African Journal of Biotechnology Vol. 11(70), pp. 13477-13489, 30 August, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1968
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

In vitro effects of salicylic acid, calcium and copper ions on growth and sporulation of *Ganoderma boninense*

Rahamah Bivi M.[1], Siti Noor Farhana M. D.[1], Khairulmazmi A.[1,3]*, Idris A.[2], O.H. Ahmed[1,3], Zamri R[3] and Sariah M.[4]

[1]Department of Crop Sciences, Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia, Bintulu Campus, Sarawak, Malaysia.
[2]Ganoderma and Disease Research Unit, Malaysian Palm Oil Board (MPOB) Bangi, Malaysia.
[3]Center for Tropical Forest Ecosystem Science, Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia, Bintulu Campus, Sarawak, Malaysia.
[4]Department of Plant Protection, Faculty of Agriculture. Universiti Putra Malaysia Serdang Selangor, Malaysia.

Accepted 3 August, 2012

The *in vitro* effects of single and combined application of calcium ion (Ca^{2+}), copper ion (Cu^{2+}) and salicylic acid (SA) were evaluated on growth and sporulation of *Ganoderma boninense.* In poison medium test, T7-(Ca+Cu+SA) showed effective control of *G. boninense in-vitro* with EC_{50} and EC_{90} values of 1500+150+150 ppm and 2000+200+200 ppm, respectively. However, in dipping test, T3-(SA) showed effective control for *G. boninense in-vitro* with EC_{50} and EC_{90} values of 50 ppm and 200 ppm, respectively. Interestingly, T7 (Ca+Cu+SA) indicate effective control of *G. boninense* at low concentration, 500+50+50 pmm as shown by EC_{90} analysis. Pre-treatment of *Ganoderma* -infected rubber wood block with 500 ppm Ca + 50 ppm Cu + 50 ppm SA reduced the number (20.14%), size (1.20 cm^2) and weight of basidiocarp (0.80 g) compared to the control. This was followed by a significant reduction in weight loss of the *Ganoderma*–infected rubber wood block (41.85%) suggesting the inhibition of the degradative enzymatic activity of the fungus. The mixture of Cu, Ca and SA had potential to suppress growth of *G. boninense in vitro.*

Key words: *Ganoderma boninense,* calcium chloride, copper- ethylenediaminetetraacetic acid (EDTA), salicylic acid, EC_{50} and EC_{90}.

INTRODUCTION

Ganoderma basal stem rot (BSR) fungal disease has caused the significant loss in South East Asia's oil palm industry (Flood et al., 2000). *Ganoderma* BSR is a major concern in Malaysia as palm oil is the major cash crop in this country. In 2010, the export earnings from oil palm products reached about US$19.50 billion. With a total oil palm area of 4.85 million ha, potential (30 to 70%) losses of oil palm occur due to BSR could have an adverse effect on the oil palm industry. This disease is believed to be the most serious disease of oil palm in Malaysia and other parts of South East Asia (Susanto, 2009). The disease has also been recorded in Africa, Papua New Guinea (Turner, 1981), Honduras (Chinchilla and Richardson, 1987), Colombia (Nieto, 1995) and Thailand (Tummakate and Likhitekaraj, 1994). In Peninsular Malaysia, most of the oil palm estates detect this disease at the second or the third replanting. About 90% of the estates in West Malaysia have been infested with *G. boninense* (Khairuddin and Chong, 2008).

*Corresponding author. E-mail: kcik@hotmail.com. Tel: Phone: 6086-855409 Fax: 6086-855415.

In Sabah and Sarawak, most of the estates are in their first cycle of planting, providing alternative hypothesis for the lower relative incidence of pathogen attacked in these regions. Thus, *G. boninense* has a significant effect on the lifespan of affected trees and adverse effect on yield (Corley and Tinker, 2003).

Primary infection of palms by *Ganoderma* species has been thought to occur by contact of living palm roots with colonized debris within the soil (Idris et al., 2002). Secondary spread of inoculums has been assumed to be in contact with living palm roots with each other (De Oliveira et al., 2005). Besides, basidiospores released in this early stage play no part in the disease progression (Sanderson, 2005). As brackets are formed in the later stage, basidiospores are produced which later infect the wounded parts of the tree. However, Sanderson (2005) reported that spores are scarcely to be considered as the mode of disease transmission as they are rarely seen. In addition, some speculated that these spores are carried by insects linking to BSR infection and development (Chung et al., 1998). To control BSR, chemical, biological, cultural, and mechanical control was conducted. However, no control practices have been proved satisfactory (Susanto et al., 2005). The disease does not cause external symptoms until it is too far advanced and at a stage when trees cannot respond to treatment anymore. Therefore, treatment such as nutrient and plant hormone should be applied on selected concentration to the seedling stage of oil palm in order to make them resistance towards BSR disease. To study that initially we tried *in vitro* test in this report. *In vitro* bioassays often correlate well with field trials. Holloman and Young (1951) reported that laboratory assay was an authentic criterion for field performance to control of Botrytis leaf spot on gladioli. Besides, Klomparens and Vaughn (1952) reported field and laboratory trials very consistent for control of *Helminthosporium* on bent grass.

The nutritional status of a plant has a major impact on disease susceptibility, and this has been exploited for suppressing a variety of diseases (Engelhard, 1989). Previous studies tested, calcium nitrate suppressed BSR symptoms on clonal materials (Sariah and Zakaria, 2000). Besides, copper has played a significant role in organic and conventional systems for battling some fungal diseases. *Ganoderma* wilts disease caused by *G. applanatum* and *G. lucidum* in coconut trees has been controlled by copper-based fungicides (Nambiar et al., 1992). Recently, salicylic acid (SA) a naturally occuring plant hormone received attention after it was determined that it can induce resistance to patho-gens and abiotic stress tolerance in plants (Gautam and Singh 2009; Pieterse et al., 2009; Ramirez et al., 2009). Calcium, magnesium, copper, carbon, salicylic acid and nitrogen are critical variables for plants and so modifying these as a control method may prevent *Ganoderma* attack. Those nutrients and plant hormone could conceivably be supplied by soil application to control the disease in addition to them being used as fertilizers. Fertilizers are, added which comprise some of these minerals but the point here is to control disease not to improve the growth of the oil palm. The existing literature is silent on controlling *G. boninense* although the uses of soil amendments have been suggested by Sariah and Zakaria (2000). Due to the increasing economic significance of this disease in Malaysia, effective and feasible management strategies need to be established.

In this study, we evaluated the most ideal concentration of Ca^{2+}, Cu^{2+} and salicylic acid (SA) required on suppression of the growth of *G. boninense in-vitro* and subsequently to demonstrate in block studies.

MATERIALS AND METHODS

Ganoderma boninense culture

Pure fungal cultures of *G. boninense*, PER 71 were obtained from the fungal collection of Malaysian Palm Oil Board (MPOB), Bangi. Those plates of *G. boninense* culture were then sub-cultured to mass multiply a pure culture of the pathogen for this study. They were afterwards, maintained in a potato dextrose agar (PDA) medium (Merck, Darmstadt, Germany) at room temperature (28 ± 2 °C).

Preparation of treatments

There were seven treatments with five concentrations for each and 0 ppm serves as a control plate (T8) (Table 1).

Poison medium test

Five concentrations of calcium chloride anhydrous powder (Merck, Darmstadt, Germany) were prepared at 0, 500, 1000, 1500 and 2000 ppm (Table 1). These concentrations were amended into a melted PDA medium in Petri dishes (Favorit®, Malaysia) with four replicates for every concentration. The Petri dishes were shaken gently and horizontally to allow Ca^{2+} to distribute evenly on the PDA medium. After solidification, the plates were inoculated by placing 5 mm diameter disc of three days old PDA culture of *G. boninense* which was taken from the margin of fungal colonies and transferred to the centre of the new Petri dishes (Grover and Moore, 1962). The Petri dishes were then sealed with Parafilm® (Pechiney, Chicago, IL) and incubated at room temperature. Data on the radial colony diameter were recorded five days after incubation or as the growth of the control treatment was completely covered by the plate. Radi of the colonies on PDA on each concentration was measured from the bottom side of the Petri dishes. Percentage inhibition of radial growth (PIRG) value was computed by measuring the radius growth of *G. boninense* in the control plate (R1) and the radius of fungus in nutrients treated plates (R2) using the formula developed by Skidmore and Dickson (1976),

Represents, $\text{PIRG (\%)} = \frac{R1-R2}{R1} \times 100$

The same method was used for ethylenediamine tetraacetic acid copper disodium salt (Copper-EDTA) (Merck, Darmstadt, Germany), 2-Hydroxybenzenecarboxylic acid (Salicylic acid) (May

Table 1. Preparation of treatments for poison and dipping test method.

Treatment	Concentration, (ppm)
T1 (Ca)	500 1000 1500 2000
T2 (Cu)	50 100 150 200
T3 (SA)	50 100 150 200
T4 (Ca+Cu)	500 + 50 1000 + 100 1500 + 150 2000 + 200
T5 (Ca+SA)	500 + 50 1000 + 100 1500 + 150 2000 + 200
T6 (Cu+SA)	50 + 50 100 + 100 150 + 150 200 + 200
T7 (Ca+Cu+SA)	500 + 50 + 50 1000 + 100 + 100 1500 + 150 + 150 2000 + 200 + 200
T8	Control

and Baker, England) and its combination.

Dipping test

Similar concentration as the aforementioned study was tested in this study. Initially, 1 cm plugs of *G. boninense* were taken from new mycelia margin of active growing cultures and dipped in each treatment. Each treatment was replicated four times. Another set of discs was dipped in sterile distilled water to serve as a control (0 ppm). The prepared discs were air dried in a laminar chamber (Esco® Smart Programme, Malaysia) and firmly applied to the surface of PDA medium after which the plates were incubated at 28 ± 2°C for at least five days incubation period for observation of radial inhibition of mycelia growth (Dikin et al., 2003; Yang and Clausen, 2007). Percentage inhibition of radial growth PIRG value was computed using the formula developed by Skidmore and Dickson (1976). The EC_{50} and EC_{90} values were determined for each treatment by calculating percent inhibition which obtained from poison medium and dipping methods. These data were then subjected to probit analysis (StatPlus 2009 Professional 5.8.4) by linear regressions of the percent inhibition of mycelia growth versus the $\log_{10}$ for each concentrations of each treatment. EC_{50} values for treatment concentrations were obtained from the graphed regression line at the probit zero (50% reduction) point and alike for EC_{90} values. The inhibition mycelia growth at all concentrations were evaluated and groups were denominated as effective (E), least effective (LE) and not effective (NE), respectively.

Preparation of wood blocks for block treatment experiment

Rubber wood blocks were obtained from Universiti Putra Malaysia Bintulu Campus, Sarawak and treated using a method adapted from Adaskaveg et al. (1990) and Rees et al. (2007). Blocks of rubber wood (6 × 6 × 6 cm) were washed and autoclaved (Hirayama, Japan) at 121°C, 15 psi for 45 min. Each block was placed in heat-resistant polypropylene bags (7.5 cm x 33 cm x 0.05 mm thick material). Afterwards, a 100 ml of hot molten Malt Extract Agar (MEA) (HiMedia, India) was poured over the blocks as a supplementary nutrient for *G. boninense* and these were re-sterilized. After sterilization and cooling, the rubber wood block in the polypropylene bag was rotated to ensure that it was well covered with the agar before the latter solidified. The cooled blocks containing malt-extract agar (MEA) were inoculated with six 10 mm plugs of agar taken from the leading edge of *G. boninense* cultures growing on PDA and inoculated with each of the rubber wood blocks. Inoculated blocks were incubated at room temperature (28 ± 2°C) in a dark cabinet for about 90 days then the inoculum blocks were weighed to obtain an initial weight (M1). These colonized blocks were then used as inoculum sources for the subsequent block treatment experiment in a net house.

Rubber wood block treatment

Two kilogram of soil mixtures were placed in a poly bag. Eight treatments with 10 replicates were used in this study. The inoculums blocks were half buried in the soil. For every treatment, the best concentration was chosen is based on the previous *in-vitro* study. Therefore, T1- 1000 ppm (Ca), T2- 50 ppm (Cu), T3- 100 ppm (SA), T4- 500+50 ppm (Ca+Cu), T5- 500+50 ppm (Ca+SA), T6- 50+50 ppm (Cu+SA), T7- 500+50+50 ppm (Ca+Cu+SA) and T8- control (distilled water) were tested. About 100 ml of nutrient solution was sprayed over the block of each treatment. Treatments were applied for every 14 days and stop after 90 days of incubation periods. Data such as formation of basidiocarp in each poly bag was taken for every treatment and size of basidiocarp was recorded every 30 days. The biomass of basidiocarp and inoculum blocks was weighed after 90 days of incubation periods.

Measurement of size of basidiocarp

The basidiocarp size (width x length) formed on the rubber wood blocks on each treatment was measured monthly using vinyl caliper up to 3 months after incubation periods.

Measurement of biomass of basidiocarp

Ninety days after treatment, the basidiocarp was collected, air dried

and weighed using electronic balance to measure their fresh weight. Then, they were oven dried at 70°C for 2 days. Following this, dry weight of basidiocarp was again measured using electronic balance to measure their dry weight.

Measurement of weight loss of the rubber wood block

All rubber wood blocks were taken out from the soil and cleaned up using tap water 90 days after treatment. For measurement of weight loss of the rubber wood block due to decomposition activity by *G. boninense* final weight (M1) of rubber wood block was measured individually for each treatment. Weight percent loss (WPL) value was computed using the following formula. For initial weight (M0) of rubber wood block, it was measured before treatments were applied to the rubber wood block using balance.

$$\text{Represents, WPL (\%)} = \frac{M0 - M1}{M0} \times 100$$

Measurement of size of basidiospores

Spore samples were taken from spore deposits of *G. boninense* for control and treated inoculum blocks. The spores were then covered with Lactophenol blue (Merck, Darmstadt, Germany) and viewed under a light microscope (LEICA DM 2500, USA) followed by the image capturing that used a camera microscope (JENOPTIK Optical systems GmbH, Germany). The size (width and length) of spores were measured randomly on 30 spores for each specimen.

Effect of treatments on mycelia morphology

About five basidiocarps of *G. boninense* were collected from control and treated inoculum blocks. Internal tissues of basidiocarp were excised and cultured onto PDA medium. After seven days, the *G. boninense* culture was subculture to obtain a pure culture. Hyphal strands at the end of the fungal colony of *G. boninense* pure culture plate was removed and examined under a light microscope for abnormalities growth.

Data analysis

The experiments for the poison medium test and dipping test were conducted in a completely randomized design (CRD) with four replicates. Recorded data were tested using analysis of variance (ANOVA) and Tukey's Test ($P \leq 0.05$) using the statistical software package SAS V9.0 (SAS Institute, Carey, North Carolina, USA) program. In block treatment study, the experiment was arranged in CRD with ten replicates. For the growth rate of basidiocarp alone; the percentage data were Arc Sine transformed (Gomez and Gomez, 1984) before subjected to analysis of variance (ANOVA) and analysed by descriptive statistics using SAS V9.0. The mean values obtained were compared using Tukey's Test at ($p \leq 0.05$).

RESULTS

Suppression of the growth of *G. boninense* by *in-vitro* test

Poison medium test showed inhibitory effect of more than 50% for T7 (2000 ppm Ca + 200 ppm Cu+200 ppm SA) and T5 (2000 ppm Ca + 200 ppm SA) with 83.33 and 55.83%, respectively (Table 2). There were significant differences for all treatment tested however greater inhibition of fungal growth was observed at higher concentration for most of the treatments whereas the lowest concentration had little inhibitory effect. The EC_{50} and EC_{90} values could not be estimated for almost all the nutrient treatment except for Ca+Cu+SA (T7) and Ca+SA (T5) in poison medium test. Those nutrients were denominated as least effective (LE) as shown in (Table 3). For the T7 denominated as effective (E), the EC_{50} was (1500 ppm Ca + 150 ppm Cu + 150 ppm SA) with 45.00% and EC_{90} was (2000 ppm Ca + 200 ppm Cu + 200 ppm SA) with 83.33% of inhibition rate. For T5 designated as effective likewise for EC_{50} and it was 2000+ 200 + 200 ppm of Ca+SA with 55.83% inhibition of mycelial growth. However in dipping test, SA (T3), Ca+SA (T5), Cu+SA (T6) and Ca+Cu+SA (T7) showed percentage inhibition of radial growth (PIRG) values greater than 50% for all their concentrations. Therefore, it shows those nutrients have the potential to inhibit the mycelial growth of *G. boninense* greatly. There was a significant difference at inhibitory effect at ($p \leq 0.05$) for calcium (T1) and salicylic acid (T3) but there were no significant differences for copper (T2) and combined treatment concentration. T7 had highest efficacy with an average of 95.00, 96.67, 100.00 and 100.00% for concentration 500 + 50 + 50, 1000 + 100 + 100, 1500 + 150 + 150, 2000 +200 + 200 ppm, respectively (Figure 1) and this was closely followed by T5 with an average of 90.00, 91.67, 100.00 and 100.00% for concentration 500 + 50, 1000 + 100, 1500 + 150, and 2000 + 200 ppm, respectively (dipping test). However, Ca (T1) and Ca +Cu (T4) were found less effective in inhibiting growth of *G. boninense* compare to other treatments. The 50 and 90% effective concentration was determined by probit analysis for EC_{50} were 50 ppm of SA and (50 ppm Cu + 50 ppm SA) with 52.50 and 50.00% inhibition and EC_{90} were 200 ppm of SA, (500 ppm Ca + 50 ppm SA) and (500 ppm Ca + 50 ppm Cu + 50 ppm SA) with 84.17, 90.00 and 95.00%, respectively and the rest of nutrient concentrations were least effective in controlling mycelial growth in dipping test. For those nutrients concentrations to be effective for 50 and 90% it should be more or less than range listed in Table 4. Based on EC_{90} results of this *in vitro* study, the *G. boninense* fungus was most sensitive to T7 (Ca+Cu+SA), followed-by T5 (Ca+SA) and T3 (SA).

Suppression of formation and development of *G. boninense* by inoculum block experiment

Inhibits formation of basidiocarp

The first denotation of basidiocarp formation was the appearing of a white mycelium after 14 days of incubation in poly bags and development of whitish clump on rubber

Table 2. Effect of treatments with different concentrations on percentage inhibition of radial growth of *G. boninense* isolate in poison medium test and dipping test.

Concentration (ppm)	Mean of PIRG* value ± SE (%)	
	Poison medium test	Dipping test
Ca		
500	8.33 ± 0.83^{c}	1.67 ± 0.83^{b}
1000	10.83 ± 0.83^{b}	5.83 ± 0.83^{a}
1500	13.33 ± 0.83^{a}	7.50 ± 0.00^{a}
2000	30.83 ± 0.83^{a}	8.33 ± 0.83^{a}
Cu		
50	20.83 ± 0.83^{b}	35.00 ± 0.00^{a}
100	23.33 ± 0.83^{b}	35.83 ± 0.83^{a}
150	25.83 ± 0.83^{b}	36.67 ± 0.83^{a}
200	35.00 ± 2.50^{a}	37.50 ± 0.00^{a}
SA		
50	10.83 ± 0.83^{c}	52.50 ± 1.44^{b}
100	16.67 ± 0.83^{b}	76.67 ± 0.83^{a}
150	25.00 ± 1.44^{a}	83.33 ± 1.44^{a}
200	26.67 ± 1.67^{a}	84.17 ± 0.83^{a}
Ca + Cu		
500 + 50	1.67 ± 0.83^{b}	6.33 ± 0.67^{a}
1000 + 100	1.67 ± 0.83^{b}	6.50 ± 0.50^{a}
1500 + 150	3.33 ± 0.83^{ab}	7.00 ± 0.00^{a}
2000 + 200	4.17 ± 0.83^{a}	8.00 ± 1.00^{a}
Ca + SA		
500 + 50	17.50 ± 0.00^{c}	90.00 ± 10.00^{a}
1000 + 100	22.50 ± 2.50^{c}	91.67 ± 8.33^{a}
1500 + 150	40.83 ± 0.83^{b}	100.00 ± 0.00^{a}
2000 + 200	55.83 ± 2.20^{a}	100.00 ± 0.00^{a}
Cu + SA		
50 + 50	9.17 ± 0.83^{b}	50.00 ± 0.00^{a}
100 + 100	13.33 ± 0.83^{b}	51.67 ± 1.67^{a}
150 + 150	25.00 ± 1.44^{a}	53.33 ± 1.67^{a}
200 + 200	27.33 ± 0.83^{a}	55.00 ± 0.00^{a}
Ca + Cu + SA		
500 + 50 + 50	22.50 ± 0.00^{c}	95.00 ± 5.00^{a}
1000 + 100 +100	35.00 ± 0.00^{bc}	96.67 ± 3.33^{a}
1500 + 150 + 150	45.00 ± 2.50^{b}	100.00 ± 0.00^{a}
2000 + 200 + 200	83.33 ± 8.33^{a}	100.00 ± 0.00^{a}

Means ± (SE) in the same column with different alphabet(s) are significantly different ($p \leq 0.05$) according to Tukey's Test *Percentage inhibition of radial growth of *G.boninense* after five days of incubation.

wood blocks for all treatments, which then developed into a small, white, button-like structure at 28 days except for T7 (Ca+Cu+SA) which only formed after 42 days incubation. The top end began expanding rapidly causing bracket-like structures which were generally white when first formed, but as their length and width increased rapidly, the upper surface developed reddish-brown colour at the centre zone. Basidiocarp is the most easily

Table 3. Comparison of effective treatment concentration to inhibit 50 and 90% of mycelial growth of *G. boninense* isolate from poison medium method test.

Treatment	Class / EC_{50} (ppm)	Class / EC_{90} (ppm)
T1 (Ca)	LE / > 2000	LE / > 2000
T2 (Cu)	LE / > 200	LE / > 200
T3 (SA)	LE / > 200	LE / > 200
T4 (Ca + Cu)	LE / > 2000 + 200	LE / > 2000 + 200
T5 (Ca + SA)	E / 2000 + 200	LE / > 2000 + 200
T6 (Cu + SA)	LE / > 200 + 200	LE / > 2000 + 200
T7 (Ca + Cu + SA)	E / 1500 + 150 + 150	E / 2000 + 200 + 200

LE= Least effective; E= Effective.

identifiable structure associated with the fungus. This study was conducted to identify best treatment for controlling basidiocarp formation of *G. boninense* on rubber wood block. Figure 2 shows that there were no significant differences between second and third months for most of the treatments especially for treatments Ca (T1), Ca+Cu (T4), Ca+SA (T5), Cu+SA (T6) and Ca+Cu+SA (T7). This indicating those treatments inhibited further formation of basidiocarp. Application of Cu (T2), SA (T3) alone was not effective in inhibiting formation of basidiocarp. In this study, combination application of Ca+Cu+SA (T7) efficiently controlled the formation and development of basidiocarp compared to other treatments. T7 forming 20.14% basidiocarp on 60 and 90 days after treatment applied followed by 41.15% for the Ca+SA (T5) compared to the control treatment (T8) formed 100% of basidiocarp (Figure 3).

Inhibition of size and weight of basidiocarp

The mean size of basidiocarp ranged from 1.20 to 2.79 cm^2 (Figure 4). The smallest size of basidiocarp was obtained from treatments Ca+Cu+SA (T7), Ca+SA (T5) and SA (T3) with the size of 1.20, 1.20, and 1.21 cm^2, respectively. On the other hand, Ca (T1) and the control (T8) showed the biggest size of basidiocarp of 2.78 and 2.79 cm^2, respectively. These two treatments also produced the largest fruiting bodies. In terms of the weight of basidocarp, Figure 5 shows significant differences between treatments (T1 to T7) compared with the control treatment (T8) except for T6 (Cu+SA). The significant reduction in weight and size of basidiocarp was obtained from T7 (Ca+Cu+SA). This treatment was the best among treatments and these results suggesting that application of Ca+Cu+SA manage to suppress the growths of basidiocarp in block experiment significantly.

Weight loss of the rubber wood block

G. boninense produced manganese peroxidises (MnP), and laccases enzymes; suggesting that infection from a large inoculum in dead material allowed invasion of the living material. However, the macromolecules which would be invaded are not living tissue. There is no particular reason to suggest that the fungus needs to overcome truly living cells first as the dead wood could be completely exposed. Result from Figure 6 showed that the lowest decrease in weight of inoculum block was in Ca+Cu+SA (T7) (41.85%) followed by Ca+SA (T5) (45.57%) as compared to the control block (61.87%). The lower weight loss showed that the enzymatic activities of basidiocarp were inhibited effectively by applying these treatments which also led to lower formation of basidiocarp on the rubber wood blocks.

Effect of treatments on size of basidiospores

Microscopic basidiospores are produced in the pores present on the underside basidiocarps. When basidiospores are dropped into mass on a white surface, they will appear brownish-red in colour. Soil immediately around a basidiocarp that has dropped its spores may appear to be covered with a rusty colour dust. This often gives the impression that the area has been generously sprinkled with cocoa powder. From the microscopic observation, the treated and untreated spore sizes were similar. The basidiospores of *G. boninense* were observed under 400 magnifications of a light microscope. The widths and lengths of most basidiospores in treated and control basidiocarp were about 0.04 and 0.08 mm, respectively (Figure 7).

Effect of treatment on morphology of mycelia

The effect of the suppression of nutrient treatments against *G. boninense* was further investigated using a light microscope. The changes of hyphal tips of *G. boninense* treated with nutrients were observed under 400 magnifications. Malformation of hyphae and shrunk occurred in the presence of treatment compared with

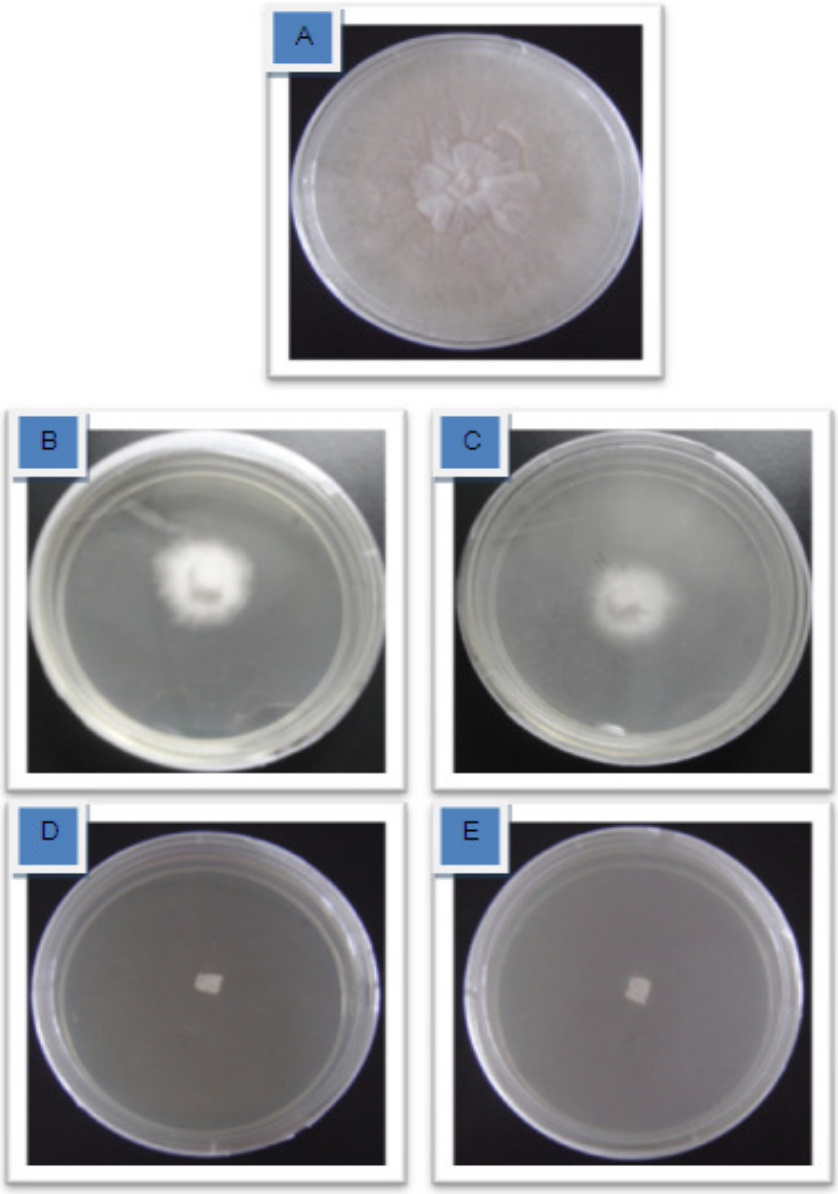

Figure 1. Inhibitory effect of mycelia growth for Ca+Cu+SA at concentration of 500 +50+50 ppm (B), 1000+100 +100 ppm (C), 1500 +150 +150 ppm (D), 2000+200 +200 ppm (E) for *in-vitro* study by dipping test. Healthy *G. boninense* colony on control plate (A).

Table 4. Comparison of effective treatment concentration to inhibit 50 and 90% of mycelial growth of *G. boninense* isolate from dipping method test.

Treatment	Class / EC_{50} (ppm)	Class / EC_{90} (ppm)
T1 (Ca)	LE / >2000	LE / >2000
T2 (Cu)	LE / >200	LE / >200
T3 (SA)	E / 50	E / 200
T4 (Ca+Cu)	LE / >2000+200	LE / >2000+200
T5 (Ca+SA)	LE / <500+50	E / 500+50
T6 (Cu+SA)	E / 50+50	NE / >200+200
T7 (Ca+Cu+SA)	LE / <500+50+50	E / 500+50+50

LE= Least effective; E= Effective; NE= Not effective.

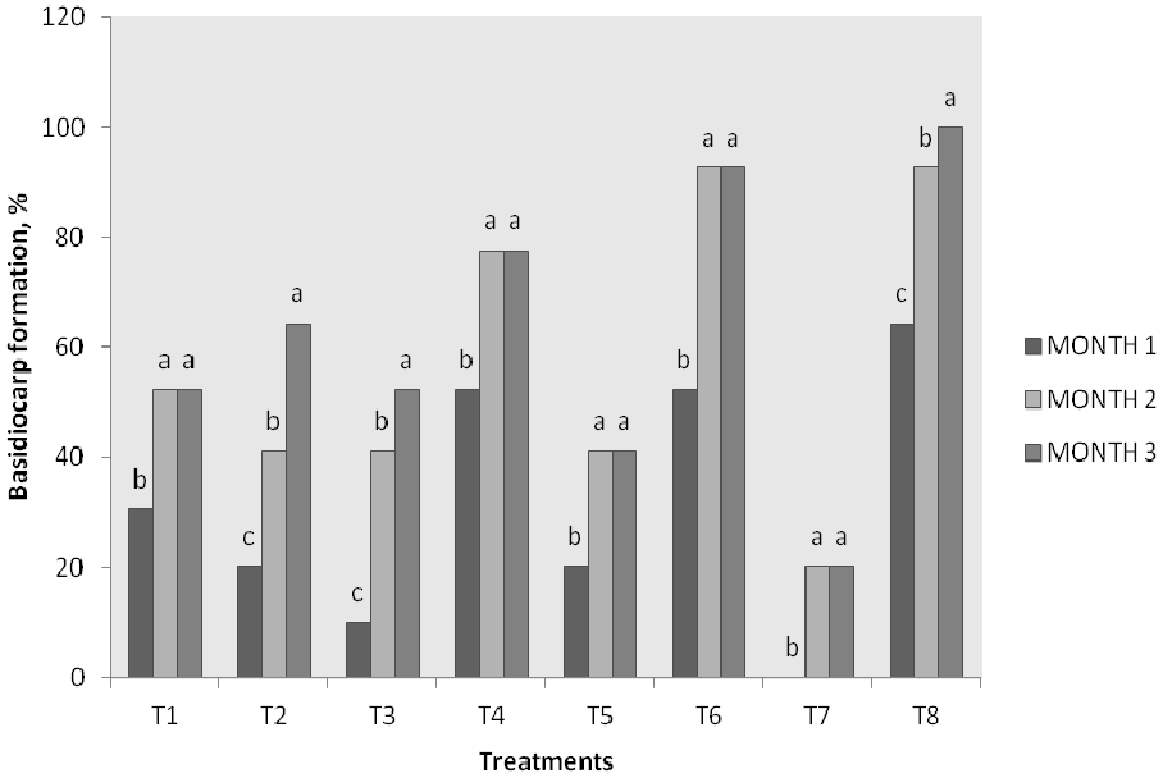

Figure 2. Effects of different treatments on percentage of basidiocarp formation at first, second and third months. T1 (1000 ppm Ca); T2 (50 ppm Cu); T3 (100 ppm SA); T4 (500 ppm Ca + 50 ppm Cu); T5 (500 ppm Ca + 50 ppm SA); T6 (50 ppm Cu + 50 ppm SA); T7 (500 ppm Ca + 50 ppm Cu + 50 ppm SA); T8 (Control). Different letters above each bar indicate significant differences between means (P ≤ 0.05) according to Tukey's test and Arc sine transformed before subjected to analysis of variance (ANOVA).

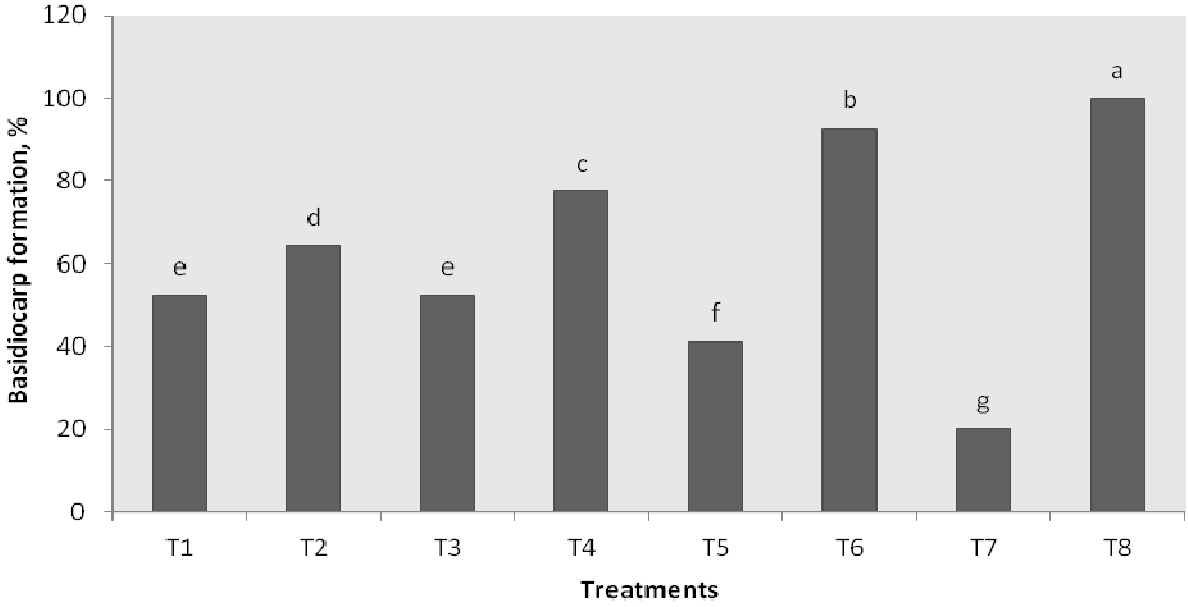

Figure 3. Effects of different treatments on percentage of basidiocarp formation at third month. T1 (1000 ppm Ca); T2 (50 ppm Cu); T3 (100 ppm SA); T4 (500 ppm Ca + 50 ppm Cu); T5 (500 ppm Ca + 50 ppm SA); T6 (50 ppm Cu + 50 ppm SA); T7 (500 ppm Ca + 50 ppm Cu + 50 ppm SA); T8 (Control). Different letters above each bar indicate significant differences between means (P ≤ 0.05) according to Tukey's test and Arc sine transformed before subjected to analysis of variance (ANOVA).

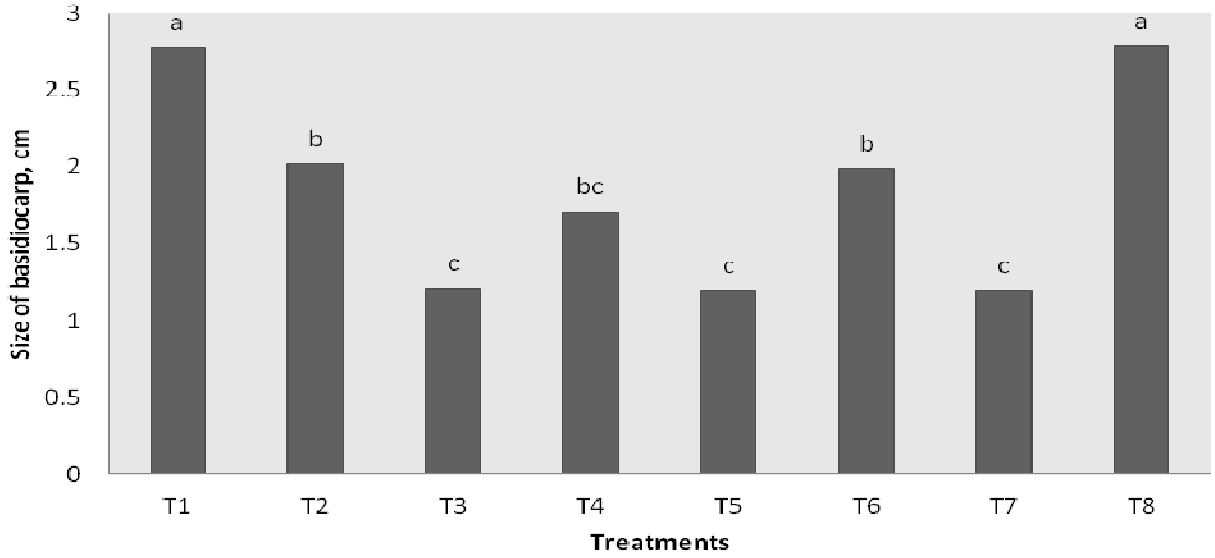

Figure 4. Effects of different treatments on size of basidiocarp formation. T1 represents (1000 ppm Ca); T2 represents (50 ppm Cu); T3 represents (100 ppm SA); T4 represents (500 ppm Ca + 50 ppm Cu); T5 represents (500 ppm Ca + 50 ppm SA); T6 represents (50 ppm Cu + 50 ppm SA); T7 represents (500 ppm Ca + 50 ppm Cu + 50 ppm SA); T8 represents (Control). Different letters above each bar indicate significant different between means (P ≤ 0.05) according to Tukey's test.

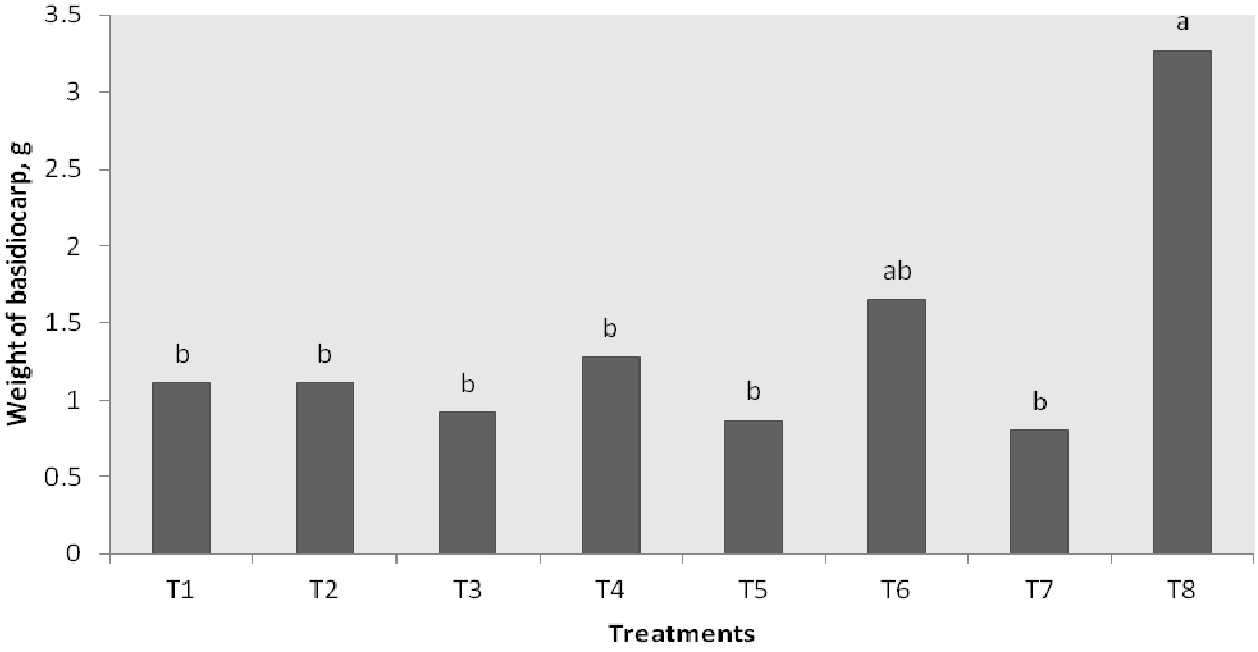

Figure 5. Effects of different treatments on weight of basidiocarp obtained. T1 (1000 ppm Ca); T2 (50 ppm Cu); T3 (100 ppm SA); T4 (500 ppm Ca + 50 ppm Cu); T5 (500 ppm Ca + 50 ppm SA); T6 (50 ppm Cu + 50 ppm SA); T7 (500 ppm Ca + 50 ppm Cu + 50 ppm SA); T8 (Control). Different letters above each bar indicate significant different between means (P ≤ 0.05) according to Tukey's test.

hyphae in the control plate. Hence, growth restriction upon application treatment was associated with frequent induction of morphological abnormalities such as lysis of hyphae (Figure 8).

DISCUSSION

Our findings in mycelia growth test showed the highest inhibitory effect by Ca, Cu and SA treatments by dipping

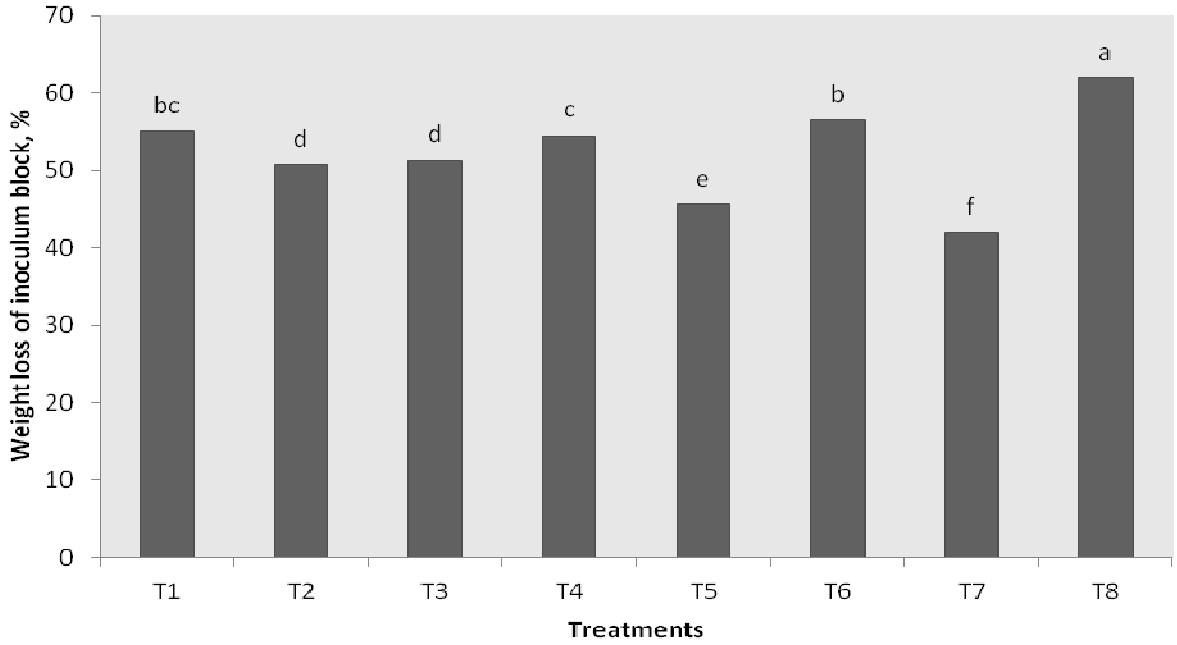

Figure 6. Effects of different treatments on weight loss of inoculum block. T1 (1000 ppm Ca); T2 (50 ppm Cu); T3 (100 ppm SA); T4 (500 ppm Ca + 50 ppm Cu); T5 s (500 ppm Ca + 50 ppm SA); T6 (50 ppm Cu + 50 ppm SA); T7 (500 ppm Ca + 50 ppm Cu + 50 ppm SA); T8 (Control). Different letters above each bar indicate significant different between means ($P \leq 0.05$) according to Tukey's test.

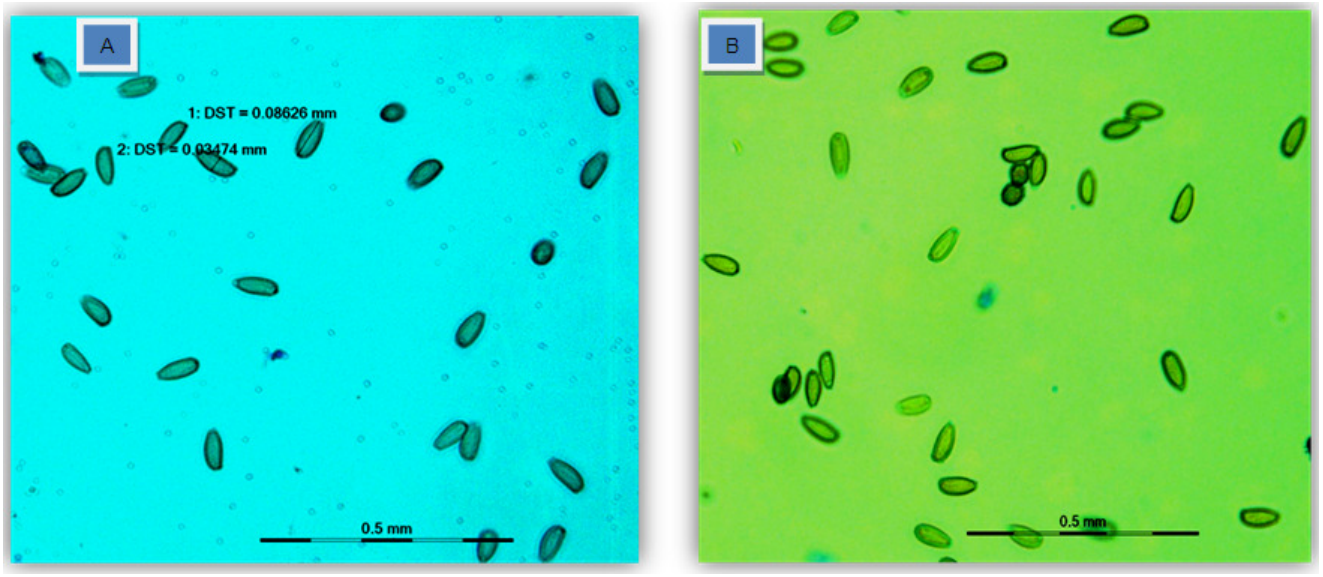

Figure 7. Observation size and morphology of *G. boninense* basidiospores at 400 magnifications under an optical microscope. The size and shape of basidiospores for control treatment (A) and treated basidiospores spores (B).

test instead of poison medium test. Based on the data from Table 2, poison medium test showed inhibitory effect of more than 50% for the T7 (2000 ppm Ca + 200 ppm Cu + 200 ppm SA) and T5 (2000 ppm Ca + 200 ppm SA) with 83.33 and 55.83%, respectively. Similar result obtained in dipping test where the highest effective value to inhibit *G. boninense* was attained by T7 (Ca+Cu+SA). Concentrations of (2000 ppm Ca + 200 ppm Cu + 200 ppm SA) and (1500 ppm Ca + 150 ppm Cu + 150 ppm SA) were 100.00% inhibition in mycelial growth. While, (1000 ppm Ca + 100 ppm Cu + 100 ppm SA) and (500 ppm Ca + 50 ppm Cu + 50 ppm SA) inhibited fungal

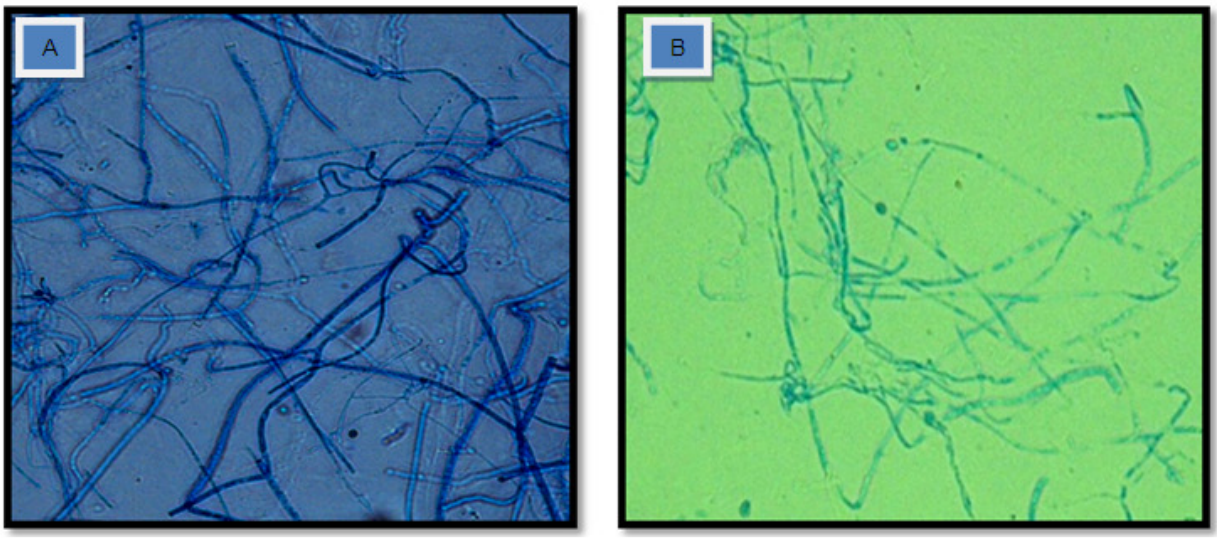

Figure 8. Observation mycelia morphology of *G. boninense* at 400 magnifications under an optical microscope. Healthy hyphae observed on control treatment (A). Treated hyphae showed morphological abnormalities such as lysis of hyphae (B).

growth by 96.67 and 95.00%, respectively. The obtained result clearly revealed that the mixture of Ca + Cu + SA (T7) was effective against *G. boninense* for both poison medium and dipping tests. This was further supported by the results of EC_{50} of T7 which was 1500 + 150 + 150 ppm and EC_{90} was 2000 + 200+ 200 ppm for poison medium test and EC_{90} was 500 + 50 + 50 ppm in dipping test.

Thereafter, further test was conducted in a net house to prove the effectiveness of the treatments tested. For every element, the best concentration was chosen from previous *in vitro* studies. Based on an inoculum block study, T7 (Ca+Cu+SA) showed encouraging results where total formation of basidiocarp was only 20.14% compared to the control (100.00%). Furthermore, the size of basidiocarp was around 1.20 cm^2 and that of the control (2.79 cm^2); the weight of basidiocarp was 0.80 g compared to the control (3.27 g) and the weight losses of inoculum block were 41.85% equated to control treatment approximately (61.87%). In previous studies, the production of lignin degrading enzymes by *G. boninense* has been reported by Ali et al. (2004) which presented that the fungus could cut down lignin in oil palm. Other report by Adaskaveg et al. (1991) showed that white rot fungi caused significant weight loss up to 63%. Of the white rot fungi, *Ganoderma colossum* caused the greatest weight loss (81%), while *Scytinostroma galactinum* caused the least (36%). Our further study was led to observed spores and mycelia growth; hence, the spore sizes do not changes much compared to the control. Although the lengths and widths of spore size appeared same, 0.04 mm in width and 0.08 mm in length. However, interesting finding was found in light microscope observation. Results show that there were significant changes in hyphal tips due to treatment effect. Mycelia treated with Ca+Cu+SA looked abnormal with shrinkage and malformation while control had normal hyphal cell walls with no swellings and vacuolations. Based on our observations on oil palm plantation in Sarawak indicated that plant stress may be due to soil types especially those planted on peat soil and other factors are soil depth and poor nutrition. These factors can elevate disease severity levels. However, until now there is no clear correlation with any single factor or with combination of factors. Moreover, the interactions of nutrition in these components are dynamic and all essential nutrients are reported to influence the incidence or severity of some diseases. According to Turner (1981), potassium increase drought resistance and disease resistance in oil palm and bunch size and number are reduced in K-deficient palms.

Evidence from soil application of calcium nitrate suppressed the development of basal stem rot. Disease severity was reduced by 25 to 30% based on foliar symptoms, number of fruiting bodies produced and number of roots with lesions. Calcium nitrate did not reduce mycelia growth of the pathogen, suggesting that the effect of calcium in reducing infection was indirectly and probably due to the formation of cell wall com-ponents resistant to degradation by *Ganoderma* (Sariah et al., 1997). The role of copper in disease suppression was two folds. The first was direct suppression of disease on the surface of the leaf. This is as fungicides. Copper containing enzymes is important for the production of polyphenol compounds that are involved in lignin biosynthesis. Other compounds are also manufactured in the process; alkaloids, the formation of brown melanotic substance that is formed when tissues are wounded.

These compounds can also act as phytoalexins, which inhibit spore germination and fungal growth. Copper sulphate is a fungicide used to prevent and control plant fungal diseases, including powdery mildew, leaf spots and blight (Meister, 1992). Another excellent example is that with peanut seedlings treated with Cu^{2+} there is significant inhibition in peanut root growth and a decrease in endogenous IAA content. The increase in the activity of anionic peroxidase (POD) isozyme P3.5 was correlated with the rise in lignin content in Cu-treated roots. The researchers suggested that the increase in anionic POD isozyme P3.5 induced by Cu might be responsible for lignin synthesis in peanut roots (Li et al., 2001). Salicylic acid which has recently been considered as one of plant hormones (Raskin, 1992) is involved in reducing the damage caused by various pathogens such as bacteria, fungi and viruses. It is also considered as the most important factor in systemic acquired resistance against aforementioned pathogens (Nie, 2006). The levels of salicylic acid increase in plants undergoing systemic acquired resistance. Different investigations have also shown that the application of salicylic acid causes the induction of resistance in plants such as Arabidopsis and tobacco against viruses and fungi (Malamy and Klessing, 1992).

In summary, the study assessed the inhibitory effect of treatments and effective concentration against pathogenic *G. boninense in vitro* and inoculum block study. The results revealed that combination of 500 ppm Ca + 50 ppm Cu + 50 ppm SA offer an effective solution to suppress the growth of *G. boninense* effectively *in-vitro* experiment. Thus, these findings are a feasible reference foundation for further tests in the field.

ACKNOWLEDGEMENTS

This project was supported by Universiti Putra Malaysia Bintulu Campus Sarawak (UPMKB). Special gratitude is expressed to Mrs. Siti Fatimah for her technical support during conducting research activities. Last but not least special appreciation to all staff of UPMKB who directly or undirectly were involved in this project.

REFERENCES

Adaskaveg JE, Gilbertson RL, Blanchette RA (1990). Comparative studies of delignification caused by *Ganoderma* species. Appl. Environ. Microbiol. 56:1932-1943.

Adaskaveg JE, Blanchette RA, Gilbertson RL (1991). Decay of date palm wood by white rot and brown rot fungi. Can. J. Bot. 69:615-629.

Ali SRA, Yaacob NS, Seman IA, Wahid MB (2004). Oil palm cellulose and lignin degradation of different *Ganoderma* sp. based on ASTM standard rotting experiment. International conference on pests and diseases of importance to the oil palm industry, Malaysian Oil Palm Board, Kuala Lumpur, Malaysia. 18-19 May 2004. pp.2-15.

Chinchilla C, Richardson DL (1987). Four potentially destructive diseases of the oil palm in Central America. Proc. of the 1987 International Oil Palm/Palm Oil Conference: Progress and Prospects - Agriculture Conference (Abdul Halim eds.). 23 - 26 June 1987. pp.468-470.

Chung GF, Cheah SS, Nur Azarina AB (1998). Some insects associated with *Ganoderma* fruiting bodies. In: Proceeding International Workshop on *Ganoderma* diseases, MARDI Serdang, Malaysia. 5-8 October 1998. (Abstr.).

Corley RHV, Tinker PB (2003). The Oil Palm. 4th Edition. Blackwell Publishing. Great Britain. pp. 27-28, 407-413.

De Oliveira FGR, Candian M, Lucchette FF (2005). A technical note on the relationship between ultrasonic velocity and moisture content of Brazilian hardwood (*Goupia glabra*). Build. Environ. 40:297-300.

Dikin A, Kamaruzaman S, Zainal Abidin MA, Idris AS (2003). Biological Control of Seedborne Pathogen of Oil Palm, *Schizophyllum commune* Fr. with Antagonistic Bacteria. Int. J. Agric. Biol. 5(4):507-512.

Engelhard AW (1989). Soilborne plant pathogens: Management of diseases with macro- and microelements. APS Press, St. Paul, Minn.

Flood J, Hasan Y, Turner PD, O' Grady EB (2000). The spread of *Ganoderma* from its infective sources in the field and its implication of management of the disease in oil palm. In: Flood J, Bridge PD, Holderness M (eds), *Ganoderma* Diseases of Perennial Crops. CABI Publishing, Wallingford, UK. pp.101-112.

Gautam S, Singh PK (2009). Salicylic acid induced salinity tolerance in corn grown under NaCl stress. Acta Physiol. Plant 31:1185-1190.

Gomez KA, Gomez AA (1984). Statistical Procedures for Agricultural Research 2nd Edition. J. Wiley, New York. p.680.

Grover RK, Moore JD (1962). Toximetric studies of fungicides against brown rot organism- *Sclerotinia fructicola* and *S. laxa*. Phytopathology 52:876-880.

Holloman AJ, Young RA (1951). Selection by laboratory assay of fungicides for field control of Botrytis leaf spot of gladiolus. Phytopathology 41:940.

Idris AS, Ismail S, Arifin D, Ahmad H (2002). Control of *Ganoderma* Infected Palm – Development of Pressure Injection and Field Applications. MPOB Inform. Series TT p. 131.

Khairuddin H, Chong TC (2008). An overview of the current status of *Ganoderma* basal stem rot and its management in a large plantation group in Malaysia. The Planter 84:469-482.

Klomparens W, Vaughn JR (1952). The correlation of laboratory screening of turf fungicides with field results. Michigan Agricultural Experiment Station Quart. Bullet. 34:425-435.

Li X, Poon C, Liu PS (2001). Heavy metal contamination of urban soils and street dusts in Hong Kong. Appl. Geochem. 16:1361-1368.

Malamy J, Klessing DF (1992). Salicylic acid and plant disease resistance. Plant J. 2:643-654.

Meister A (1992). On the antioxidant effects of ascorbic acid and glutathione. Biochem. Pharmacol. 44:1905-1915.

Nambiar KKN, Rethinam P, Varghese M (1992). Management of *Ganoderma* wilt disease of coconut in Kerala. Indian Coconut J. 22(10):6-9.

Nie X (2006). Salicylic acid suppresses Potato virus Y isolate N: O induced symptoms in tobacco plants. Phytopathology 96:255-263.

Nieto LE (1995). Incidence of oil palm stem rots in Colombia. Palmas 16:227-232.

Pieterse CMJ, Reyes AL, Ent SVD, Wees SCMV (2009). Networking by small molecule hormones in plant immunity. Nature Chem. Biol. 5(5):308-316.

Raskin I (1992). Salicylate, a new plant hormone. J. Plant Physiol. 99:799-803.

Ramírez AA, Dolores R, David R, Jesús AJ, Gregorio N, López-Climent M, Gómez-Cadenas A, Carlos N (2009). Evidence for a role of gibberellins in salicylic acid-modulated early plant responses to abiotic stress in Arabidopsis seeds. Plant Physiol. 150:1335-1344.

Rees RW, Flood J, Hasan Y, Cooper RM (2007). Low soil temperature and root inoculum contact enhance *Ganoderma* infection of oil palm; implications for late disease appearance in plantations and screening for disease resistance. Plant Pathol. 56:862-870.

Sanderson FR (2005). An insight into spore dispersal of *Ganoderma boninense* on oil palm. Mycopathologia 159:139-141.

Sariah M, Joseph H, Zakaria H (1997). Suppression of basal stem rot (BSR) of oil palm seedlings by calcium nitrate. The Planter 73:359-360.

Sariah M, Zakaria H (2000). The use of soil amendments for the control of basal stem rot of oil-palm seedlings. In: Flood J, Bridge PD,

Holderness M (eds.), Ganoderma Diseases of Perennial Crops. CABI Publishing, Wallingford, UK. pp.89-99.
Skidmore AM, Dickinson CH (1976). Colony interactions and hyphal interference between *Septoria Nodorum* and phylloplane fungi. T. Br. Mycol. Soc. 66:57-64.
Susanto A, Sudharto PS, Purba RY (2005). Enhancing biological control of basal stem rot disease (*Ganoderma boninense*) in oil palm plantations. Mycopathol. 159:153-157.
Susanto A (2009). Basal stem rot in Indonesia. Biology, economic importance, epidemiology, detection and control. Proceedings of International Workshop on Awareness, Detection and Control of Oil Palm Devastating Diseases. Kuala Lumpur Convention Centre, Malaysia. Universiti Putra Malaysia Press.
Tummakate A, Likhitakaraj S (1994). The situation of Ganoderma on oil palm in Thailand. Proc. of the First International Workshop on Perennial Crop Diseases Caused by Ganoderma (Holderness, M eds.). 28 November-3 December 1994. UPM, Serdang, Selangor. (Abstr.).
Turner PD (1981). Oil Palm Diseases and Disorders. Oxford University Press, Kuala Lumpur, Malaysia p.280.
Yang VW, Clausen CA (2007). Antifungal effect of essential oils on southern yellow pine. International Biodegradation and Biodeterioration. 59:302-306.