EXPRESSION PROFILING OF AUXIN RESPONSIVE GENES DURING INFLORESCENCE DEVELOPMENT IN BLACK PEPPER (*Piper nigrum* L.)

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"EXPRESSION PROFILING OF AUXIN RESPONSIVE GENES DURING INFLORESCENCE DEVELOPMENT IN BLACK PEPPER (*Piper nigrum* L.)"** 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 to me of any degree, diploma, associate ship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"EXPRESSION PROFILING OF AUXIN RESPONSIVE GENES DURING INFLORESCENCE DEVELOPMENT IN BLACK PEPPER (*Piper nigrum* L.)"** is a record of research work done independently by Ms. Karapareddy Sowndarya under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associate ship to her.

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Karapareddy Sowndarya

Dedicated
To

My beloved parents
(NageswarReddy and Padmalatha)

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
A	Adenine
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 260 nm wavelength
AMs	Axillary meristems
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
cm	Centimere
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
DEPC	Diethyl pyrocarbonate
EDTA	Ethylene diamine tetra acetic acid
FP	Forward Primer
FM	Floral meristems
G	Guanine
g	gram
GOI	Gene of interest
ha	Hectare
HPLC	High Performance Liquid Chromatography
IAA	Indole Acetic acid
IM	Inflorescence meristem
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
nM	Nanomolar
NPA	N-1-naphthylphthalamic acid

PAT	Polar auxin transport
PCR	Polymerase chain reaction
PPC	Plant and Prokaryote Conserved domain
PVP	Polyvinyl pyrrolidone
RP	Reverse primer
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
s	Second
SAM	Shoot apical meristem
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SSR	Simple sequence repeat
T	Thymine
TBE	Tris-borate EDTA buffer
T_m	Melting temperature
Tris HCl	Tris (hydroxy methyl) aminomethane hydrochloride
viz.	namely
w/v	weight/volume
v/v	volume/volume

LIST OF SYMBOLS

%	Percent
°C	Degree Celsius

Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum* L.) belonging to the family of *Piperaceae*, is the most important spice crop in the world. It is a perennial shrub native to Southern India. Due to its strong pungency, it is regarded as the 'King of spices' and it has valuable medicinal potency and massive trade share in the world-wide market (Srinivasan, 2007).

Geographically, *P. nigrum* is grown mainly in hot and moist conditions (Ravindran and Kallupurackal, 2012). The main black pepper growing areas are found in the Western Ghats of the South Indian Peninsula and was subsequently introduced to other countries in South and Southeast Asia (Hao *et al.*, 2012). In 2016, Vietnam was the world's leading producer of pepper (140,000 metric tonnes), followed by Indonesia (70,000 metric tonnes), India (48,500 metric tonnes), and Brazil (45,000 metric tonnes).

Black pepper is a very common kitchen spice and the pungency is due to the chemical constituent piperine (1-peperoyl piperidine, discovered in 1819 by Hans Christian), which has diverse pharmacological uses. It is commonly known as 'Kali mirch' in Urdu and Hindi, 'Marich' in Nepali, 'Pippali' in Sanskrit which means berry (Kumar *et al.*, 2011), 'Milagu' in Tamil, and Black Pepper, Peppercorn, Green pepper, White pepper, Madagascar pepper in English.

Pepper production in India has gradually decreased (Hussain *et al.*, 2017). It is estimated that by the year 2020, the world-wide demand for pepper will increase to about 2,80,000 MT to 3,60,000 MT by the year 2050 (Sooraj, 2016). Hence yield needs to be improved substantially so as to regain the past glory of Indian black pepper in the international market.

Spike architecture and spike characters *viz.*, number of spikes vine^{-1}, spike yield, length of spike and spike branching are known to have significant influence on yield of black pepper. Usually, black pepper species have unbranched spikes (Sujatha and Namboothiri, 1995). Inflorescence branching is mostly uncommon in pepper, except for two varieties *viz.*, *Aympirian* and *Uthirankotta*, which show a

tendency for branching, with one or two rudimentary branches. The pepper type *viz.*, 'Thekken', a selection of *Piper nigrum,* identified by Shri. T. T. Thomas in the forest area of Kanchiyar in Kattappana Panchayat, Idduki District, has been observed to possess profuse branching trait with more than 30 well developed branches bearing around 300 berries (Farm Innovators, 2010).

Auxin is a key hormone involved in shaping inflorescence architecture in many crop species. Genes like *Barren stalk fastigiate1 (BAF1), Barren inflorescence2 (BIF2)* and *Ramosa2 (RA2)* involved in the positioning and shaping of meristems on inflorescence axis are known to be regulated by auxin (Gallavotti, 2013).

Barren stalk fastigiate1 (*BAF1*) encodes a transcriptional regulator containing a DNA binding motif of the AT-hook. It is involved in the demarcation of the boundary region where axillary meristem is formed and plays a role in the initiation of axillary meristem (Gallavotti *et al.*, 2011).

Barren inflorescence2 (*BIF2*) in maize encodes a co-orthologous serine/threonine protein kinase to *PINOID (PID)*, and also regulates the transport of auxin through direct regulation of ZmPIN1a (Skirpan *et al.*, 2009). An intriguing speculation is that the bifunctional role of the *BIF2* kinase can provide a mechanism to induce genes necessary for the initiation of axillary meristem in response to auxin transport (Skirpan *et al.,* 2008).

Ramosa2 (*RA2*) is transiently expressed in a group of cells that predicts the position of axillary meristem formation in inflorescence. It is a lateral organ boundary transcription factor that helps to determine the meristem boundary (Bortiri *et al.*, 2006).

As flowering governs the yield parameters in black pepper, knowledge of the genes involved in floral architecture and their characterisation is of great importance. Examining the transcript modulation of these genes during inflorescence development might help in developing strategies to improve yield potential of black pepper.

Therefore, the objective of the present study proposes to profile auxin responsive genes like *Barren stalk fastigiate1*, *Barren inflorescence2* and *Ramosa2* during inflorescence development in black pepper (*Piper nigrum* L.) by RT-qPCR analysis. Comparison of the expression of these genes in different cultivars differing in inflorescence architecture, may help in identifying potential molecular resources for future yield improvement programmes.

Review of Literature

2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L.; Family: *Piperaceae*) referred to as 'King of spices' and commonly called as 'Black Gold' is one of India's most important spice trading commodity since the prehistoric period (Govindarajan and Stahl, 1977).

Black pepper was originated in the evergreen tropical forests of the Western Ghats of India. It grows successfully between 20^0 North and 20^0 South of equator and upto 1500 m above mean sea level. It is a humid tropical plant, requiring 2000-3000 mm of rainfall, tropical temperature and high relative humidity with little variation in the length of the day throughout the year (Pillay, 1988). It can be grown in a wide range of soils with a pH of 5.5 to 6.5. In its natural habitat, it thrives well in laterite red soils and optimum soil temperature for root growth is 26^0C-28^0C (Wahid and Sitepu, 1987).

Black pepper is mostly dioecious in wild form (Krishnamurthi, 1969), but in the cultivated types, the plants are mostly gynomonoecious (*i.e.*, bearing female and bisexual flowers) or trimonoecious (i.e., bearing female, male and bisexual flowers). Bisexual flowers are protogynous and stigmas are exerted 3-8 days before dehiscence of the anthers (Martin and Gregory, 1962).

India is one of the leading producer, consumer and exporter of black pepper followed by Indonesia, Malaysia, Sri Lanka and Brazil in the world (Ravindran, 2003). It is grown mainly in Kerala, Karnataka and Tamil Nadu and to some extent in Maharashtra, North Eastern states and Andaman and Nicobar Islands. Small amounts of pepper are also produced in the states of Goa, Andhra Pradesh, Orissa and Assam.

Spike architecture and spike characters *viz.*, number of spikes vine^{-1}, spike yield, length of spike and spike branching are known to have significant influence on yield of black pepper (Gallavotti, 2013).

The uncovering of genetic pathways leading to spike branching can help in taking up crop improvement programmes. Auxin is a key hormone involved in shaping inflorescence architecture in many crop species (Gallavotti, 2013). The present study was taken up with the objective to study the expression of auxin responsive genes such as *Barren stalk fastigiate1 (BAF1), Barren inflorescence2 (BIF2) and Ramosa2 (RA2)* during inflorescence development in black pepper (*Piper nigrum* L.) by quantitative Real Time PCR (RT q-PCR) with different cultivars of black pepper.

In this chapter, the literature concerned with black pepper, role of auxin responsive genes during inflorescence development of different crop plants and the molecular techniques for gene expression analysis have been presented.

2.1 GENERAL MORPHOLOGY OF THE BLACK PEPPER PLANT

Black pepper is a woody perennial climber and produces two types of branches *viz*., orthotropic branches (main stem) and plagiotropic branches (fruiting stem). The orthotropic branches are straight, upward growing with monopodial growth habit, having swollen nodes with 10-15 short adventitious roots and one leaf per node. At the axil of the leaf lies an axillary bud which develops into a plagiotropic branch. The plagiotropic branches lack aerial roots that grow laterally with sympodial growth habit, and produces flowers and fruits. As the shoot grows, the terminal bud gets modified into a spike, and the axillary bud continues to grow more (Ravindran *et al*., 2000).

2.2 INFLORESCENCE AND FLOWER DEVELOPMENT IN BLACK PEPPER

Flowers (50-150) are borne in the axils of ovate, fleshy bracts of the long hanging spikes that appear opposite to the leaves on the plagiotropic branches. The apical bud of plagiotropic branches is transformed into an inflorescence (spike). The inflorescence in its early stages of development has a convex apical meristem, supported by a leaf and a bract (Tucker, 1982). The meristem of the inflorescence appears to be terminal so that the growth is sympodial.

Flowers are borne on solitary spikes opposite to the leaves, morphologically tenninal and bisexual or unisexual. Among the cultivars, variations occur with respect to the relative proportion of male, female and bisexual flowers in spikes. The composition of flowers in the spikes is governed mainly by the genotype. However, environmental factors also play an important role. The effect of climate on productivity of black pepper is more related to local weather rather than global climate patterns. In general, a high percentage of bisexual flowers is essential for effective pollination and fruit formation (Ravindran *et al.*, 2000).

2.3 VARIETIES AND CULTIVARS OF BLACK PEPPER

2.3.1 Karimunda and Panniyur-1

In India 75 cultivars/varieties of black pepper are grown. Black pepper cultivars originated from wild cultivars and the diversity of cultivars is richest in the state of Kerala. More than hundred cultivars are known and a few of them are still popular (Ravindran *et al.*, 2000).

'Karimunda' is one of the most popular among the cultivated varieties of black pepper. Other important varieties are Kottanadan, Narayakodi, Aimpiriyan, Neelamundi, Kuthiravally, Balancotta, and Kalluvally in Kerala and Billimalligesara, Karimalligesara, Doddigya, Mottakare and Uddagare in Karnataka. Some of these cultivars have been used for selection of high yielding varieties and for hybridization programmes. The first hybrid, 'Panniyur-1' was produced at Panniyur Pepper Research Station in Kerala more than three decades ago. This hybrid variety is very popular among pepper producers in India. Panniyur-1 has large berries compared to the Karimunda variety. Average yield of Karimunda is 3-5 kg per vine and that of Panniyur-1 is 1242 kg ha^{-1}.

2.3.2 Thekken: A spike branching black pepper type

Mutation in the floral meristem of black pepper could lead to the proliferation of inflorescence. A few years ago, a farmer, Shri T. T. Thomas, spotted a new spike variant of 'Thekken' with 100% of the proliferating spikes

growing in the Idukki district of Kerala (Plate 1). The vine, closely resembles the popular black pepper cultivar 'Karimunda'. The length of the spike measured 18 cm with about 150 berries per spike compared to the length of the 7-10 cm and about 30-70 berries in the normal spike (Farm Innovators, 2010).

2.4 DIVERSITY IN INFLORESCENCE ARCHITECTURE

The diversity in inflorescence architecture has not only attracted artists and plant scientists, but has also drawn the attention of plant breeders, because inflorescence traits directly related to crop yields (Doebley *et al.*, 1997). Selection for three dimensional flower arrangement has been widely exploited in many domesticated crops to increase the yield, particularly in cereals.

In many plants, inflorescence architecture is observed to be highly diverse in nature (Brown *et al.*, 2006). According to Weberling (1989), inflorescence architecture can be broadly grouped into inflorescences without branching (simple) and with branching (compound). These distinct inflorescence architectures result from different developmental programs as listed below:

Inflorescence meristem initiates directly floral meristems which have determinate meristems on their flanks and this forms a simple raceme as in *Arabidopsis* (Benlloch *et al.*, 2007; Tanaka *et al.*, 2013; Teo *et al.*, 2014).

Barton, (2010) reported the shoot apical meristem (SAM) identifies the leaf, trunk, and flower buds in unique locations based on the complex temporal interactions between proteins, microRNAs, and hormones. Tanaka *et al.* (2013) reported the developmental fate of the inflorescence meristem (IM) *i.e.,* its conversion into a floral meristem (FM) determines the type of inflorescence and is species specific.

In the inflorescence of general grasses, the inflorescence meristem (IM) gives rise to several branch meristems and these branch meristems are generally indeterminate meristems. Among all plant species, grasses have different inflorescence patterns (Kellogg *et al.*, 2013).

a. Bush pepper -Thekken

b. Spike- Thekken

Plate 1. Bush pepper and spike of Thekken

Various studies on floral development and identity genes of floral meristems in maize, *Arabidopsis*, *Antirrhinum*, cauliflower, rice etc., have confirmed that floral genes are highly conserved in the plant kingdom (Goto *et al.*, 2001; Jack, 2004; Song *et al.*, 2010).

2.4.1 Genes regulating inflorescence meristem formation

The development of the *Arabidopsis* inflorescence meristem is controlled by mutual regulation of three genes *viz.*, *LEAFY* (*LFY*), *APETALA* (*AP1*) and *TERMINAL FLOWER1* (*TFL1*) (Kaufmann *et al.*, 2010; Yoo *et al.*, 2010). These genes maintain the balance between inflorescence meristem and floral meristem identity at the inflorescence apex (Kim *et al.*, 2013; Benlloch *et al.*, 2015). From the vegetative to reproductive phase, first meristem produced during transition is inflorescence meristem.

Maizel *et al.* (2005) reported *LEAFY* (*LFY*) is strongly expressed throughout the young floral meristems from the earliest stages of development. The shoot character of the *lyf* 'flowers' is more marked in the first positions in the inflorescence, while the structures formed at the apex progressively acquire a great degree of floral identity due to the independent activation of other floral meristem identity genes such as *AP1* (Huala and Sussex, 1992; Bowmann *et al.*, 1993).

APETALA (*AP1*) gene encodes a transcription factor which belongs to the MADS-box gene family. *AP1* is directly activated by *LFY* (Wafner *et al.*, 1999) and is expressed in young floral meristems.

According to Benlloch *et al.* (2015), *TERMINAL FLOWER1* (*TFL1*) in floral initiation played a role opposite to that of *LFY* and *AP1* where it prevented the inflorescence meristems from assuming the floral identity by inhibiting the expression of *FMI* genes. Therefore, while *LFY* and *AP1* specify the identity of the floral meristem, *TFL1* specify the identity of the bud.

2.4.2 Genes regulating branch meristem formation

The hierarchy and complexity of the branching depend on the species and are also affected by environmental factors, such as nutrition, light and temperature (Tanaka *et al.*, 2013; Kyozuka *et al.*, 2014; Teo *et al.*, 2014). Genetic control of branching is a primary determinant of yield, regulating seed number and harvesting ability.

Auxin is essential for the formation of secondary axes of growth and these secondary axes are established by axillary meristems that are responsible for the formation of branches and flowers. The use of polar auxin transport inhibitors, such as N-1-naphthylphthalamic acid (NPA) has determined that auxin transport is necessary to initiate leaves during vegetative development and flowers during reproductive development (Okada *et al.*, 1991; Reinhardt *et al.*, 2000; Benkova *et al.*, 2003; Krizek, 2011).

Internode patterning is a key factor in the inflorescence architecture, and is dependent in part on the overlapping activities of two transcription factors: *BREVIPEDICELLUS* (*BP*) and *PENNYWISE* (*PNY*). Mutations in *BP* cause short internodes and downward-pointing pedicels (Douglas *et al.*, 2002; Venglat *et al.*, 2002). Mutations in *PNY* cause irregular internode elongation (Byrne *et al.*, 2003; Smith and Hake, 2003).

A member of the *PIN* family of auxin efflux transporters is the *PINFORMED1* (*PIN1*) protein of *Arabidopsis* (Galweiler *et al.*, 1998; Paponov *et al.*, 2005). In maize, conserved *PIN1* expression patterns have a conserved polar auxin transport mechanism which is necessary for the formation of all axillary and lateral primordial meristems (Gallavotti *et al.*, 2008). *PIN1* protein has been reported to be involved in the early regulation of *LFY* and aerial organ development (Vernoux *et al.*, 2000; Tanaka *et al.*, 2006).

LOG (LONELY GUY) has been repeatedly observed in all meristems of the developing panicle, including panicle branch and floral meristems when it is expressed at higher level (Kurawaka *et al.*, 2007). Yoshida *et al.* (2013) reported a

rice gene *TAWAWA1* (*TAW1*) encodes a nuclear protein with an *ALOG* domain. In contrast, loss of *TAW1* function causes reduction in inflorescence meristem indeterminacy and small inflorescences.

2.5 ROLE OF AUXIN IN SHAPING INFLORESCENCE ARCHITECTURE

Growth regulators such as auxin, cytokinin and carotenoid derivatives were reported to regulate branching architecture by interfering with the signal transduction pathway of flowering (Shani *et al.,* 2006; Kyozuka, 2007).

Auxin is one of the most important hormone which has diverse role in plant growth and development (Taiz and Zeiger, 2002). Gallavotti, (2013) reported auxin influences the elongation of the stem and regulates the formation, activity, and fate of meristems, therefore it has been recognized as an important hormone in shaping architecture of the plant.

In plants among the natural auxins, indole-3-acetic acid (IAA) is the most abundant form of auxin. In plants, auxin is transported mostly via polar transport *i.e.,* Polar Auxin Transport (PAT) which controls multiple developmental processes including shaping of inflorescence/ inflorescence branching (Muller and Layser, 2011; Yue *et al.*, 2015).

2.6 ROLE OF AUXIN RESPONSIVE GENES DURING INFLORESCENCE DEVELOPMENT

The diversity of the architecture of the plant is determined by axillary meristems (AMs) (McSteen and Leyser, 2005). The appearence of axillary meristems and lateral organs is controlled by the hormone auxin, which is transported towards the site of primordial onset and generate vegetative branches and reproductive inflorescences (Delker *et al.*, 2008).

Early events include *PIN1* protein accumulation followed by induction of auxin responsive genes. Genes expressed include organ boundary gene *Cup shaped cotyledon2* (*Cuc2*) as well as *organ polarity determinants filamentous flower* (*FIL*) *and Revoluta* (*Rev*) which help in spatially confining the axillary meristem. Finally,

meristem establishment coincides with expression of Leafy (*LFY*) gene (Heisler *et al.*, 2005).

Galweiler *et al.* (1998) reported the *PIN1* mutants have strong defects in the development of the inflorescence and have pin-shaped inflorescences, hence the name *pinformed*. The *PIN* family of polar auxin transporters functions in the efflux of auxin from cells and plays various roles in growth and development. Significance of auxin transport is further established by studies using auxin transport inhibitors in maize which affected axillary meristem initiation during reproductive development (Gallavotti *et al.*, 2008).

Key genes involved in auxin responsive meristem initiation and confinement in maize are *Barren stalk fastigiate1 (Baf1), Barren inflorescence2 (Bif2) and Ramosa2 (Ra2)*. *Baf1* is adaxial side boundary determinant. *Bif2 and Ra2* are expressed within meristem (Gallavotti, 2013).

2.6.1 *Barren stalk fastigiate1* (*BAF1*)

Gallavotti *et al.* (2011) reported the identification of a new regulator of the formation of axillary meristem in maize, the *Barren stalk fastigiate1* (*Baf1*) gene. *Baf1* encodes a putative transcriptional regulator that contains a DNA binding motif of the AT hook and a conserved domain of unknown function, called the Plant and Prokaryote Conserved (PPC) domain. *BAF1* can form homo- and heterodimers with other putative AT-hook DNA binding proteins, revealing an unknown component of this protein family. It is suggested that *Baf1* is required to reach a threshold level of *BARREN STALK1 (BA1)* expression for the initiation of maize ears.

Mutations in *Baf1* give rise to plants that either lack ears or produce fusion defects, suggesting that *Baf1* interacts at the periphery where the axillary meristem forms and plays a role in the initiation of axillary meristem. The coorthologs of *Baf1* are present in different grasses, and four potential coorthologs were identified in *Arabidopsis*, although without strong support. The individual coorthologs of *Baf1* are found in syntenic regions of *Brachypodium distachyon*, rice (*Oryza*

sativa), and sorghum (*Sorghum bicolor*), suggesting that the gene is present in all cereal species (Gallavotti *et al.*, 2011).

2.6.2 *Barren inflorescence2* (*BIF2*)

Barren inflorescence2 (Bif2) in *Arabidopsis* facilitates the subcellular localization of AtPIN1, and the *bif2* mutant inflorescences has lower levels of auxin later in development (Friml *et al.*, 2003, Michniewicz *et al.*, 2007).

Skirpan *et al.* (2008) reported that *BIF2* accumulates in the meristem region and phosphorylate the transcription factor *BARREN STALK1 (BA1)*, required for axillary activation of meristem, suggesting that *BA1* is a target of *BIF2. BIF2* in maize encodes a serine/threonine protein kinase co-orthologous to *PINOID (PID)*, and also regulates transport of auxin through direct regulation of ZmPIN1a (Skirpan *et al.*, 2009).

An intriguing speculation is that the bifunctional component of the *BIF2* kinase may provide a mechanism to activate the genes necessary for axillary meristem activation in response to auxin transport. *BIF2* kinase has multiple putative targets in the regulation of axillary meristem initiation during maize inflorescence development. (Skirpan *et al.*, 2008).

2.6.3 *Ramosa2* (*RA2*)

Branches are normally either long or short and there are no intermediates in the maize tassel. The genes of the *RAMOSA* family *viz.*, *RAMOSA1*, *RAMOSA2* and *RAMOSA3* have been cloned from maize and can form a network of genes that control lateral branching production (Tanka *et al.*, 2013). *RA1* encodes a zinc-finger domain protein, a presumed transcription factor regulates the fate of most axillary meristems (Vollbrecht and Sigmon, 2005). *RA2* is a LOB domain, presumed to be a transcription factor (Bortiri *et al.*, 2006). Cloning of the putative orthologs of *Ra1* and *Ra2* from other species indicates that the evolution of this pathway may have been involved in the evolution of the morphology of the inflorescence (Vollbrecht *et al.*, 2005; Bortiri *et al.*, 2006). *RA3* encodes a metabolic protein, a trehalose-6-

phosphatase which may be trehalose-6-phosphate (*T6P*) could play a role in the regulation of development.

Ra2 is expressed transiently in a group of cells that predicts the position of axillary meristem formation in inflorescences. *Ra2* was cloned by chromosome walking and shown to encode a *LATERAL ORGAN BOUNDARY* domain transcription factor. *Ra2* mutants show remarkable phenotypic heterogeneities in the development of inflorescence, including additional branching of the tassel, spikelet multimers and disorganized rows of ear kernels. (Bortiri *et al.*, 2006).

Ramosa2 (*Ra2*) mutant of maize involved in the branching and architecture of the floral organs, increased branching with short branches replaced by long and undetermined branches. The *Ra2* expression pattern is conserved in rice (*Oryza sativa*), barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*), and maize, suggesting that *Ra2* is critical for shaping the initial steps of inflorescence architecture of grasses (Vollbrecht *et al.*, 2005; Bortiri *et al.*, 2006).

2.7 MOLECULAR STUDIES OF GENES IN BLACK PEPPER

Vimarsha *et al.* (2014a) performed RAPD analysis to study relatedness and diversity among different black pepper varieties. He found that the variety 'Vellamundi' depicted near relativeness (74 %) with 'Thekken' compared to the wide cluster formed by local cultivar 'Karimunda'.

Vimarsha *et al.* (2014b) reported presence of sequence homology for the gene *TFL1,* which indicated the possible involvement in inflorescence branching in 'Thekken'. However, two non-spike branching varieties including 'Karimunda' and 'Vellamundi' have also shown amplification for the *TFL1* gene. *TFL1* gene is a well-known regulator of gene expression and it belongs to the MADS box transcription regulator. The expression level of the *TFL1* gene rather than its presence, may be critical for the spike branching trait.

Subba (2014) performed molecular analysis of 'Thekken' using RAPD, SSR markers and SDS-PAGE. These studies showed no difference between

branched and non-branched spikes at molecular level. Studies based on candidate genes showed difference in *PIN1* and *LOG1* profile between 'Thekken' and 'Karimunda' and absence of *BP* amplification in 'Thekken' and differential amplification of *RA2* gene.

Vivekanand (2015) reported the presence of an integrase core domain in the genome of black pepper. Differential amplification of cDNA of stage II (6-8 cm) from 'Thekken' and 'Karimunda' with *RA3* primers suggests that altered expression of the region, may play a role in the induction of spike branching in 'Thekken'. He also suggests that the presence of the integrase core domain plays a possible role of retroviral integration in differential expression.

Sooraj (2016) performed sequence analysis and expression studies of *TFL1* gene in black pepper. Expression of *TFL1* gene was observed in mature leaves and in low levels in spikes. The isolated sequences were partial orthologs of *PEBP* (Phosphatidyl Ethanolamine-Binding Protein) genes from black pepper. He also identified the flowering time and floral integrator gene *FT* in black pepper.

Bhasi *et al.* (2017) reported the role of auxin in inducing branching trait in 'Thekken' through quantification of indole-3-acetic acid (IAA) content in immature spikes using HPLC and comparing it with non-branching varieties like 'Karimunda' and 'Panniyur-1'. The study revealed that the IAA content in the spikes of branching variety 'Thekken' was significantly low compared to 'Panniyur' and 'Karimunda'.

According to Bhasi (2018) the overexpression *PIN1*, an auxin efflux carrier, noticed in 'Thekken' at the transcriptome level correlated with the lower auxin content in hormonal analysis. The twenty seven fold expression of *BP* noticed in 'Thekken' correlated with the increase in cytokinin content and the emergence of differentiated meristamatic tissues that was prominent in the histological sections of stage II (6-8 cm) of Thekken.

2.8 TECHNIQUES AND TOOLS USED FOR EXPRESSION PROFILING OF GENES

Gene expression is closely linked to its function. Real-time quantitative PCR (RT-qPCR) is a powerful and sensitive methodology to analyse the expression of target genes (Dong *et al.*, 2019). Expression profiling of genes involves DNA isolation, RNA isolation, designing of primers, specificity check of primers using genomic DNA by PCR, cDNA synthesis and quantitative real-time RT-PCR (qRT-PCR).

2.8.1 Primer designing

A primer is a short oligonucleotide which is the reverse complement of a region of a DNA template and that would bind to a DNA strand to facilitate amplification of the targeted DNA sequence. Specific amplification of the intended target requires that the primers do not have matches to other targets in certain orientations and within certain distances that allow undesired amplification. The choice of appropriate primers is one of the most important factor that affect the polymerase chain reaction (PCR). (Garg *et al.*, 2008).

While designing a primer the two important criteria to be balanced are the specificity of primers to avoid mispriming and the efficiency of primers to be able to amplify a product exponentially (Dieffenbach *et al.*, 1993). Kampke *et al.* (2001) reported the primer pairs are selected such that they will be extended towards each other to cover the given target region. Primers function in pairs, the so-called forward and reverse primer. However, to avoid mispriming, primers should not be very sticky (high G/C content) on their 3'ends (Abd-Elsslam, 2003).

Wu *et al.* (1991) reported the length of primer and the annealing temperature of PCR reaction decides its specificity. In general, the shorter the primer, the more quickly it will be anneal to target DNA and form a stable double-stranded template (Ahsen *et al.*, 2001). The primer designing is determined by several parameters and the sequences of the designed primers can have an important effect on the specificity and sensitivity of the PCR reaction.

The stability of a primer template DNA duplex can be measured by its melting temperature (T_m). Melting temperature (T_m) of oligonucleotide forward and reverse primers should be almost similar for ensuring better result. The most simple, popular and roughly accurate prediction of T_m can be calculated as $T_m = 2\ (A+T) + 4\ (G+C)$, as given by Suggs *et al.* (1981), whereas melting temperature of primers can also be calculated by using the formula given by Wallace *et al.* (1979).

Stronger hydrogen bonding of G and C bases helps to promote proper primer binding if they are present at 3' end (Sheffield *et al.*, 1989; Garg *et al.*, 2008). Melting temperature and annealing temperature of primer are dependent on each other. Primers should maintain a reasonable GC content between 50 and 60% (Rychlik *et al.*, 1990; Wu *et al.*, 1991; Garg *et al.*, 2008). If GC content of a primer is higher, melting temperature will be higher, as the bond between G and C is bound by three hydrogen bonds.

Annealing temperature is usually calculated as five degree celsius lower than the estimated T_m (Dieffenbach *et al.*, 1993; Abd-Elsalam, 2003). In order to inhibit the formation of either hairpins or primer dimerization, it is important to have a minimum intra-molecular or inter-molecular homology between primers (Abd-Elsalam, 2003). Primer dimer can supress the PCR reaction with no amplification and usually primer of 20-24 bases and GC content between 45-60 percent with T_m of 52-58 ^{0}C works finest in most applications (Patel and Prakash, 2013).

2.8.2 DNA Isolation

Numerous DNA extraction protocols in plants have been reported for the purpose, but none is found to be universally applicable (Varma *et al.*, 2007). This could be primarily due to the variations in the composition of primary and secondary metabolites even among the different tissues of the same plant, which makes the isolation process requiring to be adjusted as tissue specific or plant specific (Dhanya and Sasikumar, 2010).

Methods used for DNA extraction and quality of genomic DNA acquired could affect downstream and analytical techniques (Boiteux *et al.*, 1999; Fredicks *et*

al., 2005). Several forms of contaminants in the DNA can reduce the activity of restriction endonucleases, polymerases and ligases (Shoida *et al.*, 1988; Richards *et al.*, 1988; Maltas *et al.*, 2011; Sahu *et al.*, 2012). Isolation of DNA from plant tissues with high levels of polysaccharides and polyphenol contents is very difficult.

Presence of polysaccharides in DNA samples may become problematic and it may cause inhibition of *Taq* polymerase (Fang *et al.*, 1992). Polyphenol oxidation and co-precipitation causes the browning of the DNA (Varma *et al.*, 2007; Mishra *et al.*, 2008). Antioxidants like β-mercaptoethanol, ascorbic acid, sodium azide, bovine serum albumin (BSA) and polyvinylpyrrolidone (PVP) are used in DNA isolation methods to reduce the problems related to phenolic compounds during DNA isolation (Horne *et al.*, 2004). Oxidation of polyphenols causes browning of DNA samples which can be avoided by the use of PVP and it removes polyphenols from mature, damaged and improperly stored leaf tissues.

Dhanya *et al.* (2007) reported that isolation of DNA from black pepper berries used potassium acetate (5 M) which helped in eliminating most of secondary metabolites and polysaccharides from the DNA and the use of PEG for precipitation of DNA resulted in comparatively purified homogenous DNA without proteins and polysaccharides.

Lodhi *et al.* (1994) reported that the modified protocol reported by Doyle and Doyle (1990) including high concentration of NaCl in the buffer can remove polysaccharides. The addition of NaCl at concentrations greater than 0.5 M, together with Cetyl trimethyl ammoniumbromide (CTAB), removes polysaccharides during DNA extraction (Paterson *et al.*, 1993; Moreira and Oliveira, 2011; Sahu *et al.*, 2012). Sambrook and Russell, (2001) reported CTAB does not precipitate nucleic acids in high ionic strength solutions and form complex with proteins. Therefore CTAB is useful for the purification of nucleic acids from plants with large amounts of polysaccharides

Subba *et al.* (2014) isolated genomic DNA from mature leaves and spikes of black pepper by using modified CTAB method using high concentrations of CTAB,

NaCl, EDTA and PVP in DNA extraction buffer. Isolated DNA was of good quality and high concentration.

2.8.3 RNA Isolation

RNA is highly susceptible to degradation, hence the isolation requires special care and precautions (Kojima and Ozawa, 2002; Buckingham and Flaws, 2007). The extraction of good quality RNA depends on the RNAse free technique and good laboratory practices.

RNase is a thermo stable enzyme and is present ubiquitously and renatures after heat denaturation making RNA very unstable (Buckingham and Flaws, 2007; Tadokoro and Kanaya, 2009). RNases problem can be eliminated by using RNase free equipment, glassware and chemicals used for RNA extraction. They should be maintained and stored separate from common lab equipment and treated with harsh chemicals that destroy RNase (Kansal *et al.*, 2008; Rezadoost *et al.*, 2016). Strong denaturants used to inhibit endogenous RNases are mostly harmful and corrosive agents.

Several methods of RNA extraction are based on the denaturation, separation and elimination of proteins, polyphenols and polysaccharides (Hu *et al.*, 2002; Rai *et al.*, 2010; Ghawana *et al.*, 2011; Dash, 2012). Portillo *et al.* (2006) reported efficiency of RNA extraction depends on the type of reagents and homogenisation procedures used. In general, the extraction protocols use reagents such as cetyl-trimethyl ammonium bromide (CTAB), acidic guanidinium thiocynate, Sodium dodecyl sulfate (SDS), phenol, chloroform, sodium acetate, lithium chloride among other reagents, inorder to obtain pure RNA samples without proteins, polysaccharides, and other polyphenolic compounds (Gesteira *et al.*, 2003; Rezadoost *et al.*, 2016).

RNA samples can be isolated by using TRIzol reagent (Ahmann *et al.*, 2008). During sample homogenization, TRIzol reagent disrupts cells and dissolves cell components and maintains the integrity of RNA. The mixture separates into an aqueous phase and an organic phase. RNA remains in the aqueous phase after

addition of chloroform followed by centrifugation. Aqueous phase is transferred to fresh tube and RNA can be precipitated with isopropanol (Chomczynski, 1993). Liu *et al.* (2018) used polyvinyl pyrrolidone (PVP) during the extraction procedure to reduce phenolic compounds and polysaccharides.

2.8.4 Quantity and purity of nucleic acids

Spectrophotometry can be used to detect concentrations and purity of the DNA (Sambrook *et al.*, 1989). This method of identification of quantification and assessment of purity of DNA samples uses absorbance values at 260 nm and 280 nm (Glasel, 1995). Pure DNA would give concentration of 50 µg ml^{-1} and RNA of 40 µg ml^{-1} when the absorbance value at 260 nm is 1.0. Maniatis *et al.* (1982) reported that the ratio of absorbance at 260 nm and 280 nm for DNA sample should be around 1.8 and for RNA it should be around 2.0.

2.8.5 PCR (Polymerase Chain Reaction)

Polymerase chain reaction (PCR) is a very sensitive technique and can be used to detect and amplify nucleic acids from any source, regardless of quantity and combination (Joshi *et al.*, 2010; Krohn-Molt *et al.*, 2013; Culley *et al.*, 2014; McCall *et al.*, 2015). Mullis *et al.* (1986) reported that PCR uses *in vitro* enzymatic reaction to amplify specific DNA fragment and the process is an exponential amplification of DNA fragments because the products of each cycle serve as templates for the next cycle. The technique uses multiple cycle of template denaturation, primer annealing, and primer extension and after 20 to 40 such cycles, enough amplified product is generated and can be visualized on an agarose gel using a specific staining method.

Components of PCR reaction includes template, primers, magnesium ion, dNTPs, buffer for PCR reaction, and thermostable DNA polymerase enzyme (Mullis *et al.*, 1986). The template used can be DNA; RNA or cDNA. Primers decide length of the amplicons (Saiki *et al.*, 1985). Zamft *et al.* (2012) reported that *Taq* DNA polymerase is the most commonly used thermostable DNA polymerase and it is suitable for routine amplifications. The magnesium ion acts as cofactor for *Taq*

polymerase. Its concentration affects the enzyme activity, primer annealing, melting temperature of the template and the PCR Product.

2.8.6 Real time *PCR*

Real-time quantitative PCR (RT-qPCR) is a sensitive and accurate method for detecting expression levels of gene transcripts in various organisms. Unlike other quantitative PCR methods, real-time PCR does not require the handling of the post-PCR sample, which avoids possible contamination of the PCR product and generates much faster er and higher throughput assays. Compared to traditional PCR, which is based on observing the brightness of the electrophoretic PCR band, RT-qPCR is a real-time quantitative measure for the PCR product, allowing amplification and detection simultaneously. The real-time PCR method has a very large dynamic range of initial determination of the target molecule (at least five orders of magnitude) (Bustin 2002; Wang *et al.*, 2015).

Prior to using real time quantitative PCR to quantify a target message, care must be taken to optimise RNA isolation, primer design, and PCR reaction conditions so that accurate and reliable measurements can be made (Mazaika and Homsy, 2014; Armstrong and Schulz, 2015).

Gilliland *et al.* (1990) reported to minimize the errors and correct the sample to sample variation, a cellular RNA is instantaneously amplified with the product, which serves as an internal reference against which other RNA values can be normalised. For normalisation the most common genes used are housekeeping genes. For each specific experiment, selection of housekeeping gene should be made very carefully, as the reliability of the results according to the cells of interest and specific experimental treatments depends on the choice of the most relevant house-keeping gene. Theoretically these genes should be expressed at a constant level among different tissues of an organism at all stages of development, and their expression levels should be constant in different experimental conditions.

Actin is a major component of the plant cytoskeleton. Also the most widely used reference gene for normalization of data in real time quantitative PCR

(Pohjanvitra *et al.*, 2006). Gines *et al.* (2018) predicted the expression stability of most commonly used housekeeping 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. *Actin* has been found to be the most stable gene in different tissues and also under heat and drought stress. Jaiswal *et al.* (2019) evaluated the expression stability of ten candidate reference genes in various tissues of *Cyamopsis tetragonoloba* for gene expression analysis of RT-qPCR during seed development and under abiotic stress conditions.

Ririe *et al.* (1997) reported that the presence of any dsDNA generates fluorescence, specificity of this assay decreases considerably due to the amplification of non-specific PCR products and primer-dimers. The software is configured to acquire fluorescence above the melting temperature of primer-dimers' but below that of the target. Longer amplicons creates a stronger signal. In single plex reactions generally, SYBR Green is used; however, multiplex reactions can be used when coupled with melting point analysis (Arya *et al.*, 2005).

Morrison *et al.* (1998) reported that SYBR Green 1 is a non-sequence specific fluorogenic minor groove DNA-binding dye that intercalates into dsDNA. Fluorescent measurements are performed at the end of elongation step of each PCR cycle to control the increasing amount of amplified DNA. SYBR green 1 exhibits little fluorescence when it does not bind to the solution, but emits a strong fluorescent signal upon binding to the dsDNA. An increase in the fluorescent signal occurs sduring polymerisation and this decreases when DNA is denatured. The advantage of this technique is that it is relatively cheap as it can be used with any pair of primers for any target. Silvar *et al.* (2005) reported that real-time polymerase chain reaction (RT- qPCR) assay with SYBR Green was found to be sensitive and robust enough to determine *Phytophthora capsici* infection and resistance to *Phytophthora* root rot in different genotypes of black pepper.

During melt curve analysis, the real-time machine continuously monitors the fluorescence of each sample as it is slowly heated from a user defined temperature

below the T_m of the products to a temperature above their melting point. Fluorescent dye is released upon melting (denaturation) of the double-stranded DNA, providing accurate T_m data for every single amplified product. Melting peaks are calculated by taking the differential (the first negative derivative (–dF/dT) of the melt curve. These peaks are analogous to the bands on an electrophoresis gel and allow for the qualitative monitoring of products at the end of a run. Short primer dimers will melt at lower temperature than longer, target amplicon products (Nolan *et al.*, 2006).

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Expression profiling of auxin responsive genes during inflorescence development in black pepper (*Piper nigrum* L.)" 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.

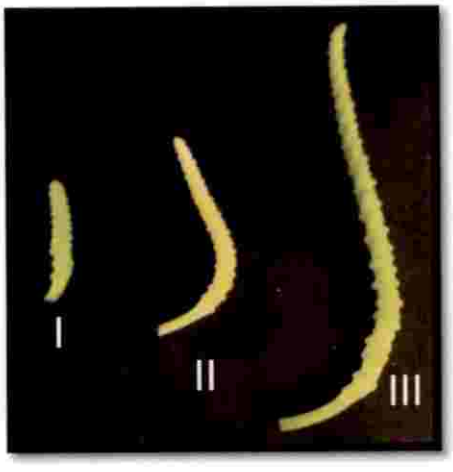
3.1 COLLECTION OF PLANT SAMPLES

Spike samples of 'Thekken' was collected from Idukki district and spikes of non-branching varieties *viz*., 'Karimunda' and 'Panniyur-1' were collected from Coconut Research Station (CRS), Balaramapuram, Thiruvananthapuram at three different developmental stages *viz*., stage 1 (1-2 cm), stage 2 (6-8 cm) and stage 3 (9-12 cm) from two different plants of each cultivar (Plate 2).

3.2 PRIMER DESIGNING

Nucleotide sequences of black pepper transcriptome available at Rajiv Gandhi Institute of Biotechnology, Poojappura, Thiruvananthapuram were used for designing of primers (Fig 1, 2 and 3). The transcriptome sequences were analysed using "Blastn software" for designing gene specific primers for *Barren stalk fastigiate1 (Baf1), Barren inflorescence2 (Bif2) and Ramosa2 (Ra2)* (Table 1). The forward primer designed was subjected to '*Oligos*' for synthesising its reverse primer. Factors such as primer length, annealing temperature, GC content, potential hair pin formation and 3' complementarity were analysed by using 'Primer Express' and 'mfold Server'. Primers were synthesised at Xetra Bio Solutions, Poojappura, Thiruvananthapuram. Amplification efficiency of the gene specific primers was determined by standard curve analysis and "Lin Reg" software.

a. Thekken **b. Karimunda**



c. Panniyur-1

I- 1st Stage
II- 2nd Stage
III - 3rd Stage

Plate 2. Spike samples at different developmental stages of varieties/cultivars

>2464340

GCACATGTTATAGCTGCACTTCACATTAATCCAAAGCACAATAATGCA
TAGACAATTATTCAAACAAGCTGAGAAATAGCTGGAAAATAATATTA
TTCTTACAGATCAGATTATAAATATAATTAAGAGCAACATTAATATAC
TTATCATCATGATCATCTCAAAGGCCTCGTCTTTCTTTACTCCGGCAAC
GCCATCTATGTCCTCCAACTCCAACCAAGGTATAGAAGTTCTGATCAA
AAGGGTGGCCTTGCATGGCTCCACCCATACCCGTCGTGAGCCAGCTGC
GCCACATTAGCCAAAAGGTTCGGCGGCAGGTTATACACCGGCAACGC
CGATGGGTCTTGCATGCCATGCCCGCCGCCGATCCCCGGCGGCGACCC
GCCGTCCCCTCCACCGCCTCCTCCCCCCATTCCGCCGCTGCTCCCGGC
GTCCTCCTCCTCGTCCAACGGCAGCCGCTCATAGGTCGCGTTGGCAAA
AGTGGCGGCGATCACCATCACCGGCCCCGACGCCACCAAAGGCCCGA
CAACGCTGCCTCCGACGACCTGCCCCTGCCCGCCAGCGAGATAGACG
GTGAGCCCCGTCGAGCCCGGCGGCGCCGGGCCCGGCAAGAAGGAGCC
GGTGAGGGAGAGGATTTCGAACCGGCCTTGGAGGGCGACGACGGCGC
CGGGTGCGGTGGGCTGCCGGAGGGTCACGTTGGCGACGGCGCCGGTG
CCACTCAAGACGCAGACGCCGCGCTGGCGGCGGCGGGCTAACTGGGC
GACGCAGTCGGCGATGTCGGCTCCGCTGGCCACCTCCATGACGTTGCT
ACGAAGGGAGTTGGGGCTTTCCCTGGTGACGAAGATGGGCGGCTTGG
GCCTGTTCTTCGAGCCAGGCGGTCGCCCTCTGGGTCGGCGGGTGCCGA
CCTCGACGGCTCCCTCCTTGGGCTCGTCGTTGTTGTCCCGCTCTTCGTC
TTCGACGTTGCCTCGGTCGTTGGTGTTGGTGTTGGTGTTGCTGCTTTCC
GGGTCGGTGAGTGGCTCCATATTCTTCATGCCGAGACGTTCGGCCCTG
AGGTCGACGCTGGCGAGGCCCGGAGGCCCCGCCCACCACCGCGCCGC
CAGTGTTCCTATCTAGCTTGGCGAACCGAGCTCCCCTTCCCGAAGCTA
GCTTAGGCAGGTTGCGATTGAAGATCAGATAGATAGAGAATTCTCCTC
AATTCTTGATCATCTGTCACACCTCCAATTAGTGGTACTG

Fig. 1. FASTA format of 143 bp sequence of *BAF1*

>2455011

TTTAGATTGCTTAAACGGCTTGGCTGTGGGGATATTGGCAGTGTATAT
TTATCTGAGTTGAGTGGAACAAGATGCTATTTTGCAATGAAGGTAATG
GACAAAGCATCACTTGCCAGTCGGAAGAAGTTGCCAAGGGCCCAGAC
TGAAAGAGAGATTTTACAGCTTCTGGATCACCCTTTTTTACCAACATT
ATATACACATTTTGAGACAGACAGATTCTCATGTTTGGTCATGGAGTA
TTGCCCAGGAGGTGATTTGCACACTTTGAGGCAGCGACAGGCAGGAA
AGCACTTCCCTGAATTTGTAGCTAGGTTCTACGCAGCGGAGATTCTGT
TGGCTCTTGAGTATCTTCACATGCTTGGAGTTGTTTACAGGGATTTGA

AGCCAGAAAATGTTCTAGTACGTGATGATGGTCACATAATGCTATCTG
ACTTTGACCTCTCTCTTAGATGTGCAGTCTCCCCAACCCTCATAAAGTC
ATCCTCTGATTCTGATCCTTCTAAACGAGGCTTGGGAGGTTTCTGTGT
ACAGCCAATTTGCATTGAACCTGGTTCTGTTTGCATACAGCCTGCGTG
CTTCATGCCGAAGATGTTCTCTTCCAAGGAAAAAAAGAAGAATCAAA
AACCACGTCCTGAACCTGGGCCAATCTCCTCCACTTCTTTGCCAGAGC
TCATAGTAGAGCCTACTACAGCACGGTCGATGTCATTTGTGGGTACCC
ACGAGTACCTTGCTCCAGAAATTATCAAGGGAGAAGGCCATGGGAGT
GCAGTAGATTGGTGGACCTTTGGCATTTTTCTCTATGAGCTCTTGTATG
GAAAGACACCATTCAAGGGCTCTGGTAACCGAGCTACACTGTTCAAT
GTAGTAGGACAGCAGCTCAGATTTCCTGAATCACCATCAACAAGTTAC
TCAAGCAGAGATTTGATAAGGGGGCT

Fig. 2. FASTA format of 125 bp sequence of *BIF2*

>2450025
TTGAATTGCTTTCACTGATATTACTTCTCGAGTTATACAGTTGCTACAT
TAACTCTTGGCACAAGATTACAAATGAAAGGTGATGAATCCTCATTAG
CCGCATCACCCCTTAATTTGCTACCACTAGTACCAATCCTAATTTAGTC
CTAATCAATTTTAGTCTCCTTATTAGAAAATGCAAGTTTAAGGCTCTG
GATGACAAGTTCATGTCCATAGTGGTTCCCAGGCCGTGGAGCCGAAG
CCCGGGGAGTCGACGTCGAATCCTGCGGCTGCACCGCTGCTCGGGGG
GCTGGCCGAGTAGTAGTTTATGTCGGCATAAGAAGGGGGGGTCAGGG
AATAGACAATGGCAATGAGGCTATCTTGTTGGGTCTTCACTCCCACCA
CCTCCGCCTGCGCCTTGGCCAGCTCCTCCTGCAACTGGCTCACTTGCTT
CTGCAGCTGGAACACCACCCCCGCGCAGCCGTAGACCGGATCGCGAA
TTCGGGCGTTGGCCTCGTACACCATGCTGCTCACGGCATCCGCCCGCT
GCTCCTCCTTCAGTTCTTGAAGCATCTTGATAATGTTGCTGGCACCGA
ACACCCGGTGCGCGGTGGCGAACTTGAGCGGCTCGGAAGGGGGGAAG
TAGGGCGCGAGCACGCACTTCTCGACGCACCGCCGTCGGAGGATCTT
GCATGCGGCGCAGGGGCTGACGACCACCAGCGGTGGAGGGGACGGC
GACAAAGACGGCGACCTAGGAGTGTGTGCTGCAGCTGGGGATGTTGC
ACTCCCTTCCATGGACTCCATTTTTGTTGACACAGACCAACCTCCCAT
GGACTCAAGGTAGCTACCACTCCAAACACTGACTCTTT

Fig. 3. FASTA format of 163 bp sequence of *RAMOSA2*

Table 1. Accession number of *Barren stalk fastigiate1 (BAF1), Barren inflorescence2 (BIF2)* and *Ramosa2 (RA2)* from related species

Primer	Accession number
Barren stalk fastigiate1 (*BAF1*)	*Elaeis guineensis* XM_019855371.2
Barren inflorescence2 (*BIF2*)	*Juglans regia* XM_018999065.1
Ramosa2 (*RA2*)	*Ananas comosus* XM_020231650.1

3.3 ISOLATION OF GENOMIC DNA

Genomic DNA was isolated from spike samples of Thekken, Panniyur-1 and Karimunda by CTAB (Cetyl trimethyl ammonium bromide) method (Subba *et al.*, 2014).

Spike samples of different cultivars were cryogenically ground in a mortar and pestle after chilling in liquid nitrogen. CTAB extraction buffer (Appendix-1) containing 0.2 % β-mercaptoethanol and 2 % PVP (Poly Vinyl Pyrrolidone) was added to the crushed spike samples. The homogenized tissue was transferred to sterile 2 ml microfuge tubes and incubated for 30 min at 60 ^{0}C in a recirculating water bath. After incubation, CTAB/plant extract mixture was centrifuged at 10000 rpm for 10 min to remove the cell debris. Then the supernatant was transferred to clean microfuge tubes.

To eliminate protein contamination, 500 µl of chloroform-isoamyl alcohol (24: 1) was added to each tube and the solution was mixed by gentle inversion. After mixing, the tubes were centrifuged at 10,000 rpm for 10 min. The upper aqueous phase containing DNA was transferred to clean microfuge tubes.

For the precipitation of DNA, an equal volume of ice cold absolute ethanol was added to each tube. It was mixed well and incubated at -20 ^{0}C for 30 min. The precipitate was pelleted by spinning the tube at 10000 rpm for 7 min to form a pellet. The supernatant was removed and the DNA pellet was washed by adding ice cold 70 % ethanol. After washing, the DNA was spun into a pellet by centrifuging at 10000 rpm for 5 min. The supernatant was removed and the DNA pellet was allowed to dry for15 min. The DNA was resuspended in 50 µl sterile DNase free water and stored at -20 ^{0}C.

3.3.1 Quantity and Quality of DNA

The quantity and quality of isolated DNA were measured by using agarose gel electrophoresis and taking the absorbance value using a

Spectrophotometer (EPPENDORF Bio spectromer) at wavelengths 260 nm and 280 nm. The DNA samples were diluted before taking absorbance, using nuclease free water.

An absorbance value of 1.0 at 260 nm indicates that 50 ng μl^{-1} of DNA is present in the solution. The concentration of DNA in the sample was determined by the formula:

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

Ratio of absorbance at 260nm to 280nm (A_{260}/A_{280}) indicated DNA purity.

3.3.2 Agarose gel electrophoresis

Quality of the isolated DNA was assessed by Agarose gel electrophoresis in a horizontal gel electrophoresis unit (BIO-RAD, USA). Agarose (0.8 Percent) was prepared by boiling in 1X TBE (Tris Borate EDTA) buffer (Appendix II). Once the agarose was cooled, ethidium bromide was added at a final concentration of 0.5 µg/ml. It was mixed well and the solution was poured into gel tray with comb placed in gel casting apparatus. After 20-30 min the solidified gel was placed in the buffer tank and completely immersed in TBE buffer. The comb was removed carefully to form wells. DNA (5 µl) mixed with 6X gel loading dye (2 µl) and nuclease free water (5 µl) was poured 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 (BIO-RAD) using the software 'Image Lab'.

3.3.3 Specificity check of primers

The genomic DNA isolated from the spikes of Thekken, Panniyur-1 and Karimunda were amplified using gene specific primers for *Barren stalk fastigiate1 (Baf1), Barren inflorescence2 (Bif2)* and *Ramosa2 (Ra2)*. A standard PCR mixture was prepared for 20 µl total volume containing 50 ng of template

DNA, 0.2 mM dNTPs, and 400 nM each of primers, 1 unit of Taq polymerase and 1X PCR buffer (Table 2).

The amplified products were separated on agarose gel (1.2 percent) and observed in a gel documentation system.

3.4 ISOLATION OF RNA

From spikes at different developmental stages of Thekken, Panniyur-1 and Karimunda, the total RNA was isolated by using TRIzol reagent. Mortar and pestle, microtips, microfuge tubes, forceps and reagents were sterilised by double autoclaving. DEPC (Diethyl pyrocarbonate) treated water was used for reagent preparation.

RNase away and RNase Zap were used for removing RNase contamination. Chilled mortar and pestle wiped with RNase away was used for grinding the spike samples of different stages into a fine powder using liquid nitrogen. 1 ml of TRIzol reagent was added to the powdered tissue in mortar and gently mixed to homogenize the mixture and incubated at room temperature for 5 min. The homogenate was transferred to a 2 ml pre chilled microfuge tube. To remove protein contamination, 0.2 ml chloroform was added and shaken vigorously for 15 sec and incubated at room temperature for 5 min. The sample was kept on ice for 10 min and then centrifuged at 12000 g for 15 min at 4 ^{0}C. Aqueous phase of the sample was transferred to a fresh tube. Then 0.5 ml ice cold isopropanol (100 %) was added to each tube and incubated at room temperature for 10 min for precipitation of RNA. The tubes were mixed by inverting slowly and the sample were centrifuged at 12000 g for 10 min at 4^{0}C. The supernatant was removed and pellet was washed with 1 ml of 75 % alcohol (in DEPC treated water). The sample was vortexed briefly and centrifuged at 7500 g for 5 min at 4 ^{0}C and dried for 30-40 min in RNase free laminar air flow chamber. The RNA pellet was re suspended in 30 µl RNase free water and incubated at 55-60 ^{0}C for 10 min and stored at -20 ^{0}C.

Table 2. Thermal profile for amplification of genes (*Baf1*, *Bif2* and *Ra2*)

Step	Stage	Temperature (^{0}C)	Duration
1	Initial denaturation	95^{0}C	2min
2	Denaturation	92^{0}C	15sec
3	Annealing	55^{0}C	15sec
4	Extension	72^{0}C	45sec
5	Final extension	72^{0}C	5min

The steps 2-4 were allowed to repeat 40 times.

Quality of the total RNA was estimated by agarose gel electrophoresis. RNA (5 µl) mixed with 6X gel loading dye (2 µl) and DEPC treated water (5 µl) was poured into wells using a micropipette on agarose gel (1.2 percent). 1 kb marker was added to know the size of different RNA bands.

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

$$\text{Concentration of RNA (ng } \mu l^{-1}) = A_{260} \times 40 \times \text{dilution factor}$$

RNA purity was estimated by taking the ratio of absorbance at 260 nm to 280 nm (A_{260}/A_{280}).

3.5 PREPARATION OF cDNA

cDNA was synthesised from the isolated total RNA (Verso cDNA synthesis kit, Thermo Fisher Scientific). It includes verso reverse transcriptase enzyme, RNase inhibitor, oligo dT primer, Random hexamer and RT enhancer for removing DNA contamination.

The composition of reaction mixture (20 µl) for cDNA synthesis is as follows:

5X cDNA buffer	:	4 µl
dNTP mix	:	2 µl
Oligo dT	:	0.5 µl
Random Hexamer	:	0.5 µl
RT enhancer	:	1 µl
Verso enzyme mix	:	1 µl
RNA	:	4µl

Nuclease free water : 7 µl

Total volume : 20 µl

The contents were mixed well and incubated at 42 ^{0}C for 30 min followed by another incubation at 92 ^{0}C for 2 min in order to inactivate the RT enhancer and the cDNA samples were stored at -20 ^{0}C.

3.5.1 Quality check of cDNA

cDNA synthesised was checked by PCR using gene specific primers for *Barren stalk fastigiate1 (Baf1), Barren inflorescence2 (Bif2)* and *Ramosa2 (Ra2).* A standard PCR mix was prepared for 20 µl total volume containing 50 ng of template DNA, 0.2 mM dNTPs, and 400 nM each of primers, 1 unit of Taq polymerase and 1X PCR buffer as per Table 2.

The amplified products were separated on agarose gel (1.2 percent) and observed in a gel documentation system.

3.6 DIFFERENTIAL EXPRESSION ANALYSIS USING REAL-TIME QUANTITATIVE PCR

To study the differential expression of auxin responsive genes *viz., BAF1*, *BIF2* and *RAMOSA2* in the above mentioned cultivars in different developmental stages, real-time quantitative PCR was done. Housekeeping gene *Actin* and non-template (without cDNA) were kept as control.

SYBR green, an intercalating dye, was used for generating fluorescence. Reaction mixture (20 µl) was prepared as follows:

2X Real time PCR Smart Mix : 10 µl

Forward primer (10 pmol/µl) : 0.6 µl

Reverse primer (10 pmol/µl) : 0.6 µl

Template cDNA : 3.0 µl

Nuclease free water : 5.8 µl

The thermal profile of the real time PCR programme was set with initial denaturation temperature of 95 ^{0}C for 2 min, followed by 40 cycles of denaturation at 92 ^{0}C for 15 sec, annealing at 55 ^{0}C (wrt primer) for 15 sec, and extension at 72 ^{0}C for 45 sec. The final extension was set at 72^{0}C for 5 min.

The data generated was analysed using the "qbase plus" software using *Actin* as the reference gene. The data was subjected to melt curve analysis and the results were interpreted based on the most repeated pattern noticed among the two replications.

Results

4. RESULTS

The results of the study entitled "Expression profiling of auxin responsive genes during inflorescence development in black pepper (*Piper nigrum* L.)" carried out at the Department of the Plant Biotechnology, College of Agriculture, Vellayani, during 2017-2019 are presented in this chapter.

4.1 PRIMER DESIGNING

Nucleotide sequences of black pepper transcriptome was analysed using "Blastn software" for designing gene specific primers *viz., Barren stalk fastigiate1 (Baf1), Barren inflorescence2 (Bif2)* and *Ramosa2 (Ra2)* genes of black pepper.

The designed forward primer was subjected to '*Oligos*' for synthesising its reverse primer. Factors such as primer length, annealing temperature, GC Content, potential hair pin formation and 3' complementarity were analysed by using "Primer Express" and "mfold Server" and none of the designed primers exhibited hairpin formation and 3' complementarity. Sequence of primers designed for *BAF1, BIF2* and *RAMOSA2* are shown in Table 3.

4.2 ISOLATION OF GENOMIC DNA

Genomic DNA was isolated from spike samples of 'Thekken', 'Panniyur-1' and 'Karimunda' using modified CTAB method. Extracted genomic DNA showed the presence of unsheared DNA bands by agarose gel electrophoresis (Plate 3).

4.2.1 Quantity and Quality of Isolated DNA

Quality and quantity of the extracted genomic DNA analysed using the absorbance value, using the spectrophotometric method are presented in Table 4.

4.2.2 Specificity check of primers with genomic DNA

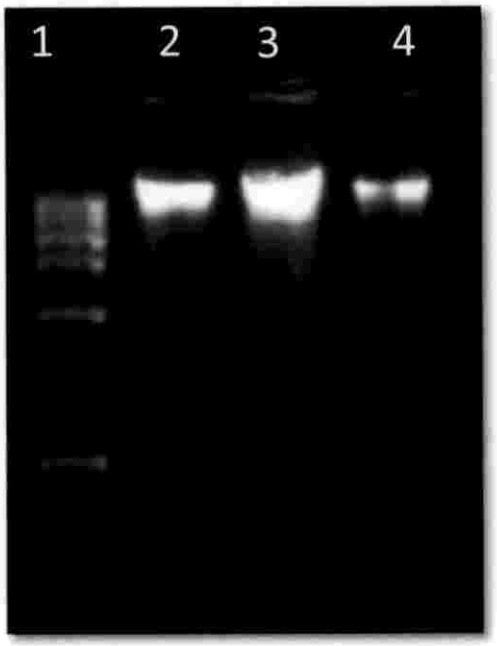
Specificity of the designed gene specific primers for auxin responsive genes *BAF1, BIF2* and *RAMOSA2* was checked by PCR using genomic DNA.

Table 3. Forward and Reverse primers designed for *BAF1*, *BIF2* and *RAMOSA2*

Primer	5'....3' gene sequence	Tm (^{0}C)	GC content (%)	Amplicon size (bp)
BAF1	FP: ACGAGCCCAAGGAGGGAG	60.5	67	143
	RP: CTGGCCACCTCCATGACGT	61.0	63	
BIF2	FP: CCAGGAGGTGATTTGCACACT	59.8	52	125
	RP: CAACTCCAAGCATGTGAAGATACTC	61.3	44	
RAMOSA2	FP: CGTCGAGAAGTGCGTGCTC	61.0	63	163
	RP: CGTTGGCCTCGTACACCAT	58.8	58	

Table 4. Quality and Quantity of isolated genomic DNA

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



Lane1: 1 kb ladder
Lane2: Thekken
Lane3: Panniyur-1
Lane4: Karimunda

Plate 3. Gel Profile showing genomic DNA of varieties/cultivar

Amplification of the genomic DNA with primer designed for *BAF1, BIF2* and *RAMOSA2* produced an amplicon of expected size 143 bp, 125 bp and 163 bp respectively (Plate 4). But in case of *RAMOSA2*, one more additional band of 300bp was obtained.

4.3 AMPLIFICATION EFFECIENCY OF PRIMERS

Data generated was in cluster by standard curve analysis. Amplification efficiency of the designed primers was determined by "Lin Reg software" and the details are presented in Table 5. All the primers exhibited cent per cent amplification efficiency.

4.4 ISOLATION OF RNA

Total RNA was isolated from the spikes of three developmental stages of 'Thekken', 'Panniyur-1' and 'Karimunda' using TRIzol method. Three distinct intact rRNA bands with no apparent degradation were observed on agarose gel (2 %) (Plate 5).

4.4.1 Quantity and quality of isolated RNA

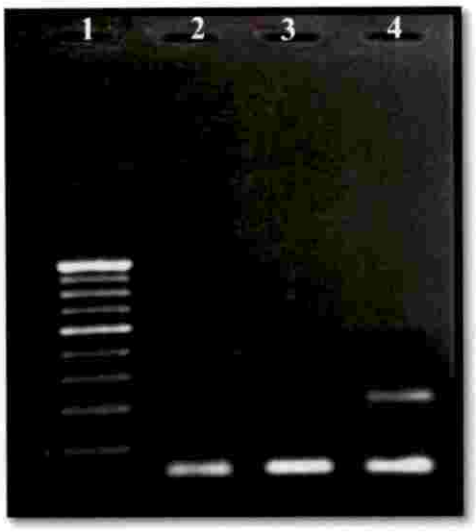
Quality and quantity of the isolated RNA analysed using the absorbance value, using the spectrophotometric method are presented in Table 6.

4.5 PREPARATION OF cDNA

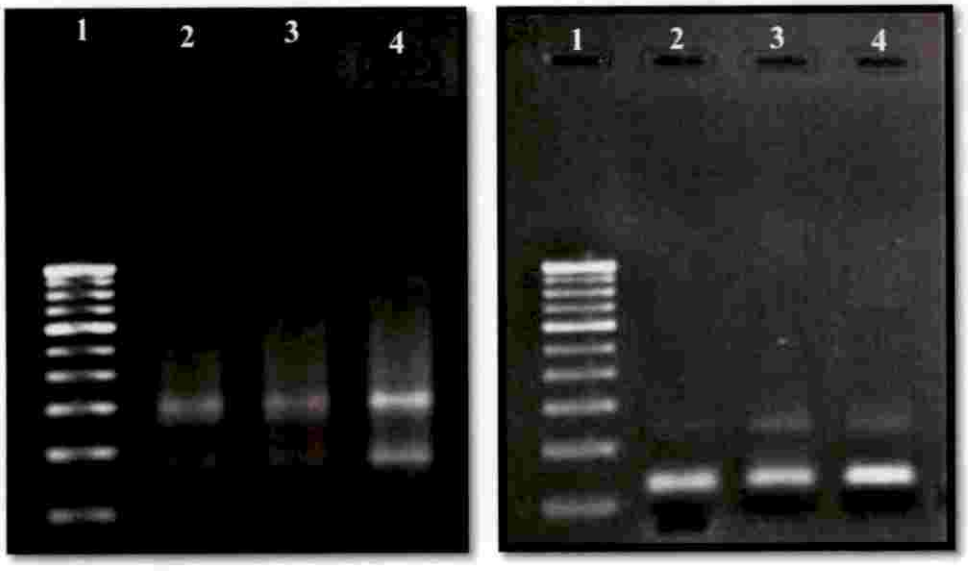
Isolated RNA was reverse transcribed to cDNA using cDNA Verso synthesis kit (Thermo Fisher Scientific). Quality check of cDNA was done by PCR using all the primers.

4.5.1 Quality check of cDNA

Quality of cDNA synthesised was checked by PCR using *Actin* primer (Plate 6) and primers for auxin responsive genes *viz.*, *BAF1*, *BIF2* and *RAMOSA2* (Plate 7, 8 and 9). Amplification of cDNA with all the gene primers produced amplicons of expected size indicating specificity of the synthesised primers



a. Amplicon obtained from genomic DNA using primer *BAF1*



b. Amplicon obtained from genomic DNA using primer *BIF2* and *RAMOSA2*

Lane1: 100 bp ladder
Lane2: Panniyur
Lane3: Karimunda
Lane4: Thekken

Plate 4. Gel Profile showing PCR amplicon obtained from genomic DNA using the primer *BAF1*, *BIF2* and *RAMOSA2*

Table 5. Amplification efficiency of primers using "Lin Reg" software

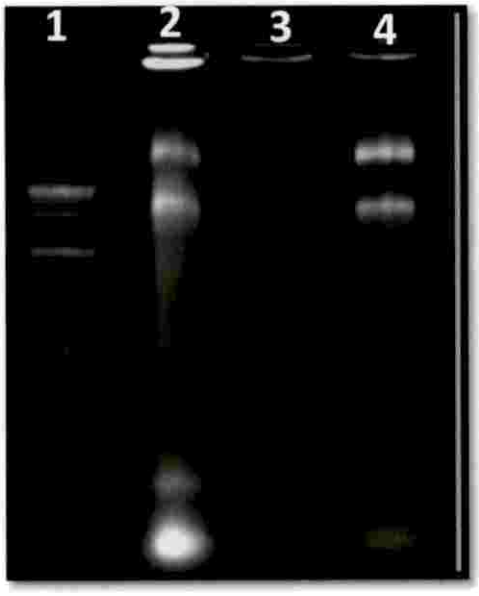
GENES	AMPLIFICATION EFFECIENCY
Barren stalk fastigiate1 (BAF1)	1.964
Barren inflorescence2 (BIF2)	1.960
Ramosa2 (RA2)	2.030

Table 6. Quality and quantity of isolated total RNA

Stage	Variety	RNA concentration (ng/µl)	A_{260}/A_{280}
1	Thekken	1792.8	1.85
2	Thekken	2570.2	1.80
3	Thekken	1120.0	1.90
1	Panniyur-1	2147.8	1.90
2	Panniyur-1	1175.3	1.80
3	Panniyur-1	1674.0	2.00
1	Karimunda	2560.7	1.80
2	Karimunda	1760.5	1.85
3	Karimunda	1178.6	1.90

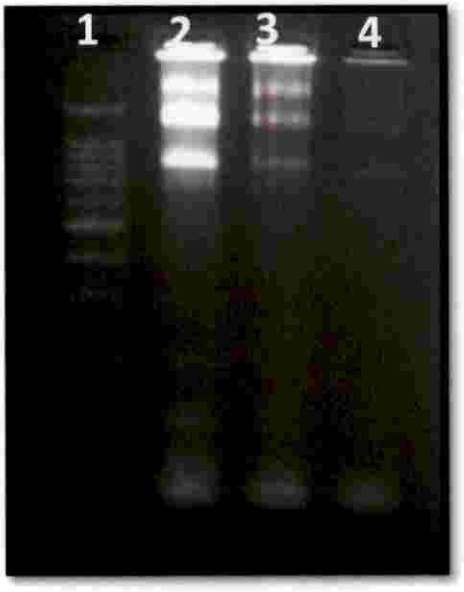
Lane1: 100 bp Ladder
Lane2: RNA of Thekken stage 1
Lane3: RNA of Thekken stage 2
Lane4: RNA of Thekken stage 3

a. Thekken



b. Panniyur-1

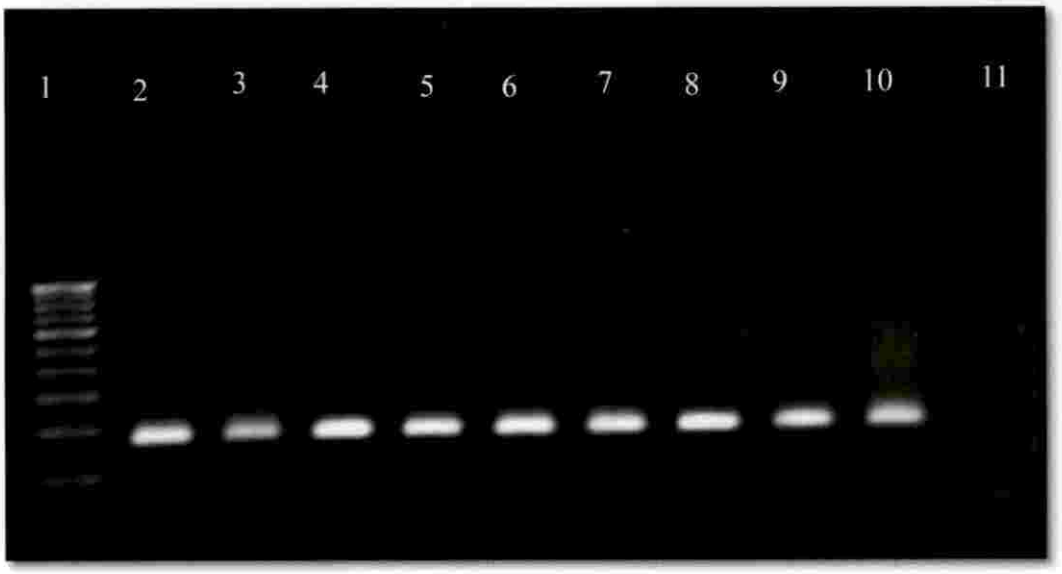
Lane1: 100 bp Ladder
Lane2: RNA of Panniyur-1 stage 1
Lane3: RNA of Panniyur-1 stage 2
Lane4: RNA of Panniyur-1 stage 3



c. Karimunda

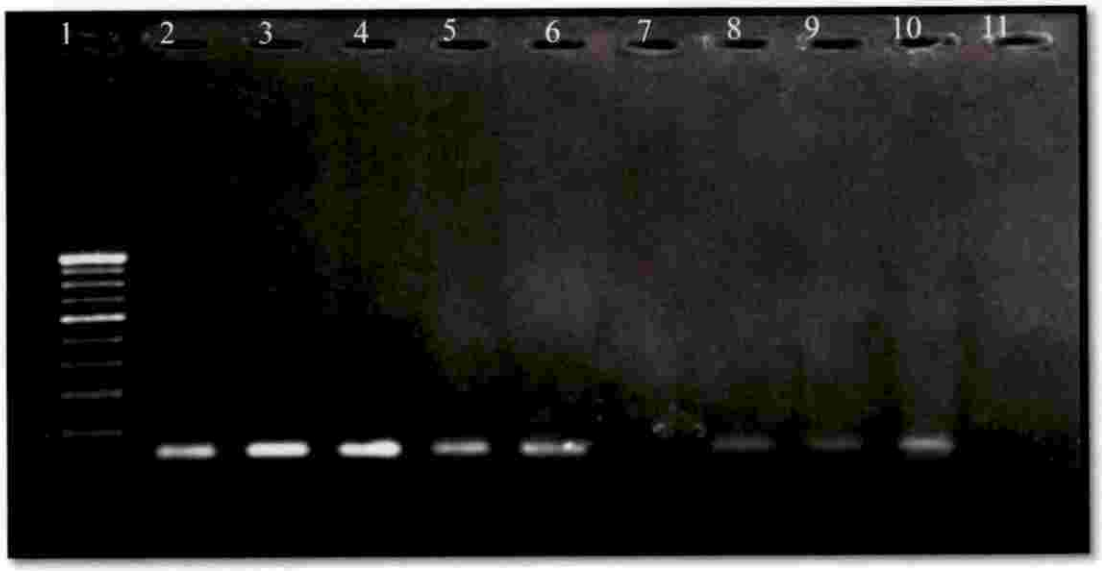
Lane1: 100 bp Ladder
Lane2: RNA of Karimunda stage 1
Lane3: RNA of Karimunda stage 2
Lane4: RNA of Karimunda stage 3

Plate 5. Gel Profile showing total RNA isolated from different stages of Thekken, Panniyur-1 and Karimunda



Lane1: 100 bp ladder	Lane5: Panniyur-1 stage 1	Lane8: Thekken stage 1
Lane2: Karimunda stage 1	Lane6: Panniyur-1 stage 2	Lane9: Thekken stage 2
Lane3: Karimunda stage 2	Lane7: Panniyur-1 stage 3	Lane 10: Thekken stage 3
Lane4: Karimunda stage 3		Lane 11: Blank

Plate 6. Gel Profile showing PCR amplicon obtained from cDNA at three developmental stages of spikes using the primer *Actin*

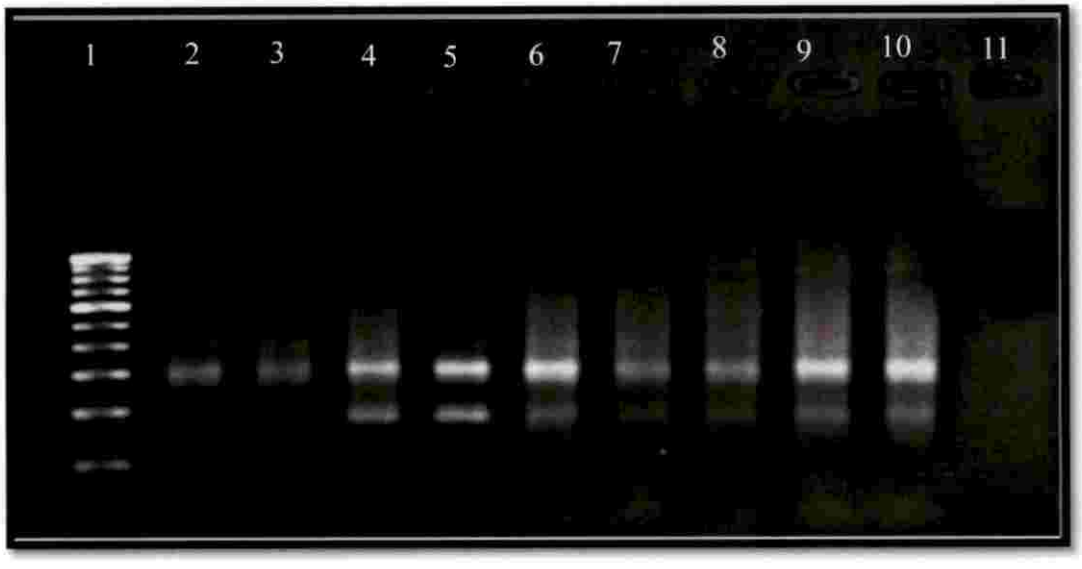


Lane1: 100 bp ladder	Lane5: Panniyur-1 stage 1	Lane8: Thekken stage 1
Lane2: Karimunda stage 1	Lane6: Panniyur-1 stage 2	Lane9: Thekken stage 2
Lane3: Karimunda stage 2	Lane7: Panniyur-1 stage 3	Lane10: Thekken stage 3
Lane4: Karimunda stage 3		Lane11: Blank

Plate 7. Gel Profile showing PCR amplicon obtained from cDNA at three developmental stages of spikes using the primer *BAF1*

Lane1: 100 bp ladder	Lane5: Panniyur-1 stage 1	Lane9: Karimunda stage 1
Lane2: Thekken stage 1	Lane6: Panniyur-1 stage 2	Lane10: Karimunda stage 2
Lane3: Thekken stage 2	Lane7: Panniyur-1 stage 3	Lane11: Karimunda stage 3
Lane4: Thekken stage 3	Lane8: Blank	

Plate 8. Gel Profile showing PCR amplicon obtained from cDNA at three developmental stages of spikes using the primer *BIF2*



Lane1: 100 bp ladder	Lane5: Panniyur-1 stage 1	Lane8: Karimunda stage 1
Lane2: Thekken stage 1	Lane6: Panniyur-1 stage 2	Lane9: Karimunda stage 2
Lane3: Thekken stage 2	Lane7: Panniyur-1 stage 3	Lane10: Karimunda stage 3
Lane4: Thekken stage 3		Lane11: Blank

Plate 9. Gel Profile showing PCR amplicon obtained from cDNA at three developmental stages of spikes using the primer *RAMOSA2*

4.6 q-RT PCR/ REAL TIME PCR

Real-time PCR (RT-qPCR) was done in the above mentioned cultivars in different spike developmental stages using primers for auxin responsive genes with *Actin* as reference gene. SYBR green, an intercalating dye, was used for generating fluorescence.

Amplification plot and melt curve was generated by the 'CFX Maestro Software'. The raw expression data generated from the amplification plot of *BAF1*, *BIF2* and *RAMOSA2* are shown in Fig 4, 5 and 6. Melt curves of *BAF1*, *BIF2* and *RAMOSA2* are shown in Fig 7, 8 and 9. Average Cq values generated by RT- qPCR of *BAF1*, *BIF2* and *RAMOSA2* are presented in Table 7 and Fig 10, 11 and 12.

4.7 ANALYSIS OF RT-qPCR DATA

The Cq values generated by RT-qPCR was analysed in 'qbase plus' software to generate relative expression values for target genes normalized with reference gene *Actin* in different cultivars *viz*., 'Thekken', 'Panniyur-1' and 'Karimunda' (Table 8; Fig 13, 14 and 15). Amplification factor was taken two for all the genes.

Differential expression of the three genes was noticed in all the stages of inflorescence development in the three cultivars using RT-qPCR. Relative expression values of *BAF1*, *BIF2* and *RAMOSA2* with respect to first stage taken as control for each variety are shown in Table 9. The expression pattern of *BAF1* was downregulated at stage 3 of 'Panniyur-1' and 'Karimunda' (Fig 16 and 17) whereas the expression of *BAF1* was upregulated in stage 3 of 'Thekken' (Fig 18).

The expression pattern of the *BIF2* was maximum at stage 2 of spike development in 'Karimunda' and 'Panniyur-1' (Fig 19 and 20), whereas the expression of the *BIF2* was maximum at stage 3 in 'Thekken' (Fig 21). The expression of *RAMOSA2* was downregulated at stage 3 of 'Panniyur-1' and 'Karimunda' (Fig 22 and 23), but it was upregulated in stage 3 of 'Thekken' (Fig 24).

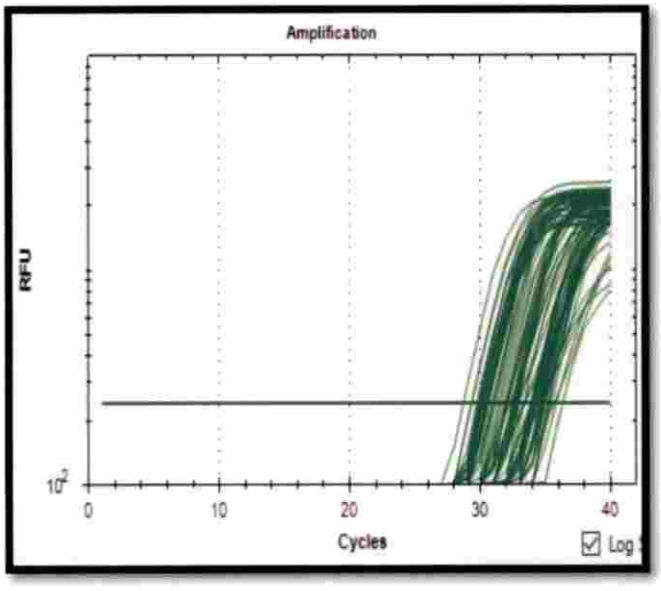


Fig. 4. Amplification plot of *BAF1* by RT-qPCR

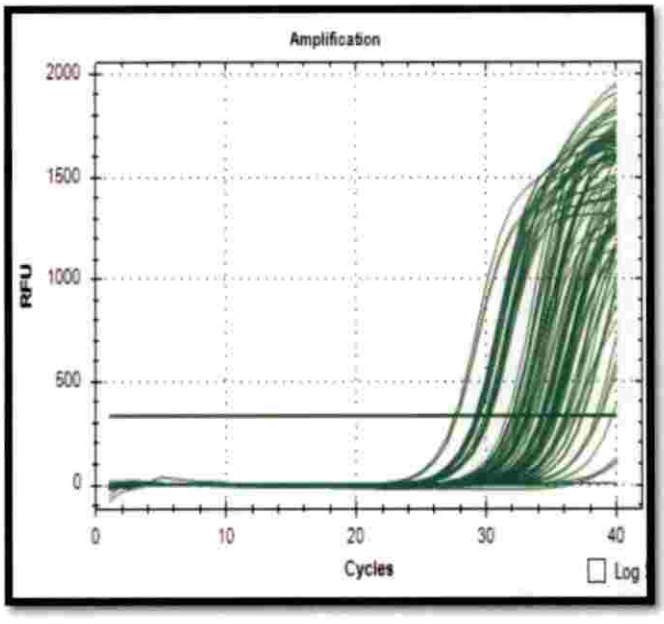


Fig. 5. Amplification plot of *BIF2* by RT-qPCR

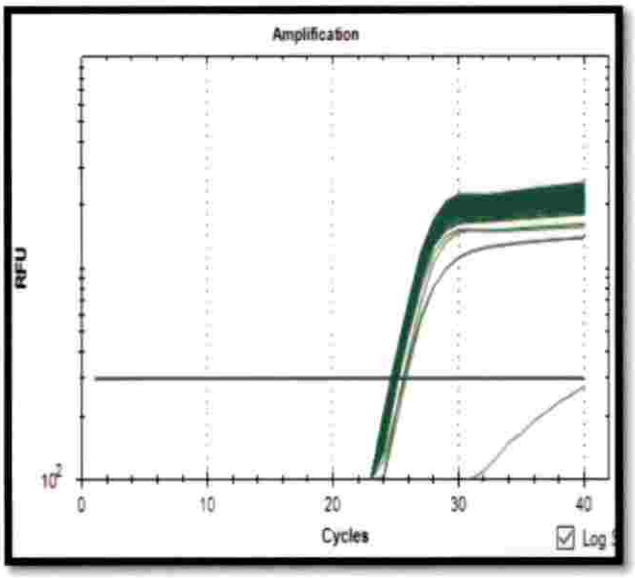


Fig. 6. Amplification plot of *RAMOSA2* by RT-qPCR

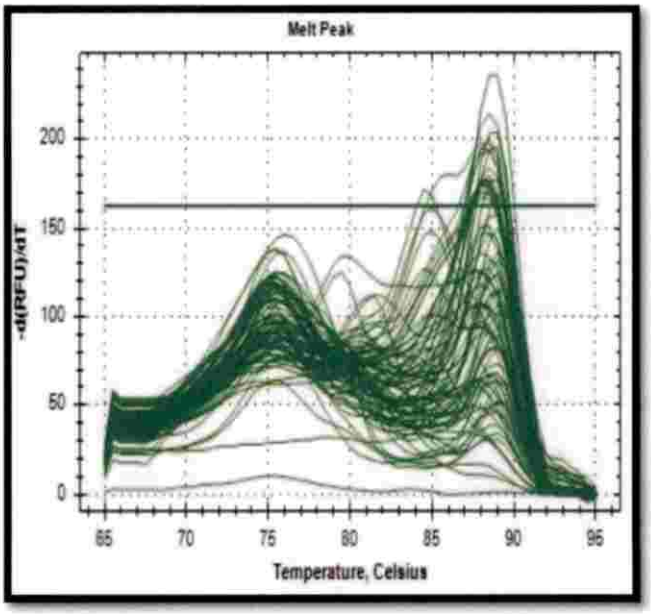


Fig. 7. Melt curve of *BAF1* by RT-qPCR

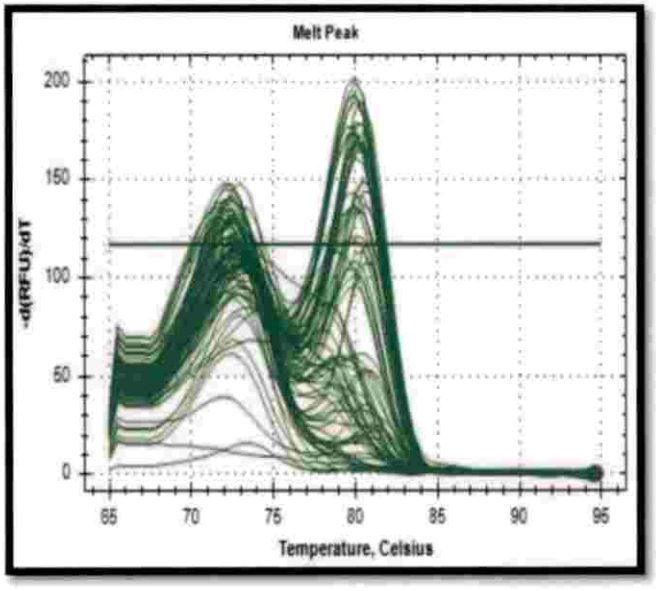


Fig. 8. Melt curve of *BIF2* by RT-qPCR

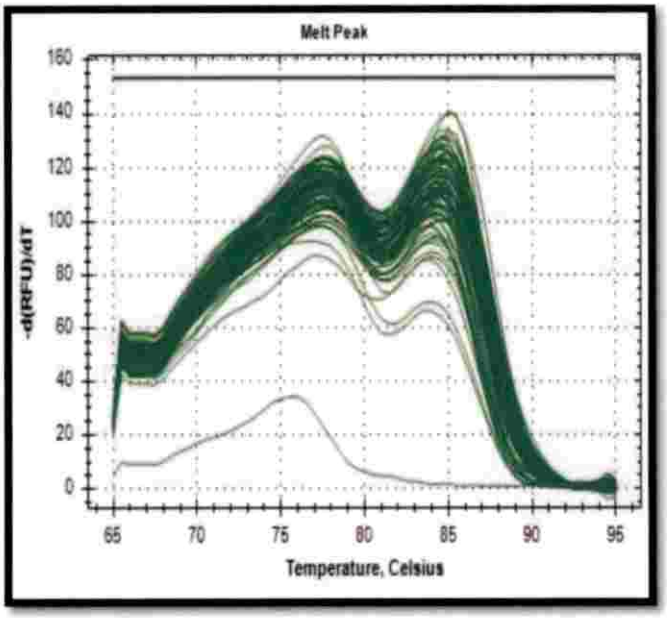


Fig. 9. Melt curve of *RAMOSA2* by RT-qPCR

Table 7. Average Cq values generated by RT-qPCR

SAMPLES	*BAF1*	*BIF2*	*RAMOSA2*
K1	35.32	36.58	24.92
K2	34.40	29.27	24.74
K3	33.58	35.59	24.83
P1	33.54	38.99	24.84
P2	34.83	35.82	25.32
P3	35.22	37.77	24.85
T1	30.03	31.19	24.78
T2	31.05	33.18	24.93
T3	31.51	30.60	24.71

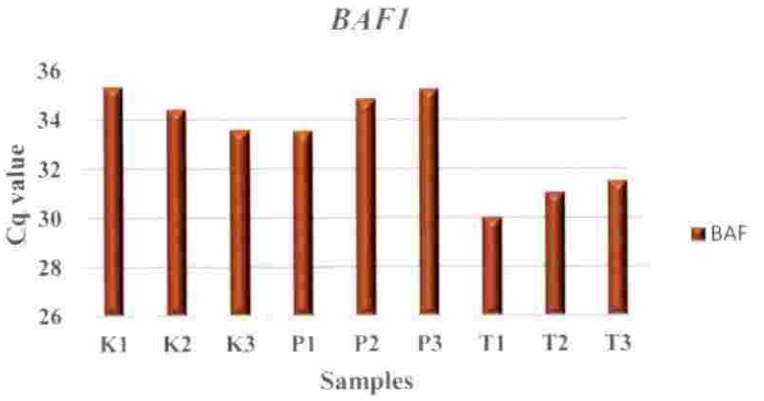


Fig. 10. Average Cq values generated by RT-qPCR of *BAF1*

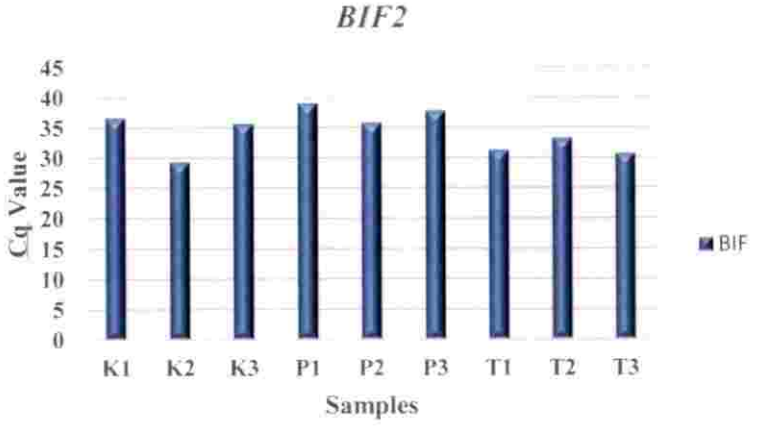


Fig. 11. Average Cq values generated by RT-qPCR of *BIF2*

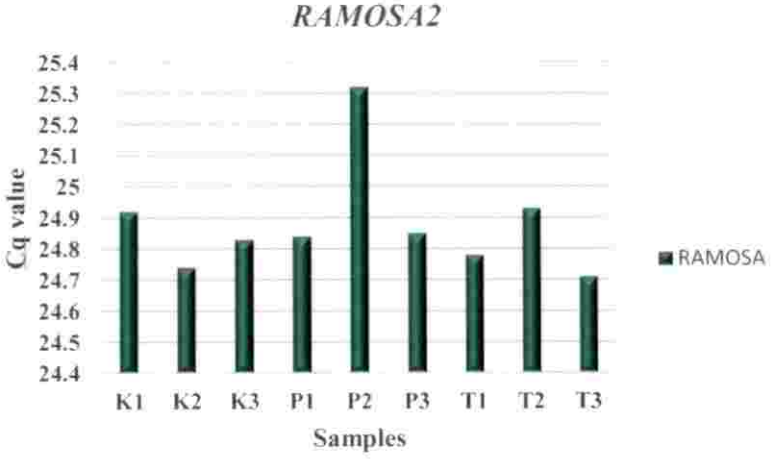


Fig. 12. Average Cq values generated by RT-qPCR of *RAMOSA2*

Table 8. Relative expression values for target genes normalized with reference gene in different black pepper cultivars

Samples	*BAF1* NRQ	*BAF1* STD	*BIF2* NRQ	*BIF2* STD	*RAMOSA* NRQ	*RAMOSA* STD
K1	0.08	0.042385	0.15	0.143137	2.52	0.009
K2	1.05	0.0594	26.4	0.64	17.32	0.335
K3	0.76	0.07185	0.28	0.09545	12.84	1.32
P1	4.01	1.1795	0.09	0.040995	44.81	1.005
P2	1.46	0.80115	1.91	1.81195	29.58	15.665
P3	0.76	0.10715	0.33	0.301865	28.03	0.405
T1	1.00	0.0875	0.51	0.13855	1.00	0.169
T2	2.44	2.2788	1.00	0.38365	4.39	1.263
T3	3.00	2.0973	1.77	0.7135	15.55	15.26555

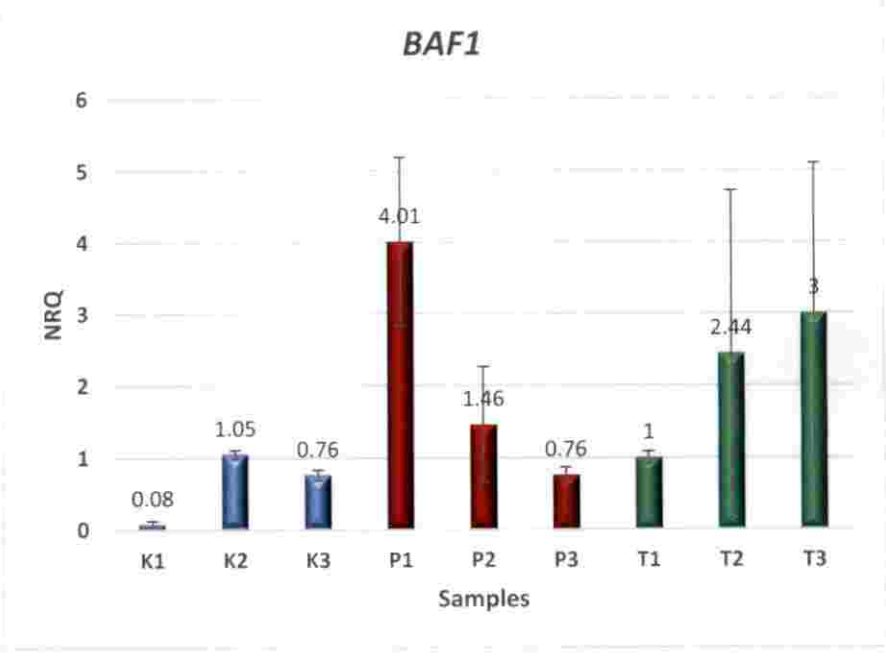


Fig. 13. Relative Expression values of *BAF1*

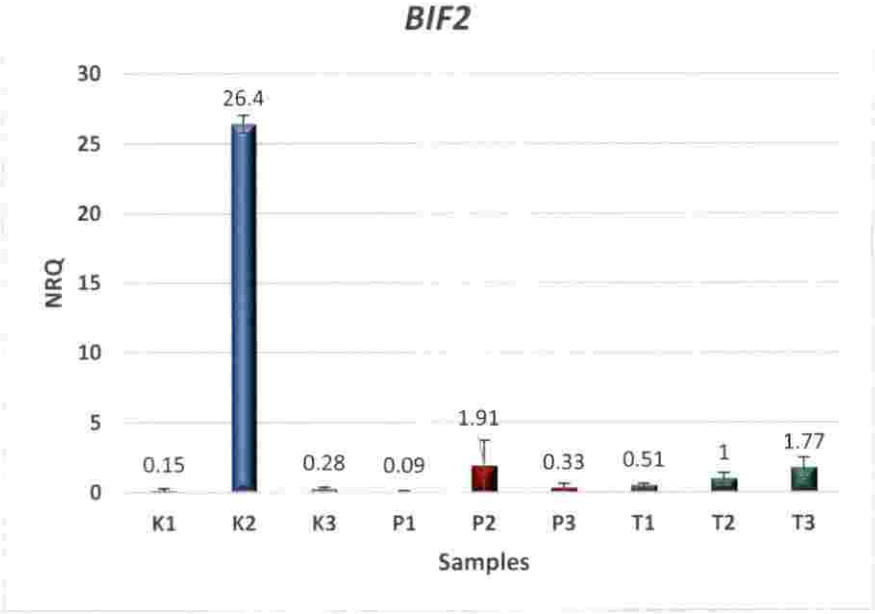


Fig. 14. Relative Expression values of *BIF2*

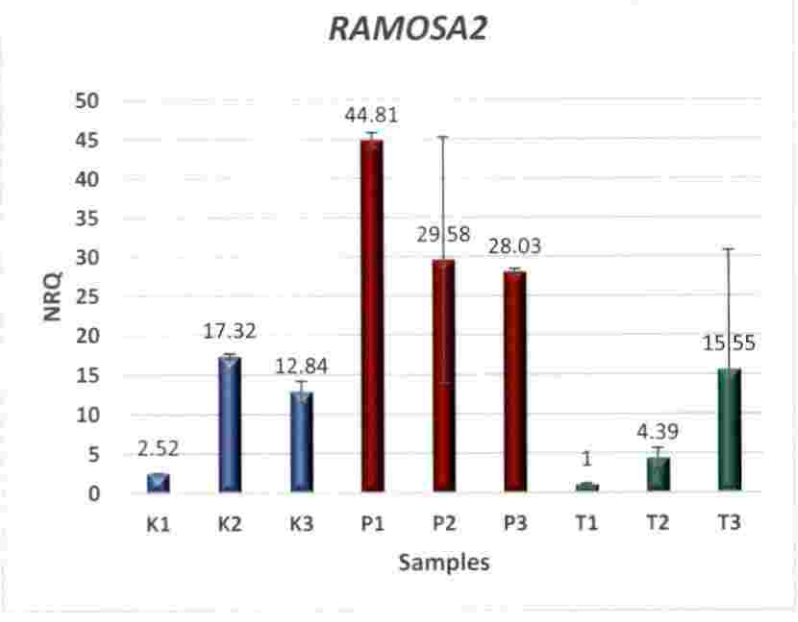


Fig. 15. Relative Expression values of *RAMOSA2*

Table 9. Relative expression values of *BAF1*, *BIF2* and *RAMOSA2* with respect to first stage taken as control for each variety

Samples	*BAF1* NRQ	*BAF1* STD	*BIF2* NRQ	*BIF2* STD	*RAMOSA* NRQ	*RAMOSA* STD
K1	1.00	0.54	1.00	0.98	1.00	0.004
K2	13.33	0.75	181	4.39	6.85	0.14
K3	9.66	0.91	1.9	0.66	5.08	0.53
P1	1.00	0.29	1.00	0.42	1.00	0.02
P2	0.37	0.20	19.82	18.73	0.66	0.35
P3	0.19	0.03	3.39	3.12	0.63	0.009
T1	1.00	0.08	1.00	0.62	1.00	0.15
T2	2.24	2.1	0.82	0.23	3.75	1.08
T3	2.76	1.93	2.86	1.16	13.34	13.15

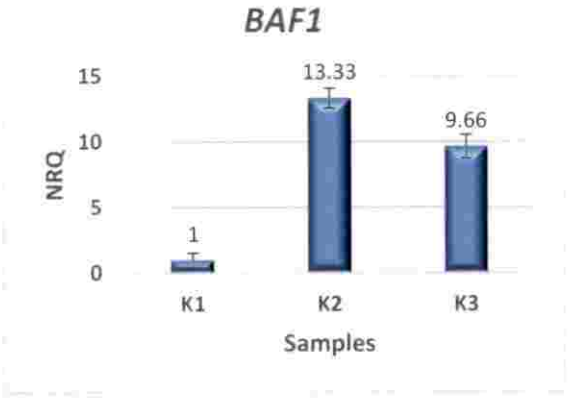


Fig. 16. Relative Expression values of *BAF1* in Karimunda

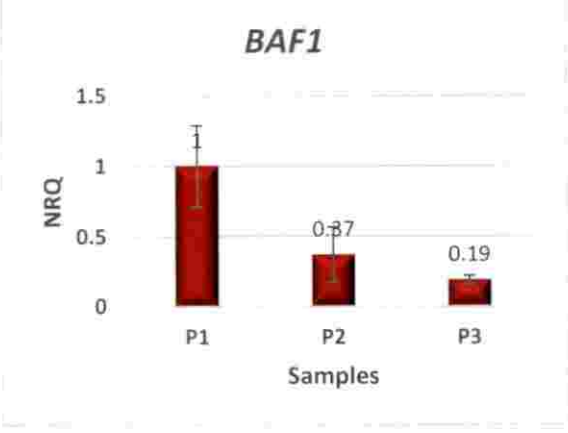


Fig. 17. Relative Expression values of *BAF1* in Panniyur-1

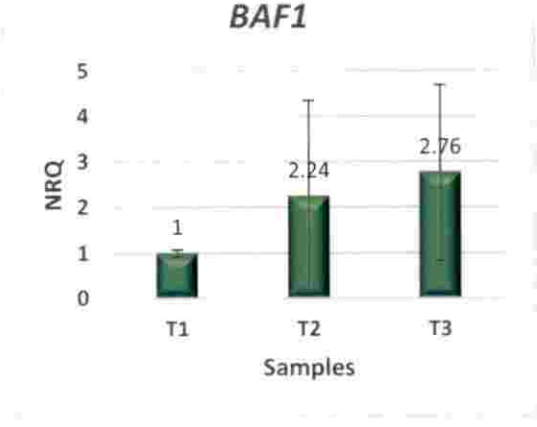


Fig. 18. Relative Expression values of *BAF1* in Thekken

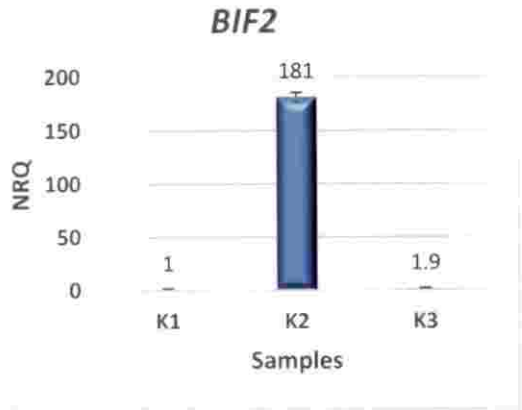


Fig. 19. Relative Expression values of *BIF2* in Karimunda

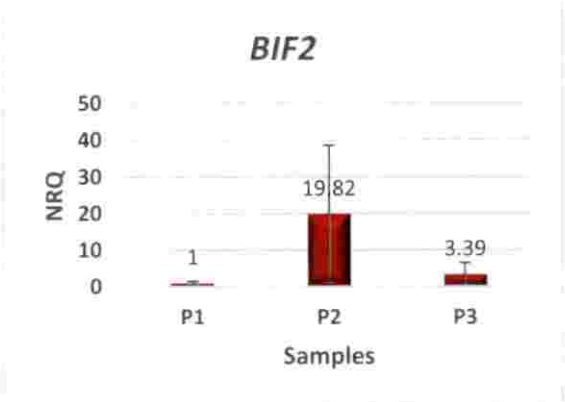


Fig. 20. Relative Expression values of *BIF2* in Panniyur-1

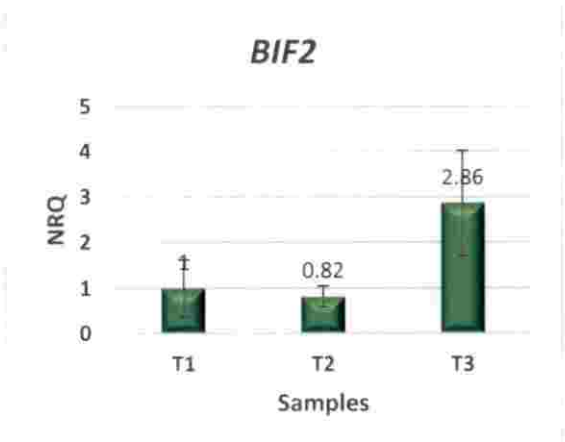


Fig. 21. Relative Expression values of *BIF2* in Thekken

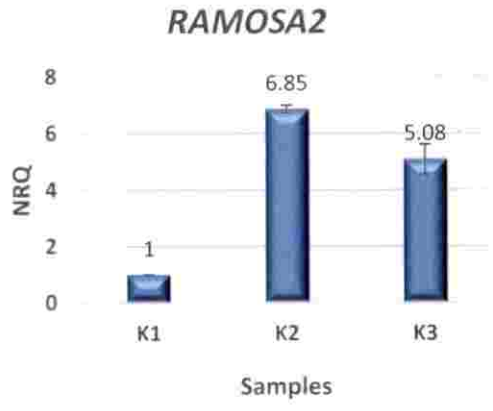


Fig. 22. Relative Expression values of *RAMOSA2* in Karimunda

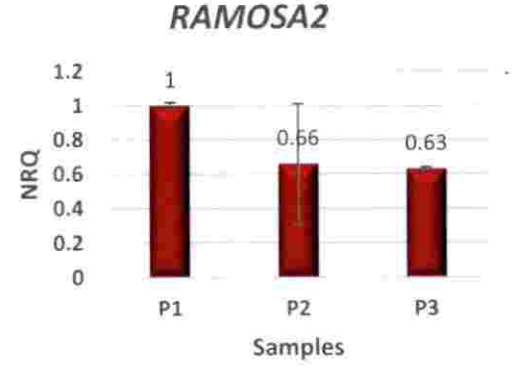


Fig. 23. Relative Expression values of *RAMOSA2* in Panniyur-1

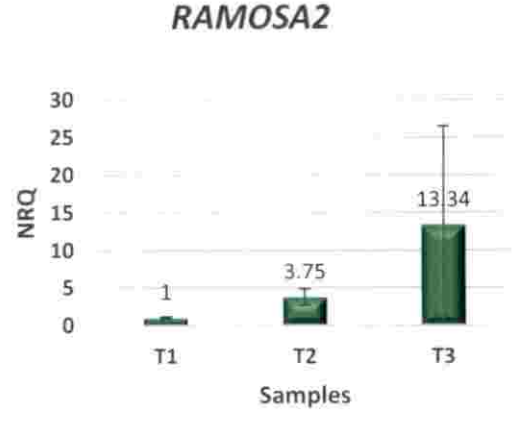


Fig. 24. Relative Expression values of *RAMOSA2* in Thekken

Discussion

5. DISCUSSION

Piper nigrum is believed to have originated in the Western Ghats. Kerala which forms part of the Western Ghats holds the richest species diversity and cultivar diversity in black pepper. It harbours a major share of the gene pool of this crop. Wide variability has been observed among the cultivars of black pepper with respect to economically important traits like spike length, number of berries per spike and size of berries. As branching is a trait of high commercial value that can contribute to increase in yield, the study entitled "Expression profiling of auxin responsive genes during inflorescence development in black pepper (*Piper nigrum* L.)" was carried out to analyse the expression of genes at the molecular level by comparing branching with non-branching varieties.

For studying the auxin responsive genes *viz.*, *BAF1, BIF2* and *RAMOSA2* spikes of black pepper were used as experimental samples. Black pepper vines are usually grown using runner shoots and begin to flower only after the third or fourth year of planting and the flowering period is from June to July. It is reported that bush pepper plants bloom throughout the year (Bhattacharya *et al.*, 2018). In order to assure the continuous availability of spikes throughout the study, bush pepper plants of 'Thekken', 'Panniyur-1' and 'Karimunda' were maintained at the College of Agriculture, Vellayani. Spike samples at the three developmental stages *viz.* stage 1 (1-2cm), stage 2 (6-8cm) and stage 3 (9-12cm) from two different plants of each cultivar/variety were used for analysing gene expression (Le *et al.*, 2012; Zeng *et al.*, 2017). The spike samples were subjected to molecular (genome and transcriptome levels) analysis.

5.2 GENOME AND TRANSCRIPTOME ANALYSIS

Genome is specific to respective organism and DNA-based techniques are used in differentiating the varieties and identifying the genes (Kaur *et al.*, 2015). Quality of isolated DNA is an important factor, as it can affect molecular techniques (Henderson *et al.*, 2013).

Dhanya *et al.* (2007) reported that the extraction of DNA from black pepper is challenging due to the presence of large amount of polyphenolic compounds, polysaccharides and other secondary metabolites. Vimarsha *et al.* (2014a) isolated genomic DNA from eight black pepper varieties, by using the protocol reported by Doyle and Doyle (1990) and the absorbance ratio (A_{260}/A_{280}) ranged between 1.71 and 1.88. Subba *et al.* (2014) modified the protocol (Doyle and Doyle, 1990) to isolate genomic DNA from mature spikes and berries for obtaining better quality DNA.

In the present study, genomic DNA isolation by modified CTAB method developed by Subba *et al.* (2014) using high concentrations of CTAB, NaCl, EDTA and PVP in DNA extraction buffer gave an absorbance ratio (A_{260}/A_{280}) in the range of 1.70 to 1.88 indicating good quality DNA (Weising *et al.*, 2005), from different spike samples of different developmental stages. The purity was also confirmed by obtaining single distinct bands in agarose gel electrophoresis (Plate 2).

In the present study, TRIzol method was used to isolate total RNA from the different spike samples of 'Thekken', 'Panniyur-1' and 'Karimunda'. George *et al.* (2005b) have reported that strong denaturing buffer containing PVP and guanidinium thiocyanate produces a good quality RNA suitable for reverse transcription experiments. In the present study, the RNA yield varied from 1120 ng/µl to 2570 ng/µl in different cultivars. Spectrophotometric absorbance ratio (A_{260}/A_{280}) ranging from 1.70 to 2.00 is considered to be pure RNA (Accerbi *et al.*, 2010). The absorbance ratio ranging from 1.80 to 2.00 in this study indicated that the extracted RNA was of good quality. Ribosomal RNA is reported to signify more than 90% of the total RNAs isolated and RNA degradation during preparation can appear as smear or indistinct bands (Asif *et al.*, 2000). Different bands of 28S, 18S and 5S eukaryotic ribosomal RNA bands were obtained without smear on agarose gel indicating that RNA isolated was of good quality in the present study (Plate 4).

The isolated total RNA was reverse transcribed to cDNA using Verso enzyme kit which included DNase that degrades DNA, ensuring that the PCR product is obtained from the cDNA and not from the DNA.

The gene sequence of *BAF1, BIF2* and *RAMOSA2* of black pepper was not available in the NCBI database. Hence, transcript sequence was obtained from RGCB, Poojappura and two set of primers were designed for these genes. Designing of an appropriate and specific pair of primers is extremely crucial for the correct estimation of the transcript abundance of any gene in a given sample (Singh and Pandey, 2015). Primers should maintain a reasonable GC contents between 50 and 60 %, melting temperature and the annealing temperature of the forward and reverse primers are dependent on each other (Rychlik *et al.*, 1990; Wu *et al.*, 1991; Garg *et al.*, 2008). Primer of 20-24 bases and GC content between 45-60 percent with T_m of 52-58^{0}C works finest in most applications (Patel and Prakash, 2013). In the present study, the T_m of forward and reverse primers of *BAF1* was 60.5^{0}C and 61.0^{0}C respectively and the GC content was 67% and 63% respectively. For *BIF2* the T_m of forward and reverse primer was 59.8^{0}C and 61.3^{0}C respectively and the GC content was 52% and 44% respectively. For *RAMOSA2* the T_m of forward and reverse primer was 61.0^{0}C and 58.8^{0}C respectively and the GC content was 63% and 58% respectively. Good quality primers of desired T_m and GC content could be designed using "Primer express software" in the present study. Absence of secondary structure of the designed primers at the binding site was confirmed by "mfold" web server (Zuker, 2003).

For checking the specificity of the primers designed for the auxin responsive genes *viz.*, *BAF1, BIF2* and *RAMOSA2*, PCR was performed with the isolated genomic DNA and cDNA using gene specific primers. Amplicon of expected size was obtained using genomic DNA and cDNA in PCR using all the designed primers. However, an additional amplicon of 300 bp in case of *RAMOSA2* may be due to alternative splicing (Plate 8).

Standard curve analysis is generally used to determine amplification efficiency (Ruijter *et al.*, 2009). However, in the present study the data generated formed clusters in standard curve analysis. According to Ramakers *et al.* (2003), amplification efficiency of the designed primers can be determined by "Lin Reg

software". In the present study, all the designed primers exhibited cent per cent amplification efficiency using "LinReg software" (Table 5).

BAF1, BIF2 and *RAMOSA2* genes are reported to have significant role in the axillary meristem initiation during inflorescence development (Bortiri *et al.*, 2006; Skirpan *et al.*, 2008; Gallavotti *et al.*, 2011). In the present study, attempts were made to analyse the differential expression of these auxin responsive genes during inflorescence development in branching variety 'Thekken' and non-branching variety 'Panniyur-1' and 'Karimunda'.

To quantitate the expression of *BAF1, BIF2* and *RAMOSA2* real-time PCR (RT-qPCR) was carried out. RT-qPCR is a powerful methodology to analyse the expression of target genes (Dong *et al.*, 2019). According to Wang *et al.* (2019) RT-qPCR is attractive due to its high sensitivity in gene expression analysis. SYBR Green I is reported to be the most commonly used dsDNA specific dye in real-time PCR (Navarro *et al.*, 2015; Kuang *et al.*, 2018) and therefore, was also used in the present study. The problem of the binding of SYBR green with primer dimers and non-specific PCR products can be addressed by the analysis of the melting curve, dependent on the content of G + C % and the length of the amplification product (Espy *et al.*, 2006).

The relative expression approach is commonly used to normalize the data (Niaz *et al.*, 2019). In the present study, the gene of interest (GOI) was compared in relation to the reference gene *Actin*. The housekeeping gene *Actin* was included as it is reported to be a simple and convenient method to correct the variation from one sample to another in most of the real-time PCR studies (Huggett *et al.*, 2005). Expression analysis of the genes was performed using "qbase plus" software (Hellemans *et al.*, 2007; Rajpur and Barrett, 2019) and the amplification factor was taken as two for all the genes.

The first report of *BAF1,* a new regulator of the formation of axillary meristem in maize. *Barren stalk fastigiate1* (*BAF1)* involved in the demarcation of the boundary region where axillary meristem is formed and plays a role in the initiation

of axillary meristem (Gallavotti *et al.*, 2011). The present study is the first report of the expression of *BAF1* in black pepper.

The present study is the first report of the expression of *BIF2* in black pepper. *BIF2* in maize encodes a serine/threonine protein kinase co-orthologous to *PINOID (PID)*, and also regulates auxin transport through direct regulation of ZmPIN1a (Skirpan *et al.*, 2009). *BIF2* kinase has multiple putative targets in the regulation of axillary meristem initiation during maize inflorescence development (Skirpan *et al.*, 2008). The present study is the first report of the expression of *BIF2* in black pepper.

Ramosa2 (*Ra2*) mutant of maize involved in floral organ branching and architecture. *Ra2* was cloned by chromosome walking and shown to encode a *LATERAL ORGAN BOUNDARY* domain transcription factor (Bortiri *et al.*, 2006).

Ramosa2 is transiently expressed in a group of cells that predicts the position of axillary meristem formation in inflorescences. Expression in different mutant backgrounds places *Ra2* upstream of other genes that regulate branch formation. The early expression of *Ra2* suggests that it functions in the patterning of stem cells in axillary meristems. *Ramosa2* expression pattern is conserved in rice (*Oryza sativa*), barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*), and maize, suggesting that *Ra2* is critical for shaping the initial steps of grass inflorescence architecture (Vollbrecht *et al.*, 2005; Bortiri *et al.*, 2006).

Vivekanand (2015) reported that an amplicon of 450 bp was obtained for *RA2* primers in 'Thekken' when analysed at the genomic level. According to him, *RA1* primers produced four amplicons (600 bp, 550 bp, 400 bp and 200 bp) and *RA3* primers produced two amplicons (650 bp and 450 bp). He did not notice any difference in the gel profile in 'Thekken' and 'Karimunda'. In addition, when examining the cDNA, the primers designed for the *RA1* and *RA2* genes showed no amplification in both 'Thekken' and 'Karimunda'. However, the primers designed for *RA3* gene by him showed a differential expression in 'Thekken' and 'Karimunda'.

In the present study auxin responsive genes *viz*., *BAF1, BIF2* and *RA2* showed differential expression pattern in branching 'Thekken' and non-branching varieties 'Panniyur-1' and 'Karimunda' in different developmental stages.

The normalized expression data for the present study showed that the pattern of expression of *BAF1* was downregulated at stage 3 of 'Panniyur-1' and 'Karimunda', whereas the expression of *BAF1* was upregulated in stage 3 of 'Thekken'. The expression pattern of *BIF2* was maximal in stage 2 of spike development in 'Karimunda' and 'Panniyur-1', while the expression of *BIF2* was maximal in stage 3 in 'Thekken'. The expression of *RAMOSA2* was downregulated at stage 3 of 'Panniyur-1' and 'Karimunda' but it was upregulated in stage 3 of 'Thekken'.

All the auxin responsive genes showed higher levels of expression in the first two stages of 'Karimunda' and 'Panniyur-1', while in stage 3 the expression levels decreased. However, the initial stages of the spike development of 'Thekken' had a lower level of gene expression and the third stage showed the highest level of expression. The level of expression in stage 3 in 'Thekken' was considerably higher compared to the other two cultivars 'Karimunda' and 'Panniyur-1'.

Differential expression of the three genes *BAF1, BIF2* and *RA2* was noticed in all the stages of inflorescence development in the three cultivars in the present study. The delayed induction of genes that respond to auxin in 'Thekken' indicates a probable role of auxin signalling in the branching of the inflorescence. Moreover, the difference in the levels of auxin recorded in 'Thekken' compared to 'Karimunda' and 'Panniyur-1' correlates with earlier reports of low auxin content in the spikes of branching variety 'Thekken' compared to 'Panniyur-1' and 'Karimunda' (Bhasi *et al*., 2017).

5.3 FUTURE LINE OF WORK

Other auxin responsive genes need to be evaluated for confirming the role of auxin signalling during inflorescence development in black pepper.

Summary

6. SUMMARY

The study entitled "Expression profiling of auxin responsive genes during inflorescence development in black pepper (*Piper nigrum* L.)" was conducted during 2017-2019 at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram. The objective was to study the expression of auxin responsive genes such as *Barren stalk fastigiate1* (*BAF1*), *Barren inflorescence2* (*BIF2*) *and Ramosa2* (*RA2*) during inflorescence development in black pepper (*Piper nigrum* L.) by Reverse Transcription quantitative PCR (RT-qPCR).

Spike samples of three cultivars of black pepper *viz*., 'Thekken', 'Karimunda' and 'Panniyur-1' were used for the study. Samples were collected at three different developmental stages *viz*., stage 1 (1-2 cm), stage 2 (6-8 cm) and stage 3 (9-12 cm) from two different plants of each cultivar.

Primers were designed for *Barren stalk fastigiate1* (*BAF1*), *Barren inflorescence2* (*BIF2*) and *Ramosa2* (*RA2*) 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. All the primers produced amplicons of expected size in PCR, 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.

Genomic DNA was extracted by modified Cetyl Trimethyl Ammonium Bromide (CTAB) method and RNA by TRIzol method respectively. Isolated total RNA from the spike samples was reverse transcribed to cDNA by "Verso Enzyme Kit" which included DNase that degrades DNA ensuring that the PCR product is obtained from the cDNA not the DNA and the quality was confirmed by PCR and agarose gel electrophoresis. Specificity check of cDNA was also checked by PCR with designed gene specific primers. All the primers produced amplicons of expected size in PCR using cDNA.

cDNA was used for RT-qPCR using SYBR® Green an intercalating dye, for generating fluorescence. 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 and Cq values were obtained by RT-qPCR.

Gene expression analysis was performed using "qbase plus" software using *Actin* as the reference gene and the amplification factor as two for all the genes. The Cq values were used for generating relative expression values using the software.

The results of the present study *i.e.,* auxin responsive genes *BAF1, BIF2* and *RA2* showed higher levels of expression in the first two stages of 'Karimunda' and 'Panniyur-1' whereas in stage 3 the levels were decreased. However, initial stages of spike development in 'Thekken' had a lower level of gene expression and the third stage showed the highest level of gene expression. Expression levels at stage 3 of spike development in 'Thekken' was considerably higher compared to the other two cultivars 'Karimunda' and 'Panniyur-1'.

Differential expression of the three genes was noticed in all the stages of inflorescence development in the three cultivars. The expression of *BAF1* and *RA2* was downregulated at stage 3 of spike development of 'Panniyur-1' and 'Karimunda' and upregulated in stage 3 of spike development of 'Thekken'. The expression of *BIF2* was maximum at stage 2 of spike development in 'Karimunda' and 'Panniyur-1', whereas in 'Thekken' it was at stage 3 of spike development.

Delayed induction of auxin responsive genes *BAF1, BIF2* and *RA2* in 'Thekken' indicate a probable role of auxin signalling in inflorescence branching. Moreover, the difference in the levels of auxin recorded in 'Thekken' compared to 'Karimunda' and 'Panniyur-1' correlates with earlier reports of low auxin content in the spikes of branching variety 'Thekken' compared to 'Panniyur-1' and 'Karimunda' (Bhasi *et al.*, 2017).

Expression profiling of other auxin responsive genes also need to be evaluated in future for confirming the role of auxin signalling during inflorescence development in 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)	freshly added prior to DNA extraction
PVP	4 % (w/v)	

APPENDIX II

TBE Buffer (5X) for 1 liter solution

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

EXPRESSION PROFILING OF AUXIN RESPONSIVE GENES DURING INFLORESCENCE DEVELOPMENT IN BLACK PEPPER (*Piper nigrum* L.)

by

KARAPAREDDY SOWNDARYA

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ABSTRACT

The study entitled "Expression profiling of auxin responsive genes during inflorescence development in black pepper (*Piper nigrum* L.)" was conducted during 2017-2019 at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram. The objective was to study the expression of auxin responsive genes such as *Barren stalk fastigiate1* (*BAF1*), *Barren inflorescence2* (*BIF2*) and *Ramosa2* (*RA2*) during inflorescence development in black pepper (*Piper nigrum* L.) by Reverse Transcription quantitative PCR (RT-qPCR).

Auxin signalling plays an important role in positioning of axillary meristems and determining the inflorescence architecture. The auxin responsive genes *Barren stalk fastigiate1* (*BAF1*), *Barren inflorescence2* (*BIF2*) and *Ramosa2* (*RA2*) are key genes governing inflorescence architecture.

Spike samples of three cultivars of black pepper *viz*., 'Thekken', 'Karimunda' and 'Panniyur-1' were used for the study. Samples were collected at three different developmental stages *viz*., stage 1 (1-2 cm), stage 2 (6-8 cm) and stage 3 (9-12 cm) 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 *Barren stalk fastigiate1* (*BAF1*), *Barren inflorescence2* (*BIF2*) and *Ramosa2* (*RA2*) 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. All the primers produced amplicons of expected size in PCR, 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 and Cq values were obtained.

Gene expression analysis was performed using "qbase plus" software using *Actin* as the reference gene and the amplification factor as two for all the genes. Differential expression of the three genes was noticed in all the stages of inflorescence development in the three cultivars. The expression of *BIF2* was maximum at stage 2 of spike development in 'Karimunda' and 'Panniyur-1', whereas in 'Thekken' it was at stage 3. The expression of *BAF1* and *RA2* was downregulated at stage 3 of 'Panniyur-1' and 'Karimunda' and upregulated in stage 3 of 'Thekken'.

To conclude, auxin responsive genes *BAF1, BIF2* and *RA2* showed higher levels of expression in the first two stages of 'Karimunda' and 'Panniyur-1' whereas in stage 3 the levels were decreased. However, initial stages of spike development in 'Thekken' had a lower level of gene expression and the third stage showed the highest level of expression. Expression levels at stage 3 in 'Thekken' was considerably higher compared to the other two cultivars. Delayed induction of auxin responsive genes in 'Thekken' indicate a probable role of auxin signalling in inflorescence branching. Further studies involving other auxin responsive genes are needed for confirming the role of auxin signalling during inflorescence development in black pepper.