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MOLECULAR PCR ASSAYS FOR DETECTION OF *Ganoderma* PATHOGENIC TO OIL PALM IN MALAYSIA

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ABSTRACT

Ganoderma boninense is a fungal pathogen that causes basal stem rot (BSR) disease in oil palm. Being a serious disease problem to the oil palm industry, monitoring and detecting the pathogen is of the utmost importance to reduce disease spread and facilitate effective management strategies. Because the traditional culture-based assay is time-consuming, labor-intensive, and required special skills in mycology, plant pathologists are turning to more accurate, sensitive, and fast methods such as molecular techniques. In this study, polymerase chain reaction (PCR) assays were developed to detect pathogenic *Ganoderma* species causing BSR disease in oil palm using a primer designed based on the ribosomal DNA internal transcribed spacer (ITS) region. The effectiveness of conventional and real-time PCR assays was analyzed compared to the traditional isolation-based assay. For artificially inoculated oil palm plantlets, consistent detection of *G. boninense* was observed. Real-time PCR assay has shown to be more sensitive and rapid in detecting *G. boninense* in field samples and could potentially serve as a validation tool to other detection techniques for the implementation of effective disease control measures.

Key words: Basal stem rot disease, detection, *Ganoderma*, oil palm, PCR technology

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is an important commodity crop to Malaysia. In 2020, the crop has reached an export revenue of RM 73.25 billion with a total cultivation area of over 5.87 million hectares (Parveez *et al*., 2021). Being a monoculture plantation crop, oil palm is vulnerable to serious threats from pests and diseases. Basal stem rot (BSR) disease, prevalently caused by *Ganoderma boninense*, is the most economically devastating disease reported with an estimated yield loss of 68.73% (USD 4000) yearly (Kamu *et al*., 2021). BSR disease infection occurs through root contact with inoculum sources in the soil. The disease is manifested by progressive decay of roots that compromises water and nutrient uptake which eventually resulted in frond wilting and yellowing, flattening of the crown, un-opening of spear leaves, and formation of fruiting bodies on the lower stem, which ultimately caused the oil palm stand to collapse (GiatFee, 2011). The symptoms of BSR infection in the early stage are not visible and the earliest symptom can only be recognized once the infection reaches 60-70% (Chong *et al*., 2017). Thus, the development of a rapid and accurate detection method of BSR disease is needed so that infected oil palms can be treated and prolong their economic lifespan.

The primary aim of diagnostics is to detect and identify the causal agent of a disease that is rapid, accurate, and reliable. Traditionally, diagnosis and identification were based on direct observation of macro-and microscopic structures of fungi by a skilled observer. This isolation-based method is time-consuming, lack of resolution and sensitivity has oriented plant pathologists to move towards molecular techniques, essentially the polymerase chain reaction (PCR) technology. The basic culture-based method relies upon physical characteristics, while PCR is based on genetic characteristics that are rapid, sensitive, highly specific, and potentially more accurate diagnostics. In the PCR-based method, identifying organisms does not require culturing, thus removing the bias against slow-growing or non-culturable organisms (Schaad *et al*., 2003). While conventional PCR amplifies genomic DNA exponentially by a DNA polymerase using specific primers, in real-time PCR, the amplification reaction is achieved by a non-specific detection strategy independent of the target sequence, essentially fluorescent dyes that have special fluorescent properties when bound to double-stranded DNA (Gachon *et al*., 2004). This has allowed the detection and quantification to be completed in a single assay and gives higher sensitivity and specificity (Babu *et al*., 2011). Real-time PCR has also eliminated the post-PCR steps and hazards relative to ethidium bromide staining in conventional PCR and has indirectly improved the robustness of the system.

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The inception of molecular tools, particularly PCR technology, has revolutionized fungal diagnostics. The PCR-based method has been employed in numerous detection and identification of fungal pathogens using diverse target sequences to distinguish the target phytopathogen from non-target microbes under laboratory and field conditions (Hughes *et al*., 2006; Tomlinson *et al*., 2007; Ioos *et al*., 2009; Ioos *et al*., 2010; Sikdar *et al*., 2014; Luchi *et al*., 2018; Schneider *et al*., 2019). The ribosomal DNA internal transcribed spacer (ITS) region characterized by White *et al*. (1990), has been generally used to develop species-specific PCR primers for the detection of fungal pathogens because ITS is commonly conserved within species and varies between fungal species and genera (Li *et al*., 2011; Schoch *et al*., 2012). Previous studies on the utilization of ITS for detection of *Ganoderma* isolates pathogenic to oil palm had been reported (Bridge *et al*., 2000; Utomo & Niepold, 2000; Idris *et al*., 2003; Panchal & Bridge; 2005; Utomo *et al*., 2005; Mandal *et al*., 2014). However, these studies were conducted using conventional PCR and no attempt was made on real-time PCR.

In this study, the specificity, sensitivity, and effectiveness of conventional PCR and real-time PCR to detect pathogenic *Ganoderma* species using laboratory and field samples were analyzed. The culture-based and PCR-based diagnostic effectiveness were compared using purified fungal DNA, oil palm plantlets inoculated with *G. boninense,* and oil palm field samples, i.e., fruiting bodies, trunk, and root tissues.

MATERIALS AND METHODS

Fungal isolates and DNA isolation

Species of *Ganoderma* pathogenic to oil palm, i.e., *G. boninense*, *G. zonatum,* and *G. miniatocinctum* were included in this study (Idris *et al*., 2016). Non-pathogenic *Ganoderma tornatum* and other non-target fungi were also included (Table 1). All of the fungi were obtained from the internal culture collection of the Malaysian Palm Oil Board (MPOB, Bangi, Selangor, Malaysia). Fungi cultures were grown on potato dextrose agar (PDA) (Oxoid; Thermo Fisher Scientific, Waltham, MA, USA) (pH 5.5) and incubated in the dark for 7 days at 28 ± 2 ºC. Fungal mycelia were transferred to potato dextrose broth (PDB) (Difco; Difco Laboratories, Sparks, MD, USA) and grown on static culture at 28 ± 2 ºC in the dark for 7 days. DNA extraction of 7-day-old PDB fungal cultures was carried out using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. DNA concentration and purity were measured using Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). DNA integrity was examined through 0.8% agarose gel electrophoresis at 80 V for 30 min in 1× TAE running buffer.

Primer annealing temperature and specificity test using conventional PCR

The primer pair used in this study were forward primer GB4F (5'-CGTTCGTTTGACGAG TTTGC-3') and reverse GB4R (5'-GGTTGGTTTCTTTTCCT-3'), designed based on specific sequence data for the ITS region. Universal fungal primer pair, ITS1, and ITS4 (White *et al*., 1990) were employed to check for successful DNA isolation across samples tested. The optimum annealing temperature of the GB4 primer pair was determined by a gradient PCR performed at an annealing temperature range of 50 ºC to 60 ºC. The genomic DNA of *G. boninense* isolate PER71 was used in this initial PCR. PCR was performed in 10-µL reaction mixtures containing 5 ng of genomic DNA, 0.25 µL (10 µM) each primer, 5 µL of 2× MyTaq Red mix (Bioline) and sterile deionized water (dH_2O). Non-template control (NTC) was also included in the PCR reaction. PCR amplification was conducted using a T100 thermal cycler (Bio-Rad) with the following cycling program: initial denaturation at 95 ºC for 2 min; followed by 35 cycles of denaturation at 95 ºC for 30 s, annealing at gradient temperature of 50ºC to 60 ºC for 30 s, and extension at 72 ºC for 1 min; and final extension at 72 ºC for 10 min. The PCR products were resolved by electrophoresis on 1% (w/v) agarose (1st Base) gels, pre-stained with RedSafe™ (Intron Biotechnology) in 1× Tris-acetate-EDTA running buffer at 80 V for 30 min. DNA bands were visualized under UV and photographed with a Chemi 410 UVP BioSpectrum imaging system (Upland, CA, USA).

Primer sensitivity was evaluated with serial diluted *G. boninense* DNA concentrations from 5 ng μL^{-1} to 5 fg μL^{-1}. Primer specificity was tested using DNA from target and non-target fungi listed in Table 1. The PCR reagent volumes and concentrations were as described above. The optimized annealing temperature was used during the PCR amplification with the rest of the parameters remaining the same as described before. PCR products were then electrophoresed and visualized.

Sensitivity, amplification efficiency, and standard curve of real-time PCR assays

The sensitivity of the GB4 primer pair to detect pathogenic *Ganoderma* species was estimated through the generation of standard curves. The quantification cycle (C_q) values were plotted on the y-axis against the log of starting DNA concentration on the x-axis. The standard curve was constructed from seven 10-fold serial dilutions of purified DNA extract of *G. boninense*, with concentrations of 5 ng μL^{-1} to 5 fg μL^{-1}. To investigate any PCR inhibition by host extracts, healthy oil palm root DNA (2 ng μL^{-1}) was spiked with *G. boninense* pure culture DNA ranging from 5 ng μL^{-1} to 5 fg μL^{-1} to construct oil palm root extract standard curve. The PCR amplification efficiency (*E*)

in the presence of oil palm extract was determined from the equation $E = 10^{-1/slope} - 1 \times 100$, with the theoretical maximum of 100% producing a doubling in the number of amplicon molecules (Bustin *et al*., 2000). The standard curves generated using purified fungal DNA extract were compared to oil palm extract spiked with fungal DNA.

Real-time PCR was performed to analyze primer specificity using target fungal DNA. Each 10 µL PCR reaction was prepared using 4.5 µL of 2× SensiFAST™ SYBR No-ROX mix (Bioline), 0.2 µL of 10 µM of each forward and reverse primer, 5 ng of DNA and dH_2O. NTC that includes all the amplification reagents but no template DNA was also set up. Thermocycling was conducted using a CFX96 Touch Real-Time PCR (Bio-Rad) initiated by a 2 min incubation at 95 ºC, followed by 35 cycles of denaturation at 95 ºC for 30 s, annealing at 58.7 ºC for 30 s and extension at 72 ºC for 1 min. The melting temperature (T_m) of the amplicon was determined by fluorescence reading taken after the annealing step in each cycle. To confirm amplification specificity and lack of primer dimers, a melting curve analysis was completed after each run by raising the reaction temperature from 50 ºC to 90 ºC in 0.1 ºC increments, with fluorescence data captured every 0.5 s. The real-time PCR products were then electrophoresed and visualized as described previously to confirm that the amplicons are of the expected size. C_q values and amplicon T_m were determined by the CFX Manager 3.1 Software (Bio-Rad) and exported into MS Excel workbook (Microsoft Inc.) for data analysis.

Validation of PCR assays using artificially inoculated oil palm plantlets with *G. boninense*

To validate the PCR assay amplification and detection of *G. boninense* DNA, forty-five one-month-old oil palm plantlets (*Dura* × *Pisifera*, D×P) artificially infected with *G. boninense* were assayed. Oil palm plantlets were infected according to the artificial pathogenicity technique established by Sundram *et al*. (2020). The fungal inoculum was prepared from rubberwood sawdust mixture inoculated with seven days old *G. boninense* culture and allowed to colonize in the dark at 28 ºC for 7 to 10 days. Physiological and morphological of the oil palm plantlets were observed to examine the progress of disease symptoms development. After 4 weeks of incubation, total DNA was extracted (Qiagen DNeasy Plant Mini Kit) and used as a template for the PCR assays. In the PCR assays, a negative (sterile distilled water) and positive (genomic DNA of *G. boninense*) were included in each PCR reaction. *G. boniense* was reisolated using *Ganoderma* selective medium (GSM) according to the culture-based technique described by Idris *et al*. (2016). The culture was then transferred to PDA after incubation in the dark at 28 ºC for 3 to 5 days.

PCR detection of *Ganoderma* in naturally infected oil palm field samples

Fruiting bodies, oil palm trunks, and root tissues were sampled in the oil palm plantation located at the Malaysian Palm Oil Board (MPOB) Research Station in Keratong, Pahang, Malaysia. In total, thirty healthy-looking oil palms with mild or early infection, where white mycelium or fruiting body of *Ganoderma* is present but without any foliar symptoms or stem rotting at the base were surveyed and selected for sampling. Thirty fruiting bodies were sampled at the base of the oil palm. Oil palm trunk was drilled with a motorized drill bit to 15 cm in-depth, 1 to 1.5 m above ground to obtain 10 g of sample tissues. Drill bits used were sterilized with 80% alcohol before sampling the next oil palm tree to avoid sample cross-contamination. Ten grams of primary roots were collected near the white mycelium or fruiting body on the oil palm. Trunk and root tissues were sampled in three replicates from each oil palm. All samples were maintained on the ice during transportation. Total DNA was extracted (Qiagen DNeasy Plant Mini Kit) and tested for PCR assays. Three controls were included in the real-time PCR assay, (1) non-template control (i.e., sterile distilled water); (2) positive control (i.e., DNA of *G. boninense*); and (3) negative control (i.e., DNA of healthy oil palm). A piece of tissue from the fruiting body, trunk, and root sample was excised using a sterile scalpel and cultured on GSM for isolation of fungal pathogens (Idris *et al*., 2016). GSM plates with sample tissues were incubated in the dark at 28 ºC for 3 to 5 days to observe for mycelium growth. The pure culture of *Ganoderma* was then transferred to PDA to be maintained for further verification through PCR assays.

Data analysis

Raw data generated from the real-time PCR analysis were extracted and imported into MS Excel workbook (Microsoft Inc.). DNA concentrations were log-transformed and plotted against C_q values on the y-axis to calculate the slope of real-time PCR standard curves. The PCR amplification efficiency, (E), is determined using the formula $E = 10^{-1/slope} - 1 \times 100$, according to Rutledge and Côté (2003). DNA band results from the PCR assays using artificially inoculated oil palm plantlets with *Ganoderma* and field samples were scored as either success (positive) or failure (negative), according to Bernoulli trials where the probabilities of the two possible outcomes for each trial remain the same throughout the trials (Saitou, 2013). A binomial test was performed using IBM SPSS Statistic (version 20) software to analyze the efficiency of *Ganoderma* detection between PCR assays and culture-based methods.

Table 1. List of target oil palm fungal pathogens and non-target fungi included in the PCR assays

Fungus	Isolate code	Fungus	Isolate code
Ganoderma boninense	PER71	*T. virens*	GC1BRT
G. boninense	PER73	*Fusarium oxysporum*	Foe
G. boninense	PER74	*F. solani*	Fos
G. miniatocinctum	337035	*Phythium spelendens*	G10
G. miniatocinctum	337036	*Os-CCCVd*	B1PV251
G. zonatum	POR68	*Phytophthora palmivora*	PPM1
G. zonatum	POR69	*P. palmivora*	PPM4
G. tornatum	POR57	*Marasmius palmivorus*	MPS3
G. tornatum	NPG1	*M. palmivorus*	MPS28
Hendersonia toruloidea,	GanoEF1	*Curvularia reesii*	GC3
Streptomyces hygroscopicus,	Actino347	*C. senegalensis*	GC2
Streptomyces sp.	GanoSA1	*Exserohilum rostratum*	GC1
Schizophyllum commune	Basidio18	*Neopestalotiopsis formicarum*	GG1
S. commune	Basidio24	*Nigrospora lacticolonia*	GF1
Pycnoporus cinnabarinus	Pyc1	*N. oryzae*	GE1
Hypoxylom rickii	Hyp1	*Phoma herbarum*	GB1
Bacillus sp.	EB1sp	*Colletotrichum gloeosporioides*	GD1
Pseudomonas aeruginosa	EB1	*C. gleosporiodes*	GF1(1)
Bacillus sp.	EB2	*Duportella trigonosperma*	GA2CRT
Trichoderma asperellum	G15	*Grammothele fuligo*	GD1CRT
T. harzianum	GE9RT		

RESULTS

Conventional PCR assay

Gradient PCR conducted with *G. boninense* PER71 as a template showed 58.7 ºC as the optimum annealing temperature for amplification by GB4 primer pair (Figure 1a). The highest temperature that produced distinct intense DNA band was chosen to ensure PCR conditions were more stringent and thus reduce non-specific amplification due to internal single-base mismatches or partial annealing. Both PCR sensitivity tests using *G. boninense* and oil palm root extract spiked with *G. boninense* DNA could detect the lowest DNA concentration of 0.5 ng μL^{-1} (Figure 1b). To test the specificity of the GB4 primer pair, *G. boninense* and other non-target fungi were tested using the established PCR assay. As shown in Figure 1c, PCR products of 500 bp in size are detected in all pathogenic *Ganoderma* species whereas the other non-target fungal species and non-pathogenic (*G. tornatum*) give negative amplifications.

Real-time PCR assays

Real-time PCR assays showed that the limit of detection of *G. boninense* was 50 fg μL^{-1}, with the lowest C_q value of 15 (Figure 2a). The limit of detection for *G. boninense* in the presence of DNA extracted from oil palm roots was 500 fg μL^{-1}, with the lowest C_q value of 16 (Figure 2b). The cut-off C_q value for the real-time PCR assays was 32 as there were no amplification signals seen. The amplification efficiencies for real-time PCR assays calculated from *G. boninense* DNA in water and the same DNA extract spiked with oil palm root extract were 81% and 119.8%, respectively. The melting curve peak was observed at 85.2 ºC for both real-time PCR using *G. boninense* DNA in water and spiked with oil palm root extract. A single sharp peak was displayed, thus, confirming the specificity of primer annealing. Gel electrophoresis analysis of the real-time PCR products showed the presence of the expected PCR products of 500 bp in size. There were no cross-reaction and no amplification signals detected in assays using DNA extracted from any of the non-target fungal species and non-pathogenic *G. tornatum*.

Validation of PCR assays using artificially inoculated oil palm plantlets with *G. boninense*

The leaves on oil palm plantlets inoculated *in vitro* with *G. boninense* turned brown on the area where colonization occurred. The bole area of oil palm plantlets was examined and decay was observed after 4 weeks of incubation. Total DNA isolated from the artificially inoculated oil palm plantlets was subjected to conventional and real-time PCR assays. Figure 3 summarises the comparison of traditional isolation-

based and PCR-based for detection of *Ganoderma* using inoculated oil palm plantlets. *G. boninense* was reisolated 80% from the inoculated oil palm plantlets using the traditional culture-based method (Figure 3). Conventional and real-time PCR assays had detected *G. boninense* 93.3% and 97.8%, respectively, from the total inoculated oil palm plantlets (Figure 3). The C_q values of real-time PCR analysis range from 18.8 to 29.1 (Table 2). Both conventional and real-time PCR products resolved through gel electrophoresis analysis showed the presence of a single PCR product of 500 bp in size. Real-time PCR showed a significantly (P=0.001) higher percentage of *G. boninense* than the GSM isolation-based method. However, the detection rate was not significantly different (P=0.167) compared to conventional PCR assay. Additionally, no significant difference (P=0.13) was observed between the culture-based method and PCR assay in artificially inoculated oil palm plantlets.

PCR analysis in oil palm field samples naturally infected with *Ganoderma*

Figure 4 shows the comparison of conventional isolation-based and PCR-based detection of the fungal pathogen using different field samples. Real-time PCR assay showed the highest detection rate of *G. boninense* in fruiting bodies, trunk, and root tissues with 80%, 70%, and 33.3%, respectively, compared to culture-based and PCR assays (Table 2; Figure 4). The C_q values for real-time PCR analysis range from 15.0 to 31.9 (Table 2). In fruiting bodies samples, the detection percentage of *G. boninense* using the culture-based and conventional PCR (P=0.262) and real-time PCR (P=0.572) were not statistically significant. However, when the binomial test was carried out on trunk tissues analysis, real-time PCR detected a significantly (P=0.009) higher percentage of *G. boninense* compared to the conventional PCR assay, but the real-time PCR detection was not significant (P=0.104) to the culture-based method. A similar observation was seen in root tissues where real-time PCR assay detected *G. boninense* 33.3% from total samples analyzed, which was significantly higher (P=0.003) than that analyzed by using conventional PCR assay (13.3%; Table 2). However, there were no statistical differences (P=0.22) between culture-based and real-time PCR assays in root tissues tested.

Table 2. Comparison between culture-based and PCR-based assays for detection of pathogenic *Ganoderma* in oil palm plantlets (artificially inoculated with *G. boninense*) and field samples (naturally infected with *Ganoderma*)

Sample	Number of samples	*Ganoderma* isolated on GSM* (%)	PCR assay (%)	Real-time PCR assay (%)	C_q* range for real-time PCR
Oil palm plantlets	45	80	93.33	97.7	18.8 – 29.1
Fruiting bodies	30	73.3	80	80	15.0 – 30.3
Trunk tissues	30	56.7	46.7	70	19.2 – 31.6
Root tissues	30	16.7	13.3	33.3	29.3 – 31.9

*Abbreviations: GSM, *Ganoderma* selective media; C_q, quantification cycle.

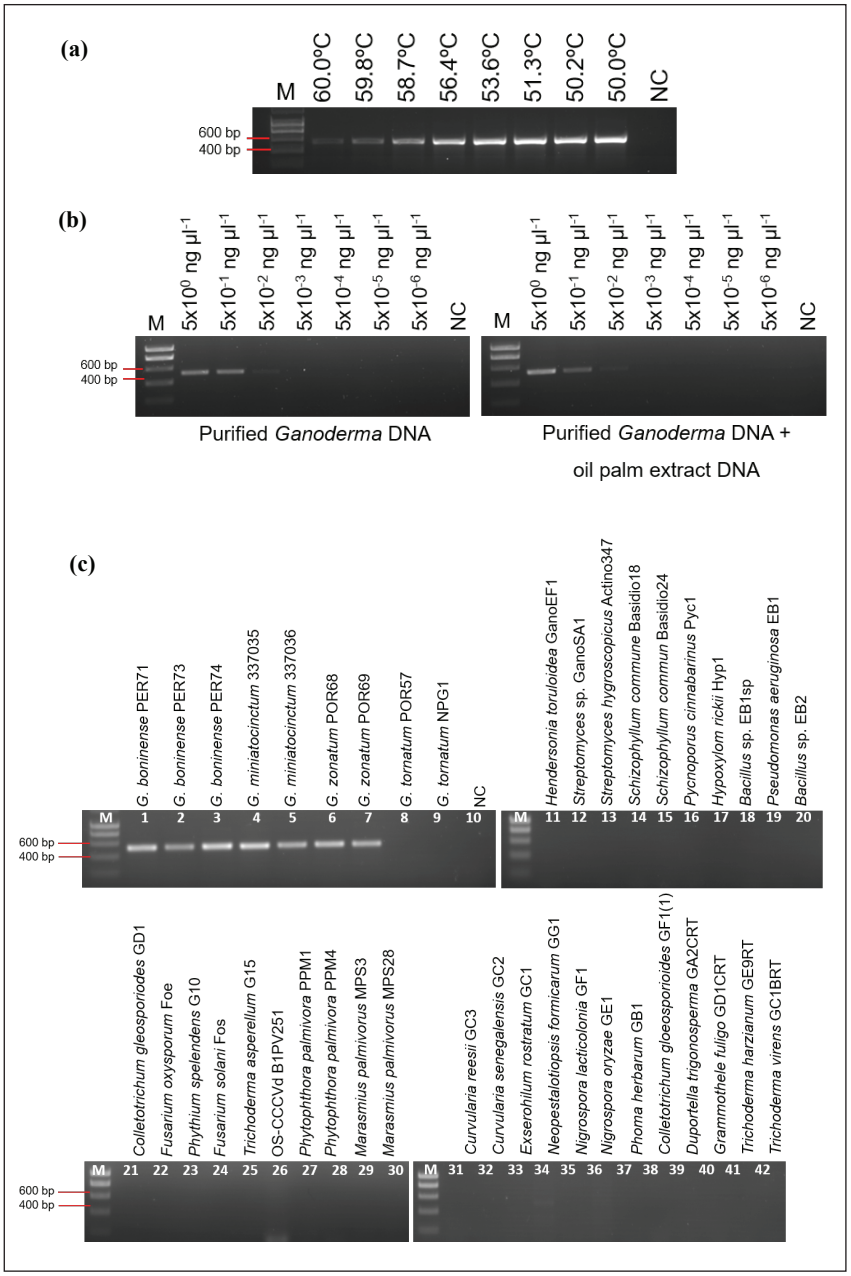

Fig. 1. Evaluation of GB4 primer using conventional PCR. **(a)**, Gradient PCR to determine the optimum primer annealing temperature; **(b)**, Sensitivity testing on serial diluted *G. boninense* DNA concentrations; **(c)**, Specificity testing across target pathogens and non-target fungi. Notes: M, DNA molecular ladder; NC, negative control.

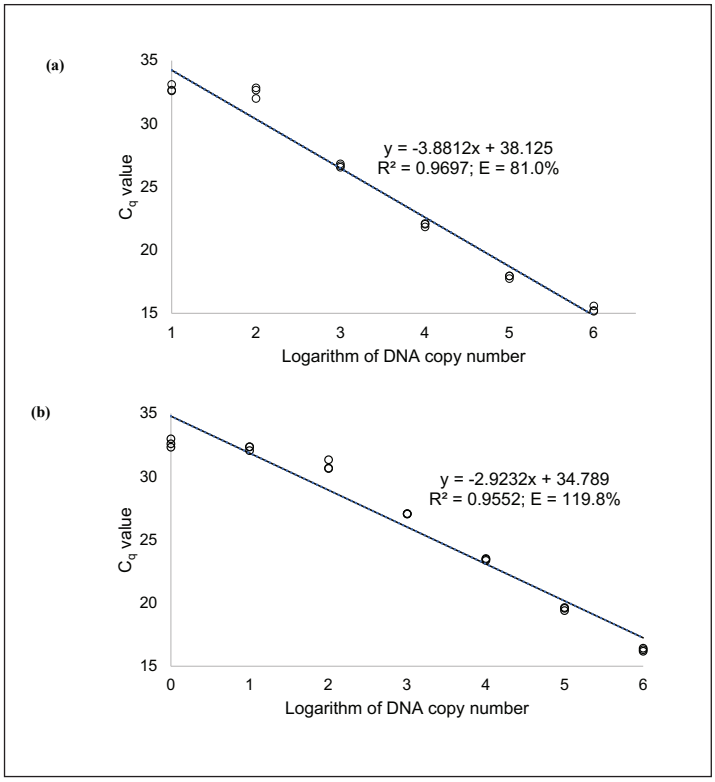

Fig. 2. Standard curves generated from real-time PCR assay using purified *G. boninense* DNA; (a) and oil palm extract DNA spiked with *G. boninense* DNA (b). DNA concentrations were tested from 5 ng μL^{-1} to 5 fg μL^{-1}. Notes: E, PCR amplification efficiency; C_q, quantification cycle.

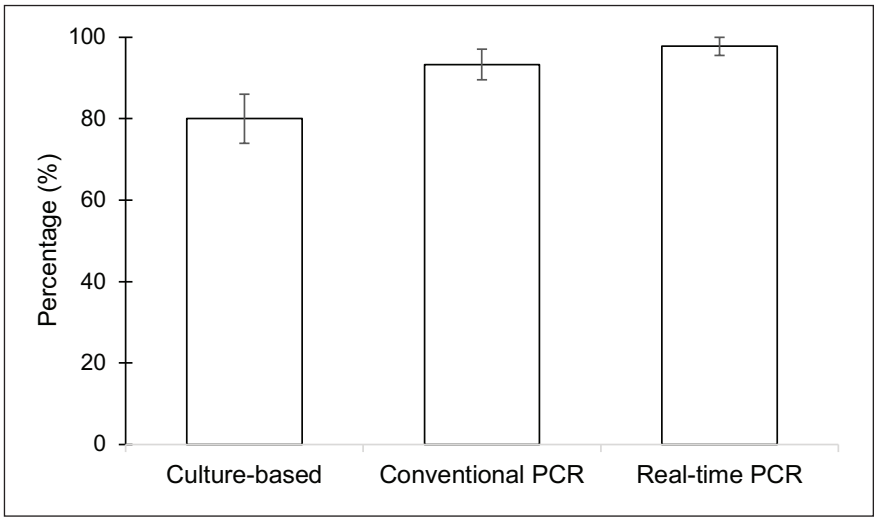

Fig. 3. Percentage of pathogenic *Ganoderma* detected in artificially infected oil palm plantlets by culture-based, conventional PCR and real-time PCR assays. Error bars represent the standard error of the mean (SEM).

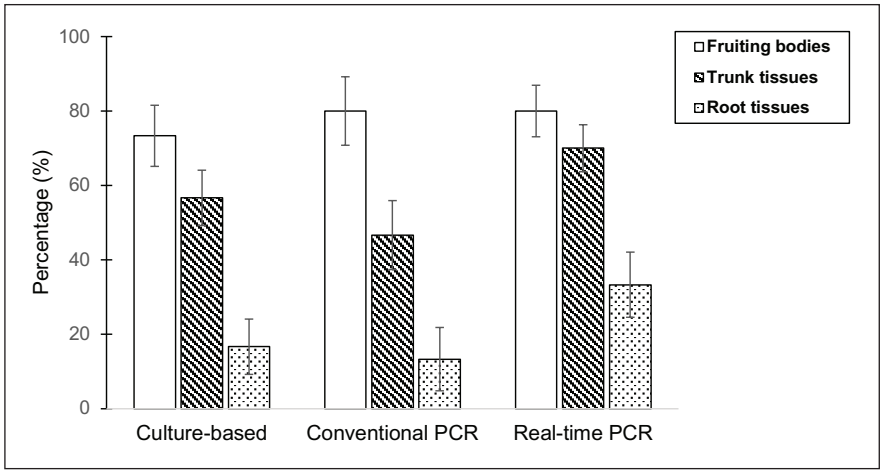

Fig. 4. Percentage of pathogenic *Ganoderma* detected in oil palm field samples, i.e., fruiting bodies, trunk, and root tissues by culture-based and PCR-based assays. Error bars represent the standard error of the mean (SEM).

DISCUSSION

Efficient diagnoses of pathogenic *Ganoderma* species in oil palm were developed using conventional and real-time PCR assays. In this study, conventional PCR assays had efficiently amplified the ITS region of pathogenic *Ganoderma* species, i.e., *G. boninense, G. zonatum,* and *G. miniatocinctum* but did not amplify DNA from non-pathogenic and other non-target fungi as reported by Idris *et al*. (2003). This shows that the polymorphisms present in the ITS region of pathogenic *Ganoderma* species have enough stringency to differentiate them from others. Results observed here were supported by previous reports on the use of ITS for *Ganoderma* detection using conventional PCR (Bridge *et al*., 2000; Utomo & Niepold, 2000; Panchal & Bridge; 2005; Utomo *et al*., 2005). The ITS variations were also extensively exploited in *Ganoderma* taxonomy and genetic diversity studies (Jing *et al*., 2015; Midot *et al*., 2019; Fryssouli *et al*., 2020). Being a fast-evolving region, the nuclear ribosomal DNA ITS region is generally used as genotypic character to detect and identify different taxonomic groups (White *et al*., 1990).

Under *in vitro* conditions, the GB4 marker were detectable at 0.5 ng μL^{-1} of purified *G. boninense* DNA using conventional PCR. This was shown to be sufficient to detect pathogen DNA in artificial inoculated oil palm expressing disease symptoms. However, pathogen DNA is usually present at substantially low levels or unevenly distributed in naturally infected plant samples and conventional PCR is ineffective for consistent and accurate diagnosis of field and environmental samples. The real-time PCR assay in this study had a minimum detection limit of 50 fg μL^{-1} from pure cultures and 500 fg μL^{-1} from extracts of oil palm tissue. The high degree of correlation (r^2=0.9552) of *G. boninense* DNA quantified in oil palm extracts indicated that the GB4 marker performed reliably over the range of dilutions tested. The difference in sensitivity of PCR assays has been reported in other pathogens detection from various plant hosts and samples as summarised by Okubara *et al*. (2005). This may be due to the complexity of DNAs found in natural samples and the presence of natural inhibitors found in matured hardy plants that may affect the PCR amplification efficiency.

In artificially infected oil palm plantlets, both culture-based and PCR-based assays show more than 80% detection of *G. boninense* (Figure 3) possibly due to the high density of pathogen mycelia. Oil palm plantlets were grown under sterile conditions on MS (Murashige and Skoogs, 1962) and Y3 (Eeuwens, 1976) nutrient media with a known composition that made them delicate and less resilient to biotic and abiotic stresses. *In vitro* inoculation of oil palm plantlets with *G. boninense* in this study has been speculated to permit substantial growth of *G. boninense* and resulted in high pathogen DNA yield. Similarly, high detection of *Ganoderma* in fruiting bodies using both isolation-based and PCR-based was also observed due to the high presence of pathogen DNA. However, a lower detection rate was seen in PCR assays of oil palm trunk and root samples, probably due to low levels of pathogen DNA, uneven distribution of the pathogen in the field, or decayed tissues samples with no recovery of pathogen DNA. Further analysis using PCR assays of some of the fungal pathogen isolated from the GSM culture method from the trunk and root samples did not generate PCR products specific for pathogenic *Ganoderma*, suggesting false positives can be avoided where decayed was not due to BSR disease.

The unavailability of a fast and accurate detection method to detect and identify pathogens in the field has made the management of many root and stem diseases very challenging. Detection of fungal

pathogens in samples without the need for culturing has made PCR-based assays a valuable tool to assist in the monitoring disease spread and reinforcement of border biosecurity. While the conventional PCR method gives specific amplification of targeted DNA, it lacks sensitivity for field disease detection. Madihah *et al*. (2018) reported *Ganoderma* detection using another method that combines isothermal DNA amplification and bioluminescence detection known as loop-mediated isothermal amplification (LAMP) to be faster and more reliable compared to conventional PCR. However, the method was not yet tested as a field assay. A comparative study by Khan *et al*. (2018) had shown that real-time PCR is the most sensitive compared to other PCR-based and LAMP assays evaluated. The improved form of conventional PCR known as real-time PCR is more sensitive and rapid as DNA can be quantified along with the amplification (Mackay *et al*., 2004). Identification and quantification of fungal pathogens such as *Aspergillus versicolor, Cladosporium cladosporoiodes, Stachybotrys chartarum*, and *Alternaria alternate* using real-time PCR had been demonstrated by Black (2009). Oak *et al*. (2008) reported an extensive monitoring survey of *Phytophthora ramorum* in 3 years where more than 12,000 samples were tested from 44 hosts in the United States forests using nested and real-time PCR. The developed real-time PCR reported here could facilitate another promising diagnostic method such as the emerging sensor technology using spectral of RGB (red, green, blue), multispectral, and hyperspectral that measure reflectance, fluorescence, and radiation emission (Oerke, 2020). Several studies have reported the use of remote sensing techniques for the early detection of BSR disease in oil palm (Shafri *et al*., 2012; Liagat *et al*., 2014; Izzuddin *et al*., 2018; Noor Azmi *et al*., 2020).

CONCLUSION

In summary, this study demonstrated the development and application of a DNA-based marker for the detection of pathogenic *Ganoderma* species using PCR assays, with optimal specificity, sensitivity, and rapidity. The real-time PCR assay is valuable for detecting *Ganoderma* species in causing BSR disease in field samples as it is shown to be more sensitive than conventional PCR for this particular assay reported here. Real-time PCR could also be used to examine the ecology and epidemiology of soilborne pathogen, essentially *Ganoderma*, as quantification can be made based on fungal inoculum that is static or not spread a great distance from one planting year to another in oil palm cultivation (Rees *et al*., 2007). The developed assays could also provide a useful validation tool, alongside other emerging detection techniques available such as remote sensing, in monitoring oil palms and detecting pathogenic *Ganoderma* species to reduce disease spread and benefit the decision-making for suitable control management of BSR disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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