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Infection potential of vegetative incompatible *Ganoderma boninense* isolates with known ligninolytic enzyme production

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Ganoderma boninense **produces ligninolytic enzymes namely lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase to degrade the lignin composition in plant cell walls. The present study aimed to evaluate the infection potential of vegetative incompatible isolates of *G. boninense* in causing Basal Stem Rot (BSR) disease in oil palm through the production of ligninolytic enzymes. Vegetative incompatibility test was carried out to test the antagonistic relationship of *G. boninense* isolates. *G. boninense* isolates with strong antagonistic reaction were selected for ligninolytic enzymes analyses. *In vitro* infection of oil palm seedlings and downstream analyses were then carried out. Control experiment was carried out with GBLS, a known *G. boninense* isolate. From this study, *G. boninense* isolates *a1* and *h2* showed the highest laccase (43.07 and 40.44 UI^{-1}) and MnP (14.80 and 16.21 UI^{-1}) enzymes production. Oil palm seedlings infected by isolates *a1* and *h2* resulted in relatively high percentage of disease severity index (DSI) (42.50 and 56.25% respectively). GBLS had lower laccase and MnP enzyme activities (24.31 and 9.27 UI^{-1} respectively) and obtained the lowest DSI value (29.55%). Overall, a direct relationship was observed between the production of ligninolytic enzymes and the infection potential in vegetative incompatible *G. boninense* isolates.**

Key words: Oil palm, Basal Stem Rot (BSR), vegetative incompatible, ligninolytic enzymes, *in vitro* infection.

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is an erect monoecious plant that belongs to *Elaeis* genus of Palmae family. It offers the highest yields of oil per hectare of land as compared to other oil producers up to date (Abdullah and Wahid, 2011). Palm oil is not only crucial as source of food and energy supplied to developing countries, but also scientifically proven to have nutritional values that benefit human health (Sundram et al., 2003). Before World War II, oil palms were largely free from serious diseases and disorders (Hartley, 1988). As the crop plantation area has expanded, there have been serious outbreak of oil palm diseases in different parts of the world. In the Far East severely affected areas, more than 50% of oil palms might surrender to *Ganoderma* spp. According to Su'ud et al. (2007), *Ganoderma boninense* can cause lethal effects in oil palm by degrading its xylem

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Abbreviation: BSR, Basal stem rot; **LiP,** Lignin peroxidase; **MnP,** Manganese peroxidase; **VP,** Versatile peroxidase; **CWDEs,** Cell wall degrading enzymes; **AAR,** Applied Agricultural Resources; **ABTS,** 3-ethylbenzothiazoline-6-sulphonic acid; **PDA,** Potato dextrose agar; **MS,** Murashige and Skoog; **MEB,** Malt extract broth; **DSI,** Disease severity index; **PCR,** Polymerase chain reaction; **ANOVA,** Analysis of variance; **SEM,** Standard error of mean; **ITS,** Internal transcribed spacer.

and affect the allocation of water and nutrients to other parts of palm trees. This disease is believed to be a major problem for oil palm cultivation because loss in crop yield can reach 80 % after constant planting cycles. Although different strategies and controls, such as cultural practices, mechanical and chemical treatments, have been applied on this fungus in order to inhibit its infection, the results were not satisfactory (Moller and Schultz, 1997). This may be due to poor understanding of the biology and behavior of *G. boninense* upon infecting host plants, the mechanisms of disease establishment, development and spread. Such understanding may include the potential of vegetative incompatible isolates of *G. boninense*in producing ligninolytic enzymes that break down the cell wall of the host plant.

Basal Stem Rot (BSR) infection in oil palm by *G. boninense* appeared to involve a few stages of developmental switches as described by Rees et al. (2009). The first stage involved the biotrophic phase in the infected root cortex or stem base where the intracellular layers of oil palm are greatly colonised by *G. boninense* hyphae. This phase of invasion mode will switch to an aggressive necrotropic phase which involves extensive oil palm cell wall degradation through the production of a series of cell wall degrading enzymes (CWDEs). Ligninolytic enzymes such as manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP) and laccase are examples of CWDEs used by *G. boninense* to degrade oil palm's lignin polymers that have high level of biodegradability (Polaina and MacCabe, 2007). Oil palm's lignin is comprised of a high percentage of aryl ether-linked syringyl (S) units (Paterson et al., 2009). In addition, significant amount of *p*-hydroxybenzoic acid and small amounts of syringic, vanillic acids and esterified *p*-hydroxybenzoic acid are also present in the lignin of oil palm (Suzuki et al., 1998). This composition is the main reason that oil palm's lignin is more vulnerable when compared with wood lignin that contains high guaiacyl (G) units (Paterson et al., 2009).

The compatibility relationship is a unique characteristic of basidiomycetes, including *G. boninense*. This relationship is related to the life cycle of these fungi and is often described as the ability between two isolates of single fungal species to exist concurrently without conflict. Pilotti et al. (2002) discovered that *G. boninense* is heterothallic with multiple alleles at both mating type loci and possess bifactorial incompatibility (tetrapolar) that favours out-crossing within a population. Hence, somatic incompatibility that allows sexual recombination to promote out-breeding amongst individuals is common in *G. boninense*. Vegetative incompatibility systems in *Ganoderma* are allelic in nature; they mainly act to limit the transfer of nuclear and cytoplasmic components when fusion (anastomosis) of two incompatible fungal hyphae takes place. Antagonistic interaction between somatically incompatible dikaryon isolates can be distinguished by the occurrence of thin line or pigmented clear zone where the mycelium of two cultures meet (Qi et al., 2003), and this solid demarcation line is called the "barrage" by Pilotti et al. (2002). According to Burgess et al. (2009), vegetative compatibility in fungi reveals the phenotypic differences among individual isolates from similar species.

It is believed that different isolates of a single fungal species may have different strength of compatibility to cause infection to a different degree of severity on similar host. It was hypothesized that the vegetative incompatible *G. boninense* isolates with higher amount of ligninolytic enzymes production are more aggressive and have a higher potential to cause BSR disease in oil palm. Thus, this study aimed to detemine the relationship between the ligninolytic enzymes activities of each *G. boninense* isolates and the degree of infection caused by particular isolates on the oil palm through artificial *in vitro* infection approaches to conclude the aggressiveness of incompatible *G. boninense* isolates in causing disease in oil palm.

MATERIALS AND METHODS

Sources of fungal isolates and growth

In this study, *Ganoderma boninense* isolate GBLS was served as the positive fungal control. This isolate was previously characterized by amplifying its DNA fragment with GbITS1 and GBITS4 primers and confirmed as *G. boninense* after sequencing (GenBank KF164430.1). The mycelium pure culture of this isolate was obtained from Plant Pathology Laboratory, School of Biosciences, University of Nottingham Malaysia Campus. Another six *G. boninense* isolates (*g1, a1, c3, d4, e2* and *h2*) in the form of pure culture mycelium were also initiated from a single basidiocarp (fruiting body), termed as T4G1 through sexual recombination of monokaryotic mycelium from previous study (Chai, 2011). These isolates were obtained from a basidiocarp on an infected 24 year-old oil palm within Plot No. PM86B of the Boustead Balau Estate, Semenyih, Selangor, Malaysia. Pure culture mycelium of *G. boninense* isolates were maintained by sub-culturing on fresh potato dextrose agar (PDA; Merck) at 25°C for every three weeks to provide optimum conditions for the fungal growth.

Oil palm seedlings

One month old oil palm seedlings used for *in vitro* infection studies in this paper were supplied by Applied Agricultural Resources (AAR) Sdn. Bhd. They were grown in 72 × 72 × 100 mm^3 sterilised Incu Tissue Culture Jars (SPL) containing 40 ml Murashige and Skoog (MS) medium (Duchefa), in a growth chamber (Conviron, CMP 6010) at 27°C, 16 h of light intensity and 50% relative humidity.

Vegetative incompatibility test

Vegetative incompatibility test among the *G. boninense* isolates was carried out as described by Pilotti et al. (2002). *G. boninense* isolates *g1, a1, c3, d4, e2* and *h2* were paired among each other, together with the GBLS isolate. Self-pairings of each isolate were served as controls in this test. Mycelial interactions between each culture were assessed and recorded at 7 days intervals (day 0, 7, 14, 21 and 28). The antagonistic relationship

Table 1. Scores of the symptoms of oil palm seedlings on a disease scale of 0-3.

Disease class	Symptoms of Infection
0	Healthy plants with green leaves without appearance of fungal mycelium on any part of plants
1	Appearance of white fungal mass on infected region (stem) without chlorotic leaves
2	Appearance of white fungal mass on infected region (stem) with chlorotic leaves (1-3 yellowing leaves)
3	Appearance of white fungal mass on infected region (stem) with chlorotic leaves (1-3 browning leaves)

between the isolates were categorised based on the level of antagonism (strong, medium, weak) as illustrated by Pilotti et al. (2002).

Quantification of *G. boninense* ligninolytic enzymes by colorimetric assays

Five mycelia discs (5 mm²) of each fungal strain (GBLS, *a1*, *e2* and *h2*) grown on PDA medium were transferred into 250 ml Erlenmeyer flasks containing 30 ml of malt extract broth (MEB; Merck) and 5.00 g of sterile rubber wood chips. The cultures were then agitated at 120 rpm for 7 days at room temperature for the induction of ligninolytic enzymes. After incubation period, 0.1 M of sodium acetate (SYSTERM®) was added to the liquid medium at a ratio volume of 1:1. The mixture solution was then agitated at 120 rpm for 4 to 5 h before incubating overnight at 4°C. Ligninolytic enzymes produced from each isolates were harvested after the incubation period by filtering through a Whatman Grade 4 filter paper. The enzymes solution was centrifuged (Eppendorf, 5810R) twice at 6000 rpm for 30 min, the supernatants were collected in new tubes as enzyme sources and stored at -20°C.

Laccase activity was verified by the oxidation rate of 0.03% (w/v) 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, ~98 %) as the substrate at 30 °C as described by Murugesan et al. (2007). The assay mixture (100 µl) contained 10 µl of 100 mM sodium acetate buffer (pH 5.0; SYSTERM®), 80 µl of 0.03% (w/v) ABTS (Sigma-Aldrich) and 10 µl of crude enzyme. Absorbance values of the assay mixture were read at 420 nm (E_{420} = 36.0 $mM^{-1}cm^{-1}$) at 1 min intervals for 5 min.

Lignin peroxidase (LiP) activity was quantified by monitoring the oxidation of veratryl alcohol (~96 %) as described by Tien and Kirk (1988). The reaction mixture (200 µl) comprised of 80 µl of 125 mM sodium tartarate buffer (pH 3.0; QRëC), 40 µl of 10 mM veratryl alcohol (Sigma-Aldrich) and 40 µl of crude enzyme. The mixture was incubated at 30°C for 2 min before adding 40 µl of 2 mM hydrogen peroxide (Ajax Finechem). Absorbance values of the reaction mixture were monitored at 310 nm (E_{310} = 9.3 $mM^{-1}cm^{-1}$) at 1 min intervals for 5 min.

Manganese peroxidase (MnP) activity was assayed by the oxidation of guaiacol as enzyme substrate (Patrick et al., 2011). The assay mixture (180 µl) consisted of 20 µl of 0.5 M sodium succinate buffer (pH 4.5; SYSTERM®), 20 µl of 4 mM of guaiacol (Acros Organics), 20 µl of 1 mM manganese (II) sulphate (SYSTERM®), 80 µl of distilled water and 20 µl of crude enzyme. The mixture was incubated at 30°C for 2 min before adding 20 µl of 1 mM hydrogen peroxide. Absorbance values of the reaction mixture were monitored at 465 nm (E_{465} = 12.1 $mM^{-1}cm^{-1}$) at 1 min intervals for 5 min.

For blank reaction mixture (control), equal volume of distilled water was used to replace crude enzyme in these three enzyme assays. Reaction mixtures for each assay were pipetted into individual wells of a 96-flat test plate in triplicates and each enzyme assays were repeated for three times. The absorbance values of these mixtures were measured using a 96-well microplate reader (Thermo Scientific, Varioskan Flash) at respective wavelengths. For all enzymes under evaluation, one activity unit (U) was defined as "the amount of enzyme necessary to produce 1 µmol of product per minute at 30°C" (Murugesan et al., 2007).

Artificial *in vitro* infection technique

The *in vitro* infection technique designed for inoculating oil palm stems with *G. boninense* isolates (GBLS, *a1, e2* and *h2*) in this study is the first report of its use in this plant-fungal interaction. Artificial wounds were first made on the stem regions of healthy oil palm seedlings at approximately 0.50 cm above the crown region by puncturing with sterilised needles (18G × 1.5", Terumo). Mycelium of each *G. boninense* isolate were scraped off the surface of 9 cm² Petri dish cultures and applied directly onto the wounded seedlings. A control treatment was comprised of wounded but non-inoculated seedlings. For treatments, *in vitro* infection of oil palm seedlings was conducted using four replicates with 3 units for each replicate. All treated seedlings were then replanted in growth medium and grown in the growth chamber for three weeks at 27°C, 16 h of light intensity and 50% relative humidity. Three weeks after inoculation, the infected oil palm seedlings were removed from the culture jars and the external *G. boninense* mycelium were washed away with 20% bleach solution, before proceeding with further analytical tests.

Disease severity scale

The symptoms developed from treated oil palm seedlings were observed and evaluated based on the formation of white mycelium and number of chlorotic leaves as derived from the disease severity scale presented by Izzati and Abdullah (2008). The scale comprises four categories, ranging from 0 to 3 as described in Table 1 and Figure 1. Photographs of the infected seedlings were taken using a camera (Panasonic, Lumix LX5) for later assessment on the disease severity index (DSI) of these seedlings. The degree of disease severity caused by *G. boninense* isolates on replicated oil palm seedlings was assessed by calculating the DSI value as shown in the formula below:

$$\text{Disease severity index (DSI)} = \frac{\sum(\text{A X B})\text{x } 100}{\sum \text{B x } 4}$$

Where, A is the disease class ranging from 0, 1, 2, and 3 according to the symptoms of infection (Table 1) and B is the number of plants showing that disease class per treatment (in this case, per isolate).

Microscopic observation of the internal tissues of treated oil palm seedlings

In order to observe the severity of internal tissue damage and the extent of decay on the site of infection on oil palm seedlings, the stem regions were cut into longitudinal slices. A drop of lactophenol

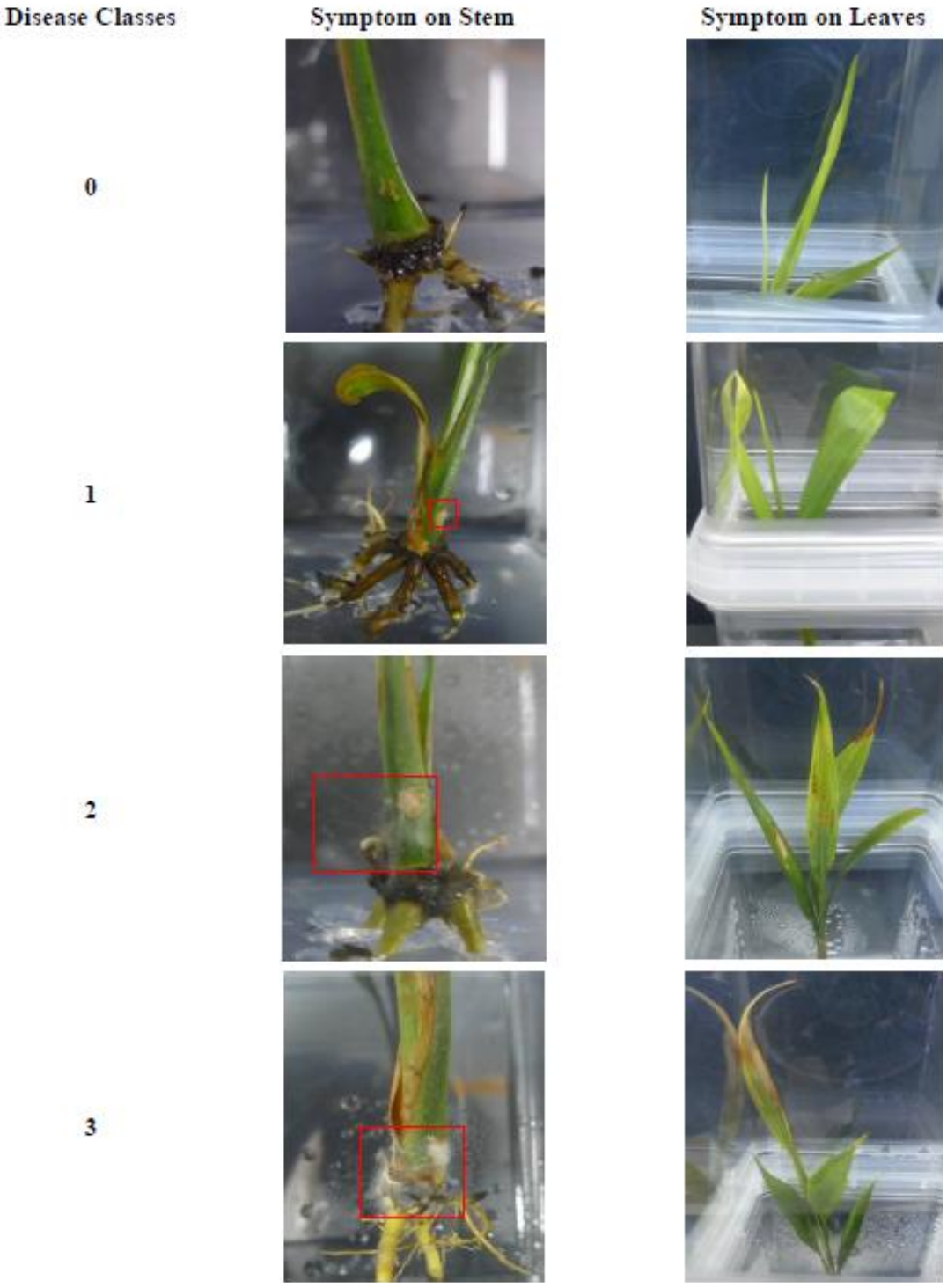

Figure 1. Illustration of the symptoms developed in treated oil palm seedlings of different disease classes.

blue dye was placed onto the samples to stain the *G. boninense* fungal cell walls. The stained stem regions were observed under a light microscope (Nikon, AZ100) using 1x objective lenses and the magnification was adjusted to 30x, 60x and 80x. Photographs for cross sections of seedlings'stem were captured using a camera system (Nikon, DS-Ri1 and NIS Element software).

***G. boninense* mycelia harvesting for DNA extraction and purification**

G. boninense isolates (GBLS, *a1*, *e2* and *h2*) were grown in MEB solution based on a technique modified from Idris et al. (2003). Five mycelial plugs of each *G. boninense* isolate were obtained from the

Table 2. Antagonistic relationships observed between *G. boninense* isolates at Day 21. (X indicated compatible interaction; W, M and S indicated weak, medium and strong incompatible interactions respectively).

Isolate	GBLS	*g1*	*a1*	*c3*	*d4*	*e2*	*h2*
GBLS	X	M	S	W	M	M	S
g1		X	M	W	M	M	X
a1			X	X	M	S	S
c3				X	M	W	M
d4					X	W	X
e2						X	S
h2							X

actively growing region using a sterilised cork borer. These plugs were then transferred into a 50 ml falcon tube containing 25 ml of MEB. The mycelium culture were incubated and remained agitated at 120 rpm for 7 days at room temperature. After incubation, the mycelium cultures were harvested through centrifugation at 10,000 rpm for 20 min. The supernatant was discarded in bleach solution and the pellets were air-dried before grinding with liquid nitrogen.

G. boninense and Oil Palm DNA extraction and purification

Extraction of *G. boninense* and oil palm DNA were carried out based on conventional methods described by Góes-Neto et al. (2005) and Moller et al. (1992) respectively. Before amplifying the extracted DNA from *G. boninense* mycelium and oil palm seedlings through polymerase chain reaction (PCR), the quantity and quality of extracted DNA were measured with a nanodrop spectrophotometer (Thermo Scientific, ND-1000). The quantity of DNA extracted was measured in ng/µl and the quality (purity) was determined by using the ratio of absorbance value at 260/280 nm.

Molecular diagnostic of DNA extracted

PCR amplification technique was carried out to increase the amount of extracted DNA from *G. boninense* mycelium and treated oil palm seedlings. According to Karthikeyan et al. (2006), two 18 mers were selected as the forward and reverse primers in this study to yield a DNA fragment at 167 bp.

DNA of *G. boninense* isolates (GBLS, *a1*, *e2* and *h2*) served as the positive control for oil palm seedlings infected by respective *G. boninense* and distilled water served as the negative control. A PCR thermocycler (G-storm, GS-1) was programmed as follows; 5 min of pre-heating at 95°C followed by 48 cycles consisting of denaturation at 94°C for 40 s, annealing at 45°C for 40 s and extension for 45 s at 72°C with a final extension for 72°C for 12 min.

After PCR reaction, PCR products were analysed by electrophoresis on a 1.5 % (w/v) agarose gel (1st Base) that stained with SyBr Safe (Invitrogen) in 1x TBE buffer (1st Base). GeneRuler 100 bp Plus DNA Ladder (Fermentas) was used as marker. The gel was run at 80 V (Biorad, PowerPad® Basic) for 1.5 h, after which the DNA bands were visualized and photographed under UV transilluminator (Biorad, Gel Documentation XR System).

The DNA bands were then excised and purified by using GF-1 nucleic acid extraction kit (Vivantis) as described in the manufacturer's instructions. Purified DNA samples and the forward primer were sent for single pass sequencing (1st Base) to identify the sequences of the amplified fragment.

Statistical analysis

Data of this study were statistically analysed by one-way analysis of variance (ANOVA), except for the percentage of RBBR dye decolourisation which were analysed by two-way ANOVA. The significant differences between isolates and treatments were detected by Tukey Multiple Comparison Test using GraphPad Prism programme version 5.02.

RESULTS

Vegetative incompatibility test

All self-pairing controls in this test showed compatible reactions (Table 2). Among the 28 antagonistic reactions of different isolates, three of them showed complete compatibilities, five showed strong incompatibilities, 9 showed medium incompatibilities and another 4 showed weak incompatibilities. The morphological illustrations of the compatible, weak, medium and strong antagonistic barrage formed on the plates between these isolates are shown in Figure 2.

Quantification of *G. boninense* ligninolytic enzymes by colorimetric assays

According to the laccase assay (Figure 3A), isolate *a1* possessed the highest level of laccase activity (43.07 UL^{-1}). In contrast, isolate *e2* had the lowest laccase activity (19.19 UL^{-1}). Statistically, the laccase activity between all isolates were significantly different ($P < 0.05$), except for laccase activity between isolates *a1* vs. *h2*.

In MnP assay (Figure 3B), all *G. boninense* isolates tested gave positive MnP results. Isolates *h2* had highest level of MnP activity (16.22 UL^{-1}), signifying that this isolate produced the highest amount of MnP enzyme. The MnP activity detected in isolate *e2* was the lowest. Statistically, the MnP activity between isolates GBLS vs. *e2* and *a1* vs. *h2* were not significantly different ($P < 0.05$) from one another.

Based on the LiP assay graph (Figure 3C), all *G.*

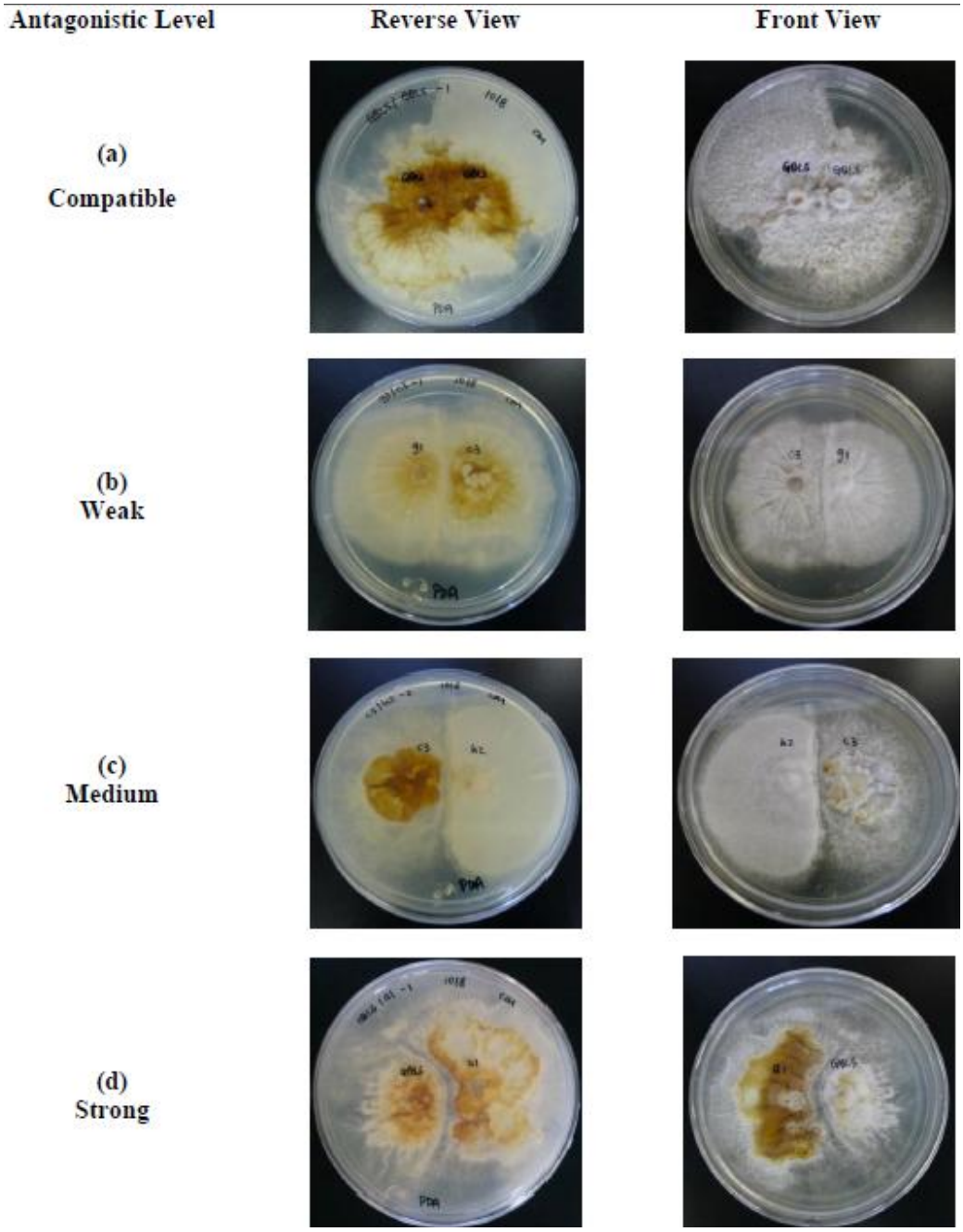

Figure 2. Front and reverse view of the (a) compatible, (b) weak, (c) medium, (d) strong antagonistic relationship among *G. boninense* isolates.

boninense isolates showed no lignin peroxidase activity, except for isolate *e2* that showed a relatively low level of activity (0.27 UL^{-1}), signifying that this isolate produced a low amount of lignin peroxidase enzyme.

Disease severity scale

Oil palm seedlings infected with *G. boninense* isolates showed different disease severity index (DSI) values (Table 3) after three weeks of inoculation. In contrast, non-infected oil palm seedlings which served as the control, remained healthy with green leaves and DSI value of 0.00%. The highest DSI value of 56.25% was in the oil palm seedlings infected with *h2* isolate, followed by *e2* and *a1* isolates. GBLS isolate, which served as the control *G. boninense* isolate, had the lowest value of DSI of 29.55%.

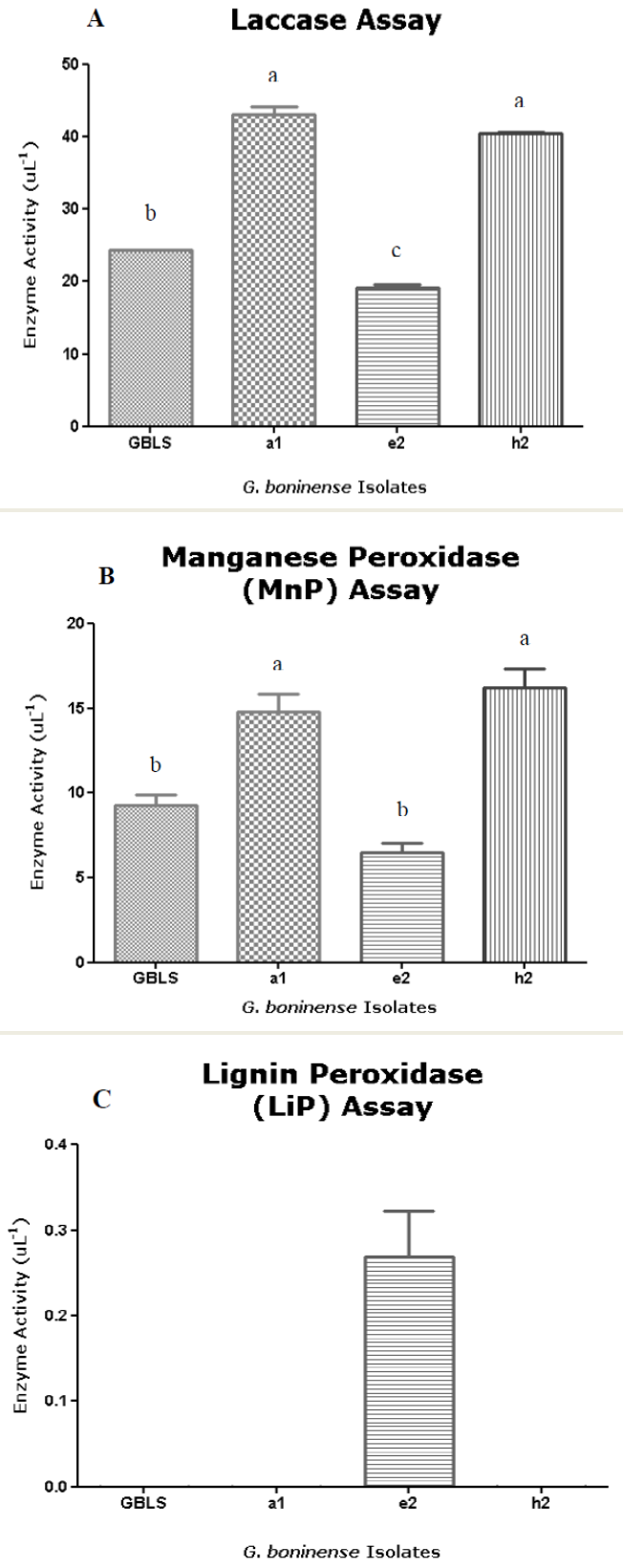

Figure 3. (A-C) Ligninolytic enzyme assays in *G. boninense* isolates. Bars indicated the standard error of mean (SEM) for triplicate culture plates. Means tagged with the different alphabetic letter are significantly different at $P< 0.05$.

Microscopic observation of the internal tissues of treated oil palm seedlings

Before staining with lactophenol blue dye, the cross-section of all oil palm seedlings stem, including control seedlings, showed a necrotic lesion at the wounded region (Figure 4). The necrotic lesions in all infected oil palm seedlings were more severe as compared with the mild lesion in control seedlings, which was believed to be due to the mechanical injury exerted on the stem region during the wounding process. After staining, intense blue mass of fungal mycelium was found around the wounded region of infected seedlings. In contrast, the wounded region of control seedling was unstained and showed no indication of fungal mycelium.

Molecular Assessment of G. boninense DNA in Treated Oil Palm Seedlings

All the DNA samples showed amplified bands at 167 bp (Figure 5), except for the DNA of control uninfected oil palm seedlings (lanes 2 to 4). There was no DNA band amplified in the negative control lane, indicating no contamination in PCR products during the PCR amplification process.

Sequencing confirmed that the isolates used in this study (GBLS, *a1*, *e2*, *h2*) as *G. boninense* after BLAST analysis using the NCBI database. The isolates' sequences recognized by the forward primer (GbF) are as followed:

5'_GATTTTTTCCATTTAGAAAATACTGCTCTCCACTCT
ACACCTGTGCACTTACTGTGGGTTATAGATCGTGTG
GAGCGAGCTCGTTCGTTTGACGAGTTCGCGAAGCGC
GTCTGTGCCTGCGTTTTATCACAAACACTATAAAGTA
TTAGAATGTGTATTGCGATGTAACGC_3'

From the results of BLAST analysis, all the isolates shared 99% similarity with the *G. boninense* sampled by Utomo et al. (2005) in Indonesia.

DISCUSSION

Based on the results obtained in vegetative incompatibility test, variations in antagonistic relationship can be explained by the degree of genetic heterogeneity which affects the reception or rejection of a nucleus within the interacting fungal hyphae of vegetative incompatible isolates (Kope, 1992). Since majority of the pairings of *G. boninense* isolates resulted in weak and medium incompatibility, it suggests that these isolates were genetically related. This assumption was supported in a study conducted by Pilotti et al. (2002), where increasing genetic relatedness of incompatible isolates was linked with a decrease in their somatic interactions. Nevertheless, strong antagonistic responses were still

Table 3. Number of treated oil palm seedlings in different disease classes and their disease severity index (%) after three weeks of inoculation.

Isolate	Disease class (No. of treated oil palm seedlings)					Disease Severity Index* (DSI; %)
	0	1	2	3	Contamination	
Control	12	0	0	0	0	0.00 ± 0.00^{c}
GBLS	3	3	5	0	1	29.55 ± 9.24^{b}
a1	0	4	5	1	2	42.50 ± 4.30^{ab}
e2	0	1	7	2	2	52.50 ± 4.26^{a}
h2	0	1	7	4	0	56.25 ± 3.99^{a}

All values of DSI represent mean ± standard error of mean (SEM) for 4 replicates. *Disease severity index with different alphabetic letters was significantly different at $P < 0.05$ by Tukey multiple comparison test.

found in a small portion of pairings in this experiment, indicating the presence of genetic diversity in *G. boninense* isolates within a single fruiting body. Apart from the self-pairing controls, three pairs of *G. boninense* isolates also showed complete compatible interaction. Lack of antagonism in these three pairings could be either due to non-self recognition in compatible mating, or "switching off" of putative incompatibility genes in these isolates after fusion of their hyphae by unknown underlying mechanisms (Pilotti et al., 2002). From these results, it can be concluded that *G. boninense* is highly heterozygous and the assumption of individualism among the isolates was confirmed.

In this study, laccase and MnP enzymes were produced in greater quantity in all *G. boninense* isolates, ranging from 19.09 UL^{-1} to 43.07 $UL^{-1}$and 6.52 UL^{-1} to 16.21 UL^{-1} respectively. In contrast, the LiP production was significantly lower or undetected in all *G. boninense* isolates. These results obtained from the colorimetric assays were complemented with the findings of Pelaez et al. (1996), where laccase and MnP enzymes were more broadly distributed in white rot fungi including *G. boninense* as compared to LiP. Insignificant amounts of LiP detected in this study may be due to the fact that LiP activity is often restricted to the culturing method utilised for the induction of ligninolytic enzymes (Reddy, 1993). Previous reports also stated that LiP was very difficult to be detected in lignocellulose extracts and only a few reports found the secretion of LiP in their natural substrates, such as wood and straw (Vares et al., 1995; Mester et al., 1998). Furthermore, it was suggested that the production of LiP and MnP enzymes are often inhibited by agitation of submerged fungal mycelium in liquid culture as their production are optimal at high oxygen tension, whereas laccase production can be enhanced by agitation (Wesenberg et al., 2003). Since the induction of ligninolytic enzymes in this experiment was done by agitating the mycelial culture in liquid broth, as predicted the laccase production was predominant in all *G. boninense* isolates.

In addition, negligible or low amount of LiP enzymes produced in all *G. boninense* could be also due to the timing of the onset of production of LiP enzyme (Lankinen, 2004). According to his study, there was no sign of LiP activity during the first 5 days of *Phlebia radiate*'s cultivation and often peaked on day 8 or 9 of growing period. Initial production of LiP in this fungus was most probably masked by interfering compounds in the crude liquid culture, which tend to compete with veratryl alcohol as the substrates and inhibit LiP activity. Hence, it was assumed that the LiP in *G. boninense* isolates after 7 days of cultivation in this study may not reach its peak of production. In fact, RBBR decolourisation test was initially used to perform an early detection and evaluation of ligninolytic enzymes production in *G. boninense* isolates GBLS, *a1*, *e2* and *h2.* However, the results for this experiment were not shown as they were contradicting with the main findings of colorimetric assays.

On the other hand*, in vitro* infection of oil palm seedlings showed that disease symptoms only developed in infected oil palm seedlings, but not in the control seedlings. Since the entire experiment was done in an *in vitro* approach and the treated oil palms seedling were incubated in similar controlled conditions, the effects of environmental factors and biotic stress agents such as insects were not considered (Kozai et al., 1997). Furthermore, there was very little possibility that these symptoms were due to wound-induced injuries as all the control seedlings (wounded but non-infected) remained healthy. Hence, it was deduced that the external symptoms found on the infected oil palm seedlings were solely based on the effects of *G. boninense* inocula. In addition, the time required for disease symptom development in this study was found to be much shorter (three weeks) as compared with some previous studies in which oil palms were artificially infected using rubber wood blocks *in vivo*. According to Izzati and Abdullah (2008), the first disease-induced symptom was found after 14 weeks with 12.50% DSI value. Similarly, another recent study done by Naher et al. (2012) showed that disease symptoms were only detected on the *G. boninense* infected oil palm after five weeks after

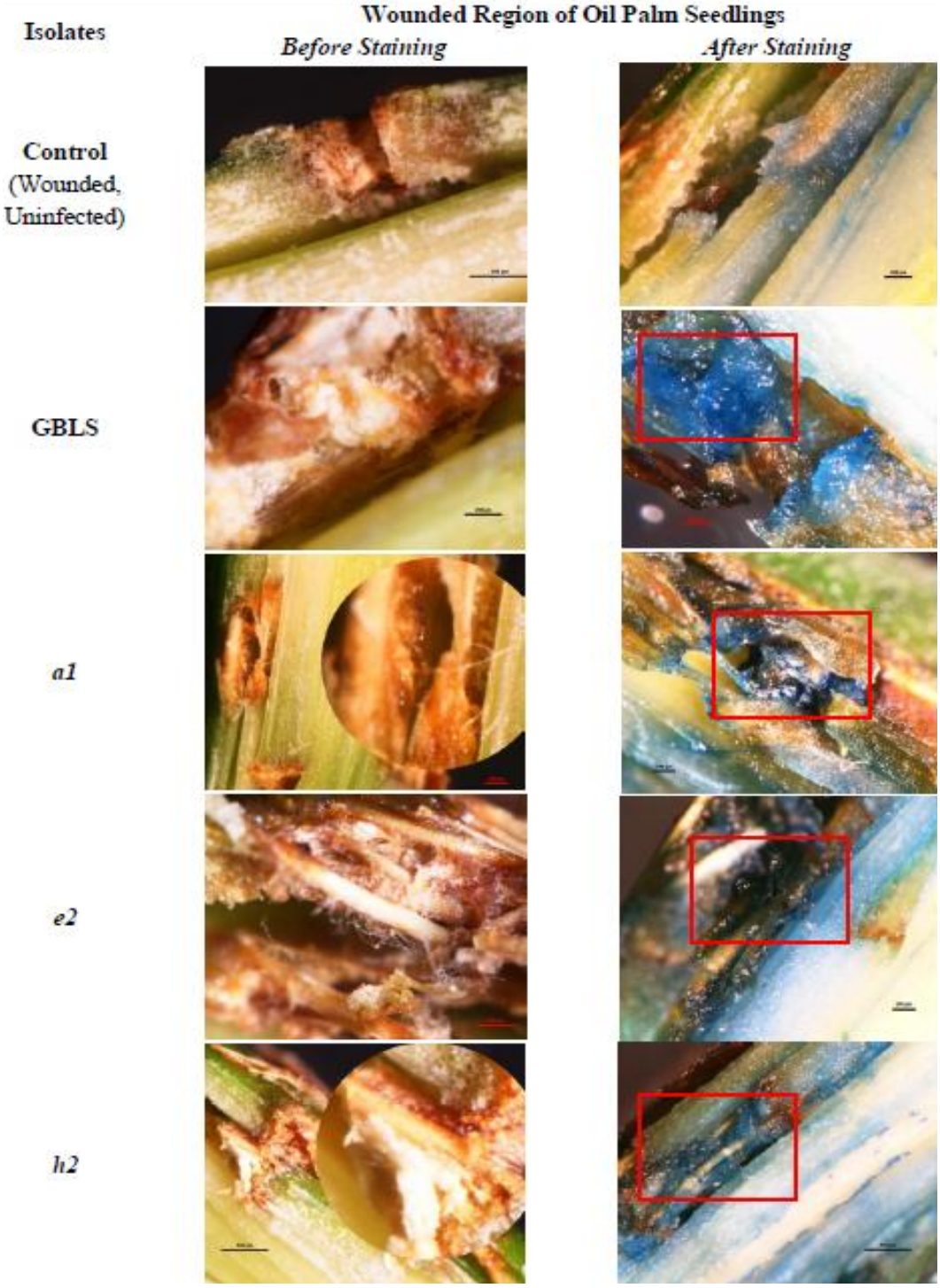

Figure 4. Microscopic observation of the infected region of oil palm seedlings before and after staining with lactophenol blue dye.

inoculation at 8.30% DSI value.

All *G. boninense*-infected oil palm samples and *G. boninense* mycelia DNA showed amplification at location 167 bp, indicating the presence of *Ganoderma* in all parts of infected oil palm seedlings (leaves, stems and roots). These results were complemented with previous findings by Karthikeyan et al. (2006; 2007) that they adopted similar primer sequences (Gan1 and Gan2) which amplified a DNA fragment of the size of 167 bp internal transcribe spacer (ITS) region when *Ganoderma* isolates

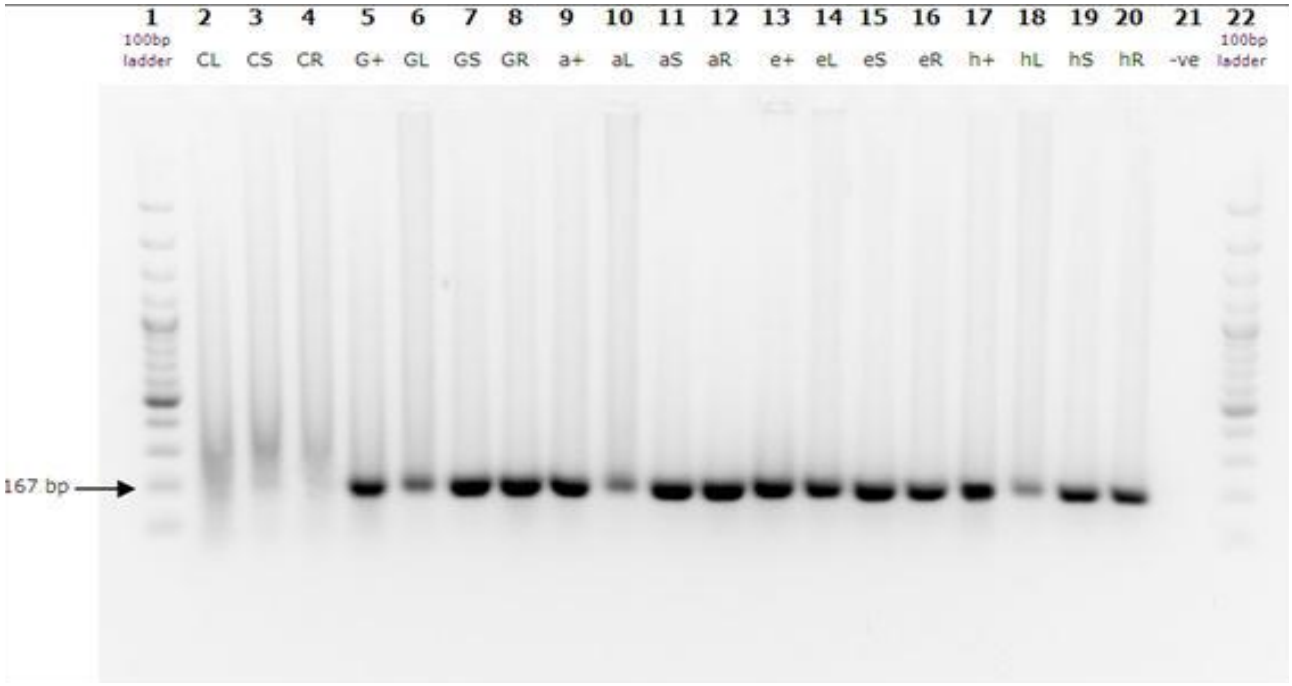

Figure 5. PCR amplification of the DNA of infected oil palm seedling with *G. boninense* isolates using GbF and GbR primers. Lane 1 and 22: 100 bp DNA ladders; Lane 5, 9, 13 and 17: Positive control of GBLS (G), *a1* (a), *e2* (e) and *h2* (h) fungal DNA; Lane 2-4: Leaves (L), stems (S) and roots (R) DNA samples of control uninfected oil palm seedlings; Lane 6-8, 10-12, 14-16 and 18-20: DNA samples of infected oil palm seedlings; Lane 21: Negative control.

were used for amplification.

From this study, all of the *G. boninense* isolates (GBLS, *a1*, *e2* and *h2*) were vegetatively incompatible with strong antagonistic relationships. Isolates *a1*, *e2* and *h2* originated from a similar fruiting body, whereas isolates GBLS was isolated from another fruiting body. According to the results of disease severity, the DSI values in oil palm seedlings infected with isolates *a1*, *e2* and *h2* were not significantly different ($P < 0.05$) from one other, but were significantly different ($P < 0.05$) from the seedlings infected with isolate GBLS. This suggested that *G. boninense* isolates originated from similar fruiting body had relatively similar level of infection potential, although they showed strong level of vegetative incompatibility. This phenomenon was probably because of isolates originated from a single fruiting body are genetically related (Pilotti et al., 2002).

Overall, *G. boninense* isolates (*a1* and *h2*) with high ligninolytic enzymes production resulted in relatively high DSI values (42.50 and 56.25%, respectively) when these isolated were used to infect oil palm seedlings through *in vitro* infection. In contrast, seedlings infected by isolate GBLS with lower laccase and MnP enzyme activities (24.31 and 9.27 UL^{-1}, respectively) obtained the lowest DSI value (29.55%) after the inoculation period. These results indicated that *G. boninense* isolates with higher laccase and MnP production had higher infection potential as they can cause more severity on the oil palm seedlings when these particular isolates were used to infect the seedlings *in vitro*. Hence, it was deduced that there was a direct relationship between the ligninolytic enzyme production and the infection potential in vegetative incompatible *G. boninense* isolates. This relationship is supported by Ali et al. (2004), who clearly indicated that pathogenic *G. boninense* produced lignin degrading enzymes (ligninolytic enzymes) and diminished the lignin content in dead oil palm samples *in vitro* when they were used to infect oil palm. Although a direct relationship between the ligninolytic enzyme production and infection potential cannot be drawn from the results for isolate *e2*, one explanation could be due to the present of LiP enzymes produced by this isolate. According to Paterson (2007), LiP is capable to attack wider range of linkages, including the oxidation in both phenolic and non-phenolic compounds, suggesting its strong impact in lignin degradation even if it is present at low level. Since LiP is also a type of ligninolytic enzyme, it was deduced that the ligninolytic enzymes in isolate *e2* directly affect its infection potential in oil palm seedlings as well.

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