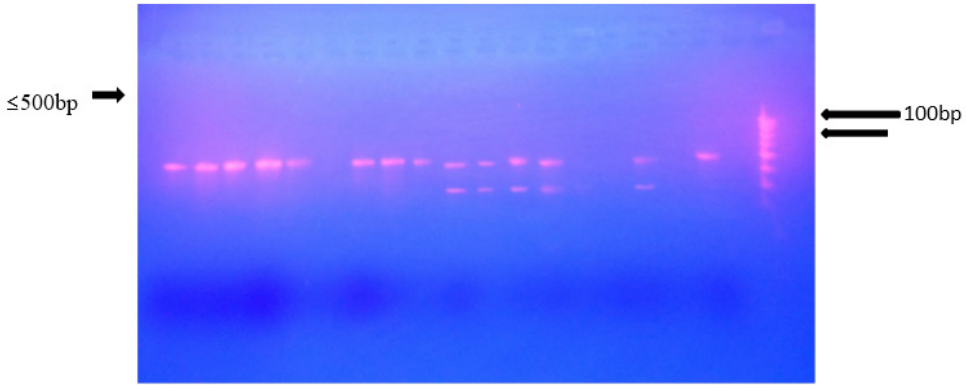

Figure 01: PCR amplified DNA of ITS regions and *Alu*I digested DNA of ITS regions. Lane 1: undigested isolate 1; Lane 2: undigested isolate 2; Lane 3: undigested isolate 3; Lane 4: undigested isolate 4; Lane 5: undigested isolate 5; Lane 7: undigested isolate 7; Lane 8: undigested isolate 8; Lane 9: undigested isolate 9; Lane 10: *Alu*I digested isolate 1; Lane 11: *Alu*I digested isolate 2; Lane 12: *Alu*I digested isolate 3; Lane 13: *Alu*I digested isolate 4; Lane 16: *Alu*I digested isolate 7; Lane 18: *Alu*I undigested isolate 9; Lane 20: 100bp DNA ladder.

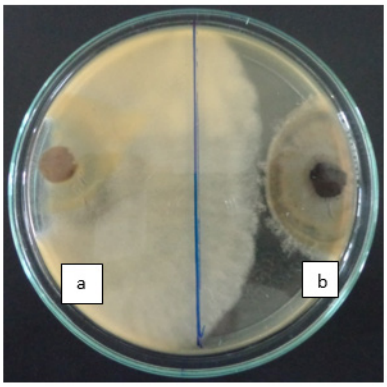

Figure 02: Four days old dual culture plate of *T. viridae* (a) and *C. paradoxa* (b) in PDA.

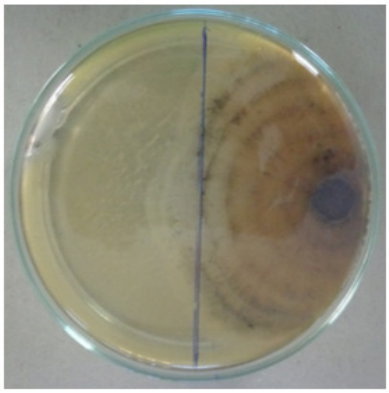

Figure 03: Four days old single culture plate of *C. paradoxa* in PDA

Table 04: Percentage inhibition of *Ceratocystis paradoxa* in the dual culture with *Trichoderma*

Trichoderma isolate used in the dual culture	Percentage inhibition of C. paradoxa in dual culture plate (%)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1- *T. harzianum*	7.41	17.5	25	46.84	55.79	61.82	69.12
2- *T. viridae*	3.70	15	23.21	39.24	47.37	52.73	61.76
3- *T. harzianum*	11.11	12.5	21.43	21.52	26.32	30	43.38
4- *T. viridae*	7.41	12.5	32.14	45.57	53.68	60	67.65
5- *T. polysporum*	11.11	15	23.21	37.97	47.37	52.73	61.76
6- *T. harzianum*	11.11	15	19.64	21.52	26.32	34.55	47.06
7- *T. viridae*	7.41	10	33.93	44.30	52.63	54.55	63.24
8- *T. harzianum*	7.41	20	25	45.57	54.74	60.91	68.38
9- *T. viridae*	3.70	12.5	25	39.24	47.37	52.73	61.76
10- *T. harzianum*	11.11	15	19.64	20.25	28.42	31.82	44.85

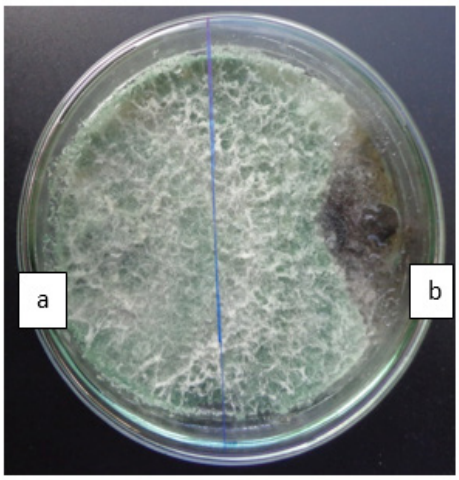

Figure 04: Six days old dual culture plate of *T. viridae* (a) and *C. paradoxa* (b) in PDA.

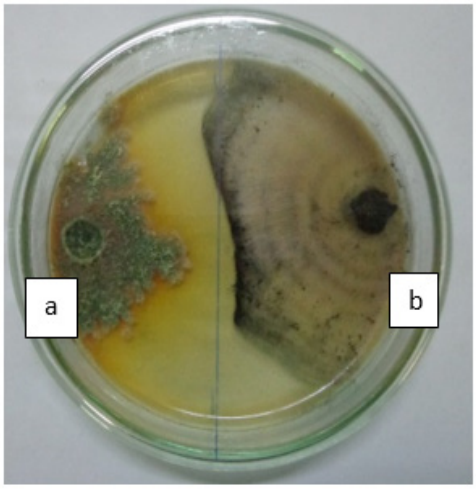

Figure 05: Four days old dual culture plate of *T. harzianum* (a) and *C. paradoxa* (b) in PDA.

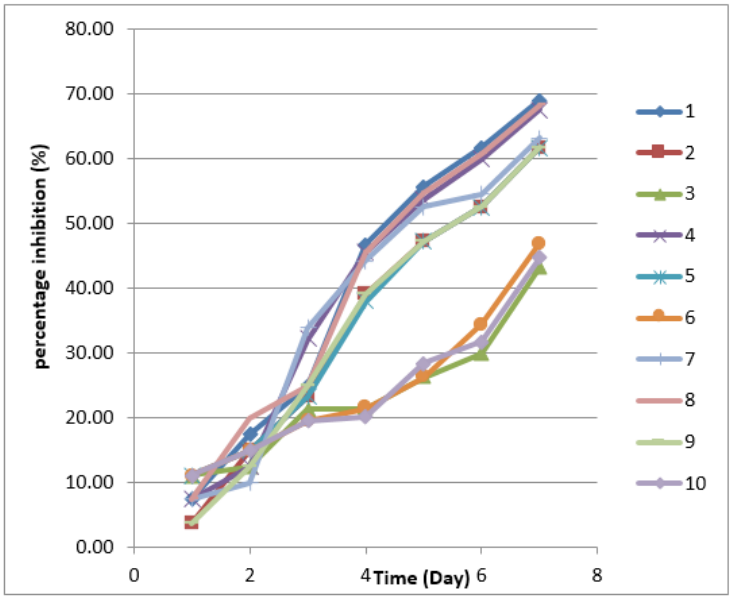

Figure 06: Percentage inhibition of *C. paradoxa* cultured with *Trichoderma* isolates in dual culture

T. harzianum in isolates 1 and 8, and *T. viridae* in isolates 4 and 7 had relatively low percentage inhibition at the beginning while showing high percentage inhibitions later (Figure 05). Therefore, those isolates have the highest rate of inhibition of the pathogen. That makes them very effective BCAs because with time their biological control potentials increase. *T. polysporum* (isolate 5) and *T. viridae* isolates 2 and 9) have exhibited moderate rate of inhibition among the all isolates (Figure 06).

In this study, it was also found that the biological control abilities of different isolates that belong to the same assigned species vary. The previous studies have also reported that even the same *Trichoderma* species can exhibit different abilities to control the same pathogen in different occasions. In 1995 Jinantara have reported that *T. harzianum* possess different abilities to control *Sclerotium rolfsii* (Imtiaj and Lee, 2008).

The presence of different inhibition mechanisms in the same species at different situations has also been reported. This phenomenon could be due to the distinctive biological control mechanisms established by the isolates (Anees *et al*., 2010).

Another possible explanation for this situation is that the responsible gene expression efficiency of biological control abilities may be different in different isolates. The isolates that can express these genes more rapidly and efficiently are usually better biological control agents than the other isolates (Herman, 2006).

Although an attempt was made to observe hyphal interactions between the pathogen and the BCA in a dual culture by using slide culture method, it was unsuccessful because there were no significant hyphal interactions observed. But it has been reported that the mycelium intersections and the subsequent overlapping of hyphae of both BCA and the pathogen *C. paradoxa* began to form after 3 to 4 days and penetration in to the hyphae of pathogen began subsequently (Eziashi *et al*., 2007).

T. harzianum has been reported to have the ability to penetrate into the cell wall of resting spores and hyphae of other fungi which is attributed to the production of enzymes that catalyze the breakdown of chitin, a primary component of fungal cell walls of the pathogen (Brimner and Boland, 2003).

CONCLUSION

This investigation sheds new light on the ways for the use of *Trichoderma* spp. in controlling the plant pathogen *C. paradoxa* which is one of the serious fungal pathogens which develops the stem bleeding in *Cocos nusifera*. The present results also reveal that the *T. viridae*, *T. polysporum*, and *T. harzianum* are capable of controlling the growth of *C. paradoxa*.

The precise benefits and consequences of the present findings open several avenues for future research in the field of biocontrol and biotechnology.

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