

Research Article

Assessing the Suitability of Reference Genes for Normalising Gene Expression in *Ganoderma boninense*-Infected Oil Palm

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ABSTRACT

Oil palm is a major agricultural commodity in Southeast Asia, particularly in Malaysia and Indonesia. Its productivity, however, is being jeopardised by climate change and disease outbreaks, particularly basal stem rot (BSR) caused by *Ganoderma boninense*. Monoculture practices exacerbate disease spread, calling for innovative and sustainable solutions. Beyond current integrated plant management (IPM) practices, developing disease-tolerant plant material could enhance disease management. For that purpose, efforts have been initiated to identify potential genetic markers associated with *G. boninense* tolerance; include *in vitro* screening (IVS) using oil palm clonal material is employed, where artificial infection is conducted under a controlled environment. However, the reliability of such studies depend on accurate quantification of gene expression, which requires the use of reliable reference genes (RGs). Quantitative PCR (qPCR) is a powerful tool for measuring gene expression levels; however, reliable normalisation depends on RGs that maintain consistent expression across tissues and experimental conditions. Consistent RG expression is essential for minimising technical and biological variation, ensuring that observed changes in target gene expression reflect true biological responses rather than variation arising from the normalisation process. Therefore, this project evaluated ten potential RGs selected from the literature for gene expression analysis in young oil palm ramets under controlled *G. boninense*-infected conditions. Among the ten RGs evaluated, *ubi* and *fbox* were consistently identified as the most stable and reliable RGs for *in vitro* expression analysis under controlled disease-stressed conditions, based on geNorm, NormFinder, BestKeeper, and RefFinder analyses. In contrast, *gapdh* was unsuitable as an RG under these conditions. The RGs identified and validated in this study are anticipated to enhance the accuracy of gene expression analysis in *G. boninense*-infected oil palm ramets under controlled *in vitro* conditions. This approach will be instrumental in identifying potential defence-related genes associated with *G. boninense* tolerance through comprehensive gene expression profiling.

Key words: Gene expression, oil palm ramet, RT-qPCR, reference genes

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INTRODUCTION

The African oil palm (*Elaeis guineensis* Jacq.) is a crucial commodity of local use, providing a tremendous socio-economic advantage in terms of commerce and job opportunities, and meeting the continuously expanding global need for edible oil, oleochemicals, and biofuel (Kwan *et al.*, 2016). This sector serves as the largest revenue generator, and oil palm is a crucial plantation crop in Malaysia, with approximately 5.67 million hectares of cultivation area recorded in 2023 (Parveez *et al.*, 2024). Therefore, control of diseases that affect oil palm productivity is of great interest in this region. While monoculture practices are a major contributor to the prevalence of basal stem rot (BSR) diseases caused by *Ganoderma boninense*, its widespread occurrence is also linked to high soil inoculum, poor site drainage and environmental pressures (Zakaria, 2023). This pathogen initiates infection in the roots, progressively

colonizing the vascular tissues and reaching the stem base, where it disrupts water and nutrient transport within the plant. The resulting physiological stress weakens overall plant health, accelerates canopy decline, and can substantially reduce fresh fruit bunch (FFB) production (Kwan *et al.*, 2016; Woittiez *et al.*, 2017; Zakaria, 2023).

BSR disease was first reported in 1931 (Turner, 1965; Abas & Seman, 2012). Early on, the economic losses associated with the infection were deemed minimal as the disease was observed to afflict older palms, typically those over 25 years of age (Zakaria, 2023). Over the past three decades, infection rates have surged across all stages of the oil palm life cycle—driven by climate change, monoculture cultivation, and inadequate disease control—alongside the aggressive scaling of oil palm into a dominant commercial crop (Murphy *et al.*, 2021). Commercialisation of the oil palm industry in Malaysia expanded rapidly, with oil palm cultivation increases from 54,000 ha

in the 1960s to 800,000 ha by 1980 under diversification policies and FELDA settlement initiatives (Rahmat, 2016). The rapid expansion of oil palm plantations increased the extent of areas susceptible to *G. boninense* infection, thereby amplifying its impact on plantation productivity and causing substantial economic losses. The presence of *G. boninense* in plantation areas can significantly reduce oil palm fresh fruit bunch (FFB) production within just 2–3 years of initial infection, with productivity losses reaching up to 80% in severely infected palms. Consequently, this notorious infection has contributed to annual productivity losses up to USD 500 million a year (Zakaria, 2023).

Oil palm populations originating from diverse geographical regions and genetic lineages exhibit varying susceptibility to *G. boninense*, reflecting differences in tolerance likely shaped by each palm's unique genetic makeup—an effect supported by multi-omics reviews (Bharudin *et al.*, 2022; Saputra *et al.*, 2026) and RNA-seq studies highlighting differential gene expression among seedlings from varied origins (Shafiq Saipol *et al.*, 2024). Although numerous approaches to control BSR have been developed, their effectiveness varies considerably, potentially due to the diverse genetic makeup of *G. boninense* populations resulting from genetic recombination through its tetrapolar mating system (Ab Wahab *et al.*, 2024). This genetic diversity is closely associated with its dimorphic mycelial life cycle, in which compatible monokaryons undergo mating to form dikaryotic secondary infective mycelia capable of activating virulence-related genes and causing basal stem rot (BSR), while the monokaryotic mycelia remain primarily saprophytic (Chan *et al.*, 2011; Angel *et al.*, 2026).

As part of the ongoing strategy to combat basal stem rot (BSR) and given the limited reports on potential molecular biomarker candidates associated with *G. boninense* tolerance, a more comprehensive understanding of the host response is needed. In particular, investigating genes involved in the host–pathogen interaction may provide valuable insights into disease progression and support the identification of molecular biomarkers for screening tolerant planting materials. To address this gap, the present study was initiated using an *in vitro* infection assay to minimise the influence of external environmental factors and enable a more controlled investigation of host responses to *G. boninense* infection.

Whole-transcriptome RNA sequencing (RNA-seq) and targeted amplicon RNA-seq provide high-throughput approaches for profiling transcript abundance and identifying genes and biological pathways associated with host–pathogen interactions (Wang *et al.*, 2009; Ma *et al.*, 2018). While transcriptomic approaches enable comprehensive characterisation of gene expression across biological, spatial, and temporal conditions, they generally require greater experimental, computational, and data-analysis resources (Westermann *et al.*, 2017). In contrast, quantitative real-time PCR (qPCR) is a sensitive, specific, and cost-effective approach for targeted quantification of selected transcripts and is widely used to validate candidate genes identified through transcriptomic analyses (Bustin *et al.*, 2009; Vijayakumar & Sakuntala, 2024). However, reliable qPCR analysis depends on appropriate normalisation using reference genes with stable expression, as RG stability is highly dependent on the biological material and experimental conditions (Xia *et al.*, 2014).

Several reference genes (RGs) have been identified for specific oil palm tissues and infection conditions. Amiruddin *et al.* (2022) reported *egref_5*, *egref_7*, and *egref_11* as stable across various mature tissues and tissue culture stages, while Mohd Zuhar *et al.* (2019) identified *Gibberellin-responsive protein 2*, *GRAS*, and *actin* as suitable RGs in infected seedlings, and *GAPDH* and *Mn-SOD/GVHK* in infected mature palms. However, RG stability is highly

dependent on the biological material and experimental conditions (Xia *et al.*, 2014), and no study has specifically evaluated RGs in young oil palm tissue-culture ramets under *in vitro* *G. boninense* infection. Therefore, this study aims to identify and validate stable RGs for qPCR-based gene expression analysis in root tissues across infection stages up to 8 weeks post-inoculation (wpi). Establishing reliable RGs will provide a foundation for subsequent host defence gene profiling and the identification of potential biomarkers for *G. boninense* tolerance.

MATERIALS AND METHODS

Selection of planting material

Young oil palm ramets used in this study were sourced from the Tissue Culture Department, FGV R&D Sdn. Bhd (Negeri Sembilan, Malaysia). Two-month-old post-rooting ramets with at least three green leaves and three roots. Two ramets were used as technical replicates for each infection time point, while another two uninfected ramets served as negative controls. The *in vitro* infection assay was conducted at eight time points, ranging from 1 to 8 wpi, with samples collected weekly to capture disease progression over time. The observation period was limited to 8 wpi, as severely infected susceptible planting materials were mostly dead by week 9–10, whereas tolerant material generally remained healthy (Supplementary data A). Inoculation was performed using 5 mm agar plugs containing five-day-old *G. boninense* mycelia grown on half-strength potato dextrose agar (PDA). The mycelial agar plugs were transferred into test tubes containing semi-solid medium and incubated for 3 days to allow fungal colonisation of the medium. Subsequently, one oil palm ramet was introduced into each test tube containing semi-solid medium colonised by *G. boninense*. The *in vitro* infection assays were maintained in a Conviron growth chamber (Model Gen100) at 28°C and 70% relative humidity (RH) under an 8 hr light/16 hr dark photoperiod. The light source consisted of LED lights (6500 K Cool Daylight, 60 mm, 220–240 V, 50/60 Hz, 70 mA; Model 9290011848C) set at 50% intensity, with a distance of 3.5 cm between the light source and the test tube lids. Infected root tissues were harvested at the respective time points and stored at –80°C until further analysis. The schematic figure of the assay is illustrated in Figure 1.

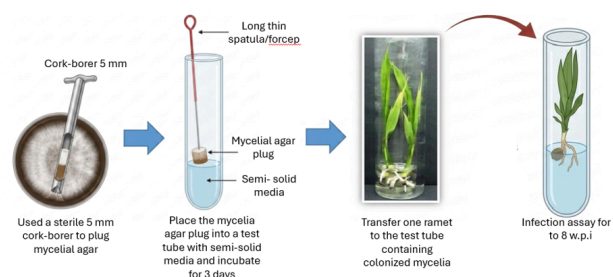


Fig. 1. Schematic representation of the laboratory-scale protocol for *G. boninense* infection on *in vitro* oil palm ramet samples. Duplicate infected samples were harvested weekly from 1-week post-infection (wpi) to 8 wpi.

RNA extraction and cDNA synthesis

Oil palm root samples were cryogenically ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted from both infected and non-infected root tissues using the RNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's protocol. Genomic DNA contamination was subsequently removed by DNase I treatment (Qiagen, Germany). The concentration and purity of the extracted RNA were measured using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), while RNA integrity was evaluated by electrophoresis on a 1.0% (w/v) agarose gel in 1× TBE buffer, run at 80 V for 30 min. For

Table 1. List of primers used in qPCR analysis.

Gene Name	Abbreviation	Function	Expected size (bp)	Primer Sequences (5'>3')
Alpha-tubulin	α -tub	Cell growth and elongation	192	F – CAACACCTTCTTCAGTGAGACC R – CTCCTTCCCAGATTGTGTAATGG
Beta-tubulin	β -tub	Cytoskeleton structural protein	185	F – CCTACAACGCCACCTTATCTG R – TTCAAGTCTCCAAAGCTGGG
Cyclophilin	cyc	Peptidyl prolyl isomerase activity	138	F – GAGGCGATCAGACTCAATCC R – CATCCAGTCCCTTCAAGCACG
Elongation factor	eef	Elongation	123	F – CACCCGTATCCAAAGATTCTG R – GAAATAATTGTCTGCCCACTCTC
F-box domain containing protein	f-box	Signal transduction	168	F – GTTTATCATCCATCACCCTG R – CATTCTGTCCACCTCCTTCCA
Glyceraldehyde-3-phosphate	gapdh	Energy provider	198	F – GGCATTAGGAACCTGAGGA R – GTCAGACTTGTATTTCATGCTCGT
Mitogen-activated protein kinase	mapk	Immunity and stress response	174	F – TAGTCAAGTCTCTGCATTTACC R – GAACTTGGTTATCTCCTTAGCC
Manganese superoxide dismutase	mnsod	Oxidative stress control	151	F – CGGACTATGGTTCAGTGGAG R – ACAAGTGGATCCTGATTTCG
40S ribosomal	r40s	Protein synthesis	136	F – GAGGCTAAGTTCAAGCTATGCA R – CCAGATCAAGCTTAATAGTGTCTG
Ubiquitin conjugating protein	ubi	Abiotic stress	181	F – GCCTTCATAGGTGGATCATCG R – GGATGGAGGAAGATGACCTG

cDNA synthesis, 1 μ g of total RNA from each oil palm sample was reverse-transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen, USA) with oligo(dT) primers in a final reaction volume of 20 μ L, following the manufacturer's instructions. The resulting cDNA was stored at -80°C until further use.

Primer design and amplification efficiency

Ten commonly used RGs in oil palm gene expression studies— *α -tubulin*, *β -tubulin*, *cyclophilin*, *elongation factor (eef)*, *F-box protein (fbox)*, *glyceraldehyde-3-phosphate dehydrogenase (gapdh)*, *mitogen-activated protein kinase (mapk)*, *manganese superoxide dismutase (mnsod)*, *ribosomal protein 40S (r40s)*, and *ubiquitin-conjugating protein (ubi)*—were selected based on prior studies across different oil palm growth and development stages (Chan *et al.*, 2014; Muhamad Afiq *et al.*, 2019). These genes represent diverse biological functions, minimising the likelihood of co-regulation and providing a broad panel for stability assessment. Although other RGs are available, these 10 RGs were selected because they have previously been evaluated in oil palm at both nursery and mature stages, providing an opportunity to identify overlapping RGs with stable expression for the present *in vitro* infection study and potentially for application across other developmental stages (Zuhair *et al.*, 2019; Muhamad Afiq *et al.*, 2019).

To confirm their relevance to this study, the selected genes were further cross-validated using our previous in-house transcriptome database (unpublished data), derived from *G. boninense*-infected oil palm root tissues subjected to controlled *in vitro* infection, covering selected time points from 12 hr post-inoculation to 21 days post-inoculation. This validation step confirmed that all candidate genes were expressed within the specific pathosystem, supporting their suitability for subsequent stability assessment in this study. In addition, the Iso-Seq transcriptome databases were used to examine potential transcript isoforms, thereby facilitating the identification of suitable and specific regions for qPCR primer design. Primer pairs were designed using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) with *Elaeis guineensis* (taxid:51953) as

the reference organism. Primer specificity was optimised to target exon–exon junctions, with melting temperatures between 55 – 60°C , primer lengths of 20–24 bp, GC content ranging from 40–60%, and amplicon sizes between 90–200 bp (Table 1). Primer specificity was evaluated through melt curve analysis using the LightCycler® 480 system (Roche, Germany) and further confirmed by visualisation on a 1.5% agarose gel electrophoresed in $1\times$ TAE buffer, run at 100 V for 30 min. Primer efficiency was determined using a standard linear curve generated from five serial 10-fold dilutions of the target cDNA template, prepared using nuclease-free water. Primer efficiency (E) and correlation coefficient (R^2) were calculated from standard curve slopes using the formula: $E = [10^{-(1/\text{slope})} - 1] \times 100\%$. Only primers with efficiencies between 90 – 110% were selected for subsequent analysis (Bustin *et al.*, 2009; Svec *et al.*, 2015).

Reverse Transcription Quantitative PCR (RT-qPCR) reaction

RT-qPCR analysis was carried out using the FastStart Essential DNA Green Master kit (Roche, Switzerland), following the manufacturer's protocol. Each reaction contained 1 μ L of 50-fold diluted cDNA as the template. Amplification was conducted in LightCycler® 480 Multiwell Plate 96 (Roche, Germany) 96-well plates using the LightCycler® 480 System. The thermal cycling protocol began following the thermal profile shown in Table 2. All reactions were performed in triplicate, and a no-template control (NTC) was included to monitor for contamination. The quantification cycle (Cq) values were automatically recorded and used for subsequent gene expression stability analysis.

Analysis and validation of expression stability

The raw qPCR data were processed using the LightCycler® 480 software (Roche), and Cq values were converted into linear scale data suitable for downstream analysis using multiple gene stability evaluation tools. The expression stability of candidate RGs across all sample groups was assessed using three widely adopted algorithms: geNorm (based on pairwise comparison) (Vandesompele *et al.*, 2002), NormFinder (model-based approach) (Andersen *et al.*, 2004), and BestKeeper (descriptive statistical analysis) (Pfaffl *et al.*, 2004),

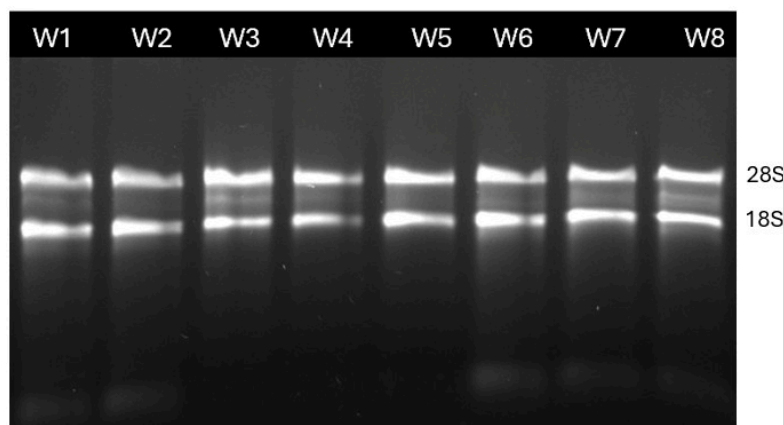


Fig. 2. Agarose gel electrophoresis of total RNA from *G. boninense*-infected oil palm roots. Lanes 1–8: total RNA (500 ng) from individual samples. Electrophoresis was performed on a 1.0% agarose gel in 1× TBE buffer containing RedSafe™ Nucleic Acid Staining Solution at 80 V for 30 min. RNA integrity is indicated by the presence of distinct and well-defined 28S and 18S rRNA bands with minimal degradation.

each executed according to the developers' recommended protocols with default settings. Given that each algorithm evaluates gene stability from a distinct methodological perspective, integrating their outputs using RefFinder (Xie *et al.*, 2023), a comprehensive web-based tool, enables a more robust and consensus-driven ranking of RG stability. This combined approach enhances the reliability and accuracy of selecting the most suitable RGs for normalisation in qPCR studies (Vandesompele *et al.*, 2009).

RESULTS

RNA quality

Purified RNA samples had $A_{260/280}$ and $A_{260/230}$ ratios in the ranges of 1.80 – 2.00 and 2.00–2.40, indicating that the extracted RNA samples were pure with negligible protein and organic contaminants. Two intact 18S and 28S ribosomal RNA bands were assessed through agarose gel electrophoresis (Figure 2). The purified RNA samples that had good quality and highly intact, fulfilling the minimal sample requirement for RT-qPCR analysis.

Evaluation of RG-specific primer pairs

To assess the suitability of the primer pairs for RT-qPCR analysis, each RG-specific primer pair was evaluated by conventional PCR using cDNA as the template (Table 2). Agarose gel electrophoresis showed a single amplification product for all ten candidate RGs – *α-tub*, *β-tub*, *cyc*, *eef*, *f-box*, *gapdh*, *mapk*, *mnsod*, *r40s*, and *ubi*—indicating specific amplification (Figure 3). Each primer pair produced a clear amplification product without visible primer-dimer formation, indicating their suitability for subsequent qPCR analysis. In addition, RT-qPCR was performed using cDNA templates, and the resulting melting curves were evaluated to further confirm amplification specificity. The melting curves of the ten candidate RGs all produced dissociating curves with single peaks, indicating specific amplification capability (Supplementary data B). Since the samples were prepared under co-culture conditions involving oil palm ramets and *G. boninense*, the PCR products were cloned, sequenced, and subjected to BLAST analysis against the NCBI databases to

verify the specificity of the designed primer sets. This confirmation was necessary due to the possibility of unintended amplification or false priming, which could result in amplification of *G. boninense* sequences rather than the intended oil palm reference genes. The summarised sequence information of each isolated cDNA encoding potential RGs was shown in Table 3. The efficiency of primer amplification (*E*) was calculated based on the slope (*K*) and correlation coefficient (*R*²) derived from the standard curve. The amplification of the candidate genes varied from 90.8% for *mnsod* to 110% for *ubi*; this is the acceptable range for most of the efficiency studies (Bustin *et al.*, 2009), while fitting a regression line to the data points resulted in correlation coefficients for the candidate genes ranging from 0.97 – 0.99 (Table 4).

Expression profile of RGs

To assess the consistency of transcript abundance of the ten candidate RGs across different infection time points, their quantification cycle (Cq) values were determined using RT-qPCR. At this stage, the Cq values provide an indication of the amount of target transcript detected in each sample prior to normalisation against a housekeeping gene (HK). As an initial assessment of candidate RG suitability, both the transcript abundance and consistency of the Cq values were considered. Candidate RGs with extremely low Cq values, indicating very high transcript abundance, or extremely high Cq values, indicating relatively low transcript abundance, may be less suitable for normalisation because substantial differences in transcript abundance between the target and RG can affect the reliability of subsequent normalisation (Köhler *et al.*, 2020). Therefore, candidate RGs with moderate and consistent Cq values across the tested samples are generally preferable. Among the genes analysed, *cyc* and *f-box* exhibited the narrowest Cq ranges, with differences of 2.31 and 2.46 cycles, respectively, between their highest and lowest Cq values, whereas *gapdh* showed the widest Cq range, with a difference of 8.12 cycles (Table 5). Under prolonged disease conditions, *cyc* exhibited the lowest coefficient of variation (CV) at

Table 2. Thermal cycling profile used for primer assessment and RT-qPCR.

Step	Temperature	Time	Cycles
Initial denaturation	95°C	5 min	1×
Denaturation	95°C	10 s	45×
Annealing	55°C	20 s	45×
Extension	72°C	10 s	45×
Hold	4°C	Forever	1×

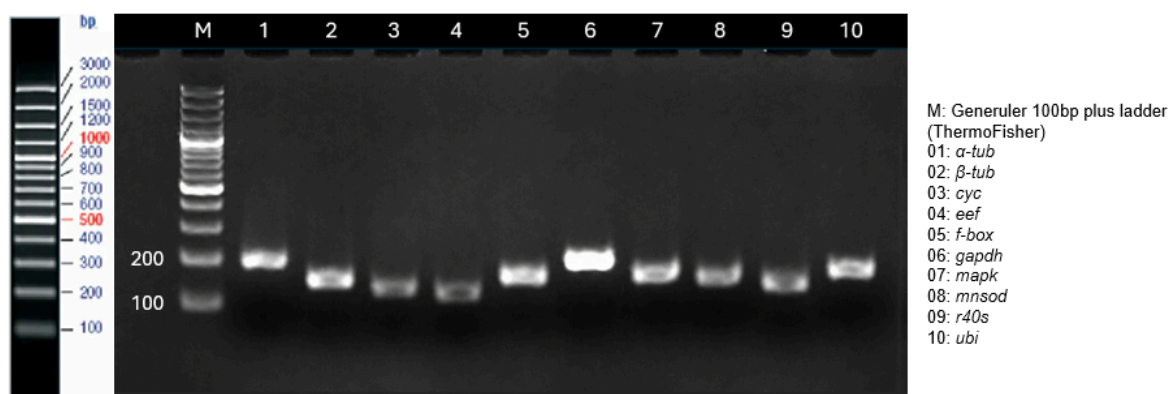


Fig. 3. Specificity analysis of ten candidate RG primers of oil palm.

Table 3. Summarised information on cloning sequences of partial-length cDNA encoding potential RGs from oil palm.

No	Gene name	Partial-length sequence (bp)	Coding region (bp)	Species	Accession No.	Similarity (%)
01	α -tub	192	192	<i>E. guineensis</i> tubulin alpha chain	XM_010941239.3	100
02	β -tub	185	185	<i>E. guineensis</i> tubulin beta-2 chain	XM_010907071.3	100
03	cyc	138	138	<i>E. guineensis</i> peptidyl-prolyl cis-trans isomerase	XM_010915523.3	100
04	eef	123	123	<i>E. guineensis</i> elongation factor G	XM_010917697.3	100
05	F-box	168	168	<i>E. guineensis</i> F-box only protein 6	XM_010928518.3	100
06	gapdh	198	198	<i>E. guineensis</i> glyceraldehyde-3-phosphate dehydrogenase	XM_010912093.1	100
07	mapk	174	174	<i>E. guineensis</i> mitogen-activated protein kinase	XM_010918251.3	100
08	mnsod	151	151	<i>E. guineensis</i> superoxide dismutase [Mn]	XM_010939703.3	100
09	ubi	136	136	<i>E. guineensis</i> probable ubiquitin-conjugating enzyme	XM_010919381.3	100
10	r40s	181	181	<i>E. guineensis</i> 40S ribosomal protein S4-3	XM_010940121.3	100

2.38%, whereas *gapdh* showed the highest CV at 12.07%. Overall, the mean Cq value across all candidate RGs was 30.6 cycles, with more than 80% of the Cq values falling between 25 and 35 cycles (Figure 4). Based on the mean Cq values, the candidate RGs were ranked from the highest to lowest apparent transcript abundance

across the 8-week infection period as follows: *gapdh* > *ubi* > *mnsod* > *r40s* > *f-box* > *mapk* > *eef* > *cyc* > *α -tub* > *β -tub*. *gapdh* had the lowest mean Cq value of 26.1 cycles, whereas *β -tub* and *α -tub* had the highest mean Cq values, at 34.6 and 34.27 cycles, respectively. These differences in Cq values provide an indication of the apparent

Table 4. Summary of primer amplification efficiency test of the RGs from *G. boninense*-infected oil palm root.

Gene	E (%)	R ²	Slope
α -tub	103.5	0.99	-3.241
β -tub	97.2	0.99	-3.391
cyc	98.8	0.97	-3.351
eef	101.1	0.99	-3.297
fbox	108.9	0.99	-3.126
gapdh	91.18	0.99	-3.554
mapk	101.6	0.99	-3.2834
mnsod	90.8	0.99	-3.564
r40s	103.3	0.99	-3.2443
ubi	110	0.99	-3.101

Table 5. Mean Cq values of the ten candidate RGs across multiple time points following inoculation.

Sample	α -tub	β -tub	Cyc	eef	f-box	gapdh	mapk	mnsod	ubi	r40s
1 wpi	33.52±0.89	34.17±1.27	33.83±0.21	31.34±1.08	29.65±0.29	25.46±1.95	31.79±1.79	27.41±1.35	29.58±0.61	26.90±1.26
2 wpi	35.89±0.72	37.11±0.58	34.29±0.13	34.91±1.50	30.81±0.54	31.20±2.23	36.28±1.62	30.89±1.18	31.42±0.61	30.36±1.36
3 wpi	36.00±0.18	36.82±0.71	33.99±0.37	33.74±1.18	30.76±0.16	28.09±2.20	32.88±1.97	29.23±1.11	29.35±1.21	29.89±0.57
4 wpi	36.39±0.31	36.17±0.42	33.96±0.08	30.96±0.09	29.96±0.06	23.08±0.07	28.54±0.10	26.91±0.11	27.00±0.08	28.40±0.08
5 wpi	36.92±0.01	36.83±0.85	34.33±0.02	30.87±0.03	29.61±0.08	23.16±0.04	28.72±0.06	27.09±0.07	27.12±0.05	28.49±0.03
6 wpi	33.78±1.33	34.58±1.45	33.61±0.30	29.75±0.57	28.91±0.27	23.32±0.14	28.25±0.28	26.61±0.25	26.38±0.38	28.86±0.30
7 wpi	30.86±0.07	30.53±0.20	32.78±0.27	28.88±0.30	28.35±0.07	24.81±1.78	28.92±1.70	27.14±1.51	26.09±0.75	29.70±0.58
8 wpi	30.83±0.03	30.63±0.02	32.02±0.14	29.70±0.30	28.41±0.01	29.55±1.70	34.08±2.36	31.14±1.54	28.18±0.74	31.05±0.39
Cq variation	6.09	6.58	2.31	6.03	2.46	8.12	8.03	4.54	5.34	4.15
Overall mean Cq	34.27	34.60	33.60	31.27	29.56	26.08	31.18	28.30	28.14	29.21
CV (%)	7.09	7.82	2.38	6.63	3.23	12.07	9.73	6.55	6.58	4.49

Mean Cq value: The average Cq value across biological replicates at each time point $\pm$ standard deviation. Cq variation: the difference between the highest and lowest mean Cq values recorded for each gene across all time points. CV (%): coefficient of variation, calculated as (standard deviation / overall mean Cq) $\times$ 100.

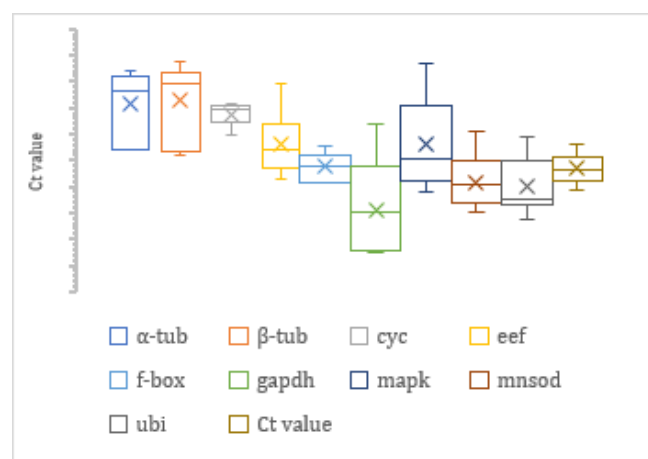


Fig. 4. Expression level of 10 candidate RGs across 10 weeks of disease treatment in *E. guineensis*. Y-axis values are given in the form of qPCR threshold cycle (Ct) values. The boxes represent the distribution of the Cq value, and bars indicate the standard deviation of the Cq value in all experimental samples.

transcript abundance of the candidate RGs; however, they do not represent normalised gene expression levels because the data have not yet been normalised against an HK. The observed Cq ranges and coefficients of variation therefore provide an initial assessment of transcript abundance and its consistency across the infected root samples. While the Cq values indicate differences in the apparent abundance of the candidate transcripts, the suitability of each RG cannot be determined based on Cq values alone. Subsequent normalisation and reference-gene stability analyses will be used to determine the most suitable RGs for downstream RT-qPCR analysis under prolonged disease conditions.

Evaluation of the expression stability of RGs

Since the ten candidate RGs exhibited significant divergence across different time points, several software tools were employed to rank the genes based on their stability. These programs could offer complementary approaches, providing a well-rounded picture of candidate RGs' suitability by evaluating the expression stability of RGs from different aspects. NormFinder program utilises a model-based approach, assessing gene expression stability using linear scale data. It accounts for both inter- and intra-group variability in its stability calculation. The assigned S (stability) value represents

a weighted measure of expression consistency, with lower S values indicating higher stability (Sundaram *et al.*, 2019). In the *in vitro* infection, *G. boninense* study on 2-month oil palm ramets, *ubi* displayed the highest stability with NormFinder, followed by *eef* with S values of 0.663 and 0.694, respectively, and *gapdh* being the least stable gene (S value: 2.436).

Excel-based BestKeeper program analysis evaluates the expression stability of the RGs based on the variability of their Cq values across different samples. This is accomplished by calculating standard deviations (SD) of the original Cq value under various coefficients of variation (CV) settings. The gene exhibiting the least variation (lowest SD & CV) in the parameters is deemed to be the most stable and reliable candidate for normalisation purposes (Muhamad Afiq *et al.*, 2019). Analytical results with BestKeeper software suggested *cyc*, followed by *f-box* and *r40s*, as the most stable genes among the 10 potential RGs.

The geNorm analysis evaluates the stability of candidate reference genes by calculating the M value, which represents the expression stability of each of the ten candidate RGs under prolonged disease conditions. A lower M value indicates greater expression stability,

Table 6. Gene expression stability under disease-stress treatment ranked by geNorm, NormFinder, BestKeeper and RefFinder.

Rank	RefFinder Gene	RefFinder Stability Value	geNorm Gene	geNorm Stability Value	NormFinder Gene	NormFinder Stability Value	BestKeeper Gene	BestKeeper Stability Value	Delta Ct Gene	Delta Ct Stability Value
1	fbox	1.57	fbox	0.584	ubi	0.663	cyc	0.624	f-box	1.79
2	cyc	2.24	cyc		eef	0.694	f-box	0.766	ubi	1.83
3	ubi	2.38	eef	1.182	f-box	0.834	r40s	1.044	eef	1.85
4	eef	3.22	ubi	1.285	mnsod	1.295	ubi	1.555	mnsod	2.03
5	mnsod	4.47	mnsod	1.535	cyc	1.404	mnsod	1.596	cyc	2.04
6	r40s	5.05	r40s	1.629	r40s	1.602	eef	1.631	r40s	2.28
7	α -tub	7.48	α -tub	1.837	mapk	2.181	α -tub	2.082	mapk	2.49
8	mapk	7.94	β -tub	1.930	α -tub	2.254	β -tub	2.312	α -tub	2.58
9	β -tub	8.49	mapk	2.112	β -tub	2.323	mapk	2.658	β -tub	2.63
10	gapdh	10.00	gapdh	2.228	gapdh	2.436	gapdh	2.741	gapdh	2.67

geNorm ranks the two most stable genes as a tied top pair with a shared M value, hence no distinct rank 2 entry.

with genes having an M value <1.5 generally considered suitable for use as reference genes. The geNorm algorithm is a statistical approach widely used to identify stable reference genes for qPCR normalisation (Vandesompele *et al.*, 2002). As shown in Table 6, the candidate RGs were ranked according to their M values as follows: *f-box* = *cyc* > *eef* > *ubi*. The “=” sign indicates that *f-box* and *cyc* were assigned the same ranking position based on their comparable M values and were identified as the most stable candidate RGs. *eef* and *ubi* followed in the subsequent ranking positions, indicating comparatively lower stability.

The stability of each RG was also evaluated by Δ Cq, with the gene(s) that exhibit the lowest mean standard deviation considered the most stable. The Δ Cq analysis indicated that *f-box* was the most stable gene across different infection time points, while *gapdh* was identified as the least stable RG across all samples. To address the potential discrepancies in evaluating RGs across different software, RefFinder presents a valuable solution (Table 6). This web-based integrated tool leverages the strengths of multiple programs to provide a reliable assessment of RG suitability for RT-qPCR studies (Xie *et al.*, 2023). The comprehensive stability ranking across the 8-week infection period revealed that *f-box* was suggested as the best RG for normalising the expression of target genes, while *gapdh* was consistently identified as the least stable gene by all software. The comprehensive stability ranking for all ten potential RGs was as follows: *f-box* > *cyc* > *ubi* > *eef* > *mnsod* > *r40s* > α -*tub* > *mapk* > β -*tub* > *gapdh*.

Validation of the potential RGs

RefFinder analysis identified *f-box*, *cyc*, *ubi*, and *eef* as the four most stable RGs in this study. The stability of these RGs was further validated based on $\Delta\Delta$ Cq variation by comparing the consistency of each gene's expression relative to the others. Theoretically, an ideal RG should demonstrate minimal variability in expression relative to the other RGs, while the least stable gene is expected to exhibit the greatest fluctuations (Vandesompele *et al.*, 2002). Figure 5 illustrates the $\Delta\Delta$ Cq values for the four most stable RGs relative to the other RGs. The $\Delta\Delta$ Cq value represents the change in the normalized Cq value of a sample relative to the selected calibrator. On the y-axis, the $\Delta\Delta$ Cq value is plotted, which is inversely proportional to gene expression stability—meaning that lower $\Delta\Delta$ Cq values correspond to higher stability and less variation across different time points. Based on the data presented in Figure 5, the *ubi* gene exhibits the least variation in expression. The rank for the validation analysis

was as follows: *ubi* > *f-box* > *eef* > *cyc*. The expression variation of each gene is available in Supplementary data C.

DISCUSSION

BSR caused by *G. boninense* severely threatens the oil palm industry in Malaysia, impacting profitability and sustainability, with annual losses reaching up to USD 500 million (Ibrahim *et al.*, 2020). With current advancements in transcriptome data analysis, it is now possible to systematically unravel the molecular architecture of oil palm responses to fungal infection (Zuhar *et al.*, 2021). However, the widely known, cost-effective yet precise technique, qPCR, is still regarded as an effective approach for evaluating gene expression associated with the plant defence system. RGs serve as the cornerstone for accurate normalisation in qPCR, enabling researchers to obtain expression data sets with minimal variation arising from sample preparation and reaction conditions (Bustin *et al.*, 2009). Evidence from some studies suggests that the expression stability of RGs can fluctuate when subjected to various stressors (Orrego *et al.*, 2022; Xing *et al.*, 2025). As a result, research aimed at identifying suitable RGs across various oil palm studies has been spurred. Over the years, many suitable RGs have been identified in oil palm, with investigations spanning different tissue types (Zuhar *et al.*, 2019), genome-wide analyses (Xia *et al.*, 2014), and developmental stages (Chan *et al.*, 2014). This research sought to determine suitable RGs for gene expression of *in vitro* oil palm ramets across different stages of *G. boninense* root infection, a system that has yet to be established. The presence of fungal RNA within the total RNA extracted poses a key obstacle to *in vitro* expression analysis of oil palm genes. Although a total of 1 μ g of RNA was used for cDNA synthesis for each sample, this RNA does not necessarily represent oil palm-derived RNA alone, as the samples were collected under co-culture (*in vitro*) conditions. As *G. boninense* was suspected to have entered the oil palm roots during infection, a proportion of the extracted RNA may have originated from the fungus. Consequently, the relative proportions of plant- and fungus-derived RNA may vary as infection progresses, potentially affecting the accuracy of subsequent normalisation. Similar observations, where RNA extracted from infected plant tissues may contain a mixture of host- and pathogen-derived transcripts, have also been reported in previous studies (Orrego *et al.*, 2022).

Among the ten candidate RGs analysed, β -*tub* exhibited the highest mean Cq value, whereas *gapdh* showed the lowest mean Cq value. As Cq values are inversely related to the amount of target transcript detected, the lower mean Cq value observed for *gapdh* indicates

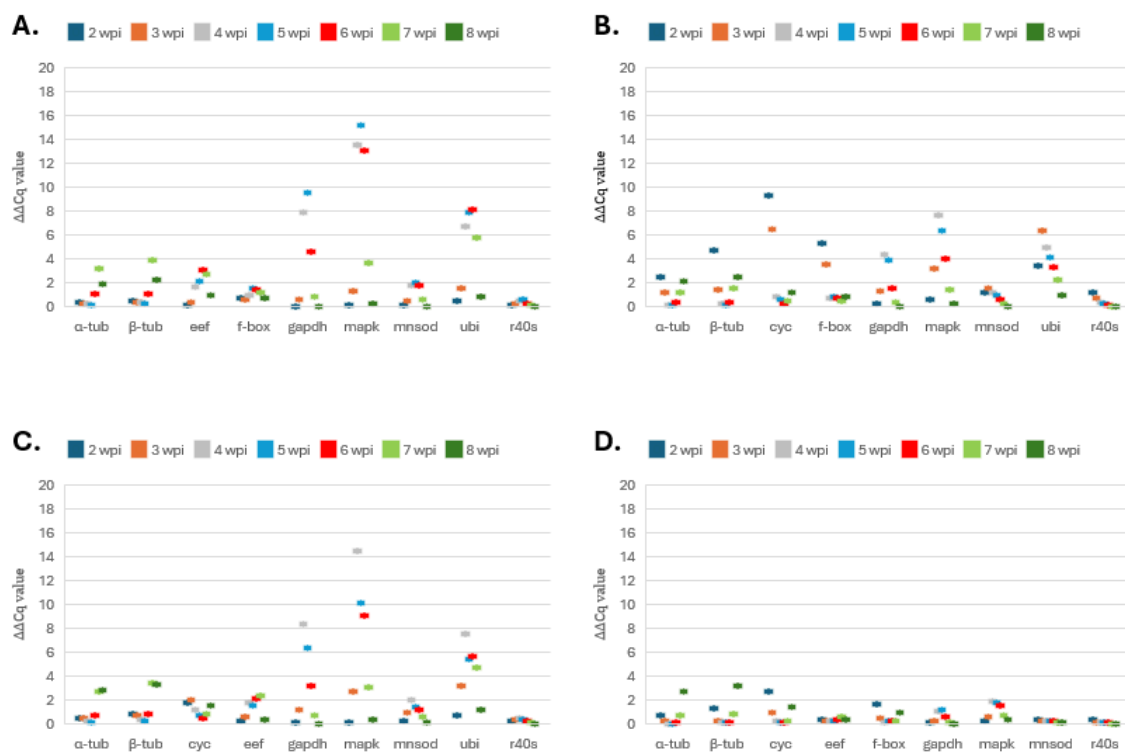


Fig. 5. The $\Delta\Delta Cq$ value for the four most stable RGs across *G. boninense* infection time points. Panels A, B, C and D represent *cyc*, *eef*, *f-box* and *ubi*, respectively. Each panel displays the $\Delta\Delta Cq$ value for various RG candidates. Different colored data points correspond to various *G. boninense* infection times (1–8 wpi).

a higher apparent transcript abundance compared with the other candidate RGs. However, these Cq values represent unnormalised measurements and therefore should not be interpreted as relative gene expression levels. The smallest expression variability was observed in *cyc* (2.31) and *f-box* (2.46), in contrast to *gapdh* (8.12), which had the highest variation across samples (Table 5). In this study, geNorm, NormFinder, and BestKeeper were employed to evaluate the stability of RGs under disease-stress conditions. The results exhibited some discrepancy in RG stability across different software tools, possibly due to the different algorithms each program employs (Wu *et al.*, 2025). Similar inconsistent trends have been documented in other oil palm RGs studies (Xia *et al.*, 2014) as well as in other species such as *Dioscorea opposita* (Chinese yam) (Xing *et al.*, 2025). In this study, geNorm ranked *f-box* and *cyc* as the most stable RGs across different infection stages, while NormFinder, BestKeeper and Delta Cq ranked *ubi*, *cyc* and *f-box*, respectively. Conversely, *gapdh* was consistently ranked as the least stable gene across all four algorithm programs, making it the most unreliable choice as an internal RG for gene expression analysis on *in vitro* *G. boninense*-infected oil palm roots.

To address discrepancies resulting from the use of multiple software tools, RefFinder, an integrated analysis platform, was employed to consolidate and evaluate their results. RefFinder functions by weighting each RG and calculating the geometric mean of these weights to establish a comprehensive ranking for stability. For RefFinder, genes with the smallest geometric mean values are regarded as the most stable (Wu *et al.*, 2025). In the present work, RefFinder analysis ranked a different set of most stable RGs, in which *f-box* and *cyc* genes are the most reliable RGs to be used in gene expression studies involving *in vitro* *G. boninense*-infected oil palm ramets. Meanwhile, *gapdh* remained the least stable RG even by the RefFinder algorithm. By comparing the stability rankings produced by four algorithms—geNorm, NormFinder, BestKeeper,

and the delta Cq method—all programs were in consensus in ranking *f-box* among the top four most stable RGs and *gapdh* as the least stable, demonstrating strong correlation among these tools. Additionally, *cyc* exhibited considerable stability, indicating its potential as a suitable RG under specific conditions.

The validation analysis was conducted on *cyc*, *eef*, *f-box*, and *ubi*, which are the top four most stable RGs based on the RefFinder platform. Their stability was further validated by assessing each gene's expression in relation to the other RGs. Ideally, this analysis should reveal the variability in the stability of the selected RGs across the different infection time points. In the present analysis, *ubi* exhibited the most stable expression pattern, suggesting that it may serve as the most suitable RG for normalising target gene expression in *in vitro* oil palm–*G. boninense* infection studies. Meanwhile, *f-box*, which was consistently identified as a stable RG across all three platforms, may serve as an alternative reference gene. Since optimal normalisation of target gene expression may require the use of more than one RG, the combination of multiple validated RGs may further improve the accuracy and reliability of target gene expression normalisation (Muhammed Afiq *et al.*, 2019).

Ubi displayed stable gene expression, which is consistent with several other findings from earlier studies on monocot plants such as Chinese yam (*Dioscorea opposita*) (Xing *et al.*, 2025), common wheat (*Triticum aestivum*) (Dudziak *et al.*, 2020), and rice (*Oryza sativa*) (Auler *et al.*, 2017). This result also aligns with studies conducted on *G. boninense*-infected 3-month-old oil palm nursery seedlings, which similarly identified *ubi* as one of the RGs with the highest expression stability despite differences in developmental stages (Kwan *et al.*, 2016). This stability may be attributed to the fact that the *ubi* gene belongs to the ubiquitin gene family, which plays a critical role in several essential biological processes, including cell cycle regulation, growth, apoptosis, signal transduction, and immune responses (Wang *et al.*, 2024). Although these studies support the stability

of *ubi* across different plant species and developmental conditions, discrepancies have also been reported in other *G. boninense*-oil palm studies. For instance, software analysis by Zuhar *et al.* (2019) concluded that *gapdh* and *gwhk* were more suitable RGs for *G. boninense*-infected 15-year-old mature oil palm field samples. This is the closest experimental comparison, and the discrepancy may be attributed to the significant difference in developmental stages between the samples.

The stability evaluation in this study highlighted that no single RG was suitable for all experimental conditions. The most stable RG varied across species and even between developmental stages and tissue types within the same species. Therefore, this study recommends that RG selection be tailored to each specific experimental setup to ensure accurate normalisation, despite the additional effort and time required.

CONCLUSION

This study systematically identified and screened ideal RGs for RT-qPCR analysis of target gene expression for *in vitro* 2-month-old oil palm ramets under disease-stress *G. boninense*-infection conditions. Our findings revealed that two consistently stable genes, *ubi* and *f-box*, are suitable for accurately normalising target gene expression in *in vitro* *G. boninense*-infected oil palm ramets, while all four platforms consistently identified *gapdh* as unsuitable for accurate normalisation. This study also recommended validating candidate RGs in the context of the specific experimental condition under investigation, as well as implementing more than one internal RG to optimally minimise the variation.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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