MORPHOLOGICAL CHANGES OF *G. boninense* MYCELIA AFTER BEING CHALLENGED WITH THIRAM, ZNO NPS AND LUCSIN

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Abstract. Basal Stem Rot (BSR) disease is one of the major threats to the oil palm industry in South East Asia. This paper aims to find control to BSR using thiram, Zinc Oxide nano-particles (ZnO NPs) and Lucsin. The study was carried out *in vitro* to assess the antifungal effect of these agents by percentage inhibition radius growth with the observation of their morphology under Scanning Electron Microscope (SEM) after treatment. From our observation, thiram could fully inhibit *G. boninense* radial growth, while ZnO NPs up to 80%. No inhibition was recorded with Lucsin. Observation under SEM confirmed *G. boninense* mycelia after treated with thiram shown abnormality, distorted and damaged hyphal which had resulted in less branches of the fungal mat. There are also evidences of less branches of fungal mat as well as malformation structures of *G. boninense* hyphae after being treated by ZnO NPs. Only minor ruptures was found on the hyphae, while the hyphal branch were shriveled, distorted and flattened when challenged with Lucsin. In summary, thiram shows potential for complete inhibition of *G. boninense* while ZnO NPs has slightly lower inhibition potential but Lucsin is incapable of providing significant control to the growth of *G. boninense*.

Keywords: *Ganoderma boninense*, thiram, ZnO NPs, Lucsin, morphological disruption

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Introduction

Basal stem rot (BSR) is a severe disease caused by a white rot fungus called *Ganoderma boninense* that may be the most detrimental disesase to the South East Asian oil palm sector [1]. In Malaysia, the economic losses due to BSR were estimated to be between RM225 million and 1.5 billion per year [2] as the disease reduces the yield of the infected palms either by direct loss due to falling of the infected palms, or indirect loss by limiting the yield, decreasing the weight or reducing the number of fresh fruit bunch (FFB) in infected but still living palms. Numerous strategies have been tried to reduce BSR, yet none of them have provided effective control to *G.boninense* [3-4] since most control measures just prolong the productive lives of diseased palms without curing them. Thus, alternative control methods may be needed to reduce the losses caused by the disease. This paper is presented an investigation on the use of some reported fungal control agents, but either was not yet tested on *G.boninense* or was not clearly documented on their inhibition capability, to assess their potential application to control *G.boninense* infection in oil palm.

Tetramethylthiuram disulfide (thiram) is a non-systemic fungicide (chemical formula: $C_6H_{12}N_2S_4$) that belong to the group of dimethyl dithiocarbamate compounds categorized as not likely to be carcinogenic to humans. Thiram has been used for crop preventation from damage, to preserve harvested crops from deteriorating during storage or transportation, as a seed treatment to protect against fungal infections, and as a foliar application to manage diseases on vegetable and fruits crops, turf and lawns. The application of thiram in Malaysia's oil palm plantation has been reported by Kuntom *et al.* since 1999 where thiram was mentioned as one of the most common fungicides being used [5]. Thiram is a protectant chemical used to treat the open surfaces of *G.boninense* infected oil palm trees after surgery to prevent further decay. However, to this point of time, no formal report could be found on fungicide activity of thiram on *G.boninense*, and therefore justify the investigation carried out in this paper.

Zinc Oxide Nanoparticles (ZnO NPs) has a unique physical and chemical characteristic, making it the most significant metal oxide nanoparticles that is extensively utilized in various fields. ZnO NPs have several importance in the industrial and medical sectors including food packaging, healthcare, medical care, and also in decoration and construction. Recently, some studies demonstrated the antimicrobial activities of nanoparticles (NP) materials of ZnO. Amongst them, ZnO NPs were reported to have excellent antibacterial activity against bacteria like *Klebsiella pneumonia* and *Staphylococcus aureus* [6], effective in killing both Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*S. aureus* and *Bacillus subtilis*) [7] as well as inhibiting fungal growth of *Aspergillus niger* fungus [8]. Unfortunately, the use of ZnO NPs against *G.boninense* have not been reported in any paper yet and thus it is quite intriguing to do so at least at the *in vitro* stage.

On the other hand, *Lactobacillus* (Lb) which is one of lactic acid bacteria genera, probably the most predominant genus of gram-positive, anaerobes or microaerophilic, aetolerant, rod-shaped and non-spore-forming bacteria. Lactic acid bacteria are thought to have antimicrobial activities due to the production of low-molecular-weight substances such as lactic acid, hydrogen peroxide, acetic acid, amino acids, reutericyclin, diacetyl, benzoic acid, 3-phenyl lactic acid, methylhydantoin, benzene acetic acid, mevalonolactone, 2-propenyl ester, 2,6-diphenyl-peperidine, as well as cyclic dipeptides [9]. Cyclic dipeptides and their structures have been broadly researched in their biological activity, specifically their antibacterial and antifungal properties [10]. Magnusson *et al.* reported the suppression of fungal growth by cyclic dipeptides, and their antifungal action was not exclusively linked to the production of

organic acids [11]. In the populous Lb, *L. plantarum* is the most versatile strain with useful properties and is commonly used in various fermentation of food products such as in industrial fermentation and raw foods processing as it is listed as as generally recognized as safe (GRAS) and has qualified presumption of safety (QPS) status. Cycle (Gly-Leu) from *L. plantarum* was found to be antifungal substances against plant fungal pathogen *Fusarium avenaceum* (*Gibberella avenacea*) VTT-D-80147 [12]. Presently, *L. plantarum* LBP-K10 was found as the most effective antifungal strain from traditional fermented vegetables in Korea. Previous research by Kwak *et al.* reported that the isolated *cis*-cyclo(L-Leu-L-Pro) and *cis*-cyclo (L-Phe-L-Pro) from the culture filtrate of LBP-K10 had demonstrated impressive antifungal activity against *G. boninense* [13].

These three agents were chosen in this investigation based on anti-fungal activities reported in the literatures but without firm evidence on *G. boninense*. It is hypothesized that these agents would have some inhibitions capibility on *G. boninense* as it has on other fungus or bacteria, and therefore worth to be investigated.

Materials and Methods

G. boninense. Pure culture of *G. boninense* was obtained from the Genetic Laboratory of Faculty Science and Natural Resources, Universiti Malaysia Sabah (UMS). The identification of the culture was confirmed earlier [14]. The culture was then sub-cultured on potato dextrose agar (PDA) and maintained at 25 ˚C until further use.

Thiram. Tetramethylthiuram disulfide (Sigma-Aldrich), commercially available and known as thiram, 97%, with molecular weight of 240.416 g/mol in the form of powder, was first obtained from a local supplier. A series concentration (0.010. 0.012, 0.014, 0.016, 0.018, 0.020, and 0.030) mg/ml of thiram was prepared by incorporating the respective thiram solution into PDA, with first thiram were dissolved in sterilized distilled water (5 ml for each concentration) before being incorporated into the media [15]. The media with the thiram solution were then poured into sterile petri dishes (9 cm in diameter).

ZnO NPs. A series of ZnO NPs (Sigma-Aldrich) with the concentraion of 2, 4, 6, 8, 10, and 20 mg/ml were prepared by incorporating the respective ZnO NPs solution into the PDA, with ZnO first dissolved in sterile distilled water (5 mL) before incorporated into the media. The media were then poured into sterile petri dishes (9 cm in diameter).

Lucsin. Lucsin product, the proline-cyclic dipeptides from the culture filtrate of LBP-K10 was obtained from Dr. Min-Kyu Kwak of Eulji University, South Korea. The product was autoclaved for 15 minutes at 121 ˚C and left to cool down. Lucsin was prepared for the treatment by incorporating the respective volume of Lucsin into the media with a series of concentrations in percentage (0.5%, 2.5%, 5%, and 10%) (Example: 0.1 ml of Lucsin dissolved in 19.9 ml of PDA to make up a 0.5% v/v). The media was then poured into sterile petri dishes.

Treatment on G. boninense. *G. boninense* plugs (0.8 cm diameter) were taken from the edge of seven days old cultures using the end of the sterile pipette tip and placed onto the center of each treatment plate, including the control plates. Agar plates with the PDA only served as control. All plates were incubated for 7 days at room temperature. All the experiments were performed in triplicates for each concentration.

Measurement of the percentage inhibition radial growth (PIRG). The radial growth of the colony was measured daily for 7 days. The degree of the inhibition was calculated based on the percentage inhibition of radial growth (PIRG) using the formula described below:

$$\text{PIRG} = \frac{R_1 - R_2}{R_1} \times 100 \quad (1)$$

where PIRG is the Percent Inhibition of Radial Growth, R1 is the Radial growth of *G. boninense* alone (control) and R2 is the Radial growth of *G. boninense* with treatment.

Sample preparation for Scanning Electron Microscopy (SEM). To observe the morphological changes of *G. boninense* hyphae after the different treatments, selected samples were examined using Zeiss EVO® 10 MA SEM at Biotechnology Research Institute (BRI), UMS. The agar plates with *G. boninense* fungal mycelia were first excised with a scalpel and trimmed 10 mm x 10 mm in size, and as thin as possible to minimize moisture. It was then air-dried at room temperature in laminar flow for about 3 to 4 hours. After mounting the samples on aluminium stubs with conductive double-sided adhesive carbon tabs (NISSHIN EM. CO. LTD), it was sputter-coated with gold-palladium (Emitech K550x carbon coater) from different angles and viewed at 15 kV accelerating voltages.

Statistical analysis. All treatments were carried out in in triplicates. Thus, the PIRG obtained in the experiments were analysed and their significant in inhibiting *G. boninense* were compared using one-way analysis of variance (ANOVA) with the aid of Statistical Package for Social Science (SPSS) 22.0 software.

Results and Discussion

The pictorial growth of *G. boninense* after being treated with thiram, ZnO NPs and Lucsin is shown in Figure 1. It was found that lower concentration of thiram (0.010 mg/ml) failed to show inhibitory effect on *G. boninense*, while increasing the concentration from 0.012 mg/ml to 0.020 mg/ml gave low inhibitory effect to the mycelia of the pathogen, which resulted in a decrease mycelia radial growth (Figure 1(a)). The highest tested concentration of thiram in this experiment (0.030 mg/ml) gave a total inhibition to *G. boninense*.

Figure 1(b) shows the radial growth of *G. boninense* when challenged with ZnO NPs, where the radial growths were suppressed as the concentrations of ZnO NPs increased. All tested concentrations of ZnO NPs gave a significant inhibitory effect on *G. boninense*. However, for Lucsin, there is no inhibition occurs as the radial growth of the *G. boninense* were same with the control at day 7 (Figure 1(c)).

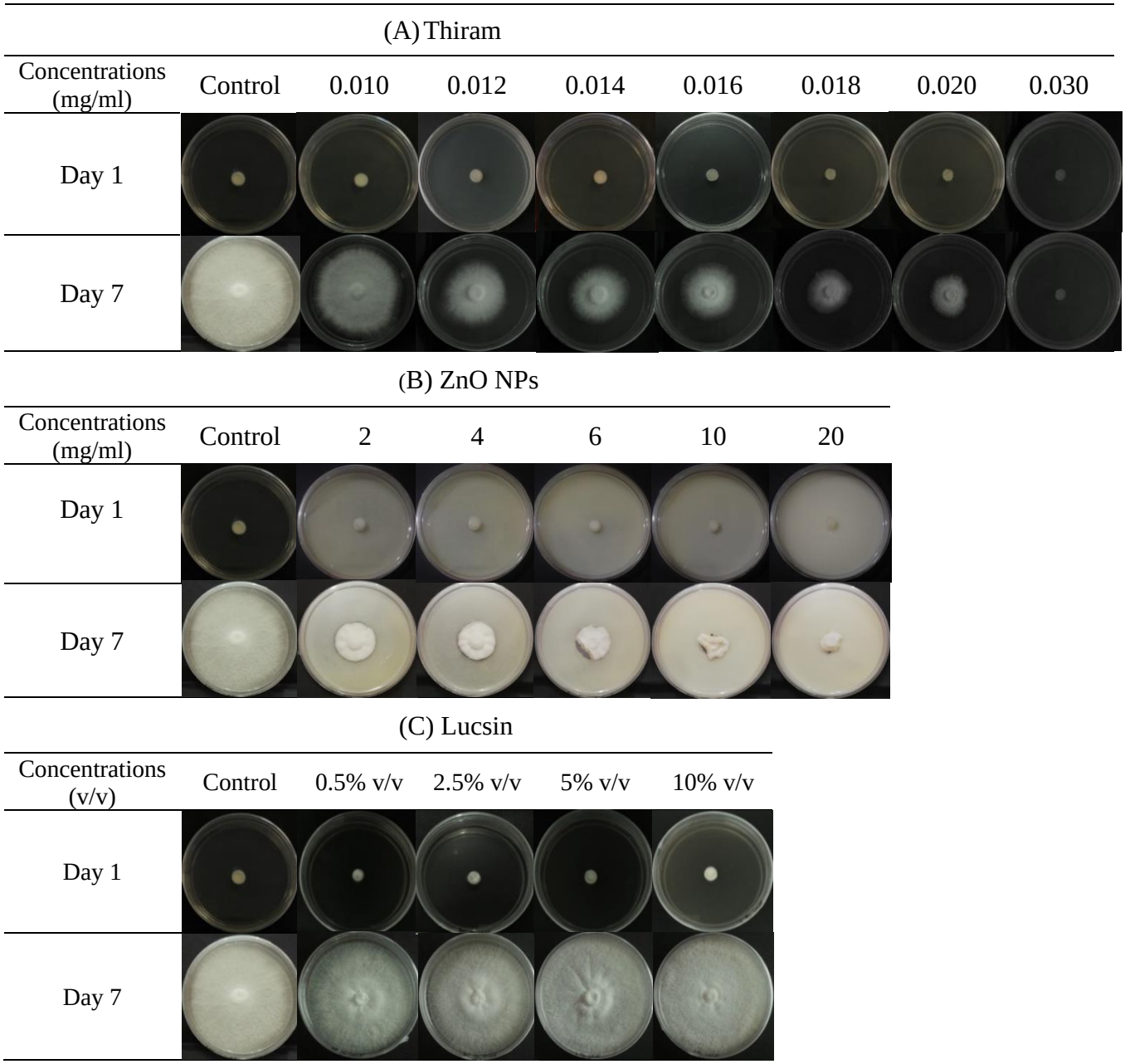

Figure 1. Growth of *G. boninense* mycelia from day 1 and day 7 under different concentrations of (a) thiram, (b) ZnO NPs and (c) Lucsin

The PIRG of *G. boninense* with different treatments at day 7 was recorded and are shown in Table 1. The highest tested concentration of thiram in this experiment (0.030 mg/ml) was found to have a 100% PIRG on *G. boninense.* Investigation under SEM shows that *G. boninense* after being challenged with thiram resulted in less branches of the fungal mat, damaged and distorted hyphae as shown in Figure 2(B) compared to control in Figure 2(A). This observation is similar to the results report by [15-16]. Meanwhile, all different tested concentrations of ZnO NPs showed an inhibitory effect of more than 50%, with the highest tested concentration (20 mg/ml) giving a PIRG up to 80.37% (Table 1). Similarly, *G. boninense* challenged with ZnO NPs also resulted with less fungal mat and the strand of the hyphae shows a malformation structure compared to the control as shown in Figure 2(C). In addition, some parts of the hyphae branch were shriveled.

Treatment with Lucsin shows no inhibition effect on *G. boninense* mycelia with 0 % of PIRG (Table 1). Investigation under SEM however shows that the morphological structures of the pathogen did show some structural changes as shown in Figure 2(D) compared with the control (Figure 2(A)). There were many small viods on the hyphae branches showing a small ruptured on the branch (marked with arrow) and the hyphal branches were shriveled, distorted and flattened as shown in the figure.

Table 1. Inhibitory effect of different treatments against *Ganoderma boninense* mycelia growth

Treatments	Concentrations	Day 7	
		Radial Growth (cm)	% PIRG
Control	0	4.50 ± 0.00^{d}	0.00±0.00
Thiram	0.010 mg/ml	4.50 ± 0.00^{d}	0.00±0.00
	0.012 mg/ml	3.43 ± 0.09^{c}	23.70±1.97
	0.014 mg/ml	3.17 ± 0.09^{c}	29.76±2.10
	0.016 mg/ml	2.80 ± 0.10^{b}	37.76±2.31
	0.018 mg/ml	2.25 ± 0.08^{a}	50.73±2.04
	0.020 mg/ml	2.10 ± 0.10^{a}	53.33±2.23
	0.030 mg/ml	0.00±0.00	100.00±0.00
ZnO NPs	2.0 mg/ml	1.80 ± 0.10^{d}	60.00±2.20
	4.0 mg/ml	1.61 ± 0.04^{cd}	64.03±1.68
	6.0 mg/ml	1.50 ± 0.06^{bc}	66.67±2.25
	10.0 mg/ml	1.33 ± 0.04^{b}	70.37±1.68
	20.0 mg/ml	0.88 ± 0.02^{a}	80.37±0.64
Lucsin	0.5 % v/v	4.50±0.00	0.00±0.00
	2.5 % v/v	4.50±0.00	0.00±0.00
	5 % v/v	4.50±0.00	0.00±0.00
	10 % v/v	4.50±0.00	0.00±0.00

* Values presented are means of three replicates ± standard deviation

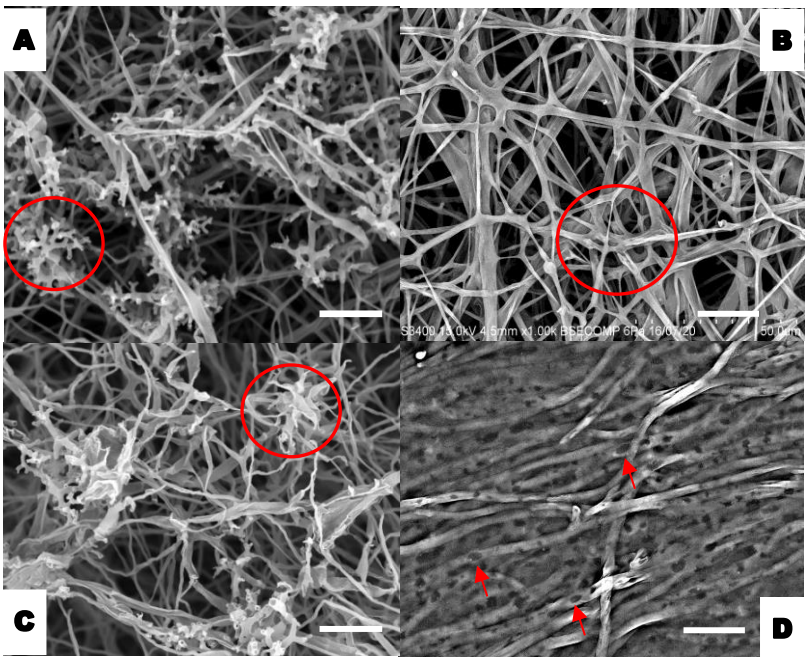

Figure 2. Antifungal effects of different treatments on mycelia of *G.boninense* at day 7. (A) The healthy dense of mycelium branches in control, (B) less fungal mat with distorted and damaged hyphal after treated with thiram (0.020 mg/ml), (C) less fungal mat with malformation structure of hyphal strand after challenged with ZnO NPs and (D) Mycelia with the presence of many holes on the hyphae branches after being treated by Lucsin. Scale bar: 50 µm.

Thiram is popularly known as a contact fungicide that acts by inhibiting the growth of the fungal mycelia and several thiol-containing enzymes in the respiration of the fungi. Thiram affects multiple target sites and interferes with various fungus metabolic processes, therefore thiram has a very low risk of causing fungicide resistance where it is unlikely for the fungus to develop all the mutations necessary for the resistance simultaneously. Thiram also affects the depletion of glutathione (GSH) and intracellular inactivation of GSH reductase because of the disulfide bridge in the structures of the fungicide [17]. Lack of GSH could lead to disturbance in such cellular processes and could make the fungi cells more vulnerable. At lower thiram concentration (0.010 mg/ml), the depletion of GSH may be insufficient to disturb the cellular processes; therefore the *G. boninense is* steadily similar to the control. However, thiram with concentration of 0.012-0.020 mg/ml showed a significant increase of inhibitory effect which suggests that thiram is capable of depleting the GSH of the pathogen, but at higher concentration. The highest tested concentration of thiram (0.030 mg/ml) had caused a major destruction and further damage to the cellular antioxidant system of the pathogen which led to a total inhibition of *G. boninense* as observed on day 7.

As for ZnO NPs, when identifying the antifungal compound of the nanoparticles, the element to consider is the "action site" which includes the cell wall, cell membrane, RNA and DNA, as well as the surface properties of the targeted fungus. The cell wall of the fungus might be the target and the ZnO, however, has to take account on the surface physiochemical of ZnO which is the size and shape that might affect its toxicity [18]. ZnO NPs may cause damage to cellular of microbial cell membranes, resulting in cytoplasm leakage and bacterial cells death [19]**.** Other researchers also reported that the production of reaction oxygen species (ROS) is the primary mechanism responsible for the antifungal activity of ZnO NPs. The generation of ROS caused oxidative stress, which may cause damage to the cell membrane, cellular proteins,

and nucleic acids that may eventually resulted in cell death. This is because the synthesis of β-1 3-D-glucan synthase (FKs1p) is particularly important in fungus cell walls, and/or on *N*-acetylglucosamine (*N*-acetyl-D-glucose-2-amine), which is implicated in the synthesis of the chitin (a most importance of polysaccharide in the cell wall structures) [20]. As the concentration of ZnO NPs increases, the chances of smaller size of ZnO particles to enter the cell wall of the pathogen is higher, disturb the cellular-life activity and therefore the generation of ROS give rise to the oxidative stress that eventually interferes the growth of the pathogen and inhibit it. Observation under SEM suggests similar actions in the interactions between ZnO NPs with *G. boninense* mycelia which contributed to the final inhibition.

When *G. boninense* challenged with Lucsin product, there was no inhibitory effect shown on its radial growth. However, as shown under SEM investigation, some changes in the morphology were observed compared to the control. As it was reported, the *L. plantarum* LBP-K10 isolated from Korean kimchi, or Lucsin, is the most efficient antifungal strain and may be capale to dissipating pyruvate, the end product of glycolysis, which an important branch point in the most organism' sugar metabolism [21]. Cells are known to excrete numerous secondary metabolites for their use, which act as antifungal compounds [22]. Therefore, culture filtrate was selected to be investigated for its possibility to act as antifungal agents. The isolated cyclic dipeptides of *cis*-cyclo(L-Val-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) from the culture filtrate were later reported by Kwak *et al*. as the most potent anti-*Ganoderma* compound [22]. In this paper however, this antifungal activity of Lusin was not shown on PIRG. Nontheless, its antifungal potential is shown under electron microscopy as disturbance of mycelia branches compared to the control. Amongst the potential reasons for this is that the concentration of Lucsin used in this investigation was not high enough and therefore the actual inhibition could not be observed (not shown in PIRG).

Conclusion

In-vitro experiments confirmed thiram and ZnO NPs have the capability to inhibit *G. boninense*. The higher concentration of thiram (0.030 mg/ml) can kill the pathogen while increasing the concentration of ZnO NPs up to 20 mg/ml gave a PIRG of 80.37% against this pathogen. Investigation under SEM supports the antifungal activities on *G. boninense* shown in PIRG where less branches of the fungal mat is observed with damaged and distorted hyphae after being challanged using thiram, while ZnO NPs treatment reduces the number of fungal mat and the strand of the hyphae shows a malformation structure. Although the inhibitory effect of Lucsin was not notable from the PIRG assessment, some antifungal activities of the treatment is shown under SEM with some structural changes are observed such as small voids on the hyphae branches, as well as shriveled, distorted and flattened hyphal branches.

Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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