SHORT COMMUNICATION

Journal of Oil Palm Research Vol. 37 (1) March 2025 p. 181-186
DOI: https://doi.org/10.21894/jopr.2024.0040

DEVELOPMENT OF qPCR PRIMER FOR *Ganoderma boninense* DETECTION IN SOIL

HUN JIAT, TUNG[1]*; YIT KHENG, GOH[1] and WEI CHEE, WONG[1]

ABSTRACT

Quantitation of pathogenic Ganoderma *species presence in oil palm plantation soil is crucial for basal stem rot (BSR) disease management where implemented disease control practices could be validated. Quantitative polymerase chain reaction (qPCR) has been widely applied in quantification of various pathogens in soil and plant tissues due to its high sensitivity. In this study, primers designed based on internal transcribed spacer (ITS) sequences were utilised for detection of pathogenic* Ganoderma *species. Its specificity and detection efficiency were tested using* Ganoderma *inoculated and non-inoculated soil samples. As for qPCR applications, primer IGbF-R was proven to be specific to* Ganoderma boninense*, and capable of amplifying its synonyms (*G. orbiforme *and* G. miniatocinctum*) in* Ganoderma *inoculated soil.*

Keywords: basal stem rot, DNA quantification, *Elaeis guineensis*, soil.

Received: 3 January 2024**; Accepted:** 9 July 2024**; Published online:** 28 August 2024.

INTRODUCTION

The commercially cultivated oil palm (*Elaeis guineensis*) is vulnerable to the attacks by various important fungal diseases, particularly vascular wilt (*Fusarium oxysporum*), bud rot (*Phytophthora palmivora*) and BSR (*Ganoderma boninense*) in different continents of the world (Corley & Tinker, 2016). Root-to-inoculum sources had been proposed as one of the routes of disease transmission by *G. boninense,* in particularly replanting of oil palm at areas previously infected by BSR (Rees *et al*., 2009). Appearance of the multiple unopened spears, skirting or downward snapping of the green fronds and yellowing of the fronds are some of the common visual foliar symptoms observed in the oil palms with BSR (Rees *et al*., 2009). Disease rotting of the stem bole and manifestation of fungal masses or fruiting bodies are some of the common visual symptoms associated with BSR (Rees *et al*., 2007). Unfortunately, once either one of the above symptoms' manifests, BSR disease is well-established and at a late stage.

[1] Advanced Agriecological Research Sdn. Bhd., 11, Jalan Teknologi 3/6, Taman Sains, 47810 Petaling Jaya, Selangor, Malaysia.

* *Corresponding author e-mail: tunghj@aarsb.com.my*

Detection of *G. boninense* in the oil palm tissues have been studied through non-molecular and molecular approaches (Chong *et al*., 2017; Naher *et al*., 2013). Some of the non-molecular methods explored are a) *Ganoderma*-selective medium (GSM) for pure culture isolation (Ariffin & Idris, 1992); and b) detection of *G. boninense* through ergosterol analysis (Chong *et al*., 2017). Other detection approaches, namely e-nose system, spectroscopy, remote sensing, and ultrasonic, have also been explored and reviewed (Naher *et al*., 2013; Siddiqui *et al*., 2021). For molecular diagnostic methods, techniques such as PCR and loop-mediated isothermal amplification (LAMP) were reported. For instance, PER44-123 and LR1 primers (amplicon size: ~580 bp) were capable of amplifying *G. boninense, G. miniatocinctum* and *G. zonatum* (pathogenic *Ganoderma* species in oil palm plantations), but not in other tested *Ganoderma* species (Idris *et al*., 2003). In a separate study, Gan1 and Gan2 primers (amplicon size: 167 bp) had been developed to detect *Ganoderma* DNA. Unfortunately, this primer-set will also amplify other saprophytes (*Aspergillus*, *Rhizopus* and *Penicillium* species) of oil palms with a range of DNA fragment sizes (Utomo & Niepold, 2000). The LAMP method, albeit published, the primer sequences were not reported.

Quantification of the pathogens with qPCR in plants has been reported (Luchi *et al.,* 2020). This method has also been adopted for quantifying *Ganoderma* DNA in ramets or clonal materials under semi-controlled and sterile conditions (Chow *et al*., 2017; Goh *et al*., 2016). However, successful application of qPCR for *Ganoderma* DNA quantification in environmental soil samples is limited. Thus, in this study, we reported the development of specific qPCR primers for *Ganoderma* DNA quantification in oil palm plantation soil samples.

MATERIALS AND METHODS

Fungal, Bacterial and Actinomycetous Isolates and Growth Conditions

A total of seven fungal, one bacterial and five actinomycetous cultures were isolated from Typic Endoaquept (3°55′33.4″N, 100°48′51.7″E) and Typic Quartzipsamments (3°55′44.6″N, 100°48′36.2″E) series soils of an oil palm estate in Perak, Malaysia (*Table 1*). They were maintained and inoculated on malt extract agar (MEA, Difco) (for fungi), trypticase soy agar (TSA, Difco) (for bacteria) and actinomycetes isolation agar (AIA, Difco) (for actinomycetes) prior to DNA extraction. Ten *Ganoderma* spp. isolates and one *Heterobasidion annosum* isolate (*Table 1*) were purchased from CBS-KNAW Fungal Biodiversity Centre, National Agriculture and Food Research Organization (NARO) and Centre for Agriculture and Bioscience International (CABI). All the fungal cultures were incubated at 24°C, while bacterial and actinomycetous cultures were incubated at 28°C, both in the dark. Genomic DNAs of all the pure cultures of fungi, bacteria and actinomycetes were extracted with the FastDNA Spin Kit (MP Biomedicals, USA).

Soil Samples from the Oil Palm Field

Two different soils, namely Typic Kandiudults (coordinates: 3°16′13.36″N; 101°27′18.62″E) and Typic Quartzipsamments (coordinates: 3°55′44.6″N, 100°48′36.2″E) were collected from inter-palm areas at the soil depth of approximately 0-15 cm and portion of the soils were autoclaved twice at 121°C for 30 min (Rusli *et al*., 2016). Both autoclaved and non-autoclaved soils were inoculated with *G. boninense* colonised rubber wood block (RWB) for nursery infection studies and all the treatments were in triplicates following the procedures outlined previously (Goh *et al.,* 2022). The soil samples in polybags were collected and subjected to DNA extraction after five months of post-nursery-infection. Briefly, soil samples were collected approximately 1 cm from the *Ganoderma*-inoculated or non-inoculated RWB and 1 cm from the soil surface using sterilised spatula. Soil samples were then processed by breaking them into small pieces and removing the intact shells, root fragments and wood debris using sterilised forceps prior to sieving through the 2 mm sieve and homogenisation (Goh *et al*., 2020; Strohm, 2015). The processed soils were then stored at -80°C prior to DNA extraction. Prior to DNA extraction, the soil samples were then treated with propidium monoazide (PMA) (Biotium, CA, USA) following previously described protocols (Joo *et al*., 2019). For each replicate, soil total DNA was extracted from approximately 0.4 g soil sample using DNeasy PowerSoil Kit (Qiagen, Inc., CA, USA) and three extractions were conducted for the respective samples. The extracted DNAs were then pooled into one composite prior to qPCR analyses.

Primer Design

Alignment of internal transcribed spacer (ITS) region for 12 *G. boninense* isolates sampled from across Peninsular Malaysia was performed using Geneious 11.1.5 (https://www.geneious.com). From the alignment, constant region (defined as no SNPs) between the 12 isolates were identified and submitted to PrimerQuest (https://www.idtdna.com/SciTools) for primer design. The parameters for qPCR primers design were: a) Optimal primer Tm at 62°C (± 3°C), b) GC at 50% (± 15%) and c) primer size at 22 bp (± 3 bp). The range of amplicon size was set from 75-150 bp. The primer set which satisfied the qPCR parameters were selected for further testing.

Quantitative Real-time Polymerase Chain Reaction (qPCR)

In addition to the selected primer set in this study, another *Ganoderma*-specific primer set *i.e.*, Gan1 (5′-TTGACTGGGTTGTAGCTG-3′) and Gan2 (5′-GCGTTACATCGCAATACA-3′) (Utomo & Niepold, 2000) were included for comparison in qPCR assays using genomic DNAs from the microbial isolates (*Table 1*) and soil DNAs. The qPCR reactions were performed in QuantStudio™ 6 Flex system (Applied Biosystems, USA). Reaction mixture comprised 10 µL KAPA Sybr Fast qPCR kit (KAPA BioSystems, USA), 0.5 µL of 10 µM each forward and reverse primers, 7 µL sterile water, and 2 µL of 10 ng/µL DNA template. Assays were performed in three technical replicates. The qPCR thermal cycling was performed with pre-incubation at 95°C for 3 min, followed by 40 cycles of 95°C for 3 s and 60°C for 20 s with fluorescence data acquisition at the end of each cycle. The dissociation curve was carried out at 95°C for 15 s,

60°C for 1 min and 95°C for 15 s. Additionally, melt curve analysis was carried out at 60°C for 1 min and 95°C for 15 s. Standard curve was constructed using the cycle threshold (Ct) values of a series of ten-fold diluted *G. boninense* PER71 DNA (between 335 ng/µL and 0.003 ng/µL). Detection and quantification of *G. boninense* DNA using both pure microbial cultures and soil samples were performed by comparing the Ct values for the respective samples against the standard curve. The melt curve was generated to examine the specificity of amplification.

RESULTS AND DISCUSSION

The forward primer IGbF (5′- GCAGCGAAAT GCGATAAGTAATG -3′) and reverse primer IGbR (5′-CCGCAAAGAGATTGTAGGTTGA-3′) (produces 140 bp amplicon) found to be the only primer set that fulfilled the criteria for qPCR primer design. When the amplicons of primer IGbF-R and Gan1-2 from three isolates of *G. boninense* DNA were visualised on 2.50% (w/v) agarose gel, both primer sets yielded one single band with the expected amplicon size. Correspondingly, the melt curve plot displayed a single peak in both IGbF-R and Gan1-2. The amplification efficiency determined by standard curve for IGbF-R was 95.74% followed by Gan1-2 with 68.32% (*Figure 1*). Amplification of other fungi and bacteria was not observed for both sets of primers. Primers IGbF-R showed amplification only for *G. boninense* and its synonyms (*G. orbiforme* and *G. miniatocinctum*). In a separate study, the pathogenicity of *G. orbiforme* IMI375255 and *G. miniatocinctum* IMI337035 in oil palm seedlings was established with nursery infection (unpublished data). The disease index and disease severity index were comparable with *G. boninense* control. The melting point of IGbF-R for *G. boninense*, *G. miniatocinctum* and *G. orbiforme* are similar at 80.62°C, given that the sequences of region amplified are identical.

For PCR amplification of *G. boninense* pure cultures, 28 isolates obtained from a single oil palm estate showed positive amplification using IGbF-R primer set. Subsequent testing using DNAs extracted from soil samples (oil palm nursery polybag), with and without *Ganoderma* inoculum were quantified using both primers IGbF-R and Gan1-2 for comparison of the qPCR

TABLE 1. FUNGAL, BACTERIAL AND ACTINOMYCETOUS ISOLATES INCLUDED FOR DETERMINATION OF PRIMER SPECIFICITY TEST

Microbial isolates	Isolate/Culture	GenBank accession	Culture collection*	Amplification	
				IgbF-R	Gan1-2
Ganoderma applanatum	IMI344119	N/A	CABI	X	√
G. tropicum	CBS128582	N/A	CBS-KNAW	X	√
G. weberianum	CBS219.36	MH855780.1	CBS-KNAW	X	√
G. miniatocinctum[#]	IMI337035	MN490055.1	CABI	√	√
G. colossus	CBS216.36	N/A	CBS-KNAW	X	√
G. orbiforme[#]	IMI375255	N/A	CABI	√	√
G. boninense	MAFF305601	N/A	NARO	√	√
Ganoderma sp.	MAFF243200	N/A	NARO	X	√
G. gibbosum	MAFF244236	N/A	NARO	X	√
G. applanatum	MAFF305600	N/A	NARO	X	√
G. boninense	PER71	MN490049.1	MPOB	√	√
Heterobasidion annosum	CBS869.87	N/A	CBS-KNAW	X	X
Trichoderma harzianum	D10	OK584118	AAR	X	X
T. simmonsii	D15	OK584471	AAR	X	X
T. yunnanense	D27	OK584679	AAR	X	X
T. simmonsii	B3	OM978939	AAR	X	X
T. yunnanense	D6	OK584477	AAR	X	X
Aspergillus ochraceopetaliformis	D11	OK584475	AAR	X	X
A. protuberus	D8	OK576909	AAR	X	X
Terrabacter koreensis	B15	N/A	AAR	X	X
T. koreensis	208	OM919535	AAR	X	X
Streptomyces abikoensis	M24	OK576703	AAR	X	X
S. abikoensis	M30	OK576707	AAR	X	X
Pseudomonas aeruginosa	D25	OK576699	AAR	X	X
Nocardia bhagyanarayanae	209	OK602688	AAR	X	X

Note: Positive amplification is denoted by "√" and negative amplification denoted by "x". *Ganoderma* sp. denoted with "#" are synonymous with *G. boninense*. CABI - Centre for Agriculture and Bioscience International; CBS-KNAW - Fungal Biodiversity Centre, NARO - National Agriculture and Food Research Organization; MPOB - Malaysian Palm Oil Board; and AAR - Advanced Agriecological Research Sdn. Bhd.; N/A - not available.

detection efficiency and sensitivity. Both primers showed positive detection of *Ganoderma* DNA in all inoculated soil samples with mean Ct value ranging from 17.04 to 23.70 (*Table 2*). Inoculated soils that were sterilised prior to nursery infection experiment showed slightly lower mean Ct value (*i.e*., early amplification of target) compared to non-sterilised inoculated samples (ranges from 1.0%-8.5% difference) in both Typic Kandiudults and Typic Quartzipsamments soils. This observation indicated a higher *Ganoderma* population in the sterilised soil compared to non-sterilised soil. This observation agrees with Rees *et al*. (2009) study where *G. boninense* is shown to be a poor competitor in non-sterile soil. In this study, all primer sets were able to amplify *Ganoderma* DNA in two different soil types tested. Typic Kandiudults have sandy clay texture with approximate pH 4.5. Meanwhile, Typic Quartzipsamments have coarse sandy texture with shell deposits, higher pH and total calcium in comparison to Typic Kandiudults (Goh *et al*., 2022). In general, the IGbF-R primer set was more sensitive in detection when compared to Gan1-2 as the mean Ct value is lower in all samples tested. There was no amplification in non-inoculated soil samples regardless of sterilisation status (mean Ct value >27.00). Detection of pathogenic *Ganoderma* in soil by qPCR primers developed in this study could potentially be used for rapid pathogen screening in oil palm plantations. The primers designed using ITS region which are known to have several copies in genome increased the sensitivity of the primers for detection. Although the primers were shown to be able to detect *G. boninense* in inoculated soil, the inoculum size used in this nursery study was substantial (rubber wood block inoculum is approximately 8.42% of soil volume in polybags). Hence, it may not necessarily reflect true detection sensitivity for soil in oil palm plantations. Nevertheless, the detection of pathogenic *Ganoderma* sp. is still plausible when optimised soil DNA extraction protocol is incorporated into the workflow. Additionally, the primers developed are not suited for absolute, but limited to relative quantification. This is due to the multiple copies of ITS present in *Ganoderma* genome and the primers could amplify several pathogenic *Ganoderma* species.

CONCLUSION

A reliable and economical repeating test to determine the presence of *Ganoderma* species is prerequisite for effective BSR disease management. However, a precise *G. boninense* detection method is scarced due to low sequence variability for designing of primer and a wide collection of microbial cultures is required for its specificity

TABLE 2. MEAN Ct VALUE OF *Ganoderma* DNA IN POLYBAG'S SOIL

Ganoderma inoculation	Sterilisation	Mean Ct value	
		IGbF-R	Gan1-2
With	Non-sterilised	19.72	21.47
		20.09	21.83
		18.08	21.60
		17.84	19.28
		17.04	18.09
		21.41	22.89
	Average	**19.03**	**20.86**
	Sterilised	22.41	23.13
		20.38	21.88
		23.23	23.70
		19.43	21.12
		21.93	22.22
		17.50	19.37
	Average	**20.81**	**21.90**
Without	Non-sterilised	30.54	30.19
		29.52	27.90
		33.50	32.12
		32.58	32.03
		29.03	27.08
		29.21	28.02
	Average	**30.73**	**29.56**
	Sterilised	33.03	32.12
		28.52	27.62
		33.27	32.39
		35.18	34.09
		31.50	29.61
		34.68	31.79
	Average	**32.70**	**31.27**

Note: Total of six replicates from each parameter (inoculation status and sterilisation) were quantified via qPCR.

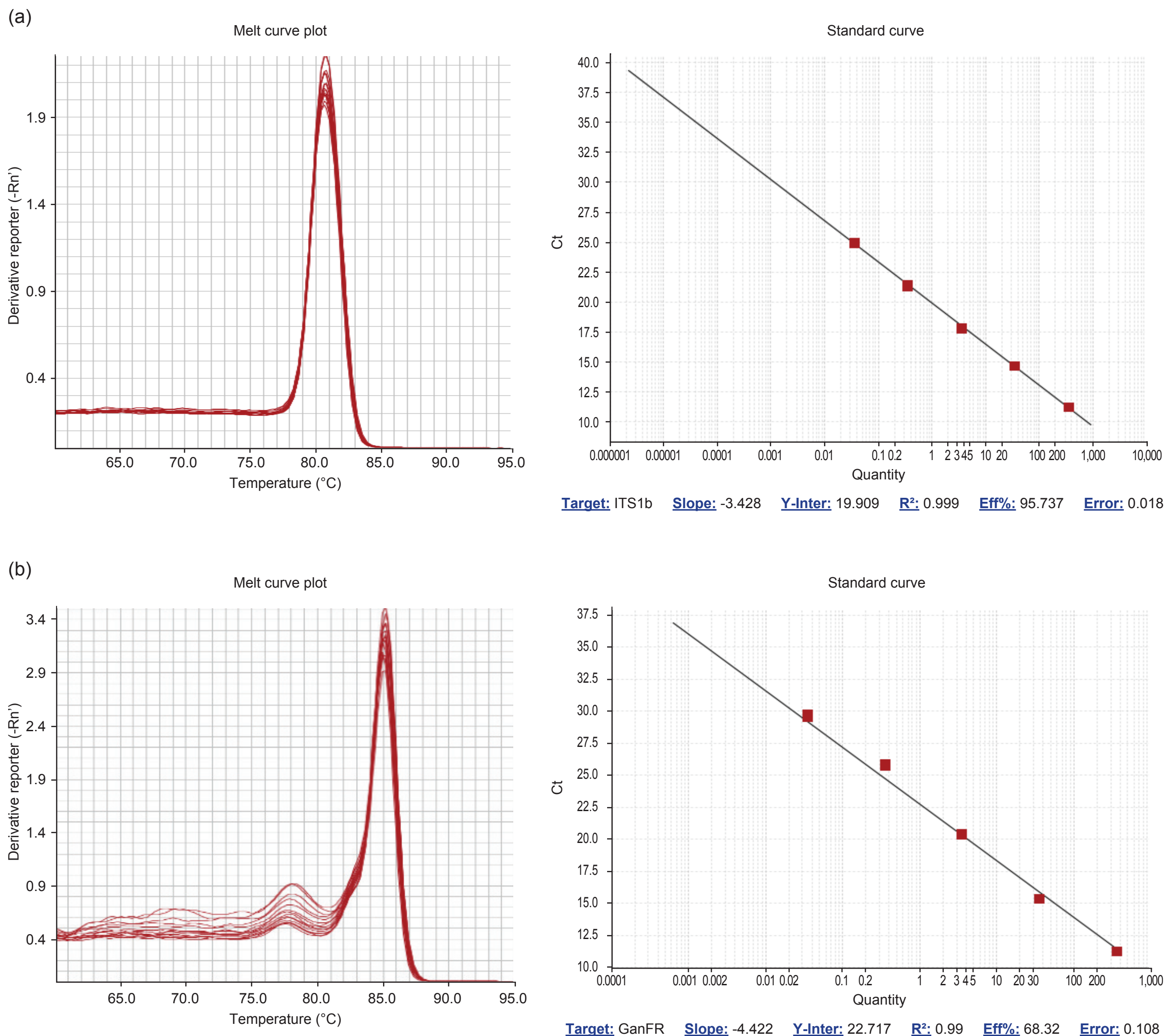

Figure 1. Melt curve plot and standard curve of (a) IGbF-R and (b) Gan1-2 constructed using G. boninense *pure culture DNA.*

evaluation. In this study, primer IGbF-R was developed based on the ITS sequences of *G. boninense* isolates sampled from different regions of Peninsular Malaysia. The primer specificity in detecting *G. boninense* and its synonym *G. orbiforme* and *G. miniatocinctum* was proven by verifying using 11 *Ganoderma* spp., and a collection of fungal and bacteria isolated from oil palm plantation soil. The effectiveness of IGbF-R was confirmed through successful amplification of the DNAs of *G. boninense* in two different soil types *i.e.*, Typic Kandiudults and Typic Quartzipsamments under nursery conditions which inoculum of *G. boninense* was present. Current application of IGbF-R is limited for nursery experimental trial. A robust IGbF-R-based qPCR system could be developed in near future to aid in detecting *G. boninense*, and quantitative monitoring of this fungal pathogen in oil palm plantations over generation of plantings.

ACKNOWLEDGEMENT

H.J.T. isolated and PCRed *G. boninense* isolates in this study, performed data analysis and prepared manuscript. Y.K.G. performed all qPCR related works and procured other *Ganoderma* spp and soil sample. W.C.W. designed the primer, edited and reviewed the manuscript. The authors would like to thank AAR Principals, Boustead Plantations Berhad and Kuala Lumpur Kepong Berhad for funding this research, and for their permission to publish the data in this article.

REFERENCES

Ariffin, D., & Idris, A. S. (1992). *The* Ganoderma *selective medium (GSM)*. PORIM Information Series No. 8. MPOB.

Chong, K. P., Dayou, J., & Alexander, A. (2017). Current detection methods of *G. boninense* infection in oil palm. In K. P. Chong, J. Dayou, & A. Alexander (Eds.), *Detection and control of* Ganoderma boninense *in oil palm crop* (pp. 13-20). Springer International Publishing.

Chow, Y. Y., Rahman, S., & Ting, A. S. Y. (2017). Understanding colonization and proliferation potential of endophytes and pathogens in planta via plating, polymerase chain reaction, and ergosterol assay. *Journal of Advanced Research, 8*, 13–21. https://doi.org/10.1016/j.jare.2016.10.008

Corley, R. H. V., & Tinker, P. B. (2016). Diseases of the oil palm. In R. H. V. Corley & P. B. Tinker (Eds.), *The oil palm* (pp. 399-436). Blackwell Publishing.

Goh, K. M., Dickinson, M., Alderson, P., Yap, L. V., & Supramaniam, C. V. (2016). Development of an *in planta* infection system for the early detection of *Ganoderma* spp. in oil palm. *Journal of Plant Pathology, 98*, 255–264. https://doi.org/10.4454/jpp.v98I2.019

Goh, Y. K., Zoqratt, M. Z. H. M., Goh, Y. K., Ayub, Q., & Ting, A. S. Y. (2020). Determining soil microbial communities and their influence on *Ganoderma* disease incidences in oil palm (*Elaeis guineensis*) via high-throughput sequencing. *Biology, 9*(12), 424. https://doi.org/10.3390/biology9120424

Goh, Y. K., Zoqratt, M. Z. H. M., Goh, Y. K., Ayub, Q., & Ting, A. S. Y. (2022). Discovering naturally-occurring microbiota in disease-suppressive soil: Potential role of biological elements in suppressing *Ganoderma boninense*. *Biological Control, 165*, 104787. https://doi.org/10.1016/j.biocontrol.2021.104787

Idris, A. S., Yamaoka, M., Hayakawa, S., Basri, M. W., Noorhasimah, I., & Ariffin, D. (2003). *PCR technique for detection of Ganoderma*. MPOB Information Series No. 188. MPOB.

Joo, S., Park, P., & Park, S. (2019). Applicability of propidium monoazide (PMA) for discrimination between living and dead phytoplankton cells. *PLoS ONE, 14*, e0218924. https://doi.org/10.1371/journal.pone.0218924

Luchi, N., Santini, A., Salvianti, F., & Pinzani, P. (2019). Early detection of fungal plant pathogens by real-time quantitative PCR: The case of *Diplodia sapinea* on pine. *Methods in Molecular Biology*, 95–104. https://doi.org/10.1007/978-1-4939-9833-3_8

Naher, L., Yusuf, U. K., Ismail, A., Tan, S. G., & Mondal, M. M. A. (2013). Ecological status of Ganoderma and basal stem rot disease of oil palms (*Elaeis guineensis* Jacq.). *Australian Journal of Crop Science*, *7*(11), 1723–1727. http://www.cropj.com/naher_7_11_2013_1723_1727.pdf

Rees, R. W., Flood, J., Hasan, Y., & Cooper, R. M. (2007). Effects of inoculum potential, shading and soil temperature on root infection of oil palm seedlings by the basal stem rot pathogen *Ganoderma boninense*. *Plant Pathology*, *56*(5), 862–870. https://doi.org/10.1111/j.1365-3059.2007.01621.x

Rees, R. W., Flood, J., Hasan, Y., Potter, U., & Cooper, R. M. (2009). Basal stem rot of oil palm (*Elaeis guineensis*); mode of root infection and lower stem invasion by *Ganoderma boninense*. *Plant Pathology*, *58*(5), 982–989. https://doi.org/10.1111/j.1365-3059.2009.02100.x

Rusli, M. H., Idris, A. S., & Cooper, R. M. (2016). Evaluation of Malaysian soils for potential suppressiveness of Fusarium wilt of oil palm caused by *Fusarium oxysporum* f. sp. *elaeidis*. *Journal of Microbial and Biochemical Technology, 8*, 459–464. https://doi.org/10.4172/1948-5948.1000325

Siddiqui, Y., Surendran, A., Paterson, R. R. M., Ali, A., & Ahmad, K. (2021). Current strategies and perspectives in detection and control of basal stem rot of oil palm. *Saudi Journal of Biological Sciences*, *28*(5), 2840–2849. https://doi.org/10.1016/j.sjbs.2021.02.016

Strohm, J. H. T. (2015). *The influence of agricultural tillage practices on soil biodiversity* (Master's thesis). The University of Guelph.

Utomo, C., & Niepold, F. (2000). Development of diagnostic methods for detecting Ganoderma-infected oil palms. *Journal of Phytopathology*, *148*(9–10), 507–514. https://doi.org/10.1046/j.1439-0434.2000.00478.x