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Suppression of Basal Stem Rot Disease Progress in Oil Palm (*Elaeis guineensis*) after Copper and Calcium Supplementation

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ABSTRACT

The effects of copper and calcium nutrient supplementation on basal stem rot disease caused by *Ganoderma boninense* were evaluated in oil palm during a 6-month glasshouse study. Nutrients were supplemented when seedlings were at the 2- to 3-leaf stage. The aim of the study was to assess the effects of copper and calcium on the suppression of basal stem rot. Nutrient supplementation, with copper at 2.0 mg/L and calcium at 4000 mg/L alone or in combination, significantly reduced *G. boninense* infection in oil palm roots. The treated seedlings did not escape the disease, but it developed more gradually than in the untreated seedlings. The seedlings supplemented with a combination of calcium and copper remained free from any symptom for a longer period and developed the disease at a later stage than the controls. The supplementations of calcium and copper could have triggered oil palm mechanism of resistance by enhancing the production of peroxidase and lignin during fungal penetration. These findings suggested that copper and calcium supplementations could be used to reduce the severity of basal stem rot in oil palm.

Keywords: Copper, Calcium, Basal stem rot, *Ganoderma boninense*, *Elaeis guineensis*

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INTRODUCTION

Oil palm is of major economic importance in southeast Asia, where it is grown extensively in Malaysia and Indonesia for the production of vegetable oil used in foods, cosmetics, and biodiesel. Nonetheless, oil palm is

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vulnerable to various diseases, among which basal stem rot (BSR) which is caused by *Ganoderma*, is of particular significance, e.g., to Malaysia (Paterson *et al*., 2008). *Ganoderma boninense*, the causal pathogen of BSR disease, is a white rot basidiomycetous fungus. The fungus is spread by root contact between infected and healthy tissues. The pathogen that remains in the soil infects oil palm primarily through the roots and degrades the lignin component of wood, leaving white cellulose exposed. For this reason, it is called white rot fungus (Paterson, 2007). Eventually, the degradation of lignin will weaken the tree and make it susceptible to wind damage. Based on an understanding of the mode of infection of *G. boninense* on oil palm, the ideal solution to slow the emergence of basal stem rot disease is to enhance oil palm's natural defences through lignin manipulation resulting from the administration of plant nutrients.

Lignin is believed to be one of the products of the evolution of phenol metabolism. More pecifically, it is considered as a product of phenylpropanoid pathway. This secondary metabolic pathway is hypothesised to be important in the adaptation of plants to land. It is considered to provide several benefits, including protection against UV light and pathogens (Mazza *et al*., 2000). Peroxidase (POD) within the cell wall has been shown to be involved in monolignol polymerisation. Therefore, it appears to participate in lignification (Fry, 2004). Laccase is responsible for the early stage of lignin biosynthesis. Subsequently, laccase and POD function cooperatively in lignin biosynthesis (Lin *et al*., 2005) and become important enzymes during lignification. Copper (Cu) has been reported to be involved in the enhancement of peroxidase in rice leaves (Fang & Kao, 2000), lignin biosynthesis in soybean roots (Lin *et al*., 2005), and enhancement of peroxidase activity and lignin content in *Raphanus* (Chen *et al*., 2002). Cu in low concentrations may not cause significant damage to cell membranes but it may also lead to accumulation of peroxide compounds (Weckx & Clijsters, 1996). In contrast, calcium (Ca) augmented soluble POD activity (Kolupaev *et al*., 2005) or reduced phenolic compounds (Ruiz *et al*., 2003). Meanwhile, deposition of Ca pectate is crucial for the plant to undergo lignification, which later provides a barrier against pathogen attack (Willats *et al*., 2001). In view of these findings, this study was conducted to evaluate the effects of Cu and Ca on the suppression of *G. boninense* infection in oil palm seedlings.

MATERIALS AND METHODS

Planting Materials and Experimental Design

Three-month-old oil palm seedlings were used for the infection studies. The seedlings were DxP crosses supplied by Sime Darby Sdn. Bhd., Banting, Selangor. The seedlings were grown in 12 x 15 cm white polythene bags in standard plantation soil, mixed with river sand in a ratio of 7:3. In Cu and Ca supplementations, 2 mg/L Cu (as $CuSO_4.5H_2O$) and 4000 mg/L Ca (as $CaNO_3$)

were amended into the basal fertilizer (Table 1). These concentrations were chosen as the optimum levels of Cu and Ca required for oil palm under field conditions (Goh & Hardter, 2000). Each supplementation was replicated five times (with four seedlings per replicate) and arranged in a Completely Randomised Design (CRD). The seedlings were watered twice daily, and 7g 15/6/4 NPK fertilizer per seedling was applied once a month to serve as basal fertilizer (BF). The seedlings received Cu and Ca supplementations at monthly intervals for three months.

TABLE 1: Treatments with Cu and Ca applied to oil palm seedlings inoculated with *G. boninense*

Treatment	Descriptions
T1	Control *(BF)
T2	BF + 4000 mg/L $CaNO_3$
T3	BF + 2 mg/L $CuSO_4$
T4	BF + 4000 mg/L $CaNO_3$ + 2 mg/L $CuSO_4$

**Basal Fertilizer*

Preparation of Ganoderma Inoculum on Rubber Wood Blocks

Rubber wood blocks measuring 12 cm x 12 cm x 12 cm were obtained from Kilang Kayu Getah Wah Heng Sdn. Bhd., Semenyih, Selangor. These blocks were sterilised (121°C; 1.04 kg cm^{-2} pressure) for 30 min. The blocks (one block per bag) were placed in heat-resistant polypropylene bags (15 cm x 33 cm x 0.05 mm thick material), and 100 mL of molten malt extract agar (MEA) was added as a supplementary nutrient for *G. boninense*. The bags, containing a rubber wood block and molten MEA, were autoclaved at 121°C for 30 minutes. After sterilisation 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. After the agar had been solidified, the rubber wood blocks were inoculated with 14-day-old *G. boninense* culture at a half plate per block and incubated for four weeks under dark conditions at room temperature (28 ± 2°C) until they were fully colonised with *G. boninense*. The *G. boninense* isolate was obtained from a basidiomata of an infected oil palm trunk growing in Banting, Selangor, Malaysia. Ganoderma-selective medium (Ariffin & Idris, 1991) was used to obtain the isolate. The identification was confirmed based on spore morphology and cultural characteristics.

Inoculation of Oil Palm Seedlings with G. boninense-*infected Rubber Wood Blocks*

Three months after Cu and Ca supplementation, the seedlings were challenged with *G. boninense*-colonised rubber wood blocks placed in contact with the roots (Sariah *et al*., 1994). The plant-cum-inoculum was placed in a polythene bag filled with one-third soil mixture (3:2:1 v/v/v topsoil:peat:sand). More soil (10 kg) was then added to cover the roots and inoculum. The progress of colonisation over 6 months was monitored based on the development of the macromorphological symptoms of BSR.

Disease Assessment

Disease development was monitored monthly by measuring the percentage of Disease Incidence (DI). The DI is the number of visibly diseased seedlings (chlorosis and necrosis of leaves, with or without sporophore production) relative to the total number of seedlings and is assessed using the following formula (modified from Campbell & Madden, 1990):

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

A reduction in the disease incidence compared with the control would be a measure of the effectiveness of the treatment in suppressing the disease. This value was assessed by plotting the data in the form of a disease progress curve. The Area under the Disease Progress Curve (AUDPC) was calculated using the following formula from Campbell and Madden (1990):

$$AUDPC = \sum_{i}^{n-1} \left(\frac{Y_i + Yi_{i+1}}{2} \right) \left(t_{i+1} - t_i \right)$$

where n is the number of assessment times, Y is the disease incidence and t is the observation time. The slope of the curve was obtained by transforming the DI data with the monomolecular model (Monit) in Campbell and Madden (1990).

The development of disease in the seedlings was also rated in terms of Disease Severity (DS). DS refers to the total area or volume of plant tissue that is diseased (Kranz, 1988). DS is calculated based on the symptoms appearing on the foliage, root and bole on a 0-4 assessment scale (modified from Breton *et al*. 2005), as follows:

0 = Healthy; 1 = Yellowing of lower leaves and formation of rhizomorph at base of bole; 2 = Necrosis of lower leaves and emergence of button-like sporophore at the base of bole; 3 = > 50% necrosis of leaves and production of sporophore at the base of bole; 4 = Total necrosis and production of sporophore at the base of bole.

The DS for the foliar symptoms was calculated using the formula derived from Liu *et al*. (1995), as follows:

$$DS_{(external)} = \frac{\text{Number of seedlings in the rating} \times \text{rating number}}{\text{Total number of seedlings assessed} \times \text{highest rating}} \times 100$$

At the end of the experiment (i.e. 6 months after the seedlings were inoculated with *G. boninense*), the internal symptoms were recorded. The seedlings were uprooted carefully, split longitudinally and visually assessed for the severity of the internal symptoms based on the rating of the bole-tissue damage produced by *G. boninense*. This assessment was based on the following scale (modified from Breton *et al*., 2005):

0- Healthy; 1- Up to 20% rotting of bole tissues; 2- From 20% to 50% rotting of bole tissues; 3- Over 50% rotting of bole tissues; 4- Over 90% rotting of bole tissues.

Meanwhile, Disease Severity (DS) for the internal symptoms of the bole tissues was calculated based on the following formula derived from Liu *et al*. (1995):

$$DS_{(internal)} = \frac{\text{Number of seedlings in the rating} \times \text{rating number}}{\text{Total number of seedlings assessed} \times \text{highest rating}} \times 100$$

Statistical Analysis

Statistical analysis was performed using SAS (version 9) software. The significance of the differences between means was determined with a one-way analysis of variance (ANOVA) at 95% confidence level. Products with the same letter have no significant difference with $p>0.05$.

RESULTS AND DISCUSSION

The external symptoms of BSR infection were observed. These symptoms included the progressive yellowing of the lower leaves, the subsequent desiccation from the oldest to the younger leaves, and the rapid development of the sporophore, which assumed a button-like form. The symptoms followed the typical pattern of infection, which had previously been described by Sariah *et al*. (1994) (see Fig.1).

The Disease Incidence (DI) based on foliar symptoms in seedlings pre-supplemented with Cu, with Ca and with a combination of Cu and Ca progressed more slowly than in the control. The appearance of disease symptoms between treatments

Fig.1: Progressive development of the BSR symptoms in Ganoderma-inoculated oil palm seedlings; (A) yellowing of lower leaves, (B) dessication of lower leaves, (C) extensive necrosis, and (D) dead seedling with sporophore production.

was observed 3 months after the inoculation of the seedlings with *G. boninense*. Disease suppression is indicated by a lower DI value. Three months after the inoculation of the seedlings with *G. boninense*, the DI was found to be 0% for the seedlings pre-supplemented with Treatment 4 (a mixture of 2 mg/L Cu and 4000 mg/L Ca). This indicated that the seedlings had achieved partial or complete disease suppression (Fig.2). Meanwhile, the yellowing of the leaves was observed 4 months after the inoculation of the seedlings. The formation of a sporophore was only observed 6 months after the inoculation of the seedlings. At the end of the experiment, the DI was only 28.3%. This result suggested that the combined supplementation of Cu and Ca produced a good level of disease suppression.

The seedlings pre-supplemented with 4000 mg/L Ca alone (Treatment 2) and 2 mg/L Cu alone (Treatment 3) also showed a DI reduction relative to the control. However, the amount of disease suppression was less than that produced by Treatment 4. At six months after the inoculation of the seedlings with *G. boninense*, the DI values for Treatment 2 and Treatment 3 were 43.3% and 48.5%, respectively. These values were not significantly different. As expected, the control seedlings (Treatment1) recorded the highest DI of 88.3% six months after they had been inoculated with *G. boninense*. Although the foliar symptoms in the infected seedlings were clearly distinguishable, foliar symptoms could not indicate the extent of damage to the roots and in the bole region.

The Cu and Ca supplementation was considered effective in suppressing the onset of BSR infection because the DS was significantly reduced. The DS, assessed from foliar symptoms, developed rapidly in the absence of Cu and Ca supplementation and reached a level of 4 (on a 0-4 scale) in the control 6 months after the inoculation of the seedlings with *G. boninense*. At 6 months after they had been inoculated with

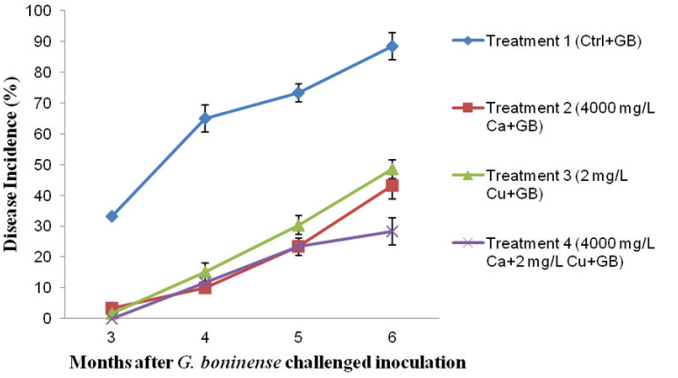

Fig.2: Basal Stem Rot Disease incidence (DI) on *G. boninense* inoculated oil palm seedlings based on chlorosis and necrosis of leaves, with and without production of sporophore. Values are the means of five replicates. Bars represent standard deviation.

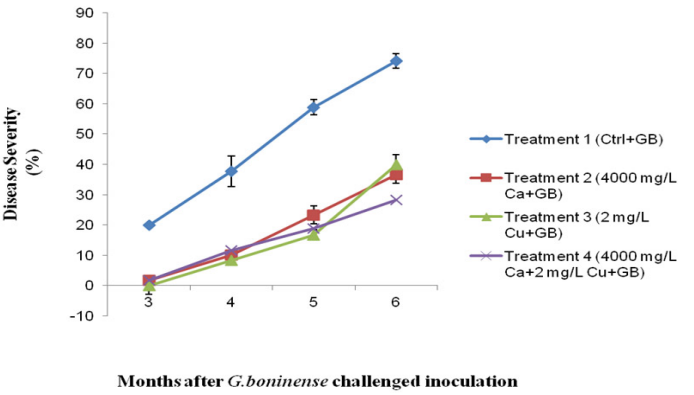

Fig.3: Disease severity (DS) over time observed on *G. boninense* inoculated oil palm seedlings based on foliar symptoms. Values are the means of five replicates. Bars represent standard deviation.

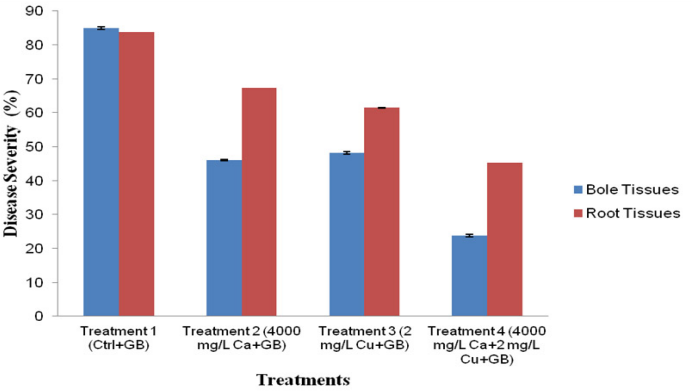

Fig.4: Disease severity (DS) observed on root and bole tissues of oil palm seedlings 6 months after *G. boninense* inoculation. Values are the means of five replicates. Bars represent standard deviation.

G. boninense, the seedlings in Treatment 2 (4000 mg/L Ca) had an external DS of 36.7% (DS scale ≥ 2). Treatment 3 (2 mg/L Cu) showed a DS of 40.0% (DS scale ≥ 2), and Treatment 4 (mixture of 2 mg/L Cu and 4000 mg/L Ca) showed a DS of 28.3% (DS scale ≥ 1). In contrast, Treatment 1 (control) showed a DS of 74.2% (DS scale ≥ 3) (Fig.3) at six months after the inoculation of the seedlings with *G. boninense*.

Destructive sampling was carried out at the end of the experiment (i.e. 6 months after the inoculation of the seedlings with *G. boninense*) to assess the extent of root rot and bole decay. The highest level of root rot and decay, with extensive colonisation of fungal masses on the root surface, was observed in Treatment 1 (control), in which 83.8% of the primary roots showed brown discolouration as compared to Treatment 2 (67.3%), Treatment 3 (61.5%) and Treatment 4 (45.3%). Meanwhile, the longitudinal sections of the infected boles showed brown lesions marked by an irregular, darker zone.

The disease severity, based on the extent of lesions in the bole, was also affected by Cu and Ca supplementation (Fig.4). In the control seedlings, the severity was significantly higher (87.0%) than in the supplemented seedlings. The seedlings that were pre-supplemented with the combination of Cu and Ca (Treatment 4) showed the lowest percentage of disease severity (23.8%). Treatment 2 (46.1%), with 4000 mg/L Ca, did not differ from Treatment 3 (48.2%), with 2 mg/L Cu. However, both the treatments differed significantly from the control. These results suggest that Treatment 4 effectively slowed the penetration and spread of *G. boninense* to the vascular system.

The Area under the Disease Progress Curve (AUDPC) is a quantitative summary of the disease intensity over time and can be used to compare management strategies. The percentage of disease reduction (%DR) was derived from the values of the Area under Disease Progress Curve (AUDPC), as shown in Table 2. AUDPC was calculated based on the DI or DS of the foliar symptoms or the DS of the bole tissues. Higher AUDPC values indicated a greater susceptibility of the seedlings to the disease. Seedlings pre-supplemented with 4000 mg/L Ca + 2 mg/L Cu (Treatment 4) showed the lowest AUDPC values for DI (49.17), DS_{foliar} (48.06) and DS_{bole} (489.09). Therefore, the mixture of Cu and Ca was the most effective in slowing down the infection of *G. boninense* because it also showed the highest percentage of disease reduction based on DI (71.67%), DS_{foliar} (71.66) and DS_{bole} (76.17) as compared to the other treatments. In addition, it also showed the lowest epidemic rates based on DI (0.545 unit/month) and DS_{foliar} (0.548 unit/month).

The lower DI%, DS%, AUDPC and ER and higher values of DR in the seedlings pre-supplemented with Ca and Cu singly or as a mixture (Treatment 2, Treatment 3 and Treatment 4) relative to the control suggested that the nutrient supplementation had caused the former seedlings to acquire a level of tolerance to the physical damage caused by *G. boninense*. In this study, the combination of Cu and Ca supplementation yielded the best disease control of all the supplementation treatments. Disease suppression has been associated with the activation of plant defence mechanisms such as the induction of phenolic compounds (Gross, 1980), the presence of laccase and POD and the alteration in physical barriers, where the latter is associated with the formation of lignin (Walter, 1992) and suberin (Espelie & Kolattukudy, 1986) in roots. The result of the current study has also shown that the presence of H_2O_2 and the enzymatic activities of PODs and laccases are strongly correlated with the onset of lignification in the roots of oil palm seedlings that were treated with Cu and Ca (Nur Sabrina *et al*., 2012).

Cu has been reported to influence many plant diseases, primarily by decreasing the spread of the disease (Evans *et al*., 2007). A study by Chmielowska *et al*. (2009) showed that pepper plants stressed by Cu were less symptomatic if challenged with *Verticillium dahlia*; wilt pathogen. In addition, their

study also showed that plants stressed by Cu had fewer numbers of wilted leaves and lower reduction in the length of stem, which were the symptoms of Verticillium disease, than plants which were not supplemented with Cu.

Meanwhile, Cu plays an essential role in photosynthesis, respiration, antioxidant activity, cell wall metabolism, hormone perception (Pilon *et al*., 2006) and the induction of POD (Ros Barceló, 1995). POD is known to be induced by both abiotic and biotic stresses, including heavy metal stress and pathogen attack (Passardi *et al*., 2005). POD may play several roles in the plant, such as the functions related to resistance to pathogens. In addition, POD can produce massive amounts of reactive oxygen species (oxidative burst) that are involved in plant cell signalling and that also create a highly toxic environment for pathogens. Moreover, POD is involved in the deposition of materials such as lignin and suberin, which strengthen the cell wall by forming a mechanical barrier against pathogenic agents. A higher value of DR% in the plants pre-supplemented with Cu, singly or in combination, suggested that Cu plays an important role during lignification by producing laccase and POD, which later act as an important substrate for monolignol polymerisation. These lignified cell walls subsequently serve as a barrier to *G. boninense* penetration. It has previously been demonstrated that the induction of POD activity in pepper by Cu stress is related to lignin accumulation (Diaz *et al*., 2001) and that lignification confers tolerance to *Verticillium dahlia* in pepper plants (Pomar *et al*., 2004). Finally, another possible role for Cu-induced POD in plant defence is a direct and intrinsic antifungal activity, which has been reported for POD from several plant sources (Caruso *et al*., 2001; Ghosh, 2006). Even though Cu is often used as an active ingredient in fungicide, in this study, there was no direct assessment made on the growth of Ganoderma as monitoring was carried out on disease development on inoculated plants.

Ca significantly suppressed disease incidence and delayed the onset of Phytophtora stem rot in soybean (Sugimoto *et al*., 2008). These results indicate that Ca-rich areas may be more resistant to invasion by *P. sojae* and that calcium crystals may play an important role in Ca ion storage and its availability to allow the plant tissues to maintain long-term field resistance. In fact, Ca may harden plant primary cell walls by cross-linking of pectic polymers and confer resistance to pathogen attack (Akai & Fukutomi, 1980). For lignification to be successful, the middle lamella and the cell wall corners must be rich in calcium pectate because these regions are the primary sites of lignification (Lewis & Sarkanen, 1999). Our preliminary observations showed that Ca supplementation increased the production of the lignin-related enzymes POD and laccase and thus amplified lignin production. Sufficient Ca content decreases the rate of breakdown in pathogenic disease so that when *G. boninense* attempts to breach and invade the plant cell, the barrier reinforces the wall. This effect can be

observed if the DR% is higher in plants pre-supplemented with Ca singly or in combination. Furthermore, Ca naturally slows the reactions involving pathogenic enzymes during cell decomposition. The beneficial effects of Ca also include the improvement of the structure of the soil, the stabilisation of the cell membranes, and an increase in the pH of the soil. These effects decrease the probability of *G. boninense* attachment.

CONCLUSION

The interactions between Cu and Ca have profound effects that serve to suppress disease progression in oil palm seedlings. The suppression of disease, as indicated by the observations of DI, DS, AUDPC, and ER, showed that the mixture of 2 mg/L Cu and 4000 mg/L Ca is the best supplementation treatment for slowing the emergence of BSR. However, field studies are still needed to explain the effectiveness of Cu and Ca supplementation against *G. boninense* attack by conducting analysis on the increase of the cell wall components. Moreover, it could be more useful to integrate the use of ergosterol as a tool to measure infection, as proposed by Mohd As'wad *et al*. (2011). There is also a need to determine the best method of application for the management of BSR.

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