

Full Length Research Paper

Assessment and validation of reference genes for qRT-PCR normalization in local cowpea (*Vigna unguiculata* L.) varieties in response to sterilized and unsterilized soil conditions

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Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is widely employed as a mandatory analysis in biological work to analyze and validate gene expression; however, its precision is greatly hampered by the robustness of the internal control genes employed for normalization. In this study, we selected suitable housekeeping genes in leaves of cowpea for normalization of expression analysis when exclusively exposed to sterilized and unsterilized soil conditions. To validate appropriate internal control genes for qRT-PCR for three cowpea varieties grown under sterilized and unsterilized soil conditions, we investigated the expression stability of five conventional reference genes in cowpea leaves using four approaches: BestKeeper, Delta Ct, geNorm and NormFinder, incorporated in the RefFinder web-based software. The four algorithms employed revealed that *Actin* is the reference gene with the highest precision when used as an internal control, with *EF1- α* and *18S rRNA* being classified as optimal housekeeping genes for local cowpea under both soil conditions. However, *GAPDH* and β -tubulin exhibited highly unstable expression patterns in cowpea under both soil conditions based on the standardized algorithms. These analyzed genes shed light on the molecular physiology of cowpea plants in response to varied soil conditions and suggested standardized reference genes for laboratories.

Key words: *Vigna unguiculata*, reference gene, qRT-PCR, unsterilized condition, nodule.

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp.) is an ancient and versatile crop, serving as a source of food for humans

and livestock, as well as a valuable product for consumers (Langyintuo et al., 2003; Nkomo, 2020;

Timko and Singh, 2008). It has been cultivated since the Neolithic era (Agyeman et al., 2015; Summerfield et al., 1974) and belongs to the Fabaceae family and Faboideae sub-family (Appiah et al., 2011; Horn et al., 2022). Cowpea originates from Africa and is predominantly grown in semi-arid regions of Africa, Asia, and South America (Sani et al., 2014). It is a staple food crop in tropical and subtropical regions (Alemu et al., 2016; Chinma et al., 2008), known for its low-fat content of 1%, high carbohydrate content of 60%, protein content of 23 to 30%, and other essential minerals such as iron, zinc, phosphorus, calcium, and vitamins that improve human nutrition and overall health (Choudhary, 2019). Over 26 cowpea varieties have been released in Ghana, including Sima, Ayeyi, Soronko, Nhyira, Aduapa, Asomdwe, Zamzam, and Tona, among many others (MOFA, 2019). Environmentally, cowpea plays a significant role in managing soil fertility, especially in cereal-based intercropping and rotational cropping systems in sub-Saharan Africa (Sánchez-Navarro et al., 2019a, b). It contributes to nutrient enrichment in the soil, exhibits resistance to certain pests, and enhances agricultural practices (Sánchez-Navarro et al., 2019a, b). Furthermore, cowpea helps improve soil fertility, particularly in smallholder farming systems with limited fertilizer use (Kyei-Boahen et al., 2017). It serves as soil cover and green manure, providing additional agricultural benefits and suppressing weed growth (Beshir et al., 2019). Biologically, the cowpea plant possesses a distinctive ability to capture atmospheric nitrogen through its nodules via a symbiotic relationship with soil-dwelling bacteria called rhizobia, aided by nitrogenase enzymes (Hamza and Alebejo, 2017; Makanjuola et al., 2023) when grown in unsterilized soil or exogenously inoculated.

RT-qPCR is a widely employed procedure in molecular biology for the real-time quantification of genes in recent times (Lü et al., 2018; Vandesompele et al., 2002). It allows for the quantification of gene expression levels in specific genes across a wide range of molecular operations in various fields of life science studies (Lü et al., 2018). RT-qPCR is mostly used in gene expression profiling due to its notable characteristics such as high sensitivity and specificity, excellent reproducibility, and a broad dynamic range for quantification (Ma et al., 2021; Pfaffl, 2007). In general, RT-qPCR pertains to the quantification of expression levels of target genes relative to a set of internal control genes that are known to be stable (Bustin et al., 2013). The technology has been used in various biological studies for the determination of both target and reference genes. For instance, Yang et al. (2016) utilized RT-qPCR to identify robust housekeeping genes for quantifying miRNA levels in sugarcane plants in response to low temperatures. Qi et al. (2016) employed

RT-qPCR to determine stable reference genes during flower development in *Chrysanthemum* species. Additionally, studies have utilized RT-qPCR and the SYBR Green method for reference gene selection in insect gene expression research (Lü et al., 2018; Rodrigues et al., 2017). However, RT-qPCR relies on solid and reliable housekeeping genes for error-free determination of results (Qi et al., 2016). In the context of RT-qPCR analysis, choosing internal control genes with consistent expression levels across various biological conditions is crucial for normalization (Bustin et al., 2009; Tang et al., 2017). Since there is no universal control gene, multiple reference genes are typically employed to effectively standardize the RT-qPCR results (Tang et al., 2017; Udvardi et al., 2008). Several studies have reported finding suitable internal control genes for normalizing expression data in cowpea and other legumes in response to varied stressors (Amorim et al., 2018; Borges et al., 2012; Da Silva et al., 2015; Gao et al., 2017). However, finding a dependable internal control gene with high reliability and consistent expression in specific plants belonging to a locality is essential, as no acceptable and standardized internal control genes have been used for all crop and plant species globally.

A reference gene, typically a housekeeping gene, is expressed in all cells and performs essential functions in cell structure or metabolism. Examples of such reference genes include actin, ribosomal protein L14 (RPL14), ubiquitin (UBQ), elongation factor-1 α (EF1- α), phosphoglycerate kinase gene (PGK), 18S rRNA, tubulin (TUB), S-adenosylmethionine synthase (SAMS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), among others (Czechowski et al., 2005; Zheng et al., 2018). According to Da Silva et al. (2015), the best reference genes for cowpea are in the order VuPp2A, VuUbq28, VuYls8, and VuPolyP for qPCR standardization after exposure to drought stress. However, the stability of these commonly used internal control genes can vary across different conditions, plant species, tissues, growth and developmental stages, as well as experimental treatments (Gao et al., 2012; Li et al., 2021). Validating and finding suitable reference genes for local cowpea varieties and other locally adapted horticultural crops for normalization of RT-PCR under sterilized and unsterilized soil is obscure in Ghana.

In scientific experiments, especially in plant science, plants are either grown under sterile conditions or under sterilized or unsterilized soil conditions, depending on the purpose of the study. The cultivation of plants under sterilized or unsterilized soil conditions induces and alters growth, nodulation, and regulation of gene expression in plants differently. Since most experiments are carried out

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under these two conditions, validating possible internal control genes for normalizing expression data in nodulating local cowpea (specifically in Ghana) would be of great importance for other researchers. We report, for the first time, the identification and confirmation of internal control genes in the leaves of cowpea plants cultivated under unsterilized and sterilized soil conditions, exclusively dependent on symbiotic nitrogen fixation (SNF) for nitrogen. This study also evaluates the effects of these conditions on growth, biomass, and nodulation of local cowpea varieties. The expression stability was evaluated using five widely used statistical algorithms: geNorm, NormFinder, BestKeeper, Comprehensive Ranking, and the Delta Ct (Δ Ct) method. The general profiling of expression of the various housekeeping genes was determined using analysis of variance (ANOVA). The selected reference genes from this study will serve as a basis for gene expression studies in cowpea and related species in the Molecular Biology Department of the Centre for Scientific and Industrial Research-Crops Research Institute (CSIR-CRI) and other researchers globally. These reference genes were selected because they are the five most frequently used and available internal control genes for normalization in the Molecular Biology Laboratory of CSIR-CRI. Therefore, identifying the best and worst reference genes among these five will help reduce unspecific normalization in subsequent experiments in the laboratory.

MATERIALS AND METHODS

Study site and conditions

The experiment was conducted in a screenhouse of the CSIR-CRI's Biotechnology laboratory, Ashanti-Ghana. The screenhouse provided natural conditions, with daytime maximum temperatures ranging from 31 to 37°C and nighttime minimum temperatures ranging from 18 to 24°C. The relative humidity averaged between 65 and 70%.

Experimental materials and soil preparation

Seeds of three cowpea varieties, viz., Tona, Asomdwe, and Zamzam, were generously provided by the Legumes and Oil Seeds Division of the CSIR-Crops Research Institute in Fumesua, Ghana. Nitrogen-deficient loamy soil was sourced from heavy nitrogen-feeding crop fields at the CSIR-Crops Research Institute for the study. Part of the collected soil was sterilized at 121°C for three times to get rid of all inherent microorganisms, and the other part was left unsterilized. Plants in this study were cultivated under sterilized or unsterilized soil conditions to induce nodulation and regulate gene expression in cowpea plants.

Pot experimental design and growth of cowpea

A complete randomized block design with four replications was employed for this study. A 2 kg of soil was weighed and placed in twelve plastic pots, each for both the sterilized and unsterilized soil conditions. The experiments were placed on steel (1 m width and 3 m length) benches half a meter off the ground. In each pot, four

surface-sterilized (with a NaClO₄ solution of 5-7% v/v) seeds were sown at a depth of 2.5 cm. After germination, the plants were thinned down to two plants per pot. The pots were randomly distributed in the treatment groups and watered two to three days, depending on the stage of growth, to maintain the moisture level.

Data collection and plant harvesting

One plant per pot was tagged, and the plant growth (height) was measured at approximately one-week intervals from the base of the shoot as a reference point to the tip of the apex leaf using a ruler (tape measure). After a growth period of five weeks, the final plant height was measured using a ruler, and the plants were harvested. Following the harvest, the plants were fractioned into three parts: roots, shoots, and nodules. Fresh weight measurements were obtained using a balance (Highland® Portable Precision Balance – HCB 602). The fractions were then oven-dried at 60°C for 72 h. Dry biomass was determined separately for roots and shoots using a balance (Highland® Portable Precision Balance – HCB 602). Nodules were separated from the roots, and fresh weight was determined, oven-dried and dry weight determined. Fresh materials were frozen and stored at -80°C for molecular analysis.

Total nucleic acid isolation and cDNA synthesis

Total RNA was extracted from leaf tissues using the Total RNA Extraction Miniprep System Kit (Viogen, USA) following the manufacturer's protocol. The purity and concentration were measured with a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, NanoDrop Technologies, Wilmington, DE, USA) at a 260/280 nm ratio within the range of 2.03–2.33. The extracted RNA was assessed on a 0.8% agarose gel to confirm the integrity of the isolated nucleic acid. Following the manufacturer's instructions, 1 µg of total RNA was used for first-strand cDNA synthesis using the Quantitect RT reagent Kit with gDNA Wipeout (Qiagen, USA). The generated cDNA products were stored at -20°C and used for qRT-PCR analyses.

Selection of reference genes and quantitative real-time polymerase chain reaction (qRT-PCR)

Primers of five reference genes (Table S1), pre-validated at the CSIR-CRI Molecular Laboratory, were selected as candidate reference genes. The primers used met the following conditions: melting temperatures (T_m) of 55 to 60°C, primer lengths of 17 to 24 bp, 40 to 60% GC content, and amplicon lengths ranging from 100 to 224 bp. qRT-PCR was conducted using the LUNA 2X SYBR qPCR Kit (New England Biolabs) in a 36-well plate with a Rotor-Gene real-time PCR machine (Qiagen, Germany). PCR reactions were performed in a total volume of 10 µl, containing 20 ng cDNA, 2X RT PCR MasterMix, 10 mM forward and reverse primers and nuclease-free sterile water. All reactions were performed in duplicates in 36-well reaction plates. The thermal cycling program was as follows: 95°C for 10 min, 45 cycles of 95°C for 15 s, 60°C for 60 s, and 72°C for 60 s. The melting curves were analyzed at 60 to 95°C with a heating rate of 0.1°C per second, followed by a cooling step at 4°C.

Statistical analyses of reference genes expression stability

The expression stability of each reference gene was determined by using five varied approaches using the RefFinder (<http://bloge.cn/RefFinder/>) as elaborated (Amorim et al., 2018). Briefly, the geNorm method determines an expression stability value (M) for each reference gene (Vandesompele et al., 2002). The

Table 1. Plant growth (height, cm plant⁻¹) and fresh matter accumulation (shoot, root and total; g plant⁻¹) of three cowpea varieties cultivated under sterilized and unsterilized soil over a period of five weeks.

Variety	Plant height		Shoot fresh weight		Root fresh weight		Total fresh weight	
	Sterilized	Unsterilized	Sterilized	Unsterilized	Sterilized	Unsterilized	Sterilized	Unsterilized
Tona	73.38±1.25 ^b	79.50±1.29 ^a	18.29±0.29 ^c	24.05±0.86 ^a	6.02±0.77 ^{ab}	4.88±0.37 ^b	24.31±1.06 ^{cd}	28.92±0.57 ^a
Zamzam	45.75±1.71 ^f	51.13±0.85 ^e	15.71±0.33 ^d	19.17±1.11 ^c	5.55±0.39 ^{ab}	6.63±0.63 ^a	21.25±0.41 ^e	25.80±0.82 ^{bc}
Asomdwe	56.75±3.40 ^d	61.50±1.29 ^c	17.59±0.70 ^c	21.44±0.65 ^b	4.91±0.92 ^b	6.61±0.86 ^a	22.50±1.57 ^{de}	28.05±0.96 ^{ab}
Mean	58.63±1.12 ^B	64.04±1.14 ^A	17.19±0.44 ^B	21.55±0.87 ^A	5.49±0.69 ^B	6.04±0.62 ^A	22.69±1.01 ^B	27.59±0.78 ^A
P _V		***		***		ns		***
P _T		***		***		*		***
P _{V×T}		ns		*		***		ns
CV		2.78		3.95		9.80		4.01

Data presented means, standard deviations, and ANOVA for varieties (V), treatment (T), V×T interaction, and coefficient of variation (CV, %) of four replicates. Different capital letters indicate significant differences between the treatments for all varieties (Tukey's test; $p < 0.05$). Different small letters indicate significant differences among the varieties (Tukey's test; $p < 0.05$). Asterisks indicate significant differences as determined by Tukey's test (* $p < 0.05$; *** $p < 0.001$); ns, nonsignificant.

NormFinder algorithm reveals a stability value (SV) for each reference gene, as described (Andersen et al., 2004). In comparison with geNorm, the NormFinder analyzes data by juxtaposing the differences in treatments. The SV is determined by a merging computation of intra- and inter-group-disparity relating to the reference gene expression. The smaller the SV, the more accurate the reference gene expressed. The BestKeeper algorithm evaluates the most stable gene up to ten reference genes (Pfaffl et al., 2004). The average of the Cq values for each data in various reference genes is amalgamated to obtain the BestKeeper index. The gene is juxtaposed in a pairwise trend by the Pearson correlation coefficient to the BestKeeper index. The gene with the greatest coefficient of correlation with the BestKeeper index shows the greatest reliability, and the highest-ranked gene is the most stable. The delta CT (Δ CT) method contrasts the relative expression of all pairwise combinations of reference genes within each condition. This helps reveal which pairs show less difference. It will determine the gene that has the most stably expressed level by determining the average standard deviation (SD) of the relative expression of the pair of genes. The lower the mean SD, the more reliable the reference gene (Silver et al., 2006). The Comprehensive Ranking method also ranks the expression of each internal control gene. The

gene with the least value is regarded the most suitable gene and vice versa.

Validation of identified reference genes

The early Nodulin-like genes (*ENODL2*, *ENODL3*) are nodulin-encoding genes that are expressed during the development of symbiotic root nodules (Fang and Hirsch, 1998) and malate dehydrogenase 1 (*MDH-1*) genes were used as genes of interest to substantiate the validity of our study, with primers attached (Table S1) and designed using Primer3plus (<https://www.primer3plus.com/index.html>). The most, optimal and least stable internal control genes were utilized respectively, to normalize the expression of *ENODL2*, 3 and *MDH-1* in the leaves of cowpea varieties grown under sterilized and unsterilized soil conditions. The relative expression was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Data were analysed using a 2-way analysis of variance (ANOVA). The significance between treatment and variety means for each measured trait was determined using a

Tukey's test HSD (Honestly Significant Difference). P-values < 0.05 were regarded significant, and $p < 0.01$ and $p < 0.001$ as highly significant. Data analyses and figures were performed using R Statistical Software (Version 4.2.2., R Core Team 2022).

RESULTS

Growth, nodulation and biomass accumulation in cowpea

Cowpea varieties exposed to sterilized and unsterilized soils revealed significant variations in treatments and varieties, as depicted in Tables 1 and 2, and Figures 1 and 2. The weekly growth of cowpea varieties observed significant differences between the varieties ($P < 0.001$), but not in treatments or treatment-variety interactions (Figure 1). The Tona variety under unsterilized conditions recorded the highest growth rate between weeks 1 and 3. However, Tona under sterilized conditions took over from weeks 4 to 5, with Zamzam under

Table 2. Dry matter accumulation (shoot, root and total; g plant⁻¹) and root-shoot ratio (g g⁻¹) of three cowpea varieties cultivated under sterilized and unsterilized soil over a period of five weeks.

Variety	Shoot dry weight (g plant ⁻¹)		Root dry weight (g plant ⁻¹)		Total dry weight (g pot ⁻¹)		Root-shoot ratio (g g ⁻¹)	
	Sterilized	Unsterilized	Sterilized	Unsterilized	Sterilized	Unsterilized	Sterilized	Unsterilized
Tona	2.46±0.34 ^c	3.87±0.75 ^a	1.01±0.13 ^a	0.80±0.01 ^b	3.47±0.35 ^c	4.66±0.76 ^a	0.42±0.08 ^a	0.21±0.04 ^{cd}
Zamzam	2.57±0.14 ^c	3.65±0.18 ^a	0.91±0.12 ^{ab}	0.61±0.02 ^c	3.48±0.24 ^{bc}	4.26±0.18 ^{abc}	0.35±0.04 ^{ab}	0.17±0.01 ^d
Asomdwe	2.65±0.38 ^{bc}	3.48±0.40 ^{ab}	0.99±0.06 ^a	0.94±0.06 ^{ab}	3.64±0.42 ^{bc}	4.42±0.35 ^{ab}	0.38±0.05 ^a	0.28±0.05 ^{bc}
Mean	2.56±0.29 ^B	3.67±0.44 ^A	0.97±0.10 ^A	0.78±0.03 ^B	3.53±0.34 ^B	4.453±0.43 ^A	0.38±0.06 ^A	0.22±0.03 ^B
P _V		ns		***		ns		*
P _T		***		***		***		***
P _{V×T}		ns		*		ns		ns
CV		12.42		9.03		10.36		14.23

Data presented means, standard deviations, and ANOVA for varieties (V), treatment (T), V×T interaction, and coefficient of variation (CV, %) of four replicates. Different capital letters indicate significant differences between the treatments for all varieties (Tukey's test; $p < 0.05$). Different small letters indicate significant differences among the varieties (Tukey's test; $p < 0.05$). Asterisks indicate significant differences as determined by Tukey's test (* $p < 0.05$; *** $p < 0.001$); ns, nonsignificant.

unsterilized condition recording the least growth rate. Cowpea varieties observed significant differences in both varieties and treatments ($P < 0.001$), but non-significant in the treatment-variety interaction in terms of plant height (PH) (Table 1). All varieties under both treatments differed significantly from each other with Tona and Zamzam under unsterilized and sterilized conditions obtaining the highest and lowest growth (PH), respectively (Table 1).

Similarly, shoot fresh weight (SFW), root dry weight (RDW), root-shoot-ratio (RSR) and total fresh weight (TFW) also revealed highly significant differences in both treatments and varieties ($P < 0.001$), except in shoot dry weight (SDW), root fresh weight (RFW) and total dry weight (TDW), where it was observed that varieties demonstrated non-significant variations statistically (Tables 1 and 2). The results showed that varieties under unsterilized conditions exhibited the highest in traits such as PH, SFW, RFW, TFW, SDW, TDW than sterilized soil conditions, except in RDW and root-shoot-ratio (RSR). The grand fresh biomass

(TFW) revealed that Tona and Zamzam varieties recorded the maximum and minimum biomass under unsterilized and sterilized conditions, respectively (Table 1). However, in TDW, Tona under both treatments exhibited the highest and least dry biomass accumulation (Table 2). Tona under sterilized conditions recorded the highest RSR. Treatment-variety interactions ($P_{V×T}$) were highly significant in parameters such as SFW, RFW and RDW, but were non-significant in PH, TFW, SDW, TDW and RSR (Table 1).

Meanwhile, nodulation parameters measured also revealed significant variations, with highly significant differences between ($P < 0.001$) varieties only under unsterilized conditions (Figure 2). This is because no nodule growth was observed under sterilized soil conditions. All varieties differed significantly from each other. The results indicated that Asomdwe variety was outstanding and obtained the highest biomass in both nodule fresh weight (NFW) and dry weight (NDW) (Figure 2). Zamzam and Tona varieties exhibited the least biomass under NFW and NDW, respectively.

To further evaluate the effects of treatments on varieties, a heatmap of Pearson correlation analysis was performed (Figure 3). The results reveal highly significant correlations among measured traits. For example, RSR significantly correlated negatively with TDW ($r = -0.82$), but positively with RDW ($r = 0.78$). Meanwhile, TDW correlated positively and significantly with TFW and SDW ($r = 0.80, 0.98$) (Figure 3). Principal component analysis (PCA) further exemplified the variations in treatments (Figure 4). The first PCA explained 57.1% of the variations in treatments, with the second PCA explaining 21.8%.

Threshold cycle (Ct) profiling of reference genes in cowpea varieties under sterilized and unsterilized soil conditions

Different reference genes Ct values were profiled using ANOVA under unsterilized and sterilized conditions in three cowpea varieties to ascertain their expression (mRNA) levels (Figure 5). The results of this study clearly indicated highly

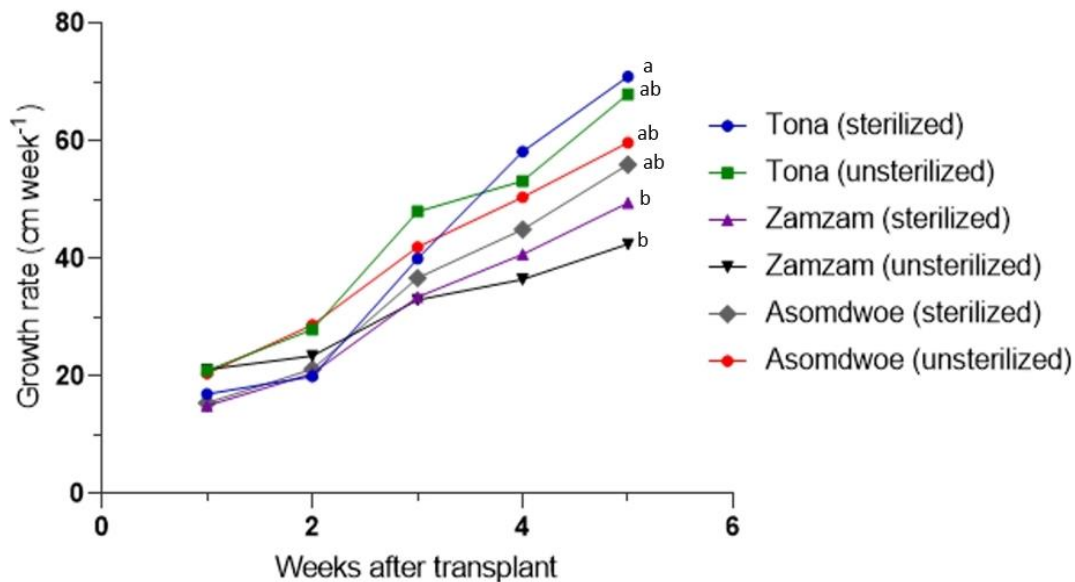


Figure 1. Growth rate of cowpea (Tona, Zamzam and Asomdwoe) varieties cultivated under sterilized and unsterilized soil conditions over a period of five weeks. Data represent the means of four biological replicates. Data with different letters are significantly different as determined by Tukey's test ($P \leq 0.05$).

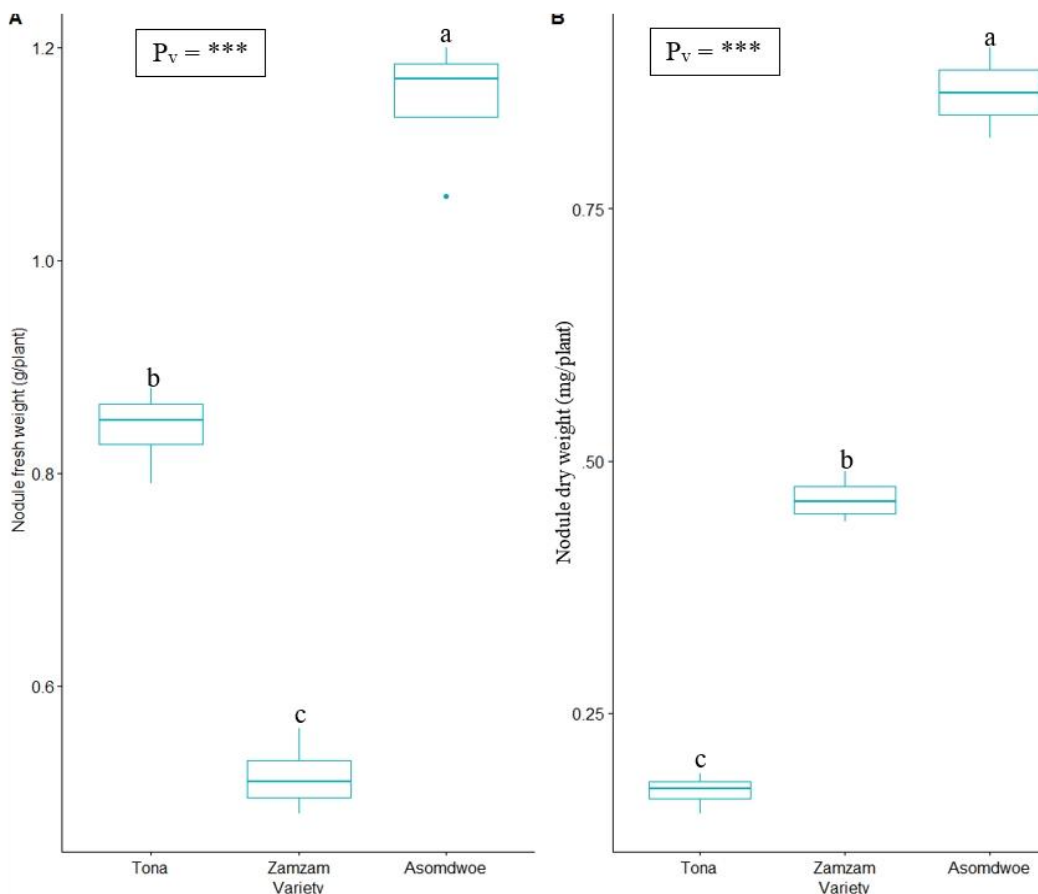


Figure 2. Nodule fresh and dry biomass of cowpea (Tona, Zamzam and Asomdwoe) varieties grown in unsterilized soil over a period of five weeks. (A) Nodule fresh biomass (B) nodule dry biomass. Data represent the means of four biological replicates. Data with different letters are significantly different as determined by Tukey's test ($P \leq 0.05$).

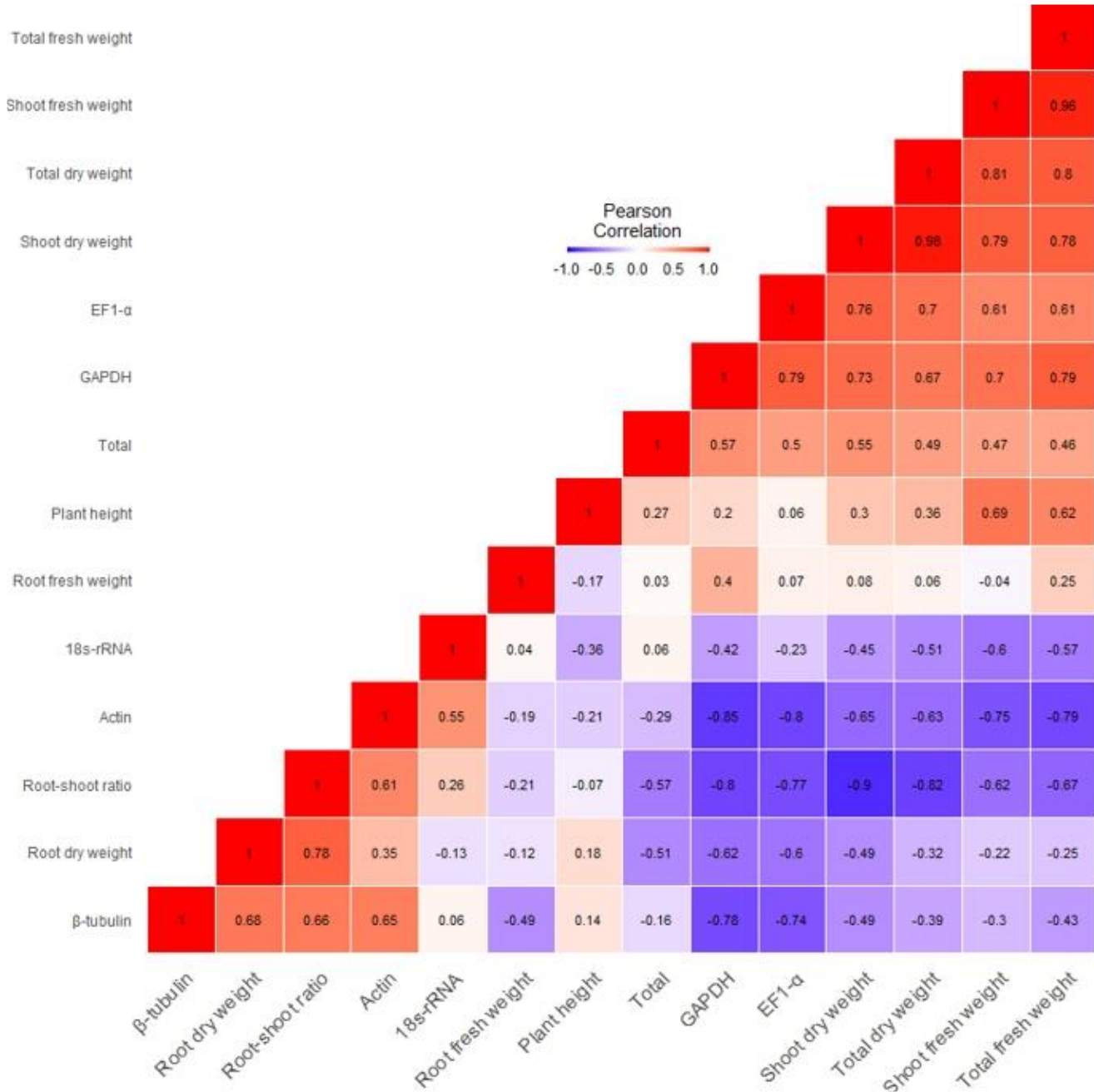


Figure 3. Heatmap of Pearson correlation coefficient of morpho-physiological traits and reference genes of cowpea varieties grown for five weeks under two conditions (sterilized and unsterilized). Total: total Ct values.

significant variations in both treatments and varieties ($P < 0.001$) in all reference genes. However, treatment-variety interaction did not show any significant variation in *Actin*, *Elongation factor 1-alpha (EF1- α)* and *18S ribosomal RNA (18S rRNA)*, but highly substantial variations were obtained in *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* and *Beta (β)-tubulin*. On the individual levels, Asomdwe under unsterilized conditions noticed the highest expressions, with Tona under sterilized conditions

witnessing the least expression in *Actin* (Figure 5A). Meanwhile, the expression of *Actin* was higher under unsterilized compared with sterilized conditions.

For *GAPDH*, the reverse expression was observed between both treatments, with the sterilized condition revealing an almost 2-fold higher expression than the unsterilized (Figure 5B). Interestingly, Asomdwe under sterilized conditions recorded the highest in *GAPDH*, with Tona under unsterilized conditions obtaining the least

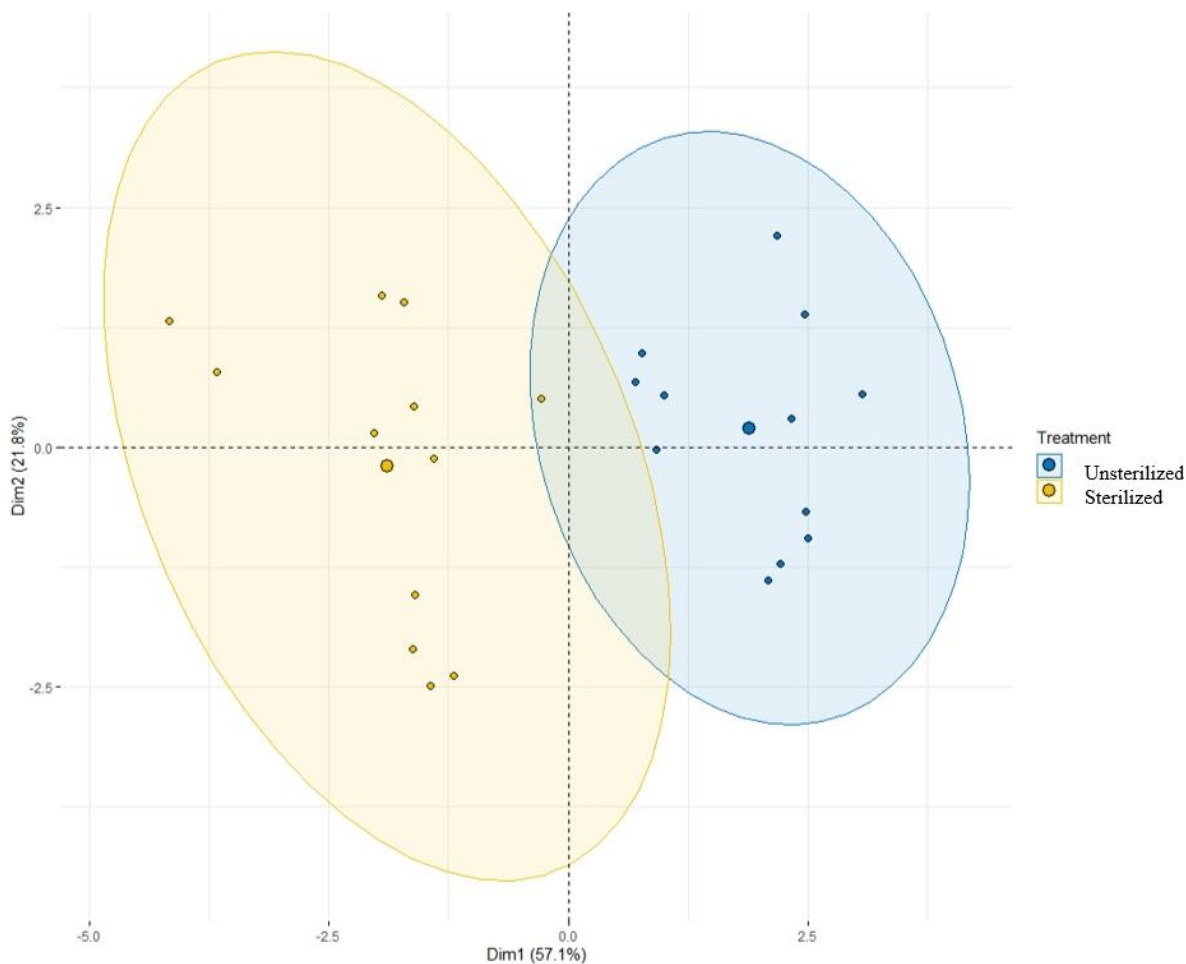


Figure 4. Principal component analysis (PCA) separating treatment on growth and biomass traits measured in cowpea varieties over a period of five weeks under two different conditions (sterilized and unsterilized).

expression. Under β -*tubulin*, unsterilized condition differed significantly from sterilized conditions in all varieties (Figure 5C). Remarkably, Asomdwe under unsterilized conditions prevailed again as the best-performing variety in β -*tubulin* (Figure 5C); however, the same variety under the opposite condition recorded the lowest expression. The expression of *EF1- α* exhibited similar expression as *GAPDH* in the treatment comparison (Figure 5D), with varieties under sterilized conditions recording the greatest expression. At the varietal level, the Asomdwe variety under sterilized conditions accumulated the highest expression, but differed significantly from the same variety under unsterilized conditions, and even recording the minimum expression (Figure 5D). Additionally, the results showed that varieties under unsterilized conditions increased expression and differed significantly from the sterilized treatment in *18S rRNA* (Figure 5E), with Asomdwe variety under unsterilized conditions again accounting for the best expression comparatively. However, when all the expressions in the five reference genes were summed, varieties under sterilized were the

highest and differed significantly from the unsterilized conditions (Figure 5F). Interestingly, the Zamzam variety under sterilized conditions recorded the highest expression, followed by Asomdwe under the same condition in total expression. Tona under unsterilized soil conditions obtained the least expression in grand expression (Figure 5F).

To estimate the error rates of these reference genes Ct values, the standard deviations (SD) and coefficient of variations (CV) of each gene were determined (Table 3). The results indicated that *Actin* accounted for the least SD, with *EF1- α* revealing the highest. However, *GAPDH* exhibited the lowest CV value, with *18S rRNA* being the highest. The contention was between *Actin* and *GAPDH*, obtaining contrasting SD and CV values (Table 3). Significant correlations among growth parameters and reference genes were observed in this study (Figure 3). For instance, β -*tubulin* correlated positively with *Actin* ($r = 0.65$), and negatively with *GAPDH* ($r = -0.78$) and *EF1- α* ($r = -0.74$). *Actin* on the other hand, correlated negatively with growth parameters such as SDW ($r = -0.65$), TDW (r

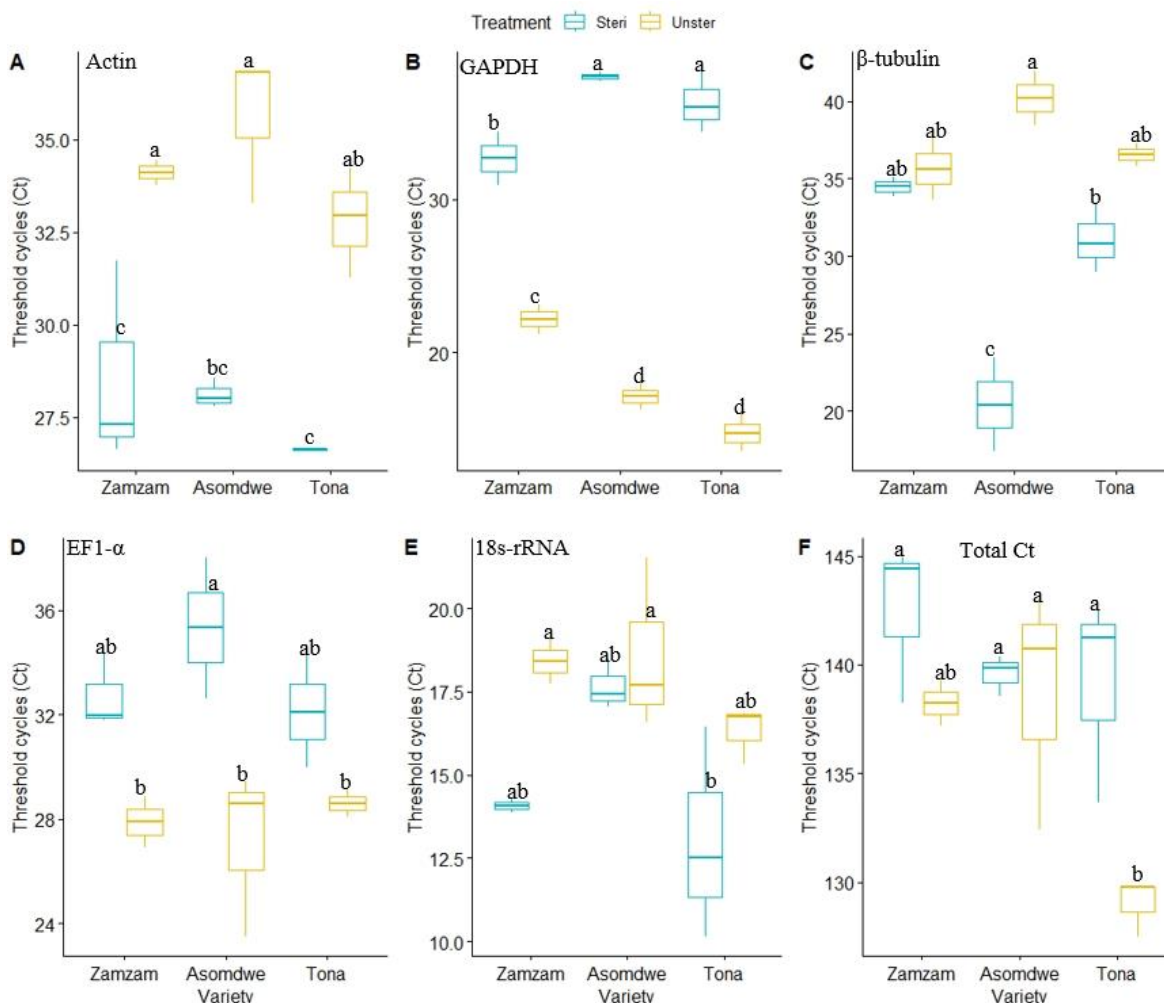


Figure 5. Threshold cycle (Ct) values of five reference genes in leaves of three cowpea varieties. (A) Actin (B) GAPDH (C) β -tubulin (D) EF1- α (E) 18S rRNA and (E) total Ct. Data represent the mean values of three biological replicates. Different letters indicate significant differences among the varieties. Blue and yellow colour represents sterilized (denoted as Steri) and unsterilized (denoted as Unster).

Table 3. Standard deviation (SD) and coefficient of variation (CV, %) of the threshold cycles (Ct) values of the five reference genes in cowpea varieties grown under sterilized and unsterilized conditions.

Reference gene	Threshold cycle (Ct)	
	SD	CV (%)
Actin	1.12	5.04
GAPDH	1.16	4.30
β -tubulin	1.72	6.27
EF1- α	1.84	6.99
18S rRNA	1.38	11.39

= -0.63), SFW ($r = -0.75$), TFW ($r = -0.79$), but positively with RSR ($r = 0.61$). Interestingly, *GAPDH* portrayed a highly significant and positive association with the

mentioned growth traits ($r = 0.73, 0.67, 0.70,$ and 0.79 , respectively), but a negative association with RSR ($r = -0.80$) (Figure 3).

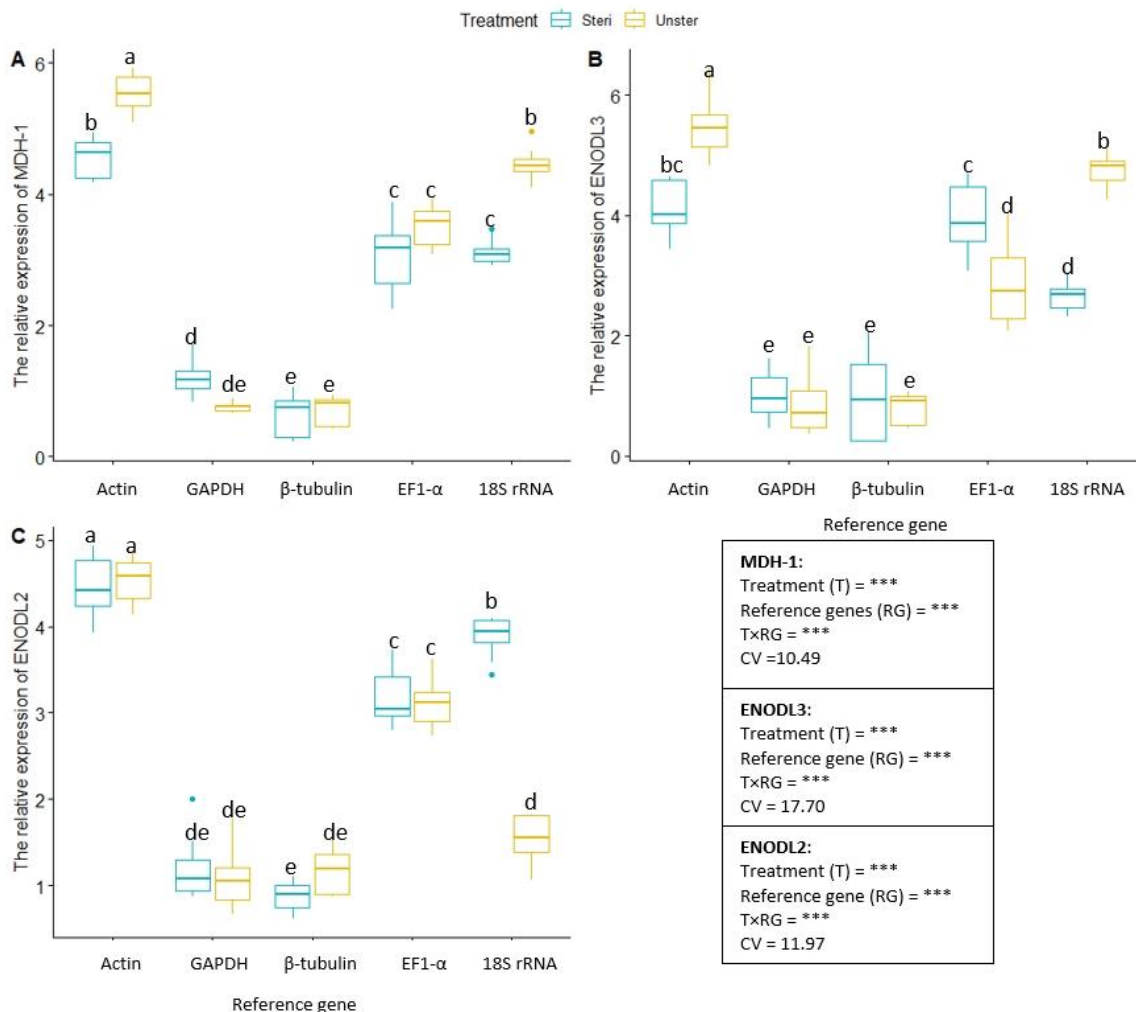


Figure 6. Relative quantification of *MDH-1*, *ENODL2* and *ENODL3* genes in leaves of cowpea varieties grown under sterilized and unsterilized conditions. The expression levels of these target genes were normalized using both the best stable reference genes *Actin/18S rRNA*, the intermediary stable gene *EF1- α* , and the highly unstable reference genes *β -tubulin* and *GAPDH* that changed the most in tissues. Values are means \pm SE. Different letters indicate significant differences in gene expression among treatments separated at ($P < 0.05$). ***, represents highly significant differences between treatments (T), reference genes (RGs) and the interactions of the two (TxRG) at $p < 0.001$. CV, coefficient of variation.

Validation of reference genes

The main purpose for growing the cowpea plants under these two conditions was to induce nodulation.

Therefore, we selected nodulating- and carbon-fixing-genes as the target genes to confirm the accuracy of the various internal control genes in ours. As could be shown, *MDH-1* and *ENODL2*, *ENODL3* genes were expressed in leaves under both conditions (Figure 6). The highest expression was recorded when target genes were normalized with *Actin* followed by *18S rRNA* (Figure 6A-C). The relative expression levels of all target genes were shown not to be biased when *EF1- α* was introduced (Figure 6). However, the mRNA expression levels of all the genes of interest varied drastically when the least stable genes, *GAPDH* and *β -tubulin*, were employed to normalize

the target genes (Figure 6). Generally, statistically significant differences ($p < 0.001$) were demonstrated among treatments, reference genes and their interactions. These results, however, confirmed the accuracy of the recommended internal control genes, which provides theoretical basis for cowpea plants gene expression patterns under these two conditions.

Expression stability of reference genes based on five different statistical algorithms under unsterilized soil conditions

In the present study, the expression robustness of all five internal control genes was analyzed in three varieties of cowpea under unsterilized soil conditions, using five different methods: geNorm, NormFinder, BestKeeper,

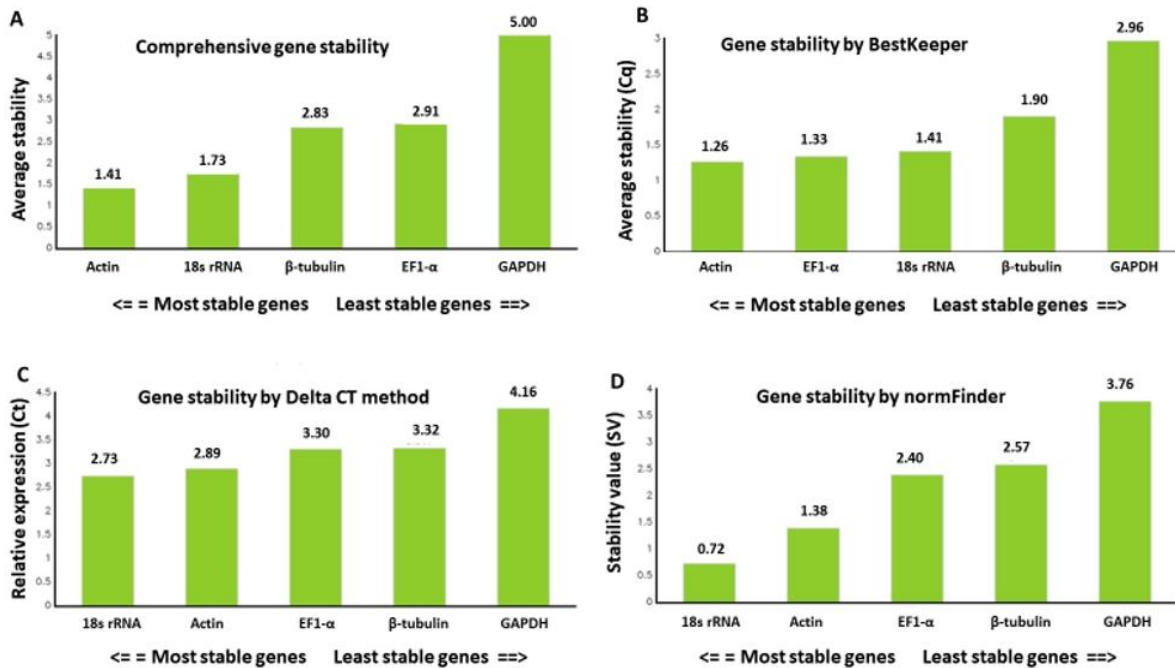


Figure 7. RefFinder analysis showing the average expression stability of all five reference genes in cowpea varieties under unsterilized soil conditions. (A) Comprehensive gene stability ranking (B) gene stability analyzed by BestKeeper (C) gene stability by Delta CT method (D) gene stability analyzed by normFinder. The values on top of each graph are the averages of the Ct values as calculated and ranked from lowest to the highest by each statistical algorithm. The most stably expressed genes present lower values. The lower the value, the more stable the gene.

Comprehensive Ranking and the delta Ct (Δ Ct) method (Figures 7 and 9). All five of these algorithms were determined by the RefFinder web-based software. Under unsterilized soil condition, the Comprehensive gene ranking showed that the stability ranking of the various internal control genes are from most to least stable, with *Actin* and *18S rRNA* being the most stably expressed internal control genes (Figure 7A). The stability expression analysis by BestKeeper (CP) presented similar expression as the Comprehensive ranking (Figure 7B). The result showed that *Actin*, *EF1- α* and *18S rRNA* exhibited the highest stability, with *β -tubulin* and *GAPDH* being the least stable reference genes under unsterilized soil conditions. The BestKeeper algorithm calculates the Pearson correlation coefficient for the BestKeeper index. The internal control gene with the greatest Pearson correlation coefficient indicates the greatest stability (Table 4). From the Pearson correlation coefficient table (Table 4), the analysis revealed *18S rRNA* (0.798), *GAPDH* (0.772), and *Actin* (0.331) as the three most stable reference genes, with *β -tubulin* (0.175) and *EF1- α* (0.001) representing the least stable genes for qPCR normalization under unsterilized soil conditions. The Δ Ct approach relies on the juxtaposition of all genes using the widely known delta Ct method. This approach compares all internal control genes to each other, with genes being ranked based on the average standard deviation (SD) according to the relative expression of the pair of genes. The SD with the

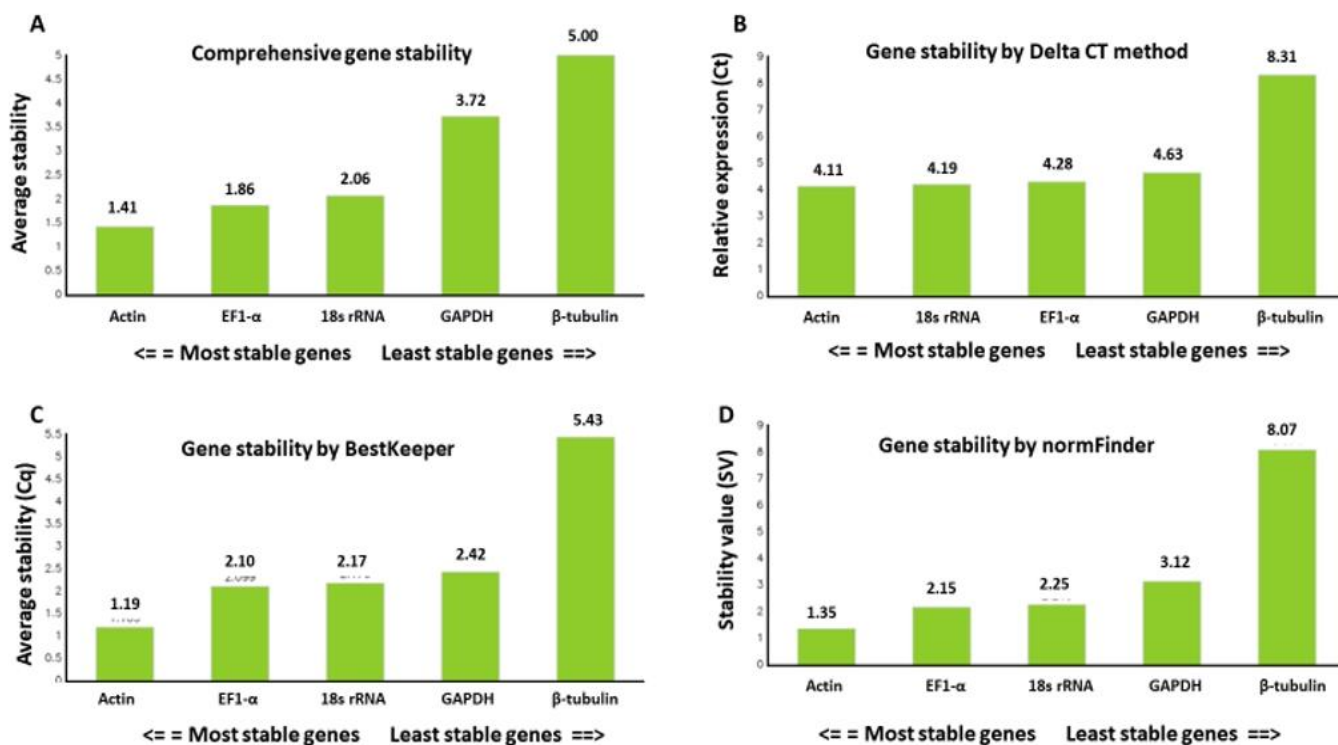
lowest mean is regarded more stable. Under unsterilized soil conditions, the stability ranking under this method presented stability from the highest to the lowest as follows: *18S rRNA* < *Actin* < *EF1- α* < *β -tubulin* < *GAPDH* (Figure 7C). This means that *18S rRNA* exhibited the most stability (with the lowest SD value), followed by *Actin*, with *GAPDH* recording the least stability (with the highest SD value) (Figure 7C). The normFinder analysis presented the same pattern of stability as the delta Ct approach (Figure 7D). The results of both algorithms identified *18S rRNA*, *Actin* and *EF1- α* as the three most stable internal control genes under unsterilized soil condition. Finally, the geNorm computes the ideal number of internal control vital for reliable normalization. The top three most stable reference genes identified by RefFinder were *Actin*/ *β -tubulin* < *18S rRNA*, which also revealed low M values (the lowest three) by geNorm analysis (Figure 9A), with *EF1- α* and *GAPDH* exhibiting the least stability under unsterilized soil conditions. *Actin* and *β -tubulin* revealed co-expression pattern and therefore require an additional reference gene under sterilized conditions.

Expression stability of reference genes based on five different statistical algorithms under sterilized soil conditions

The expression stability of all five internal control genes

Table 4. Pearson correlation coefficient for each reference gene as analyzed by the RefFinder web-based software using the BestKeeper method under unsterilized soil condition.

BestKeeper vs.	<i>Actin</i>	<i>GAPDH</i>	β - <i>tubulin</i>	<i>EF1-α</i>	<i>18S rRNA</i>
coeff. of corr. [r]	0.331	0.772	0.175	0.001	0.798
p-value	0.384	0.015	0.652	0.980	0.010

**Figure 8.** RefFinder analysis showing the average expression stability of all five reference genes in cowpea varieties under sterilized soil conditions. (A) Comprehensive gene stability ranking (B) gene stability by Delta Ct method (C) gene stability analyzed by BestKeeper (D) gene stability analyzed by normFinder. The values on top of each graph are the averages of the Ct values as calculated and ranked from lowest to the highest by each statistical algorithm. The most stably expressed genes present lower values. The lower the value, the more stable gene.

was analyzed in three varieties of cowpea under sterilized soil conditions using the same methods: geNorm, NormFinder, BestKeeper, Comprehensive ranking and delta Ct (ΔC_t) method. Under sterilized soil conditions, the results consistently revealed the direct opposite of what is presented in the unsterilized soil conditions (Figures 8 and 9). Based on the Comprehensive gene stability analysis, *Actin*, *EF1- α* and *18S rRNA* exhibited the most stable reference genes for normalization (Figure 8A), with *GAPDH* and β -*tubulin* being classified as the least stable reference genes under sterilized soil conditions. However, the exploitation of the ΔC_t method demonstrated slight variations compared with the Comprehensive gene stability analysis, with *Actin*, *18S rRNA* and *EF1- α* presenting the highest stability (lowest SD) under sterilized

soil conditions (Figure 8B). Interestingly, the BestKeeper and normFinder approaches, as well as the Comprehensive gene stability analysis, presented similar quantifications for all genes in this study. The gene stability analysis by BestKeeper identifies that *Actin*, *EF1- α* and *18S rRNA* were classified as the most stable genes, with *GAPDH* and β -*tubulin* recording the least stability (Figure 8C). The Pearson coefficient from the BestKeeper index showed that *18S rRNA* (0.564) demonstrated the highest coefficient of correlation, followed by *Actin* (0.398) and *EF1- α* (0.294), with *GAPDH* and β -*tubulin* presenting the least coefficients (Table 5). Additionally, the gene stability analysis revealed *Actin*, *EF1- α* and *18S rRNA* as the most stable genes, with β -*tubulin* emerging as the least stable (Figure 8D) using the normFinder. The gene stability

Table 5. Pearson correlation coefficient for each reference gene as analyzed by the RefFinder web-based software using the BestKeeper method under sterilized soil condition.

BestKeeper vs.	<i>Actin</i>	<i>GAPDH</i>	<i>β-tubulin</i>	<i>EF1-α</i>	<i>18S rRNA</i>
coeff. of corr. [r]	0.398	0.001	0.052	0.294	0.564
p-value	0.289	0.909	0.895	0.443	0.114

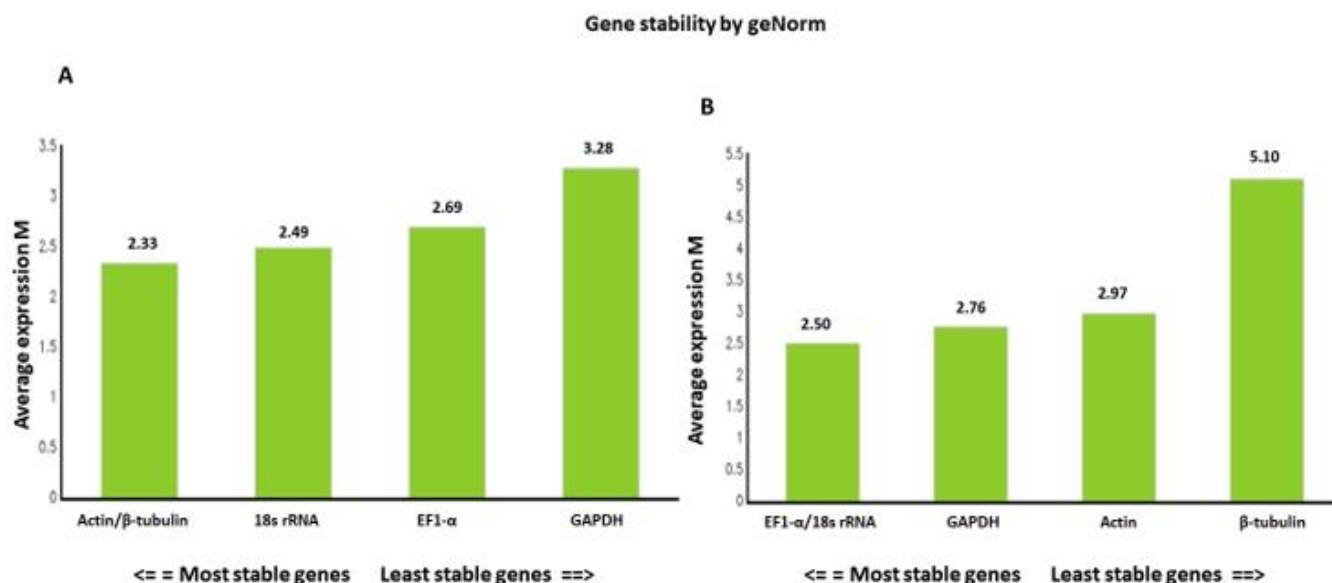


Figure 9. RefFinder analysis showing the mean expression stability by geNorm (M value) of all five reference genes in cowpea varieties under (A) unsterilized and (B) sterilized soil conditions. The values on top of each graph are the averages of the Ct values as calculated and ranked from lowest to the highest by each statistical algorithm. The most stably expressed genes present lower M values. The lower the M value, the more stable the gene.

analysis by geNorm presented a co-expression pattern between *EF1- α* and *18S rRNA* and so required the addition of different reference genes under sterilized conditions (Figure 9B). The geNorm analysis under sterilized was the reverse of the unsterilized soil conditions (Figure 9B). *EF1- α /18S rRNA* and *GAPDH* were the highly stable reference genes under sterilized soil conditions compared to unsterilized. However, *Actin* and *β -tubulin* were considered the least stable genes for qPCR normalization under sterilized soil conditions using geNorm.

DISCUSSION

It's been reported that growth of plants is significantly increased in the presence of soil microorganisms relative to soils devoid of such microorganisms (Makanjuola et al., 2023; Nyaga et al., 2020; Oruru et al., 2018). This means that the presence of soil microorganisms has a higher possibility of impacting the physiological and molecular mechanisms (regulation and expression of genes) of

plants. The presence of soil microorganisms does not only induce growth, but also enhances nutrient solubility and nodulation in legumes. As of now, miniature consciousness has been paid to the systematic study of normalization procedures, especially in developing countries, where molecular studies remain infantile. Therefore, the quest to find accurate and suitable reference genes for normalizing locally adapted crops grown in the presence and absence of microorganisms and when plants are nodulating is imperative. Several studies have chronicled the significance of exploiting more than one algorithm and statistical approach for internal control gene stability assessment in plants (Amorim et al., 2018; Da Silva et al., 2015; Zhu et al., 2013). There is a high tendency that the comparison using diverse statistical algorithms might ensure reliable and accurate evaluation of the reference gene set under a given experimental condition (Amorim et al., 2018; Da Silva et al., 2015). With this concept, we adopted the five commonly used methods, viz., geNorm, NormFinder, BestKeeper, Δ Ct method and Comprehensive Gene Stability to ascertain a set of reference genes for qPCR normalization of cowpea

varieties grown under sterilized and unsterilized soil conditions. The RefFinder (a web-based tool) integrates all five statistical algorithms to rank the overall stability of candidate housekeeping genes in this work and is known to produce accurate results (Xie et al., 2012). We employed the conventional analysis of variance (ANOVA) to determine the growth and expression profiling of the various reference genes, but we did not consider them in our discussion because ANOVA has not been used as a standardized analysis for the determination and confirmation of gene stability in plants.

In this study, five candidate internal control genes (*EF1- α* , *18S rRNA*, *Actin*, *GAPDH*, and *β -tubulin*) were chosen for normalization of gene expression under sterilized and unsterilized soil conditions. Some of these genes have been established as stable and unstable in varied crops under different experimental conditions. The findings of our work demonstrated that the reference gene transcript expressions differ with the conditions stated above (Figures 7 to 9). From the results analyzed with the five algorithms under sterilized soil conditions, four of these approaches (that is, Comprehensive Gene Stability, NormFinder, BestKeeper; and Δ Ct method) consistently justified *Actin* as the most stable internal control gene, with contrasting responses exhibited under unsterilized soil conditions between *18S rRNA* (declared as best by NormFinder and Δ Ct method) and *Actin* being declared as best by Comprehensive Gene Stability, BestKeeper and geNorm (Figures 7 to 9). All five algorithms declared *Actin* and *EF1- α /18S rRNA* as the two top-ranked genes, with minimal variations in their respective sequence under the two experimental conditions. On the contrary, our results demonstrated that *GAPDH* and *β -tubulin* showed the least stability as declared by four of the algorithms except geNorm, which exhibited contrasting rankings.

Interestingly, *Actin* has been declared and recommended as the most stable internal control gene in cowpea under root dehydration (Amorim et al., 2018), cotton under different stages of growth (Artico et al., 2010), rice under salt stress (Caldana et al., 2007), peanut under biotic and abiotic stresses (Morgante et al. 2011), common bean under biotic and abiotic stresses (Borges et al., 2012) and *Eustoma grandiflorum* under floral development stages (Xue et al., 2022). Indeed, the observation of *Actin* as the most stable reference gene for standardization supports the above premise, especially under sterilized soil conditions (Figure 8). Similarly, under unsterilized soil conditions, it was recommended as a stable reference gene using all four major algorithms applied in this study (Figures 7A, B, and 9A). The similarities observed under both soil conditions exclusively indicate that *Actin* can be pronounced as the most stably expressed and reliable reference gene that has the propensity of increasing the precision and accuracy of gene expression analysis in cowpea, mostly under sterilized conditions and probably under unsterilized conditions too, which is consistent with the validation result in all target genes (Figure 6). Yu et al.

(2019) ranked *Actin* (*ACT1*) as the most stably expressed reference gene among the eight genes in Ramie tissues across eleven abiotic and biotic conditions. This phenomenon was further reported when soybean plants were evaluated under nitrogen stress among different cultivars (Wan et al., 2017). Adding to the above, *Actin* was further classified as the second most stable reference gene in soybean under aluminum toxicity (Gao et al., 2017), confirming the findings of this current study.

The *18S ribosomal RNA* (*18S rRNA*) is a central part of ribosomal and structural RNA for small constituent of eukaryotic cytoplasmic ribosomes. *18S rRNA* has been used as a reference gene and recommended as a stable reference gene in *Spinacia oleracea* under varied abiotic stresses (Chen et al., 2012; Xie et al., 2021). *EF1- α* , on the other hand, has been used as a reference gene for legumes under osmotic, salt, cold, and heat stress, as reported (Zhu et al., 2013). Our results consistently revealed *EF1- α* and *18S rRNA* as the second most ranked and stable reference genes in cowpea plants under both soil conditions, as demonstrated by all the algorithms, but were contrastingly regarded as the most stable reference genes as identified by geNorm algorithms under sterilized soil conditions (Figures 7, 8 and 9). Interestingly, both reference genes revealed co-regulation under sterilized conditions, as shown by the geNorm algorithms. It is tempting to suggest that both *EF1- α* and *18S rRNA* can be recommended as the second most preferential and stable reference gene, which has the capability to completely remove the non-specific variations when used as an internal control gene for normalization of cowpea under unsterilized soil conditions. It is indeed clear that both genes could perfectly serve as direct alternatives in cases where *Actin* is unavailable. Notwithstanding, Xie et al. (2021) observed *18S rRNA* as an optimal reference gene in roots, stems, leaves, flowers, and seedlings in response to salinity stress. This observation is in accordance with our report, which declared *18S rRNA* as an optimally stable reference gene in cowpea under both conditions by all algorithms. Additionally, *18S rRNA* exhibited higher stability compared to other candidate reference genes when *Cephalotaxus hainanensis* was exposed to six stimuli treatments (Sun et al., 2019). The finding that highlights *EF1- α* as an optimally stable gene in our study is in line with what was reported (Wan et al., 2017; Xue et al., 2022; Yu et al., 2019; Zhu et al., 2013) under different stresses, but at odds with those established (Amorim et al., 2018), where *EF1- α* was classified as the least stable reference gene under root dehydration or salinity stress. It's quite plausible to emphatically suggest that *EF1- α* and *18S rRNA* can be recommended as suitable alternative reference genes to *Actin* for normalization of target genes in qPCR in plants grown under any of the conceivable soil conditions. To confirm the above findings, the validation analyses revealed these two reference genes as the second and third most recommended genes for accurate normalization of target genes, viz., *MDH-1*, *ENODL2*, and

and *ENODL3* genes, as depicted (Figure 6).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme in the glycolytic pathway that converts glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate with the concomitant reduction of NAD^+ to NADH_2 , whereas β -*tubulin* is also involved in plant morphogenesis, cell division, the direction of cell expansion, and the deposition of cell wall material (Gavazzi et al., 2017). The results of this study have clearly shown that under both soil conditions, *GAPDH* and β -*tubulin* were exclusively and consistently revealed as the least and most unstable reference genes and therefore cannot be recommended as preferential reference genes for reliable normalization of qPCR data in cowpea varieties (Figures 7 and 9A). Apparently, all four algorithms intermittently ranked *GAPDH* and β -*tubulin* as the most unstable reference genes in this study (Figures 7-9). Similar studies in rice (Auler et al., 2017) and *Ophraella communa* (Zhang et al., 2020) to establish the stability of reference genes have revealed *GAPDH* and β -*tubulin* as the most highly unstable reference genes based on the applied algorithms. Their findings are in accordance with what is reported in our current study. The results apparently confirm that *GAPDH* and β -*tubulin* are highly unspecific with high error rates and cannot remove non-specific variations when used as internal control genes for cowpea under unsterilized soil conditions. A similar report of instability of *GAPDH* was displayed in the leaves and roots of Ramie plants exposed to varied stresses (Yu et al., 2019) and *Ananas comosus* in response to hormone stimuli (Mao et al., 2021). Interestingly, β -*tubulin* was ranked as the second most stable gene for the normalization of qPCR analysis only under unsterilized soil conditions, as identified by the geNorm algorithm in this study (Figure 9A). Meanwhile, contrasting results using different algorithms have been reported (Amorim et al., 2018) and are apparently exhibited in this study. The use of the geNorm approach alone cannot adequately be employed in analysis to declare a reference gene as stable for competent normalization of qPCR analysis. For a reference gene to be considered stable, it must be ranked the most stable or optimally stable by at least three out of the five approaches employed in the conceivable analysis. Adding to this, *GAPDH* is often not recommended as a reference gene due to its variability of expression caused by specific experimental factors and because it is known to participate not only in the basic processes of cell, but also significantly impact other processes occurring during the experiment (Kozera and Rapacz, 2013). Furthermore, to authenticate the trustworthiness of the reference genes in cowpea, the relative transcriptional expression level of two *early nodulation-like genes (ENODL2, 3)* and the *carbon-fixing gene (MDH-1)* were evaluated in leaves using qPCR. Consistent with the recommendations from the algorithms, when *Actin*, *EF1- α* , and *18S rRNA* were used as internal controls, the transcriptional expressions of *ENODL2*, *ENODL3*, and *MDH-1* were found to be highly

elevated in contrast to *GAPDH* and β -*tubulin* (Figure 6). This means that different reference genes resulted in varied judgements. Therefore, we strongly advocate compulsory confirmation of the stability of an internal control preceding any RT-qPCR experiments.

Conclusions

The presence of microorganisms in soil not only improved growth, biomass accumulation, and nodulation but also altered the expression of reference genes differently compared to soil devoid of microbes in different cowpea varieties. Based on the different algorithms applied herein, it can be concluded that *Actin* and *EF1- α /18S rRNA* exhibited exceptional stability qualities among the candidate internal control genes, with *GAPDH* and β -*tubulin* recognized as the most highly unstable reference genes in cowpea. The validation of the relative expression of the three target genes (*ENODL2*, *ENODL3*, and *MDH-1*) confirms high expressions with *Actin*, followed by *EF1- α* and *18S rRNA*, relative to the drastically low expressions when *GAPDH* and β -*tubulin* were introduced. The variations observed under the two soil conditions signify the impact of the presence and absence of microbes on growth, nodulation, and transcriptional expression in cowpea varieties. This provides a primary premise to analyze qPCR data in cowpea under both sterilized and unsterilized soil and offers a significant research basis for other crops and plant species.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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