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Selection of stable housekeeping genes for gene expression studies in different varieties of black pepper (*Piper nigrum* L.)

Nasreena C[1], Swapna Alex[1]*, Soni KB[1], Manjushri Dinkar Dongare[1], Kiran AG[1] & Manju RV[2]

[1]Department of Plant Biotechnology, College of Agriculture Vellayani, Kerala Agricultural University, Thiruvananthapuram Kerala-695 522, India
[2]Department of Plant Physiology, College of Agriculture, Vellayani, Kerala Agricultural University, Thiruvananthapuram Kerala-695 522, India

*Email: swapna.alex@kau.in

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Abstract

Real Time quantitative PCR (RT-qPCR) is a widely used technique to study the transcript level modulation of genes during developmental stages of crop plants as well as in stress responses. Suitable reference genes have not been validated in many plants including black pepper. In this study, expression stability of six commonly used housekeeping genes *viz*., actin, β-tubulin, elongation factor, initiation factor, ubiquitin and glyceraldehyde 3- phosphate dehydrogenase were evaluated by RT-qPCR during the growth of the black pepper inflorescence of varieties *viz*., Panniyur 1, Karimunda and Thekken. The results were analyzed using geNorm and Normfinder statistical algorithms. Stable reference gene is critical for the accurate normalization of target gene data in RT-qPCR. In this study actin, elongation factor and initiation factor were identified as the most stable housekeeping gene in different black pepper varieties *viz.,* Thekken, Panniyur 1 and Karimunda respectively. Actin in combination with GAPDH and elongation factor were obtained as optimal reference genes for Thekken. It is the first report on identification of stable housekeeping gene in different varieties of black pepper and can aid in expression studies in black pepper for yield improvement. The study will aid in normalization of gene expression studies in different varieties of black pepper.

Keywords

Normalization, Reference gene, RT-qPCR

Introduction

Real-time PCR technique is an effective and sensitive method to measure transcript level modulation of genes and provides significant quantitative information on gene expression (1-2). Although the conventional PCR method can measure a small difference in the level of transcription, a series of optimization and post PCR techniques like agarose gel electrophoresis are required. Even though real-time PCR is the most widely used technique for transcriptome analysis, there are several problems associated with it. The major problem is the lack of proper normalization of data (3). It is important to normalize data for an accurate result. The RT-qPCR data can be normalised using a variety of methods, including sample size/tissue volume, total RNA amount, ribosomal RNA, alien molecules (RNA spike), reference gene(s) etc. Normalization to sample size/tissue volume, is the first step in minimizing experimental error and ensures that a similar sample size is obtained by sampling tissues of comparable volume or

weight. It is rather simple; however, sample size and tissue volume estimation may be challenging and/or may not accurately reflect biological diversity. In total RNA amount method of normalization, a reliable method of evaluating quality and quantity is needed. However, it assumes no fluctuation in the rRNA/mRNA ratio and does not correct for error introduced at the PCR or reverse transcription processes. Internal controls known as ribosomal RNAs (rRNA) are subject to the same restrictions as the RNA of interest. As there is no poly A tail present in this instance, oligo dT priming of RNA for reverse transcription will not be effective with rRNA. However, it needs to be verified using the same experimental samples, and the resolution of that is determined by the inaccuracy of the reference gene (rRNA). An alternative internal control method uses an artificially incorporated molecule called an alien molecule that is cloned and *in vitro* transcribed from another species or generated synthetically. Compared to the RNA of interest, this RNA is not isolated from cells and needs to be more fully characterized and made commercially available. Among these proposed methods, normalization using reference genes is the most frequently used method to control the possible experimental errors (1).

The expression of the reference gene is anticipated to be constant for particular cell, tissues, and experimental settings. For specific cell, tissues and experimental settings, the expression of the reference gene is anticipated to be steady. Housekeeping genes, which control fundamental physiological processes such as the cytoskeleton, glycolytic pathway, protein folding, production of ribosome subunits, electron transport and protein degradation, are frequently used as references (1). There is no perfect housekeeping gene that has a consistent expression level under all experimental circumstances, according to prior studies, and the expression levels of the regularly employed housekeeping genes are not always stable (2-4). However, normalization using reference gene has received less attention in plant studies, and the housekeeping genes are being used as reference genes without any validation (2). The best acceptable reference gene(s) have been identified using a variety of statistical techniques or software tools, including geNorm (4) and NormFinder (5).

Piperaceae is one of the largest families among flowering plants, which includes species like *Piper nigrum, P. mullesua, P. longum, P. betel, P. retrofactum* and *P. chaba*. Black pepper, which belongs to the species *Piper nigrum* L., is the most significant and popular spice in the world (6). It is also referred as 'Black Gold' and has prime place in the international market due to its high quality. The major constraint in black pepper production is yield reduction. Biotechnological interventions in black pepper are mainly focused on overcoming biotic and abiotic stress tolerance. Yield and quality improvement studies in black pepper are also required. To date different black pepper varieties have been reported for their specific feature but Panniyur 1 is the high yielding first hybrid variety in the world developed by P. K. Venugopalan Nambiar (1924-1996) at the Head of Pepper Research Station, Panniyur in Kerala and released for commercial cultivation in 1971. Thekken is the novel mutant variety which have a remarkable branching character of spikes can contribute to high yield. Karimunda is a local variety of pepper wildly used for cultivation over the Kerala state. In this context, screening of suitable reference genes in different black pepper varieties at inflorescence development can aid in expression studies in yield improvement programmes. Therefore, the objective of this study was to evaluate appropriate reference genes for RT-qPCR gene expression studies across different black pepper varieties *viz.,* Panniyur 1, Karimunda and Thekken. Actin, β-tubulin, elongation factor, initiation factor, ubiquitin and glyceraldehyde 3-phosphate dehydrogenase were examined here as six frequently used housekeeping genes during the growth of the black pepper inflorescence.

Materials and Methods

Plant Material

Inflorescence samples of 3 varieties of black pepper *viz*., Panniyur 1, Karimunda and Thekken were used for the study. Bush pepper plants, maintained in College of Agriculture, Vellayani, Kerala, India were used for the experiments. Samples were taken from two distinct plants of each variety during the developmental stages of inflorescences like stage I (1-2 cm), stage II (6-8 cm) and stage III (9 -12 cm) (Fig. 1).

Total RNA extraction and cDNA synthesis

Inflorescence samples were collected and ground to fine powder in liquid nitrogen using pre-cooled mortar and pestle. Total RNA was extracted using TRIzol reagent (Invitrogen) according to manufacturer's instruction. Agarose gel electrophoresis was used to evaluate the RNA's structural integrity and the A_{260}/A_{280} ratio was used to measure purity. A UV spectrophotometer was used to measure the concentration of RNA. Only good quality RNA was used for subsequent steps. Single-stranded cDNA was synthesized from 1 µg of total RNA by Verso cDNA synthesis kit (Thermo Scientific™, Cat No. AB1453A) according to manufacturer's instruction.

Primer design and RT-qPCR

Actin, β-tubulin, elongation factor, initiation factor, ubiquitin and glyceraldehyde 3-phosphate dehydrogenase were chosen as the 6 genes that were most consistently expressed during the development of the inflorescence in black pepper cultivars. These genes were selected as candidate genes for the study since their use as a stable reference gene are reported at different experimental conditions (7-14). Full length genome sequence of black pepper is not available yet; hence primers were designed for the six reference genes using the sequences as detailed in Table 1. Primer express 3.0 software was used for primer design with the default settings. MFold software was subsequently used to evaluate the formation of secondary structure at the primer binding site. Details of designed primers are shown in Table 2. The Bio-Rad Real Time System (CRX96TM Real-Time System) was used to perform real-

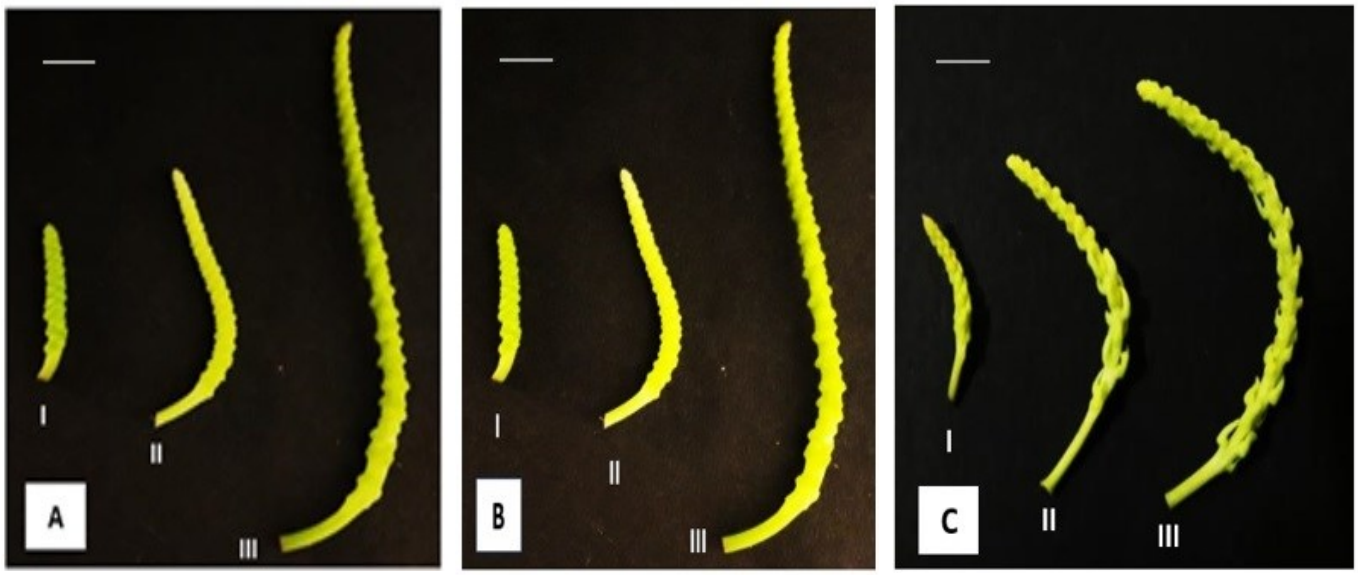


Fig. 1. Inflorescence samples at three different stages *viz*., stage I (1-2cm), stage II (6-8cm) and stage III (9-12cm) of black pepper varieties *viz.,* Panniyur 1 (**A**), Karimunda (**B**) and Thekken (**C**); Scale bar =1cm.

Table 1. Candidate reference genes and their related sequence ID used for primer designing.

Gene	Sequence ID
GADPH	*Manihot esculenta* XM_021775719.1
Elongation Factor 1 α	*Rosa chinensis* XM_024304812.1
Actin	*Quercus suber* XM_024037498.1
Initiation Factor 4A1	*Quercus suber* XM_024029585.1
β Tubulin	*Nicotiana attenuata* XM_019378551.1
Ubiquitin	*Citrus sinensis* XM_015529274.2

GAPDH-Glyceraldehyde-3-phosphate dehydrogenase

specificity of the amplicon was confirmed. RT-qPCR for every gene was performed with two biological replicates with three technical replicates.

Data analysis

Two statistical algorithms geNorm (4) and NormFinder (5) were used to evaluate the stability of the six candidate reference genes. Expression levels of the tested reference genes were determined by Cq values. Relative quantities were created using the Cq values of all reference genes utilized in the geNorm and NormFinder. Based on the average pair-wise alterations in the expression level, the

Table 2. Nucleotide sequence of primers designed for RT-qPCR (Real time quantitative polymerase chain reaction) of housekeeping genes.

Primer	Nucleotide sequences (5'-3')	Tm (°C)	GC content (%)	Expected size of amplicon (bp)
ACT	F-GCCACACGGTGCCTATCTAT	59.8	43	190
	R-AAATGCGAGAGCTTCTCCTTCA	60.0	48	
eEF	F-CTTCAGGATGTGTACAAGATTGGTG	58.6	44	171
	R-GTCACCAGGAAGAGCCTCCTG	59.8	62	
GAPDH	F-ATTGTTGAGGGTTTGATGACCACT	58.1	38	210
	R-ATCGACGGTAGGAACACGGA	60.6	57	
eIF	F-TCATGCCTGTGTTGGTGGAA	59.5	50	133
	R-TAATGTGATCAGGGCGAAGTGA	59.2	45	
TUB	F-GCTCTTTATGACATTTGCTTCCGA	60.5	42	200
	R-GGAGCAAAGCCAACCATGAA	59.6	50	
UBQ	F-GTGGAGAGCTCCGATACCATTGAT	61.4	50	174
	R-ACGCAGACGCAGCACCAA	61.2	61	

ACT-Actin; eEF-Eukaryotic Elongation Factor; GAPDH-Glyceraldehyde-3-phosphate dehydrogenase; eIF-Eukaryotic Initiation Factor; TUB-Tubulin and UBQ-Ubiquitin.

time PCRs. 0.3 mM of forward and reverse primers and 10 µL of 2X SYBR Green PCR master solution was used in a total volume of 20 µl. For RT-qPCR, the temperature was 95 °C for 2 min, then 40 cycles of 95 °C for 15 sec, 55 °C for 15 sec and 72 °C for 45 sec. By using agarose gel electrophoresis and melting curve analysis after 40 cycles, the geNorm algorithm estimated the expression stability value (M) for each candidate reference gene. The minimum number of reference genes for the appropriate normalization was computed using the pairwise variation (Vn/Vn+1) using the geNorm programme. By integrating the inter- and intra- group differences, NormFinder, another Microsoft Excel-based software programme, computed the

expression stabilities of the potential reference genes.

Results and discussion

Expression levels and variation of the candidate reference genes

In quantitative real-time PCR, normalization of data is crucial to minimize the effect of non-biological variation. For accurate and reliable real time PCR results, an appropriate reference gene must be determined. The use of an unstable, nonspecific reference gene could add more bias to the result. On that account, it is important to validate reference gene according to each experimental condition. Actin, β-tubulin, elongation factor, initiation factor, ubiquitin and glyceraldehyde 3-phosphate dehydrogenase were 6 potential genes whose expression profiles were examined throughout the development of the inflorescence in 3 black pepper varieties. Cq values of all candidate genes were obtained using Biorad CFX Maestero software and are given in Table 3. In MFold software analysis, secondary structures were not found at primer binding site of the sequences. Melting curve analysis was performed to check the specificity of primers designed for selected housekeeping genes. Single peak in the melting curve analysis proved specificity (Fig. 2).

Table 3. Ct value of six reference genes generated using RT-qPCR.

Samples	Inflorescences Stage	Reference genes					
		EF	Actin	GAPDH	IF	TUB	UBQ
Karimunda	Stage 1	29.15	29.62	30.25	26.99	28.29	28.14
	Stage 2	26.09	26.62	29.7	23.99	23.02	25.57
	Stage 3	31.07	30.53	37.53	28.34	28.45	28.62
Panniyur 1	Stage 1	34.32	35.03	37.77	31.23	37.17	40.00
	Stage 2	28.24	28.51	29.90	26.71	25.15	28.68
	Stage 3	36.16	36.69	40.00	32.57	40.00	40.00
Thekken	Stage 1	27.05	27.66	26.98	26.30	23.90	27.69
	Stage 2	30.38	30.65	30.59	28.71	28.30	28.89
	Stage 3	25.11	24.75	24.39	22.90	22.78	25.95

ACT-Actin; eEF-Eukaryotic Elongation Factor; GAPDH-Glyceraldehyde-3-phosphate dehydrogenase; eIF-Eukaryotic Initiation Factor; TUB-Tubulin and UBQ-Ubiquitin.

Data analysis using geNorm and Normfinder software

geNorm software program used to analyse expression stability calculates gene-stability measure M which is the average pairwise variation of a particular gene with all the other genes. Genes with the lowest M value has the most stable transcription and the most stable gene can be used as a reference gene for normalization in RT-qPCR (4). The most stable combination of reference genes is determined by the geNorm V value, which is calculated by the geNorm software. In order to determine the ideal number of reference genes to be utilized for precise normalization, pairwise variation is calculated using n/n+1 reference genes. n number of reference gene can be considered sufficient for normalization based on the 0.15 cut-off for the pairwise variation (15). If the geNorm V value is less than 0.15, the addition of one more reference gene provides no improvement in the fidelity (12).

M values for all the genes were generated by geNorm when varieties were analysed together as well as separately (Table 4). It was found that actin was the most stable gene among the selected genes for the study, when all the varieties were analysed together (Fig. 3A). But the geNorm M value for all the genes were more than the default limit of 1.5 (4). Thus, it is necessary to use combination of reference gene for more accurate normalization of data (5).

When varieties were analyzed separately, actin was found to be the most stable reference gene in Thekken with M value 0.325 (Fig. 3D). Actin is the most widely used reference gene for normalization of data in RT-qPCR (14, 16-18). It is reported to be the most stable reference gene in many other plant systems including *Plukenetia volubilis* during flower development (19) and *Cyamopsis tetragonoloba* during seed development and abiotic stress conditions (20).

The pairwise variation results in present study are shown in (Fig. 4). In Panniyur1 and Karimunda geNorm V value were more than 0.15 (Fig. 4B & 4C). The optimal number of reference genes for gene expression studies in Thekken was 3 (Fig. 4A) (geNorm V < 0.15 when comparing a normalization factor based on 3 or 4 most stable targets). Actin, GAPDH and elongation factor can be used to accurately normalize data for gene expression investigations during the development of inflorescence in Thekken. It is reported that in bell pepper actin was used in combination with elongation factor as stable reference gene during expression studies after hormonal treatments (22) and actin in combination with tubulin is reported to be the reference gene for expression studies under drought stress in carrot (23).

Elongation factor and actin were observed to be the most stable gene in Panniyur 1 whereas initiation factor and elongation factor recorded as most stable genes in Karimunda when varieties were analysed separately (Fig. 3B & 3C). However, the geNorm M value for all the genes were above the default limit of M = 1.5 indicating lesser

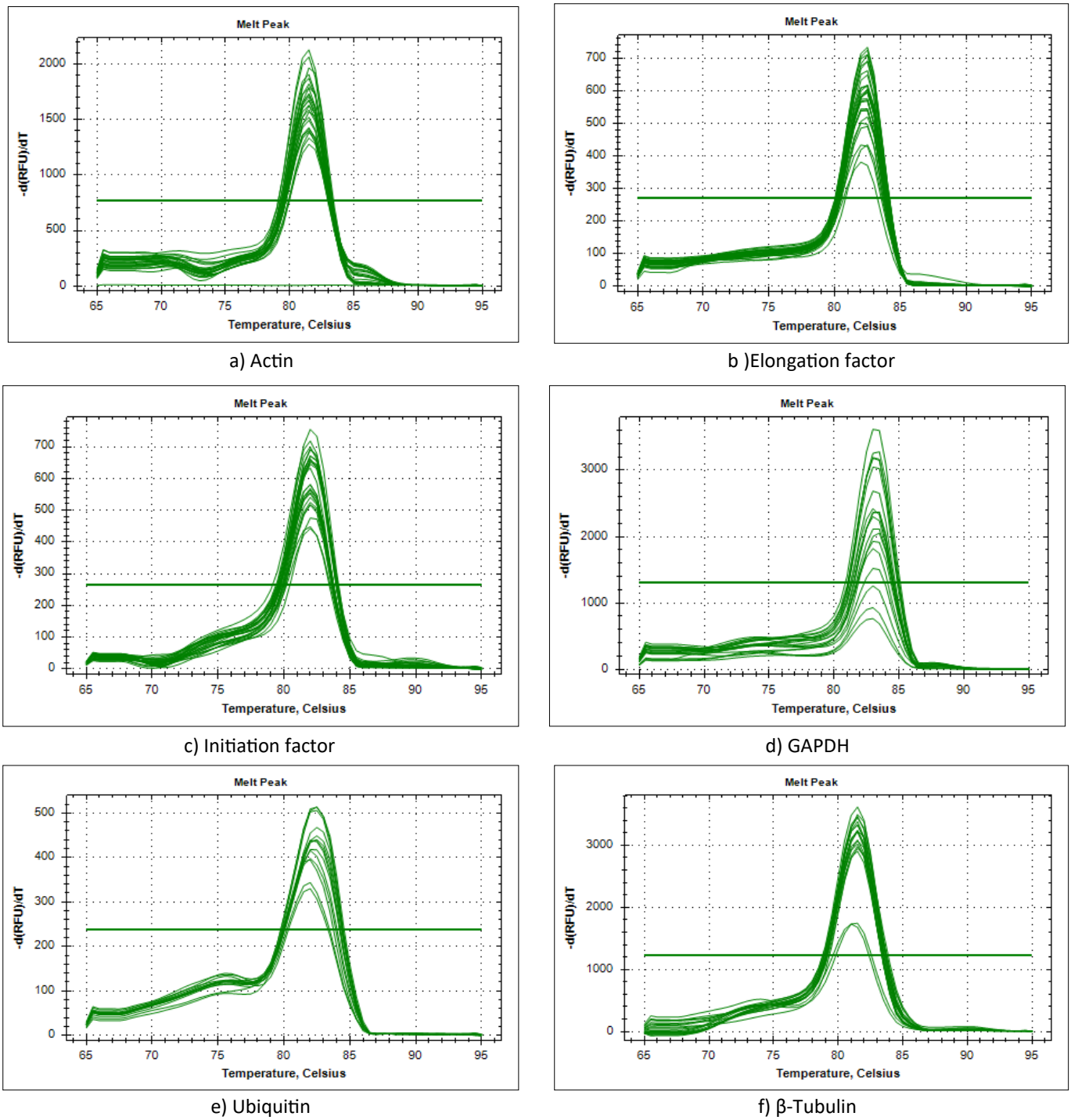


Fig. 2. Specificity of RT-qPCR amplification of six candidate reference genes (actin, elongation factor, GAPDH, initiation factor, β-tubulin and ubiquitin) with single peak generated from all amplicons.

Table 4. Expression stability value (M value) of reference genes calculated by using geNorm in different varieties of black pepper.

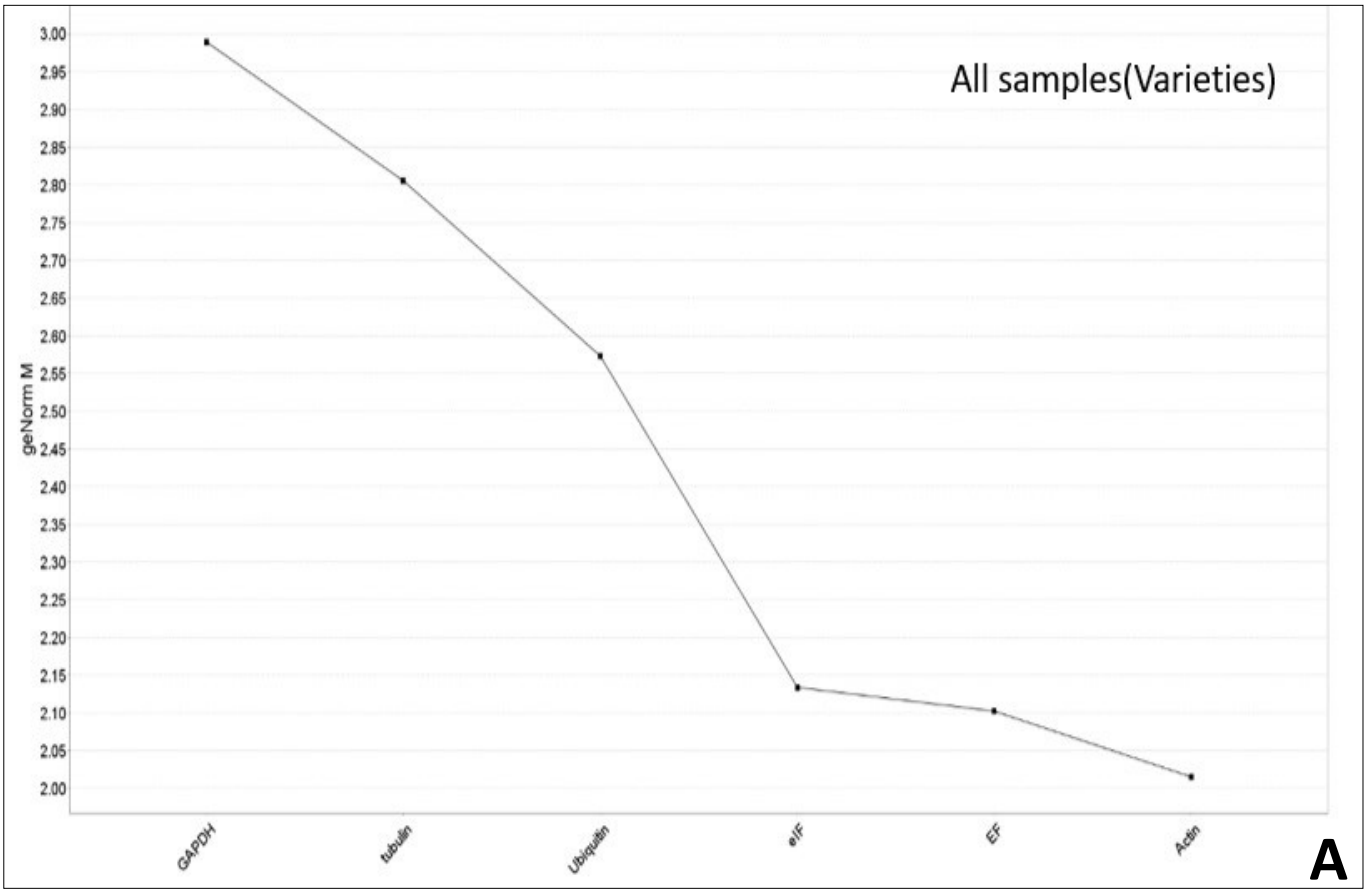
Gene	Expression stability value (M)			
	Thekken, Panniyur1 and Karimunda samples analysed together	Thekken	Panniyur 1	Karimunda
Actin	2.00	0.325	1.85	1.89
Elongation factor	2.10	0.400	1.83	1.58
GAPDH	2.97	0.360	1.95	2.49
Initiation factor	2.13	0.430	2.19	1.50
β-Tubulin	2.90	0.570	2.65	3.25
Ubiquitin	2.57	0.760	2.79	2.98

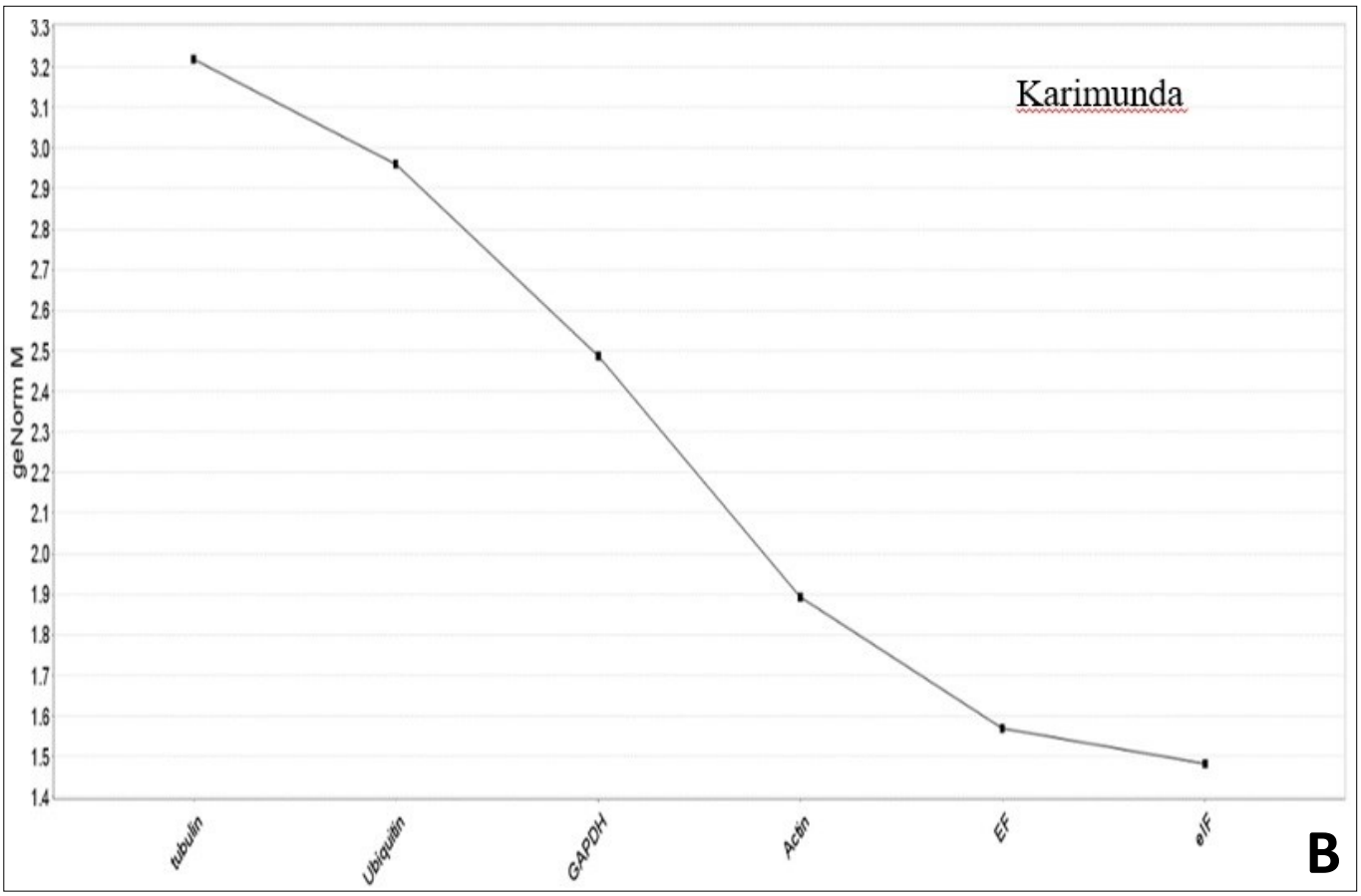
GAPDH- Glyceraldehyde-3-phosphate dehydrogenase

stability of these genes (5). Similar reports have been

observed, in peach (*Prunus persica*) exhibiting variation in stability of reference genes across different varieties during fruit ripening and softening respectively (23).

Normfinder is another excel based tool used to identify stable reference gene. Relative expression values were given as input data. Stability values were calculated for all the genes by software. It ranks candidate reference gene based on their expression stability in a given set of samples and given experimental condition. Low average expression stability values represent genes with stable





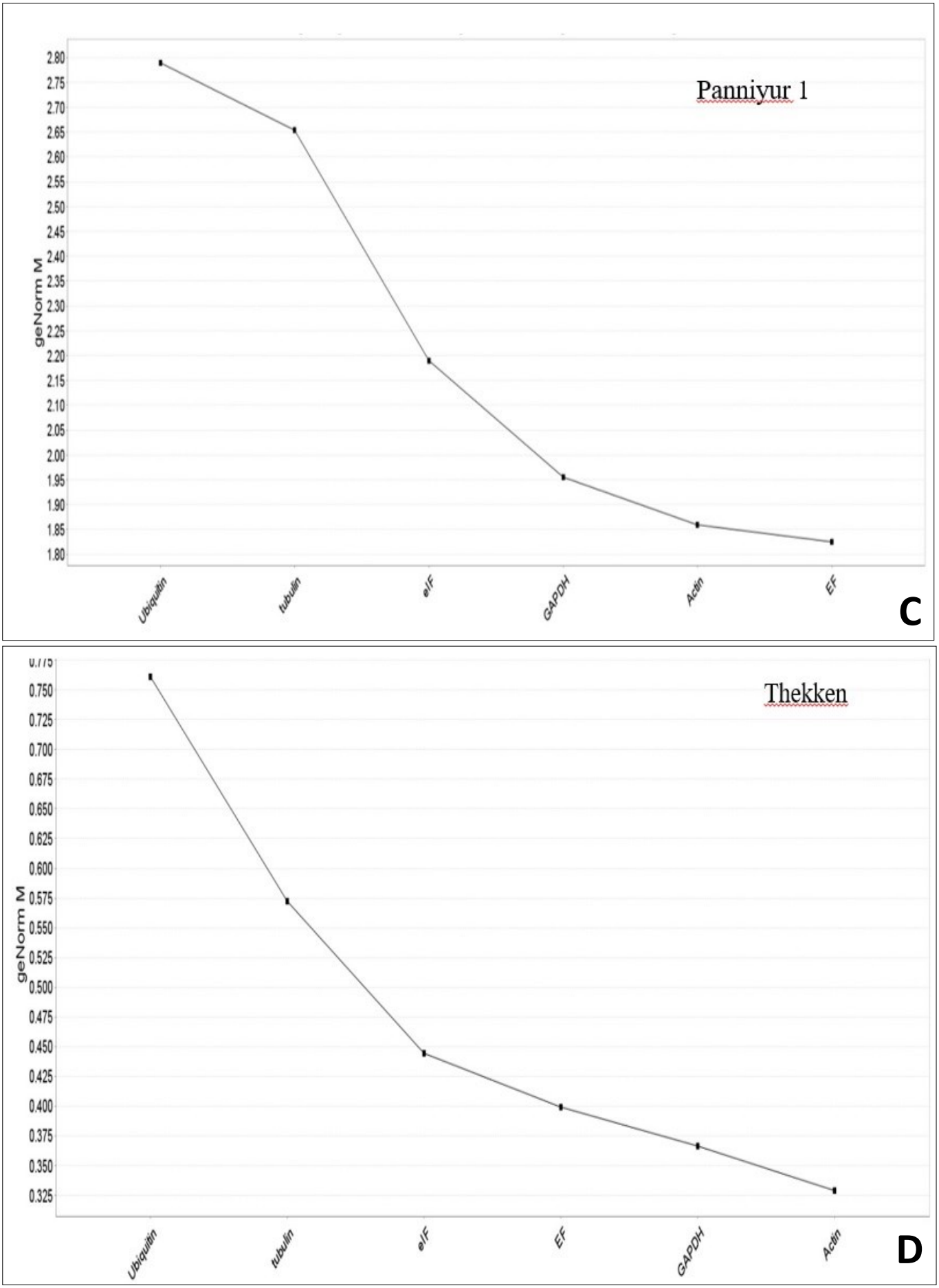


Fig. 3. Average expression stability value (M) of six candidate reference genes generated by geNorm software in black pepper (*Piper nigrum* L.) samples (varieties) (**A**) All samples (varieties) together (**B**) Karimunda (**C**) Panniyur 1and (**D**) Thekken.

expression (5). According to stability value calculated by Normfinder software (Table 5) actin was the most stable

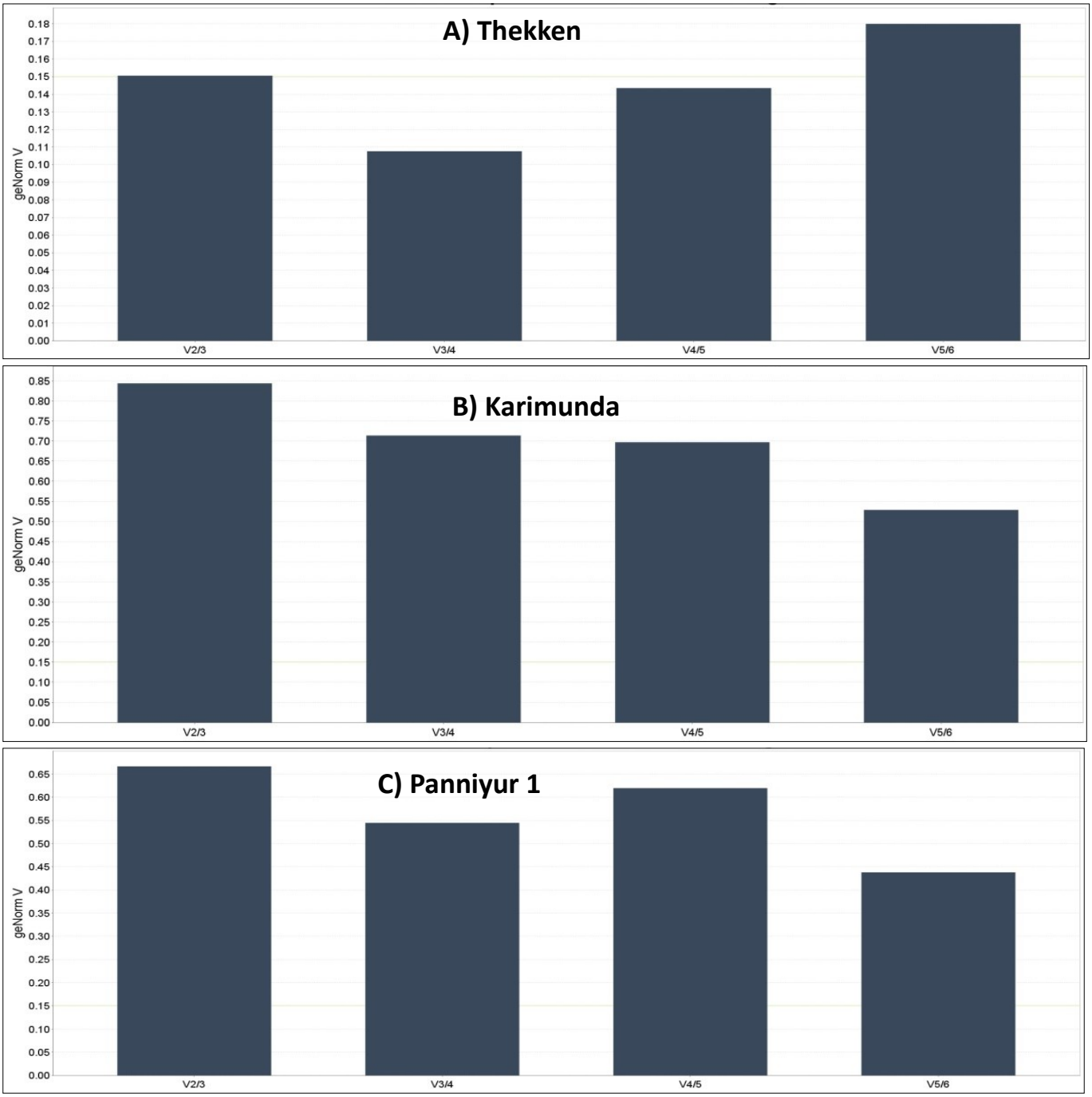


Fig. 4. Pairwise variation analysis to determine optimal number of reference genes in three varieties of black pepper *viz.,* Thekken (**A**), Karimunda (**B**) and Panniyur 1 (**C**) using geNorm software. V2/3 is the pairwise variation between the two most stable genes and the three most stable genes. The three most stable genes are compared to the four most stable genes in V3/4. V4/5 is the pairwise variation between the four most stable genes and the five most stable genes. The five most stable genes are compared to the six most stable genes in V5/6. Line indicates cut-off value of 0.15, below which additional reference genes are not necessary for normalisation.

Table 5. Stability values of reference genes generated by Normfinder analysis in different varieties of black pepper (*Piper nigrum* L.).

Gene	Stability values			
	Thekken, Panniyur1 and Karimunda samples analysed together	Thekken	Panniyur 1	Karimunda
Actin	0.842	0.260	0.647	0.846
Elongation factor	1.137	0.260	1.120	1.355
GAPDH	1.450	0.387	1.047	1.283
Ubiquitin	1.497	0.689	1.387	1.620
β-Tubulin	1.588	0.492	1.508	1.804
Initiation factor	1.729	0.442	1.385	1.341

GAPDH- Glyceraldehyde-3-phosphate dehydrogenase

gene in all the 3 varieties *viz.,* Thekken, Karimunda and Panniyur 1, when analysed together as well as separately. Variation in the expression of genes in Thekken variety was comparatively less. But in case of Panniyur 1 and Karimunda high variation was observed for all the genes. The expression stability of different reference genes varied between cultivars and these variations were noted in *Paeonia suffruticosa*, *Panax ginseng* and strawberry (24-26).

According to geNorm, elongation factor and actin were most stable reference genes in Paniyur 1; initiation factor and elongation factor were the most suitable reference genes in Karimunda whereas Normfinder predicted actin as the most stable gene in both the varieties. Such variations are reported earlier in other crops (12, 14, 27-29). In chrysanthemum variation is reported in stability of the reference genes among different varieties of same crop by correlating with complexity of the genetic background (30).

Elongation factor is a commonly used reference gene in many plants including tomato under nitrogen stress (31), pearl millet under abiotic stress (32), cucumber under viral stress (12) and Bedstraw weed (*Galium aparine* L.) in biotic and abiotic conditions (33). In the present study elongation factor was the most stable housekeeping gene only in Panniyur 1 and Karimunda varieties of pepper.

The normalisation of RT-qPCR in *Stipagrostis pennata* under stress conditions has used the GAPDH and Initiation factor genes as internal controls. GAPDH and Initiation factor genes displayed the most constant expression levels in *S. pennata* under PEG treatment and rhizosheath development (34). Also, GAPDH observed to be the best expression stability in different tissues and organs of *Saccharum* sp. (35). Similarly in developing fruit of *Lycium barbarum* GAPDH along with elongation factor were found to exhibit expression stability and were used as optimal reference gene (36). In, animal models GAPDH is also found to be stable internal reference gene for gene expression study in cancer biology (37). In the present study, GAPDH showed an intermediate expression compared to other genes in different varieties of black pepper. This is in agreement with the reports in cucumber under biotic stress (12).

Initiation factor is a protein that plays an important role in initiating the translation of the mRNA molecule into peptide. Initiation factor are reported to be the most stable gene in in rice seeds at different growth stages (38). Additionally, it was discovered that the initiation factor gene was expressed in a stable manner in stem segments of *Populus tomentosa* (39). Our study identified initiation factor as the most stable reference gene in Karimunda and Thekken.

β-tubulin and ubiquitin are reported to be stable in many plants (7, 8, 33, 40, 41). However, in the present study they exhibited least stability of expression in all the varieties and hence they were not suitable for normalization of data. Similar results have been reported in cucumber wherein β-tubulin exhibited high variability in expression levels when subjected to growth regulators and abiotic stress (42) and in *Actimidia deliciosa* under biotic stress (43).

Actin was found to be the most stable housekeeping gene compared to β-tubulin, elongation factor, initiation factor, ubiquitin and glyceraldehyde 3-phosphate dehydrogenase when the genes were examined collectively by utilizing geNorm and Normfinder. Analysis using Normfinder software alone also identified actin as the most stable gene when the different varieties were analysed in combination and separately. However, when samples were analyzed separately, using geNorm software, actin, elongation factor and initiation factor were observed to be the stable genes in Thekken, Panniyur 1 and Karimunda respectively. In Panniyur 1 and Karimunda variation in stability of housekeeping genes was observed to be higher.

The outcomes demonstrated the differences in candidate reference gene stability and expression levels among the various black pepper varieties. The results highlight the importance of validation of reference genes while undertaking yield improvement studies in different varieties of black pepper.

Conclusion

To conclude, actin was found to be the most stable housekeeping gene compared to β-tubulin, elongation factor, initiating factor, ubiquitin and glyceraldehyde 3-phosphate dehydrogenase in all varieties of black pepper. Actin combined with GAPDH and elongation factor were predicted as optimal reference gene for Thekken variety. Selection of most appropriate reference gene with respect to different varieties of crop plants is a key step in gene expression studies using RT-qPCR. The results offer a foundation for choosing reference genes in different varieties of black pepper for crop improvement programmes.

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Authors contributions

NC carried out the research work, SA, SKB, participated in the design and co-ordination of the experiment, MDD drafted the manuscript, KAG and MRV participated in the analysis of result. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical issues: None.

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