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Polyclonal antibodies of *Ganoderma boninense* isolated from Malaysian oil palm for detection of basal stem rot disease

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Basal stem rot (BSR) disease caused by the fungus, *Ganoderma boninense* has become a serious threat to the oil palm industry, especially in Southeast Asia (SEA). A highly selective and sensitive diagnostic tool for BSR is extremely required for early detection, and thus, development of immunological test using enzyme-linked immunosorbent assay-polyclonal antibody (ELISA-PAb) was evaluated. Results indicate that ELISA-PAb shows recognition of *Ganoderma* species associated with BSR except for *G. tornatum*. Cross-reactivity test with fungi commonly found in oil palm plantation revealed observation of some cross-reactions with some saprophytic fungi. ELISA-PAb shows better detection as compared to cultural-based method, *Ganoderma* selective medium (GSM) with an improvement of 18% at nursery trial. The present study also demonstrates sensitive detection on ELISA-PAb with an increment of 30% as compared to GSM test at field trial using oil palm roots and stems. Polyclonal antibodies raised against *G. boninense* with positive signals was achieved, however, not specific enough for detection of BSR disease caused by *Ganoderma*.

Key words: *Ganoderma boninense*, basal stem rot, polyclonal antibodies, enzyme-linked immunosorbent assay (ELISA).

INTRODUCTION

Palm oil is an important commodity to the world's largest palm oil producing countries, Malaysia and Indonesia. In Malaysia, the oil palm industry has significantly contributed to the rapid economic development of the

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Abbreviations: BSR, Basal stem rot; **ELISA,** enzyme-linked immunosorbent assay; **OD,** optical density; **MAb,** monoclonal antibody; **PAb,** polyclonal antibody; **EDTA,** ethylene diamine tetraacetic acid; **PBS,** Phosphate buffered saline; **PBST,** Phosphate buffered saline with tween 20; **HRP,** horseradish peroxidase; **IgG,** immunoglobulin; **ABTS,** 2,2'-azino-di-ethyl-benzothiazoline-6 sulfonic acid; **SDS-PAGE,** sodium dodecyl sulphate-polyacrylamide gel electrophoresis; **GSM,** *Ganoderma* selective medium.

country. Demand on the palm oil has lead to the increment of oil palm planted area in Malaysia which reached 5.08 million hectares, with an increase of 1.5% in 2012 against 5 million hectares recorded in 2011 (MPOB, 2013). Nevertheless, rapid growth of the oil palm industry has contributed to the fast movement and distribution of pests and diseases from other regions. Among others disease, basal stem rot (BSR) has pose a serious threat to oil palm plantation where infection can kill up to 80% stand palms in replanted areas (Ariffin et al., 2000; Turner and Gillbanks, 2003) or under planted areas with coconut palms (Idris et al., 2000; Turner, 1965).

In recent years, much attention has been given to BSR disease as it being the most destructive disease infecting oil palm in Southeast Asia (Turner, 1981). Earlier studies have made clear that, at least four different *Ganoderma* species is associated to BSR disease with *G. boninense* being the most pathogenic against oil palm (Idris, 1999). This disease has become a serious threat to oil palm industries in Malaysia which causes great losses of stand palm due to death (Ariffin et al., 2000). The disease can only be recognized at a very late stage with serious symptoms of foliar chlorosis and breakage at older fronds, presence of decayed tissues at palm base and production of fruiting bodies (Utomo and Niepold, 2000). The stem rotting caused restriction of water and nutrients uptake to the fronds; thus, promote the collapsing of palm trunk (Turner, 1981). In older palms, the disease was easily spread to neighbouring palms by root to root contact (Singh, 1991). BSR was also found in younger palms aged 10-15 years old; resulted to an unopened sheath leaves symptom (Turner, 1981). Once BSR was identified, younger palms normally died within 6-24 months, whereas, the matured palms survived lesser than two to three years (Idris, 1999, 2011). BSR disease was highly found in area replanted from coconuts and oil palms in inland area (Turner, 1965) and peat area (Azahar et al., 2011).

To date, there is effective controlling method or robust diagnostic tools for detecting the BSR disease at early stage. Generally, the detection of the disease at early stage is done in three conventional methods using drilling technique (Ariffin et al., 1993), chemo diagnostic test using ethylene diamine tetraacetic acid (EDTA) which was done to diagnose Thanjavur wilt disease caused by *Ganoderma lucidium* (Natarajan et al., 1986) and semi selective media for *Ganoderma* cultivation on agar plates (Ariffin et al., 1993). However, these methods were time consuming and gave low accuracy, hence, a rapid, economical and accurate method were urgently required to optimise fungicide use for prolonging the life span of the infected oil palm as the curative treatments currently are unavailable. A nucleic acid-based technique developed by Utomo and Niepold (2000), requisite on detection of specific DNA sequences in the genome and proper laboratory environment was required (McCartney et al., 2003). This method may produce false positive results if the sterilization and aseptic techniques are not practised correctly. Due to limitation of the sample preparation, specific antibodies offer more rapid diagnostic than nucleic acid-based techniques (Ward et al., 2004).

Immunological methods by manipulating antibodies have widely been used in detecting bacteria, viruses (López et al., 2003), fungi in roots, soil and plant materials (Cotado-Sampayo et al., 2008; Safarnejad et al., 2011; Walcott, 2003). Mostly antibodies produced by manipulating animals such as rabbits, mice and chicken, and most recently, recombinant antibodies produced by mammalian cell line was discovered (Frenzel et al., 2013). Antibodies are used by the immune system to identify and nullified foreign objects andn have been used to investigate presence of various fungi with different degrees of specificity (Thornton and Wills, 2013) as a diagnostic tool in various fields such as plant pathology, pharmaceutical and medicine (Alvarez, 2004).

The use of monoclonal and polyclonal antibodies in immunochemical techniques such as enzyme-linked immunosorbent assay (ELISA) offer greater simplicity and fast diagnostic than DNA probe analysis such as PCR (Bridge et al*.,* 2000; Darmono, 2000). Monoclonal antibodies are mostly more specific and sensitive than polyclonal antibodies in determining the target pathogen even in low concentration with a high degree of accuracy (Tsai et al., 1992). Successful works on monoclonal and polyclonal antibodies by ELISA has been reported previously. Diagnostic by monoclonal antibody (MAB) in mycology studies was carried out for the detection of *Puccinia striiformis* urediniospores that caused yellow rust disease in wheat plants (Skottrup et al., 2007), and for the detection on *Spiroplasma citri* and *S. kunkelii*, the plant pathogen for citrus stubborn disease and corn stunt disease (Jordan et al., 1989). Other successful detection using polyclonal antibody (PAB) was reported on *Ganoderma lucidum* from coconut palm (Rajendran et al., 2009), *Alternaria alternate* in tomato and potato plants (Smith, 1993) and also detection of *Aspergillus parasiticus* in contaminated corn, rice, wheat and peanut (Guo-Jane and Shou-Chin, 1999) and detection of Streptomyces species in soil samples (Sangdee et al., 2012). Polyclonal antibodies was commonly used for detection of human infection as in production of Tas transactivator for detection of foamy virus (Qiu et al., 2012), detection of *Escherichia coli* using Shiga Toxin 2 in human (He et al., 2013) and to study human collectin 11 (CL-11) levels somewhat related to human diseases and symptoms (Selman et al., 2011).

Presently, detection of *G. boninense* using immunological methods neither have not broadly been practiced nor utilised for screening of BSR disease. Development of polyclonal and monoclonal antibodies against *G. boninense* isolated from Indonesia were reported, which showed unevenness of detection (Utomo

and Niepold, 2000; Darmono, 2000). Study by Shamala et al. (2006) has successfully produced monoclonal antibody (MAb) against *G. boninense* using Malaysian oil palm isolate; however cross-reactivity highly occurred. Hence, in this study, our aim was to develop polyclonal antibodies against *G. boninense* using the vast virulent isolate to oil palm, which was discovered in highly infected oil palm plantation with BSR disease in Malaysia. In this paper, we describe the production and application of specific PAbs against *G. boninense* for BSR disease detection using modified ELISA method. The results from the experiments conducted in the nurseries and fields in Malaysian oil palm plantations describe their diagnostic potential.

MATERIALS AND METHODS

Preparation of *Ganoderma* antigen

Pure culture of *G. boninense* isolate PER71 was obtained from culture collection of GanoDROP unit, Malaysian Palm Oil Board, Bangi, Malaysia. Potato dextrose agar (PDA) was used for culture maintenance according to Wagner et al. (2003) in *G. lucidum* study. After 7-10 days of incubation, the actively growing mycelium was cut and transferred to sterile conical flasks containing 100 mL potato dextrose broth (PDB) and incubated at 28°C for 14 days. The mycelia cultures was harvested by vacuum filtration, subsequently rinsed with distilled water and blotted dry using sterile Whatman No.1 filter paper. Mycelium (0.5 g) was ground using a pre-cooled sterile mortar and pestle in the presence of liquid nitrogen. Then, suspended in 1.5 mL phosphate buffer saline (PBS: 8 gL^{-1} NaCl, 0.2 gL^{-1} KCl, 2.9 gL^{-1} Na_2HPO_4, 0.2 gL^{-1} KH_2PO_4, pH 7.4), vortexes thoroughly for a few seconds and centrifuged at 9000 rpm for 20 min at 4°C. Supernatant was separated and purified using ammonium sulphate (70%) precipitation. Precipitated protein was referred to as antigen and suspended in PBS buffer for further analysis.

SDS-PAGE analysis and protein profilling

Protein concentration was determined using Bradford assay (Bradford, 1976) based on bovine serum albumin (BSA) standard. Protein molecular mass was determined using 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Protein was run in equal concentration in the SDS-PAGE gel. Gel was stained with Coomassie Brilliant Blue G 250 and destained with destained-buffer (Blakesley and Boezi, 1977). Protein profiling of *G. boninense* was also conducted using Liquid Chromatography Mass Spectrophotometry (LC-MS) analysis to identify the amino acid profiling. Amino acid analysis was provided by Chemical Engineering Pilot Plant (CEPP), Universiti Teknologi Malaysia (UTM), Skudai, Johor, Malaysia.

Immunization and polyclonal antibodies (PABs) production

Ganoderma antigen was prepared in PBS and concentration was adjusted to 200 µg/mL for the injection. Three adult New Zealand white rabbits initially were given four intramuscular injections in 1:1 (v/v) Freuds complete adjuvant (FCA, Difco, USA). Further boosting immunization was done two weeks later with another injection of 200 µg/mL in 1:1 (v/v) Freuds incomplete adjuvant (FIA, Difco, USA). Rabbits received injections in each treatment from Day-0 until Week-18. Blood (20 mL) was taken from the rabbits two weeks after each injection, subsequently the titre of anti-serum was analysed for immunoreactivity towards *G. boninense* and detected by indirect ELISA. Blood samples were allowed to clot at 37°C for 1 h and stood overnight at 4°C to retract. Anti-serum was collected after a centrifugation at 1500 rpm for 20 min to remove the remaining red blood cells. Harvested anti-serum was stored at -20°C for further analysis.

Enzyme-linked immunosorbent assay (ELISA)

Fifty microliters of anti-serum (2 µg/mL) diluted in coating buffer, PBS (pH 7.4) was incubated overnight in the ELISA plate at 4°C. The plate was washed with 200 µL phosphate buffer saline with tween 20 (PBST) three times, blocked with 5% skim milk at 37°C for 2 h, and washed again with PBST three times. About 50 µL of anti-serum at different dilutions (1:10, 1:100, 1:1000 and 1:10,000) was incubated per well at 37°C for 1 h. After washing with PBST three times, 50 µL horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (IgG) (JacksonImmunoLab, New York) at 1:5000 dilution was added to each well and incubated at 37°C for another 1 h. Plate was washed another three times with PBST. Colour reaction was developed by adding the 50 µL/well azino benzothiazoline sulfonic (ABTS; 2, 2'-azino-di-[3-ethyl-benzothiazoline-6 sulfonic acid) and reaction was stopped by the addition of 50 µL/well of 2 M H_2SO_4. Hydrolysed substrate was read at 405 nm with microplate reader according to optical density (OD). Analyses on the data was done by plotted the standard curve from the series of concentration serial dilutions of serum (X-axis/log scale) against the absorbance (Y-axis/linear). All statistical analysis was done through analysis of variance (ANOVA) with the mean compared by the Least Significant Difference (LSD) at P-value ≤ 0.05 using Statistical Analysis System (SAS) software.

Cross-reactivity test with fungi associated in oil palm plantation

Specificity was determined by ELISA assay using the fungi commonly found in the oil palm plantation in Malaysia. Pure culture of fungi tested in this study was obtained from the culture collection of GanoDROP unit, Malaysia. Antigen preparation of each fungus was obtained according to the *Ganoderma* extraction as mentioned previously. Equal concentration of protein was prepared for ELISA-PAb test against *Ganoderma*. Fungi used for cross-reactivity test are *G. zonatum*, *G. miniatocinctum* and *G. tornatum*. Others fungi commonly found in oil palm plantations were also tested, these are *Penicillium sp,. Marasmius palmivorus*, *Thielaviopsis paradoxa*, *Trichoderma* spp., *Aspergillus niger, Trichoderma virens, Trichoderma harzianum, Curvularia* sp*., Helminthosporium* sp., *Pestalotiopsis* sp*., Schizophyllum* sp*., Fusarium* sp*., Botryodiplodia sp.* and *Melanconium* sp. All ELISA-PAb test on cross-reactivity was done in three replicates.

Nursery evaluation in seedlings artificially inoculated with *G. boninense*

In nursery test, oil palm (DxP) aged 3 months old, was challenged with *Ganoderma* via artificial inoculation with *G. boninense* using rubber wood block (RWB) sitting technique as described by Idris (1999). Blocks sized 6 x 6 x 12 cm, were prepared by incubating the *G. boninense* inoculum onto RWB for 3 months. A total of 30 palms were conducted in the test which consisted of two treatments: infected palms with *G. boninense* and uninfected palms (control). The experiments were laid out in completely randomized

design (CRD) with three replicates. Samples from leaves, stems and roots were collected and surface sterilization was performed prior to extraction of the protein. Preparation of the protein was done exactly according to antigen preparation. Protein concentration was then determined by using Bradford assay to define the minimum level of coating concentration of protein on wells with sufficient amount of antigen for immunization and ELISA protocol. Protein was stored in the -20°C for further analysis. This experiment was repeated in triplicates.

Field evaluation in oil palm infected with *G. boninense*

ELISA-PAb test was also carried out for evaluation of field samples. A total of 120 matured palm with healthy-looking and symptoms of *Ganoderma* incidence (presence of some *Ganoderma* symptoms such as basidiomycetes fruiting bodies, yellowish leaves, broken fronds on the petiole and skirting around the palm trunk, production of stunted shoots or unopened spear leaves) were spotted randomly and collected from three different oil palm plantations: Teluk Intan, Perak; Kluang, Johor; and Sepang, Selangor. Samples from leaves, stems and roots were collected and surface sterilization was done to minimize the contamination. Cultural-based technique using GSM (Ariffin and Idris, 1991) was done subjected to obtain pure culture of fungi from each sample. Tissue samples were ground, suspended in PBS buffer, filtered and precipitated prior to getting the protein. Protein concentrations were determined by Bradford assay and subsequently continued to ELISA-PAb test.

RESULTS AND DISCUSSION

Polyclonal antibodies

Crude protein of *G. boninense* was extracted with total concentration of 1.60-2.58 mg/mL. SDS-PAGE image of the crude protein revealed that *G. boninense* consists of protein ranging from 10-220 kDa. Native protein size in this study was relatively higher than a study reported by Darmono (2000) with 70 kDa of *Ganoderma*'s protein from Indonesian isolates. Wide range of protein sizes might be due to collation of extracellular, intracellular enzymes and others protein since *Ganoderma* can colonise oil palm hard-fibre with alterations in cellulose, hemicellulose and lignin contents (Abe et al., 2013). However, the enzymes mechanism of oil palm was not clearly explained.

A total of 16 amino acids were determined from crude protein of *G. boninense* by using LC-MS and amino acid analyser. Protein analysis showed that the proline (Pro) was the most abundant amino acid in *G. boninense* at 40.15 µmol/mL followed by glycine (Gly) at 30.0 µmol/mL, glutamic acid (Glu) at 28.5 µmol/mL and valine (Val) at 26.65 µmol/mL (Figure 1). However, ammonia and cyctein (Cys) were undetectable. A high amount of proline residue identified from crude protein of *G. boninense* may become a key answer to the aggressiveness and noxiousness of *G. boninense* to oil palm. As been described by Szabados and Savouré (2009), proline produced highly in plants during environmental stress such as drought, salinity and biotic stress and was important for its tolerance towards stress conditions. Presence of proline was considered as protection of subcellular structure and macromolecule against environment and natural enemies for recovery purposes. Hence, proline accumulation in most plants, demonstrated that, it has diverse role to confer osmotic tolerance and adverse effects as plant protection and development by scavenging reactive oxygen species (Kishor et al., 2005; Matysik et al., 2002; Rhodes et al., 1999). In mutualistic fungi, it was proposed that proline help plants to notice the stress at soonest by activating the plant biochemical reactions that lessen the stress impacts (Rodriguez et al., 2004). Meanwhile, study by Chen and Dickman (2005) on a fungal pathogen, *Colletotrichum trifolii*, reported that proline protects *C. trifolii* against stresses including UV light, hydrogen peroxide, salt, and heat. Interestingly, the restoration of pathogen requires only proline that protect pathogen from death.

Thus, this gave a suggestion that in *G. boninense*, proline might have a role in response adaptation and support the organism to withstand the plant's biological counterattack or other good fungal pathogen in order to initiate the host. Transgenic plants which are unable to produce proline, proved to have significantly lower stress tolerance (Kishor et al., 2005).

Crude protein of *G. boninense* was used as antigen to obtain specific antibodies from rabbits. ELISA test was applied to evaluate the optimal polyclonal antibody titre. Antibody titre is defined as the lowest dilution to bind significantly to the antigen and as a simplest method to assess whether an immune response has occurred in the immunised animals against *Ganoderma*'s specific antigen.

Result shows that low PAb concentration at dilution of 1:10,000 was sensitive enough for the detection (Figure 2). Result also suggests that, at weeks-8, the antibody was sufficiently being detected by the ELISA-PAb. In related study, higher titres of polyclonal were found with 1:15,000 of *Ganoderma* from Indonesian isolates (Utomo and Niepold, 2000) and 1:256,000 of banana streak virus from Nigerian isolate (Agindotan et al., 2003).

Three trials done for specificity test resulted to the detection of four species of *Ganoderma viz*. *G. boninense*, *G. miniatocinctum, G. zonatum* which generally were found associated with BSR disease in oil palm with 100% of identification except for *G. tornatum* (Table 1). All three *Ganoderma* excluding *G. tornatum*, were reported as pathogenic to oil palm after a Koch Postulate analysis (Idris, 1999).

It was suggested that, all pathogenic *Ganoderma* have high similarity of recognition site in the antigen-antibody interaction, both acted as a key (antigen) and lock (antibody) conformation. Meanwhile, the non-pathogenic, *G. tornatum* offers partially conserved fragment since the percentage of detection is lesser at 66.7% on ELISA-PAb test but none detection was obtained from GSM. The specificity test conducted in this study, suggested that the pathogenic and non-pathogenic *Ganoderma* cannot be

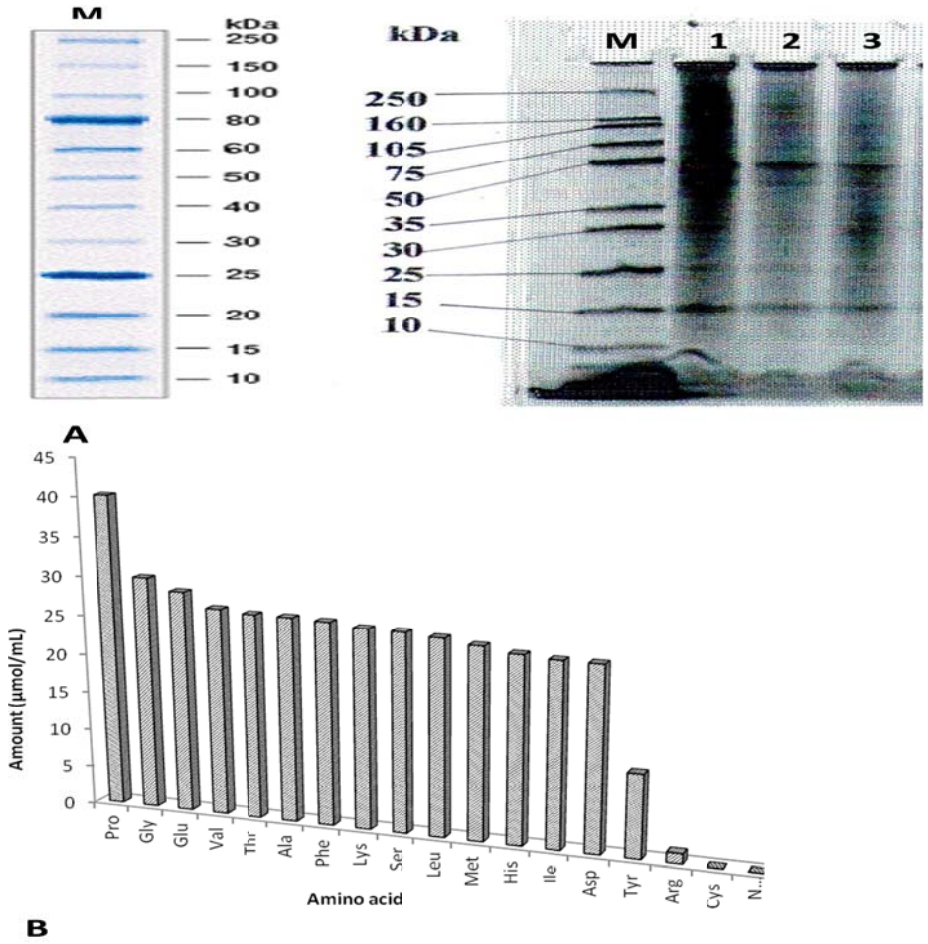

Figure 1. Characterization of *G. boninense* protein. **(A)** SDS-PAGE profile of *Ganoderma* antigen with the protein amount indicated in kilo Dalton (kDA). **(B)** Amino acid profiling using LC-MS analysis with the value indicated in µmol/mL.

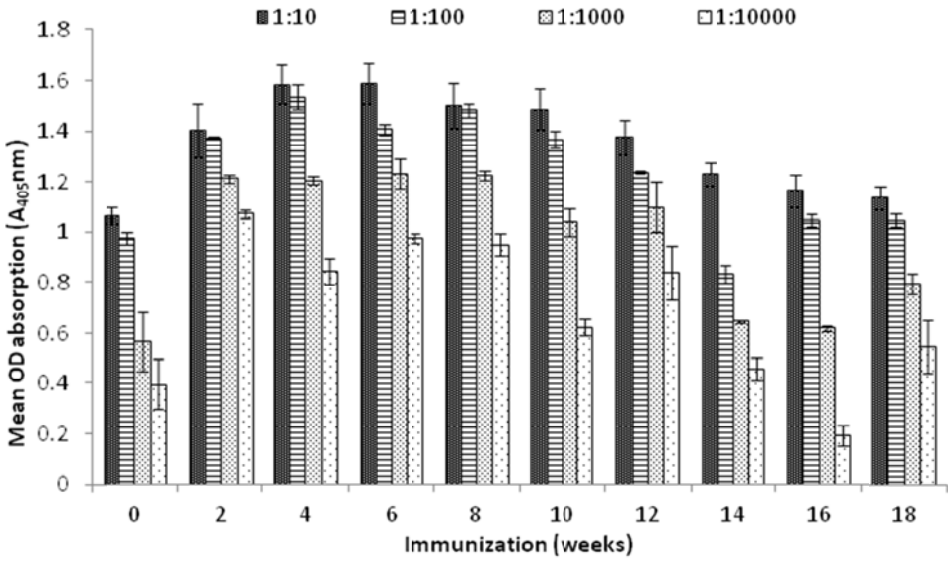

Figure 2. Determination of polyclonal antibodies titre. Substrate incubation was for 1 h at 37°C. All tests were done in triplicates.

Table 1. Cross-reactivity test of polyclonal antibodies using ELISA-PAb and GSM against various fungi isolated from oil palm plantations; N= 30.

Isolate	Pathogenicity test to oil palm	Mean of detection (%)	
		ELISA-PAb (%)	GSM (%)
G. boninense	Pathogenic (field disease)	100 ± 0[a]	100 ± 0[a]
G. zonatum	Pathogenic (field disease)	100 ± 0[a]	100 ± 0[a]
G. miniatocinctum	Pathogenic (field disease)	100 ± 0[a]	100 ± 0[a]
G. tornatum	Not pathogenic	66.7 ± 0.58[b]	100 ± 0[a]
Aspergillus niger	Not pathogenic	0 ± 0[c]	0 ± 0[b]
Penicillium spp.	Not pathogenic	100 ± 0[a]	0 ± 0[b]
Trichoderma virens	Not pathogenic	0 ± 0[c]	0 ± 0[b]
Trichoderma harzianum	Not pathogenic	0 ± 0[c]	0 ± 0[b]
Curvularia sp.	Pathogenic (leaf disease)	0 ± 0[c]	0 ± 0[b]
Helminthosporium sp.	Pathogenic (leaf disease)	0 ± 0[c]	0 ± 0[b]
Pestalotiopsis sp.	Pathogenic (leaf disease)	0 ± 0[c]	0 ± 0[b]
Schizophyllum sp.	Pathogenic (leaf disease)	0 ± 0[c]	0 ± 0[b]
Fusarium sp.	Not pathogenic	0 ± 0[c]	0 ± 0[b]
Marasmius palmivorus	Pathogenic (field disease)	100 ± 0[a]	0 ± 0[b]
Thielaviopsis paradoxa	Pathogenic (field disease)	100 ± 0[a]	0 ± 0[b]
Botryodiplodia sp.	Pathogenic (field disease)	0 ± 0[c]	0 ± 0[b]
Melanconium sp.	Pathogenic (field disease)	0 ± 0[c]	0 ± 0[b]

Means with different letters within a column are significantly different according to the t-test at $p<0.05$ using least significant difference (LSD). Note: PAb, polyclonal antibody; GSM, *Ganoderma* selective medium.

distinguished by using ELISA-PAb.

In this study, cross-reactivity test done using 17 various saprophyte fungi found in oil palm plantations revealed that *Penicillium* sp., *Marasmius palmivorus* and *Thielaviopsis paradoxa* were detected significantly using ELISA-PAb (Table 1). However, extensive cross-reactivity throughout *Ganoderma* and various fungi demonstrated the ability of false-positive values on unrelated fungus isolates. The occurrence of false-positive reaction is a serious drawback in the use of polyclonal antibodies (Griep, 1999; Utomo and Niepold, 2000).

In most cases of polyclonal antibodies as immune-assay especially for *Ganoderma* disease, cross-reactivity with saprophytic fungi is well-known since the fungi classified as complex organism comes with numerous antigen and may share with other unrelated or closely related fungi (Utomo and Niepold, 2000). However, the positive results on cross-reactivity to others fungi, might be because they were prominent fungi that commonly attack oil palm in a minor cases such as basal stem trunk caused by *Thielaviopsis paradoxa* and *Marasmius palmivorus,* causal of crown disease in oil palm (Turner, 1981). Meanwhile, *Penicillium* sp. known as ubiquitous fungi might be presence in the test due to the attribution of the antigen or cross-contamination since it was easily found in the nature environment.

The preparation of sufficiently polyclonal antibodies specifically to *Ganoderma* is very difficult as there is strong serological relationship with saprophytic fungi. Either for *Ganoderma* polyclonal or monoclonal anti-bodies, the illustration of the cross-reactivity with some fungus isolates have been reported by Shamala et al. (2006) and Utomo and Niepold (2000). By some reasons, the *Ganoderma* polyclonal antibodies failed to induce antibody response towards specific target protein which may be due to the poor antigenicity of an antigen produced and conservation of the peptide sequence in some species. It is particularly true for anti-peptide anti-bodies and in certain cases, high titre of antibodies generated against antigen may not recognize the peptide full-length either in Western or immunoassay (Biomatik, 2011).

Nursery evaluation

Samples taken from roots, stems and leaves were tested for *Ganoderma* infection using ELISA-PAb test and in-parallel with GSM method (Ariffin and Idris, 1991). *Ganoderma* PAbs produced in this study was found sensitive in distinguishing all field samples in roots and stem tissue. In the nursery trial, a total of 30 palms were tested and showed an average of 88.9% (ELISA-PAb) and 71.1% (GSM) of detection from roots samples in infected palms against healthy palm (0%) ($p<0.05$) (Figure 3). Similar results were observed from stem samples with an average of 82.2% using ELISA-PAb as

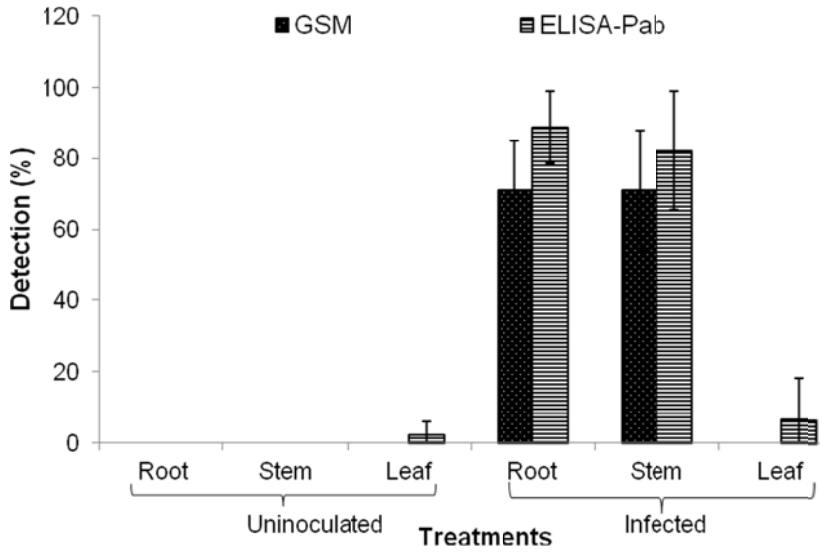

Figure 3. Nursery trial in detection of *Ganoderma* disease in oil palm seedlings using GSM and ELISA-PAb against uninoculated and artificially infected palms by *G. boninense*; N=90. GSM, *Ganoderma* Selective Medium.

compared with 71.1% using GSM ($p<0.05$) for artificially infected palms. For the remaining palms, no detection on the control (non-inoculated seedlings with *Ganoderma*) was observed. Results persisted negative or almost negative using leaves sample for both treated and non-treated palms.

Among the treatments, ELISA-PAb and GSM analysis only showed the highest percentage of detection on roots and stems but almost none using leaves samples. This may indicate that the slighter response of antibody in the leaves was observed compared to the roots and stems. As the antigen produced from the pure culture of *G. boninense* and not from systemic response of plant towards *Ganoderma*, hence, the response on leaves was lesser and not specific enough to be recognized by ELISA-PAb. The response of plants elicited by *Ganoderma* did not occur synchronously as the infection occurred based on root-to-root contact and subsequently, colonized the plant bole after the infection take place (Ariffin and Idris, 1991). During the infection, *Ganoderma* was either localized to the initial point of contact or completely enveloped the root at the point of contact through epidermis and exodermises (Flood et al., 2010). Rapid colonization of *Ganoderma* was observed through roots and into lower stem or bole by production of brown discoloration at infected area (Darmono, 1998, 2000; Flood et al., 2010).

To achieve accurate results, it was suggested that the sensitivity and accuracy may be obtained from chromatography methods such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC), as well as by molecular methods (Pirestani et al., 2011).

Field evaluation

For field evaluation, *Ganoderma* detection in apparently healthy-looking palms from three different plantations was done for an early diagnosis of diseased palms. Similar pattern of ELISA-PAb detection was also observed using samples taken from mature palms collected from three oil palm plantation at MPOB Research Station: Teluk Intan, Perak; Kluang, Johor and Sepang, Selangor (Table 2). A total of 120 palms were tested using ELISA-PAB concomitantly with GSM. Findings revealed that the positive signal was found in roots and stems collected from Teluk Intan, Perak for ELISA-PAb (100%) and GSM (70-90%) at $p<0.05$. Results on the samples accumulated from Kluang, Johor was also found similar to those of Teluk Intan, Perak as roots and stems were detected using ELISA-PAb (100%) against GSM (70-80%) at $p<0.05$.

Detection of ELISA-PAB and GSM were also found comparable for Sepang, Selangor; resulted in the detection of 100% (ELISA-PAb) and 80% (GSM) at $p<0.05$. Hence, this indicated that, ELISA-PAb was more sensitive and accurate as compared to GSM in detecting presence of *G. boninense* using both roots and stems sample. Conversely, ELISA-PAb and GSM failed to be detected in leaves. However, in order to increase the accuracy and consistency of *Ganoderma* detection, culture-based method, GSM is still needed to be applied in-parallel as a reconfirmation procedure. Effort is being focused on using high concentration of antiserum (1:100 or 1:1000) as to direct the target protein to be probed by antiserum itself, hence, remove most of the backgrounds. ELISA offers an easy, inexpensive and rapid assay as

Table 2. Field trial on detection of *Ganoderma* disease in matured oil palm using GSM and ELISA-PAb for healthy-looking palms in three different *Ganoderma* infected areas from different sample tissues; N=120.

Sample	Oil palm plantation	Mean of detection (%)	
		GSM	ELISA-PAb
Root	Teluk Intan, Perak	70 ± 4.83[a]	100 ±0[a]
Stem		90 ± 3.16[a]	100 ± 0[a]
Leaf		0 ± 0[b]	10 ± 3.16[b]
Root	Kluang, Johor	70 ± 4.83[a]	100 ± 0[a]
Stem		80 ± 4.22[a]	100 ± 0[a]
Leaf		0 ± 0[b]	0 ± 0[b]
Root	Sepang, Selangor	80 ± 4.22[a]	90 ± 3.16[a]
Stem		80 ± 4.22[a]	100 ± 0[a]
Leaf		0 ± 0[b]	0 ± 0[b]

Means with different letters within a column are significantly different according to the t-test at $p<0.05$ using Least Significant Difference (LSD). PAb, Polyclonal antibody; GSM, *Ganoderma* selective medium.

it requires small amount of sample tissues. Thus, ELISA polyclonal might be useful as pre-scan to handle many samples in time. Detection of *Ganoderma* disease in speciously infected oil palms is possible and strongly achieved with a combination of immunoassay, culture-based technique and molecular works.

Conclusion

This article provides an overview of polyclonal antibody approach and its application in detection of *Ganoderma* disease is one of decision-making tool for an early detection in nursery and field. The study is conducted as a preliminary research in developing polyclonal anti-bodies of *G. boninense*.

The findings from this study could be useful for future research work. Polyclonal antibodies of *G. boninense* can be produced, beforehand; more research needs to be carried out to achieve highly confidence of the generated polyclonal. Studies on biological and epidemiological aspects on the pathogen itself are essential in providing a better understanding of the natural occurrence of the disease. In future, provision of immunoassay-based kits would be helpful in the detection and development at nursery and field level and this would certainly mostly help the implementation of Integrated *Ganoderma* Management (IGM) against *G. boninense* disease in oil palm.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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