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Control of Basal Stem Rot Disease in Oil Palm by Supplementation of Calcium, Copper, and Salicylic Acid

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Continuous supplementation of mineral nutrients and salicylic acid (SA) as foliar application could improve efficacy in controlling basal stem rot (BSR) disease in oil palm seedling. It is revealed from the results that the highest disease severity index (58.3%) was recorded in T8 treatments at 9 months after inoculation. The best disease control was achieved by T7 treatments (calcium/copper/SA [Ca/Cu/SA]) (5.0%) followed by T1 (5.5%), T5 (5.8%), T3 (8.3%), T6 (8.3%), T4 (13.3%), and T2 (15.8%) treatments. Continuous supplementation of Ca/Cu/SA was found to be the most effective in controlling the disease and the high performance liquid chromatography results showed the detection of ergosterol at very low concentration in the treated samples. Moreover, the transmission electron microscopy analysis results clearly indicated that T7 treatment was also enhancing lignification, which was responsible for the thickness of the secondary cell walls and middle lamella compared to untreated samples. It was therefore, concluded that continuous supplementation of minerals nutrients and SA could effectively suppress disease severity by reducing ergosterol activity and also improve the process of lignification in the treated plants. Furthermore, this treatment also managed to delay the onset of BSR symptoms and promote the growth of the seedlings and eventually suppress the BSR disease.

Keywords **:** basal stem rot, copper, *Ganoderma boninense*, induced resistance, salicylic acid

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World demand for oil and fat is on yearly increase and as a consequence, the area planted with oil palm in Malaysia tremendously increased from 0.3 million hectares (ha) to 4.49 million ha in 1970 and 2008, respectively (Mohd Basri, 2009). In Southeast Asia, basal stem rot (BSR) disease has remained one of the major obstacles in oil palm cultivation. It is caused by the white-rot fungus, *Ganoderma boninense* which cuts down the oil palm yield in most production areas in Malaysia as well as Indonesia. To date, BSR is controlled by using cultural practices, biological control agents such as *Tricoderma* spp. and selected systemic chemical fungicides. However, till now, no single control proven to effectively control BSR in the field was reported (Susanto et al., 2005). The difficulty managing this disease is due to not exhibiting any external symptoms on mature palms until advanced stage. When it comes to this stage, the infected trees may not be able to respond to any treatment given. Therefore, enhanced nutritional programmes (ENPs) by using mineral nutrients and plant hormone, appropriate dosage application at seedling stage should be done, in order to make them resistant towards BSR disease when they are transplanted in the field.

Nutritional status of a plant has a major impact on dis-

ease resistance. In this regards, optimum nutrient uptake is very important to prevent nutrient deficiency in plants. In general, mineral nutrients are the primary lines of plant defence against pathogens and it is directly influenced by the three components of the plant disease triangle, the hosts, the pathogens and the environmental factors. The mineral nutrients may affect the disease resistance through inducible defences, which includes the production of toxins, metabolites and lignification (Engelhard, 1989). Earlier reports from the past researchers showed that calcium nitrate was used to suppress BSR symptoms on oil palm (Sariah and Zakaria, 2000; Sariah et al., 1998). The findings of the studies are in agreements with the findings of Nur Sabrina et al. (2012) and Rahamah Bivi et al. (2014). They showed that foliar application of combined calcium (Ca) and copper (Cu) on oil palm seedlings enhanced resistance towards *Ganoderma* infection under glasshouse condition. Moreover, salicylic acid (SA) is also associated with the induction of disease resistance against various phytopathogens as well as abiotic stress (Gautam and Singh, 2009; Pieterse et al., 2009). SA which has recently been conceived as one of plant hormones (Raskin, 1992), is needed in reducing the damage induced by several pathogens such as bacteria, fungi and viruses. It is also believed to be the most important component in systemic acquired resistance against the above pathogens (Nie, 2006). Therefore, the objective of the present work is to assess the roles of SA, Ca, and Cu supplementations on oil palm seedling against *G. boninense* infection under glasshouse condition.

Materials and Methods

Preparation of rubber wood blocks. Rubber wood blocks were obtained from Universiti Putra Malaysia, Bintulu Campus, Sarawak, Malaysia and treated following the method of Rees et al. (2007). The rubber wood blocks (6 × 6 × 6 cm) were gently washed and autoclaved at 121°C, 100 kPa for 45 min. Each block was then placed in heat-resistant polypropylene bags. Subsequently, 100 ml of molten Malt Extract Agar (MEA; Hi-Media, Mumbai, India) was poured over the blocks as a supplementary nutrient for the fungus and these blocks were re-sterilized. After cooling, the rubber wood blocks in the polypropylene bags were rotated to ensure that they were well covered with the agar before solidified. The cooled blocks containing MEA were inoculated with six plugs (10 mm) taken from the leading edge of *G. boninense* and inoculated blocks were incubated at room temperature (28 ± 2°C) in dark cabinet for about 90 days.

Immunisation of oil palm seedlings with Ca, Cu, and SA. Oil palm seedling (about 5 months old) supplied by Malaysian Palm Oil Board (MPOB) Saratok, Sarawak, Malaysia was used. All the oil palm seedlings were then grown in polybags (20 × 25 cm) containing soil mixture (3:2:1 v/v/v; soil:peat:sand). The oil palm seedling was pre-treated weekly for a month with 250 ml/seedling of different dosages of treatments as listed in Table 1, as foliar spray (Rahamah Bivi et al., 2012). After pre-treatment, the oil palm seedlings were then inoculated with *G. boninense* by transplanting them into new polybags (25 × 30 cm) containing infected rubber wood block. The oil palm seedling was treated continuously with the same treatments monthly for about eight months. In this study, T9 served as a negative control and T8 was positive control treatment. The experiment was conducted twice in a glasshouse for nine months, arranged in a randomized complete block design with nine treatments. In total, three blocks were used and for each treatment consisting of 15 replicates. The plants were watered daily and the standard fertilization and pest control programs were applied throughout the experimental period.

Table 1. Type of the treatments and dosage used in the glasshouse trial

Treatment	Nutrient/plant hormone	Concentration (ppm)
T1	Calcium chloride/*Ganoderma boninense*	1,000
T2	Copper-EDTA/*G. boninense*	50
T3	Salicylic acid/*G. boninense*	100
T4	Calcium chloride/copper-EDTA/*G. boninense*	500/50
T5	Calcium chloride/salicylic acid/*G. boninense*	500/50
T6	Copper-EDTA/salicylic acid/*G. boninense*	50/50
T7	Calcium chloride/copper-EDTA salicylic acid/*G. boninense*	500/50/50
T8	Positive control (*G. boninense*)	
T9	Negative control (distilled water)	

EDTA, ethylenediaminetetraacetic acid.

Disease assessment. Disease development was assessed based on percentage of disease incidence (DI), using the formula of Campbell and Madden (1990).

$$\text{DI (\%)} = \frac{\text{Number of infected seedlings}}{\text{Total number of the seedlings assessed}} \times 100$$

DSI. Data on disease severity index (DSI) of the treated seedling was assessed based on disease score (0 to 4 scales) as listed in Table 2. DSI was computed according to the formula described by Abdullah et al. (2003). DSI was transformed by arcsine and the mean of each treatment was compared by the Tukey's test at $P \leq 0.05$ using SAS software version 9.0 (SAS Institute, Cary, NC, USA). The DSI was computed for every month to a period of nine months.

$$\text{DSI (\%)} = \frac{\sum ab}{\text{N.K}} \times 100$$

Where,
$\sum ab$ = sum of the product of assessed plants with their corresponding score scale
N = total number of assessed plants
K = highest score scale

At the end of the experiment, the treated seedling was cut longitudinally to observe the internal symptoms of root and stem decay. Assessment of the disease was based on the scale as described by Breton et al. (2005): 0 = healthy, no internal rot; 1 = 1% to 20% rotting of tissues; 2 = 21% to 50% rotting of tissues; 3 = 51% to 90% rotting of tissues; 4 = > 90% rotting of tissues.

DSI for internal symptoms of bole tissue was estimated based on the formula drew by Abdullah et al. (2003) and Ilias (2000). Percent disease reduction (PDR) was calculated based on the formula below,

$$\text{DR (\%)} = \frac{\text{DI of positive control} - \text{DI of treated seedling}}{\text{DI of positive control seedling}} \times 100$$

Determination of ergosterol activity using HPLC. *Extraction of ergosterol from oil palm bole:* Bole part of oil palm seedling was cut into smaller pieces by using a machete and was then ground to obtain fine powder using grinder (1.5 mm). Each treatment consisted of three replications and the sample was kept in –20°C freezer prior extraction. About 1 g of sample was placed into a Pyrex test tube with Teflon screw cap and 2 ml of methanol (chromatography grade, Merck) was added thereafter. The test tube was tightly closed, shaken by using vortex and subsequently put into culture jar and placed at the centre of a conventional microwave (Panasonic Dimension4 NN-C988W; Panasonic, Osaka, Japan) for 40 s at 'medium-high' power setting. The solutions were cooled for a few seconds and neutralized by using different concentration of hydrochloric acid. A 70 ml of 2 M sodium hydroxide was added into a test tube and mixed thoroughly. After which, 5 ml, 2 ml, and 1.7 ml of hydrochloric acid to stabilize the solution. A 2 ml of pentane (analytical reagent grade; Fisher Scientific, Fair Lawn, NJ, USA) was added to form supernatant (two layers solution) and was repeated three times. Combines pentane extract were placed in a set flask before the solutions are evaporated to dryness using Bucho rotary evaporator (Buchi Rotavapor R-215; Cole-Parmer, Vernon Hills, IL, USA) at temperature of 60°C of heating water bath (Buchi Heating Bath B-491) and 158° rotation. After the solutions dried, 500 µl of methanol was added into the set flask before the solutions were pipetted into micro centrifuge tube. The solutions are placed into centrifuge tube and were kept in –20°C freezer and used for detection and quantification of ergosterol by using high performance liquid chromatography (HPLC) (Muniroh et al., 2014).

HPLC. An Agilent 1100 series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a Diode Array Detector (G1315B), a pump (G1311A), and an auto sampler (G1313A) was used for detection and quantification of ergosterol using an Ascentis® Express C18, 2.7 µm HPLC Column reverse-phase column (Supelco, St. Louis, MO, USA). Operating conditions consisted of an isocratic

Table 2. The scored (0–4 scales) of the disease class on the external signs and symptoms of the treated plants*

Disease class	Signs and symptoms
0	Healthy plants with green leaves without appearing of fungal mycelium on any part of plants
1	Appearing of white fungal mass on any part of plants, with or without chlorotic leaves
2	Appearing of basidioma on any part of plants with chlorotic leaves (1 to 3 leaves)
3	Formation of basidioma of any part of plants with chlorotic leaves (> 3 leaves)
4	Formation of a well-developed basidioma and the plants dried

*Described by Abdullah et al. (2003) (*Res. Bull. Sci. Putra* 11:31–33) and Ilias (2000).

HPLC-grade methanol mobile phase at a flow rate of 1 ml min^{-1}. The mobile phase was degassed for 30 min in an ultrasonicator (Cole-Parmer) at full power. The ultraviolet (UV) detection was at 282 nm and injection volume of 10 µl per sample was set for detection and quantification. The average retention time of ergosterol was 7 min. An ergosterol standard was prepared for constructing the standard curve. The ergosterol peak was determined by comparison of the retention time and UV absorbance at 282 nm against the pure ergosterol standard. The ergosterol concentration for each run was determined by comparison against the ergosterol standard calibration curve. All ergosterol concentrations are reported as per unit weight basis and each sample was analysed in triplicate.

TEM analysis. The root specimens were cut into a number of 1 mm^3 slices, then placed in separate vials and fixed in fixative of 4% glutaraldehyde for 24 to 48 h at 4°C. After that they were washed with 0.1 M sodium cacodylate buffer for 3 changes of 30 min each and post fixed in 1% osmium tetraoxide for 2 h at 4°C. The samples were then washed again with 0.1 M of sodium cacodylate buffer for 3 changes of 30 min each and they were dehydrated in a serial dilution of acetone (35%, 50%, 75%, and 95%) for 30 min in each dilution and in 100% acetone for 1 h for 3 changes. Later on, the specimen was infiltrated with acetone and resin mixture and the specimen were placed into beam capsules and filled up with resin. The specimen was polymerized in an oven at 60°C, for 24 to 48 h. Glass knife and ultramicrotome were used to cut 1 µm thick section, placed onto a glass slide, stained with toluidine blue, dry on a hot plate, washed the stain and examined under light microscope. Area of concern was selected and chopped into ultrathin sections, silver or golden sections were selected and picked up sections with a grid and dried out using filter paper. Then, they were stained with uranyl acetate for 15 min and washed with double distilled water and stained with lead for 10 min, washed with double distilled water again and finally viewed under transmission electron microscope (TEM).

Determination of plant vigour. The effects of Ca and Cu and SA treatments on plant vigour were assessed for every month. Greater increment in plant height, bole diameter and root mass would show improvement in plant vigour, and a positive effect of the treatments on the plant health. The rate of increment in plant height (cm) and bole diameter (mm) was measured by using measuring tape and digital calliper, respectively. Height was measured from one cm above the soil level to the tip of the leaves. The bole diameter was taken at the same height above the ground. At the end of the experiment, root mass was measured by weighing the total fresh roots of each oil palm seedling using a digital balance. All samples were oven dried for two days at 70°C.

Statistical analysis. The disease assessment data, ergosterol concentration data and plant growth data were compared by analysing the variance and comparing the averages with ANOVA and a multiple range test, Tukey's at $P \leq 0.05$. Analyses were conducted using the SAS software.

Results

Effect of Ca, Cu, and SA supplementation on disease severity. It is evident from our results that the analysis of the internal disease severity (based on the level of bole decay) showed that the seedlings treated with T7 had significantly lower disease severity (0.8%) as compared with T8 (Fig. 1). The results suggested that combination of Ca, Cu, and SA have potentials to suppress the development of BSR pathogen, *G. boninense*. The positive control suffered the highest bole rot and developed the serious necrotic lesions with > 50%. The Ca, Cu, and SA were the effective treatments in reduction of BSR DSI under glasshouse conditions. However, the assessment on external symptoms showed various degrees of DSI (Table 3). The highest DSI value (58.3%) was noticed in T8 (positive control) at 9 months after inoculation (9MAI). The best disease control (5.0%) was recorded in T7 treatment followed by T1 (5.5%), T5 (5.8%), T3 (8.3%), T6 (8.3%), T4 (13.3%), and T2 (15.8%) at 9MAI, respectively, while T9 treatment (negative control) remains healthy along the

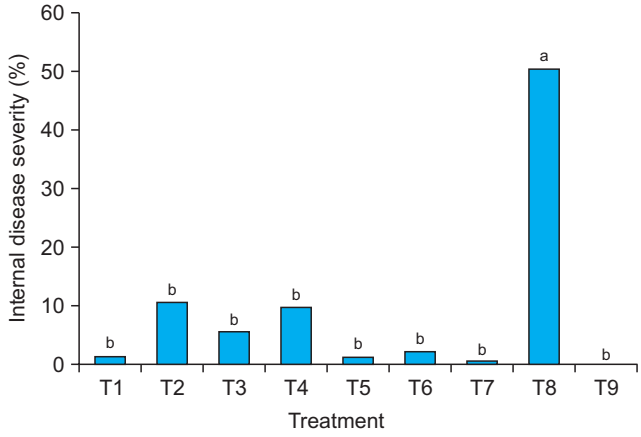

Fig. 1. Internal disease severity of bole tissue was assessed 9 months after inoculation. Different letters above each bar indicate significant differences between means ($P \leq 0.05$) according to Tukey's test. T1, calcium (Ca); T2, copper (Cu); T3, salicylic acid (SA); T4, Ca/Cu; T5, Ca/SA; T6, Cu/SA; T7, Ca/Cu/SA; T8, positive control; T9, healthy control.

Table 3. Effect of treatments on percentage of disease severity (external symptoms) in oil palm seedlings was assessed at 0–9 MAI

Treatment	Disease severity index (%)					
	4MAI	5MAI	6MAI	7MAI	8MAI	9MAI
T1 (Ca)	0.0^{b}	0.8^{b}	1.7^{b}	2.5^{c}	2.5^{c}	5.5^{c}
T2 (Cu)	2.5^{b}	3.3^{b}	5.0^{b}	11.7^{b}	12.5^{b}	15.8^{b}
T3 (SA)	0.8^{b}	0.8^{b}	3.3^{b}	5.0^{c}	6.7^{bc}	8.3^{bc}
T4 (Ca/Cu)	3.3^{b}	5.0^{b}	6.7^{b}	8.3^{b}	11.7^{b}	13.3^{b}
T5 (Ca/SA)	0.0^{b}	0.8^{b}	1.7^{b}	2.5^{c}	3.3^{c}	5.8^{c}
T6 (Cu/SA)	0.0^{b}	1.7^{b}	2.5^{b}	5.0^{c}	5.8^{c}	8.3^{bc}
T7 (Ca/Cu/SA)	0.0^{b}	0.0^{b}	0.8^{b}	0.8^{c}	3.3^{c}	5.0^{c}
T8 (positive control)	10.8^{a}	19.2^{a}	28.3^{a}	37.5^{a}	50.0^{a}	58.3^{a}
T9 (negative control)	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{c}	0.0^{c}	0.0^{c}

Different letters in the same column indicate significant differences between means ($P \leq 0.05$) according to Tukey's test.
MAI, months after inoculation; Ca, calcium; Cu, copper; SA, salicylic acid.

Table 4. Percent disease reduction of basal stem rot disease on different treatments at 9 months after inoculation

Treatment	Percent disease reduction (%)
T1 (Ca/*Ganoderma boninense*)	76.1
T2 (Cu/*G. boninense*)	38.1
T3 (SA/*G. boninense*)	71.4
T4 (Ca/Cu/*G. boninense*)	52.4
T5 (Ca/SA/*G. boninense*)	76.1
T6 (Cu/SA/*G. boninense*)	76.1
T7 (Ca/Cu/SA/*G. boninense*)	81.0
T8 (positive control)	-
T9 (negative control)	-

Ca, calcium; Cu, copper; SA, salicylic acid.

Table 5. Effect of Ca^{2+}, Cu^{2+}, and SA application at epidemic rate of *Ganoderma boninense* at 9 months after inoculation*

Treatment	Epidemic rate (slope) at 9 mo (unit/mo)
T1 (Ca/*G. boninense*)	2.152
T2 (Cu/*G. boninense*)	5.485
T3 (SA/*G. boninense*)	2.849
T4 (Ca/Cu/*G. boninense*)	4.242
T5 (Ca/SA/*G. boninense*)	1.939
T6 (Cu/SA/*G. boninense*)	2.606
T7 (Ca/Cu/SA/*G. boninense*)	1.485
T8 (positive control)	9.970
T9 (negative control)	0

Ca, calcium; Cu, copper; SA, salicylic acid.
*Each value means of 10 replicates.

study period. The infected plants leaves showed a chlorotic and necrotic pattern with a white colour fungal mass at the basal portion of the plant, which may later form basidioma.

The PDR during the glasshouse experiment is shown in Table 4. It is revealed from our results that the application of Ca/Cu/SA was the most effective in the suppression of BSR disease in T7 treatment with highest PDR (81.0%) value. While, T1, T5, and T6 were also effective treatment with PDR (76.1%) value; however, the lowest PDR (38.1%) was recorded in T2 treatment. In addition, the epidemic rate of disease development was assessed as listed in Table 5. The highest epidemic rate was recorded in T8 ($r_m = 9.970$) treatment, while, the lowest was found in T7 ($r_m = 1.485$) treatment.

Effect of Ca, Cu, and SA supplementation on ergosterol activities. The concentrations of ergosterol for various treatments in the *G. boninense* infected plants were recorded (Table 6, Fig. 2). Our studies showed that ergosterol concentration among the treatments were significantly different compared to T9 (positive control). The positive control (T8 treatment) showed the highest concentrations of ergosterol (420.947 μg g^{-1}), while the negative control (T9 treatment) has lowest ergosterol concentrations (7.947 μg g^{-1}). Among the various tested treatments, T4 was found to be the best treatment (16.416 μg g^{-1}) to supress ergosterol activity. Other treatments such as T6 (19.094 μg g^{-1}), T1 (21.263 μg g^{-1}), T7 (21.959 μg g^{-1}) and T5 (22.381 μg g^{-1}) have the same capability to supress *G. boninense* activity.

Effect of Ca, Cu, and SA supplementation on cell wall modification. TEM is a unique tool for the inspection of ultrastructure of cell wall. TEM analysis revealed that oil palm cell walls were composed of an intracellular

Table 6. Concentration of ergosterol in the treated oil palm samples as detected by HPLC*

Treatment	Peak area	Ergosterol concentration (μg g^{-1})
T1 (Ca/*Ganoderma boninense*)	8,059	21.263[d]
T2 (Cu/*G. boninense*)	116,953	308.574[b]
T3 (SA/*G. boninense*)	19,720	52.030[c]
T4 (Ca/Cu/*G. boninense*)	6,222	16.416[d]
T5 (Ca/SA/*G. boninense*)	8,483	22.381[d]
T6 (Cu/SA/*G. boninense*)	7,237	19.094[d]
T7 (Ca/Cu/SA/*G. boninense*)	8,323	21.959[d]
T8 (pure culture of *G. boninense*)	197,761	420.947[a]
T9 (negative control)	3,012	7.947[e]

Different letters in the same column indicate significant differences between means ($P \leq 0.05$) according to Tukey's test.
HPLC, high performance liquid chromatography; Ca, calcium; Cu, copper; SA, salicylic acid.
*Each value means of three replicates.

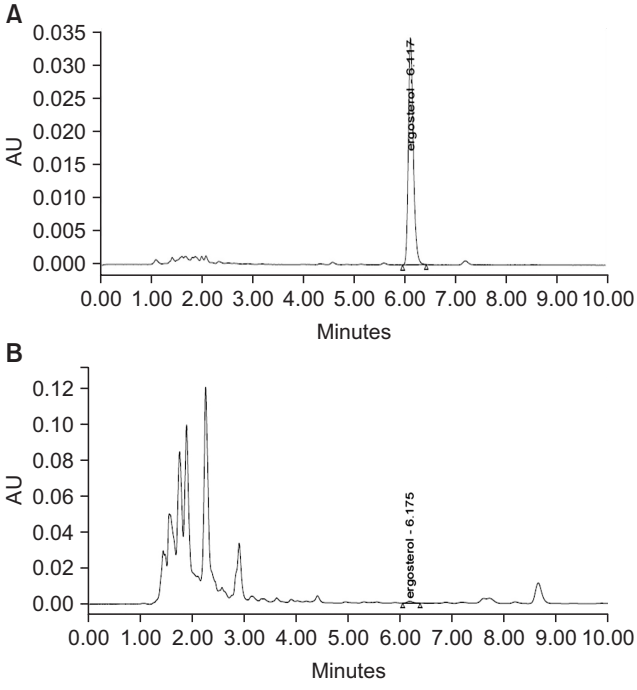

Fig. 2. Comparison of retention time between pure ergosterol standard (positive control) (A) and asymptomatic oil palm sample (negative control) (B) by using high performance liquid chromatography.

layer, a primary and secondary walls. The primary cell wall is a thin layer developed by cell division and later grow into xylem mother cells, while the secondary wall is a thick layer posited within the primary wall as noted in this study (Fig. 3). The middle lamellae represented a clear transition to the neighbouring primary wall layer. Random measurements in the T9 treatment showed that the thickness of secondary cell wall and middle lamellae were in the range of 1.47 to 1.76 μm and 0.15 to 0.29 μm, respectively. However, it was recorded in T7 treatment from 1.64 to 2.07 μm and 0.21 to 0.31 μm, respectively. Similarly, T8 treatment showed penetration of the outer barrier layers by *G. boninense*. The colonization of this pathogenic fungi occurred mainly through the inner thin walled cortex (Fig. 4). During this event the cell wall degraded at various places and the fungal hyphae invaded through these entries and infect the cortical region of the host plant.

Effect of Ca, Cu, and SA supplementation on plant vigour. The results revealed that the treated seedlings showed positive responses in terms of increment in plant height (Table 7); however, the magnitude of growth rate varies among tested treatments. T7 treatment showed the highest growth rate, while T8 treatment had lowest growth rate at 9MAI. Applications of Ca, Cu, and SA alone or in combination remarkably increased the plant height at $P < 0.05$. A similar trend was also observed for the growth rate of seedling girth (Table 8). The T7 treatment showed the highest increment in the plant girth throughout the experimental periods followed by T5, T1, T6, T3, T4, T2 treatments. However, a noticeable decreased in the growth rate of plant girth from 6MAI was observed in T8 treatment at $P < 0.05$, but later no increment was recorded at 8MAI and 9MAI. Moreover, our findings suggested that T7 treatment has highest fresh root biomass at 9MAI followed by T5, T1, T6, T3, T2, T4, T9, and T8 (Fig. 5).

Discussion

Artificial inoculation of plant by contact with the inoculum block carrying *G. boninense* is the most efficient strategy for inducing the infection in oil palm. Immunization by using Ca/Cu/SA (T7) was found able to suppress

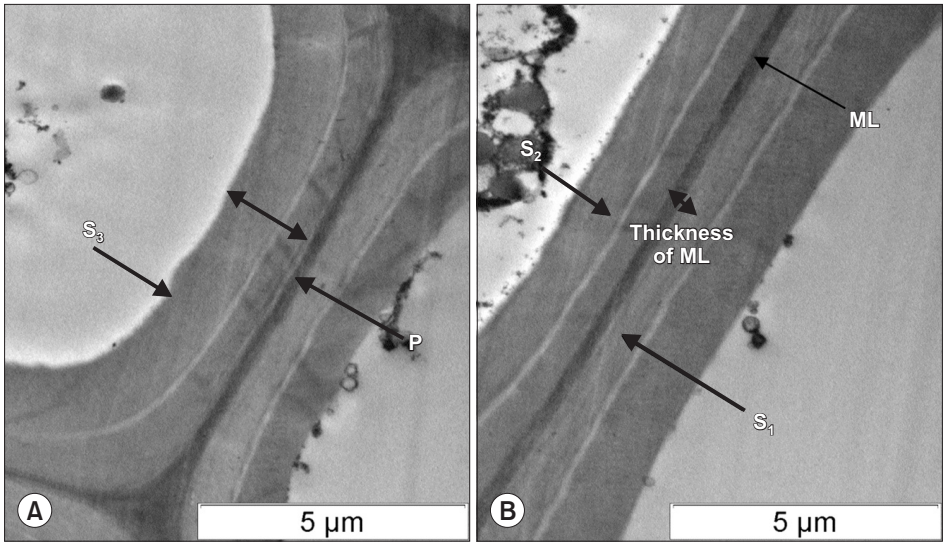

Fig. 3. Transmission electron micrograph of ultrathin sections of oil palm seedlings cell wall after being stained with uranyl acetate and lead citrate at a magnification (× 6,000). (A) T9, healthy control. (B) T7, Ca/Cu/SA tissues. Ca, calcium; Cu, copper; SA, salicylic acid; S_1, S_2, and S_3, secondary wall sub layers; P, primary wall; ML, middle lamellae.

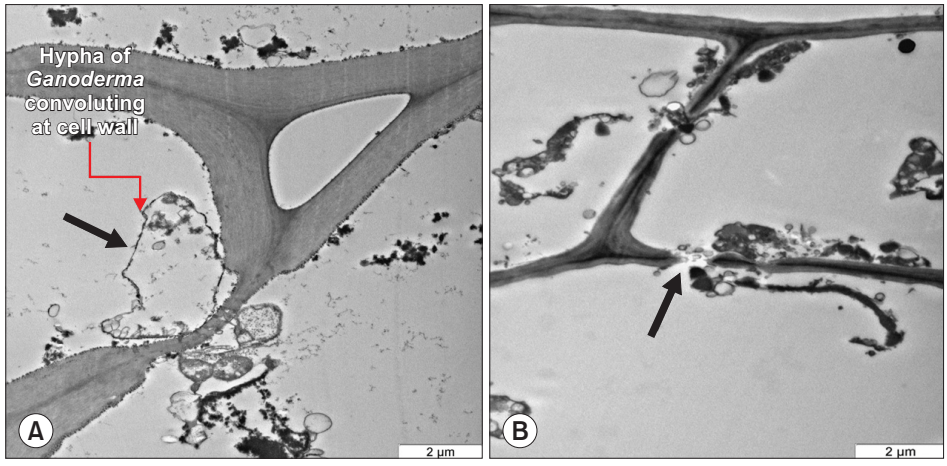

Fig. 4. Transmission electron microscopy of cell wall degradation by *Ganoderma boninense* during infection in root tissues of T8. (A) *Ganoderma* hyphae convoluting plant cell wall (arrows). (B) Cell wall degraded severely by *G. boninense* (arrow).

Table 7. Changes of growth rate on the seedling height was assessed from 2 to 9 MAI

Treatment	Changes of growth rate (cm)							
	2MAI	3MAI	4MAI	5MAI	6MAI	7MAI	8MAI	9MAI
T1 (Ca)	4.6^{ab}	9.0^{a}	15.1^{ab}	21.9^{abc}	33.9^{a}	35.2^{abc}	34.8^{ab}	39.9^{a}
T2 (Cu)	3.9^{ab}	8.9^{a}	14.4^{ab}	19.9^{d}	30.8^{a}	29.4^{a}	27.2^{cd}	20.6^{b}
T3 (SA)	4.1^{ab}	8.9^{a}	14.5^{ab}	21.1^{bcd}	34.1^{a}	33.9^{abc}	31.9^{abc}	34.2^{ab}
T4 (Ca/Cu)	4.4^{ab}	8.9^{a}	14.5^{ab}	20.4^{cd}	31.5^{a}	30.2^{bc}	28.3^{bc}	22.8^{b}
T5 (Ca/SA)	4.6^{ab}	9.2^{a}	15.6^{ab}	22.7^{ab}	35.3^{a}	36.4^{ab}	36.4^{a}	41.6^{a}
T6 (Cu/SA)	4.3^{ab}	8.9^{a}	14.8^{ab}	22.1^{abc}	34.5^{a}	35.4^{abc}	35.1^{a}	39.2^{a}
T7 (Ca/Cu/SA)	4.9^{a}	9.5^{a}	16.0^{a}	23.5^{a}	36.4^{a}	37.6^{a}	37.9^{a}	44.4^{a}
T8 (positive control)	3.2^{b}	7.7^{a}	12.4^{b}	15.0^{f}	9.2^{c}	4.4^{e}	1.2^{e}	0^{c}
T9 (negative control)	3.2^{b}	7.8^{a}	13.3^{ab}	17.0^{e}	21.3^{b}	22.5^{d}	20.5^{d}	22.8^{b}

Different letters in the same column indicate significant differences between means ($P \leq 0.05$) according to Tukey's test.
MAI, months after inoculation; Ca, calcium; Cu, copper; SA, salicylic acid.

Table 8. Changes of growth rate on seedling girth were assessed from 2 to 9 MAI

Treatment	Changes of growth rate (mm)							
	2MAI	3MAI	4MAI	5MAI	6MAI	7MAI	8MAI	9MAI
T1 (Ca)	5.2[a]	6.7[ab]	12.2[bc]	13.9[ab]	16.8[ab]	16.5[a]	21.4[ab]	23.5[ab]
T2 (Cu)	3.3[a]	4.4[b]	7.6[def]	7.8[cd]	10.2[cde]	13.1[ab]	13.2[b]	12.1[d]
T3 (SA)	4.8[a]	8.5[ab]	9.7[bcd]	9.0[cd]	12.5[cd]	16.6[a]	15.8[ab]	16.0[bcd]
T4 (Ca/Cu)	3.8[a]	4.9[ab]	8.9[cde]	8.7[cd]	11.9[cd]	16.8[a]	15.2[ab]	13.5[cd]
T5 (Ca/SA)	5.8[a]	8.0[ab]	13.7[ab]	14.3[ab]	18.4[a]	19.8[a]	22.7[a]	24.3[ab]
T6 (Cu/SA)	4.6[a]	5.5[ab]	9.1[cde]	11.1[bc]	13.9[bc]	19.2[a]	19.9[ab]	21.7[abc]
T7 (Ca/Cu/SA)	6.6[a]	9.4[a]	16.4[a]	18.0[a]	19.6[a]	21.8[a]	24.6[a]	27.2[a]
T8 (positive control)	3.7[a]	3.4[b]	4.6[f]	6.4[cd]	6.3[e]	4.1[b]	0.0[c]	0.0[e]
T9 (negative control)	3.0[a]	4.4[b]	5.2[ef]	5.7[d]	8.9[de]	11.1[ab]	12.4[b]	11.7[d]

Different letters in the same column indicate significant differences between means ($P \leq 0.05$) according to Tukey's test.
MAI, months after inoculation; Ca, calcium; Cu, copper; SA, salicylic acid.

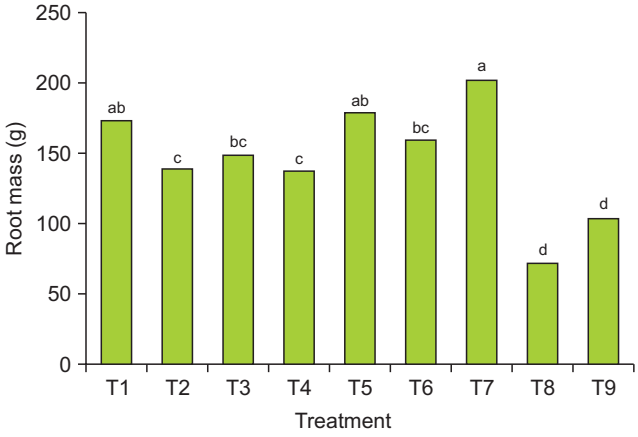

Fig. 5. Effect of calcium (Ca), copper (Cu), and salicylic acid (SA) on the root mass of oil palm seedling was assessed 9 months after inoculation. Different letters above each bar indicate significant differences between means ($P \leq 0.05$) according to Tukey's test. T1, Ca; T2, Cu; T3, SA; T4, Ca/Cu; T5, Ca/SA; T6, Cu/SA; T7, Ca/Cu/SA; T8, positive control; T9, healthy control.

BSR disease development significantly in the glasshouse trials. A similar result of trials on the efficacy of T7 was determined in *in vitro* study as reported by Rahamah Bivi et al. (2012). Mineral nutrients are significant elements for the growth of plants as well as other microorganisms and they also play crucial parts in disease control (Agrios, 2005). Similarly, plant growth regulator (SA) was subsequently found to play significant roles in inducing pathogenesis related genes expression or resistance to fungal, bacterial and viral infection in many plant species (Vlot et al., 2009).

Findings of this study suggested that continuous supplementation of Ca/Cu/SA could enhance disease resistance in oil palm and also suppress the growth of *G. boninense*. The reduction of %DSI in the oil palm seedlings pretreated with Ca/Cu/SA before and after challenging with *Ganoderma* suggest that the mineral nutrients and plant growth regulator could play important roles in limiting the penetration of *Ganoderma* infection into plant tissues. This was supported by observation in TEM which showed Ca/Cu/SA promoted lignification processes in the cell wall, which thickened the secondary cell walls and middle lamella compared to untreated plant. It can be concluded that cell wall tissues of the treated oil palm seedling are modified and this maybe the possible answer on why the treated seedlings are more resistant towards mechanical pressure used by pathogens throughout the penetration process and also more water resistant, thereby less accessible to cell wall degrading enzymes. Internal examination of infection of *G. boninense* revealed that root infection occurred subsequent to firm attachment of *Ganoderma* hyphae to the root surface. The attachment was either localised to the initial point of contact or sometimes the fungus completely enclosed the root at the point of contact. In T8, cell wall tissues are severely degraded with hyphae, but often at some distance from invading point. However, in treated samples, their secondary walls and middle lamella developed more thickly and this resulted in less damaging of the cell wall tissues as the lignification process was enhanced.

Lignification is a process whereby plant cell walls are sealed by lignin deposition (Grabber et al., 1998). It is one of the mechanisms of disease resistance in plants (Nicholson and Hammerschmidt, 1992; Vance et al., 1980). This process take place when the phenolic monomer unit polymerize, producing radicals and conjugating them with other monomer radicals. Lignification involves three

main phases, firstly lignin compounds accumulate around middle lamellae, otherwise known as cellulose microfibrils (CMLs) and cell corners with deposits of pectate and arabinose-galactose rich in hydrocarbons (HCs). Subsequently, CMLs and HCs are deposited in secondary cell wall and finally, majority of polysaccharides are deposited (Fukushima and Terashima, 1991). Therefore, lignification is considered as the first line defence against successful penetration of invading pathogens. Lignification provides the cell walls to be more resistant to chemical injuries (Grabber et al., 1998) and mechanical pressure applied throughout penetration by fungal aspersoria, as well as more water resistance and therefore less accessible to cell wall-degrading enzymes (Nicholson and Hammerschmidt, 1992; Vance et al., 1980; Zeyen et al., 2002). Similarly, Mazza et al. (2000) reported that lignification could restrain the growth of fungus and inhibits fungal proliferation. There are two types of lignin present in plant cell walls i.e., guaiacyl lignin and guaiacyl-syringl. Guaiacyl lignin is less susceptible to biological degradation and chemical breakdown. On the other hand, guaiacyl-syringlis is more resistant to biological degradation. Moreover, Ca is a structural component of cell walls and plays a crucial role in the integrity and function of the structure. Nur Sabrina (2011) reported that continuous supplementation of Ca and Cu could significantly improve lignification in oil palm root. A shortage of Ca results in plant structures which are susceptible to infection of *G. boninense*. This finding is in agreement with the findings of Sariah et al. (1998) and Sariah and Zakaria (2000), which have also shown that continuous calcium nitrate supplementation can suppress BSR symptoms on oil palm.

Continuous supplementation of Ca/Cu/SA after being inoculated with *G. boninense* may also be a possible key to enhance disease resistance in oil palm. This was proven in this study whereby %DSI of T7 was lower up to 5.0% at 9MAI compared to T8 of 58.3% at the same assessment time. Furthermore, PDR of 81.0% was recorded for T7. The external symptoms and internal symptoms of oil palm seedlings on T7 shows they are not much affected. This event can be explained by significant reduction of esgosterol activities of *G. boninense* in the treated samples as detected by HPLC. Ergosterol concentration is successfully used as bioindicator of fungal biomass in correlation with total fungal hyphal length and living fungal hyphal length in soil (Stahl and Parkin, 1996). The positive effects of Ca/Cu/SA on plant growth are always correlated with a remarkable increase in the root morphology, such as lateral root length, root hair number, and also shoot length. Hence, application of Ca/Cu/SA supported a great increase in plant growth and reduced the %DSI. When a plant is infected by a pathogen it is physiologically impaired, particularly in relation to nutrient uptake, translocation, assimilation from the root to the shoot and utilization (Marscher, 1995). Plant hormone syntheses have been shown to induce root growth. The level of nutrients can regulate the plant growth, which can affect the microclimate, thus affecting infection and sporulation of the pathogen (Marscher, 1995). In this study, Ca/Cu/SA significantly increased the seedling plant growth and root biomass.

The use of ENPs to minimize the deleterious effects of huanglongbing (HLB) disease has been a hot topic of discussion worldwide (Tian et al., 2014). According to Gottwald et al. (2012) revealed that ENP conducted in Florida do not sustain tree health, yield or fruit quality of HLB-infected plants. Similar findings was reported by Razi et al. (2011), which shows no relationship between nutritional deficiency status and HLB incidence in citrus and Zn was significantly higher in HLB-infected trees, even though ENP provide minor improvement for HLB-infected tree. However, according to Pustika et al. (2011) showed that by application of foliar fertilizer containing N and minerals, are able to reduce 40% symptom expression of HLB infected trees. On the other hand, fertilizers applied through soil did not show any improvement. This finding showed that foliar application might prolong tree life and reduce yield loss. Study by Webb (2006) also showed positive effect of ENP, when Zn or Cu ions in combination with Ca delayed DI and severity. Findings similar to ours were reported on rice, according to Rahimizadeh et al. (2007), the application of micronutrients fertilizer in rice field increases the activities of enzymes in the plants. Findings by Liew et al. (2010) showed application of K (29.9 kg/ha), Mg (2.4 kg/ha), Zn (4 kg/ha), Cu (4 kg/ha), Mn (3.6 kg/ha), and B (0.25 kg/ha) managed to enhance rice production by 27% from 4,620 to 5,870 kg/ha. This field trial was conducted for two seasons at Sawah Sempadan, Tajung Karang, Malaysia. Moreover, the area has been experiencing low rice yields with mean production being 4,500 kg/ha with severe infection of brown spot and sheath blight diseases. Finding of this study was parallel with Zayed et al. (2011) where application of micronutrients significantly improved harvest index. A field study conducted in Kelantan, Malaysia, using Zn (5 kg/ha), Cu (4 kg/ha), and Mo (0.5 kg/ha) along with recommended N, P, K, and S at 120 kg/ha, 30 kg/ha, 50 kg/ha, and 10 kg/ha, respectively managed to improve rice plant growth, rice yield, grain quality and antioxidant activity (Panhwar et al., 2015). He also reported that combined application of micronutrients particularly Cu and Zn would increase phenolic compound and flavonoid content in rice grain by 40% and 71.4%, respectively. In

addition, recent studies have shown the impact of metal ions towards the activity of mitochondria cells by inducing the oxidative stress on metal binding proteins (Tan et al., 2010).

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