Effect of secondary metabolites of *Trichoderma* spp. in inhibiting *Phytophthora palmivora* growth in cacao (*Theobroma cacao* L.)

R Harni¹*, W Amaria¹ and A H Mahsunah²

¹ Indonesian Industrial and Beverage Crops Research Institute, Jalan Raya Pakuwon km 2, Parungkuda, Sukabumi 43357, Indonesia
² Center for Biotechnology, The Agency for the Assessment and Implementation of Technology, Kawasan PUSPITEK Serpong - Gedung 630, Tangerang Selatan 15314, Indonesia

*Email: rita_harni@yahoo.co.id*

**Abstract.** Secondary metabolites are organic compounds produced by microorganisms or plants which are not directly involved to development and reproduction. The secondary metabolites produced by *Trichoderma* spp. are antibiotic compounds, enzymes, toxins, and hormones that inhibit the growth of plant pathogens. *Phytophthora palmivora* is a pathogen that causes black pod, leaf blight, and stem canker in cacao, which potentially reduce production up to 40%. This study was aimed to determine the inhibitory effect of *Trichoderma* spp. secondary metabolites against *P. palmivora*. Isolates of *Trichoderma* spp., i.e. *T. virens* LP1, *T. hamatum* LP2, *T. amazonicum* LP3, *T. atroviride* JB2, and *T. viride* PRD were cultured on vegetative and generative medium, then their secondary metabolites was extracted using butanol and ethyl acetate. Analysis of the extracts of secondary metabolite was performed using HPLC to determine their chromatogram profile. The secondary metabolites obtained were tested against *P. palmivora* using poisoned-medium method and cacao seedlings. *In vitro* inhibition activity, disease severity, and growth of cacao seedlings were observed. The results showed that extraction of the secondary metabolite using butanol produces more metabolites than those using ethyl acetate. Secondary metabolites of *T. virens* LP1 and *T. amazonicum* LP3, significantly inhibited *P. palmivora* growth up to 90.98%. In general, secondary metabolites of *Trichoderma* spp. can suppress *P. palmivora* infection (60.02%–70.00%) in cacao seedlings.

1. Introduction

*Trichoderma* is a biocontrol agent that are widely used as a bio pesticides in controlling plant diseases [1]. The main advantages of *Trichoderma* as biocontrol agents are the high capability as rhizospheric and saprophytic fungi, antagonistic to many pathogens, can be easily propagated, have a broad antagonistic spectrum, environmentally friendly, compatible with other biological agents [2], and also can increase plant growth, as well as its ability to tolerate abiotic stresses such as drought for several *Trichoderma* strains [1].
Trichoderma mechanisms in controlling plant diseases are varies depend on the species and pathogen, including antibiotics production [3–5], hyperparasitism activity [4, 6, 7], nutrients competition, degradative enzyme activity [3] and systemic resistance induction [8].

Some fungi from the genus Trichoderma are the best producers of secondary metabolites [9]. Based on previous research, more than 100 compounds have been produced by Trichoderma sp. [10, 11] included non-polar compounds such as pyrones, terpenoids, steroids and polyketides. Trichoderma secondary metabolites that have been used to control plant diseases included isoharzianic acid (iso-HA), which was isolated from T. harzianum to suppress Sclerotinia sclerotiorum and Rhizoctonia solani; and pyron to suppress Gaeumannomyces graminis var. tritici. Coninginin isolated from T. harzianum, T. koningii and T. atroviride showed in vitro antibiotic activity against Gaeumannomyces graminis var. tritici, R. solani, Phytophthora cinamomi, Pythium middletonii, Fusarium oxysporum and Bipolaris sorokiniana. Viridin isolated from T. koningii, T. viride, and T. virens to prevent the germination of Botrytis allii spores, Colletotrichum lines, Fusarium caeruleum, Penicillium expansum, and Aspergillus niger [9, 12, 13].

The fungus Phytophthora palmivora is an important pathogen in cacao [14, 15] which causes black pod, leaf blight, and stem canker. The infection may cause yield loss up to 40% [16–18] or higher in the rainy season [19]. The use of Trichoderma secondary metabolites to control P. palmivora has not been reported. This study was aimed to determine the inhibitory effect of Trichoderma spp. secondary metabolites against P. palmivora in cacao.

2. Methods

The research was conducted at Plant Protection Laboratory and Greenhouses of the Indonesian Industrial and Beverage Crops Research Institute (IIBCRI), Sukabumi, from June to Oct 2016.

2.1. Regeneration of Trichoderma spp.

Five isolates of Trichoderma spp. were used in the experiment, i.e. T. virens LP1, T. hamatum LP2, T. amazonicum LP3, T. atroviride JB2, and T. viride PRD. The isolates were cultured on potato dextrose agar (PDA) media.

2.2. Mass production of secondary metabolites

Secondary metabolites were produced through the vegetative and fermentative media. The vegetative stage is the multiplication of Trichoderma spp. to produce fungal population with uniform age, which then used as inoculum for the fermentation stage. Vegetative media composed of 20 g glucose; 100 g potatoes; 3.4 g Na₂HPO₄; 1.99 g NaH₂PO₄; 1 g urea; 0.2 g KCl; 0.2 g MgSO₄; 1 mg Thiamin HCl; 0.002 g MnSO₄; 0.002 g ZnSO₄; 0.002 g FeSO₄; 1,000 ml RO water. All ingredients were mixed and made up to 1000 ml by adding RO water. The pH was adjusted to 6.8–7.0 (with 20% NaOH addition), then put 100 ml each into a 500 ml erlenmeyer, then sterilized at 121 °C for 15 min. The starter preparation was begun with suspending Trichoderma spp. cultures by adding 2 ml of physiological solution of [0.9% (b/v)] sterile NaCl to the sloping agar. One ml of suspension [1% (v/v)] was put into a 500 ml erlenmeyer containing 100 ml vegetative media and incubated at 25–28 °C for 3 days using an orbital shaker at the speed of 150 rpm. Vegetative stage was carried out for 72 hr followed by fermentative stage. Material composition and preparation method of fermentative media was the same as those in the vegetative media, except for the potatoes used at this stage were 25 g. At the fermentative, 2% inoculums were added [v/v], then incubated at 25-28 °C for 4 days using an orbital shaker speed of 150 rpm. At the end of the fermentative process, packed mycelium volume (PMV) and pH were measured.

2.3. Extraction of secondary metabolites

Extraction of Trichoderma spp. secondary metabolites was conducted using butanol or ethyl acetate solvents. The extraction step was initiated by adding the butanol or ethyl acetate solvent into Trichoderma spp. fermentation at a ratio of 1:1 (v/v), then stirred at 150 rpm for 1 hr. Furthermore, the
separation of the solvent with the water phase from *Trichoderma* spp. extraction was done with 4000 rpm for 15 min centrifugation. The solvent phase (ethyl acetate or butanol) was evaporated to obtain concentrated extracts. Rotavapor ethyl acetate and butanol phase was carried out at 35 °C and 55 °C, respectively; followed by drying step using a centrifugal concentrator. Weight of secondary metabolites extracts obtained were measured and used for further testing.

2.4. Analysis of secondary metabolites
Analysis of secondary metabolite extracts was performed using high performance liquid chromatography (HPLC) to determine the chromatogram profile. Fractionation was carried out using a chromatographic column with the following specifications: internal diameter of the column 4.5 cm; Merck® Silica gel 60 (0.063–0.200 mm) column packing for column chromatography; extract weight of 2 g; silica gel 60 g (3 times the weight of the fractionated extract); column packing volume of 160 ml; eluent volume 500 ml (≈ 3 times the column packing volume); eluent of chloroform: methanol with 7 different composition, i.e. 100: 0; 99: 1; 98: 2; 95: 5; 90: 10; 80: 20; 50: 50; and 0: 100. This stage begun with the preparation of the column, namely installing the glass column on the statics pole until the glass column was mounted perpendicularly and installing cotton at the bottom of the glass column to avoid leaking of the column. Next, silica gel in chloroform was suspended and put in a glass column. A total of 2 g of extract in chloroform was dissolved with methanol, and silica gel powder was added to a homogeneous sample with silica powder. The solvent was evaporated using a rotary evaporator. After it is completely dried, the extract which was bound to silica gel was suspended into chloroform and inserted into the glass column. The sample was eluted with eluent and collected into erlenmeyer, each fraction was dried with a rotary evaporator at 30 °C and a vacuum of ± 100 mbar/hPa.

2.5. Testing of secondary metabolites inhibition in vitro
Crude extracts of secondary metabolites from 5 isolates were tested for their ability to inhibit the growth of *P. palmivora* colonies. The method used was food poisoning. The experiment used a randomized complete design with 3 replications. Each metabolite in the form of butanol and ethyl acetate extracts was mixed on PDA media with a concentration of 1 000 ppm (at 40 ºC), then the media was poured on a 9 cm diameter petri dish [9]. After media become solid, *P. palmivora* mycelium with diameter of 0.8 cm was placed in the middle of the media. Media without secondary metabolites was used as check control. The culture were incubated at 25 ºC until the growth of colonies in check control filled up the petri dish. The percentage inhibition of secondary metabolites against *P. palmivora* growth was observed by measuring the diameter of the colony on a petri dish.

2.6. Testing of secondary metabolites in cacao seedlings
The effectiveness of secondary metabolites from 5 isolates of *Trichoderma* spp. against *P. palmivora* were tested in cacao seedlings. The study used a complete randomized design with 5 replications. The treatments involved application of secondary metabolites from 5 isolates, positive control (without application of *Trichoderma* spp.) and as a comparison was application of fungicides. Application of secondary metabolites were given around the roots of 3-months old cacao seedlings. Along with the treatment of secondary metabolites, plants were inoculated with *P. palmivora*. Plant inoculation was carried out using hyphae sticks attached to young leaves. Plant maintenance was carried out in accordance with standard procedures for cacao seeds maintenance, such as watering the seeds every day, removing weeds, and controlling pests if necessary. Observations were made on the disease severity and growth of cacao seedlings (plant height, number of leaves, and stem diameter).

3. Results and discussion

3.1. Mass production and extraction of *Trichoderma* spp.
Observations on vegetative growth of T. virens, T. amazonicum, T. hamatum and T. viride were obtained at pH 6.51–6.90 and at 4.42 for T. atroviride. During the fermentative phase, the pH of each Trichoderma spp. has decreased to 6.05–6.52 for T. virens, T. amazonicum, and T. hamatum, and increased to 4.56 for T. atroviride (Table 1). The pH was related to the metabolic activity of microorganisms in the fermentative phase. This was indicated by bioconversion of glucose as a substrate and the use of sodium phosphate salt. Two factors affecting the pH of the medium are activity of the microorganism itself and production of acids by microorganisms.

The measurement of packed miselium volume (PMV) showed that each Trichoderma species has different growth rate. In the vegetative stage, T. virens LP1 has the highest growth i.e. 28.0%, followed by T. viride PRD, T. hamatum LP2 and T. amazonicum LP3, and T. atroviride i.e. 23%, 15%, 15%, and 4%, respectively. At the fermentative stage, the PMV value of T. virens and T. viride were decreased, while it was increased in T. amazonicum LP3 and T. atroviride (Table 1).

**Table 1.** Values of pH and packed miselium volume (PMV) of Trichoderma spp. at the vegetative and fermentative stages.

| Treatments        | Vegetative | Fermentative |
|-------------------|------------|--------------|
|                   | pH        | PMV (%)      | pH        | PMV (%)      |
| T. virens LP1     | 6.90      | 28.00        | 6.52      | 24.00        |
| T. hamatum LP2    | 6.59      | 15.00        | 5.80      | 22.00        |
| T. amazonicum LP3 | 6.61      | 15.00        | 6.46      | 19.00        |
| T. atroviride JB2 | 4.42      | 4.00         | 4.56      | 6.00         |
| T. viride PRD     | 6.51      | 23.00        | 6.05      | 19.00        |

The difference of PMV values of Trichoderma spp. depends on the growth ability of each Trichoderma spp., T. virens LP1, and T. viride PRD growth faster compared to the other Trichoderma species, because they have the ability to grow rapidly and easily on various media [7]. In the fermentative stage, decreased in PMV weight was observed in 2 species of Trichoderma spp. which have reached the stationary phase so that they did not develop and some might be dead.

### 3.2. Secondary metabolite extraction

Different solvents, butanol or ethyl acetate, yielded different secondary metabolites extract weight of each isolates of Trichoderma (Table 2). Secondary metabolites extracts with butanol was higher than those of ethyl acetate solvent. The use of butanol solvent produces the highest secondary metabolites in T. atroviride JB2 (2.1102 g) and the lowest in T. hamatum LP2 (0.9914 g). Extraction using butanol produced polar to semi-polar compounds, as can be observed in the chromatogram profile with the peak was reached at 0-10 min. On the other hand, extraction using ethyl acetate produced semi-polar compounds with the peak was reached at 6–15 min.

**Table 2.** Weight of the extract of secondary metabolites produced by Trichoderma spp. using butanol and ethyl acetate solvents.

| Treatments         | Extract weight (g) | Butanol | Ethyl acetate |
|--------------------|--------------------|---------|---------------|
| T. virens LP1      | 1.0162             | 0.6417  |               |
| T. hamatum LP2     | 0.9914             | 0.3633  |               |
| T. amazonicum LP3  | 1.1450             | 0.6679  |               |
| T. atroviride JB2  | 2.1102             | 0.8057  |               |
3.3. Secondary metabolite chromatogram

Based on the HPLC chromatogram profile of *T. virens* LP-1 active extracts, there was 1 similar peak, i.e. at a retention time of 8.22/8.209 min (Fig. 1). In *T. amazonicum* LP3 active extracts, the same compound in both extracts eluted at a retention time of 10.76/10.10 min (Fig. 2). It can be concluded that the compound has active compound activity. The active compounds produced by the extract *T. virens* LP1 and *T. amazonicum* LP3 were different due to its retention time. However, further analysis using LC-MS is required.

![HPLC chromatograms](image)

**Figure 1.** Chromatogram of *Trichoderma* spp. butanol extracts; (a) *T. virens*; (b) *T. hamatum*; (c) *T. amazonicum*; (d) *T. atroviride*; (e) *T. viride*.

3.4. Testing of secondary metabolite inhibition in vitro

All secondary metabolites of *Trichoderma* spp. both with butanol and ethyl acetate solvents influenced the growth diameter of *P. palmivora* colonies. Secondary metabolite extract with butanol solvent suppressed the growth of *P. palmivora* diameter especially for *T. virens* LP1 and *T. amazonicum* LP3 metabolites up to 91.11% (*P. palmivora* colonies was not growing and the average diameter was 0.8 cm) until 5 days observation. The other metabolites did not suppress the growth of *P. palmivora* (Table 3 and Fig. 3).
Figure 2. Chromatogram of *Trichoderma* spp. ethyl acetate extracts; (a) *T. virens*; (b) *T. hamatum*; (c) *T. amazonicum*; (d) *T. atroviride*; (e) *T. viride*.

Table 3. Inhibition rate of *Trichoderma* spp. secondary metabolites in butanol solvent against *P. palmivora*.

| Isolates       | Diameter of colonies (cm) | Inhibition (%) |
|----------------|---------------------------|----------------|
|                | 1  | 2  | 3  | 4  | 5  |               |
| *T. virens* LP1| 0.80 | 0.80 | 0.80 | 0.80 | 0.80 | 91.11         |
| *T. hamatum* LP2| 2.23 | 4.23 | 6.20 | 8.53 | 9.00 | 0.00          |
| *T. amazonicum* LP3| 0.80 | 0.80 | 0.80 | 0.80 | 0.80 | 91.11         |
| *T. atroviride* JB2| 0.93 | 1.33 | 1.57 | 2.13 | 2.47 | 72.56         |
| *T. viride* PRD| 1.77 | 3.23 | 5.00 | 6.90 | 8.17 | 9.22          |
| Control        | 1.93 | 3.60 | 5.73 | 7.60 | 9.00 | -             |
Secondary metabolites of *Trichoderma* spp. contain several important compounds that are potential to inhibit the growth of pathogens by cell wall degradation and lysis. The mechanism of *T. virens* LP1 and *T. amazonicum* LP3 in suppressing the growth of *R. microporus* were by parasitism and competition and this is similar to previous research [7].

![Figure 3. Performance of *P. palmivora* growth on PDA medium with application of *Trichoderma* spp. secondary metabolites; (a) *T. virens* LP1; (b) *T. hamatum* LP2; (c) *T. amazonicum* LP3; (d) *T. atroviride* JB2; (e) *T. viride* PRD; (f) Control.](image)

The effect of secondary metabolite extract in ethyl acetate solvent was not different from those in butanol solvent. Secondary metabolites caused quite high growth suppression of *P. palmivora* colonies. Secondary metabolites of *T. virens* LP1 and *T. amazonicum* LP3 inhibited the growth of *P. palmivora* up to 90.98% while *T. atroviride* JB2 77.79% and *T. hamatum* LP2 and *T. viride* PRD were 43.29% and 34.95%, respectively (Table 4 and Fig. 4).

**Table 4. Testing of secondary metabolites of *Trichoderma* spp. in ethyl acetate solvent against *P. palmivora***

| No | Isolates        | The average diameter of colonies (cm) | Inhibition (%) |
|----|-----------------|--------------------------------------|----------------|
| 1  | *T. virens* LP1 | 0.80 0.80 0.80 0.80 0.80             | 90.98          |
| 2  | *T. hamatum* LP2| 1.70 2.53 3.40 4.33 5.03             | 43.29          |
| 3  | *T. amazonicum* LP3| 0.80 0.80 0.80 0.80 0.80 | 90.98          |
| 4  | *T. atroviride* JB2| 0.80 1.23 1.47 1.73 1.97 | 77.79          |
| 5  | *T. viride* PRD | 1.87 2.87 4.03 5.07 5.77             | 34.95          |
| 7  | Control         | 1.80 3.43 5.57 7.30 8.87             | -              |
Figure 4. Testing of secondary metabolites of *Trichoderma* spp. in ethyl acetate solvent against *P. palmivora*; (a) *T. virens* LP1; (b) *T. hamatum* LP2; (c) *T. amazonicum* LP3; (d) *T. atroviride* JB2; (e) *T. viride* PRD; (f) Control.

3.5. Testing of secondary metabolites in cacao seedlings

All secondary metabolites tested can suppress the development of *P. palmivora* on cacao seedlings. The highest effect was on the secondary metabolites of *T. virens* LP1 (70%), followed by *T. amazonicum* LP3 and *T. atroviride* JB2 (60.02%). The effect was almost the same as chemical fungicides, i.e. 76.17% (Table 5). Disease severity was decreased in the treatment of *T. virens* LP1 and *T. amazonicum* LP3. This is due to the composition of secondary metabolites which are thought to contain antibiotic, enzymes, toxins, and hormones. These compounds can suppress the development of pathogens and also induce resistance and increase plant growth [13, 20]. It was reported previously that the secondary metabolites of *T. amazonicum* LP3 and *T. virens* LP1 were also effective in controlling *Cerathobacidium theobromae* with suppression of disease severity by 81.8% and 63.2% in cacao seedlings [21].

Table 5. Effect of *Trichoderma* spp. secondary metabolites on the infection of *P. palmivora* in cacao seedlings.

| No. | Isolates          | Disease severity (%) | Disease suppression (%) |
|-----|-------------------|----------------------|-------------------------|
| 1   | *T. virens* LP1   | 11.11                | 70.00                   |
| 2   | *T. hamatum* LP2  | 14.81                | 55.56                   |
| 3   | *T. amazonicum* LP3 | 14.81               | 60.02                   |
| 4   | *T. atroviride* JB2 | 14.81             | 60.02                   |
| 5   | *T. viride* PRD   | 18.52                | 44.43                   |
| 6   | Chemical fungicide | 11.11               | 76.17                   |
| 7   | Control           | 48.15                | -                       |
Based on this research, all of the secondary metabolites tested were not phytotoxic to cacao seedlings. As shown in Table 6, the increase of plant height was performed by *T. amazonicum* LP3, while the increase of number of leaves and stem diameter were performed by *T. atroviridae* JB2.

**Table 6.** Effect of *Trichoderma* spp. secondary metabolites to plant height, number of leaves and stem diameter of cacao seedlings.

| Treatments      | Increase of plant height (cm) | Increase of number of leaves | Increase of stem diameter (cm) |
|-----------------|-------------------------------|------------------------------|--------------------------------|
| *T. virens* LP1 | 3.01                          | 7.50                         | 0.59                           |
| *T. hamatum* LP2| 3.51                          | 8.00                         | 0.52                           |
| *T. amazonicum* LP3| 5.04                        | 8.20                         | 0.55                           |
| *T. atroviride* JB2| 4.39                       | 8.80                         | 0.60                           |
| *T. viride* PRD | 4.69                          | 7.60                         | 0.53                           |
| Chemical fungicide | 2.91                       | 7.80                         | 0.52                           |
| Control         | 2.81                          | 8.00                         | 0.55                           |

4. Conclusion

It is concluded from this research that the extraction of secondary metabolites by butanol solvent produced more extracts than those by ethyl acetate. Secondary metabolites of *T. virens* LP1, and *T. amazonicum* LP3, significantly inhibited the growth of *P. palmivora* compared to *T. hamatum* LP2, *T. atroviride* JB2, and *T. viride* PRD with inhibition of 90.98%, and suppressed the infection of *P. palmivora* in cacao seedlings up to 70.00% and 60.02%, respectively.

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