DEVELOPMENT OF AN *IN PLANTA* INFECTION SYSTEM FOR THE EARLY DETECTION OF *GANODERMA* SPP. IN OIL PALM

K. M. Goh[a], M. Dickinson[b], P. Alderson[b], L. V. Yap[c], C. V. Supramaniam[a, d]

[a]*School of Biosciences, Faculty of Sciences, The University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor Darul Ehsan, Malaysia.*

[b]*School of Biosciences, The University of Nottingham Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, United Kingdom.*

[c]*Foundation of Sciences, Faculty of Sciences, The University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor Darul Ehsan, Malaysia.*

[d]*Centre of Sustainable Palm Oil Research (CESPOR), The University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor Darul Ehsan, Malaysia.*

Running title: *In planta* infection of oil palm

Corresponding author: Christina Vimala Supramaniam

Fax number: +6 (03) 89248018

Email address: christina.supramaniam@nottingham.edu.my

SUMMARY

Basal stem rot (BSR) disease caused by the white rot fungus, *Ganoderma* spp. is a serious threat to the growth and production of oil palm (*Elaeis guineensis* Jacq.). Traditional *in planta* infection technique using inoculated rubber wood block can be inaccurate and time-consuming. In this study, a new *in planta* infection system was developed to detect early symptoms of BSR in young oil palm. One month old clones of oil palm plantlets were artificially infected with pathogenic fungal inoculum (*G. boninense* GBLS isolate) at three levels of treatments (control, T1; wounded but not infected, T2; wounded and infected, T3) for a period of 8 days. Significant declines in leaf chlorophyll content (from 32.59 to 12.60 SPAD), increases in disease severity index (DSI) values (from 5.56 to 70.37 %) and increased amounts of GBLS DNA (from 0.2 to 116.1 ng μl^{-1}) were progressively detected in T3 as compared to the T1 and T2 plantlets. The internal stem tissues of T3 plantlets were observed to deteriorate gradually from Day 2 post-inoculation (DPI) and were severely colonized and damaged by 8 DPI. The potential defence mechanism of total phenolic content peaked on 6 DPI (3.7 mg g^{-1}) in T3 plantlets and reduced thereafter. The data obtained is consistent with BSR symptoms reported in mature oil palm and is indicative of the reproducibility and reliability of an *in planta* infection system as an effective approach to detect early BSR symptoms in oil palm.

Keywords: Basal stem rot (BSR); real-time PCR amplification; controlled environment; *Elaeis guineensis; Ganoderma boninense*

INTRODUCTION

Oil palm (*Elaeis guineensis*) is a dynamic economic crop that offers good income and is a source of employment opportunity in Malaysia and Indonesia (Carter *et al*., 2007; Song, 2015). The palm oil is an important dietary food and an energy source for people in many developing countries. However, oil palm is highly susceptible to basal stem rot (BSR) disease caused by a white rot fungus known as *Ganoderma* spp. Among different species of *Ganoderma*, *G. boninense* has been identified as the main causal agent of BSR disease in South East Asia countries (Moncalvo, 2000). In severely affected land areas, more than 50 % of oil palm grown will be infected and loss in crop yield can reach up to 80 % after constant and repeat cycles of monoculture (Turner, 1981; Su'ud *et al*., 2007)

G. boninense causes lethal effects in oil palm by degrading the xylem and disrupting the uptake of water and nutrients to other parts of the palm tree. Initial signs of a BSR infected oil palm are identical to those of palms suffering drought condition, where lower leaves will collapse first and droop vertically downwards (Holliday, 1980). Young palms infected with BSR are usually retarded in growth and poor in foliar development (pale green foliage and one sided yellowing of lower fronds) (Kurian and Peter, 2007). Subsequently, the bottom part of the stem will become blackened and the majority of the bole tissues will decay. As the disease develops, fruiting bodies of *G. boninense* will start to emerge and eventually the trunk of an infected palm fractures at the basal region causing the palm to collapse at mid-age.

Artificial infection techniques have been established to advance the understanding of this specific plant-pathogens interaction. Previous artificial infection for BSR was conducted using rubber wood blocks (RWB) (6 cm x 6 cm x 9 cm) inoculated with *Ganoderma* spp. on healthy and injured roots of seedlings (Zainudin and Abdullah, 2008; Mohd As'wad *et al*., 2011; Alizadeh *et al*., 2011; Yeoh *et al*., 2013) or placed in contact with germinated palm oil seeds (Breton *et al*., 2006; Idris *et al*., 2006). Although RWB method has proved the

pathogenicity of *G. boninense* and fulfilled Koch's postulates, it requires the cultivation of the fungal mycelium of *G. boninense* on sterilised and fungal medium-coated wood blocks for a few weeks or months (Naher *et al*., 2011; Yeoh *et al*., 2013). After the colonization of *Ganoderma* spp. the RWB inoculum is placed in contact with primary roots of 6 to 12 month old oil palm in polythene bags, and eventual infection takes place 2 to 6 months post-inoculation.

Although the RWB inoculation technique has been widely used, it can be inaccurate and is time-consuming as it requires a minimum of one month for RWB inoculum to develop and a further 4 to 6 months to determine the symptoms of BSR on seedlings (Sariah *et al*., 1994; Idris *et al*., 2006), with the shortest period of 2 months post-inoculation reported (Kok *et al*., 2013). In addition, this technique can be inaccurate because experiments are conducted in shade houses with artificial external factors such as temperature, humidity and soil microbiota conditions, all of which could be variables affecting the disease. Furthermore, saprophytes are often present in the RWB even after several rounds of autoclaving, resulted in high level of contamination when the RWB is used as the inoculum (Chong *et al*., 2012).

This study investigates a new *in planta* system as a platform for artificial infection. Under controlled axenic environments, one-to-one interactions between plants and compatible pathogens can be evaluated more exclusively. Successful studies on model plants such as *Arabidopsis* (Govrin and Levine, 2000), tobacco (Stukkens *et al*., 2005) and rice (Xu and Hamer, 1996; Foster *et al*., 2003) have been carried out using artificial *in vitro* infection assays to infect host plants with spore cultures of compatible fungal pathogens, and in grape, mycelium plugs of *Armillaria mellia,* a white rot pathogen with a similar pathogen biology as *Ganoderma* spp. were co-cultivated with rootstocks on an agar-based medium in tissue culture boxes (Baumgartner *et al*., 2010).

Preliminary *in planta* infection assays have previously been conducted for 3 weeks using *Ganoderma* spp. (Goh *et al*., 2014). However, the extended time is not suitable to detect early responses to BSR disease development in young oil palms. As reported in the previous study, oil palm plantlets had a higher tendency to wilt and biochemical changes in both control and infected oil palm were not significantly different when measured after 3 weeks of inoculation (Goh *et al*., 2014). This could be due to the fact that the underlying biochemical events in plants are normally triggered rapidly at early stages following fungal infection.

With the need for a better understanding of the infection biology of *G. boninense* and oil palm early defence responses towards this disease, it is important to improve the existing infection system available for oil palm. Hence, the present study aimed to (1) develop an *in planta* infection system to investigate *G. boninense* infection in young oil palm and (2) analyse plant responses in oil palm within 8 days of infection for an efficient detection of early BSR disease development.

MATERIAL AND METHODS

Sources of host plant and fungal inoculum

Oil palm plantlets

One month old post-rooting tissue culture oil palm plantlets (cultivar AA68) were used for *in planta* infection studies. These plantlets were collected from Applied Agricultural Resources Sdn. Bhd. (AAR), Tissue Culture Lab located at Sungai Buloh, Selangor, Malaysia.

Ganoderma boninense

Ganoderma boninense isolate, GBLS is an aggressive isolate of the fungus that was isolated from Lian Seng Oil Palm Estate, Johor, Malaysia. GBLS isolate was confirmed as *G. boninense* by sequencing the ITS region (GenBank KF164430.1). Pure mycelium cultures of GBLS isolate were maintained on potato dextrose agar (PDA; Oxoid, UK) with a subculture prepared every fortnight by transferring a 5 mm^2 plug onto a new PDA plate. Cultures were incubated in the dark, at room temperature (25 ± 2 °C).

In planta infection of oil palm plantlets with G. boninense

Oil palm plantlets were infected with single, 14 day-old GBLS isolate mycelium at three levels of treatments; non-inoculated + non-wounded (T1), non-inoculated + wounded (T2), and inoculated + wounded (T3). T1 plantlets served as the absolute negative control for this experiment. *In planta* infection of oil palm plantlets with *G. boninense* was carried out as illustrated in Figure 1 (A-F). For all treatments, oil palm plantlets were transferred into 72 x 72 x 100 mm^3 sterilized Incu Tissue Culture Jars (SPL, Korea) containing 40 ml full strength

Murashige and Skoog (MS) medium (Duchefa, Netherlands), supplemented with 30 g l^{-1} sucrose and 1 g l^{-1} phytagel (Sigma, USA) at pH 5.8. Meanwhile, stem regions of oil palm plantlets (at approximately 0.5 cm above the crown region, Fig. 1A) were wounded by repeat piercing (5 different spots) with sterilized needles (TERUMO, 18G x 1.5") for T2 and T3 treatments. Lastly, GBLS isolate was inoculated onto wounds on oil palm stems for T3 treatment. GBLS inoculum (approximately 40 x 5 mm^2 from fully grown culture plate) was obtained by scraping the mycelium with a sterilized needle (Fig. 1B-C). The GBLS inoculum was applied onto the wounded region directly (Fig. 1D), before placing individual plantlets onto MS medium (Fig. 1E). The lid of tissue culture jar was tightened (Fig. 1F) and incubated in growth chamber (Conviron, CMP 6010) at 27 °C, 16 hours day light and 50 % relative humidity for 8 days. Plants were arranged using randomized complete block design.

In planta infection studies were conducted using nine replicates per treatment and time point, and the experiment was repeated for three times. Post-inoculation analyses for physiological, biochemical and molecular events of treated oil palm plantlets were conducted at 2 day intervals over 8 days of incubation period. At each time point, sample oil palm plantlets were removed from the culture jars and were subjected to physiological and biochemical assays. Molecular analyses were conducted on sample plants that were frozen in liquid nitrogen and stored at -80 ˚C.

Physiological assessments on oil palm plantlets

Morphology and symptoms on infected plantlets were observed and evaluated. Parameters of plant height, weight, stem diameter, root length and leaf chlorophyll content (using a SPAD-502plus meter, Konica Minolta) were measured for all oil palm plantlets. In addition, images of sample oil palm plantlets were taken using a camera (Panasonic, Lumix LX5). All results

were recorded for disease severity index (DSI). Different classes of disease (Table 1) on treated oil palm plantlets were adopted from a previous study (Izzati and Abdullah, 2008) and were modified by including the standard for SPAD value. Images of oil palm plantlets categorized into each disease class are illustrated in Figure 2.

In order to observe the severity of internal tissue and the extent of decay at the infection site, stem regions were cut into longitudinal slices using a sterilised scalpel. One drop of lactophenol blue dye was placed onto the samples to stain *G. boninense* fungal cell walls. Stained specimens were observed under a light microscope (Nikon, AZ100) using 1x objective lenses and the magnification were adjusted to 10x and 20x. Photographs for longitudinal cross sections of treated plantlets were captured using a camera (Nikon, DS-Ri1 and NIS Element software).

Estimation of total phenolic content in oil palm plantlets

Total phenolic content in oil palm plantlets was estimated spectrophotometrically by adopting Folin's method (Chong *et al*., 2012). In order to estimate total soluble phenolics, a standard curve was prepared by using gallic acid at different concentrations (0.0 mg ml^{-1}, 0.2 mg ml^{-1}, 0.4 mg ml^{-1}, 0.6 mg ml^{-1}, 0.8 mg ml^{-1} and 1.0 mg ml^{-1}). A standard curve of net absorbance vs. gallic acid concentration (mg ml^{-1}) was plotted, and unknown phenolic concentrations of oil palm plantlets were determined by comparing their net absorbance values at 765 nm against the standard curve. Total phenolic concentration in each plantlet was subsequently expressed as gallic acid equivalent in mg g^{-1}.

Molecular detection of G. boninense DNA in oil palm plantlets

Presence of *G. boninense* DNA in oil palm plantlets were confirmed by conventional PCR and quantified using real-time PCR. Genomic DNA of *G. boninense* (GBLS) and treated oil palm plantlets was extracted using a modified CTAB method (Möller *et al*., 1992). PCR amplifications were carried out according to a previous study (Utomo *et al*., 2000). PCR master mix components were prepared as 20 µl per reaction as follows: 1X Green GoTaq® Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP and 0.06 U μl^{-1} GoTaq® Flexi DNA polymerase; 500 nM forward and reverse primer; 20 ng μl^{-1} DNA samples and nuclease free water. Fungal primers GbF (5'-TTG ACT GGG TTG TAG CTG-3') and GbR (5'-GCG TTA CAT CGC AAT ACA-3') used in study were designed from Gan1 and Gan2 primers (Karthikeyan *et al*., 2006). A PCR thermocycler (Eppendorf, Mastercycler Gradient) was programmed for 5 minutes of pre-heating at 95 °C, subsequently followed by 40 cycles of 94 °C for 40 seconds, 50 °C for 30 seconds and 72 °C for 45 seconds with a final extension step at 72 °C for 10 minutes. PCR products were assessed by gel electrophoresis (Biorad, PowerPac® Basic) using 1.5 % (w/v) agarose gel.

After PCR amplification, bands of *G. boninense* DNA were excised and purified by GF-1 Nucleic Acid Extraction Kit (Vivantis, USA), before sequencing for the *Ganoderma* spp. ITS fragment (1st Base Laboratories Sdn Bhd, Singapore). Sequences were analysed by using Applied Biosystems Sequence Scanner Software v1.0, then subjected to Basal Local Alignment Search Tool (BLAST) program to search for closest matches in the NCBI GenBank database.

Absolute quantification of GBLS DNA in treated oil palm plantlets via real-time PCR amplification

Total DNA of infected oil palm plantlets was quantified (Thermo Scientific, Multiskan^{TM} GO) and diluted 20-fold in nuclease-free water to ensure the final concentration of DNA templates in real-time PCR amplification ranged from 3 – 5 ng μl^{-1}. A standard curve of 10-fold dilution series (1 x 10^{1}, 1 *x* 10^{0}, 1 *x* 10^{-1}, 1 *x* 10^{-2}, and 1 *x* 10^{-3} ng μl^{-1}) was prepared using genomic DNA from *G. boninense* (GBLS isolate) in 100 µl volumes. A volume of 90 µl nuclease free water was aliquoted into each dilution.

Real-time PCR amplifications were conducted in Eco 48-well plates (Illumina) in a total volume of 10 µl using an Eco Real-Time PCR System 110V (Illumina). Each reaction mixture contained 20-fold diluted DNA template of infected plantlets and a serially diluted GBLS genomic DNA, 200 nM GbF and GbR primer, 1X KAPA SYBR FAST qPCR Kit Master Mix Universal (Kapa Biosystems) and nuclease-free water. Non-template control (NTC) reactions contained the same mixtures with 1 µl of nuclease-free water to replace a DNA template. Reaction mixtures were pipetted into Eco 48-well plates with the support of an Eco loading dock (Illumina), and were sealed with Eco adhesives seal (Illumina). Samples were centrifuged (Eppendorf, 5810R) briefly at 1800 rpm for 2 min before applying to Eco Real-Time PCR System 110V.

Thermal cycling conditions for DNA were 95 ˚C for 3 min, followed by 40 cycles at 95 ˚C for 5 s and 60 ˚C for 30 s in order to detect and quantify the fluorescence at a temperature above the denaturation of primer-dimers. A melting curve temperature profile was obtained by programming the Eco Real-Time PCR system 110V for one cycle at 95 ˚C for 15 s, 55 ˚C for 15 s and 95 ˚C for 15 s. Four individual reactions (technical replicates) were run for each biological sample of plant and fungal DNA templates from infected plantlets and GBLS. A

221 standard curve was obtained by plotting the C_q value versus logarithm of the concentration of
222 each 10-fold dilution series of fungal genomic DNA. Total amount of GBLS DNA in infected
223 oil palm plantlets was calculated by comparing the C_q values to the crossing point values of the
224 linear regression line of the standard curve.

225 In order to confirm that only one PCR product was amplified in the real-time PCR
226 amplification system, reactions were analysed by 1.5 % agarose gel electrophoresis using 1 X
227 TBE buffer at 80 V (Biorad, PowerPac® Basic) for 1 hr. DNA bands were observed with a UV
228 transilluminator connected to gel documentation XR System (Quantity One, Biorad).

229

230 *Statistical analysis*

231 All data obtained in this study were analysed statistically by one-way analysis of variance
232 (ANOVA) using GraphPad Prism software version 5.02. Significant differences among the
233 treatments at $P < 0.05$ were determined by Tukey multiple comparison tests using Prism
234 software.

235

RESULTS & DISCUSSION

Physiological assessment on oil palm plantlets

Leaf senescence and the presence or absence of GBLS mycelium on stems was used to categorize plantlets into different disease severity classes as these were the key visible symptoms that were observed in the 8 day experimental period. Disease severity index (DSI) in treated plantlets (Fig. 3A) showed that disease scores of T3 plantlets were increased linearly to as high as 70.37 % at the end of experimental period, and was significantly higher ($P < 0.001$) than T1 (7.41 %) and T2 plantlets (25 %). This finding was supported by previous artificial infection studies of oil palms with *G. boninense* that showed the rise in disease severity in oil palms was closely associated with the presence of *G. boninense* and duration of infection (Mohd As'wad *et al*., 2011; Kok *et al*., 2013). Besides, disease scores recorded in T1 and T2 plantlets were most likely due to leaf senescence, as a result of mechanical injury during transfer of plantlets and artificial wounding (Philosoph-Hadas *et al*., 1994). Therefore, it was essential to artificially wound the oil palm plantlets in this study (T2 treatment) to differentiate the symptoms arose due to wounding effects from those due to infection by *G. boninense*.

No apparent differences ($P > 0.05$) were detected on the height, weight, length of roots and reduction of diameter of stems of oil palm plantlets under different treatments (T1, T2 and T3) during the 8 days of incubation (Fig. 3 B-E). Similar results were reported in a previous study on healthy regeneration of oil palm plantlets that were maintained in MS culture medium after 4 to 6 weeks (Suranthran *et al*., 2013). Although 8 days was not sufficient to detect significant changes in these physiological parameters of growth, stem diameter of T1 plantlets in this study were smaller as compared to T2 and T3 plantlets. This could be due to the natural differences between oil palm plantlets used in the experiment or the swelling effects from both

mechanical injury and fungal infection. Swelling effects on plant stems due to wounding and infection were also reported on *Pinus* spp. (Nagy *et al*., 2000).

Higher SPAD values represent higher levels of chlorophyll content present in oil palm leaves and vice versa (Santos, 2001). In Figure 3F, it was observed that the SPAD value of T1 plantlet was higher (SPAD 30 and above) and remained constant throughout the experimental period. In contrast, the SPAD values of T2 and T3 plantlets were reduced almost linearly, with T3 plantlets consistently having the lowest SPAD values. SPAD value of T3 plantlets were significantly lower than T1 ($P < 0.001$) and T2 plantlets ($P < 0.05$) on Day 8 of incubation. Reductions in leaf chlorophyll contents are commonly noticed in wounded (Riou *et al*., 2002) and infected plants (Milavec *et al*., 2001; Scarpari *et al*., 2005). This phenomenon is directly correlated with decline in the level of chlorophyll *a* and *b* photosynthetic pigments (Scarpari *et al*., 2005) due to degradation by H_2O_2 and phenolic compounds and increases in the carotenoid to chlorophyll ratio (Milavec *et al*., 2001). In *Ganoderma*-infected oil palms, significant reduction in chlorophyll content was reported by Haniff *et al*. (2005) and Shafri *et al*. (2011), due to the injury in oil palm root and vascular system caused by the fungus infection.

Lactophenol blue dye was used to stain chitin compounds in fungal cell wall (Saha *et al*., 1988) and was used to detect *G. boninense* mycelium in internal stem tissues of oil palm plantlets. Microscopic examination on stems (Fig. 4) revealed internal stem tissues of T1 plantlets remained healthy and unstained throughout the experimental period. In T2 plantlets, internal stem structures were disrupted and mild lesions were detected on T2 plantlets after 6 days incubation. Mild blue staining was observed in these plantlets on Day 6 and 8, which could be due to the retention of remaining dye solutions in the wounded region. In contrast, internal stem tissues of T3 plantlets gradually deteriorated from Day 2 post-inoculation as the size of fungal-induced necrotic lesions increased. On Day 8, internal structures of the stem of T3 plantlets were severely colonized and were intensively stained as compared to T1 and T2

plantlets. The observation on colonization of oil palm internal stem tissues by *G. boninense* was in agreement with previous studies on *G. boninense*-infected oil palm using RWB technique (Rees *et al*., 2007; 2009). Previous studies also described the anatomy of healthy and *G. boninense*-colonized oil palm cell wall under transmission electron microscopy (TEM) observation, where multiple cell wall layers were degraded in localized tissues associated with fungal hyphae (Rees *et al*., 2009).

Estimation of total phenolic content in oil palm plantlet

Accumulation of phenolic compounds as plant defence responses in oil palm plantlets was tested (Fig. 5). In T1 plantlets, phenolics were at relatively low level for the constitutive amounts as compared to T2 and T3 oil palm plantlets that elicited a significant rise ($P < 0.001$) in total phenolic levels. However, total phenolic contents in T2 and T3 plantlets were not significantly different from each other throughout the experiment ($P > 0.05$). These results were similar to numerous studies of total phenolic profiles in different plants, where phenolic contents normally increased upon wounding (Becerra-Moreno *et al*., 2012) and infection by compatible pathogens (Datta and Lal, 2012; Mikulic-Petkovsek *et al*., 2014). By Day 6 post-inoculation, the concentration of phenolics declined in T3 plantlets, suggesting that the presence of *G. boninense* could stimulate the production and accumulation of phenolics during early infection development, but the fungus potentially utilizes these compounds later in the time course. This could be due to the ability of *G. boninense* to metabolize phenolic acids that are present at low levels (Chong *et al*., 2012). Ability to metabolize phenolic compounds was also detected in the soft-rot fungi, *Phiuluphora mutabilis* and *Petriellidium boydii*, as they were able to break down syringic acid efficiently within 12 h after infection in host plants (Eriksson *et al*., 1984).

Polymerase chain reaction (PCR) amplification of extracted DNA

For PCR detection of fungal DNA in treated oil palm plantlets, fungal DNA (~167 bp) was amplified from infected T3 plantlets at all time points throughout the experimental period (Fig. 6), indicating that *G. boninense* was present in T3 plantlets. In contrast, there was no amplification of fungal DNA in T1 and T2 plantlets throughout the 8 days of incubation, suggesting that it was likely that *G. boninense* was absent in these plantlets. This result was in agreement with previous findings, where the ITS region of *Ganoderma* (167 bp) was amplified in infected mature oil palm roots by using Gan1 and Gan2 primers (Utomo *et al*., 2000). In *Ganoderma*-infected coconut palms, a PCR product of 167 bp was also detected in DNA samples of infected palms amplified by Gan1 and Gan2 primers (Karthikeyan *et al*., 2006).

DNA sequencing of PCR amplified GBLS gene sequences

The internal transcribed spacer (ITS) region of *Ganoderma* consists of highly conserved sequences (Moncalvo *et al*., 1995) and interspecific variation, enabling specific differentiation of *Ganoderma* from other saprophytic fungi in diseased oil palms (Karthikeyan *et al*., 2006). The PCR amplified GBLS DNA products of 167 bp were sequenced and analysed with BLASTN program. From BLASTN, the sequenced DNA length was 171 bp and scored 99 % similarity flanking over 87 % of nine different homologous *Ganoderma* DNA sequences.

Absolute quantification of GBLS DNA in infected oil palm plantlets via real-time PCR amplification

Although conventional PCR detection of GBLS ITS DNA fragment in oil palm plantlets served as an effective molecular detection approach to identify *G. boninense*, quantification of *G. boninense* genomic DNA in these plantlets was not possible using this method. Hence, absolute quantification via real-time PCR amplification (qPCR) was carried out with total DNA samples of infected plantlets and GBLS (as standard). Since no PCR product was observed in DNA samples of healthy and wounded oil palm plantlets when amplified by ITS primers, the qPCR quantification assay was specifically performed on infected plantlets.

Specificity of ITS primers in qPCR reactions was confirmed by both melting curve analysis and gel electrophoresis (results not shown). QPCR products were documented with a single, intense band of predicted length (167 bp) on high resolution gel electrophoresis. In addition, these PCR products also displayed sharp fluorescent peaks at 85 ˚C in melting curve analysis, indicating that a single product at a specific melting temperature was detected. Some non-specific fluorescence peaks at lower amplitudes were detected in melting curve analysis during amplification of genomic DNA of GBLS and T3 plantlets. These results indicated that non-specific PCR products may be present in the analyzed temperature range in this experiment. Non-specific PCR products detected from the melting curve analysis could be due to several reasons, including low melting temperature (Tm) of the fungal primers used (GbF: 51.91 °C and GbR: 50.34 °C). PCR primers with higher Tm values are normally more effective at binding to DNA templates, hence reducing the chances of binding to other DNA sequences (Chung, 2004). However, the presence of the strong fluorescent peak at 85 °C in this study clearly indicated the validity of the quantification method.

Real-time PCR amplification assays conducted in this study successfully quantified the amount of *G. boninense* (GBLS) DNA in infected plantlets on a progressive basis for 8 days of time points. From Figure 7, significant increases ($P < 0.001$) of GBLS DNA were detected

in infected oil palm plantlets on Day 6 and 8 post-inoculation at 10.97 ng μl^{-1} and 116.10 ng μl^{-1} respectively. In contrast, GBLS DNA was detected at relatively low levels in infected plantlets at Day 0, 2 and 4 post-inoculation as compared to Day 6 and 8. These results showed that GBLS mycelium accumulated in infected plantlets during the later stages of the one week long time course, indicating that *G. boninense* GBLS was able to grow within these plantlets. Similar patterns of fungal growth were also detected in *Magnaporthe oryzae*-infected susceptible rice cultivars by real-time PCR amplification within 6 days of inoculation period (Qi and Yang, 2002). In addition, an increase in *Peronospora parasitica* (downy mildew fungus) quantity in *Arabidopsis* plants was also observed after Day 4 post-inoculation via real-time PCR analysis (Brouwer *et al*., 2003). To the best of our knowledge, this is the first report of quantifying *Ganoderma* spp. growth *in planta* in oil palm at one week post-infection by using quantitative real-time PCR.

Overall, a strong correlation between the reduction in leaf chlorophyll content, deterioration of internal stem tissues, and escalation in disease severity index (DSI), total phenolic content and GBLS DNA amounts was observed in infected plantlets. This correlation was expectedly absent from the control and wounded plantlets. This signified that an initial development of BSR disease symptoms were detected in GBLS-infected oil palm plantlets via an axenic *in planta* infection assay within a relatively short period of time. Hence, the stability and reliability of the *in planta* infection assay used in this study was proven to be a time effective approach for studies on early disease development in young oil palm plantlets. The *in planta* infection assay can be improved by comparing the responses in oil palm plantlets infected with multiple pathogenic isolates of *G. boninense*, since the pathogenicity of *G. boninense* is determined genetically and may induces a different result in oil palm response.

Future studies to evaluate the infection mechanism of *G. boninense* and defence responses in oil palm during the early stages of infection are thus feasible with the application

of this *in planta* infection assay. However, results obtained from this artificial infection assay can be varied with natural infection of mature oil palm by different *G. boninense* isolates. This is because the oil palm plantlets used in this assay were at young age and their tissues composition may be different from the adult plants. Besides, oil palm could behave and response differently to *Ganoderma* infection in the nature when other biotic and abiotic stresses are presence. Therefore, it is essential to compare the responses of oil palm infected by *G. boninense* both artificially and naturally at different ages or developmental stages to have a better understanding of oil palm defence system towards BSR disease.

ACKNOWLEDGEMENTS

The authors are grateful for the financial supports from School of Biosciences, The University of Nottingham Malaysia Campus and MyBrain15, Ministry of Higher Education (MOHE) Malaysia. They are also thankful for the support of oil palm materials from Applied Agricultural Resources (AAR) Sdn. Bhd.

REFERENCES

1. Alizadeh F., Abdullah S.N.A., Khodavandi A., Abdullah F., Yusuf U.K., Chong P.P., 2011. Differential expression of oil palm pathology genes during interactions with *Ganoderma boninense* and *Trichoderma harzianum*. *Journal of Plant Physiology* **168**: 1106–1113.

2. Baumgartner K., Bhat R., Fujiyoshi P., 2010. A rapid infection assay for *Armillaria* and real-time PCR quantitation of the fungal biomass *in planta*. *Fungal Biology* **114**: 107–119.

3. Becerra-Moreno A., Benavides J., Cisneros-Zevallos L., Jacobo-Velázquez D.A., 2012. Plants as biofactories: Glyphosate-induced production of shikimic acid and phenolic antioxidants in wounded carrot tissue. *Journal of Agricultural and Food Chemistry* **60**: 11378–11386.

4. Breton F., Hasan Y., Hariadi S., Lubis Z., De Franqueville H., 2006. Characterization of parameters for the development of an early screening test for basal stem rot tolerance in oil palm progenies. *Journal of Oil Palm Research* **(spec.)**: 24–36.

5. Brouwer M., Lievens B., Hemelrijck W., Ackerveken G., Cammue B.P., Thomma B.P.H., 2003. Quantification of disease progression of several microbial pathogens on *Arabidopsis thaliana* using real-time fluorescence PCR. *FEMS Microbiology Letter* **228**: 241–248.

6. Carter C., Finley W., Fry J., Jackson D., Willis L., 2007. Palm oil markets and future supply. *European Journal of Lipid Science and Technology* **109**: 307-314.

7. Chong K.P., Atong M., Rossall S., 2012. The role of syringic acid in the interaction between oil palm and *Ganoderma boninense*, the causal agent of basal stem rot. *Plant Pathology* **61**: 953–963.

8. Chung D., 2004. The development of novel STR miniplex primer sets for the analysis of degraded and compromised DNA samples. Ph.D. Thesis. Ohio University, Ohio, United States.

9. Datta J., Lal N., 2012. Temporal and spatial changes in phenolic compounds in response *Fusarium* wilt in chickpea and pigeonpea. *Cellular and Molecular Biolog* **58**: 96–102.

10. Eriksson K., Gupta J., Nishida A., Raos M., 1984. Syringic acid metabolism by some white-rot, soft-rot and brown-rot fungi. *Journal of General Microbiology* **130**: 2457–2464.

11. Foster A.J., Jenkinson J.M., Talbot N.J., 2003. Trehalose synthesis and metabolism are required at different stages of plant infection by *Magnaporthe grisea*. *The EMBO Journal* **22**: 225–235.

12. Goh K.M., Ganeson M., Supramaniam C.V., 2014. Infection potential of vegetative incompatible *Ganoderma boninense* isolates with known ligninolytic enzyme production. *African Journal of Biotechnology* **13**: 1056-1066.

13. Govrin E.M., Levine A., 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Current Biology* **10**: 751–757.

14. Haniff M.H., Ismail S., Idris A.S., 2005. Gas exchange responses of oil palm to *Ganoderma boninense* infection. *Asian Journal of Plant Sciences* **4**: 438-444.

15. Holliday P., 1980. Fungus diseases of tropical crops. Cambridge University Press, Melbourne, Australia.

16. Idris A.S., Kushairi D., Ariffin D., Basri M.W., 2006. Technique for inoculation of oil palm germinated seeds with *Ganoderma*. *MPOB Information Series* **321**.

17. Izzati M.Z., Abdullah F., 2008. Disease suppression in *Ganoderma*-infected oil palm seedlings treated with *Trichoderma harzianum*. *Plant Protection Science* **44**: 101–107.

18. Karthikeyan M., Radhika K., Bhaskaran R., Mathiyazhagan S., Samiyappan R., Velazhahan R., 2006. Rapid detection of *Ganoderma* disease of coconut and assessment of inhibition effect of various control measures by immunoassay and PCR. *Plant Protection Science* **42**: 49–57.

19. Kok S.M., Goh Y.K., Tung H.J., Goh K.J., Wong W.C., Goh Y.K., 2013. *In vitro* growth of *Ganoderma boninense* isolates on novel palm extract medium and virulence on oil palm (*Elaeis guineensis*) seedlings. *Malaysian Journal of Microbiology* **9**: 33–42.

20. Kurian A., Peter K.V., 2007. Horticulture science series: Commercial crops technology. 8th ed. New India Publishing, New Delhi, India.

21. Mikulic-Petkovsek M., Schmitzer V., Stampar F., Veberic R., Koron D., 2014. Changes in phenolic content induced by infection with *Didymella applanata* and *Leptosphaeria coniothyrium*, the causal agents of raspberry spur and cane blight. *Plant Pathology* **63**: 185–192.

22. Milavec M., Ravnikar M., Kovač M., 2001. Peroxidases and photosynthetic pigments in susceptible potato infected with potato virus YNTN. *Plant Physiology and Biochemistry* **39**: 891–898.

23. Mohd As'wad A.W., Sariah M., Paterson R.R.M., Zainal Abidin M.A., Lima N., 2011. Ergosterol analyses of oil palm plantlets and plants infected with *Ganoderma*. *Crop Protection* **30**: 1438–1442.

24. Möller E.M., Bahnweg G., Sandermann H., Geiger H.H., 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Research* **20**: 6115–6116.

25. Moncalvo J.M., 2000. Systematics of *Ganoderma*. In Flood J., Bridge P., Holderness M., (ed.). *Ganoderma* Diseases of Perennial Crops, pp. 23–45. CAB International, Oxfordshire.

26. Moncalvo J.M., Wang H., Hseu R., 1995. Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. *Mycology* **87**: 223–238.

27. Nagy N.E., Franceschi V.R., Solheim H., Krekling T., Christiansen E., 2000. Wound-induced traumatic resin duct development in stems of Norway spruce (*Pinaceae*): Anatomy and cytochemical traits. *American Journal of Botany* **87**: 302–313.

471 28. Naher L., Ho C.L., Tan S.G., Yusuf U.K., Abdullah F., 2011. Cloning of transcripts
472 encoding chitinases from *Elaeis guineensis* Jacq. and their expression profiles in response
473 to fungal infections. *Physiological and Molecular Plant Pathology* **76**: 96–103.

474 29. Philosoph-Hadas S., Meir S., Aharoni N., 1994. Role of ethylene in senescence of
475 watercress leaves. *Physiologia Plantarum* **90**: 553–559.

476 30. Qi M., Yang Y., 2002. Quantification of *Magnaporthe grisea* during infection of rice
477 plants using real-time polymerase chain reaction and northern blot/phosphoimaging
478 analyses. *Phytopathology* **92**: 870–876

479 31. Rees R.W., Flood J., Hasan Y., Cooper R.M., 2007. Effects of inoculum potential, shading
480 and soil temperature on root infection of oil palm plantlets by the basal stem rot pathogen
481 *Ganoderma boninense*. *Plant Pathology* **56**: 862–870.

482 32. Rees R.W., Flood J., Hasan Y., Potter U., Cooper R.M., 2009. Basal stem rot of oil palm
483 (*Elaeis guineensis*): Mode of root infection and lower stem invasion by *Ganoderma*
484 *boninense*. *Plant Pathology* **58**: 982–989.

485 33. Riou C., Hervé C., Pacquit V., Dabos P., Lescure B., 2002. Expression of an *Arabidopsis*
486 lectin kinase receptor gene, *lecRK-a1*, is induced during senescence, wounding and in
487 response to oligogalacturonic acids. *Plant Physiology and Biochemistry* **40**: 431–438.

488 34. Saha D., Jackson M., Johnson-Cicalese J., 1988. A rapid staining method for detection of
489 endophytic fungi in turf and forage grasses. *The American Phytopathological Society* **78**:
490 237–239.

491 35. Santos C.L.V., 2001. *In situ* and *in vitro* senescence induced by KCl stress: Nutritional
492 imbalance, lipid peroxidation and antioxidant metabolism. *Journal of Experimental*
493 *Botany* **52**: 351–360.

494 36. Sariah M., Hussin M.Z., Miller R.N.G., Holderness M., 1994. Pathogenicity of
495 *Ganoderma boninense* tested by inoculation of oil palm plantlets. *Plant Pathology* **43**:
496 507–510.

497 37. Scarpari L.M., Meinhardt L.W., Mazzafera P., Pomella A.W.V., Schiavinato M.A.,
498 Cascardo J.C.M., Pereira G.A.G., 2005. Biochemical changes during the development of
499 witches' broom: The most important disease of cocoa in Brazil caused by *Crinipellis*
500 *perniciosa*. *Journal of Experimental Botany* **56**: 865–877.

501 38. Shafri H.Z.M., Anuar M.I., Seman I.A., Noor N.M., 2011. Spectral discrimination of
502 healthy and *Ganoderma*-infected oil palms from hyperspectral data. *International Journal*
503 *of Remote Sensing* **32**: 7111-7129.

504 39. Song E., 2015. Industry assessment palm oil and palm mill industry: An update. EXIM
505 Bank.

506 40. Stukkens Y., Bultreys A., Grec S., Trombik T., Vanham D., Boutry M., 2005. *NpPDR1*, a
507 pleiotropic drug resistance-type ATP-binding cassette transporter from *Nicotiana*
508 *plumbaginifolia*, plays a major role in plant pathogen defense. *Plant Physiology* **139**: 341–
509 352.

510 41. Su'ud M.M., Loonis P., Seman I., 2007. Towards automatic recognition and grading of
511 *Ganoderma* infection pattern using fuzzy systems. *World Academy of Science,*
512 *Engineering and Technology* **25**: 51–56.

513 42. Suranthran P., Sinniah U., Subramaniam S., Aziz M., Romzi N., Gantait S., 2013. Effect
514 of plant growth regulators and activated charcoal on *in vitro* growth and development of
515 oil palm (*Elaeis guineensis* Jacq. var. *Dura*) zygotic embryo. *African Journal of*
516 *Biotechnology* **10**: 10600-10606.

517 43. Turner P.D., 1981. Oil palm diseases and disorders. Oxford University Press, Kuala
518 Lumpur, Malaysia.

519 44. Utomo C., Niepold F., 2000. The development of diagnostic tools for *Ganoderma* in oil
520 palm. In: Flood J., Bridge P.D., Holderness M. (ed.). *Ganoderma* diseases of perennial
521 crops, pp. 236. CAB International, Oxfordshire, England.

522 45. Xu J.R., Hamer J.E., 1996. MAP kinase and cAMP signaling regulate infection structure
523 formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes &*
524 *Development* **10**: 2696–2706.

525 46. Yeoh K.A., Othman A., Meon S., Abdullah F., Ho C.L., 2013. Sequence analysis and gene
526 expression of putative oil palm chitinase and chitinase-like proteins in response to
527 colonization of *Ganoderma boninense* and *Trichoderma harzianum*. *Molecular Biology*
528 *Reports* **40**: 147–158.

529 47. Zainudin N.A.I., Abdullah F., 2008. Disease suppression in *Ganoderma*-infected oil palm
530 plantlets treated with *Trichoderma harzianum*. *Plant Protection Science* **44**: 101–107.

531

532 **TABLE**

533 **Table 1** Disease scores on a scale of 0-4 based on morphological symptoms on oil palm
534 plantlets.

Disease Class	Morphological symptoms
0	Healthy plants with green leaves (SPAD value ≥ 30), absence of fungal mycelium on any part of plants
1	Healthy plants with yellow-green leaves (SPAD value ranges from 10-30), absence of fungal mycelium on any part of plants
2	Unhealthy plants with chlorotic leaves (SPAD value ≤10), absence of fungal mycelium on any part of plants

3	Unhealthy plants with chlorotic leaves (SPAD value 10-30), presence of fungal mycelium on basal stem region
4	Unhealthy plants with chlorotic leaves (SPAD value ≤10), presence of fungal mycelium on basal stem region

535

536

FIGURES

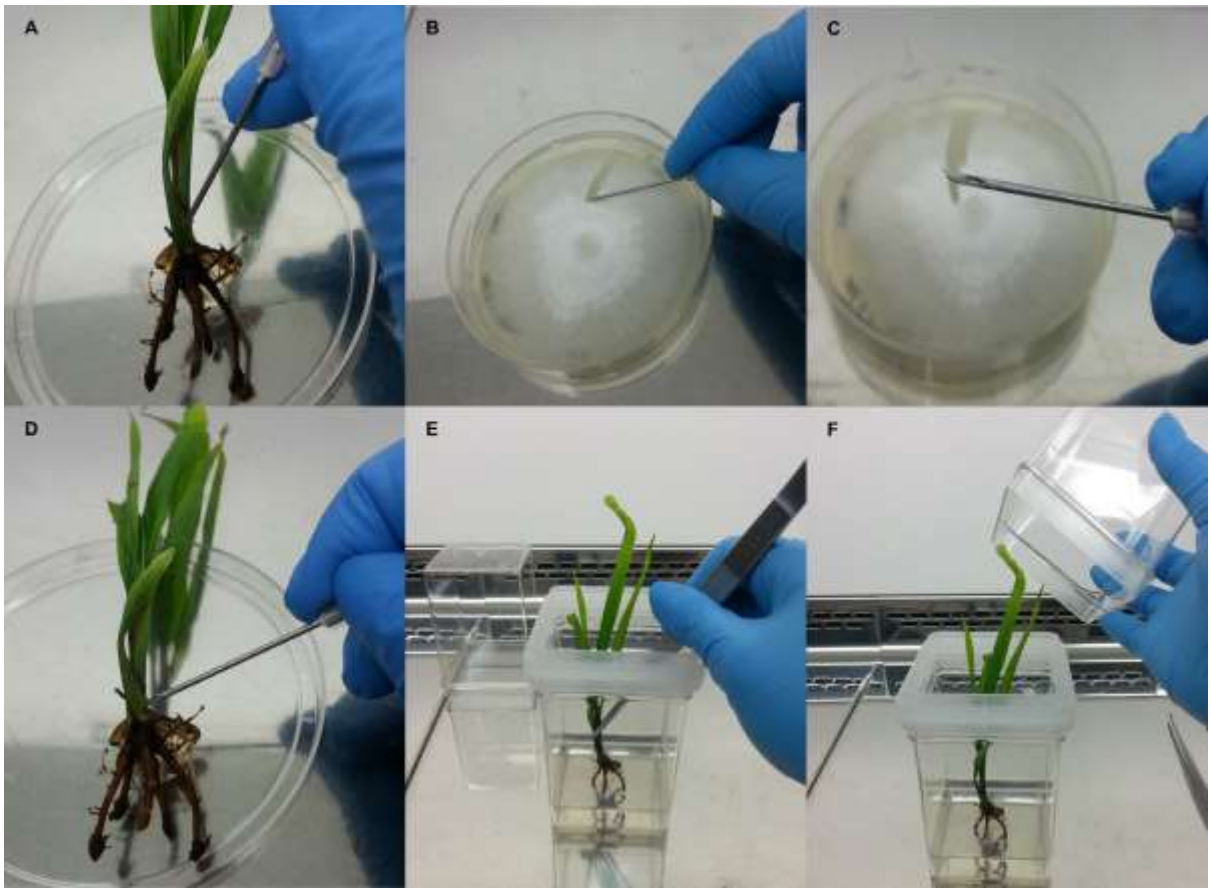
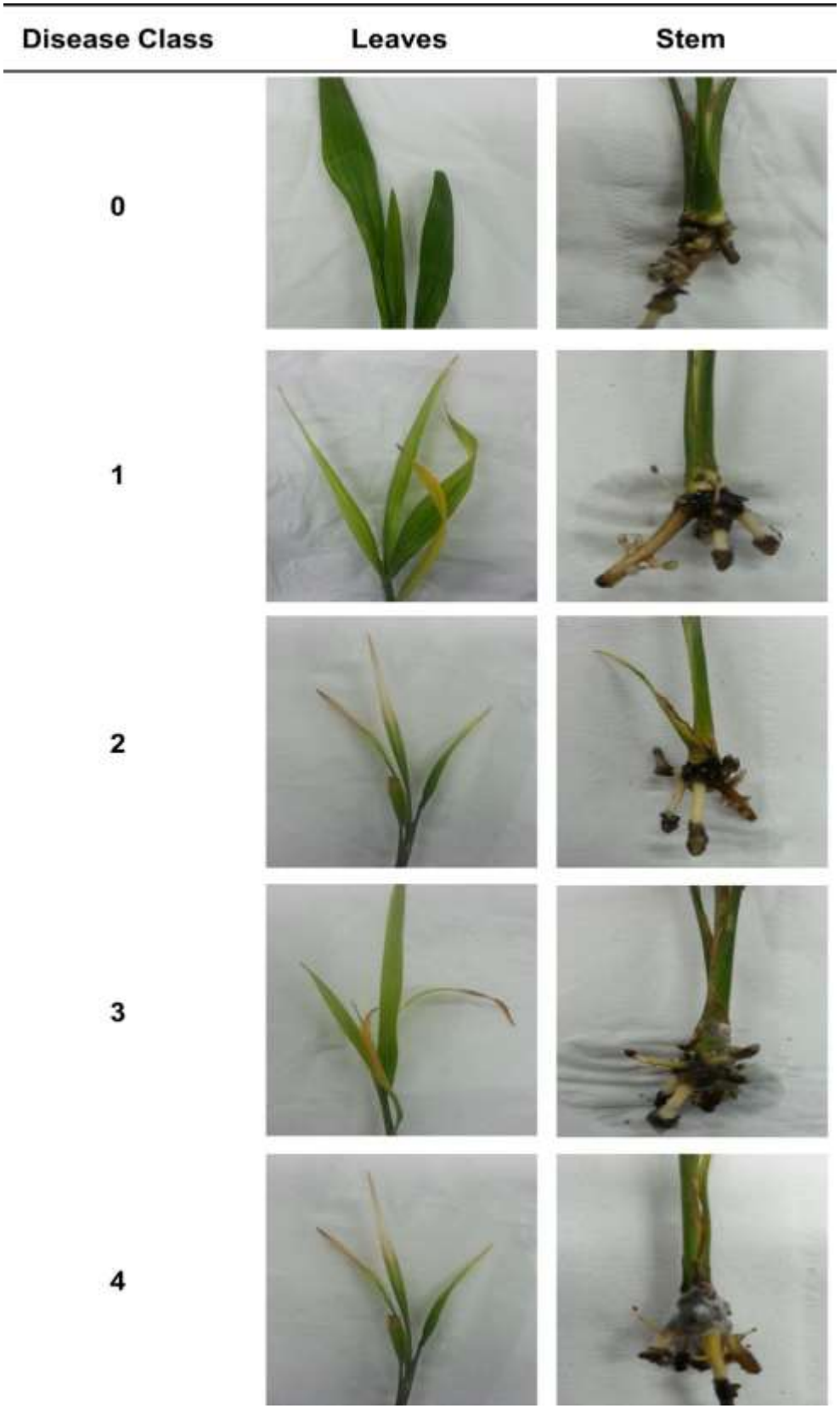
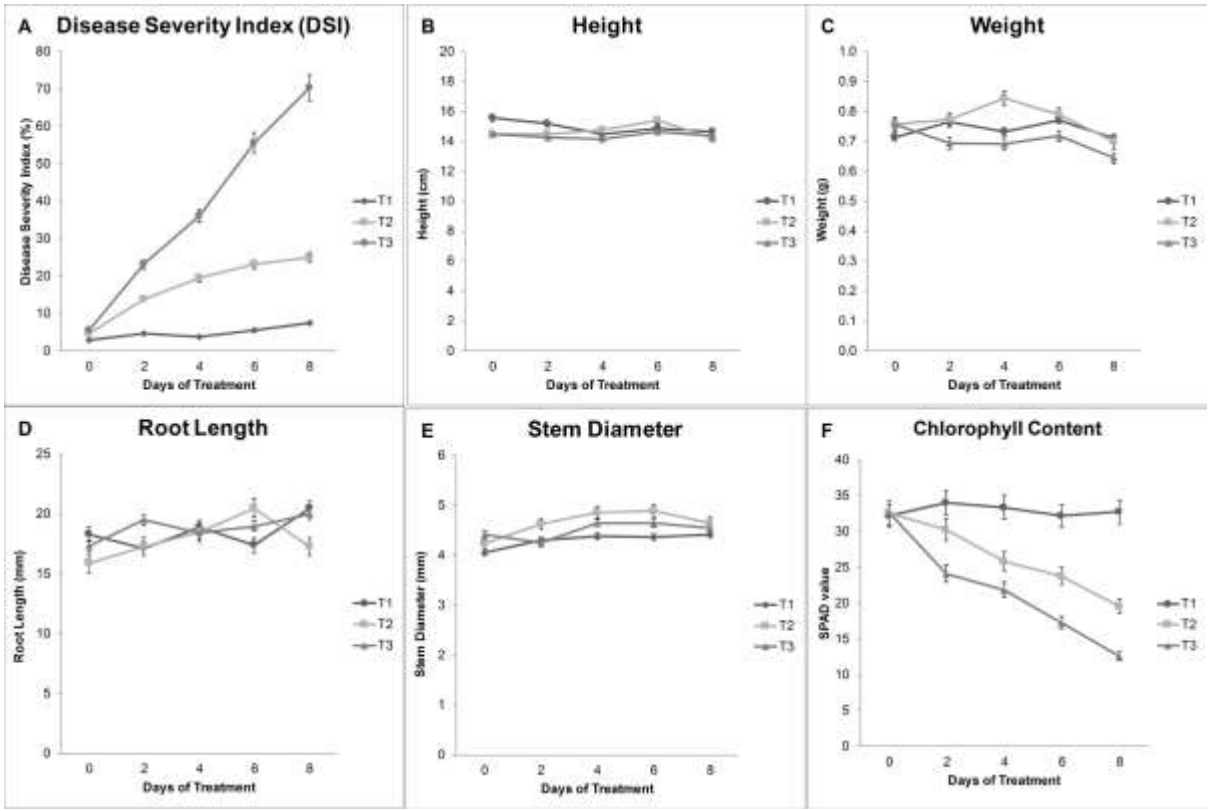


Figure 1 (A-F) *In planta* infection process of oil palm plantlets with *G. boninense.* (A) Artificial wounding on the stem region of plantlets with sterilized needle. (B-C) GBLS mycelium was obtained from nutrient medium. (D) Inoculation of GBLS mycelium on wounded region of plantlets. (E) Transfer of plantlets into Incu tissue culture jar with MS medium. (F) Assembly of tissue culture jar.



549

550 **Figure 2** Illustration of treated oil palm plantlets under different disease classes.



551

552 **Figure 3 (A-F)** (A) Disease severity (DSI) scores, (B) height, (C) weight, (D) root length, (E)
553 stem diameter and (F) chlorophyll content of oil palm plantlets within 8 days of incubation.
554 Standard error of mean (SEM) of replicate readings from three rounds of experiments were
555 represented by error bars.

556

557

558

559

560

561

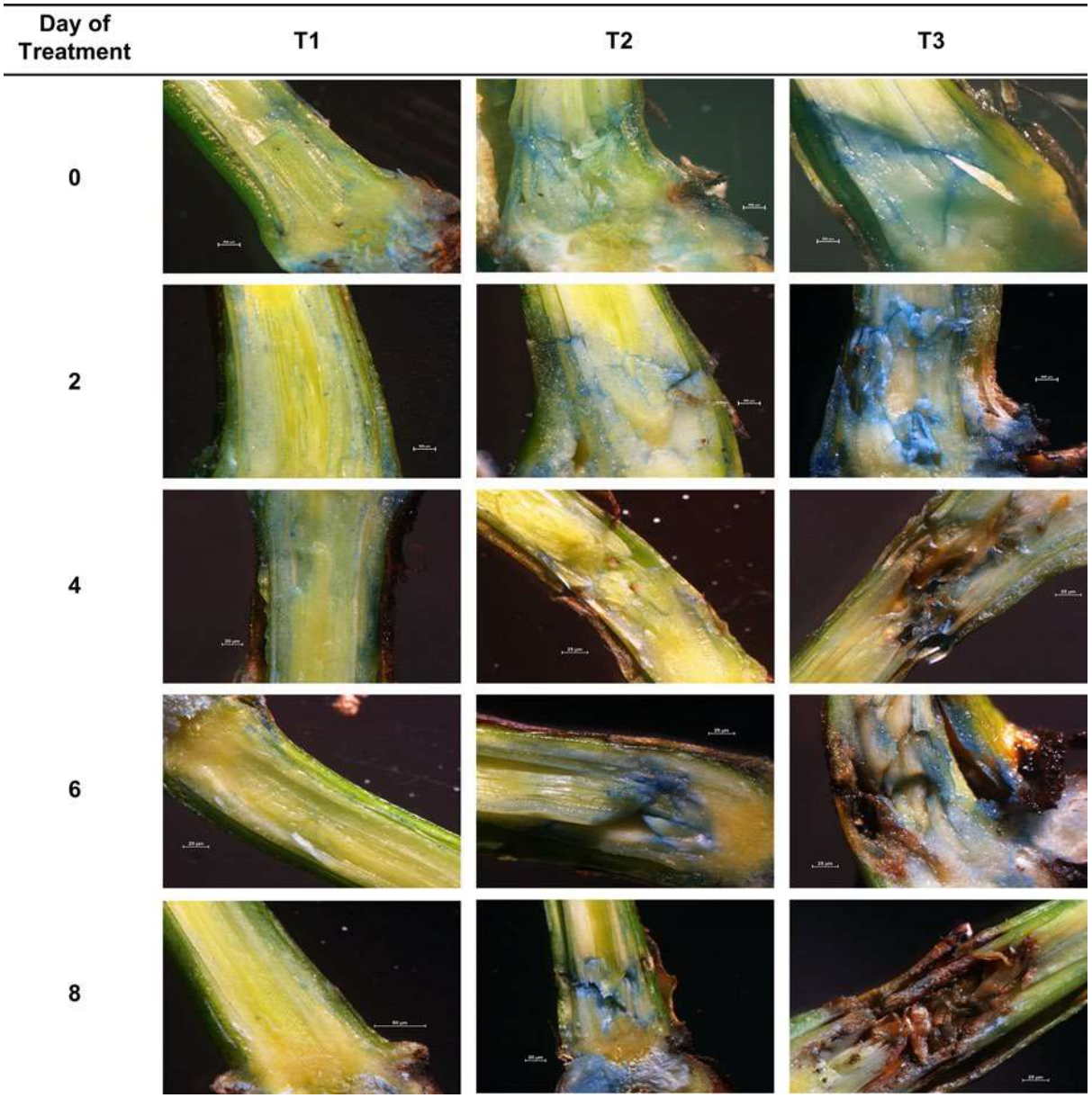


Figure 4 Microscopic observations on the stem region of oil palm plantlets after staining with lactophenol blue dye. Scale bar, 100 µm.

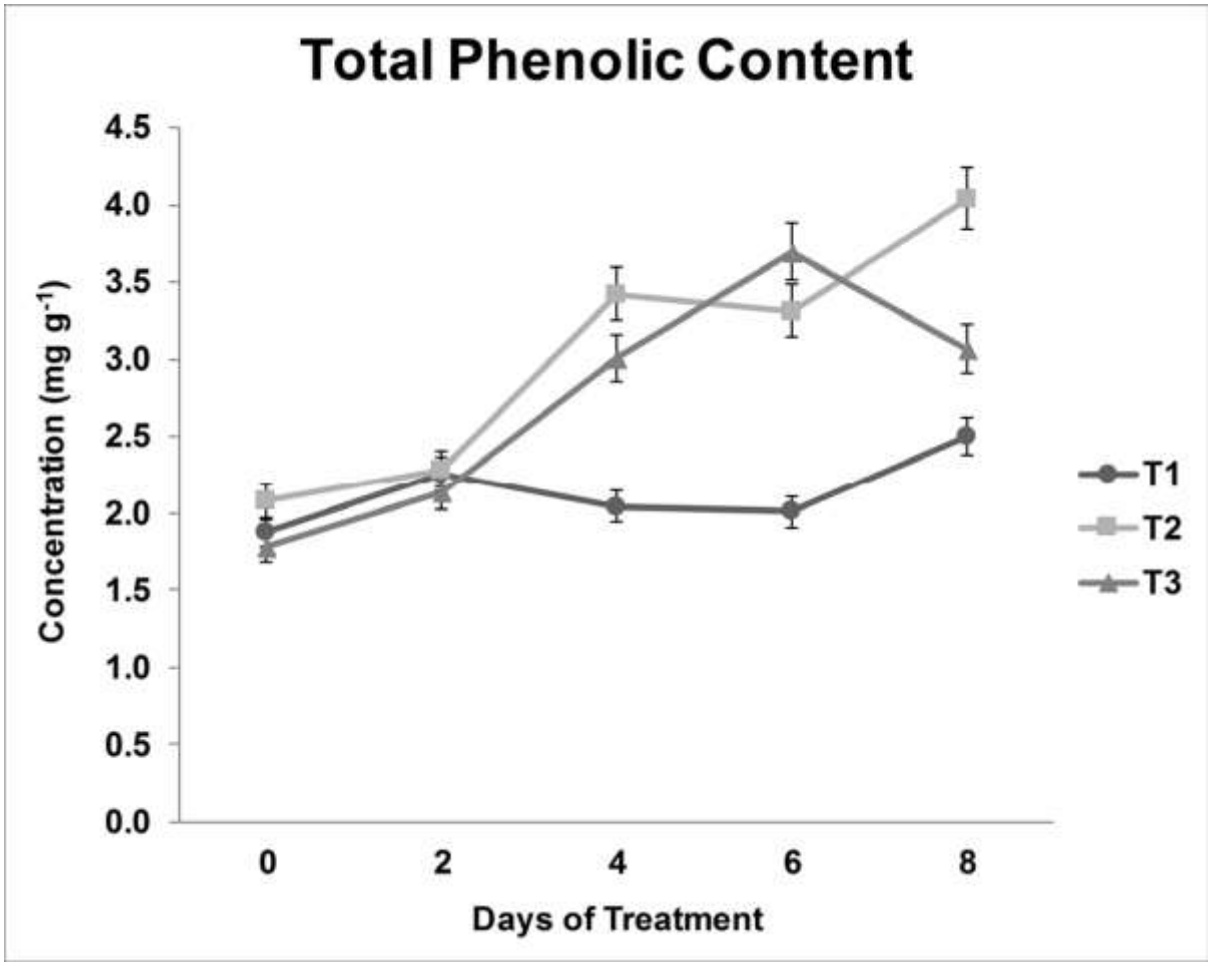
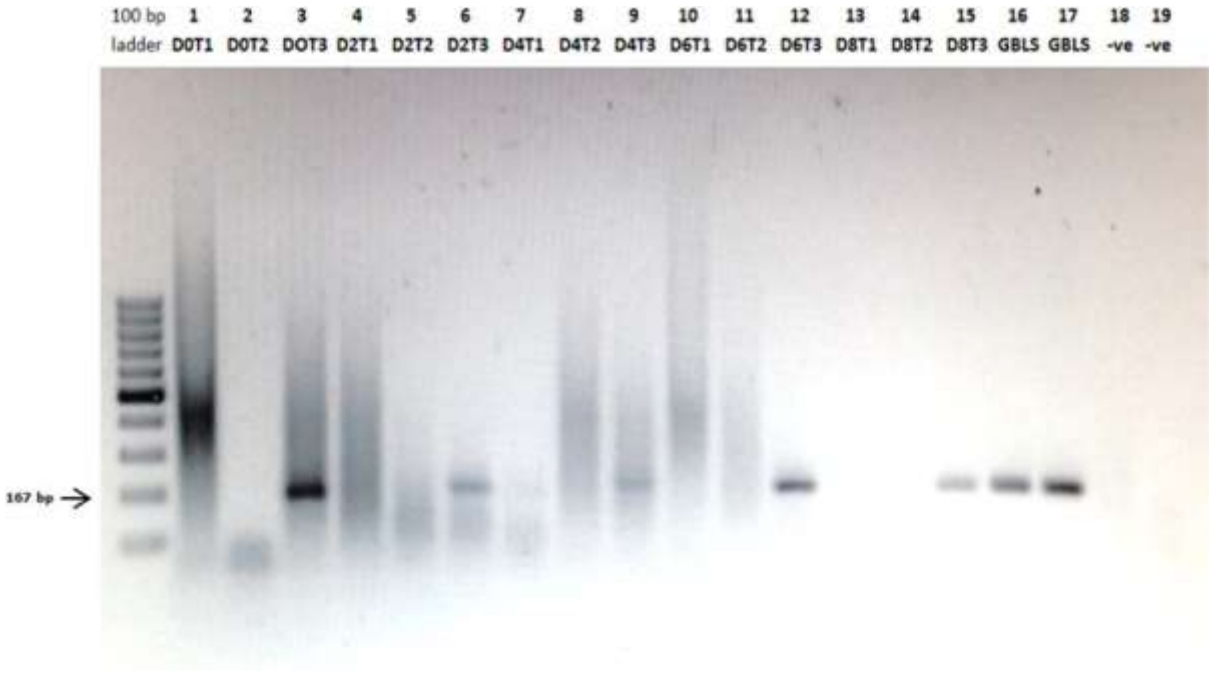


Figure 5 Total phenolic contents in oil palm plantlets within 8 days of incubation. Standard error of mean (SEM) of replicate readings from three rounds of assay were represented by error bars.



578

579 **Figure 6** PCR amplification of oil palm plantlets DNA using GbF and GbR primers. Lane 1-
580 3, 4-6, 7-9, 10-12, and 13-16: DNA samples of oil palm plantlets from Day 0, 2, 4, 6 and 8
581 experimental period respectively. Lane 16-17: Positive control, GBLS fungal DNA. Lane 18-
582 19: Negative control. Samples that showed a band at 167 bp (arrow) indicated the presence of
583 *G. boninense* DNA.

584

585

586

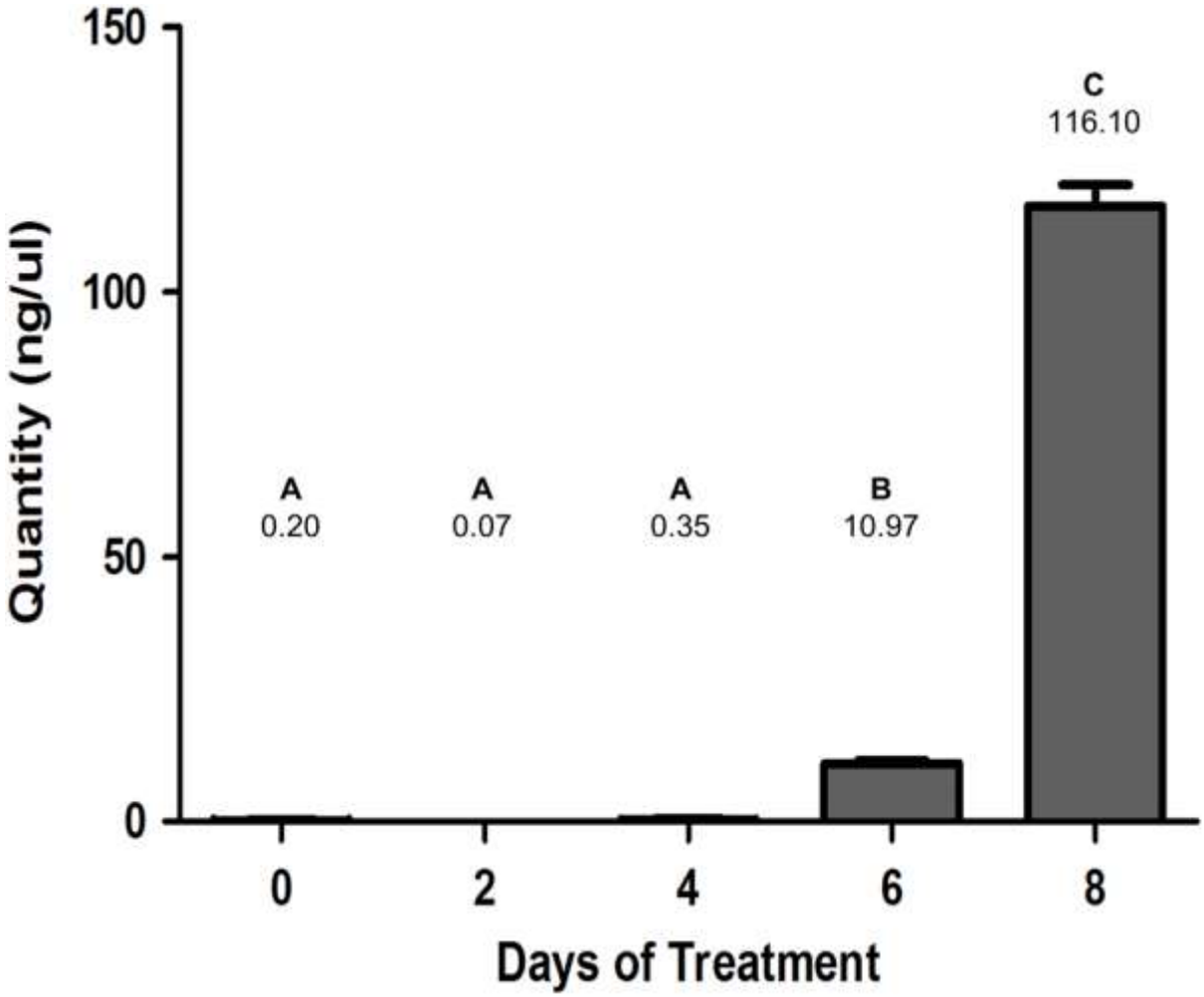
587

588

589

590

591



592

593 **Figure 7** Quantity of *G. boninense* (GBLS) DNA in infected oil palm plantlets at different
594 experimental period. Standard error of mean (SEM) of four replicate readings were represented
595 by error bars. Column with different alphabetic letters was significantly different at $P < 0.001$
596 by Tukey Multiple Comparison Test.

597