

## ISOLATION OF *Trichoderma* FROM COCOA RHIZOSPHERE AND THEIR *in vitro* ANTAGONIST ASSESSMENT AGAINST VASCULAR STREAK DIEBACK (VSD) AND BLACK POD (BP)

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**ABSTRACT** - Fungi of the genus *Trichoderma* are widely used as biocontrol agents against various plant pathogenic fungi. In present study, 29 isolates of *Trichoderma* spp. were assessed for their antibiosis properties against cocoa diseases. They were obtained from rhizosphere soils of cocoa planted in Raub, Pahang and *Trichoderma selective media (THSM)* was used for isolation. By means of poison food agar assay, they were examined for antagonism against *Ceratobasidium theobromae* and *Phytophthora palmivora*, which cause vascular streak dieback (VSD) and black pod (BP) diseases, respectively. Twelve of the isolates were found to be evidently very high antagonistic to *Ceratobasidium theobromae* with percentage inhibition of mycelial growth (PIMG) more than 75%. Meanwhile, there are 3 isolates of *Trichoderma* that showed high antagonistic to *Phytophthora palmivora* with range of PIMG from 61 to 75%. This study shows that there are good potential of using *Trichoderma* spp. as biocontrol agent for VSD and BP. However, the study need to be further tested to evaluate their potential in controlling VSD and BP on cocoa seedlings and mature trees.

**Key words:** *Trichoderma*, rhizosphere, vascular streak dieback, black pod, biocontrol

### INTRODUCTION

Cocoa (*Theobroma cacao*) is an important crop, belonging to the family Malvaceae. Its seeds, cocoa beans, are used to make chocolate, cocoa butter, cocoa solids and chocolate liquor. About 70 percent of the world's cocoa beans come from West African countries which are Cameroon, Ghana, Ivory Coast and Nigeria. The Ivory Coast and Ghana are by far the two largest producers of cocoa, accounting for more than 50 percent of the world's cocoa (Shahbandeh, 2021).

Although demand for cocoa products is increasing worldwide, the cocoa tree can be damaged by several pathogens that may threaten the sustainability of this crop. Approximately 40% of the annual cocoa harvest is lost to pathogens (Nembot *et al.*, 2018). Among the cocoa diseases in Malaysia, black pod (BP) which caused by *Phytophthora palmivora* and vascular streak dieback (VSD) which caused by *Ceratobasidium theobromae* are the most damaging diseases (Bailey and Meinhardt, 2016).

The pathogens of BP and VSD are capable of causing complete yield loss. Several practices have been applied for managing these diseases such as by using fungicides, resistant planting materials, quarantine method and cultural practices. However, none of the above approaches provide sufficient protection to cocoa trees. Moreover, these methods has led to several problems such as environmental pollution, fungicides resistance development,

uneconomical and labor-intensive (Ye *et al.*, 2020). One of such potential nonchemical alternative method is the use of microorganisms as biological control agents for environmentally friendly, cost-effective and sustainable management of plant disease (Kulkarni *et al.*, 2007).

Biological control is a component of an integrated pest and disease management strategy and is defined as the purposeful use of living organisms, their genes, and/or products, such as metabolites that reduces the pest and disease populations. *Trichoderma* spp. are soil-borne fungi and have significant antagonistic potential against a variety of phytopathogenic fungi (Elad *et al.*, 1982). They are free-living and diverse fungal microbial community known worldwide for their utility as bio-control agents in management of fungal diseases of crop plants.

The biological control exerted by *Trichoderma* spp. is due to diverse mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion (Chet, 1987; Schirmbock *et al.*, 1994) that result in augmentation of plant resistance to disease, plant growth and productivity. The potential of *Trichoderma* spp. on managing cocoa diseases mainly BP and VSD were not really well documented. Therefore, this study was executed to isolate *Trichoderma* spp. from cocoa rhizosphere. The isolates obtained were subjected to *in vitro* assays in order to assess the potential of their metabolites against *P. palmivora* and *C.*

*theobromae*, the causal fungi of BP and VSD, respectively.

## MATERIALS & METHODS

### *Isolation of pathogens*

*Phytophthora palmivora* used in this study was isolated from freshly infected cocoa pod by BP disease around Cocoa Research and Development Centre (CRDC) Jengka, Pahang. The infected pod was surface sterilized by using 70% alcohol. The isolation was done by obtained the endocarp tissue from the edge of an actively growing lesion. The tissue was then transferred into potato dextrose agar (PDA) and incubated for three days. The fungi was confirmed as *P. palmivora* based on morphological identification as conducted by Umayah and Purwantara (2006). The morphological characteristics that used for identification are colony type, hyphae enlargement, production of chlamydospores, sporangiophore branches, shape of sporangia and the diameter of pedicels and papillae.

The pathogen of VSD, *O. theobromae* was obtained from infected branches around Cocoa Research and Development Centre (CRDC) Jengka, Pahang. The branches were which shows VSD symptoms such as necrotic and chlorotic leaves, dark vascular discoloration in wood and leaf scars and three blackened vascular traces on the surface of the leaf abscission scars were selected. The samples were freshly collected from cocoa field. The petioles of infected leaves were cut and surface sterilized by using 70% alcohol. After air dried in sterile conditions, the outer layer of petiole was removed and cut into small fragments. The fragments were transferred into coconut water agar (CWA) and incubated for three days. The petiole segments were observed for hyphal growth from the area of the vascular tissue in contact with the agar. Since *C. theobromae* failed to survive in a second subculturing and cannot be maintained in pure culture, only first batch of subculture from infected petiole was used during this study. Hence, *C. theobromae* was repeatedly isolated throughout the experiment.

### *Collection of soil samples and isolation of Trichoderma spp.*

Two local cocoa plantations at Raub, Pahang were selected as sampling sites for this study. From each location, the samples were collected from 3 different cocoa trees. Three centimeter of the top soil was removed and 3 subsamples were then taken at random at a depth of 20 cm for each site under the canopy of cocoa tree. The soil samples were then kept into an ice-box and transported to the laboratory. All subsamples from one site were

mixed to yield one composite sample representing the location (Ru and Di, 2012).

Isolation of *Trichoderma* spp. from collected soil samples were done by using serial dilution technique (Arumugam *et al.*, 2013). Ten gram of soil sample was added into 90 ml sterile distilled water before agitating in an orbital shaker at 100 rpm for 10 minutes. Dilutions were made up to  $10^{-3}$  and 1 ml of final dilutions was pipetted into a petri dish. About 9 ml of *Trichoderma* selective medium (THSM) was poured into diluted soil, swirled gently and left to solid. The soil plates were examined daily and each distinct fungal colony was subcultured onto PDA. Single spore isolation was carried out on new PDA to obtain the pure culture of fungi.

### *Morphological and cultural characteristics of isolated Trichoderma spp.*

The pure culture of isolated fungi were subjected to macroscopic identification before subjecting further to *in vitro* assay. Colony features, pigmentation and sporulation pattern were observed and the growth rate was measured according to identification method by Diba *et al.* (2007).

### *Antibiosis properties – Poison food agar assay*

Poisoned food technique (Tapwal 2015; Sundram 2013) was conducted to evaluate the effect of metabolites released by the *Trichoderma* spp. on the growth of pathogens. Three mycelial plugs (8 mm diameter) of seven days old of *Trichoderma* spp. were inoculated into a conical flask containing 20ml of potato dextrose broth (PDB). After seven days of incubation, the broth was collected, filtered through whatman-I filter paper and later through syringe filter (0.22 $\mu$ m) under sterile conditions.

PDA was amended with culture filtrate (20%) just before pouring and inoculated with *P. palmivora* and *C. theobromae*, respectively. Colony diameter of pathogens was measured after seven days and compared with the growth of pathogen maintained in control petri plates amended with equal amount of distilled water. The diameter growth of pathogens was then transformed into percentage inhibition of mycelial growth (PIMG) formula as below:

$$\text{PIMG (\%)} = \frac{\text{D1} - \text{D2}}{\text{D1}} \times 100\%$$

where: D1= diameter growth of pathogen in control plate

D2= diameter growth of the pathogen in treatment plate

Descriptive assessment of antagonist activity of *Trichoderma* isolates against pathogens was scaled according to Sharfuddin and Mohanka (2012) as follows:

- > 75 PIMG = Very high antagonist activity
- 65 - 75 PIMG = High antagonist activity
- 51 - 60 PIMG = Moderate antagonist activity
- < 50 PIMG = Low antagonist activity

### Statistical analysis

All data were analyzed using SAS version 9.2 and subjected to one-way analysis of variance (ANOVA). The means were compared using Duncan test at  $p \leq 0.05$ .

## RESULTS & DISCUSSIONS

### *Trichoderma* isolates

All colonies emerging from THSM were subcultured onto PDA for macroscopic identification. Young *Trichoderma* culture appeared as faint white hyphal growth followed by green conidiation beginning from the centre of the plate on the third to fourth day after inoculation. Figure 1 shows the colonies features of *Trichoderma* spp. on PDA. Mycelial colour were whitish-green to yellowish and dark green in colors and no pigment diffused through the agar. It composed of rather loose or compact tufts, which formed 1 - 4 concentric rings of conidial zones on the surface of the colony. They produced no distinctive odour, but some isolate emitted 'coconut' odour. Colonies of *Trichoderma* spp. grew rapidly on PDA and the mycelia typically fully grown on 90 mm diameter plate within four days. Sekhar *et al.*, (2017) also successfully obtained several isolates of *Trichoderma* spp. from rhizosphere of healthy plants in groundnut field.

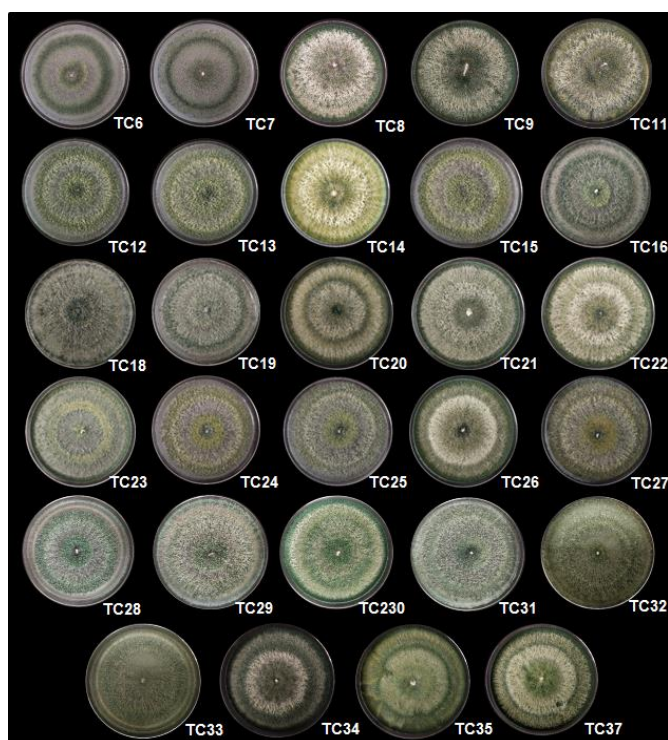


Figure 1: Colony features of different isolates of *Trichoderma* on PDA medium at 5<sup>th</sup> day after inoculation.

**Antibiosis properties**

All 29 isolates of *Trichoderma* were subjected to poison food agar assay. They were grown in PDB assuming that they will utilize the nutrients from broth and release some metabolites, which may affect the growth of pathogens. All isolates varied in their abilities to produce culture filtrate metabolites that inhibited *C. theobromae* and *P. palmivora* growth. Table 1 presented the number of *Trichoderma* isolates exhibiting inhibition towards both pathogens through the PIMG values. There are 12 isolates of *Trichoderma* that exhibited the PIMG values more than 75% against *C. theobromae*, which categorized them as very high

antagonist. Among them, culture filtrate from TC11, TC12, TC14, TC15, TC23, TC30 and TC32 were completely inhibited *C. theobromae* growth with 100% PIMG value, where the pathogen fails to grow on the culture filtrate-impregnated media. Meanwhile, there are 3 isolates of *Trichoderma* (TC37, TC14 and TC18) that showed high antagonist to *P. palmivora* with range of PIMG from 61 to 75%. For overall, antibiosis properties secreted by TC14 was the most antagonist against both pathogens, *C. theobromae* and *P. palmivora* with PIMG 100% and 63.37%, respectively.

Table 1: Mean value of percentage inhibition of mycelial growth (PIMG) exhibited by *Ceratobasidium theobromae* and *Phytophthora palmivora* against 29 isolates of *Trichoderma* spp.

Isolate no.	Mean value of PIMG (%)	
	<i>C. theobromae</i>	<i>P. Palmivora</i>
TC6	8.25 <sup>ijk</sup>	8.60 <sup>mnp</sup>
TC7	13.61 <sup>hijk</sup>	5.81 <sup>nop</sup>
TC8	74.11 <sup>abcd</sup>	46.27 <sup>cd</sup>
TC9	44.48 <sup>efg</sup>	48.35 <sup>c</sup>
TC11	100.00 <sup>a</sup>	36.31 <sup>defg</sup>
TC12	100.00 <sup>a</sup>	36.85 <sup>defg</sup>
TC13	91.14 <sup>ab</sup>	16.61 <sup>klmn</sup>
TC14	100.00 <sup>a</sup>	63.37 <sup>b</sup>
TC15	100.00 <sup>a</sup>	21.85 <sup>ijk</sup>
TC16	26.98 <sup>ghijk</sup>	38.95 <sup>cdef</sup>
TC18	2.93 <sup>jk</sup>	61.65 <sup>b</sup>
TC19	91.13 <sup>ab</sup>	1.61 <sup>op</sup>
TC20	14.34 <sup>hijk</sup>	33.51 <sup>fgh</sup>
TC21	28.02 <sup>ghij</sup>	39.25 <sup>cdef</sup>
TC22	62.85 <sup>cdef</sup>	20.89 <sup>jkl</sup>
TC23	100.00 <sup>a</sup>	32.02 <sup>fghi</sup>
TC24	47.46 <sup>defg</sup>	16.90 <sup>klmn</sup>
TC25	64.33 <sup>bcde</sup>	27.00 <sup>ghij</sup>
TC26	65.16 <sup>bcde</sup>	45.35 <sup>cde</sup>
TC27	92.01 <sup>ab</sup>	18.39 <sup>ijklm</sup>
TC28	60.56 <sup>cdef</sup>	11.81 <sup>klmno</sup>
TC29	60.93 <sup>cdef</sup>	19.15 <sup>ijklm</sup>
TC30	100.00 <sup>a</sup>	13.33 <sup>klmn</sup>
TC31	83.18 <sup>abc</sup>	9.99 <sup>lmnop</sup>
TC32	100.00 <sup>a</sup>	22.78 <sup>hijk</sup>
TC33	35.72 <sup>fgh</sup>	34.49 <sup>efg</sup>
TC34	32.62 <sup>ghi</sup>	17.36 <sup>ijklm</sup>
TC35	89.87 <sup>ab</sup>	20.29 <sup>ijklm</sup>
TC37	50.63 <sup>ddefg</sup>	74.45 <sup>a</sup>

Means for respective pathogen with same letter or symbol are not significantly different among themselves when least significant difference (LSD) test were used at 0.05 significance level

The inhibition growth of pathogens is might due to the effect metabolites secreted by *Trichoderma* spp. in PDB. It is important to highlight that *Trichoderma* spp. can produce several antibiotics such as Trichodermol,

Trichodermin, Harzianolide and Harzianum A (Hajieghrari *et al.*, 2010). There are several studies which also reported significant inhibitory activity of *Trichoderma* spp. against *C. theobromae* (Harni *et al.*, 2017) and *P. pamivora* (Harni *et al.*, 2020).

## CONCLUSIONS

It is concluded from this study that metabolites secreted by *Trichoderma* spp. has a good potential on controlling VSD and BP. However, these metabolites are more effective on inhibiting the growth of VSD pathogen under *in vitro* conditions. This study needs to be further investigated on identification of *Trichoderma* spp. into species level, and examined the metabolites effect on cocoa seedlings and mature trees.

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