In Vitro Antifungal Activity of Thiram Against *Ganoderma boninense*

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ABSTRACT Basal stem rot (BSR) which is caused by *Ganoderma boninense* (GB) is the most serious disease faced by oil palm industry, especially in Malaysia. To date, there is no satisfactory control measure for this disease, and researchers are investigating different approaches in managing the disease. The current study investigates the antifungal properties of Tetramethylthiuram disulfide or commonly known as thiram, against GB. The *in vitro* antifungal activity of thiram were expressed in inhibition of GB mycelia growth on Potato Dextrose Agar (PDA) incorporated with different concentrations of thiram (0.010, 0.012, 0.014, 0.016, 0.018, 0.020, and 0.030 mg/ml). The lower concentrations of thiram, such as 0.010 mg/ml, failed to inhibit the growth of GB completely. However, higher concentrations of thiram (0.012 to 0.020 mg/ml) significantly slow the growth rate of GB in comparison to control (without thiram). The concentration of thiram at 0.030 mg/ml completely inhibits the growth of GB. To further evaluate the effectiveness of the treatment, the GB treated mycelia were examined for their ergosterol content using HPLC. The result shows that a higher amount of ergosterol content was found in less effective treatments, and no ergosterol was found in sample when GB is completely inhibited.

KEYWORDS: *Ganoderma boninense;* Basal stem rot; Oil palm; Thiram; Ergosterol
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INTRODUCTION

The oil palm (*Elaeis guineensis*) is the most productive oil-bearing crop in Malaysia. About 5.7 million hectares of land in Malaysia has been cultivated with oil palm with an output of 17.3 million tonnes of crude palm oil, which supplies 32% of the world vegetable oil and fats (Rakib *et al*., 2017). Unfortunately, the sustainability of the oil palm production now faces the threat of a severe disease called basal stem rot (BSR) which is caused by the basidiomycete G. *boninense* and is considered the most destructive disease of oil palm in Malaysia (Susanto *et al*., 2005; Hushiarian *et al*., 2013). The disease does not show early symptom to the infected palms, where the symptoms only appear at a very late of infection when 60% of the internal tissues are already rotten, leaving no chance for the infected palms to be cured (Alexander *et al*., 2014a; Alexander *et al*., 2014b). Numerous methods have been taken to control this disease. However, to date, there is currently no effective cure for *G. boninense* infection in an existing stand. The use of fungicides in controlling BSR of oil palm is not a popular option due to its effectiveness; however, it may be further explored considering their effectiveness against many other pathogens infecting other worldwide crops. Fungicide used to control fungal diseases by destroying and inhibiting the fungus or fungal spores. Thiram (a non-systemic fungicide) with a common name of tetramethyl thiuram disulfide (chemical formula: $C_6H_{12}N_2S_4$) belongs to the group of dimethyl dithiocarbamate compounds which have melting points ranging from 155°C-156°C and a molecular mass of 240.41. Thiram is available as dust, wettable powder, water suspension formulations and in mixtures with other fungicides (Fialho *et al*., 2012). It is used to protect harvested crops from deterioration in storage or transport (USEPA, 2004; Zhang *et al*., 2018), as 'foliar application' for control of disease on fruits and vegetable crops, lawns and turf and also as 'seed treatment' to protect the latter against fungal diseases. In addition, at high doses it acts as an animal repellent to protect crops from damage by birds, rabbits, deer, and rodent in

orchards and fields (Okur, 2010). According to the USEPA Draft Proposed Guidelines for Carcinogen Risk Assessment, thiram is classified as "not likely to be carcinogenic to humans" (USEPA, 2004). Thiram is rapidly broken down by hydrolysis and photodegradation especially under acidic conditions (Thomas, 2011), also has a low to moderate persistence in soil and nearly immobile in clay soils or soils that are high in organic matter content (Sharma *et al.*, 2003). Chaurasia *et al.* (2017) has mentioned the effectiveness of thiram against *Rhizopus oryzae* fungi that causing fruit rot of brinjal. The application of thiram in oil palm plantation in Malaysia has been reported by Kuntom *et al.* (2007) since 1999 and thiram is one of the most common fungicides used. Nonetheless, there is not much research of thiram on *G. boninense* is documented to the authors' knowledge. Therefore, the current study was carried out to evaluate the potential of thiram in inhibiting the growth of *G. boninense in vitro.*

METHODOLOGY

Fungal isolate and growth

G. boninense mycelial plug (8 mm) was taken from a 10 days old pathogen pure culture stock of which was previously identified by Chong et al. (2011) and was sub-cultured and maintained on potato dextrose agar (PDA) at a temperature of 25±2˚C until further use.

Antifungal activity of thiram towards mycelial radial growth

Tetramethylthiuram disulfide ($C_6H_{12}N_2S_4$) or known as thiram, 97%, ACROS Organics™ brand, in the form of powder with a molecular weight of 240.416 g/mol was used in this study. A series of concentrations which were 0.010, 0.012, 0.014, 0.016, 0.018, 0.020 and 0.030 mg/ml were prepared by incorporation of the respective thiram solution into the PDA, with thiram being first dissolved in sterilized distilled water before incorporated into the media. The media were then poured into sterile petri dishes. Agar without thiram serves as the control. The experiment was performed in triplicates for each concentration. A fungal plug (8 mm diameter) was taken from the edge of a 10 days old culture from the section as above using a sterile pipette tip and place onto the centre of the plate. All plates were incubated at 25±2˚C for 7 days. The radial growth measurement was done by taking the diameter of the colony growth in each culture plate, and their averages were calculated. The antifungal activity of thiram against G. *boninense* was based on the percentage inhibition of radial growth (PIRG) values which were calculated using the equation as described by Siddiquee *et al., (*2009).

$$PIRG(\%) = \frac{R_1 - R_2}{R_1} x100 \quad (1)$$

where R_1 indicates radial growth of *G. boninense* in control plate and R_2 indicates radial growth of *G. boninense* treated with thiram.

Extraction of ergosterol from the mycelia of G. boninense for HPLC analysis

G. boninense mycelial including from the control plates, were extracted by scraping the mycelia on the surface of the media. Mycelia with 0.1 g of weight were immersed in 1 ml of methanol and placed in a dark place for overnight. The extract was then centrifuged and the supernatant was made up to 1.5 ml before being filtered through a 0.45 µm acetate syringe tip filter. Each extracted sample was kept in a 1.5 ml of HPLC autosampler vial until further use.

Quantification of fungal ergosterol content based on HPLC analysis

An Agilent series 1200 Chromatography System comprising of degasser G13138, Quat Pump G131A, autosampler ALS G1329A with ChemStation for data manipulation software was used with

an Agilent G1313B HPLC VWD detector for ergosterol analysis. A reversed-phase Zorbax Eclipse XDB-C_{18} 4.6mm×250mm with 5 µm particle size column was used for separation. The wavelength of UV detector was set to 282 nm and the isolated peak elution at about 7-9 min. The retention time was identified as ergosterol based on its co-chromatography and identical absorption spectrum with pure standard. The system was run isocratically with 100% methanol at the flow rate of 1.0 ml/min. A serial dilution of the ergosterol standard with a range of concentration of 100-700 µg/mL was injected into the HPLC system to develop a standard curve of ergosterol which was then to be used in ergosterol quantification of the treatment. Each sample was analyzed for 20 minutes.

Statistical Analysis

Each experiment was carried out in three replicates, and the results were expressed as mean±standard deviation (SD). The significant differences between the treatments were evaluated statistically by SD and one-way analysis of variance (ANOVA) by using Statistical Package for Sosial Science (SPSS) 22.0 programme and the value of $p<0.05$ considered statistically significant.

RESULTS AND DISCUSSION

In-vitro antifungal effect on G. boninense

Thiram with lower concentration (0.010 mg/ml) showed no inhibitory effect to *G. boninense* as the maximum mycelia growth was achieved at day 7 (4.50±0.00 cm). However, thiram with the concentration of 0.012 mg/ml to 0.020 mg/ml gave a low inhibitory effect with radius growth of 3.43±0.15 cm to 2.10±0.13 cm as the concentration of thiram increased, resulting in a smaller radius of mycelia growth. The highest concentration (0.030 mg/ml) of thiram tested in this experiment was found to be very fungitoxic to *G. boninense* as the pathogen is completely inhibited (Figure 1H).

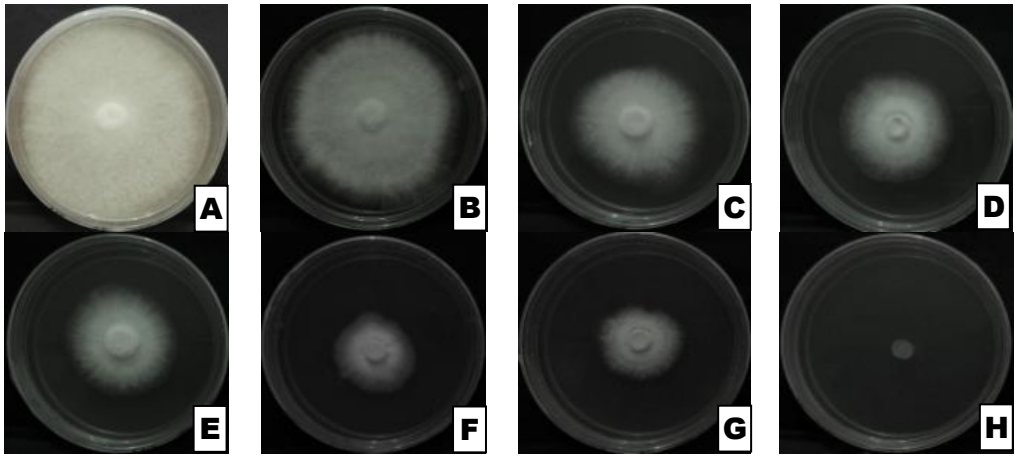

Figure 1. Antifungal effect on *G. boninense* of the (A) control, and various concentrations of thiram (B) 0.010 mg/ml, (C) 0.012 mg/ml, (D) 0.014 mg/ml, (E) 0.016 mg/ml, (F) 0.018 mg/ml, (G) 0.020 mg/ml, and (H) 0.030 mg/ml, 7 days after incubation at 28 ± 1˚C.

Figure 2 and 3 show the mycelial radial growth curve of G. *boninense* from day 1 to day 7 and the calculated percentage inhibition of mycelial radial growth (PIRG) on day 7, respectively.

All tested concentrations of thiram gave different degrees of inhibition toward the mycelial growth of *G. boninense*. Figure 3 showed the percentage of inhibition increased as the concentrations of the thiram increased, they were 0%, 20%, 24%, 34%, 48%, 51%, and 100% for 0.010, 0.012, 0.014,

0.016, 0.018, 0.020, and 0.030 mg/ml of thiram respectively. Thiram at the concentration of 0.030 mg/ml inhibited the growth of *G. boninense* most effectively among the tested concentrations.

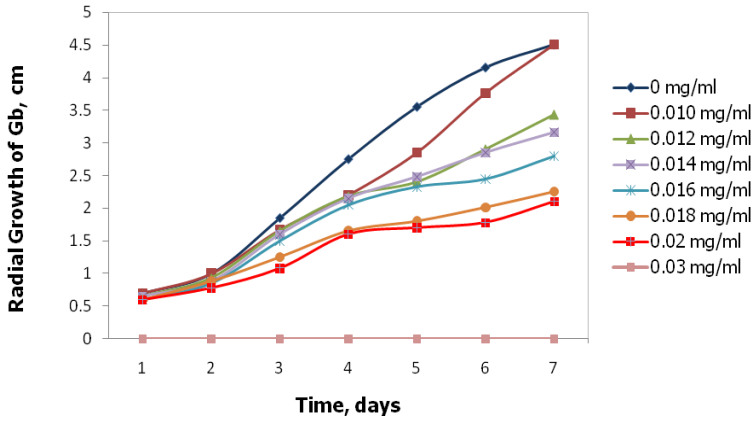

Figure 2. Comparison of *G. boninense* mycelia growth in different concentration of thiram

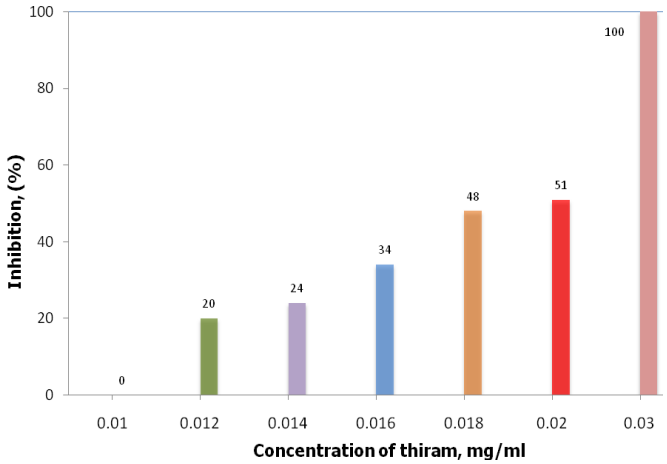

Figure 3. Percentage inhibition of radial growth (PIRG) of *G. boninense* by thiram at day 7

Ergosterol analysis and quantification

Ergosterol is the main component of the fungal membrane and it can be a useful biomarker for the quantification of fungal biomass (Toh Choon *et al.,* 2011). The result showed thiram with the highest concentration (0.030 mg/ml) appeared as the best to inhibit the G. *boninense* fungi. This was shown with the absence of ergosterol content when treated with this concentration (Figure 4), while the ergosterol content was found more in treatments with a lower concentration of thiram. However, there is a significant difference in ergosterol content between the treated and untreated G. *boninense* (control).

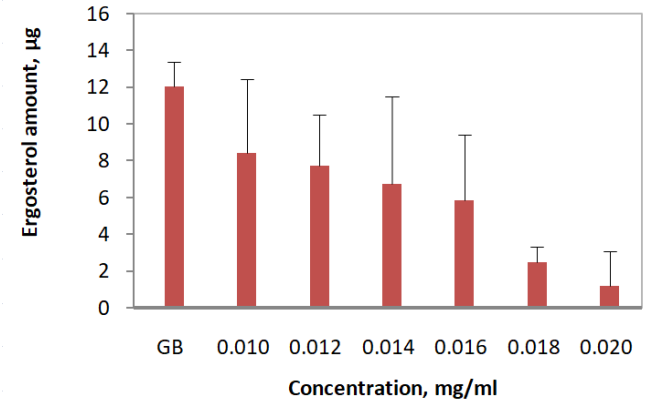

Figure 4. The amount of ergosterol content after treated with various concentration of thiram. Bar: stand. dev. (SD)

Thiram is a contact fungicide with protective properties, acts by inhibiting mycelial growth (PPDB, 2020) and several thiol-containing enzymes involved in the respiration of the fungi (Teicher, 2017). While, Yang *et al.* (2011) mentioned that thiram affects multiple targets sites and interfere concurrently with numerous fungus metabolic processes, and since it affects multiple target sites, therefore thiram is said to have a very low risk of causing fungicide resistance (Hahn, 2014), because it is very unlikely for a fungus to simultaneously develop all of the mutations necessary for resistance. Thiram mode of action was thought to be a complex process, considered one of its fungicide effects was the depletion of glutathione (GSH) (Kim *et al.*, 2008) and intracellular inactivation of GSH reductase (Elskens *et al.*, 1997) due to the disulfide bridge in the thiram structure (Xue *et al.*, 2014). GSH deals with the basic cellular functions as well as the maintenance of mitochondrial structure, membrane integrity and cell differentiation and growth (Pocsi *et al.*, 2004). Therefore, the role of GSH is important in cellular life-activity by protecting cells against the destructive effects of oxidative stress caused by reactive oxidative stress (ROS) (Meinster, 1989). Decreased GSH level resulted in more oxidative damage to the fungal cells. Moreover, GSH also claimed to modulate critical cellular processes such as cell membranes stability, transport of amino acids, modulation of gene expression and apoptosis and thus, lack of GSH could lead to disruption in such cellular processer rendering the fungi cells more vulnerable (Xue *et al.*, 2014). The depletion of GSH may be insufficient to reduce the antioxidant capability of *G. boninense* fungi at a lower concentration of thiram, but at higher concentration of thiram damaged the cellular antioxidant system adequately and resulted in total inhibition of the fungi at the concentration of 0.030 mg/ml.

CONCLUSION

In this paper, we presented the results of *in vitro* experimental investigation on the antifungal activity of thiram against *G. boninense.* From the comparison of different concentrations of thiram, the higher concentration, which is 0.030 mg/ml has successfully inhibited the growth of *G. boninense.* Meanwhile, the other concentrations gave a significant difference to mycelia growth and ergosterol content in comparison to the control. However, further investigation is necessary to evaluate the effectiveness of thiram for application in the field.

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