SELECTION OF STABLE HOUSEKEEPING GENES FOR GENE EXPRESSION STUDIES DURING INFLORESCENCE DEVELOPMENT IN BLACK PEPPER (*Piper nigrum* L.) USING REAL TIME PCR

by

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THESIS

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DECLARATION

I, hereby declare that the thesis entitled **"Selection of stable housekeeping genes for gene expression studies during inflorescence development in black pepper (*Piper nigrum* L.) using real time PCR"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award for any degree, diploma, fellowship or other similar title of any other University or Society.

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"In the name of Allah, the Most Gracious and Most Merciful"

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
A	Adenine
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 260 nm wavelength
ACT	Actin
ba1	Barren stalk1
bif 2	Barren inflorescence 2
bp	Base pair
C	Cytosine
cDNA	Complementary DNA
cm	Centimere
Cq	Quantitation cycle
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
DEPC	Diethyl pyrocarbonate
EDTA	Ethylene diamine tetra acetic acid
EF	Elongation Factor
FP	Forward Primer
G	Guanine
g	gram

GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
ha	Hectare
IF	Initiation Factor
Kb	Kilo basepair
Kg	Kilogram
M	Molar
Min	Minute
ml	Millilitre
mM	Millimolar
MT	Metric tonne
NCBI	National Center for Biotechnology Information
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometre
°C	Degree Celsius
PCR	Polymerase chain reaction
PVP	Polyvinyl pyrrolidone
Ra1	Ramosa1
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
RP	Reverse primer
rpm	Revolution per minute
RT	Reverse transcriptase
RT-qPCR	Reverse transcription-polymerase chain reaction

s	Second
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SSR	Simple sequence repeat
T	Thymine
TBE	Tris-borate EDTA buffer
TFL1	Terminal flower 1
T_m	Melting temperature
Tris HCl	Tris (hydroxy methyl) aminomethane hydrochloride
TUB	Tubulin
UBQ	Ubiquitin
U	Enzyme unit
V	Volt
viz.	namely
w/v	weight/volume
v/v	volume/volume

Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum*) commonly referred as 'Black Gold' is one of the oldest and most important spice crops in the world. It belongs to the family *Piperaceae* and is cultivated for its berries. The "King of Spices" has prime place in the international market due to its high quality. Besides its high nutritive value black pepper is also marked for its medicinal properties. It is used as an important component in traditional Ayurveda treatments (Nybe and Sujatha, 2001)

Western Ghats of Indian peninsula is considered as the centre of origin of black pepper because of the presence of highest diversity in this region (Rahiman and Nair, 1987; Mathew *et al*., 2007). In ancient time black pepper was extensively cultivated in the tropics of South East Asia and it became the important commodity of terrestrial trade between India and Europe.

Pepper is cultivated in about twenty six countries and the area under cultivation is 4, 67,708 hectares with productivity of 790.2 kg per hectare (Kumar and Swarupa, 2017). Vietnam is the largest producer and exporter of black pepper in the world followed by Indonesia and India. It constitutes 39% of the world production whereas Indonesia and India contribute 13% and 12% respectively. India is the country having largest area under pepper cultivation. In India, southern states like Kerala, Tamilnadu and Karnataka are the major producers of black pepper where in Kerala ranks first (Abraham, 2018). In the year 2000 India was the major producer of black pepper. During the subsequent years India was replaced by Vietnam and Indonesia (Yogesh and Mokshapathy, 2013). The major constraint in black pepper production is yield reduction. As flowering determines the yield parameters in black pepper, knowledge of the genes involved in floral architecture and their characterization can help significantly in taking up further yield improvement programmes. Biotechnological interventions in black pepper are mainly focused on overcoming biotic and abiotic stress tolerance. Not much work has been done for improving yield potential and quality of black pepper.

Reverse Transcription quantitative PCR (RT-qPCR) is widely used to study the transcript level modulation of genes during developmental stages of crop plants as well as in stress responses. Housekeeping genes with stable expression are critical for the accurate normalization of target gene data in RT-qPCR (Qian-Feng *et al.*, 2010; Omar *et. al.*, 2016). A housekeeping gene ideally should be stable and should not show changes under experimental conditions. There is no ideal and universal housekeeping gene. Some reference genes are highly specific for particular crop and experimental condition, so validation of reference genes is a crucial requirement before carrying out RT-qPCR experiments (Dheda *et al.*, 2004).

Housekeeping genes identified as reference genes show variability in expression levels in different experimental situations (Selvey *et al.*, 2001), emphasizing the importance of evaluation studies to identify the most stable housekeeping gene in different tissues, developmental stages or stress responses before the start of any study with real time PCR. The objective of this study was to compare the expression profile of different housekeeping genes such as *Actin,* β-*Tubulin, Elongation factor, Initiation factor, Ubiquitin* and *GAPDH* (Glyceraldehyde 3- phosphate dehydrogenase) during inflorescence development and to identity the most stable gene to be used as reference gene for RT-qPCR studies during inflorescence development in black pepper (*Piper nigrum L.*).

Review of Literature

2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L.), a woody climbing perennial is one of the most important and valuable spice in the world. The "King of Spice" is the most widely used spice and has unique and supreme position in the international market (Abraham, 2018). More than hundreds of cultivars of black pepper are known to exist in India and majority of them are in Kerala and Karnataka (Ravindran *et al.*, 1997). Western Ghats of Indian peninsula is considered as origin of black pepper.

Black pepper is mainly cultivated in tropical countries like India, Vietnam, Sri Lanka, Malaysia, Indonesia and Brazil. Hot and humid climate in this region is the main reason for highest production of black pepper (Kumar and Swarupa, 2017). Pepper is valued for its pungency and flavour due to the presence of alkaloid piperine (Ravindran *et al.*, 2000). Black pepper has been in use since centuries for its health promoting and disease preventing properties. It is the major component of ayurvedic and other traditional methods of treatments (IISR, 2015).

More than seventy five cultivars of black pepper are being cultivated in India. Among this Karimunda is the most popular cultivar in Kerala. The other important cultivars are Kottanadan, Narayakodi, Aimpiriyan, Neelamundi, Kuthiravally, Balancotta and Kalluvally. Sixteen improved varieties been released for cultivation and includes Panniyur-1, Panniyur-3, Sreekara, Panchami, IISR Shakthi etc. (Spice board, 2009).

2.1 MORPHOLOGY AND FLOWERING

Black pepper is a woody climbing vine having both adventitious and supporting aerial roots. Climbing aerial roots help the plants to attach to the surface for support. Leaves are dark green, glossy, ovate, acutely tiped and length ranges from 13-25 cm. Fruits are sessile, globose, single seeded drupe with pulpy mesocarp and exocarp become red when ripe. Elongated spikes bear minute white flowers that produce single seeded berries. Spikes are produced on the stem

opposite to the leaves. The seeds have little endosperm, minute embryo and copious perisperm which is nutritive in function (Nelson and Cannon-Eger, 2011).

Based on spike length : leaf length ratio the cultivars are classified into three groups. Those with spikes much shorter or longer than the leaf and those with spike length equal to the leaf length. Most popular cultivars fall into the third group (Ravindran *et al.*, 1997).

Predominantly black pepper is a self-pollinated crop and is dioecious in wild state and gynomonoecious or trimonoecious in cultivated forms (Thangaselvabal *et al.*, 2008). Bisexual flowers are protogynous and anther dehiscence occurs 3-8 days after stigma exertion (Martin and Gregory, 1962). Black pepper is a polyploid species and somatic chromosome number varies from 52 to 104. Most of the cultivated and many of the wild varieties have somatic chromosome number 2n =52 (Mathew, 1958).

Normally black pepper varieties are non-branching type. But a wild type black pepper reported by farmer Mr. T. T. Thomas from Idukki district, Kerala has branched inflorescence and is named as Thekken". With increase in the number of branches per spike, yield of plant will also increase. Demand for black pepper is increasing year by year in the international market. However, the production is not up to the demand (Thangaselvabal *et al.*, 2008). Since the production and productivity of black pepper in India shows a sharp decline, studies on inflorescence may be useful in increasing the production of black pepper (Hembade, 2016).

2.2 GENES INVOLVED IN INFLORESCENCE DEVELOPMENT

Plant architecture is mainly determined by shoot branching, plant height and inflorescence morphology (Wang and Li, 2006). The genetic basis of inflorescence development is highly varied between flowering species. Major studies related to genetic and molecular basis of flower development have been done in model dicotyledonous plants such as *Arabidopsis* but little is known about

monocotyledonous species (Cassani *et al*., 2006). Important progresses are made in the characterization of genes that are directly involved in the formation of plant architecture, especially that control growth of the axillary buds, the lengthening of the stems and the architecture of the inflorescences. Many of these genes are conserved in all type of plants, showing that most of the plants use same regulatory pathway for the development of plant architecture and it makes use of these genes in important crop improvement programs (Wang and Li, 2006).

The *TFL1* gene controls the architecture of the inflorescence and is conserved in many plant species. The protein encoded by the *TFL1* gene plays an important role in the architecture of the inflorescence by maintaining the correct functioning of the apical meristem (Shannon and Meeks-Wagner, 1991). Vimarsha *et al*. (2014) analysed the presence of *TFL1* homologs in different cultivars of black pepper and discovered the existence of sequence similarity for the *TFL1*gene that plays an important role in the development of the inflorescence of *Arabidopsis thaliana*.

Several mutant genes are identified in many plants that disturb the normal development of inflorescence and leads to the formation of new inflorescence architecture. In maize, *Ramosa1* (*Ra1*) gene plays a role in the inflorescence structure by determining the number of branches. *Ra1* gene influences the development of inflorescence and produces branch meristem instead of spikelet pairs. But the mechanism of the *Ra1* gene is unclear (Cassani *et al*., 2006). Hembade (2016) studied the differential expression of the genes of the RAMOSA family (*RA1, RA2, and RA3*) and their influence on the branching of spikes in black pepper and reported that the *RA2* gene exhibited differential expression in Thekken and Karimunda. The analysis was performed using RAPD, SSR markers and SDS-PAGE.

Normal black pepper plants have unbranched, single, axillary spikes. Spikes are formed by the transformation of apical bud of plagiotropic branches.

Mutation can change spike architecture by spike proliferation (Sasikumar *et al.*, 2006).

Other important genes that are involved in the plant architecture are *bif2* and *ba1*. These genes play a role in the functioning of the axillary meristem in maize (Ritter *et al.*, 2002; Gallavotti *et al.*, 2004). The barren inflorescence 2 (*bif2*) gene encodes a serine/threonine protein kinase plays a fundamental role in the onset of axillary meristem during vegetative growth and inflorescence development in maize (McSteen *et al.*, 2007).

Barren stalk1 (*ba1*) is a barren inflorescence mutant which encodes a typical basic helix-loop-helix transcription factor (Gallavotti *et al.*, 2004). *ba1* mutants affect all axillary meristem in the plant, similar to *bif2*, except the phenotype is more severe (Ritter *et al.*, 2002).

2.3 TECHNIQUES AND TOOLS USED

The measure of expression stability of genes involves designing of primers, quality and specificity check of primers, DNA isolation, RNA isolation, cDNA synthesis and RT-qPCR.

2.3.1 Reverse transcription quantitative PCR (RT-qPCR)

The 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 (Huggett *et al.*, 2005; Gutierrez *et al.*, 2008). Although the conventional PCR method can measure a small difference in the level of transcription, a series of optimization and post PCR techniques are required. These optimizations should be done for each sample because the expression of RNA varies widely between different samples. In conventional PCR, electrophoresis is used to evaluate the amplification product, while in RT-qPCR fluorescent molecules are used for the quantification. RT-qPCR is

considered as a superior method due to its sensitivity and accuracy in the quantification of mRNA transcripts (Bustin, 2002).

Real-time PCR has wide applications in different areas of functional genomics, molecular biology, biotechnology, etc. (Bustin *et al.*, 2005). In this technique, the specific region of the DNA is simultaneously amplified and quantified. The method depends on the calculation of the increase in the fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle. In the initial phase, the amount of fluorescence will be very less due to the less number of the product. With an increase in the cycle number amount of fluorescence will also increase. At a particular point, there will be a significant increase in the fluorescence and that point called threshold fluorescence. The point at which the fluorescence reaches a defined threshold is relative to gene expression. Individual reactions are characterized by the PCR cycle in which the fluorescence increases above defined background fluorescence, known as the threshold cycle (Cq). The value of Cq and the initial target concentration have an inverse relationship.

In real-time PCR, the amount of DNA is measured after each cycle via fluorescent dyes. Increase in fluorescent signal is directly proportional to the number of PCR product. Fluorescent reporters used in real-time PCR include double-stranded DNA (dsDNA) binding dyes or dye molecules attached to PCR primers or probes that hybridize with PCR products during amplification. SYBR Green is one of the fluorophores that can be used in real-time PCR, which uses a simple method for the detection and quantification of PCR products with high sensitivity (Nygard *et al.*, 2007). SYBR Green binds to double-stranded DNA and emits light after excitation. As the reaction continues, PCR products accumulate and the fluorescence increases proportionally (Pabla and Pabla, 2008).

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 normalization of data (Dheda *et al.*, 2004). It is important to

normalize data for an accurate result. Several strategies have been developed to normalize the data. One of the methods is normalization using the reference gene (Hugget *et al.*, 2005). The accuracy of the results obtained by this method depends on the normalization using stably expressed genes known as reference genes. But this method has received less attention in plant studies, where the housekeeping genes are being used as reference genes without any validation. Czechowski *et al.* (2005) showed that the expression stability of commonly used traditional housekeeping gene was less compared to other novel genes in Arabidopsis.

Podevin *et al.* (2012) conducted studies on the validation of the reference gene in non-model crop *Musa sp*. They reported that the expression stability of gene varies according to the experimental conditions. They have used *in vitro* plants, greenhouse plants, meristems and leaf disc for their studies and analysed the expression stability of six housekeeping genes. They have shown that the combination of *ACTIN*, *TUBULIN* and *Elongation factor* was most suitable for normalization of expression data from greenhouse leaf samples. But in the case of *in vitro* plant variability in gene expression was very high.

2.3.1.1 House Keeping Genes as Reference Gene

An ideal housekeeping gene should be stable and express in all cells and should not show any changes with changing experimental conditions. However, there is no ideal housekeeping gene (Vandesompele *et al.*, 2002). The housekeeping genes used as reference genes showed difference in the level of gene expression with change in experimental conditions (Selvey *et al.*, 2001), which draw attention to the importance of validation of stable housekeeping gene in studies using real-time PCR.

The most commonly used references genes in the RT-qPCR are *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Actin* (*ACT*), *Elongation factor-1a (EF-1 a), Polyubiquitin (UBQ), a-Tubulin, b-Tubulin, 18S rRNA 25S rRNA, Ubiquitin-conjugating enzyme E2 (UBC), Eukaryotic initiation factor 1 (EIF1)* and *Eukaryotic transcription factors.*

2.3.1.2 *Actin*

Actin is a major component of the plant cytoskeleton. Also the most widely used reference gene for normalization of data in RT-qPCR (Pohjanvirta *et al.*, 2006). Maroufi *et al.* (2010) showed that *ACT* and *EF* were the most stably expressed reference genes for accurate normalization of gene expression in chicory leaf and root tissue. They have analysed the expression stability of genes across root and leaf tissue by using geNorm, Normfinder, and Bestkeeper software. They also found that *GAPDH* showed relatively less stability. Wang *et al.* (2012) concluded that *Actin 1, EF 1-* α and *UEP* (Ubiquitin extension protein) genes were the suitable reference genes for reliable RT-qPCR data normalization for genetic studies related to abiotic stress and different hormonal treatment in pepper.

Tian *et al.* (2015) validated nine candidate reference genes in carrot during abiotic stresses (drought, heat, salt, and cold) and under hormonal treatment. The gene expression stability of these genes were analysed using three software *viz.*, BestKeeper, geNorm, and NormFinder. *Actin* and *Tubulin* were the most stable genes identified among the different samples studied.

Gines *et al.* (2018) predicted the expression stability of most commonly used reference genes in different cultivars of barley ('Golden Promise' and 'Harrington') across different tissues. Traditional reference gene *Actin* was found to be the most stable gene for gene expression analysis using RT-qPCR.

Jaiswal *et al.* (2019) evaluated the expression stability of ten candidate reference genes in various tissues of *Cyamopsis tetragonoloba* for RT-qPCR gene expression analysis during seed development and under abiotic stress conditions. *Actin* was found to be the most stable gene in different tissues and also under heat and drought stress.

2.3.1.3 *Elongation Factor*

The elongation factor is a protein that plays a central role in the elongation phase of protein synthesis. Nicot *et al.* (2005) analysed seven housekeeping genes to determine their expression stability in potatoes during biotic and abiotic stress conditions and to select the most stable reference gene for RT-qPCR normalization. They found that *EF 1- α* was the most stable among the seven selected housekeeping genes.

Jain *et al.* (2006) conducted experiments to identify stable housekeeping genes for gene expression analyses in rice. They analysed ten frequently used housekeeping genes under various development stages and different environmental conditions. They found that *Elongation factor* and *Ubiquitin* were the most stably expressed genes under different experimental conditions.

Dombrowski *et al.* (2009) found that *EF 1-* α and *UBQ5* were the most stably expressed genes among the commonly used housekeeping genes in *Lolium temulentum* under abiotic stress. Lovdal and Lillo (2009) reported that *EF 1- α* was the most stably expressed gene among eight candidate reference genes selected for analysis in tomato during nitrogen and cold stress.

Wan *et al.* (2010) reported that *EF 1-* α was the most stably expressed gene among ten reference genes selected for gene expression analysis in cucumber. The experiments were conducted at different conditions including biotic stress treatment with *Pseudoperenospora cubensis*, salt and drought stress conditions and hormonal treatment with salicylic acid and methyl jasmonic acid. The expression stability of selected genes was analysed using geNorm, Normfinder and Best keeper.

Shivhare and Lata (2016) examined ten candidate reference genes to identify the most suitable reference gene for gene expression analysis using RT-qPCR in pearl millet during different abiotic stress conditions. The result revealed that *EF 1-* α was the most stable gene. *EF1- α* and *GAPDH* were found to be the

most suitable reference genes for normalization of miRNA expression in cucumber under viral stress (Liang *et al.*, 2018).

2.3.1.4 *GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)*

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is a central enzyme in the glycolysis pathway. It is one of the most commonly used reference genes as an internal control in quantitative analysis of RT-qPCR because its expression is constant in some experimental conditions (Winer *et al.*, 1999). Lovdal and Lillo (2009) examined eight housekeeping genes for their potential as a reference gene in normalization in tomato during light stress and found that *GAPDH* was the most stable one.

Barsalobres-Cavallari *et al.* (2009) validated most commonly used five reference genes in *Coffea arabica* during different developmental stages of flower and found that *GAPDH* was the most stably expressed gene under different experimental conditions. Mascia *et al.* (2010) examined the expression stability of eight reference genes in root and leaf *tissue* of tomato plant under biotic stress. Tomato plants were infected with virus and the gene expression was analysed. They found that *GAPDH* and *Ubiquitin* were the most suitable reference genes for the normalization of data in both tissues. *Actin* was also stably expressed in infected tissues, but *Elongation factor* showed a variable expression.

Petriccione *et al.* (2015) reported that *Glyceraldehyde-3-phosphate dehydrogenase* was the most stable gene among the nine reference genes selected for the normalization of RT-qPCR gene expression data in *Actimidia deliciosa* leaves infected with *Pseudomonas syringae pv.* They calculated the expression stability of genes using algorithms like geNorm, Normfinder, Bestkeeper and deltaCt. They also found that *β-tubulin* and *7s-globulin* were the least stable genes.

de Andrade *et al.* (2017) conducted experiments to identify the most stable reference gene for gene expression analysis in sugarcane under water deficit

conditions. Normfinder software was used for the analysis and revealed that *GAPDH* and *Ubiquitin* were the most suitable reference genes for gene expression analysis in sugarcane under water deficit conditions.

2.3.1.5 *Initiation Factor*

Initiation factor is a protein that plays an important role in initiating the translation of the mRNA molecule into peptide. Li *et al.* (2010) studied the expression stability of nine housekeeping genes in rice seeds at different growth stages and concluded that *IF-4a* (Eukaryotic initiation factor-4a) was the most stable gene among the genes studied.

Wang *et al.* (2016) evaluated eight candidate reference genes for gene expression analysis by RT-qPCR in stem segments of *Populus tomentosa.* Analyses were done using geNorm, NormFinder, and BestKeeper. They found that *Initiation factor* was the most suitable reference gene for gene expression studies in *Populus tomentosa.*

2.3.1.6 *Tubulin*

ß-Tubulin is the major structural component and is involved in many basic cellular functions. Brunner *et al.* (2004) studied the expression stability of ten candidate reference genes in poplar (*Populus sp*). Different tissue samples were selected for the study and they reported that *Tubulin* was one of the most stably expressed gene among the ten reference genes tested. Paolacci *et al.* (2009) conducted experiments for the identification of the stable genes for normalization of data in RT-qPCR in wheat. They selected twenty novel candidate genes and twelve traditional housekeeping genes and their expression stability were analysed using geNorm and Normfinder. They found that *Tubulin* and *Actin* showed an unstable expression.

Tubulin was found to be the most suitable reference gene for gene expression analyses using RT-qPCR under abiotic stress and for hormone stimuli

treatments in *Petroselinum crispum* (Parsley). Expression stability of eight candidate reference genes were analysed using geNorm, NormFinder, BestKeeper, and RefFinder (Li *et al.*, 2016)

Knopkiewicz and Wojtaszek (2019) tested five reference genes for their expression stability in different cultivars of *Pisum sativum* under lodging condition. Results revealed that *Tubulin* was the most stably expressed gene during different development stages under lodging condition.

2.3.1.7 *Ubiquitin*

Ubiquitin is a small regulatory protein and is best known for proteasomal degradation, DNA repair, signal transduction and transcription regulation by endocytosis (Christensen *et al.*, 1992). Zhaoguo *et al.* (2009) compared the expression stability of eleven reference genes during fruit development and ripening stage in peach. *Ubiquitin* along with *Elongation factor* and *RNA polymerase II* were found to be the most suitable reference genes for normalization of data.

Artico *et al.* (2010) tested the expression stability of nine reference genes in cotton during flower and fruit development stages and concluded that *Ubiquitin* was the most stably expressed gene during flower development.

Lei *et al.* (2011) reported that *Ubiquitin* and *Ribosomal proteins* were the most stably expressed genes among twenty reference genes selected for analysis in banana. The experimental conditions include various growth stage, abiotic and biotic stress conditions, and hormonal treatments. They also found that *Actin* and *GAPDH* were not suitable as reference genes in banana.

2.3.2 Primer designing

The success of polymerase chain reaction depends largely on primer designing. A primer is a short nucleic acid sequence which aids in the initiation of DNA synthesis. In a polymerase chain reaction, forward and reverse primer helps

the amplification of a specific region of DNA by annealing to the complementary sequence (Beij *et al.*, 1991).

Several parameters must be considered while designing primers. It includes primer length, melting temperature, annealing temperature and GC content (Rychlik *et al.*, 1990; Wu *et al.*, 1991). These parameters determine the specificity and efficiency of amplification (Wu *et al.*, 1991). The specificity implies that only the target sequence is amplified and the efficiency implies that more products are amplified in less number of cycles. The specificity depends on the length of the primer and annealing temperature (Garg *et al.*, 2008). Primer length between 18-24 base pair is ideal and the short length primer can result in non-specific amplification (Lowe *et al.*, 1990). The optimum melting temperature for the polymerase chain reaction is between 60-64°C and always keeps the annealing temperature 5°C below the melting temperature (Rychlik *et al.*, 1990). The 3' end of the primer must be designed carefully, otherwise it may cause mispriming. It should contain G or C nucleotide for stronger binding. Dinucleotide repeat and continuous execution of the same bases needs to be avoided as it may result in non-specific binding (Dieffenbach *et al.*, 1993). The optimum G-C content of the primer is between 40-60% (Lowe *et al.*, 1990). The length of the amplicon also has an impact on the efficiency of amplification (Rychlik *et al.*, 1990). Secondary structures, such as hairpin loop and primer dimer can also result in no amplification or less efficiency (Sahdev *et al.*, 2007).

2.3.3 DNA Isolation

DNA isolation is a crucial step because the quality of the DNA can affect subsequent analytical techniques (Boiteux *et al.*, 1999; Fredricks *et al.*, 2005).

The method of DNA isolation can influence the quality of DNA and contaminants in the DNA can affect the activity of different enzymes such as polymerase, ligase and nucleases which directly or indirectly affect the PCR, restriction digestion, RAPD etc. (Katterman and Shattuck, 1983; Richards, 1988;

Demeke and Jenkins, 2010). A high level of polysaccharides and polyphenol in leaf tissue can interfere with the DNA isolation process (Sharma *et al*, 2000).

Several protocols have been developed for the isolation of DNA from tissues with a high level of polysaccharides and phenolic compounds. Most of these protocols include the modification of the CTAB method of DNA isolation. A higher concentration of NaCl (5 M) can increase the quality and yield of DNA and also prevent the sample from becoming viscous during grinding (Doyle and Doyle 1987; Sathelly *et al*., 2014). Use of antioxidants can control the problems related to phenolic compounds. The use of 1% PVP during DNA isolation can yield good quality DNA (Murray and Thompson, 1980; Lade *et al*., 2014).

Isolation of the DNA from the black pepper plant is a tedious process due to the presence of a high level of polysaccharides and secondary metabolites (Dhanya *et al*., 2007). Subba *et al.* (2014) modified the CTAB method of DNA isolation to isolate DNA from leaves and black pepper spike samples by increasing the concentration of CTAB, NaCl, EDTA and PVP and high-quality DNA was obtained.

2.3.4 RNA Isolation

The isolation of RNA requires special care and precautions due to the ubiquitous presence of RNAse in the environment, which makes the RNA more unstable. Strong denaturants and highly sterile conditions are required for the isolation process (Doyle, 1996).

The single-step method for RNA isolation uses 4M Guanidium thiocyanate, which is a chaotropic agent that allows the purification of RNA from cells with a high content of endogenous ribonucleases (Cox, 1968; Chomczynski and Sacchi, 1987). The Trizol reagent is a ready-to-use reagent for RNA extraction and is an improved version of the single-step method of RNA isolation. This method makes the RNA isolation process easier and fast (Simms *et al*., 1993).

The presence of secondary metabolites in certain plants may interfere with the RNA isolation process by co-precipitation or RNA binding and may result in poor yield of RNA (Gasic *et al.*, 2004). Siju *et al.* (2007) used a modified method for RNA extraction from black pepper berries for *Cucumber mosaic virus* detection and obtained good quality RNA.

2.3.5 Quantification of nucleic acid

Quantification of nucleic acid can be done using spectrophotometer (Nieman and Poulsen, 1963). It is based on the principle that DNA and RNA absorb UV light at a particular wavelength. Estimation of the concentration of nucleic acid is based on the absorbance value. The ratio of absorbance at 260 nm and 280 nm is used to determine the purity of nucleic acid. Good quality DNA has a ratio of 1.8 and for RNA ratio of 2 (Maniatis *et al.*, 1982).

2.3.6 Polymerase chain reaction

The polymerase chain reaction is a technique invented by Karry Mullis in 1983 and this opened a new era in molecular biology. PCR specifically amplifies a particular nucleotide sequence. PCR involves three steps *viz.,* heat denaturation of DNA, annealing of primer to the template and extension by DNA polymerase. The forward and reverse primers hybridize with the complementary sequence in the target and initiate DNA synthesis with the help of the DNA polymerase and the process is repeated in each cycle that duplicates the amount of DNA (Saiki *et al.*, 1988). Discovery of heat-stable *Taq* DNA polymerase from *Thermus aquaticus* bacteria made PCR more efficient (Karcher, 1995).

Basic components of the PCR reaction mix include DNA template, standard buffer, forward and reverse primer, DNA polymerase and nucleotides (Delong and Zhou, 2015).

2.4 SOFTWARES USED FOR ANALYSIS OF RT-qPCR DATA

The identification of most stable genes can be done using different software. These softwares analyse the stability of gene expression in terms of variation in the level of expression in the samples (Galiveti *et al.*, 2010).

2.4.1 geNorm Software

Vandesompele *et al.* (2002) created software to test the stability of gene expression known as geNorm, which is based on the assumption that the minimally regulated genes remain in a constant ratio with each other. The transcription ratio of two suitable reference genes must be identical in all samples, regardless of the experimental conditions. Ratio variation is inversely proportional to transcription stability. The geNorm software program calculates gene-stability measure M, which is the average pairwise variation of a particular gene with all the other genes. Gene 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.

geNorm software also calculates the geNorm V value, which determines the most stable combination of the reference genes. They calculate pairwise variation using n/n+1 reference genes to estimate the optimal number of reference genes to be used for accurate normalization. n number of reference genes can be considered sufficient for normalization based on the 0.15 cut-off for the pairwise variation (De Spiegelaere *et al.*, 2015). If the geNorm V value is less than 0.15, the addition of one more reference gene provides no improvement in the fidelity (Liang *et al.*, 2018).

2.4.2 Bestkeeper Software

Bestkeeper, is another Excel-based tool released in 2004 to find the most stable reference gene for normalization of data in RT-qPCR (Pfaffl *et al.*, 2004). Bestkeeper software uses pairwise correlation analysis. This software can

compare the level of expression of ten housekeeping genes together with ten target genes. Initially, raw cycle threshold values are computed along with standard deviation and correlation coefficient for all reference genes. Then, the Pearson correlation coefficient is calculated for each pair of reference genes. Genes that have low standard deviation (SD<1) are considered as a highly correlated reference gene and are then combined into an index value called the Bestkeeper index. The most stable reference genes which can be used for normalization are those candidate genes that are best correlated to the Bestkeeper index (Khanlou and Backestele, 2012).

2.4.3 Normfinder Software

NormFinder software was released in 2004 and is used to validate the reference genes for normalization of data in RT-qPCR (Andersen *et al.*, 2004). This software ranks candidate reference genes based on their expression stability in a given set of samples and given experimental conditions. Most stably expressed genes are indicated by low average expression stability value. Intergroup and intragroup analysis can also be performed.

2.5 STUDIES IN BLACK PEPPER USING RT-qPCR

Alex *et al.* (2008) studied the differential expression of metallothionein type-2 homologues in leaves and roots of black pepper (*Piper nigrum* L) where they used *Actin* as the reference gene for RT-qPCR analysis. Hemanth (2012) analysed floral meristem identity genes in black pepper, where *ACTIN* was used as endogenous control for RT-qPCR analysis.

Hu *et al.* (2015) conducted experiments to describe the fruit tranciptome of black pepper. They found *histone H3* gene as the most suitable reference gene for RT-qPCR analysis in black pepper fruits. Bhasi (2019) conducted experiments to characterize floral architectural genes in black peper, where *Actin* was used as reference gene for normalization gene expression analysis using RT-qPCR.

Normalization of data using the reference gene is necessary for accurate and reliable gene expression analysis using RT-qPCR. Some reference genes are highly specific for particular crop and experimental condition. Hence, validation of reference genes is a crucial requirement before carrying out RT-qPCR experiments (Dheda *et al.*, 2004).

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Selection of stable housekeeping genes for gene expression studies during inflorescence development in black pepper (*Piper nigrum* L.) using real time PCR" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2017-2019. Details of the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 COLLECTION OF SAMPLES

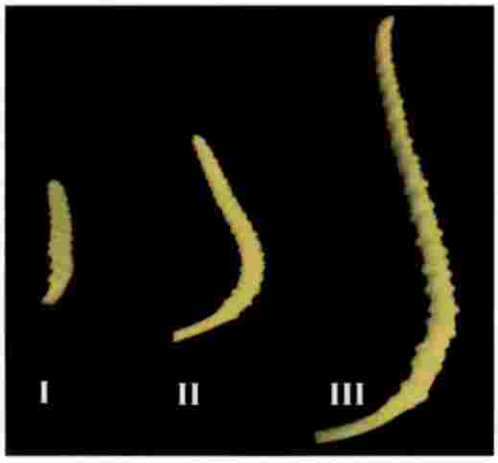
Spike samples of non-branching variety Karimunda and Panniyur 1 were collected from College of Agriculture, Vellayani. Spike samples of branching type "Thekken" were collected from farmer Mr. T. T. Thomas from Idukki district, Kerala. Samples were collected from two different plants of each cultivar. Three different development stages of spikes were used for the study (Plate 1). Criteria for the selection of different developmental stages (Stage I, Stage II and Stage III) are given in Table 1.

Table 1. Criteria for selection of developmental stages of spikes

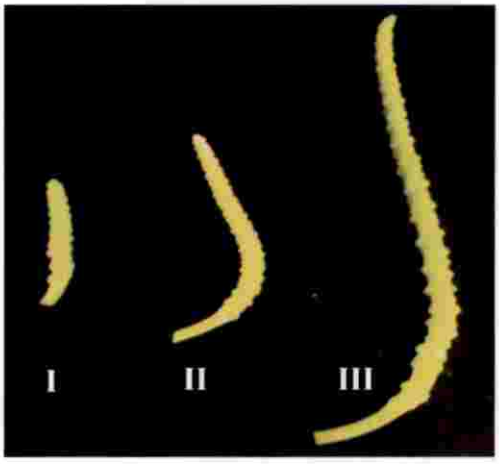
Stage	Length of spikes (cm)	Days after emergence of bud Stage
I	1 to 2	12 to 15
II	6 to 8	22 to 25
III	9 to 12	32 to 35

3.2 PRIMER DESIGNING

Primers were designed for housekeeping genes viz. *Actin, β-Tubulin, Elongation factor, Initiation factor, Ubiquitin* and *GAPDH* (*Glyceraldehyde phosphate dehydrogenase*) using Primer Express Software. Nucleotide sequence was unavailable in NCBI database. Transcriptome sequence was used for primer



a.Panniyur



b.Karimunda

c.Thekken

Stage	Length(cm)
I	1-2
II	6-8
III	9-12

Plate1. Spikes samples at different developmental stages of black pepper varieties/cultivars

designing. Transcriptome sequences are given in Fig 1 and Fig 2. Details of related sequence which were used for the designing of the primers are given in Table 2. Primers were designed according to the conserved regions. Factors such as primer length, annealing temperature, GC Content, potential hair pin formation and 3' complementarity were analysed by "Primer Express software" and absence of secondary structure formation at primer binding site was determined by "mfold web server". The Primer were synthesized at Xetra Bio solutions, Poojapura, Thiruvananthapuram.

Table 2. Sequence ID of related sequences 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

3.3 ISOLATION OF GENOMIC DNA

Genomic DNA from spike samples of Thekken, Panniyur-1 and Karimunda was isolated by modified CTAB method developed by Subba (2014).

Collected spike samples of different developmental stages were crushed in mortar and pestle using liquid Nitrogen. CTAB extraction buffer (Appendix-1) containing 0.2% β-mercaptoethanol and 2% PVP (Poly Vinyl Pyrrolidone) was added to it and homogenized with pestle and transferred to a sterile 2 ml microfuge tubes. The samples were incubated for 30 minutes at 60° C in a recirculating water bath. After incubation, CTAB/plant extract mixture was spun

>2437925 ACTIN

GCAATTCCAGGGAACATAGTAGAGCCACCACTGAGTACAATGTTACCATACAAGTCCTTCCT
GATATCAACATCACACTTCATAATTGAATTGTATGTTGTCTCGTGAATACCAGCAGCTTCCATCCCA
ATAAGGGAAGGCTGGAAAAGGACCTCAGGGCAGCGGAACCTCTCCGCTCCAATGGTGATCACTT
GGCCATCGGGCAGCTCATAGGTCTTCTCAATTGAAGAACTGCTCTTTGCAGTCTCCAGCTCTTGCT
CATAATCAAGAGCCACAAATGCGAGCTTCTCCTTCACGTCACGAACAATTTCCCGTTCGGCAGTT
GTTGTGAATGAATATCCCCTCTCAGTCAGAATTTTCATCAATGCATCTGTTAGATCACGCCCAGCA
AGGTCCAGACGTAAAATTGCATGGGGAAGGGCATACCCTTCATAGATAGGCACCGTGTGGCTCAC
TCCATCACCAGAATCCAATACAATACCAGTCGTCCGTCCACTGGCATAGAGAGAAAGAACTGCCT
GAATAGCAACATACATGGCTGGGCTATTGAATGTCTCAAACATGATCTGAGTCATCTTCTCCCTGTT
AGCCTTAGGGTTGAGAGGAGCTTCGGTCAGAAGAACAGGGTGTTCTTCAGGGGCAACACGAAG
CTCGTTGTAGAAGGTGTGATGCCAGATCTTCTCCATGTCATCCCAATTGCTCACAAT

>2435271 Elongation Factor

AGATGATTTGTTGTTGCAACAAGATGGATGCCACGACTCCCAAATACTCGAAGGCTCGTTAC
GATGAAATTGTCAAGGAAGTGTCTTCCTACCTGAAGAAGGTCGGGTACAACCCTGAGAAAATTG
CCTTTGTCCCCATTTCTGGGTTTGAAGGTGACAACATGATCGAGAGGTCGACCAACCTTGACTGG
TACAAGGGCCCAACTCTACTTGAGGCCCTTGACCAAATCCAGGAGCCCAAGAGACCATCCGACA
AGCCTCTTCGTCTCCCTCTTCAGGATGTGTACAAGATTGGTGGTATTGGAACTGTTCCTGTTGGTC
GGGTTGAGACTGGTATCATCAAGCCAGGTATGGTTGTTACGTTTGGTCCCACCGGGTTGACCACT
GAAGTCAAGTCTGTTGAGATGCACCATGAAGCACTACAGGAGGCTCTTCCTGGTGACAATGTCGG
ATTCAATGTGAAGAATGTTGCTGTCAAGGATCTCAAGCGAGGTTTTGTTGCCTCCAACTCCAAGG
ATGACCCCGCCAAGGAGGCAGCGAACTTCACTTCTCAGGTGATCATCATGAATCACCCTGGCCAG
ATCGGCAACGGATATGCACCGGTGCTTGATTGCCACACCTCTCACATTGCTGTCAAGTTCGCGGA
GATTCTCACCAAGGTTGACAGGCGATCTGGTAAGGAG

>2447851 GAPDH

GGAGGTGCTAAGAAAGTTGTTATCTCGGCCCCAAGCAAGGATGCACCAATGTTTGTTATGGG
TGTTAACGAGAAGGAATATAAGCCGGAGATTAACATTGTCTCAAATGCTAGCTGCACCACTAACT
GTCTTGCCCCGTTAGCAAAGGTCATAAATGATAGATTTGGAATTGTTGAGGGTTTGATGACCACTG
TCCACGCTATCACTGCTACCCAGAAAACCGTTGATGGTCCTTCAATGAAAGATTGGAGAGGCGGA
AGGGCTGCTAGCTTCAACATCATTCCTAGCAGCACGGGAGCTGCCAAGGCTGTTGGTAAGGTGCT
TCCGTCTTTGAATGGAAAGCTTACTGGAATGTCATTCCGTGTTCCTACCGTCGATGTTTCTGTGGT
TGACCTTACTGTGAGGCTTGAAAAAGCCGCAACATACGAGCAGATTAAGGAGGCCATCAAGGAG
GAGGCTGCGGGTAAATTGAAGGGCATTCTTGGATATACCGAAGATGATGTTGTTTCCTCTGACTTC
GTCGGTGACAATAGGTCTAGCATCTTCGATGCCAAGGCTGGAATAGCCCTCAACGAGAAGTTTGT
GAAACTTGTGGCGTGGTATGACAACGAGTTGGGCTACAGTACCCGGGTGGTCGACTTGATCCGTC
ACATGGCATCGACT

Fig. 1 Transcriptome sequence of *Actin, Elongation Factor* and *GAPDH* used for primer designing

>2451729 INITIATION FACTOR

TGATCCATGTCACCATGTGTAGCTGAGACAGTATGATCTCGACTTCTCATTTTATCGGTCAACC
AGTCAACCTTTCGCCGTGTGTTCACAAAGATGACACTTTGTGTGATGGCCAAAGTCTCATAAAGA
TCACACAAGGTATCAAGCTTCCACTCTTCCCTCTCAACGTTGACATAGAACTGTTTGATACCCTCA
AGAGTCAGTTCATCTCTCTTAACAAGAATCCGCACAGGCTTATTCATGAATTTTCGCGTGATTTCA
AGTGCTTCTGGAGGCATTGTTGCAGAGAAGACTCCCACTTGAATCTTGGGTGGGAGGAGCTGGA
ATATGTCATATATCTGATCTTTAAATCCTCTGGAAAGCATTTCGTCTGCCTCATCTAAAACAAACAT
CTTAATGTGATCAGGGCGAAGTGACTGTCTTCTCAGCATGTCAAATACTCGACCTGGGGTGCCAA
CAACCACATGAACCCCACTAGAAAGGATTCTTTGGTCCTCCCGGACACTTGTTCCACCAACACAG
GCATGAACTTTAACACCCATGTAATCACCAAGAGCTCGCATAACCTTCTCAATTTGTTGAGCCAGT
TCTCTAGTAGGGGCTAACACAAGCGCTTGGCAGTCAGTTAAATTATAGTCCAACTGCTGTAGAAC
CCCTGAGCAGAAGGTAGCAGTTTTCCCTGTACCAGATTGTGCTTGCTGAATTACGTCAAGGCCTT
TGCAGAAAGGCACAATTCCCCTCTGCTGGATTGCAGAAGGCTTTTCAAATCCATAAGCATAGATG
CCTCTCAAGAGGTTCTCATTCAAGCCCATTGAATCAAAGGTATCAAAAACCTCATTGTATTCCTTA
AAGAACCCATCTCCATCAGGTGAAAGC

>2350989 TUBULIN

GAAGTCAAGGGAGCAAAGCCAACCATGAAAAAATGCAGTCTGGGGAAGGGGATGAGATTA
ACAGCTAGCTTCCGCAGATCGGAATTAAGCTGTCCTGGAAATCGAAGACAGCAAGTCACCCCACT
CATTGTGGCAGATATAAGGTGGTTAAGATCCCCAAATGTAGGAGTTGCAAGCTTGAGTGTTCGGA
AGCAAATGTCATAAAGAGCCTCATTGTCGAGAACCATACACTCATCCGCATTTTCAACAAGCTGG
TGAACAGACAAAGTGGCATTGTAGGGCTCCACAACAGTATCAGAAACCTTGGGCGAGGGGAAAA
CAGAGAAGGTAAGCATCATGCGGTCTGGATATTCCTCTCTGATCTTCGAAATAAGAAGCGT

>2347739 UBIQUITIN

CAAGACAAGGAGGGTATCCCTCCTGACCAGCAGAGGCTTATCTTTGCTGGTAAGCAGCTGGA
GGACGGGAGGACCCTCGCTGACTATAACATTCAGAAAGAGTCCACCCTTCACCTTGTCCTGAGAC
TTAGAGGCGGGATGCAGATTTTTGTGAAGACCCTCACCGGGAAGACCATTACCTTGGAAGTGGA
GAGCTCCGATACCATTGATAACGTGAAAGCCAAAATCCAGGATAAAGAAGGAATTCCCCCAGACC
AGCAGAGGCTTATTTTTGCCGGTAAGCAGCTGGAAGATGGAAGGACCCTTGCGGATTATAATATCC
AAAAGGAGTCTACCCTCCACTTGGTGCTGCGTCTGCGTGGTGGCATGCAGAT

Fig. 2 Transcriptome sequence of *Initiation Factor*, *Tubulin* and *Ubiquitin* used for primer designing

at 10000 rpm for 10 min to remove the cell debris. The supernatant was transferred to clean microfuge tubes.

In order to remove protein contamination, 500µl of Chloroform: Iso Amyl Alcohol (24:1) was added to each tube and mixed gently by inversion. After mixing, the tubes were spun at 10000 rpm for 10 min. Upper aqueous phase containing DNA was transferred to a clean microfuge tubes.

Precipitation of DNA was done by adding equal volume of ice cold absolute ethanol to each tube and mixed gently by inverting the tube and incubated at -20°C for 30 minutes to precipitate the DNA. The tubes were centrifuged at 10000 rpm for 7 minute to form a pellet. The supernatant was removed and the DNA pellet was washed by adding ice cold 70 % ethanol. After washing, DNA pellet was formed by centrifuging at 10000 rpm for 5 min. All the supernatant were removed and allowed the DNA pellet to dry for15 min. Then the DNA was re suspended in 50 µl sterile DNase free water and stored at -20°C.

3.3.1 Quantity and quality of isolated DNA

Quantification and quality check of extracted DNA was done by UV spectrophotometer. Nuclease free water was used as blank for calibration of UV spectrophotometer at 260 nm and 280 nm. Absorbance value at both wavelengths was measured by adding 3µl of DNA to 5 ml of nuclease free water in quartz cuvette.

An absorbance value of 1.0 at 260 nm is equivalent to 50 ng μl^{-1} of DNA.

The concentration of DNA was calculated by the formula:

Concentration of DNA (ng μl^{-1}) = A_{260} x 50 x dilution factor

DNA purity was determined by A_{260}/A_{280} value. The ratio provides indication of protein contamination. Good DNA quality will have A_{260}/A_{280} values in the range of 1.8 to 2.0.

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3.3.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was done in a horizontal gel electrophoresis unit. 0.8% agarose gel was prepared by adding required amount of agarose in 1X TBE (Tris Borate EDTA) buffer (Appendix II) and was boiled in a microwave oven for uniform dissolution. After cooling ethidium bromide was added at a final concentration of 0.5µg/ml. After mixing, the solution was poured into gel tray with comb placed in a gel casting apparatus. After solidification, gel was placed in the buffer tank filled with 1X TBE running buffer. The comb was removed carefully to from gel. DNA (5µl) was mixed with 6X gel loading dye (2µl) and nuclease free water (5µl) and loaded 12 µl into wells using a micropipette and 1 kb DNA marker was added to know the size of genomic DNA band. Electrophoresis was carried out at 70 volts till the dye front reached 3/4th of the gel. Gel was taken and viewed in gel documentation system using the software Image Lab.

3.4 QUALITY CHECK OF PRIMERS

3.4.1 Specificity check of primers with genomic DNA

Specificity of primers was checked by polymerase chain reaction using genomic DNA. Genomic DNA isolated from the spikes of Thekken, Panniyur-1 and Karimunda were amplified using gene specific primers *viz., Actin, ß-Tubulin, Elongation factor, Initiation factor, Ubiquitin* and *GAPDH* (*Glyceraldehyde phosphate dehydrogenase*). PCR mix was prepared for 20µl reaction. Components of PCR master mix and thermal profile for amplification are given in Table 3 and Table 4 respectively.

Agarose gel electrophoresis (1.2%) (Appendix III) were used to check PCR products. Gel was examined by gel documentation system.

3.4.2 Amplification efficiency of primers

Amplification efficiency of primers was determined by standard curve analysis and "Lin Reg" software. Lin Reg software analyses were done using data from the exponential phase of amplification by RT-qPCR.

Table No. 3 Components, Volume and Concentration of PCR reaction mix

Sl. No.	Components	Volume for one reaction	Final concentration
1	10 X PCR buffer	2.0 µl	1X
2	dNTPs	0.4 µl	3µM
3	Primer (forward)	0.6 µl	0.3µM
4	Primer (reverse)	0.6 µl	0.3µM
5	Taq DNA polymerase	0.3 µl	1 Unit
6	Genomic DNA	4.0 µl	50 ng
7	Sterile double distilled water	12.1 µl	
	Total	20 µl	

Table No.4 Thermal profile used for PCR

Step	Stage	Temperature (^{0}C)	Duration
1	Initial denaturation	95^{0}C	2min
2	Denaturation	95^{0}C	15sec
3	Annealing	55^{0}C	15sec
4	Extension	60^{0}C	45sec
5	Final extension	60^{0}C	5min
No of cycles			40

3.5 ISOLATION OF RNA

Total RNA was isolated from spikes of different development stages using Trizol reagent. All materials used for isolation of RNA were autoclaved twice for sterilization. DEPC treated water was used for reagent preparation. During isolation process RNase away and RNase Zap were used to remove RNA contamination.

Spike samples of different development stages were ground into fine powder in chilled mortar and pestle using liquid nitrogen. 1 mL of Trizol reagent was added to the powdered tissue and gently mixed to homogenize the mixture and incubated at room temperature for 5 minutes. The homogenate was transferred to a 2 ml pre chilled microfuge tube. To remove protein contamination, 0.2 ml chloroform was added and mixed vigorously for 15 seconds and incubated at room temperature for 5 minutes. The sample was kept in ice for 10 minutes and then centrifuged at 12000g for 15 minutes at 4°C. Aqueous phase of the sample was transferred to a fresh tube. 0.5 ml ice cold isopropanol (100%) was added to each tube for RNA precipitation and incubated at room temperature for 10 minutes and mixed by inverting the tube slowly. The sample was centrifuged at 12000g for 10 minutes at 4°C. The supernatant was removed and pellet was washed with 1 ml of 75% alcohol (in DEPC treated water) .The sample was briefly vortexes and spun at 7500g for 5 minutes at 4oC and dried for 30-40 minutes in RNase free laminar air flow chamber. The RNA pellet was re suspended in 30µl RNase free water and stored at -20^{0}C.

3.5.1 Quality and quantity of isolated RNA

Quantification and quality check of extracted RNA was done by UV spectrophotometer. Nuclease free water was used as blank for calibration of UV spectrophotometer at 260 nm and 280 nm. Absorbance value at both wavelengths was measured by adding 3µl of RNA to 5 ml of nuclease free water in quartz cuvette.

An absorbance value of 1.0 at 260nm is equivalent to 40ng μl^{-1} of RNA. The concentration of RNA in the sample was determined by the formula:

Concentration of RNA (ng μl^{-1}) = A_{260} x 40 x dilution factor

RNA purity was determined by A_{260}/A_{280} value. Good quality RNA have A_{260}/A_{280} values of 2.0.

Reliability of the total RNA isolated was estimated by agarose gel electrophoresis. RNA (5µl) was added into 6X gel loading dye (2µl) and DEPC treated water (5µl) and 12 µl samples were loaded into wells on agarose gel (1.2 per cent).

3.6 cDNA SYNTHESIS

From the isolated RNA, cDNA was synthesised using Therm Scientific Verso cDNA synthesis kit. The composition of reaction mixture (20µl) for cDNA synthesis is given in Table 5.

Table 5. Composition of reaction mixture for cDNA synthesis

Components	Volume	Final concentration
5X cDNA buffer	4.0 µl	1X
dNTP mix	2.0 µl	500 µM each
Oligo Dt	0.5 µl	2 µM
Random Hexamer	0.5 µl	2 µM
RT Enhancer	1.0 µl	
Verso Enzyme Mix	1.0 µl	1 Unit
RNA	5.0 µl	1µg
Nuclease free water	6.0 µl	
Total volume	20 µl	

The contents were mixed well and incubated at 42^0C for 30 minutes for first strand synthesis. Then incubated at 92^0C for 2 min to inactivate RT enhancer and the cDNA samples were stored at -20^0C.

3.7 SPECIFICITY CHECK OF PRIMERS WITH cDNA

cDNA synthesised were amplified using gene specific primers for housekeeping genes *Actin, β-Tubulin, Elongation factor, Initiation factor, Ubiquitin* and *GAPDH* (*Glyceraldehyde phosphate dehydrogenase*). Standard PCR mix was prepared for 20µl total volume as per Table 3.

The amplified products were separated on agarose gel (1.2 percent) and viewed under gel documentation system.

3.8 REVERSE TRANSCRIPTION QUANTITATIVE PCR (RT-qPCR)

RT-qPCR was SYBR Green dye-based assay and the machine used was Biorad CFX 96. RT-qPCR for each gene was performed with three technical replicates for each sample. Amplification plot was generated by the software and Cq values were determined for all the samples. Melting curve analysis was also done to check the specificity of the reaction. Reaction mixture (20µl) for real-time PCR was given in Table 6.

Table 6. Reaction mix for Real-time PCR

Components	Volume
SYBR Green mix	10µl
Forward primer (10 pmol/µl)	0.6µl
Reverse primer (10 pmol/µl)	0.6µl
Template cDNA (4µg)	2.5 µl
Nuclease free water	6.3 µl
Total volume	20 µl

Thermal conditions for RT-qPCR were 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 45 sec.

3.9 ANALYSIS OF DATA GENERATED

Stability of the housekeeping genes in the samples were analysed using three softwares *viz*, "Bestkeeper", "geNorm" and "Normfinder". Raw Cq values were converted to relative expression values using q base plus software with amplification factor as 2.

3.9.1 Analysis using Best keeper software

Raw Cq values were given as input data. Standard deviation of Cq values of all the genes were calculated by software. Cq values having standard deviation more than one was excluded from the analysis.

3.9.2 Analysis using geNorm software

Relative expression values were given as input data. geNorm M values were generated for all the genes. Selected housekeeping genes were ranked according to their expression stability values. Best combination of genes was also given by the software.

3.9.3 Analysis using Normfinder software

Relative expression values were given as input data. Stability values were calculated for all the genes by software. Genes were ranked according to stability value.

Results

4. RESULT

The study entitled "Selection of stable housekeeping genes for gene expression studies during inflorescence development in black pepper (*Piper nigrum* L.) using real time PCR", was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2017-2019. The results related to the study are presented in this chapter.

4.1 PRIMER DESIGNING

Primers for selected housekeeping genes *viz., Actin, β-Tubulin, Elongation factor, Initiation factor, Ubiquitin* and *GAPDH (*Glyceraldehyde-3-phosphate dehydrogenase*)* were designed using primer express software using related sequence from NCBI database. Absence of secondary structure formation at primer binding site was confirmed by "mfold" web server. Details of designed primers are given below

4.1.1 Primer analysis

GC percentage and melting temperature determined by "Oligos" software showed fair values (Table 7). None of the designed primers exhibited any hairpin formation and 3' complementarity.

Table 7. Sequence of primers designed for housekeeping genes

Primer	5'.....3'sequence	Tm (°C)	GC content (%)	Expected size of amplicon (bp)
ACT F	GCCACACGGTGCCTATCTAT	59.8	43	190
ACT R	AAATGCGAGAGCTTCTCCTTCA	60.0	48	
eEF F	CTTCAGGATGTGTACAAGATTGGTG	58.6	44	171
eEF R	GTCACCAGGAAGAGCCTCCTG	59.8	62	
GAPDH	ATTGTTGAGGGTTTGATGACCACT	58.1	38	210

GAPDH	ATCGACGGTAGGAACACGGA	60.6	57	
eIF F eIF R	TCATGCCTGTGTTGGTGGAA TAATGTGATCAGGGCGAAGTGA	59.5 59.2	50 45	133
TUB F TUB R	GCTCTTTATGACATTTGCTTCCGA GGAGCAAAGCCAACCATGAA	60.5 59.6	42 50	200
UBQ F UBQ R	GTGGAGAGCTCCGATACCATTGAT ACGCAGACGCAGCACCAA	61.4 61.2	50 61	174

4.1.2 Specificity of primers

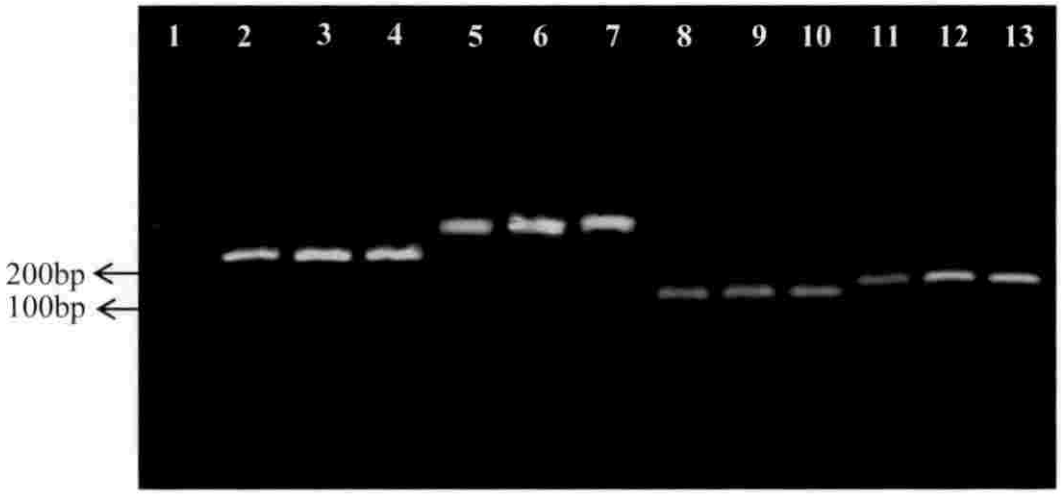
Specificty check of primers was done by agarose gel elctrophoresis using genomic DNA. Single amplicon of expected size was obtained for all the primers (Table 7). Gel picture of PCR product of *Tubulin*, *GAPDH*, *Initiation factor*, *Actin*, *Elongation factor* and *Ubiquitin* is presented in Plate 2(a) and 2(b).

4.1.3 Amplification efficiency of primers

Amplification efficiency was detrmined by standard curve analysis and Lin Reg software. Clustering of data was observed in standard curve analysis. Hence, amplification efficiency was determined using data from exponential phase of amplification by Lin Reg software and ampification value obtained are given in Table 8.

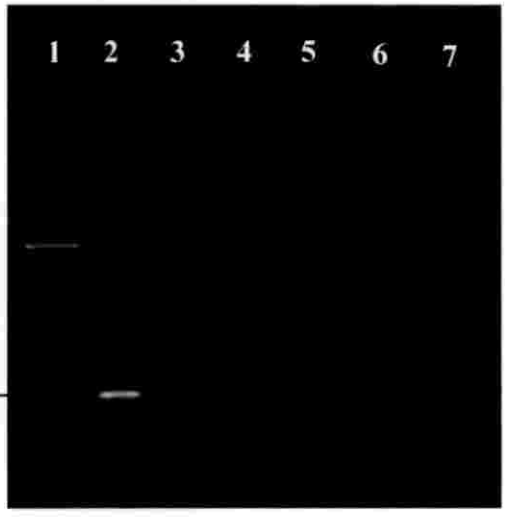
Table 8. Amplification factor of primers

Gene	Amplification factor
Actin	1.99
Elongation factor	2.02
Initiation factor	2.03
GAPDH	2.06
Tubulin	2.04
Ubiquitin	2.07



Lane1 : 100 bp DNA marker
Lane 2 to 4 : PCR product using *Tubulin* primer
Lane 5 to 7 : PCR product using *GAPDH* primer
Lane 8 to 10 : PCR product using *IF* primer
Lane 11 to 13: PCR product using *Actin* primer

Plate 2(a). Gel picture showing PCR products of genomic DNA using primers designed for housekeeping genes.



Lane 1 : 100 bp DNA Marker
Lane 2 to 4: PCR product of EF(174 bp)
Lane 5 to 7: PCR product of Ubiquitin(171 bp)

Plate 2(b). Gel picture showing PCR products of genomic DNA using primers designed for housekeeping genes.

4.2 MOLECULAR ANALYSIS AT GENOME LEVEL

4.2.1 Isolation of DNA and Agarose Gel Electrophoresis

Genomic DNA isolation was carried out from the spike samples collected at different developmental stages from the bush pepper plants of Karimunda, Panniyur 1 and Thekken. DNA was isolated by modified CTAB method. Isolated genomic DNA exhibited intact band in agarose gel (0.8%) electrophoresis (Plate 3).

4.2.2 Quantification of DNA using Spectrophotometer

Quality and quantity of isolated genomic DNA was determined by taking the absorbance reading using spectrophotometer. Value of A_{260}/A_{280} for isolated genomic DNA ranged from 1.70 to 1.88 and details are presented in Table 9.

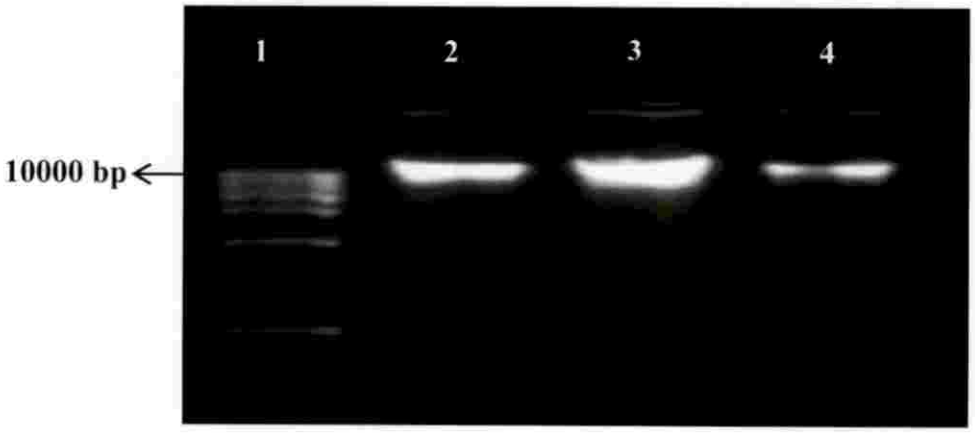
Table 9. Quantity and quality of isolated genomic DNA

Sample	Concentration (ng/µl)	A_{260}/A_{280}
Thekken	1662	1.70
Karimunda	2727	1.80
Panniyur1	1650	1.88

4.3 MOLECULAR ANALYSIS AT TRANSCRIPTOME LEVEL

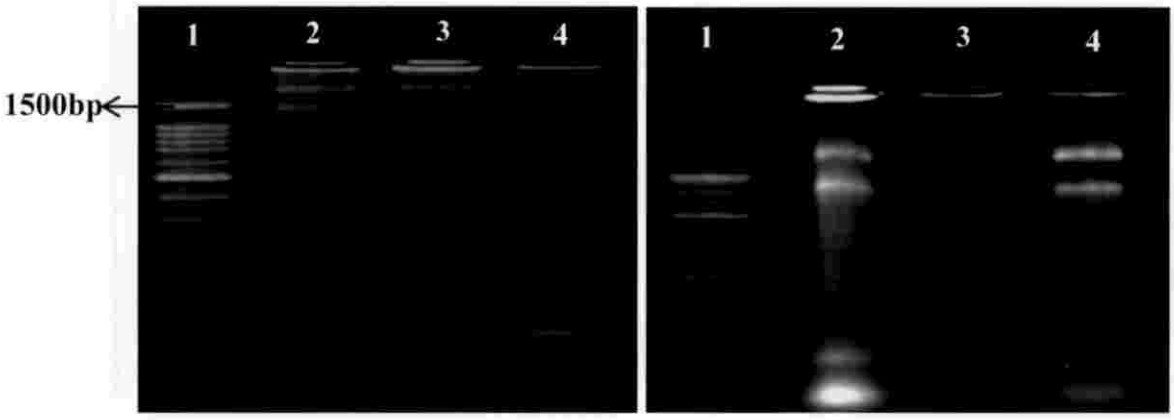
4.3.1 Isolation of RNA

RNA was isolated from collected spike samples by Trizol method. Agarose gel electrophoresis (2%) shows three bands in Karimunda and Thekken and two bands in Panniyur 1 (Plate 4).

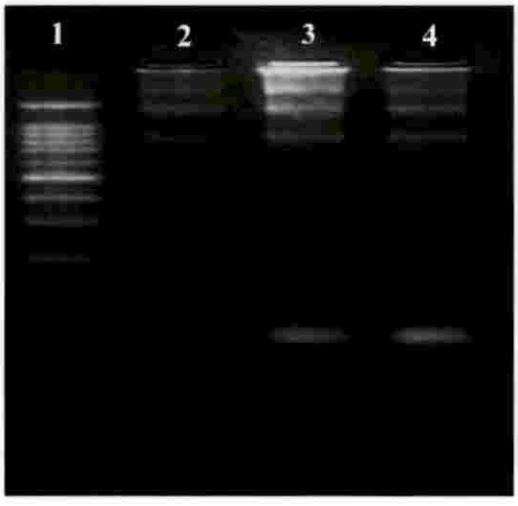


Lane 1: 1 kb ladder
Lane 2: Thekken
Lane 3: Karimunda
Lane 4: Panniyur 1

Plate 3. Gel picture showing genomic DNA isolated from black pepper cultivars/varieties



a. Panniyur 1 b. Karimunda



c. Thekken

Lane 1: 100bp DNA marker
Lane 2: First stage
Lane 3: Second stage
Lane 4: Third stage

Plate 4. Gel picture showing total RNA isolated from the inflorescence of black pepper varieties/cultivars

4.3.2 Quantification of RNA using Spectrophotometer

Quality and quantity of isolated RNA was determined by absorbance reading using spectrophotometer. Value of A_{260}/A_{280} ranged from 1.8 to 2 and details are presented as Table 10.

Table 10. Quantity and quality of total RNA isolated from spike samples

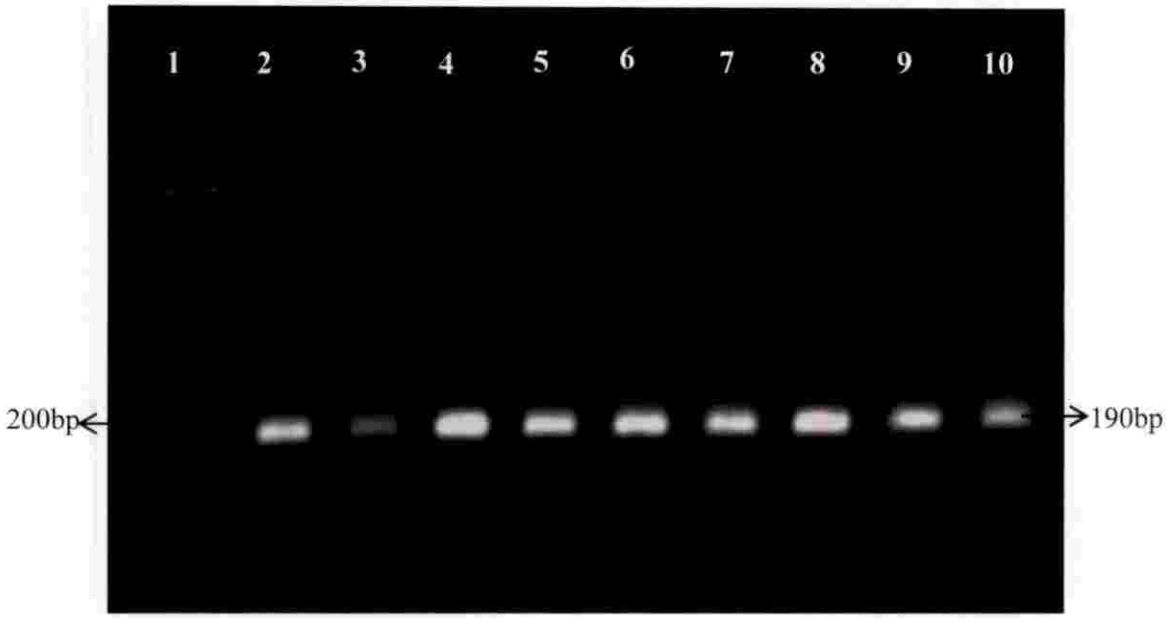
Sample	Karimunda			Panniyur 1			Thekken		
	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
Concentration (ng/µl)	2147	1475	1674	2570	1292	1720	1860	2560	1678
A_{260}/A_{280}	1.90	1.80	2.00	1.85	1.80	1.90	1.80	1.88	1.90

4.3.3 Quality check of cDNA

cDNA was synthesized by Thermo scientific Verso cDNA synthesis kit. Quality check of cDNA was done by PCR using *Actin* as the primer and agarose gel electrophoresis. Gel picture of PCR amplification product of cDNA using the primer *Actin* shows single amplicon of expected size (190 bp) in all the samples (Plate 5).

4.3.4 Reverse Transcription quantitative PCR (RT-qPCR)

cDNA from spike sample of three different developmental stages of Thekken, Karimunda and Panniyur 1 were used as template for RT- qPCR. Thermal conditions for RT-qPCR were 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 45 sec. Amplification and Melt curve plot were generated by the software. Amplification plot representing raw expression data generated for *Tubulin*, *GAPDH*, *Initiation factor*, *Actin*,



1 :100 bp ladder
2,3,4 :PCR product of actin using cDNA of Karimunda stage 1, 2 and 3 respectively
5,6,7 :PCR product of actin using cDNA of Panniyur1 stage 1, 2 and 3 respectively
8,9,10 ;PCR product of actin using cDNA of Thekken stage 1, 2 and 3 respectively

Plate 5. Gel profile of PCR product of cDNA amplified using *Actin* primer

Elongation factor and *Ubiquitin* are presented in Fig. 3 to 8. Cq values obtained using Biorad CFX Maestero software and are given in Table 11. Melt curve plot showed prominent peak for all the primers and are presented in Fig. 9 to 14.

Table 11. Cq values generated by RT-qPCR

Sample	*EF*	*ACTIN*	*GAPDH*	*IF*	*TUB*	*UBQ*
Karimunda stage 1	29.15	29.62	30.25	26.99	28.29	28.14
Karimunda stage 2	26.09	26.62	29.7	23.99	23.02	25.57
Karimunda stage 3	31.07	30.53	37.53	28.34	28.45	28.62
Panniyurl stage 1	34.32	35.03	37.77	31.23	37.17	40.00
Panniyurl stage 2	28.24	28.51	29.90	26.71	25.15	28.68
Panniyurl 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
Thekken stage 2	30.38	30.65	30.59	28.71	28.3	28.89
Thekekn stage 3	25.11	24.75	24.39	22.90	22.78	25.95

4.4 DATA ANALYSIS

4.4.1 Analysis using Bestkeeper software

Raw Cq values were given as input data of Best keeper software. Standard deviation of Cq values of all the genes were more than one. This restricted the use of Bestkeeper for further analysis. Standard deviation of Cq values of all the genes are given in Table 12.

Table 12. Standard deviation of Cq values

Gene	Standard deviation
ACTIN	4.28
EF	3.67
GAPDH	5.74
IF	2.46
TUBULIN	5.87
UBIQUITIN	5.51

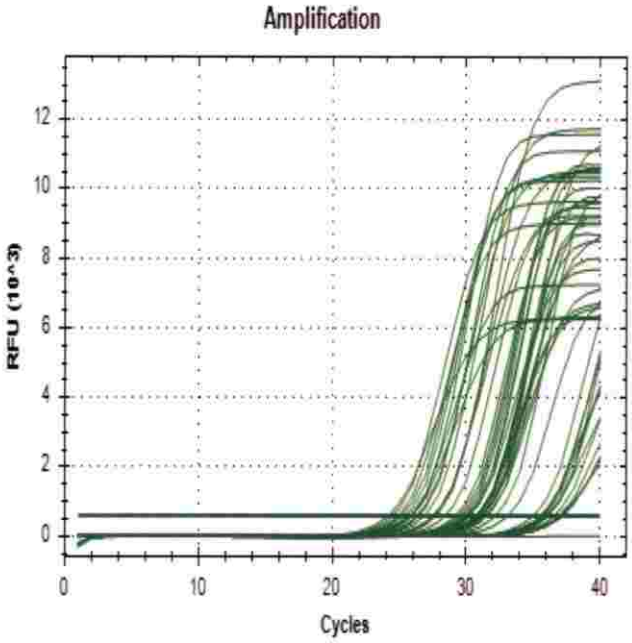


Fig. 3: Amplification plot for *Actin*

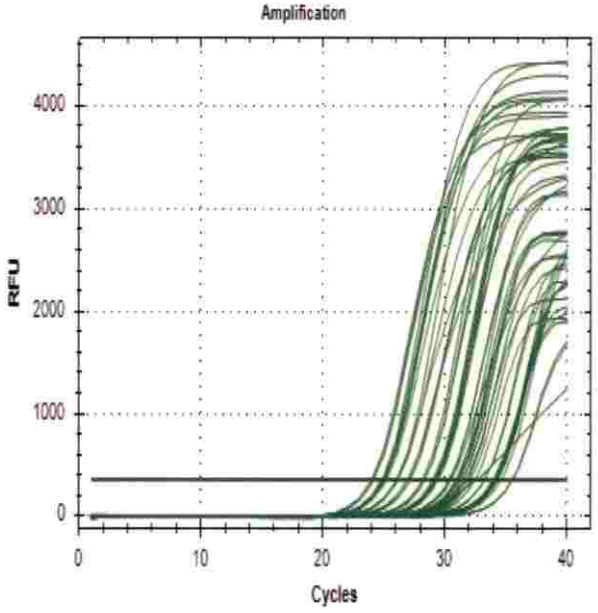


Fig. 4: Amplification plot for *Elongation Factor*

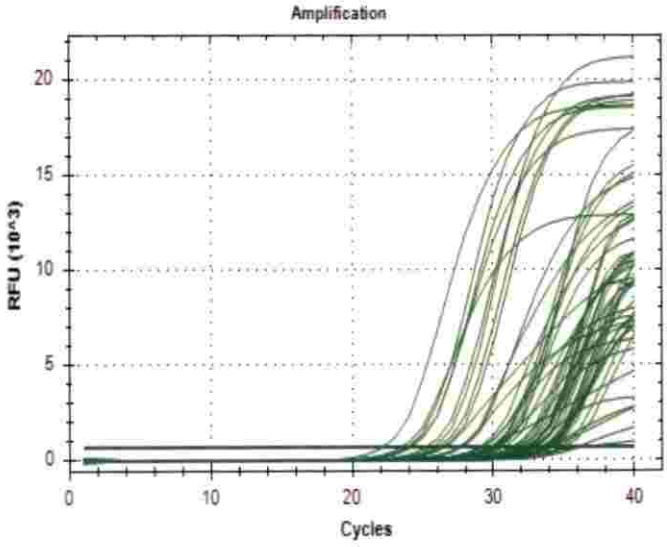


Fig. 5: Amplification plot for *GAPDH*

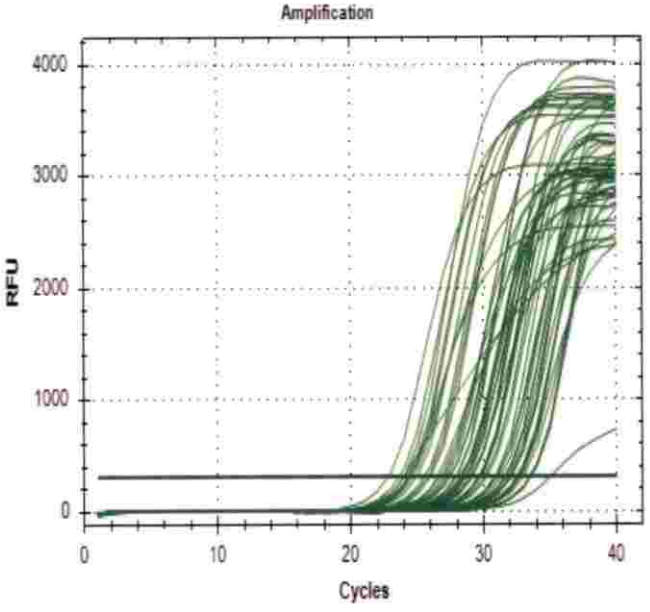


Fig. 6: Amplification plot for *Initiation Factor*

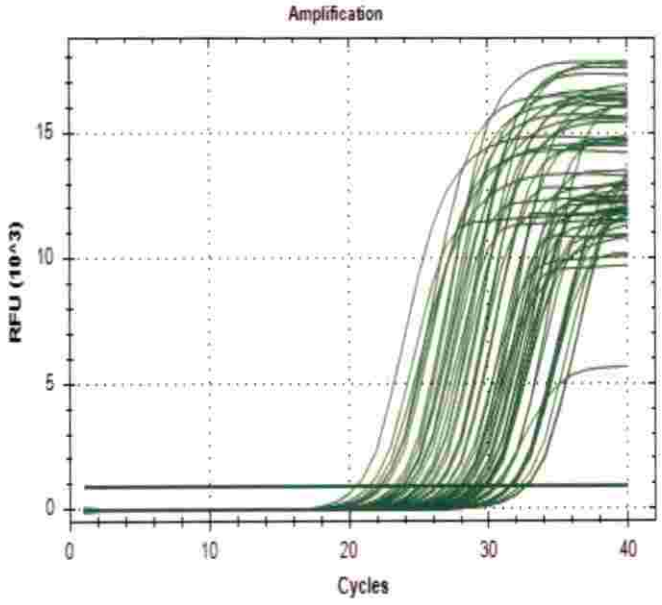


Fig. 7: Amplification plot for *TUBULIN*

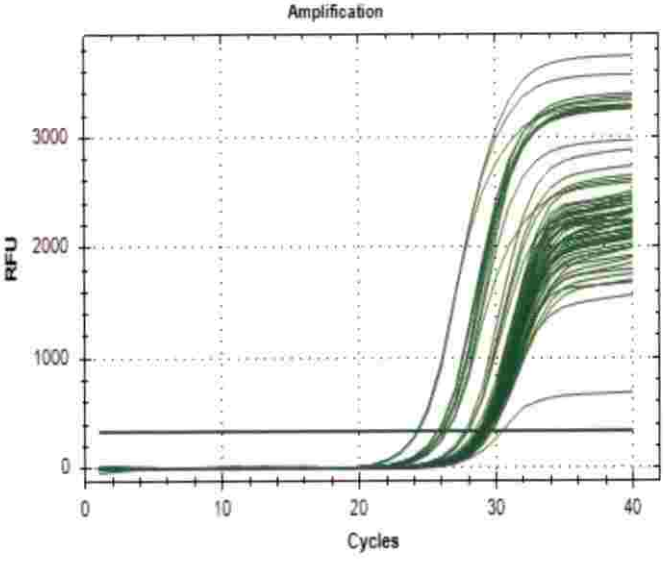


Fig. 8: Amplification plot for *UBIQUITIN*

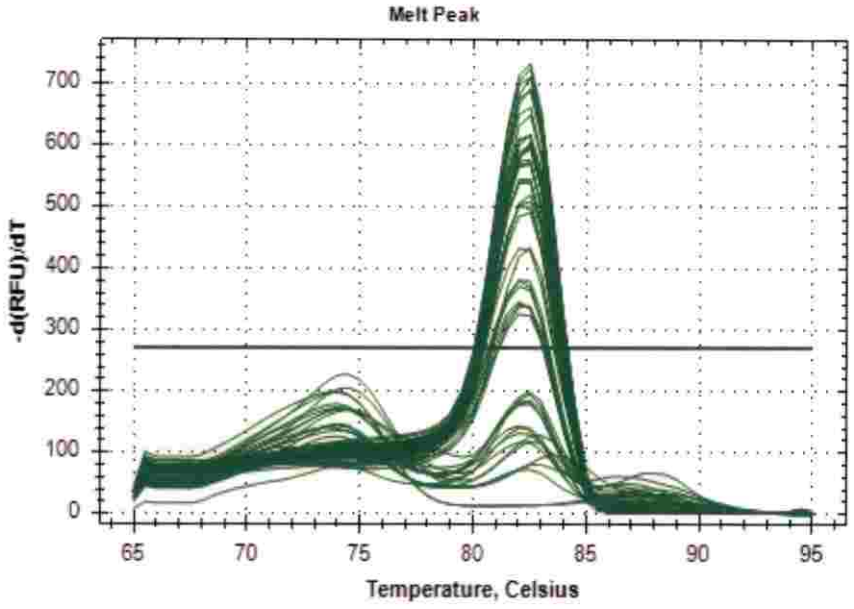


Fig. 9: Melt curve generated for *ACTIN* by RT-qPCR

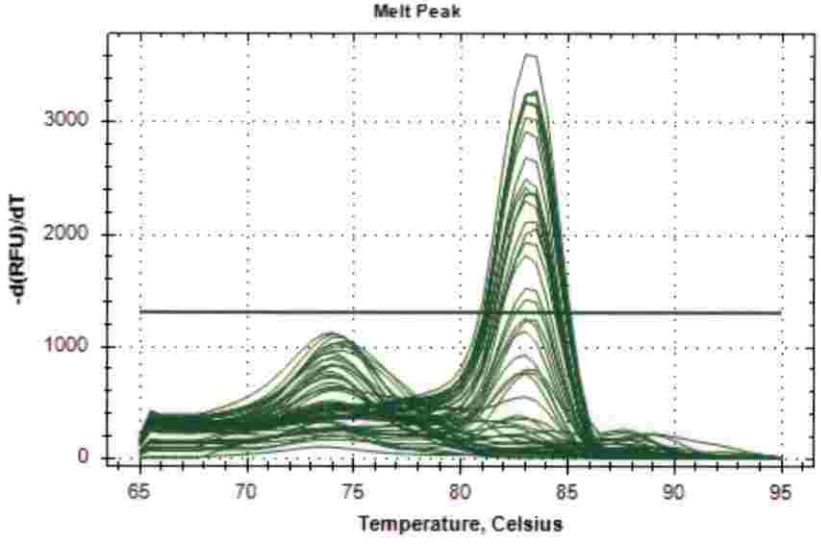


Fig. 10: Melt curve generated for *Elongation Factor* by RT-qPCR

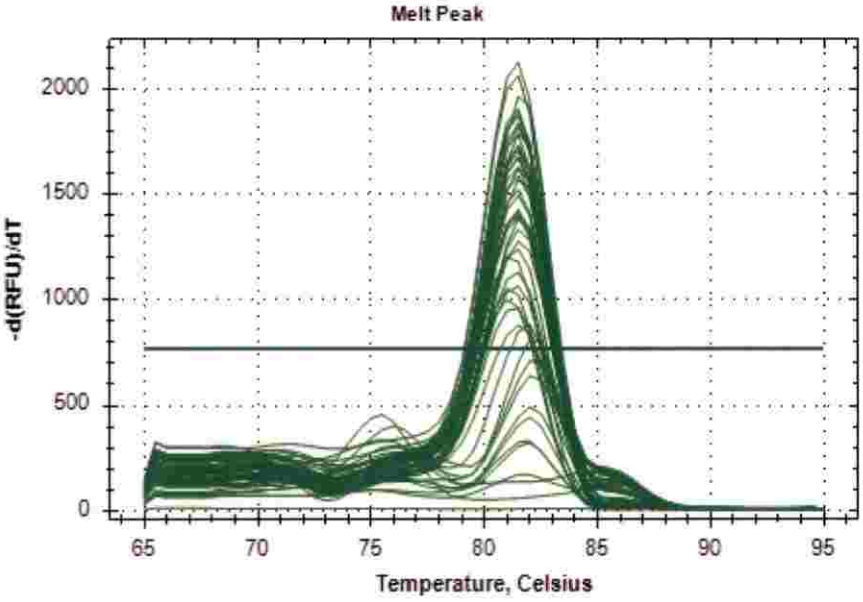


Fig. 11: Melt curve generated for *GAPDH* by RT-qPCR

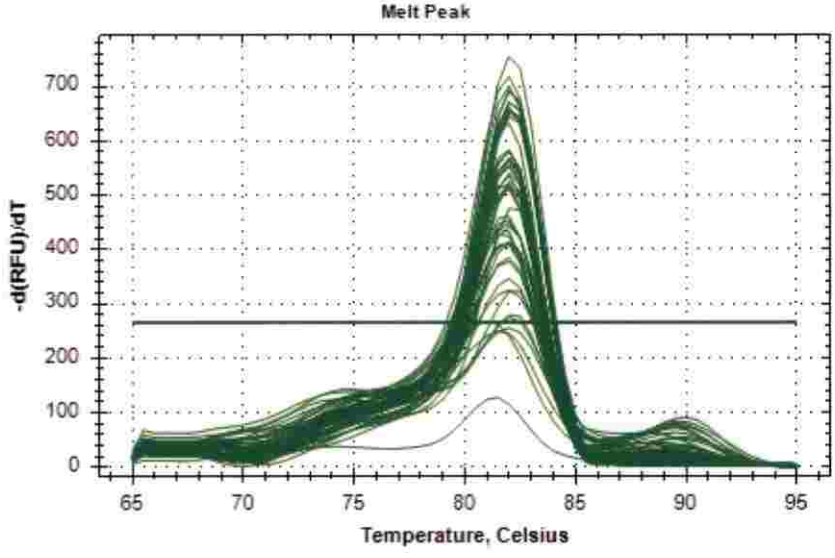


Fig. 12: Melt curve generated for *Initiation Factor* by RT-qPCR

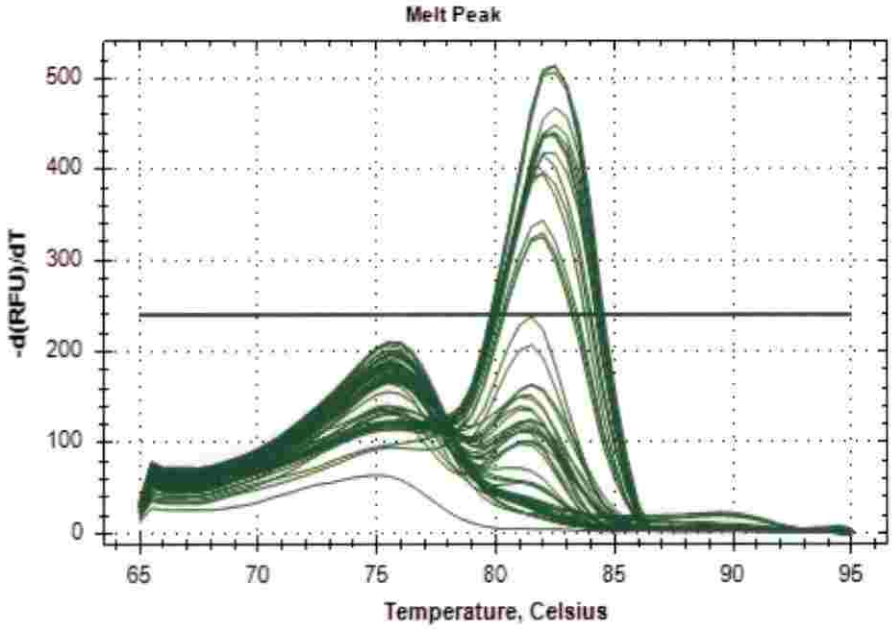


Fig. 13: Melt curve generated for *TUBULIN* by RT-qPCR

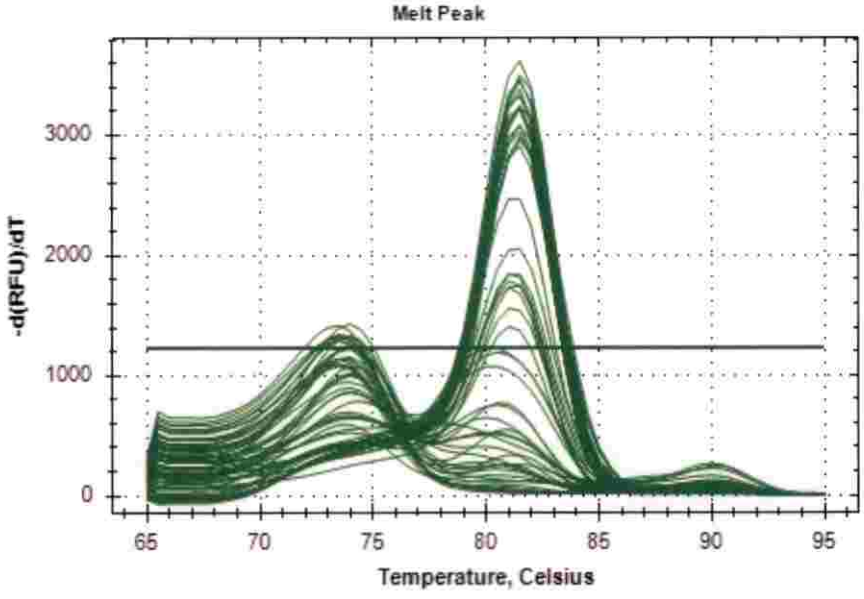


Fig. 14: Melt curve generated for *UBIQUITIN* by RT-qPCR

4.4.2 Analysis using geNorm software

Raw Cq values were converted to relative expression value using q base plus software and used as input data for geNorm software analysis. The relative expression values are given in Table 13.

Table 13. Relative expression values of house keeping genes generated by q base plus software in different cultivars of black pepper

Samples	Actin	EF	GAPDH	Ubiquitin	IF	Tubulin
Karimunda stage 1	0.5266	0.6932	2.0660	2.6000	0.4526	1.1260
Karimunda stage 2	0.8555	0.7104	0.3702	1.8910	0.4418	5.3190
Karimunda stage 3	1.5950	0.6923	0.0505	7.0500	0.6702	3.7980
Panniyur1 stage 1	1.7960	2.7930	1.6290	0.1018	3.4730	0.3462
Panniyur1 stage2	0.6194	0.7098	1.4360	0.9756	0.2996	5.4180
Panniyur1 stage 3	4.5850	2.0300	0.9051	0.2646	3.5490	0.1264
Thekken stage 1	0.4637	0.6715	4.5240	0.8054	0.1648	5.3480
Thekken stage 2	0.4595	0.5251	2.9120	2.9490	0.2440	1.9790
Thekken stage 3	0.7156	0.5319	5.5900	0.5525	0.3573	2.3810

Gene expression stability value or "M value" was calculated for all the genes by geNorm software and is given in Table 14. Among the different house keeping genes analysed, *Actin* was the most stable in geNorm analysis when Panniyur 1, Karimunda and Thekken were analysed together. *Actin*, *Elongation factor* and *Initiation factor* were found to be the most stable genes in Thekken, Panniyur1 and Karimunda respectively when the varieties were considered separately. The graph generated by geNorm software is shown in Fig. 15 to 18.

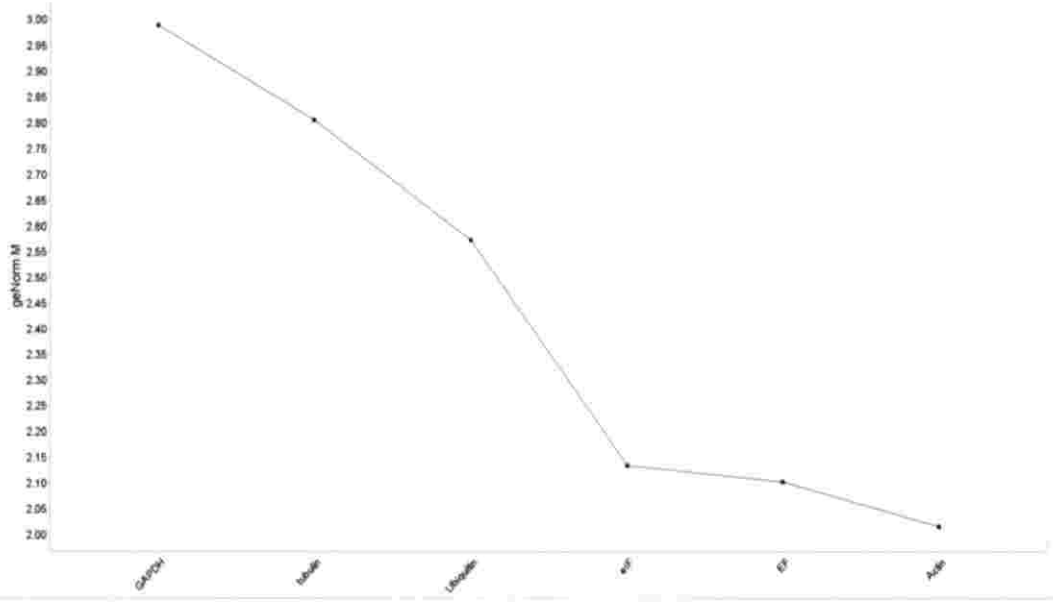


Fig. 15: Average expression stability values (M) of six candidate reference genes when all the samples were analysed together

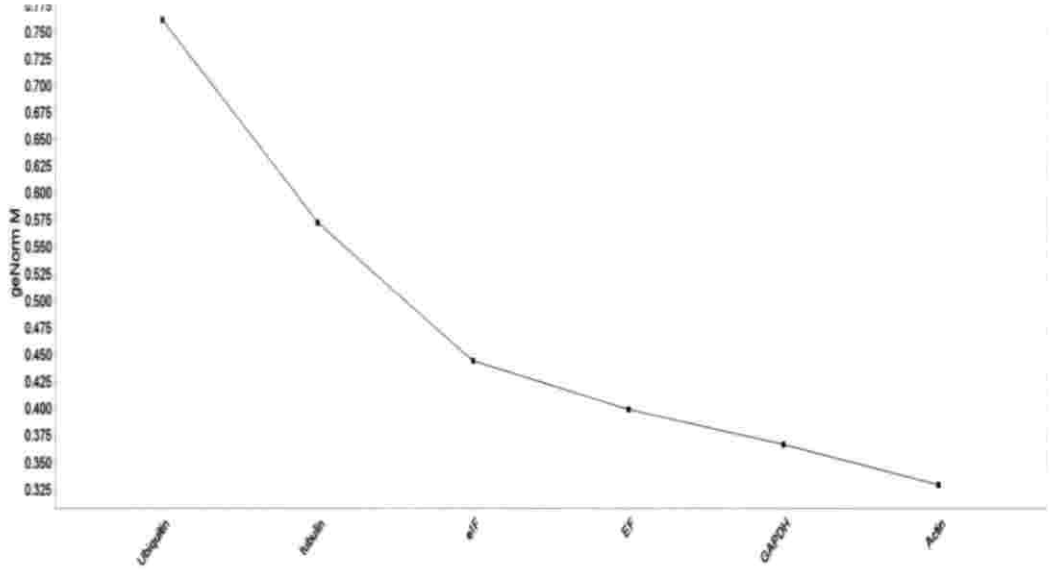


Fig. 16: Average expression stability values (M) of six candidate reference genes in Thekken

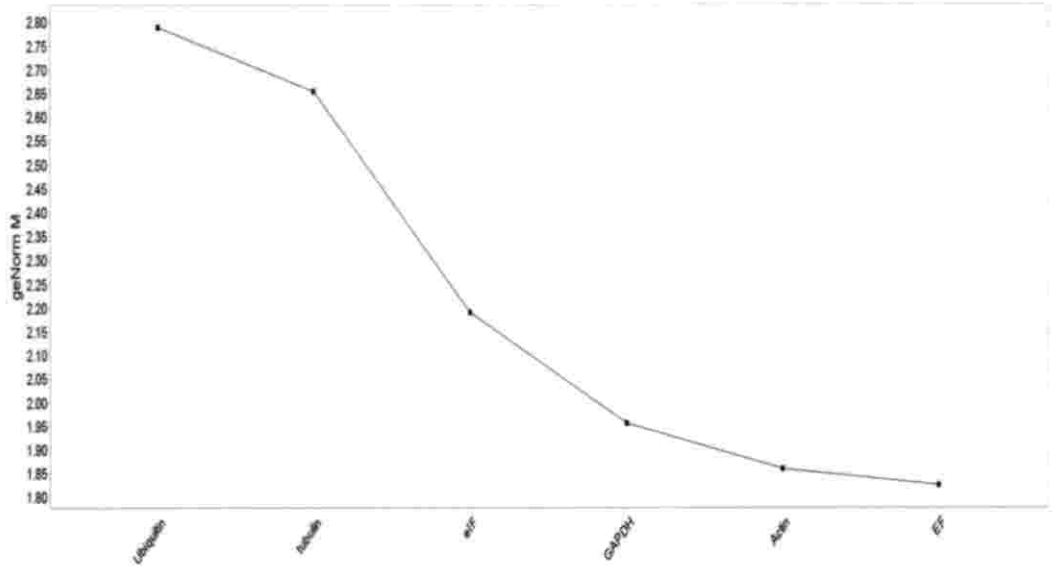


Fig. 17: Average expression stability values (M) of six candidate reference genes in Panniyur 1

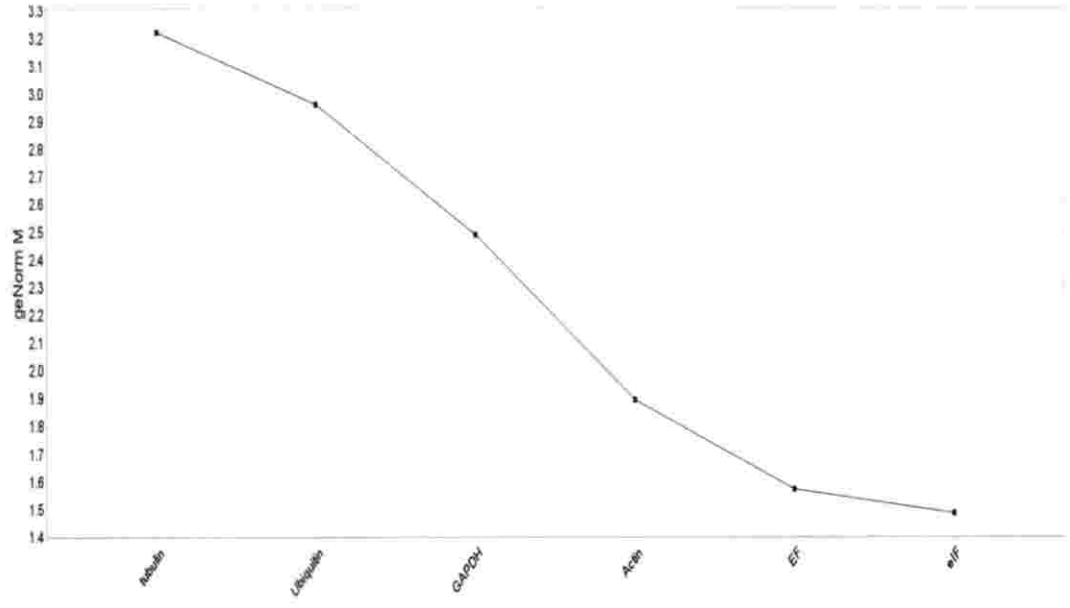


Fig. 18: Average expression stability values (M) of six candidate reference genes in Karimunda

Pair wise variation was also predicted by geNorm software(Fig. 19 to 21). In Panniyur1 and Karimunda, the variability between sequential normalization factors (based on the n and n+1 least variable reference targets) was relatively high (geNorm V > 0.15) and five reference genes with low M value *viz.Actin, Elongation Factor, Initiation Factor, GAPDH* and *Tubulin* were recommeded as reference genes.

In Thekken, the geNorm V value was less than 0.15 when comparing normalization factor based on the 3 or 4 most stable genes. Hence, in Thekken, three genes *viz.*, Actin, *Elongation Factor* and *GAPDH* were recommended as optimal reference genes.

Table 14. Expression stability value (M value) of genes by geNorm analysis in different cultivars of black pepper

Gene	Expression stability value			
	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
Tubulin	2.90	0.570	2.65	3.25
Ubiquitin	2.57	0.760	2.79	2.98

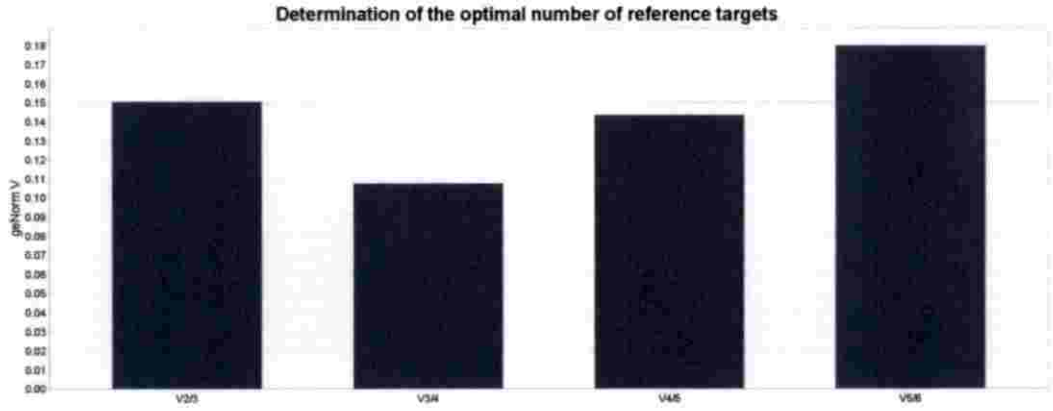


Fig.19 geNorm V value of six candidate reference genes in Thekken

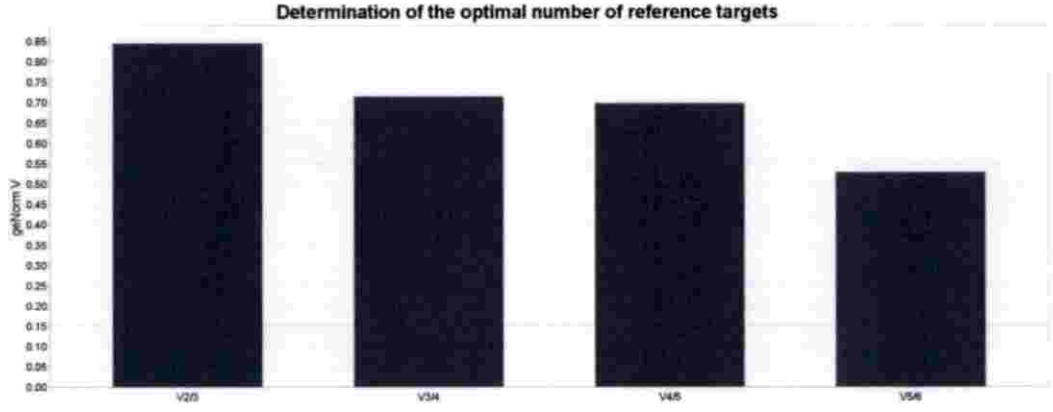


Fig.20 geNorm V value of six candidate reference genes in Karimunda

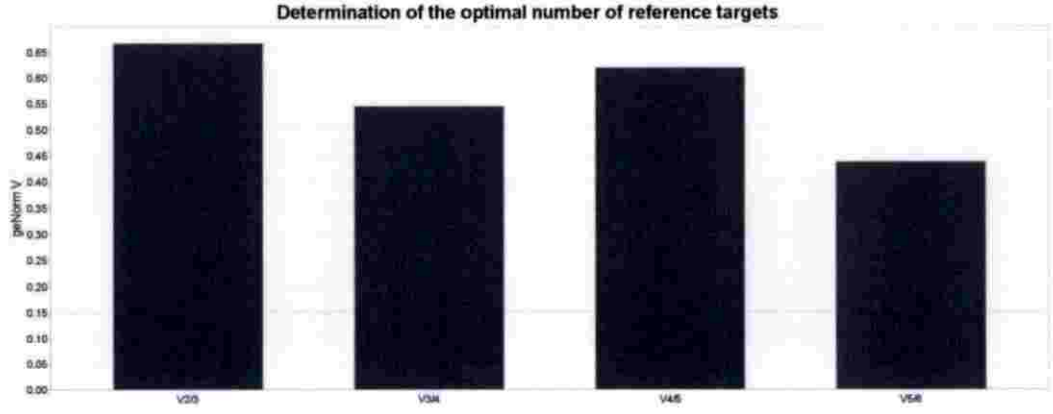


Fig.21 geNorm V value of six candidate reference genes in Panniyur 1

4.4.3 Analysis using Normfinder software

Realtive expression values generated was used as input data for analyses using Normfinder. Stability values were generated for all the genes and are presented in Table 15. Low stability value indicates stable gene expression. Normfinder software identified *Actin* as the most stable gene in all the three cultivars, when they were analysed together and separately.

Table 15. Stability values of genes generated by Normfinder analysis

Gene	Stability values			
	Thekken, Panniyur1 and Karimunda samples analysed together	**Thekken**	**Panniyur 1**	**Karimunda**
Actin	0.842	0.260	0.647	0.846
EF	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
Tubulin	1.588	0.492	1.508	1.804
IF	1.729	0.442	1.385	1.341

Discussion

5. DISCUSSION

Black pepper (*Piper nigrum*) is one of the most important and valuable spice crop in the world. Western Ghats of Indian peninsula is considered as the centre of origin of black pepper because of the presence of highest diversity in this region (Rahiman and Nair, 1987; Mathew *et al*., 2007). However, the productivity of black pepper in India is less compared to other countries. The major yield attributing character in black pepper is number of spikes (Hussain *et al*., 2017). Hence, gene expression studies related to inflorescence development can contribute to yield improvement. Real time PCR is one of the most widely used method for gene expression studies. The study entitled "Selection of stable housekeeping genes for gene expression studies during inflorescence development in black pepper (*Piper nigrum* L.) using real time PCR" was carried out at Department of Plant Biotechnology, College of agriculture, Vellayani, to identify most stable reference gene, as normalization of data with stable reference gene is necessary for accurate and reliable result in RT-qPCR.

5.1 MOLECULAR ANALYSIS AT GENOME LEVEL

In the present study, spike samples of three varieties/cultivars of black pepper *viz*., Panniyur1, Karimunda and Thekken at three different development stages *viz*., stage I (1-2cm), stage II (6-8cm) and stage III (9-12cm) were used as experimental materials. The tissues in different developmental stages are generally used for the transcriptome profiling and the gene expression analysis related to the inflorescence architecture (Feng *et al*., 2017; Yamburenko *et al*., 2017; Liu *et al*., 2019). Six housekeeping genes *viz*., *Actin, Elongation factor, GAPDH, Initiation factor, Tubulin* and *Ubiquitin* were selected as candidate genes for the study since their use as a stable reference gene are reported at different experimental conditions (Artico *et al*., 2010; Li *et al*., 2016; Wang *et al*., 2016; de Andrade *et al*., 2017; Gines *et al*., 2018; Liang *et al*., 2018)

Good quality DNA is a prerequisite for molecular analysis. Secondary metabolite contamination can hinder the downstream processes such as PCR and related molecular analyses. The most widely used method for isolation of genomic DNA in plants is CTAB method (Doyle and Doyle, 1987). High level of polysaccharides and phenols in black pepper can affect the isolation of genomic DNA (Dhanya *et al.*, 2007). In the present study, modified CTAB (Subba *et al.*, 2014) method with increased concentration of CTAB along with NaCl (CTAB- 2% (w/v), Nacl- 2 M) was used for DNA isolation to take care of the impurities. Increasing the concentration can effectively remove the polysaccharide contaminations (Syamkumar *et al.*, 2005; Sahu *et al.*, 2012). To avoid brown pigmentation in the sample and to obtain good quality DNA, PVP and β-mercaptoethanol were included in the extraction buffer since they are known to prevent the oxidation of secondary metabolites in the plant materials (Pirttilla *et al.*, 2001).

A_{260}/A_{280} value in the range of 1.8 to 2 indicates good quality DNA (Weising *et al.*, 2005). The isolated genomic DNA from the spike samples of black pepper was confirmed to be high quality with A_{260}/A_{280} values ranging from 1.7-1.88.

For amplifying gene fragments, primers were designed using Primer express software. Primer length between 18-24 base pair is ideal and the short length primer can result in non-specific amplification (Lowe *et al.*, 1990). The ideal annealing temperature for PCR reaction is always kept 5°C below the melting temperature (Rychlik *et al.*, 1990). In the present study, all the primers designed were in accordance with the mentioned criteria. Quality check of primers was done using genomic DNA. Amplification factor of two indicates 100% efficiency (Ruijter *et al.*, 2009). In the present study, amplification factor for all the primers designed was two, indicating cent per cent amplification efficiency.

Specificity of the designed gene specific primers was checked by PCR using genomic DNA. Single amplicon of expected size indicates specificity of

primers (Lorenz, 2012). Single amplicon was obtained for all the primers and it indicates the specificity of the designed primers.

5.2 MOLECULAR ANALYSIS AT TRANSCRIPTOME LEVEL

5.2.1 RNA Isolation

In the present study, Trizol method of RNA isolation was used to isolate RNA from different developmental stages of spike of black pepper cultivars. Good quality RNA without protein, genomic DNA and secondary metabolites are crucial for RT-qPCR. In black pepper, the presence of phenols and polysaccharides hinder the isolation of good quality RNA (Narayanan, 2000). Maintenance of aseptic conditions and use of sterile materials are essential to obtain good quality RNA.

The Trizol reagent is a ready-to-use reagent for the RNA extraction and is an improved version of the single-step method of RNA isolation. This method made RNA isolation process easier and fast (Simms *et al.*, 1993). Use of PVP along with extraction buffer can yield good quality RNA suitable for RT-qPCR (George *et al.*, 2005).

Ribosomal RNA represent more than 90% of the total RNA and degradation of RNA during isolation process appear as smear or indistinct band (Asif *et al.*, 2000). Plate shows the agarose gel electrophoresis picture of isolated RNA. Distinct 28S, 18S and 5S eukaryotic ribosomal RNA bands were obtained indicating that good quality of RNA was isolated without degradation. The quality of isolated RNA can be accessed by A_{260}/A_{280} value (Logemann *et al.*, 1987). A_{260}/A_{280} value of isolated RNA ranged from 1.8-2 and indicates pure RNA with less protein contamination. Concentration of RNA in different samples varied from 1292 to 2560 ng/µl.

Quality of cDNA was confirmed by polymerase chain reaction and agarose gel electrophoresis using *Actin* specific primer. Single amplicon of expected size (190) was obtained indicating good quality of cDNA.

5.2.2 Reverse Transcription quantitative PCR (RT-qPCR)

The 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 (Huggett *et al.*, 2005; Gutierrez *et al.*, 2008; Tian *et al.*, 2015). In the present study, SYBR Green dye based real-time PCR assay was done to analyse the gene expression.

SYBR Green is one of the commonly used fluorophores for the quantification and detection of the PCR products in RT-qPCR (Nygard *et al.*, 2007). The major advantages are its high sensitivity and reduced cost compared to other fluorophores (Reboucas *et al.*, 2013). Increase in the fluorescence is directly proportional to increase in the amplified product (Pable and Pabla, 2008). The point at which fluorescence exceeds the background level is known as Cq value and is dependent on the initial amount of template (Wong and Medrano 2005).

RT-qPCR is the most widely used method for transcriptome analysis in plants. The accuracy of results obtained by this method is dependent on normalization using stable reference gene (Li *et al.*, 2016). But the validation of reference gene has received less attention in plant studies, and housekeeping genes are being used as reference genes without any validation (Gutierrez *et al.*, 2008). In the present study, gene expression of six housekeeping genes were analysed by RT-qPCR to determine the most stable reference gene for the normalization of relative gene expression data in black pepper during inflorescence development.

In the present study, cDNA was synthesized by reverse transcription using mix of random hexamer and oligo(dT) primers. Combining oligo(dT) and random

primers is effective in overcoming the disadvantages associated with mechanism of priming. cDNA from different developmental stages were used as template for real-time PCR analysis. For each gene, real-time PCR was performed in triplicate and the expression levels for each sample were calculated as Cq values. geNorm and Normfinder analysis require conversion of raw Cq values to relative expression value. Raw Cq values were converted to relative expression using q base plus software.

Amplification and melt curve plot were generated for *Actin, β-Tubulin, Elongation factor, Initiation factor, Ubiquitin* and *GAPDH* by Biorad CFX Maestero software (Fig. 3 to 8). Amplification plot shows the change in relative fluorescence with respect to cycle number. Each line in the amplification plot represents the amplification curve of each sample. The lines crossing the threshold fluorescence indicates the significant increase in the fluorescence. The graph shows that the Cq values for most of the samples were in between 20-30.

Melt curve analysis was performed to verify primer specificity. Fig. 9 to 14 shows the melt curve plot for *Actin, β-Tubulin, Elongation factor, Initiation factor, Ubiquitin* and *GAPDH*. Prominent peak was obtained for all genes and it indicates the specificity of primer.

5.3 DATA ANALYSIS

In real-time PCR, normalization of data is crucial to minimize the effect of non-biological variation. Several strategies have been developed to normalize the data. One of the methods is normalization using reference gene and use of reference gene could give more accuracy and reliability compared to other methods (Hugget *et al*., 2005; Vandesompele *et al*., 2009). The use of an unstable reference gene could add more bias to the result. So it is important to validate reference gene according to each experimental conditions. To determine the most suitable reference genes, several statistical algorithms are available. Most commonly used softwares are *viz.,* geNorm, BestKeeper and NormFinder (Khanlou and Bockstaele, 2012).

5.3.1 Analysis using Bestkeeper software

Bestkeeper software is an excel based tool that predicts the ideal reference genes according to pair-wise correlation analysis among all pairs of candidate reference genes (Pfaffl *et al.*, 2004). It provides two measures that can be used for determination of stable gene for normalization of data. In the initial step, raw Cq values having standard deviation more than one will be excluded from the analysis. Also, it determines Bestkeeper index from geometric mean of remaining reference gene and performs correlation analysis (De Spiegelaere *et al.*, 2015). In the present study, standard deviation of Cq values for all the genes were more than one and which restricted the use of Bestkeeper for further analysis.

5.3.2 Analysis using geNorm software

The geNorm software program 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-PCR (Vandesompele *et al.*, 2002). geNorm software also calculates geNorm V value, which determines the most stable combination of reference gene. They calculate pairwise variation using n/n+1 reference genes to estimate the optimal number of reference genes to be used for accurate normalization. n number of reference gene can be considered sufficient for normalization based on the 0.15 cut-off for the pairwise variation (De Spiegelaere *et al.*, 2015). If the geNorm V value is less than 0.15, the addition of one more reference gene provides no improvement in the fidelity (Liang *et al.*, 2018).

M values for all the genes were generated by geNorm when analysed together as well as separately. It was found that *Actin* was the most stable gene among the genes selected for the study, when all the samples were analysed together. But the geNorm M value for all the genes were more than 1.5, which is above the default limit of M = 1.5 (Vandesompele *et al.*, 2002). So it is necessary

to use combination of reference gene for more accurate normalization of data (Andersen *et al*., 2004).

When samples of Thekken were analysed separately, *Actin* was found to be the most stable reference gene with M value 0.325. The optimal number of reference gene in the present study was recorded as 3 (geNorm V < 0.15 when comparing a normalization factor based on the 3 or 4 most stable targets). Hence, combination of *Actin*, *GAPDH* and *Elongation factor* can provide accurate normalization of data for gene expression studies during inflorescence development in Thekken. When samples of Panniyur and Karimunda were analysed separately, *Elongation factor* and *Initiation factor* respectively were observed to be the most stable gene. However, the geNorm M value for all the genes were above the default limit of M = 1.5 and hence indicates less stability of these genes (Vandesompele *et al*., 2002).

5.3.3 Analysis using Normfinder software

Normfinder is another excel based tool used to identify stable reference gene. It ranks candidate reference gene based on their expression stability in a given set of sample and given experimental condition. Most stable gene expression indicated by low average expression stability value (Andersen *et al*., 2004).

Normfinder software identified *Actin* as the most stable gene in all the three cultivars *viz.,* Thekken, Karimunda and Panniyur, when analysed together as well as separately. Variation in the expression of genes in Thekken sample was comparatively less. But in case of Panniyur and Karimunda high variation was observed for all the genes. The same result was obtained when the samples were analysed with geNorm software also.

In the case of Panniyur and Karimunda, *Elongation factor* and *Initiation factor* were the most stable genes according geNorm whereas Normfinder predicted *Actin* as the most stable gene in both the cultivars. Such variations are

reported earlier in other crops (Hong *et al.*, 2008; Huis *et al.*, 2010; Wan *et al.*, 2010; Liang *et al.*, 2018). Qui *et al.* (2016) have reported variation in stability of the reference genes among the different varieties of chrysanthemum correlating with the complexity of the genetic background of this crop.

Actin is generally used reference gene for normalization of data in real time PCR (Pohjanvirta *et al.*, 2006). In the present study also, *Actin* emerged to be the most stable reference gene among the six candidate housekeeping genes when analysed by geNorm and Normfinder softwares. It is reported to be the most stable reference gene in many other plant systems including *Plukenetia volubilis* during flower development (Niu *et al.*, 2015) and in *Cyamopsis tetragonoloba* during seed development and abiotic stress conditions (Jaiswal *et al.*, 2019). *Actin* in combination with *Elongation factor* was used as reference gene in bell pepper during hormonal treatments (Wang *et al.*, 2012) and in combination with *Tubulin* under drought stress in carrot (Tian *et al.*, 2015).

Elongation factor is a commonly used reference gene in many plants including tomato under nitrogen stress (Lovdal and Lillo, 2009), pearl millet under abiotic stress (Shivhare and Lata, 2016) and cucumber under viral stress (Liang *et al.*, 2018). In the present study also *Elongation factor* showed stable expression in all the cultivars *viz.*, Thekken, Panniyur and Karimunda.

In the present study *GAPDH* showed an intermediate expression compared to other genes in different cultivars. This is in agreement with the reports of Liang *et al.* (2018) in cucumber under biotic stress.

Tubulin and *Ubiquitin* are reported to be stable in many plants (Artico *et al.*, 2010; Li *et al.*, 2016; Knopkiewicz and Wojtaszek, 2019). However, in the present study they exhibited least stability of expression in all the cultivars and hence they were not suitable for normalization of data in RT-qPCR studies during inflorescence development in black pepepr. Similar results have been reported in cucumber wherein *Tubulin* exhibited high variability in expression levels when

subjected to growth regulators and abiotic stress (Migocka and Papierniak, 2011) and in *Actimidia deliciosa* under biotic stress (Petriccione *et al.*, 2015).

Among the different candidate genes "geNorm" and "Normfinder" software identified *Actin* as the most stable gene when all the cultivars were analysed together. "Normfinder" software identified *Actin* as the most stable gene in all the three cultivars, when they were analysed separately also. However, in the analysis using "geNorm" software, *Actin* emerged to be the most stable gene only in Thekken. In Panniyur, *Elongation factor* was the most stable gene followed by *Actin* and *Initiation factor* while in Karimunda, *Initiation factor* was the most stable gene followed by *Elongation factor* and *Actin*. When analysed using "geNorm" software, the stability value (M value) was high for all the genes in all the cultivars except in Thekken, indicating high variation for all the genes in Panniyur and Karimunda.

To conclude, among the genes studied, *Actin* was identified as the most stable housekeeping gene during inflorescence development in the genotype studied. The study also emphasizes the necessity of identifying more number of reference genes for improving the accuracy of RT-qPCR studies during inflorescence development in different cultivars of black pepper.

Summary

6. SUMMARY

The study entitled "Selection of stable housekeeping genes for gene expression studies during inflorescence development in black pepper (*Piper nigrum* L.) using real time PCR" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2017-2019 with the objective to compare the expression profile of different housekeeping genes such as *Actin, β-Tubulin, Elongation factor, Initiation factor, Ubiquitin* and *GAPDH* (Glyceraldehyde 3- phosphate dehydrogenase) during inflorescence development and to identity the most stable gene to be used as reference gene for RT-qPCR studies during inflorescence development in black pepper (*Piper nigrum L.*).

Black pepper (*Piper nigrum*) referred as 'Black Gold' is one of the oldest and most important spice crops in the world. As flowering determines the yield parameters in black pepper, knowledge of the genes involved in floral architecture and its characterization can help significantly in taking up further yield improvement programmes. RT-qPCR is an effective and sensitive technique to measure transcript level modulation of genes and provides significant quantitative information on gene expression. Accuracy of the results obtained by this technique is dependent on normalization using stably expressed genes known as reference genes. An ideal reference gene should be stable under any experimental condition.

In the present study, spike samples of three cultivars/varieties of black pepper *viz.*, Panniyur1, Karimunda and Thekken at three different development stages *viz.*, stage I (1-2cm), stage II (6-8cm) and stage III (9-12cm) were used as experimental materials. Genomic DNA extracted by modified Cetyl Trimethyl Ammonium Bromide (CTAB) method and good quality DNA was obtained. A_{260}/A_{280} value of extracted DNA ranged between 1.7 and 1.88 and yield of DNA ranged from 1650 to 2727 ng/µl. Primers for selected housekeeping gene *viz., Actin, β-Tubulin, Elongation factor, Initiation factor, Ubiquitin* and *GAPDH*

(Glyceraldehyde-3-phosphate dehydrogenase) were designed using primer express software. PCR amplification of genomic DNA was done using primers to check the specificity of primers. Single amplicon was obtained for all the primers, indicating the specificity of the designed primers. Amplification efficiency of the designed primers was determined by standard curve analysis and "Lin Reg" software. All the primers exhibited cent per cent amplification efficiency.

Total RNA was isolated from spikes of different development stages using Trizol reagent. A_{260}/A_{280} value of isolated RNA ranged from 1.8-2 indicates purity of RNA with less protein contamination. Concentration of RNA in different samples ranged from 1292 to 2560 ng/µl. RNA isolated from the spike samples was converted to cDNA and the quality was confirmed by PCR and agarose gel electrophoresis.

In the present study, SYBR Green dye based real-time PCR assay was done to analyse the gene expression. Thermal conditions for RT-qPCR were 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 45 sec. RT-qPCR for each gene was performed with three technical replicates for each sample. Amplification and melt curve plot were generated by Biorad CFX Maestero software. Prominent peak was obtained in melt curve analysis for all genes indicating the specificity of the designed primers. Cq values obtained were used for further analysis.

To determine the most suitable reference genes, several statistical algorithms are available. Most commonly used softwares are *viz.,* geNorm, BestKeeper and NormFinder. geNorm and Normfinder analysis require conversion of raw Cq values to relative expression value. Raw Cq values were converted to relative expression using q base plus software.

High standard deviation of Cq values (>1) for all the genes restricted the use of "Bestkeeper" software in the present study. "geNorm" and "Normfinder" softwares identified *Actin* as the most stable gene when all the cultivars were analysed together. "Normfinder" software identified *Actin* as the most stable gene

in all the three cultivars, when they were analysed separately also. However, in the analysis using "geNorm" software, *Actin* emerged to be the most stable gene only in Thekken. In Panniyur1, *Elongation factor* was the most stable gene followed by *Actin* and *Initiation factor* while in in Karimunda, *Initiation factor* was the most stable gene followed by *Elongation factor* and *Actin*. When analysed using "geNorm" software, the stability value (M value) was high for all the genes in all the cultivars except in Thekken, indicating high variation for all the genes in Panniyur1 and Karimunda.

The present study is the first report on selection of stable reference gene in black pepper during inflorescence development. *Actin* was identified as the most stable reference gene during inflorescence development in the genotype studied. The study also emphasizes the necessity of identifying more number of reference genes for improving the accuracy of RT-qPCR studies during inflorescence development in different cultivars of black pepper.

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Appendices

APPENDIX I

CTAB Extraction Buffer

CTAB	2 % (w/v)
Tris-HCl	100 mM
EDTA	25 mM
NaCl	2 M
β-mercaptoethanol	0.2 % (v/v)
PVP	3 % (w/v)

APPENDIX II

TBE Buffer (5X) for 1 litre solution

Tris base	54 g
Boric acid	27.5 g
0.5M EDTA (pH 8.0)	20 ml

SELECTION OF STABLE HOUSEKEEPING GENES FOR GENE EXPRESSION STUDIES DURING INFLORESCENCE DEVELOPMENT IN BLACK PEPPER (*Piper nigrum* L.) USING REAL TIME PCR

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Abstract of the thesis

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9. ABSTRACT

The project entitled "Selection of stable housekeeping genes for gene expression studies during inflorescence development in black pepper (*Piper nigrum* L.) using real time PCR" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2017-2019. The objective of the study was to compare the expression profiles of different housekeeping genes such as *Actin, β-Tubulin, Elongation factor, Initiation factor, Ubiquitin* and *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) during inflorescence development and to identify the most stable genes to be used as reference genes for Reverse Transcription quantitative PCR (RT-qPCR) studies in black pepper (*Piper nigrum L.*).

RT-qPCR is an effective and sensitive technique to measure transcript level modulation of genes and provides significant quantitative information on gene expression. Accuracy of the results obtained by this technique is dependent on normalization using stably expressed genes known as reference genes. An ideal reference gene should be stable under any experimental condition.

Spike samples of three cultivars of black pepper *viz.*, Panniyur, Karimunda and Thekken were used for the study. Samples were collected at three different developmental stages *viz.*, stage I (1-2cm), stage II (6-8cm) and stage III (9-12cm) from two different plants of each cultivar. Genomic DNA and RNA were extracted by modified Cetyl Trimethyl Ammonium Bromide (CTAB) method and Trizol method respectively.

Primers were designed for six selected housekeeping genes *viz.*, *Actin, Elongation factor, GAPDH, Initiation factor, Tubulin and Ubiquitin* using "Primer Express" software and absence of secondary structure at the primer binding site was confirmed by "mfold" web server. Specificity of the designed gene specific primers was checked by PCR using genomic DNA. Single amplicon was obtained for all the primers indicating specificity of the designed primers. Amplification efficiency of the designed primers was determined by standard

curve analysis and "Lin Reg" software. All the primers exhibited cent per cent amplification efficiency.

RNA isolated from the spike samples was converted to cDNA and the quality was confirmed by PCR and agarose gel electrophoresis. cDNA was used for RT-qPCR using SYBR Green dye-based assay. Thermal conditions for RT-qPCR were 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 45 sec. RT-qPCR for each gene was performed with three technical replicates for each sample. Cq values obtained were used for further analysis.

Stability of the housekeeping genes in the samples were analysed using three softwares *viz*, "Bestkeeper", "geNorm" and "Normfinder". Bestkeeper algorithm takes raw Cq values as input whereas "geNorm" and "Normfinder" uses relative expression values for analysis. Relative expression values were generated from Cq values using "qbase plus" software with an amplification factor of two. High standard deviation of Cq values (>1) for all the genes restricted the use of "Bestkeeper" software in the present study.

"geNorm" and "Normfinder" softwares identified *Actin* as the most stable gene when all the cultivars were analysed together. "Normfinder" software identified *Actin* as the most stable gene in all the three cultivars, when they were analysed separately also. However, in the analysis using "geNorm" software, *Actin* emerged to be the most stable gene only in Thekken. In Panniyur, *Elongation factor* was the most stable gene followed by *Actin* and *Initiation factor* while in in Karimunda, *Initiation factor* was the most stable gene followed by *Elongation factor* and *Actin*. When analysed using "geNorm" software, the stability value (M value) was high for all the genes in all the cultivars except in Thekken, indicating high variation for all the genes in Panniyur and Karimunda.

To conclude, among the genes studied, *Actin* was identified as the most stable housekeeping gene during inflorescence development in the genotypes of black pepper studied. The study also emphasizes the necessity of identifying more

number of reference genes for improving the accuracy of RT-qPCR studies during inflorescence development in different cultivars of black pepper.