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

by

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(2017-11-106)

THESIS

Submitted in partial fulfilment of the

requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

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KERALA, INDIA

2019

DECLARATION

I, hereby declare that this thesis entitled **"EXPRESSION PROFILING OF AUXIN BIOSYNTHESIS 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 BIOSYNTHESIS GENES DURING INFLORESCENCE DEVELOPMENT IN BLACK PEPPER (*Piper nigrum* L.)"** is a record of research work done independently by Ms. Arathy L. S. 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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ACKNOWLEDGEMENT

I bow my head before the Almighty God for the blessings andenlightenmentthroughout my work and to enable me to complete my thesis work successfully on time.

I feel immense pleasure and privilege to express my sincere gratitude and thankfulness to ***Dr. Swapna Alex,*** *Professor and Head, Department of Plant Biotechnologyand Chairperson of my Advisory Committee for her guidance, suggestions, constant encouragement, support and co-operation given throughout the course ofmy thesis work. It was her sincerity, dedication and perfectionism that helped me finish my work on time.*

I extend my heartful thanks to ***Dr. K. B. Soni,*** *Professor, Department of Plant Biotechnology and member of Advisory Committee, for her valuable advice, suggestionsand timely help for the successful completion of the thesis.*

I am extremely thankful to ***Dr. Roy Stephen,*** *Professor, Department of Plant Physiology and member of Advisory Committee for the support, guidance, encouragement and valuable suggestions in the pursuit of the work.*

I humbly express my gratitude to ***Dr. George Thomas,*** *Scientist F, Rajiv Gandhi Centre for Biotechnology, Poojappura and member of Advisory Committee for his encouragement, valuable suggestions and continued support throughout my research work.*

My heartful thanks and indebtness to ***Dr. A. G. Kiran****, Assistant Professor (on contract), Department of Plant Biotechnology for his selfless help, sincere support and constant encouragement rendered to me during the course of my study and research work.*

I also express my gratitude to non-teaching staff Ajithachechi for her sincere co-operation, timely helpand assistance during the lab work and throughout my course work.

I express my heartfelt thanks to my batchmates Monisha, Sowndarya, Nasreena and Athira for their unconditional support, help, encouragement, care and co-operation.

I express my sincere thanks to my seniors Mithra chechi, Bijula chechi, Smitha chechi, Elizabeth chechi, Mathew chettan, Sachin chettan and Pritam chechi for their valuable advice, suggestion and moral support and also my juniors Teenu, Amala, Nithasana, Aswathy, Sayooj and Athira for their help, love and support. Words are inadequate to express my heartful thanks to Akhilettan and Thomas Uncle who provided me with the plant samples for my research work. I would like to express my sincere thanks to Chinchu chechi and family who was there with my journey with bighearted support and unbound love.

Words cannot express my deep sense of gratitude and indebtness to my beloved mother Lathikamony J. G., beloved father L. Sathyaraj, my beloved fiancee Saju S, my beloved Mattamma, my beloved kunjas Sunitha kunja and Sudha kunja, my dear sister Deepa and my dear brother Dinesh for the encouragement, moral support and motivation rendered to me. I am also thankful to all my family members for their prayer, blessings and support throughout my hard time.

Finally I thank all those who helped and supported me in one way or another in the successful completion of thesis work.

Arathy L. S.

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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	Centimetre
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
IPA	Indole Pyruvic Acid
IM	Inflorescence meristem
Kb	Kilo basepair
Kg	Kilogram
M	Molar
min	Minute
ml	Millilitre
mM	Millimolar
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-qPCR	Reverse transcription-quantitative 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
t	Tonnes
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

%	Per cent
°C	Degree Celsius

Introduction

1. INTRODUCTION

Black pepper, botanically known as *Piper nigrum* L. belongs to the family Piperaceae. It is an important spice crop in the world. Black pepper is a perennial shrub native to Western Ghats, and has been extensively cultivated there and in other tropical regions. Due to its sturdy pungency, it is regarded as the 'King of Spices' and it has valuable medicinal properties (Srinivasan, 2007).

Vietnam is the world's largest producer and exporter of black pepper, contributing 39% of the world's total production. The major pepper producing countries include Indonesia (15%), India (10%) and Brazil (10%) (DASD, 2014). In India, black pepper is cultivated to a large extent in Kerala, Karnataka and Tamil Nadu and to a limited extent in Maharashtra, North Eastern states and Andaman and Nicobar Islands. Small quantities of pepper are also produced in Goa, Andhra Pradesh, Orissa and Assam.

In India, the productivity of black pepper is drastically reducing over last few years, even though it has large area under black pepper production. This is due to poor genetic potential of the vines, high population of senile and unproductive vines, losses caused due to biotic and abiotic stresses, non availability of quality planting material of improved varieties and non adoption of appropriate agronomic practices (DASD, 2014). It is estimated that the world-wide demand for pepper will increase extremely to about 2,80,000 t by the year 2020 and that it will increase upto 3,60,000 t by the year 2050 (Sooraj, 2016). Hence, the production and productivity of black pepper need to be improved substantially so as to regain the past glory of Indian black pepper in the international market.

Spike architecture plays a significant role in yield of black pepper *viz.*, number of spikes per vine, spike yield, length of spike and spike branching.In general all the cultivars of black pepper have non-branching type of inflorescence except for two varieties *viz.*, Aympirian and Uthirankotta (Sujatha and

Namboothiri, 1995). These two cultivars show a trend of branching, with one or two rudimentary branches. However, the black pepper type *viz.*, 'Thekken', a selection of *Piper nigrum,* identified by Shri. T. T. Thomas in forest area of Kanchiyar in Kattappana, Idukki district, has been observed to possess profuse branching trait with more than thirty well developed branches bearing three hundred berries (Farm innovators, 2010). The physiology and morphology of this spike branching is important since it decides the yield of black pepper.

Auxin plays an important role in the process of plant growth and development. It also influences stem elongation and regulates the formation, activity, and fate of meristems. It has been recognized as a major hormone in shaping plant architecture. In addition to this, auxin plays a role in the patterning and formation of lateral organs, stem elongation, and the regulation of branching. Auxin is involved in shaping inflorescence architecture in many crops (Gallavotti, 2013).

As spike branching determines the yield parameters in black pepper and auxin plays a major role in branching, knowledge of the genes involved in auxin biosynthesis and its characterization can help significantly in taking up further yield improvement programmes in black pepper. Examining the transcript modulation of these genes during inflorescence development might help in developing strategies to improve yield potential of black pepper. Transcript profiling of auxin biosynthesis genes during inflorescence development in different cultivars of black pepper might help in identifying potential molecular resources for future crop improvement programmes.

Objective:

The expression profiling of auxin biosynthesis genes like *Flavin monooxygenase* (*YUC2*) and *Tryptophan aminotransferase* (*TAA1*) during inflorescence development in black pepper (*Piper nigrum* L.) by Reverse Transcription quantitative PCR (RT-qPCR) analysis.

Review of Literature

2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L.), the 'King of Spices' is a widely used spice, known for its pungent odour which belongs to the family Piperaceae. It is a perennial crop which is native to South India, and has been extensively cultivated in other tropical regions. It is commonly called as the 'Black Gold' and is one of the important spice commodities of commerce and trade in India since the pre-historic period. It had originated in the tropical evergreen forests of the Western Ghats (Govindarajan, 1977). Vietnam is the world's largest producer and exporter of black pepper, contributing 39% of the world's total production. The major pepper producing countries include Indonesia (15%), India (10%) and Brazil (10%) (DASD, 2014). In India, black pepper is cultivated to a large extent in Kerala, Karnataka and Tamil Nadu and to a limited extent in Maharashtra, North Eastern states and Andaman and Nicobar Islands. A small quantity of black pepper is also produced in Goa, Andhra Pradesh, Orissa and Assam.

Black pepper is a perennial woody vine grown for its berries which can be used as a spice as well as medicinal purposes. The important cultivars of black pepper in India are Karimunda, Kottanadan, Narayakodi, Aympirian, Neelamundi, Kuthiravally, Balancotta, Kalluvally, Malligesara and Uddagar. Kuthiravally and Balancotta exhibit alternate bearing habit. Among these, Kottanadan has the highest oleoresin (17.8%) content followed by Aympirian (15.7%).

Black pepper grows successfully between 20°N and 20°S of equator and from sea level upto 1500 m above MSL. It grows well in areas of humid tropical climate, getting an annual rainfall of 2000-3000 mm, tropical temperature and high relative humidity with little variation in day length throughout the year. Rainfall after stress induces profuse flowering in black pepper (Pillay *et al.*, 1988). The ideal temperature for optimum growth of the crop ranges between 23°C and 32°C with an average of 28°C. It can be grown in a wide range of soils with a pH of 5.5 to 6.5, though in its natural habitat it thrives well in red laterite soils (Wahid and Sitepu, 1987).

In India, the productivity of black pepper is drastically reducing over last few years, even though it has large area under production. The low productivity is mainly due to poor genetic potential of the vines, high population of senile and unproductive vines, losses caused due to biotic and abiotic stresses, non-availability of quality planting material of improved varieties and non adoption of appropriate agronomic practices. Foot rot disease of black pepper caused by *Phytophthora* is a major production constraint in India.

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

In general, all the cultivars of black pepper have terminal solitary type of inflorescence (Jaramillo and Manos, 2001). Usually black pepper has unbranched spike. But a pepper type 'Thekken' a selection of *Piper nigrum*, collected by a farmer Shri. T. T. Thomas from the forest area of Idukki district exhibits branching character with more than thirty well developed branches bearing about three hundred berries altogether. This is about four times the yield of berries from spikes of the highest yielding varieties *viz.*, Panniyur-1, Panniyur-3 and Panniyur-5. Spike branching is an economically important trait in pepper having the potential to improve yield by three to four folds (Sasikumar *et al.*, 2007; Farm innovators, 2010).

The revealing of the genetic pathways leading to spike branching can help in taking up crop improvement programmes in black pepper. The present study was taken up with the objective to study the expression profiling of auxin biosynthesis genes such as *Flavin monooxygenase* and *Tryptophan aminotransferase* during inflorescence development in different cultivars of black pepper using RT-qPCR so that the information from the study can be used for future crop improvement programmes in black pepper.

In this chapter, the literature concerned with the morphology and flowering of black pepper, inflorescence architecture and role of auxin in shaping inflorescence architecture, the genetic pathways regulating auxin biosynthesis in model plants like *Arabidopsis thaliana*, the molecular techniques associated for identifying the genes involved in auxin biosynthesis and their expression profiling have been presented.

2.1 MORPHOLOGY OF BLACK PEPPER

Black pepper is a perennial woody vine which 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 a leaf per node. At the leaf axil, axillary bud is present which develops into a plagiotropic branch. The plagiotropic branches are not having any 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 further growth is continued by the axillary bud (Ravindran *et al*., 2000).

2.2 FLOWERING IN BLACK PEPPER

Usually 50-150 flowers are borne in the axils of ovate, fleshy bracts of the long pendant spikes appearing opposite to the leaves in the plagiotropic branches. The apical buds of this plagiotropic branches gets transformed into a spike, which is the inflorescence of black pepper. In the early stages of development, the inflorescence is having a convex apical meristem, subtended by a leaf and a bract (Tucker, 1982). The inflorescence meristem appears to be terminal so that it exhibits sympodial growth. As the inflorescence grows in length, its apical meristem diminishes. The apical meristem of the inflorescence grows in length before any organs form.

Flowers are borne on solitary spikes, morphologically terminal and bisexual or unisexual. Among the cultivars, variations occur with regard to the

relative proportion of male, female and bisexual flowers in spikes. The composition of flowers in the spikes is primarily governed by the genotype. However, environmental factors also play a major role. The effect of climate on black pepper productivity is more related to local weather rather than global climate patterns. In general, high percentage of bisexual flowers is essential for effective pollination and fruit set (Ravindran *et al.*, 2000).

A meticulous study on the inflorescence architecture and its development in black pepper has not been reported till date. The only available information is the study on inflorescence and flower development by Tucker (1982) in three *Piper* spp., namely *P. aduncum, P. amalgo and P. marginatum*. According to him, initially the *Piper* inflorescence has a convex apical meristem, subtended by a vegetative leaf and a bract and is zonate with two tunica layers, a large central initial zone, a peripheral zone and a massive pith rib meristem.

Kerala is having rich cultivar diversity of black pepper. The cultivars of black pepper have originated from their wild cultivars. More than hundred cultivars are known and a few of them are still popular (Ravindran *et al.*, 2000). Any mutation in the floral meristem of black pepper could result in inflorescence proliferation. A new spike variant 'Thekken' with 100% of proliferating spikes was collected from a farmer's field. This vine, closely resembling the popular black pepper cultivar 'Karimunda', was originally spotted by a farmer, Shri T. T. Thomas, Thekkel house, Kakkattukada, Kattappana in the Idukki district of Kerala, a few years ago. A rooted cutting of the vine procured from his nursery about three years ago and conserved at the *exsitu* gene bank of the Indian Institute of Spices Research, Calicut, India, has now spiked, confirming to its true-to-type nature. The spike length measured about 18 cm with about 150 berries per spike as against 7-10 cm length and about 30-70 berries in the normal spike. The proliferating spikes were having indeterminate growth habit. The spikes resemble grape bunches. Another feature of the spike is that it has persistent and large bracts (Vimarsha *et al.*, 2014).

Apart from many state level awards and recognitions, the farmer was also awarded Plant Genome Saviour Reward by the Protection of Plant Varieties and Farmer's Right Authority of India and the plant type has been documented as an innovation by National Innovation Foundation (NIF) at Gujarat in 2012.

According to Thangaselvabal (2008), black pepper is a self pollinated crop and it is dioecious in wild form and gynomonoecious or trimonoecious in cultivated form. Bisexual flowers are protogynous and anther dehiscence occurs 3-8 days after stigma exertion (Martin and Gregory, 1962). It is a polyploid species and the somatic chromosome number varies from 52 to 104. But most of the cultivated varieties have somatic chromosome number 2n =52 (Mathew, 1958).

2.3 INFLORESCENCE ARCHITECTURE

The inflorescence architecture in many cultivated plants is found to be highly diverse in nature (Brown *et al.*, 2006). This diversity in inflorescence architecture has drawn the attention of plant breeders, because inflorescence traits directly affect crop yields (Doebley *et al.*, 1997). The inflorescence may vary a great deal among different species and it seems to play a important role in reproductive success of the crop as it has a strong effect on pollination and fruit set (Wyatt, 1982). The aerial organs of the plants get originated from the shoot apical meristem (SAM). This meristem during the vegetative phase of the growth, generates leaves and shoots during the vegetative phase and during the reproductive phase, it becomes aninflorescence meristem and thus flowers are fashioned. The inflorescence architecture mainly depends upon the branching pattern and the position of the flowers (Benlloch *et al.*, 2007).

Inflorescences can be classified based on many criteria. Based on the complexity, they are classified as simple and compound inflorescence. Those inflorescences in which the flowers are directly formed from the main axis are called simple inflorescences (non-branching), while in compound inflorescences (branching), the flowers are formed from secondary or higher order branches

(Benlloch *et al.*, 2007; Teo *et al.*, 2014). The three dimensional flower arrangement has been widely exploited in many domesticated crops to increase the yield particularly in the case of cereals. According to Barton (2010), the shoot apical meristem (SAM) elaborates leaf, stem and flower anlagen at specific regions depending on complex temporal and spatial interactions between proteins, micro RNAs and hormones. The developmental fate of the inflorescence meristem (IM) *ie.*, its conversion into a Floral Meristem (FM) determines the type of inflorescence and it is species specific (Tanaka *et al.*, 2013).

From various studies on inflorescence development, it is known that the floral genes are highly conserved in the plant kingdom in different drops like maize, *Arabidopsis*, *Antirrhinum*, cauliflower, rice *etc.* (Goto *et al.*, 2001; Jack, 2004; Song *et al.*, 2010).

Auxin, a plant hormone 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 (Okada *et al.*, 1991; Reinhardt *et al.*, 2000; Benkova *et al.*, 2003; Krizek, 2011). 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, the conserved patterns of *PIN1* expression suggest a conserved polar auxin transport mechanism, which is necessary for the formation of all axillary meristems and lateral primordia (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).

2.4 ROLE OF AUXIN IN SHAPING INFLORESCENCE ARCHITECTURE

Growth regulators such as auxins, cytokinins and carotenoid derivatives are 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 hormones which has diverse role in plant growth and development (Taiz and Zeiger, 2002; Finet and Jaillais, 2012). Auxin influences stem elongation and regulates the formation, activity and fate of meristems and has therefore been recognized as a major hormone shaping plant architecture (Huang *et al.,* 1990; Gallavotti, 2013). Among the natural auxins available in plants, Indole-3-acetic acid (IAA) is the most abundant form of auxin. The use of polar auxin transport inhibitors such as N-1-naphthylphthalamic acid (NPA) has shown that auxin transport is required for initiation of leaves during vegetative development and flowers during reproductive development (Okada *et al.*, 1991; Reinhardt *et al.*, 2000; Benkova *et al.*, 2003). Indole-3-acetic acid, the predominant naturally occurring auxin, is implicated in almost every aspect of plant growth and development, including cell division, cell elongation, cell differentiation, flower development and vascular system patterning (Hohm *et al.*, 2013).

2.5 GENES INVOLVED IN AUXIN BIOSYNTHESIS

Even though auxin has been studied for decades, its main biosynthetic pathway in plants has only been discovered few years ago (Mashiguchi *et al.*, 2011; Won *et al.*, 2011). Information on auxin signaling pathway has greatly expanded in the last few decades, resulting in the establishment of a core model for auxin signal transduction (Chapman and Estelle, 2009). According to Philips *et al.* (2011), auxin biosynthesis is required for the formation of all primordia and is therefore essential to the development of a plant body. Indole-3-acetic acid, the natural auxin produced by plants, is known to be synthesized either by Tryptophan (Trp) dependent pathway or using a Tryptophan (Trp) independent pathway (Woodward and Bartel, 2005; Zhao *et al.*, 2012). Tryptophan dependent auxin biosynthesis is essential for embryogenesis, seedling growth, flower development, vascular pattern formation and other developmental processes (Cheng *et al.*, 2006; Stepanova *et al.*, 2008; Tao *et al.*, 2008). In contrast, the molecular components and the physiological functions of the Tryptophan independent pathway are not known clearly.

Indole Pyruvic Acid (IPA) pathway is the first complete and universally conserved auxin biosynthesis pathway in plants. According to Mano and Nemoto (2012), it is the most significant and well studied pathway among the different known pathways. It involves the conversion of Tryptophan to Indole Pyruvic Acid (IPA) by the activity of *Tryptophan aminotransferase (TAA1)* genes and the subsequent conversion of IPA to IAA by *Flavin monooxygenase (YUC2)* gene belonging to the *YUCCA* (*YUC*) gene family (Stepanova *et al.*, 2008; Tao *et al.*, 2008). Transcript induction of the biosynthetic pathway gene is reported resulting in auxin accumulation during physiological responses (Li *et al.*, 2012).

2.5.1 Tryptophan aminotransferase (*TAA1*)

In *Arabidopsis*, *Tryptophan aminotransferase* gene functions in the first step of the IPA pathway. Recombinant *TAA1* protein catalyzes the conversion of Tryptophan into Indole Pyruvic Acid *in vitro* (Mano and Nemoto, 2012). Endogenous amounts of IAA and IPA are reduced in deficient mutants of the *TAA1* family. In contrast, induction of *TAA1* increases the endogenous amounts of IPA in *Arabidopsis*. The *TAA1* gene family plays a crucial role in plant growth and development (Cohen *et al.*, 2003).

Multiple mutations in *TAA1* genes cause severe auxin deficient phenotypes in various biological processes, including embryogenesis, flower development, seedling growth, vascular patterning, lateral root formation, tropism, shade avoidance and temperature-dependent hypocotyls elongation, indicating a fundamental role of the *TAA1* family in plant development (Gallavotti *et al.*, 2013). Orthologs of the *TAA* gene family are widely distributed in vascular and nonvascular plants, indicating that *TAA* genes are evolutionarily conserved in the plant kingdom. *Vanishing tassel 2 (VT2)* and *Fish bone (FIB)* genes are co-orthologs of the *TAA1* family in maize and rice respectively, and mutations in these genes lead to decreased IAA levels and result in pleiotropic auxin deficient phenotypes. *TAA* family plays a major role in the production of IPA in Arabidopsis (Mashiguchi *et al.*, 2011).

2.5.2 Flavin monooxygenase (*YUC2*)

YUC genes were originally discovered by a genetic screen of activation tag lines in *Arabidopsis* (Gallavotti *et al.*, 2013). Gain-of-function mutants of *YUC2* showed high-auxin phenotypes such as epinastic cotyledons and long hypocotyls under light conditions. *YUC* genes encode flavin containing monooxygenases and 11 *YUC* genes are found in *Arabidopsis* (Mashiguchi *et al.*, 2011). *YUC2* gene encodes *Flavin monooxygenase* which converts Indole Pyruvic Acid to Indole Acetic Acid in IPA pathway (Zhao *et al.*, 2001). Overexpression of *YUC*2 gene leads to increased levels of IAA and results in the high-auxin phenotypes in *Arabidopsis*, suggesting that *YUC* proteins catalyze a rate-limiting step in IAA biosynthesis.

YUCCA genes have also been identified in other plant genomes such as rice (*Oryza sativa*) (Yamamoto *et al.*, 2007), maize (*Zea mays*) (Gallavotti *et al.*, 2008), *Medicago truncatula* (Tivendale *et al.*, 2010), tomato (*Solanum lycopersicum*) and *Petunia hybrida* (Santamaria *et al.*, 2002). Generally, this enzyme family exhibits low substrate specificity and it is possible that efficient substrate channeling between co-localized enzymes mitigates this problem (Muller and Weiler, 2000; Kriechbaumer *et al.*, 2006).

2.6 MOLECULAR STUDIES ON FLOWERING IN BLACK PEPPER

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

Vimarsha *et al.* (2014) also reported presence of sequence homology for the gene *TFL1* 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 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 in black pepper. These studies showed expression of *TFL1* gene was observed in mature leaves and slightly in spike with flowers and also confirmed that the isolated sequences were partial orthologs of Phosphatidyl Ethanolamine-Binding Protein (*PEBP*) genes from black pepper. He also identified the flowering time and floral integrator gene *FT* and the inflorescence meristem identity gene, *TFL1* from black pepper.

Bhasi *et al.* (2017) reported the differential auxin content in different black pepper cultivars *viz.*, Panniyur-1, Karimunda and Thekken through quantification of IAA content using HPLC. The study revealed that the IAA content in the spikes of branching variety Thekken was significantly low, that is about $1/4^{th}$ and $1/3^{rd}$ of Panniyur-1 and Karimunda.

According to Bhasi *et al.* (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* genes noticed in 'Thekken' correlated with the increase in cytokinin content and the emergence of differentiated meristematic tissues that was prominent in the histological sections of stage-2 of Thekken.

2.7 TECHNIQUES USED FOR EXPRESSION PROFILING

Gene expression is closely linked to its function. Reverse Transcription quantitative PCR or Real Time PCR is a powerful and sensitive methodology to analyse the expression of target genes. The expression profiling of genes involves several steps *viz.*, DNA isolation, RNA isolation, primer designing, cDNA synthesis, specificity check of primers using genomic DNA and cDNA by PCR and RT-qPCR.

2.7.1 Primer designing

Primer designing is a crucial step which greatly affects the success of a polymerase chain reaction. A primer is a short oligo nucleotide which is the reverse complement of a region of a DNA template and it would facilitate the amplification of the targeted DNA sequence (Garg *et al.*, 2008). In PCR, forward and reverse primer helps the amplification of specific region of DNA by annealing to the complimentary sequence (Beij *et al.*, 1991).

The important criteria to be considered for primer designing 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). The specificity implies that only the target sequence is amplified and the efficiency implies that more products are amplified in less number of cycles. The specificity depends on length of primer and annealing temperature (Garg *et al.*, 2008). Several parameters have to be considered while designing the primer which includes the length of the primer, melting temperature, annealing temperature and GC content (Wu *et al.*, 1991; Rychlik *et al.*, 1990). Primers

having length between 18-24 bp is ideal and the short length primer can result in non-specific amplification (Lowe *et al.*, 1990).

The optimum annealing temperature for polymerase chain reaction is usually kept five degree celsius lower than the estimated melting temperature (Dieffenbach *et al.*, 1993; Rychlik *et al.*, 1990: Abd-Elsalam, 2003). Inorder to avoid mispriming, the 3' end of the primer should contain G or C nucleotide for stronger binding. Dinucleotide repeats and continuous execution of same bases are to be avoided as it might result in non-specific binding (Dieffenbach *et al.*, 1993). The optimum G-C content of primer is between 40-60% (Lowe *et al.*, 1990). Length of amplicon also has impact on efficiency of amplification (Rychlik *et al.*, 1990). Secondary structures, such as hairpin loops and primer dimers can also result in no amplification or less efficiency (Sahdev *et al.*, 2007).

2.7.2 DNA isolation

In molecular techniques, DNA isolation is a critical step because the DNA quality can affect the subsequent analytical techniques (Boiteux *et al.*, 1999; Fredricks *et al.*, 2005). The contaminants in DNA can have an effect on the activity of different enzymes such as polymerase, ligase and nucleases which directly or indirectly affect PCR, restriction digestion *etc.* (Katterman and Shattuck, 1983; Richards *et al.*, 1988; Demeke and Jenkins, 2010). High level of polysaccharides and polyphenols in leaf tissue can interfere with DNA isolation process (Sharma *et al.*, 2002).

Many DNA isolation protocols have been reportedin plants, but none is found to be universally applicable (Varma *et al.*, 2007). Most of these protocols are modifications of CTAB method of DNA isolation. A higher concentration of NaCl (5 M) can increase the quality and yield of DNA and also prevent the sample from becoming viscous during grinding (Doyle and Doyle 1987; Sathelly *et al.*, 2014). The DNA isolation in black pepper is a tedious process due to the presence of polysaccharides and secondary metabolites (Dhanya *et al.*, 2007). Subba (2014) modified the CTAB method of DNA isolation to isolate DNA from

leaves and spikes of black pepper by increasing the concentration of CTAB, NaCl, EDTA and PVP.

The addition of NaCl at concentrations higher than 0.5M, along with CTAB, removes polysaccharides during DNA extraction (Paterson *et al.*, 1993; Moreira and Oliveira, 2011; Sahu *et al.*, 2012). 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 quantities of polysaccharides (Sambrook and Russel, 2001).

2.7.3 RNA isolation

According to Doyle (1996), RNA isolation requires special care and attention due to the ubiquitous presence of RNase in the environment, which degrades the RNA and therefore of sterile condition is of great importance. Isolation of good quality RNA depends maintenance mainly on good laboratory practices and RNase free technique.

The simple method for RNA isolation is the Trizol method. It uses Guanidinium thiocyanate, a chaotropic agent that allows the purification of RNA from cells (Cox, 1968; Chomczynski and Sacchi, 1987). Trizol reagent is a ready-to-use reagent for RNA extraction and it makes RNA isolation process easier and fast (Simms *et al.*, 1993). 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 and can be precipitated with isopropanol (Chomczynski, 1993).

The secondary metabolites produced by certain plants may result in poor yield of RNA (Gasic *et al.*, 2004). Siju *et al.* (2007) reported the modified method for RNA isolation from black pepper berries for *Cucumber mosaic virus* detection.Polyvinyl pyrrolidone (PVP) is used during RNA isolation to reduce phenolic compounds and polysaccharides (Liu *et al.*, 2018).

2.7.4 Quantification of nucleic acids

Sambrook *et al.* (1989) reported the use of spectrophotometry for detecting the concentration of nucleic acids. It is based on the principle that the nucleic acids absorb UV light at a particular wavelength. In this method, the absorbance values at 260 nm and 280 nm is made use for the quantification and assessment of purity of nucleic acids (Glasel, 1995). If the absorbance value at 260 nm is 1, then the concentration of DNA is 50 µg $ml^{-1}$and that of RNA is 40µg ml^{-1}. The ratio of absorbance at 260 nm and 280 nm is used to determine the purity of nucleic acid. Good quality DNA should have A_{260}/A_{280} ratio around 1.8 and for RNA it should be around 2 (Maniatis *et al.*, 1982).

2.7.5 Polymerase Chain Reaction

In 1983, Karry Mullis invented Polymerase chain reaction which opened a new era in molecular biology. It is a very sensitive technique which is used for the amplification of a specific nucleotide sequence (Culley *et al.*, 2014; McCall *et al.*, 2015). PCR consists of mainly three steps *viz.*, denaturation of DNA, annealing of primer to the template and extension by DNA polymerase. Karcher (1995) reported the discovery of heat stable DNA polymerase, Taq polymerase from the bacteria *Thermus aquaticus* that made PCR more efficient. The forward and reverse primers hybridize with the complementary sequence in the target and initiate DNA synthesis with the help of the enzyme Taq DNA polymerase and the process is repeated for several cycles. In each cycle, the DNA gets duplicated. After 20 to 40 such cycles, enough amplified product is generated and can be visualized on an agarose gel by using specific staining method (Saiki *et al.*,1985).

The basic components of PCR master mix includes template, primers, magnesium ion, dNTPs, buffer for PCR reaction, and thermostable DNA polymerase enzyme (Mullis *et al.*, 1986). The template can be DNA, RNA or CDNA. The enzyme *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 the enzyme and it affects enzyme activity, primer annealing, melting temperature etc. (Zamft *et al.*, 2012).

2.7.6 RT-qPCR or Real Time PCR

Reverse Transcription quantitative PCR or real time PCR technique is an effective and sensitive method which provides significant quantitative information on gene expression analysis (Gutierrez *et al.*, 2008; Huggett *et al.*, 2005). In conventional PCR, electrophoresis is used to evaluate the amplification product, while in real time PCR fluorescent molecules are used for the quantification. It has been considered as an advanced method for the quantification of mRNA transcripts due to its sensitivity and accuracy (Bustin, 2002). It has wide applications in different areas of functional genomics, molecular biology, biotechnology *etc.* In this technique, the specific region of the DNA is simultaneously amplified and quantified. The method depends on the calculation of the increase in the fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle (Bustin *et al.*, 2005). According to Omar *et al.* (2016), RT-qPCR has many advantages like easy performance, high sensitivity, more specificity and broad quantification range when compared to other conventional methods.

Fluorescent reporters used in real-time PCR include double-stranded DNA (dsDNA) binding dyes, or dye molecules attached to PCR primers or probes that hybridize with PCR products during amplification. SYBR Green is one of the fluorophore that can be used in real-time PCR, which uses a simple method for the detection and quantification of PCR products with high sensitivity (Nygard *et al.*, 2007). SYBR Green binds to double-stranded DNA and emits light after excitation (Pabla and Pabla, 2008; Wang *et al.*, 2015).

The melting temperature of a DNA molecule depends on both its size and its nucleotide composition, hence GC-rich amplicons have a higher T_m than those having an abundance of AT base pairs. 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 Tm 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 (Armstrong and Schulz, 2015).

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Expression profiling of auxin biosynthesis 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. The details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 COLLECTION OF PLANT SAMPLES

Spike samples at different developmental stages were collected from the black pepper cultivars Panniyur-1, Karimunda and Thekken (Plate 2) and used for the study. The criteria for selection of developmental stages *viz*., stage 1 (12-15 days after bud emergence, having length of 1-2cm), stage 2 (22-25days after bud emergence having length of 6-8cm) and stage 3 (32-35days after bud emergence having length of 9-12cm).

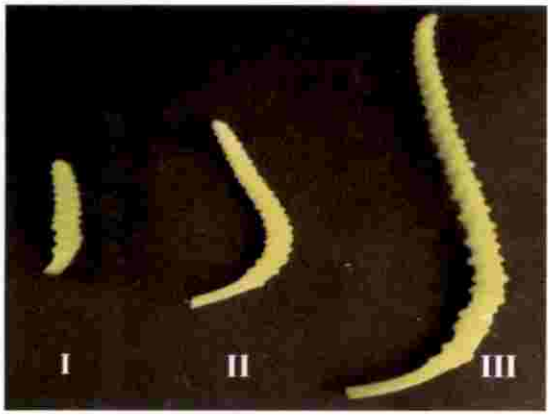
3.2 PRIMER DESIGNING

The nucleotide sequences of genes such as *Flavin monooxygenase (YUC2)* and *Tryptophan aminotransferase (TAA1)* were not available in NCBI database. Hence, the sequence from the related species was used for designing gene specific primers. The primer designing was done using "Primer Express" software. The designed forward primers were 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" web server. The primers were synthesised at Eurofins Genomics India Pvt. Ltd, Banglore.

a. Bush pepper of Thekken

b.Spike of Thekken

Plate 1. Bush pepper and spike of Thekken



a. Spike samples of Panniyur-1

b. Spike samples of Karimunda

c. Spike samples of Thekken

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

Plate 2. Spike samples of Panniyur-1, Karimunda and Thekken

Table 1. Sequence ID of *TAA1* and *YUC2* genes

Gene	Sequence ID
Tryptophan aminotransferase (TAA1)	*Prumus mume*, XM_008239250.2
Flavin monooxygenase (YUC2)	*Cucurbita maxima*, XM_023149241.1

3.3 GENOMIC DNA ISOLATION

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

Spike samples at different inflorescence development stages were cryogenically ground in a mortar and pestle after chilling in liquid nitrogen. CTAB Extraction Buffer (Appendix-I) containing 0.2% β-mercaptoethanol and 2% Polyvinyl pyrrolidone (PVP) was added to the crushed spike samples and the homogenized tissue was transferred to a sterile 2ml microfuge tubes and incubated for 30 min at 60°C in a recirculating water bath. After incubation, the CTAB/plant extract mixture was spun at 10000 rpm for 10 min to remove the cell debris and the supernatant was transferred to clean microfuge tubes.

Inorder to remove protein contamination, 500µl of chloroform-iso amyl alcohol (24:1) was added to each tube and mixed the solution by inversion. After mixing, the tubes were spun at 10000 rpm for 10 min and the upper aqueous phase containing DNA was transferred to a clean microfuge tubes.

For precipitation of DNA, to each tube, equal volume of ice cold absolute ethanol was added. It was mixed well and incubated at -20°C for 30 min to precipitate the DNA. The precipitate was isolated by spinning the tube at 10000 rpm for 7 min to form a pellet. The supernatant was removed and washed the DNA pellet by adding ice cold 70% ethanol. After washing, the DNA was made into a pellet by centrifuging at 10000 rpm for 5 min and the supernatant was

>FD758124.1
TTACATTTTCTAAAATGGGGAAGAATATGTTGACAGTTTTACAGACTTCAAACCGAGATTTTCAACGGAA
GGGACTTCGAGTGGGGAGGAGCTAGACACAGCTCAGTGGGAAAACCACAATGATAATTCCACCAAGTTCA
TCGAATTCGTGACATCTCCGAACAACCCAGATGGACATTTGAGGGAACCCAAACTCACAGGCCCCAATAC
CCAGGTGATTCATGATCGCGTCTACTACTGGCCTCATTTTACAGCAATCCCAGCTCCGGCAGACGATGAT
GTGATGATCTTCGATTTCGATCCCTTGGTAAAGCCACCGGCCATGCCGGCAGTAGATTTGGGTGGGCACT
TGTGAAAGACGAAGCTGTGTACCAGAGACTCAAGTATCAGGTGGAAATGAATGTGATCGGCATTTCACAC
GATACCCAACTGCGAGCTCTAATGATTGTCAAATCGATGTTGGGTAAAGGCGGCAGAGATTTGTTCGAGT
TTGGACACGCCGCTCTTGCCTCTTGGTCACAAGTGGGAGAGGTTGAACGAAGTAGTGTCGCAATCTAAAT
TACGCTTCTCTCTTCAGAAGATTTCTCCTCAGTACTGCACTTACTTCTGCACCGTGAGACAACCATCTCC
AGGTGATAATAATTTCTAGAGAATTCTTTTT

Figure 1. FASTA format of 266 bp sequence of *Tryptophan aminotransferase(TAA1)*

>2457047
GCCGAACTTATCATTGGAGAACGACCTTCGACCCGGCTCCCCTCTCTATTTATAACTCCCCATTCCCATTCATTCCCC
CACCACCCAACTACGACAACATTCCATTAACACCGTATTTGGCAAGCAAGCAAGGTGCTTGTGTAGTAGTACCAGT
GTTTGTGCCAAAATGGGAATGACAAGAAGTGATGATCAGCAAGAGGACGGGAAGTGGGTGTGGGTGCATGGTC
CGGTGATAGTCGGAGCAGGGCCATCTGGGTTGGCCGTGGGGGCATGTCTGAAAGAGCACGATGTCCCGTTCGTG
ATACTCGAGAGGGCAGACTGCATAGCCTCGCTGTGGCAAAAGCGCACTTATGACCGTCTCAAGCTCCACCTCCCCA
AGCAATTCTGCCAGCTCCCCGGCATGGCCTTCCCGCTTGACTTCCCGGAGTACCCATCAAAGCTGCAGTTCATCCAG
TACTTGGAGTCCTACTCTGCCCATTTCCAGCTCTCCCCTCGTTTCAACGAGTGCGTCTCCTCTGCGAAGCACGACCCT
GTCACTGGCCTGTGGCAAGTCCGCACTGCCGCCGGGGCCGAGTACATCTGCCGCTGGCTAGTCGTGGCCACCGGC
GAGAACGCCGAGATGGTCGTTCCGGAGTTCGACGGGAGTGAGCTGTTCGCCGGCAGGGTGTCGCATGCGTGCGA
GTACAAGTCCGGCGAGGACTTTCGGGGGAAGAAGGTGCTGGTTGTTGGCTGTGGGAATTCTGGCATGGAAGTCT
CGCTAGACTTGTGCAACCATAATGCTTTCCCTTCCATGGTAGTCCGTAGCAAGGTCCATGTATTGCCGAGAGAGGT
GCTGGGCAGATCGACATTCGAGATAGCAGTACTACTGTTGAAATGGCTTCCATTGTGGTTGGTGGACAAGCTACT
ACTGGTCGTGGCGTGGTTGGTGCTGGGGAACTTTGAGAAATACGGCCTGCACAGGCCGGAAGTTGGTCCTCTGCA
G

Figure 2. FASTA format of 228 bp sequence of *Flavin monooxygenase(YUC2)*

removed and the DNA pellet was allowed to dry for 15 min. Ultimately, the DNA was resuspended in 50 µl sterile DNase free water and stored at -20°C.

3.4 QUANTITY AND QUALITY CHECK OF GENOMIC DNA

The quantity and quality of isolated DNA were measured by using, the absorbance values at wavelengths 260nm and 280nm using a UV spectrophotometer by diluting the DNA samples using nuclease free water.

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

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

The ratio of absorbance at 260nm to 280nm (A_{260}/A_{280}) is taken as an indicator of DNA purity. The A_{260}/A_{280} values of the isolated genomic DNA ranges from 1.70 to 1.88 indicating its good quality.

3.5 AGAROSE GEL ELECTROPHORESIS

The quality of the isolated DNA was assessed by Agarose gel electrophoresis in a horizontal gel electrophoresis unit. Agarose (0.8%) 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 0.5µg/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 (5µl) mixed with 6X gel loading dye (2µl) and nuclease free water (5µl) was poured into wells using a micropipette and 1 kb DNA marker was also loaded to know the size of genomic DNA band. Electrophoresis was carried out at 70 V till the dye front reached 3/4th of the gel. Finally, the gel was taken and viewed in gel documentation system (BioRad inc, USA) using the software "Image Lab".

3.6 SPECIFICITY CHECK OF PRIMERS WITH GENOMIC DNA

The genomic DNA isolated from the spikes of Thekken, Panniyur-1 and Karimunda were amplified using gene specific primers for *Flavin monooxygenase* and *Tryptophan aminotransferase*. A standard PCR mix of 20µl total volume was prepared containing 50ng of template DNA, 0.2mM dNTPs and 400nM each of primers, 1 unit of Taq polymerase and 1X PCR buffer. The thermal profile for PCR was given in Table 2.

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

Table 2. Thermal profile for PCR

Step	Stage	Temperature (°C)	Duration
1	Initial denaturation	95°C	2 min
2	Denaturation	95°C	15 sec
3	Annealing	55°C	15 sec
4	Extension	60°C	45 sec
5	Final extension	60°C	5 min

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

3.7 AMPLIFICATION EFFICIENCY OF THE PRIMERS

The amplification efficiency of the designed primers were determined by using standard curve analysis and "Lin Reg" software.

3.8 RNA ISOLATION

From the spikes at different developmental stages of the pepper cultivars Thekken, Panniyur-1 and Karimunda, the total RNA was isolated by using Trizol

41

reagent. Mortar and pestle, microtips, microfuge tubes, forceps and reagents were sterilised by double autoclaving. DEPC 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 shaked vigorously for 15 s and incubated at room temperature for 5 min. The sample was kept in ice for 10 min and then centrifuged at 12000g for 15 min at 4°C. The aqueous phase of the sample was transferred to a fresh tube. Then 0.5 ml ice cold isopropanol (100%) was added to each tube for RNA precipitation and incubated at room temperature for 10 min and after that mixed by inverting the tube slowly. The sample was centrifuged at 12000g for 10 min at 4°C.

The supernatant was removed and pellet washed with 1 ml of 75% alcohol (in DEPC treated water). Then sample was briefly vortexed and spun at 7500g for 5 min at 4°C and dried for 30 - 40 min in RNase free laminar air flow chamber. The RNA pellet was resuspended in 30µl RNase free water and incubated at 55 - 60°C for 10 minutes and stored at -20°C.

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

An absorbance value of 1.0 at 260nm 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:

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

The total RNA purity was estimated by taking the ratio of absorbance at 260nm to 280nm (A_{260}/A_{280}). A_{260}/A_{280} values in the range of 1.8 to 2.0 indicates the good quality RNA.

3.9 PREPARATION OF cDNA

From the isolated RNA, cDNA was synthesised using Verso cDNA synthesis kit (Thermo Fisher Scientific). It includes 5X cDNA buffer, dNTP mix, oligo dT primer, Random hexamer, RT enhancer for removing DNA contamination and verso enzyme mix. The composition of the reaction mixture (20µl) for cDNA synthesis is as follows:

5X cDNA buffer	:	4µl
dNTP mix	:	2µl
OligodT	:	0.5µl
Random Hexamer	:	0.5µl
RT enhancer	:	1µl
Verso enzyme mix	:	1µl
RNA	:	5µl
Nuclease free water	:	6µl
Total volume	:	20µl

The contents were mixed well and incubated at 42°C for 30 min followed by another incubation of 92°C for 2 min inorder to inactivate the RT enhancer and the cDNA samples were stored at -20°C.

3.10 SPECIFICITY CHECK OF PRIMERS WITH cDNA

The cDNA synthesised was amplified using gene specific primers for*Flavin monooxygenase (YUC2)* and *Tryptophan aminotransferase (TAA1)*. A standard PCR mix was prepared of 20µl total volume containing 50ng of template DNA, 0.2mM dNTPs, and 400nM each of primers, 1 unit of Taq polymerase and 1X PCR buffer.

The amplified products were separated on agarose gel (1.2 %) and viewed under gel documentation system (BioRad inc, USA).

3.11 REAL-TIME PCR

To study the differential expression of auxin biosynthesis genes in different cultivars of black pepper during different developmental stages, Reverse Transcription quantitative PCR or Real Time PCR was done. The house keeping gene *Actin* was taken as the reference gene for RT-qPCR. SYBR green, an intercalating dye, was used for generating fluorescence. The thermal profile for RT-qPCR was given in Table 3. The reaction mixture (20µl) was prepared as follows:

2X Real time PCR Smart Mix	: 10µl
Forward primer (10 pmol/µl)	: 0.6µl
Reverse primer (10 pmol/µl)	: 0.6µl
Template cDNA	: 4 µl
Nuclease free water	: 4.8 µl
Total volume	: 20 µl

Table 3. Thermal profile for real time PCR programme

Step	Stage	Temperature (°C)	Duration
1	Initial denaturation	95°C	2 min
2	Denaturation	95°C	15 sec
3	Annealing	55°C	15 sec
4	Extension	60°C	45 sec
5	Final extension	60°C	5 min

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

RT-qPCR for each gene was performed with two biological replicates and three technical replicates for each sample. Amplification plot and melt curve plot were generated by the software. The Cq values generated by RT-qPCR was used for further analysis.

The data generated were analysed using the "qbase plus" software with *Actin* as the reference gene and two as the amplification factor for all the genes. The software converts raw Cq values into relative expression values. The results were interpreted based on the most repeated pattern noticed among the replicates.

Results

4. RESULT

The results of the study entitled "Expression profiling of auxin biosynthesis genes during inflorescence development in black pepper (*Piper nigrum* L.)" carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani, during 2017-19 are presented in this chapter.

4.1 PRIMER DESIGNING

The primers for auxin biosynthesis genes, *Tryptophan aminotransferase* and *Flavin monooxygenase* were designed using "Primer Express" software based on the sequences of related species retrieved from NCBI database. The designed primers were analysed using "mfold" web server to confirm the absence of secondary structure formation at the primer binding site. The details of the designed primers are given in Table 4.

Table 4 . Sequence of primers designed

Primer	5'.....3'sequence	Tm (°C)	GC content (%)	Amplicon size (bp)
TAAF	GGCAGTAGATTTGGGTGG	59.8	58.0	266
TAA R	AAGTAAGTGCATTATTGAGGA	58.0	52.0	
YUC F	GCTCCACCTCCCCAAGCA	60.5	66.7	228
YUC R	AGCCAGCGGCAGATGTACTC	61.4	60.0	

4.1.1 Primer analysis

GC percentage and melting temperature of the designed primers were determined by "Oligos" software and shows fair values. None of the designed primers exhibited hairpin formation and 3' complementarity.

4.1.2 Specificity of primers

Specificity of primers was checked by agarose gel elctrophoresis using genomic DNA. An amplicon of expected size obtained for all the primers indicate the specificity of the designed primers. Plate 4 shows gel profile of PCR product of *Tryptophan aminotransferase* and *Flavin monooxygenase*.

4.1.3 Amplification efficiency of primers

Amplification efficiency was determined by standard curve analysis. Clustering of data was observed in standard curve analysis. Hence amplification efficiency was determined using data from exponential phase of amplification by "Lin Reg" software and ampification value obtained are given in Table 5.

Table 5. Amplification factor of primers

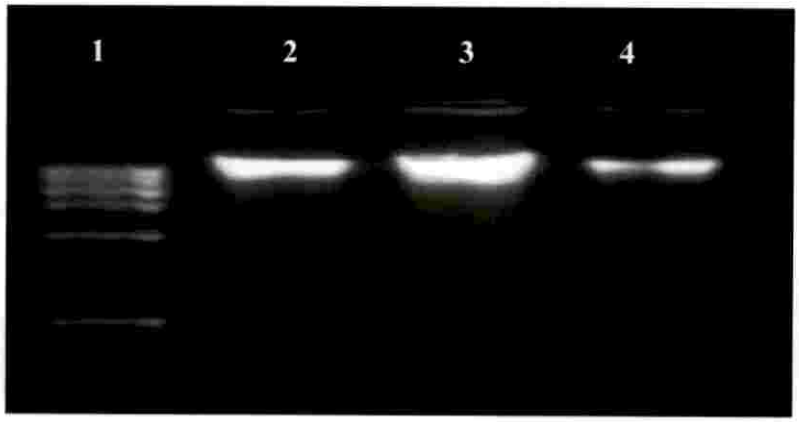
Gene	Amplification factor
Tryptophan aminotransferase	2.04
Flavin monooxygenase	2.03

4.2 MOLECULAR ANALYSIS AT GENOME LEVEL

4.2.1 Isolation of DNA and Agarose Gel Electrophoresis

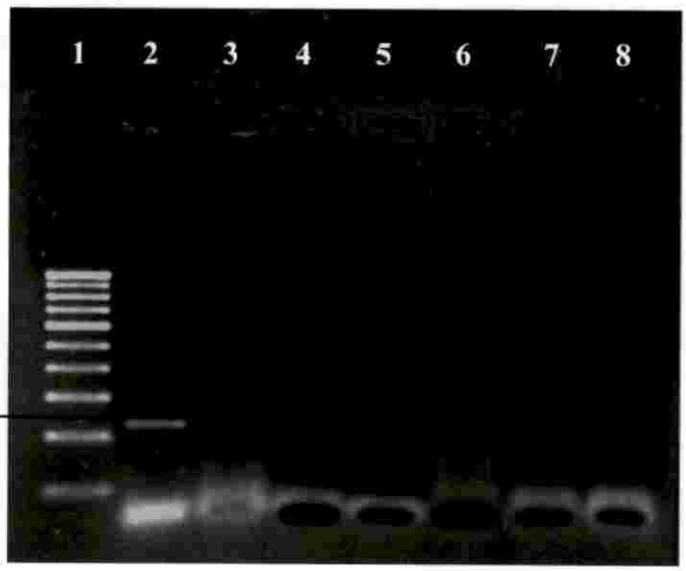
Genomic DNA was isolated from the spike samples at three developmental stages from the cultivars Thekken, Panniyur-1 and Karimunda by modified CTAB method.

The quality of the isolated DNA was assessed by agarose gel electrophoresis (0.8%). It indicated the presence of good quality DNA bands (Plate 3).



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

Plate 3. Genomic DNA isolated from black pepper cultivars



Lane1 : 100 bp DNA marker
Lane 2 to 4 : PCR amplification product of *YUC2*
Lane 5 to 7 : PCR amplification product of *TAA1*
Lane8 : Blank

Plate 4. PCR amplified products of *TAA1* and *YUC2* genes

4.2.2 Quantification of DNA using Spectrophotometer

The quantity and quality of isolated DNA were measured by using the absorbance values at wavelengths 260nm and 280nm using a UV spectrophotometer. The A_{260}/A_{280}values for isolated genomic DNA ranged from 1.70 to 1.88, indicating good quality and quantity of the isolated DNA. The details are presented in Table 6.

Table 6. Quantity and quality of isolated genomic DNA

Cultivar	Concentration (ng/µL)	A_{260}/A_{280}
Thekken	1662	1.70
Karimunda	2727	1.80
Panniyur	1650	1.88

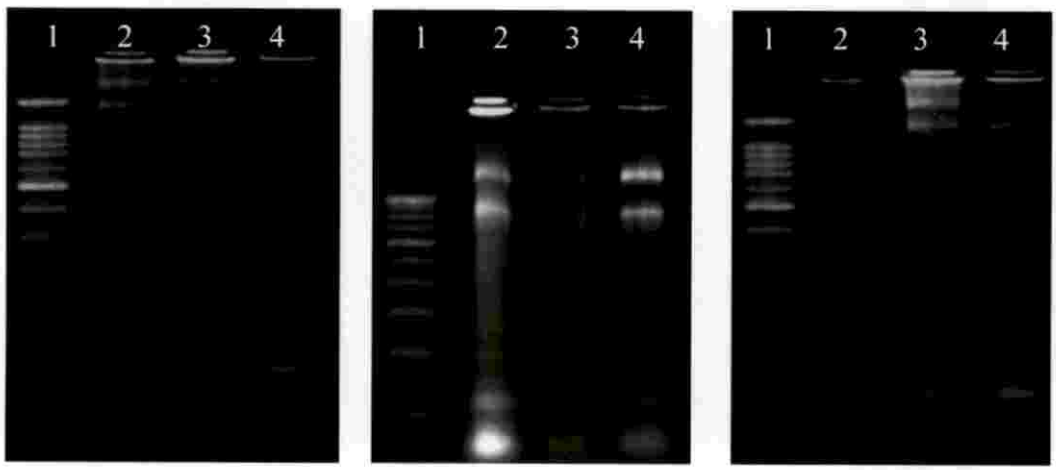
4.3 MOLECULAR ANALYSIS AT TRANSCRIPTOME LEVEL

4.3.1 Isolation of RNA

From the spikes at different developmental stages of the pepper cultivars Thekken, Panniyur-1 and Karimunda, the total RNA was isolated by using Trizol method. The reliability of the isolated RNA was observed by agarose gel electrophoresis (2%). Three intact bands were obtained in Karimunda and Thekken and two bands in Panniyur-1. The gel profile of RNA isolated from Panniyur-1, Karimunda and Thekken are shown in Plate 5.

4.3.2 Quantification of RNA using Spectrophotometer

The quantity and quality of isolated RNA were measured by using the absorbance values at wavelengths 260nm and 280nm using a UV



a.Panniyur-1 **b.Karimunda** **c.Thekken**

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

Plate 5. Total RNA isolated from the spike samples of three black pepper cultivars

spectrophotometer. The A_{260}/A_{280} values for isolated genomic DNA ranges from 1.80 to 2.00. The details are presented in Table 7.

Table 7. Quantity and quality of isolated RNA

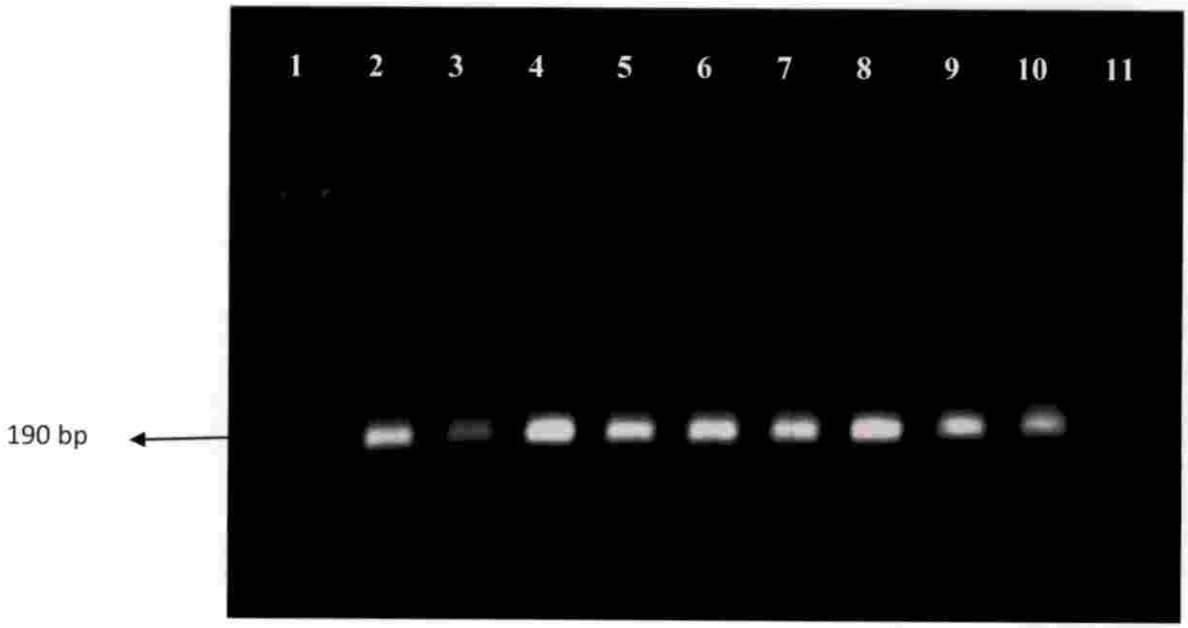
Samples	Concentration (ng/µL)	A_{260}/A_{280}
Thekken stage 1	1860	1.80
Thekken stage 2	2560	1.88
Thekken stage 3	1678	1.90
Panniyur-1 stage 1	2570	1.85
Panniyur-1 stage 2	1292	1.80
Panniyur-1 stage 3	1720	1.90
Karimunda stage 1	2147	1.90
Karimunda stage 2	1475	1.80
Karimunda stage 3	1674	2.00

4.3.3 cDNA synthesis and quality check of cDNA

From the isolated RNA, cDNA was reverse transcribed using Thermo Scientific Verso cDNA synthesis kit. The quality check of the synthesized cDNA was done by PCR using gene specific primer for *Actin* and by agarose gel electrophoresis. An amplicon of expected size of 190 bp in all the samples indicates the good quality of cDNA (Plate 6).

4.3.4 Specificity check of primers with cDNA

The cDNA synthesised was amplified using gene specific primers for*Tryptophan aminotransferase (TAA1)* and *Flavin monooxygenase (YUC2).* Amplicon of expected size was obtained in PCR amplification, which indicates the specificity of the designed primers (Plate 7 and 8).

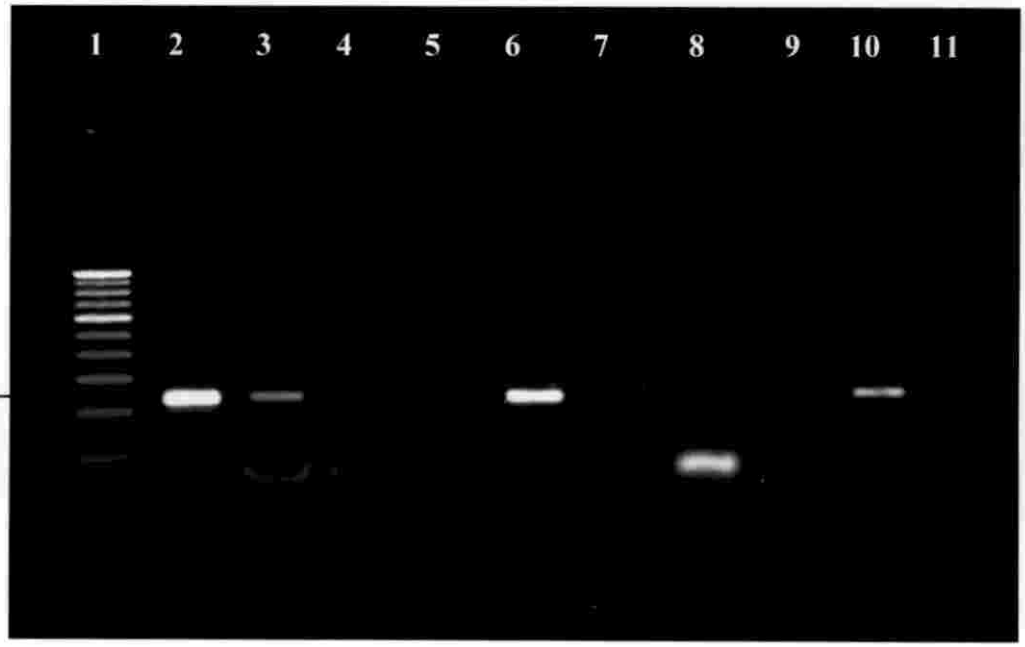


Lane1 : 100 bp DNA marker
Lane 2 to 4 : PCR amplification product of *Actin* from cDNA of Karimunda stage 1,2 and 3 respectively
Lane 5 to 7 : PCR amplification product of *Actin* from cDNA of Panniyur-1 stage 1,2 and 3 respectively
Lane 8 to 10 : PCR amplification product of *Actin* from cDNA of Thekken stage 1,2 and 3 respectively
Lane 11 : Blank

Plate 6. cDNA amplified PCR product of *Actin*

Lane1 : 100 bp DNA marker
Lane 2 to 4 : PCR amplification product of primer *TAA1* from cDNA of Karimunda stage 1, 2 and 3 respectively
Lane 5 to 7 : PCR amplification product of primer *TAA1* from cDNA of Panniyur-1 stage 1, 2 and 3 respectively
Lane 8 to 10 : PCR amplification product of primer *TAA1* from cDNA of Thekken stage 1, 2 and 3 respectively
Lane 11 : Blank

Plate 7. cDNA amplified PCR product of *TAA1*



Lane1 : 100 bp DNA marker
Lane 2 to 4 : PCR amplification product of primer *YUC2* from cDNA of Karimunda stage 1,2 and 3 respectively
Lane 5 to 7 : PCR amplification product of primer *YUC2* from cDNA of Panniyur-1 stage 1,2 and 3 respectively
Lane 8 to 10 : PCR amplification product of primer *YUC2* from cDNA of Thekken stage 1,2 and 3 respectively
Lane 11 : Blank

Plate 8. cDNA amplified PCR amplified product of *YUC2*

4.3.5 Expression profiling using RT-qPCR

To study the differential expression of auxin biosynthesis genes in different cultivars of black pepper during different developmental stages, RT-qPCR was done. The cDNA from spike samples of three different cultivars at three developmental stages were used as template for RT- qPCR.

RT-qPCR for each gene was performed with two biological replicates and three technical replicates for each sample. Amplification plot and melt curve plot were generated by the software. Amplification plot generated shows the raw expression data. The amplification plots for *Tryptophan aminotransferase and Flavin monooxygenase* are shown (Fig 3 and 4).

Melt curve plot shows prominent peak for both the primers indicating the specificity of Real time PCR reaction. Fig 5 and 6 shows melt curve generated for the primers.

The Cq values (Fig 7 and 8) generated by RT-qPCR was used for further analysis. Cq values obtained are given in Table 8.

Table 8. Cq values generated by RT-qPCR

Samples	*TAA1*	*YUC2*
Karimunda stage 1 (K1)	35.45	37.94
Karimunda stage 2 (K2)	34.75	37.83
Karimunda stage 3 (K3)	34.62	35.89
Panniyur-1 stage 1 (P1)	35.60	36.01
Panniyur-1 stage 2 (P2)	35.23	34.53
Panniyur-1 stage 3 (P3)	34.78	36.58
Thekken stage 1 (T1)	34.63	32.01
Thekken stage 2 (T2)	34.82	31.89
Thekken stage 3 (T3)	34.97	33.80

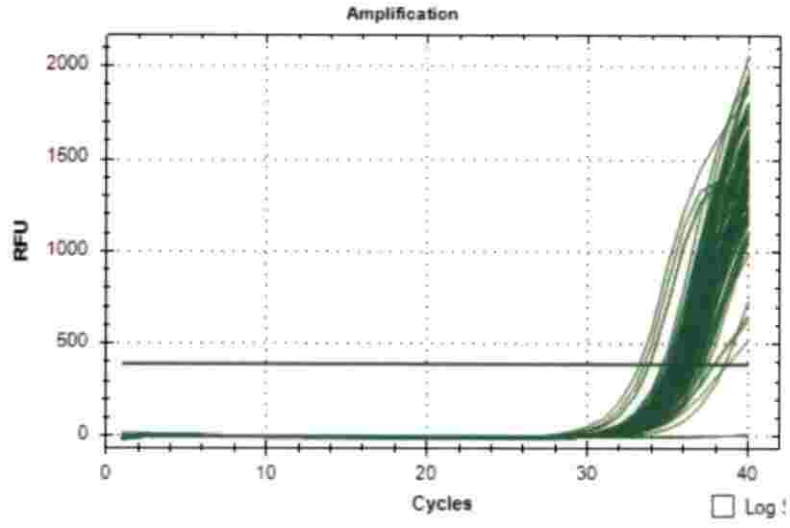


Fig. 3: Amplification plot of *TAA1* by RT-qPCR.

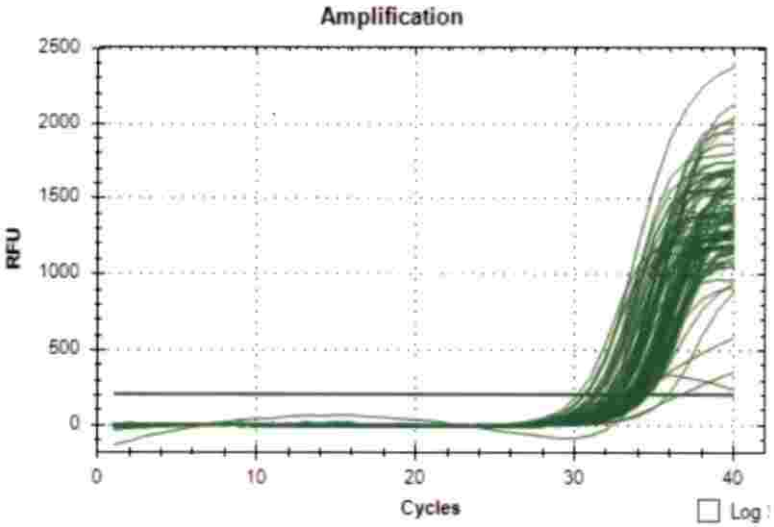


Fig. 4: Amplification plot of *YUC2* by RT-qPCR.

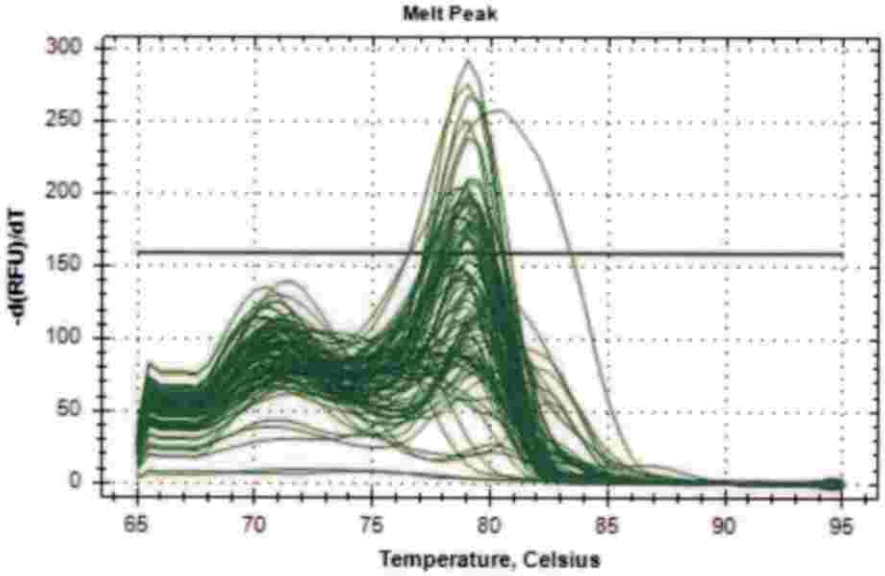


Fig 5: Melt curve of *TAA1* by RT-qPCR.

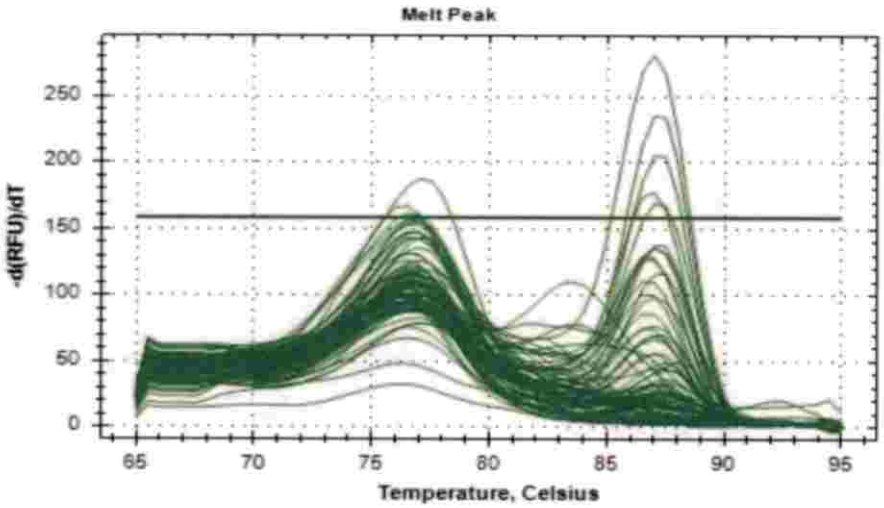


Fig 6: Melt curve of *YUC2* by RT-qPCR.

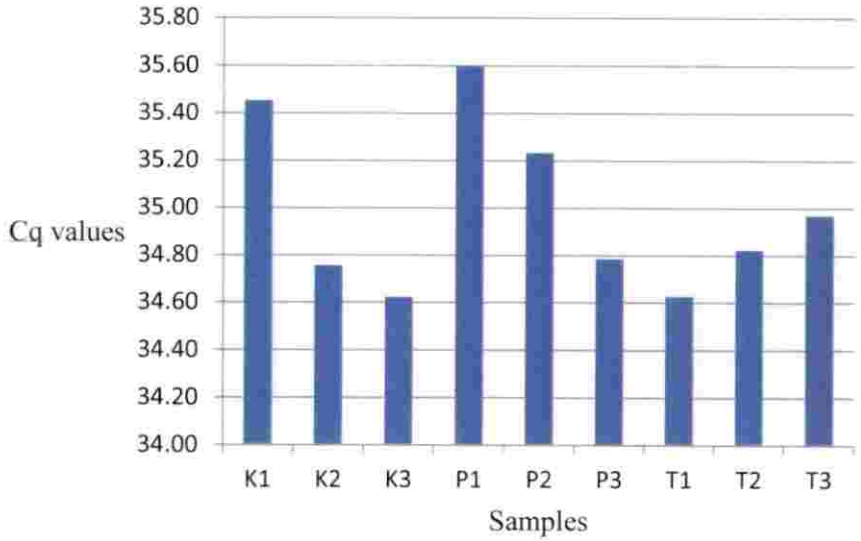


Fig. 7: Cq values obtained for *TAA1* by RT-qPCR.

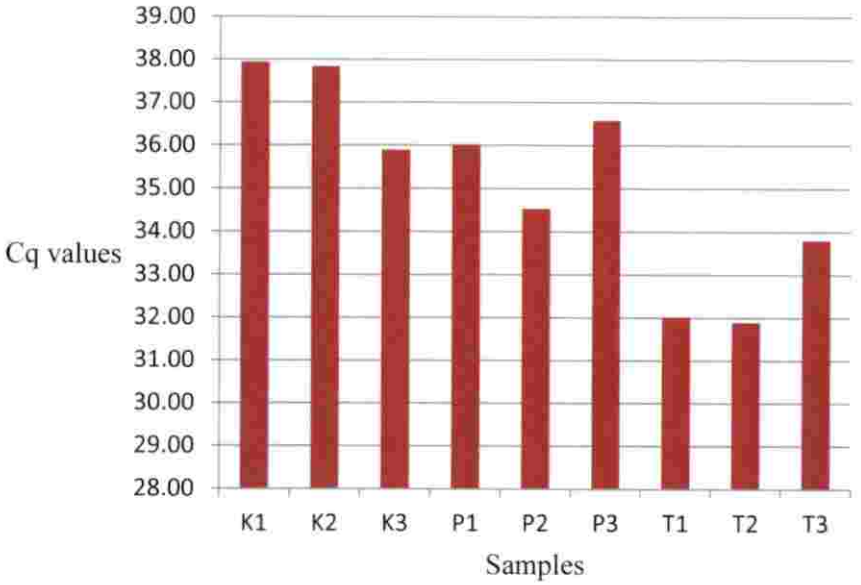


Fig. 8: Cq values obtained for *YUC2* by RT-qPCR.

4.4 DATA ANALYSIS

4.4.1 Analysis using qbase plus software

Gene expression analysis was performed using "qbase plus" software using *Actin* as the reference gene and two as the amplification factor for all the genes. Raw Cq values were converted to relative expression value by qbase plus software. The relative expression values normalized with the reference gene are given in Table 9. The relative expression of the genes with respect to the stage 1 of each varieties are shown in the Table 10.

Table 9. Relative expression of target genes normalized with reference gene

Samples	*TAA1*	*YUC2*
Karimunda stage 1	1.1324	0.9092
Karimunda stage 2	10.8462	8.0635
Karimunda stage 3	9.2942	3.4309
Panniyur-1 stage 1	17.1328	8.7334
Panniyur-1 stage 2	17.9854	16.7252
Panniyur-1 stage 3	18.5642	5.5687
Thekken stage 1	1.0000	1.0000
Thekken stage 2	3.4576	7.4358
Thekken stage 3	2.2763	2.5920

Table 10. Relative expression values of *TAA1* and *YUC2* with respect to first stage taken as control for each variety

Samples	*TAA1*	*YUC2*
Karimunda stage 1	1.0000	1.0000
Karimunda stage 2	9.5532	8.8692
Karimunda stage 3	9.2942	3.7737
Panniyur-1 stage 1	1.0000	1.0000
Panniyur-1 stage 2	1.0538	1.9151
Panniyur-1 stage 3	1.0847	0.6376
Thekken stage 1	1.0000	1.0000
Thekken stage 2	3.4482	7.4358
Thekken stage 3	2.2981	2.5920

The expression pattern of *TAA1* showed variation during inflorescence development in all the three cultivars. Higher expression was noticed in all the three stages of Panniyur-1 compared to Karimunda and Thekken. In Karimunda and Thekken, the expression was low in the first stage and peaked at stage 2 and decreased in stage 3. Expression of *TAA1* was very less in stage 2 and 3 of Thekken (Fig 9 to 12).

The expression pattern of *YUC2* also showed variation during inflorescence development in all the three cultivars. The expression pattern was similar in all the three cultivars with a peak at stage 2. The expression of *YUC2* was highest in Panniyur-1 and lowest in Thekken (Fig 13 to 16).

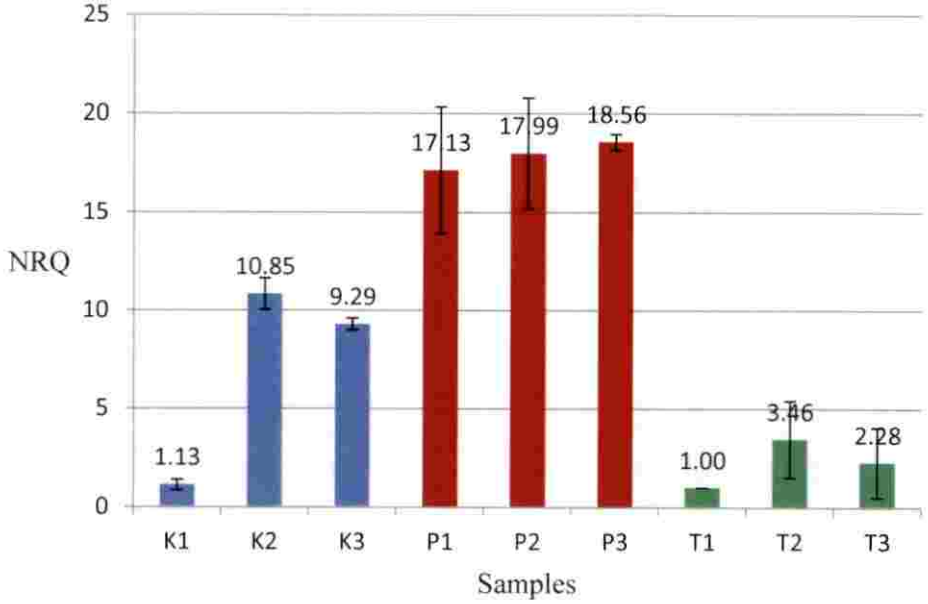


Fig. 9: Relative expression of *TAA1* normalized with *Actin*

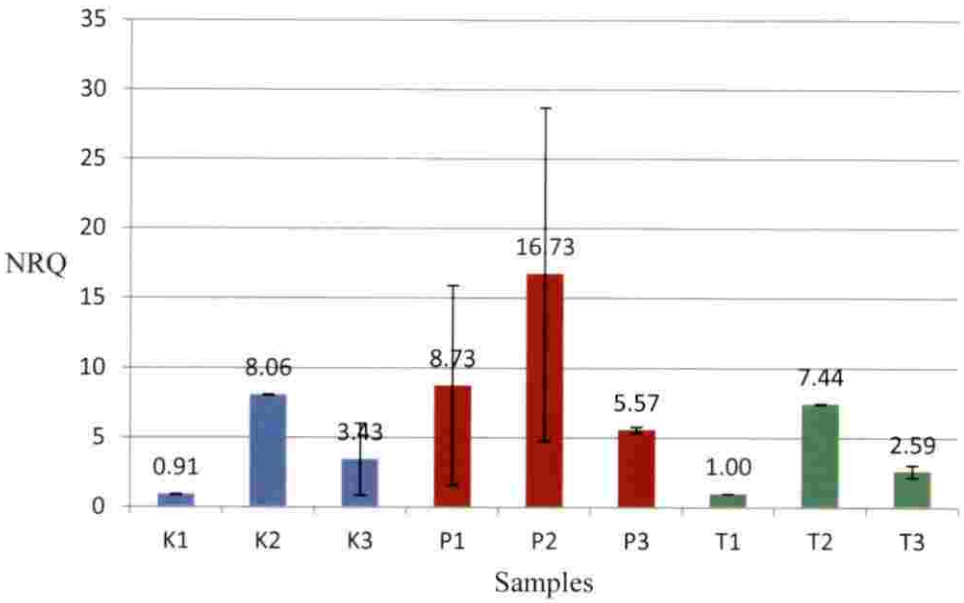


Fig. 10: Relative expression of *YUC2* normalized with *Actin*

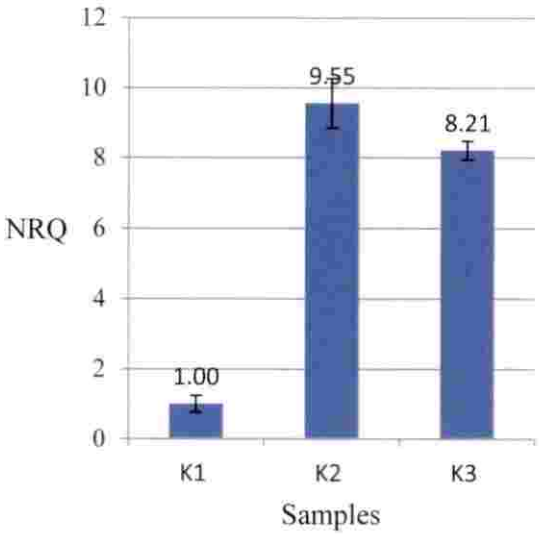


Fig.11: Relative expression values of *TAA1* in Karimunda

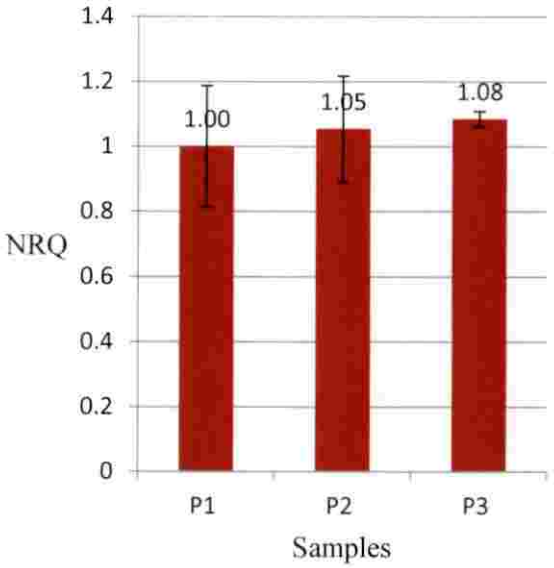


Fig.12: Relative expression values of *TAA1* in Panniyur-1

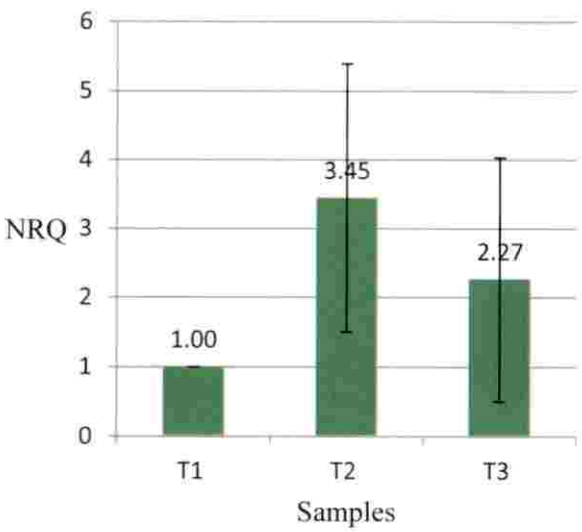


Fig. 13: Relative expression values for *TAA1* in Thekken

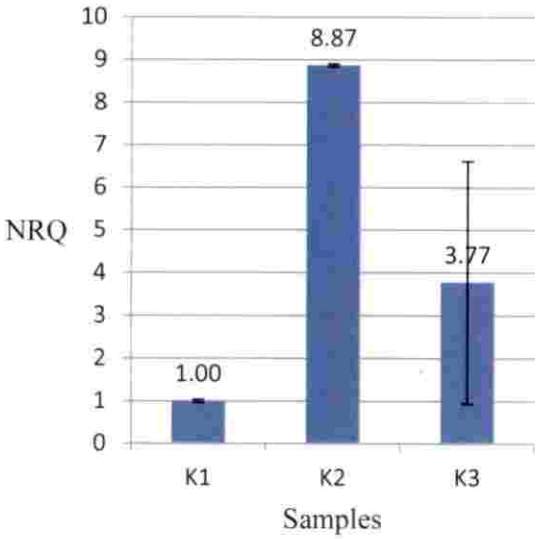


Fig.14: Relative expression values of *YUC2* in Karimunda

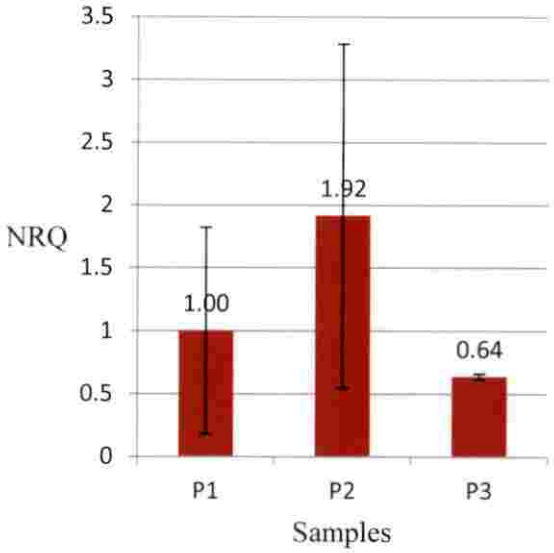


Fig.15: Relative expression values of *YUC2* in Panniyur-1

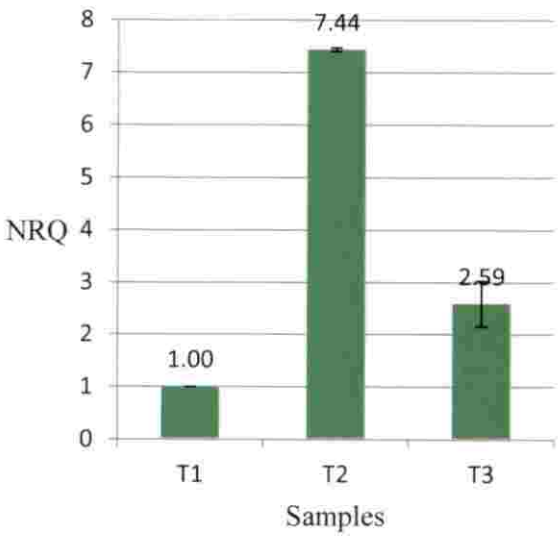


Fig.16: Relative expression values of *YUC2* in Thekken

Discussion

5. DISCUSSION

Black pepper (*Piper nigrum* L.), the 'King of Spices' which belongs to the family Piperaceae is a widely used spice, known for its pungent odour. It is commonly called as 'Black Gold' and is one of the important spice commodities of commerce and trade in India since the pre-historic period. It had originated in the tropical evergreen forests of the Western Ghats (Govindarajan, 1977). In India, black pepper is cultivated to a large extent in Kerala, Karnataka and Tamil Nadu and to a limited extent in Maharashtra, North Eastern states and Andaman and Nicobar Islands.

Usually black pepper has unbranched spike. But a pepper type 'Thekken' a selection of *Piper nigrum*, collected from the forest area of Idukki district by a farmer Shri. T. T. Thomas exhibits branching character with more than thirty well developed branches bearing about three hundred berries altogether. This is about four times the number of berries from spikes of the highest yielding varieties, Panniyur-1, Panniyur-3 and Panniyur-5. Spike branching is an economically important trait in pepper having the potential to improve yield by three to fourfold (Sasikumar *et al*., 2007; Farm innovators, 2010).

The study entitled "Expression profiling of auxin biosynthesis genes during inflorescence development in black pepper (*Piper nigrum* L.)" was carried out during 2017-2019, in the Department of Plant Biotechnology, College of Agriculture, Vellayani to compare the transcript profile of auxin biosynthesis genes such as *Flavin monooxygenase* (*YUC2*) and *Tryptophan aminotransferase* (*TAA1*) during inflorescence development in different cultivars of black pepper using Reverse Transcription quantitative PCR (RT- qPCR).

For studying these genes, spike samples of three cultivars of black pepper *viz*., Panniyur-1, Karimunda and Thekken were used. Spike samples were collected at three different developmental stages *viz*., stage 1(1-2cm; 12-15 days after bud emergence), stage 2 (6-8cm; 22-25 days after bud emergence) and stage 3 (9-12cm; 32-35 days after bud emergence) from two different plants of each

cultivar are used for analyzing the gene expression (Le *et al.*, 2012; Zeng *et al.*, 2017). Spike samples were subjected to genome and transcriptome level analysis.

5.1 GENOME AND TRANSCRIPTOME ANALYSIS

In molecular techniques, DNA isolation is a critical step because the DNA quality can affect the subsequent analytical techniques (Boiteux *et al.*, 1999; Fredricks *et al.*, 2005). CTAB method of DNA isolation (Doyle and Doyle, 1990) is the widely used method for DNA isolation from plants. In the present study, modified CTAB method (Subba *et al.*, 2014) was used for isolation of genomic DNA from the spike samples of black pepper.

The addition of NaCl at concentrations higher than 0.5M, along with Cetyl trimethyl ammonium bromide (CTAB), removes polysaccharides during DNA extraction (Paterson *et al.*, 1993; Moreira and Oliveira, 2011; Sahu *et al.*, 2012). 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 quantities of polysaccharides (Sambrook and Russel, 2001). Poly vinyl pyrrolidone (PVP) and β-mercaptoethanol were added in the extraction buffer to prevent the oxidation of secondary metabolites in the sample and to increase the quantity and quality of the isolated DNA (Prittila *et al.*, 2001; Warude *et al.*, 2003).

The quality of the isolated DNA was confirmed by obtaining distinct bands in agarose gel electrophoresis (Plate 2). According to Weising *et al.* (2005), the ratio of absorbance at 260 nm to that at 280 nm (A_{260}/A_{280}) value in the range of 1.8 to 2.0 indicates good quality DNA. In the present study, the A_{260}/A_{280} value of the DNA isolated from the spike samples ranges from 1.70 to 1.88 which indicates the good quality of DNA isolated.

According to Doyle (1996), RNA isolation requires special care and attention due to the ubiquitous presence of RNase in the environment, which degrades the RNA and therefore maintenance of sterile condition is of great

importance. In the present study, Trizol method of RNA isolation was used to isolate RNA from the spike samples at different stages of inflorescence development from Thekken, Karimunda and Panniyur-1. Sterile condition was maintained throughout the isolation process. Trizol method uses Guanidinium thiocyanate, a chaotropic agent that allows the purification of RNA from cells (Cox, 1968; Chomczynski and Sacchi, 1987). Trizol reagent is a ready-to-use reagent for RNA extraction and it makes RNA isolation process easier and fast (Simms *et al.*, 1993). It has been reported that a strong denaturing buffer containing guanidinium thiocyanate produces a good quality RNA which can be used for reverse transcription experiments (George *et al.*, 2005). The 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 and can be precipitated with isopropanol (Chomczynski, 1993). Intact RNA bands without shearing was obtained on agarose gel electrophoresis which indicates the good quality of isolated RNA (Plate 4).

The ratio of absorbance at 260 nm to that at 280 nm (A_{260}/A_{280}) value in the range of 1.7 to 2.0 indicates the purity of the isolated RNA (Acerbic *et al.*, 2010). In the present study, the A_{260}/A_{280} value of the RNA isolated from the spike samples ranged from 1.80 to 2.00 indicating the good quality of RNA isolated.

From the isolated RNA, cDNA was reverse transcribed using Thermo Scientific verso cDNA synthesis kit. The quality check of the synthesized cDNA was done by PCR using gene specific primer for *Actin* and agarose gel electrophoresis. An amplicon of expected size of 190 bp in all the samples indicates the good quality of cDNA synthesized.

As the sequence of the auxin biosynthesis genes, *Tryptophan aminotransferase (TAA1)* and *Flavin monooxygenase (YUC2)* of black pepper were unavailable in NCBI database, forward and reverse primers were designed using "Primer Express" software based on the sequence from related species. Singh and Pandey (2015) reported that primer designing is a crucial step for the

proper estimation of transcript abundance of the genes. Primers should maintain a reasonable GC content 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; Patel and Prakash, 2013). The analysis with "Oligo Calc" program revealed fair GC% and annealing temperature. None of the designed primers exhibited hairpin formation and 3' complementarity.

In the present study, the melting temperature(T_m) of forward and reverse primers of *TAA1* was 60.5°C and 61.0°C respectively and the GC content was 67% and 63% respectively. For *YUC2*, the T_m of forward and reverse primer was 59.8°C and 61.3°C respectively and the GC content was 52% and 44% respectively. Good quality gene specific primers were designed using "Primer Express" software. The absence of secondary structure at the binding site of the designed primers was confirmed by "mfold" web server.

For checking the specificity of the primers designed for the auxin biosynthesis genes *viz.*, *TAA1* and *YUC2*, PCR was performed with the isolated genomic DNA and cDNA using gene specific primers. In agarose gel electrophoresis, an amplicon of expected size was obtained for all the designed primers.

Standard curve analysis is a method used for determination of amplification efficiency using known template concentrations by RT-qPCR analysis (Bustin *et al.,* 2009; Rujiter *et al.*, 2009). However, in the present study the data generated from standard curve analysis formed clusters. Hence, amplification efficiency was determined by "Lin Reg" software (Ramakers *et al.*, 2003). In the present study, all the designed primers exhibited cent per cent amplification efficiency using "Lin Reg" software (Table 5).

Indole-3-acetic acid (IAA), the natural auxin produced by plants, is known to be synthesized either by Tryptophan (Trp) dependent pathway or using a Tryptophan (Trp) independent pathway (Zhao *et al.*, 2012). Among the

Tryptophan dependent pathway, Indole Pyruvic Acid (IPA) pathway is the first complete and universally conserved auxin biosynthesis pathway in plants (Mano and Nemoto, 2012). *TAA1* and *YUC2* genes are the two consecutive genes in IPA pathway (Gallavotti *et al.*, 2013). It involves the conversion of Tryptophan to Indole Pyruvic Acid (IPA) by the activity of *Tryptophan aminotransferase (TAA1)* genes and the subsequent conversion of IPA to Indole Acetic Acid (IAA) by *Flavin monooxygenase(YUC2)* gene belonging to the *YUCCA*(*YUC*) gene family (Zhao *et al.*,2002; Stepanova *et al.*, 2008; Tao *et al.*, 2008). In the present study, attempts were made to analyse the differential expression of these auxin biosynthesis genes during inflorescence development in Panniyur-1, Thekken and Karimunda.

For the expression profiling of auxin biosynthesis genes such as *TAA1* and *YUC2*, Reverse Transcription quantitative PCR (RT- qPCR) or Real Time PCR was carried out. Yilmaz *et al.* (2012) reported that real time PCR is one of the best technique which is used for gene expression analysis using the phenomenon of fluorescence resonance energy transfer. RT-qPCR is a powerful methodology to analyse the expression of target genes. It is attractive for its high sensitivity in gene expression analysis (Wang *et al.*, 2019).

SYBR Green dye-based assays are used to create fluorescence in the present study. It has been reported that SYBR Green is a dye which is widely used in real time PCR and it exhibits fluorescence only when it gets bound to a double stranded DNA molecule (Navarro *et al.*, 2015; Kuang *et al.*, 2018). Espy *et al.* (2006) reported 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, based on the GC content and the length of the amplification product.

RT-qPCR for each gene was performed with two biological replicates and three technical replicates for each sample. Amplification plot and melt curve plot were generated by the software. Amplification plot generated shows the raw

expression data and the melt curve plot shows a prominent peak indicating the specificity of Real time PCR reaction. The Cq values generated by RT-qPCR was used for further analysis.

Gene expression analysis was performed using "qbase plus" software (Hellemans *et al.*, 2007; Rajpur and Barrett, 2019). Normalization is very important step inorder to obtain accurate results in RT-qPCR. Several strategies have been developed to normalize the data. One of the methods is normalization using reference gene (Hugget *et al.*, 2005). The accuracy of results obtained by this method is dependent on normalization using stably expressed genes known as reference gene.

In the present study, the house keeping gene *Actin* was taken as the reference gene and two was taken as the amplification factor for all the genes. Raw Cq values generated by RT-qPCR were converted to relative expression value by qbase plus software.

The normalized expression values in the present study indicates that the expression pattern of *TAA1* showed regulation during inflorescence development in all the three cultivars. Higher expression of the gene was noticed in all the three inflorescence development stages of Panniyur-1 compared to Karimunda and Thekken. In Karimunda and Thekken, the expression was lower in the stage 1 and peaked at stage 2 and again decreased in stage 3. The expression of *TAA1* gene was very less in stage 2 and 3 of Thekken.

Similarly, the expression pattern of *YUC2* also showed differential regulation during inflorescence development in all the three cultivars. The expression pattern was similar in all the three cultivars with a peak at stage 2. Higher expression of the gene was noticed in Panniyur-1. The expression of *YUC2* was lower in the branching type Thekken.

The results of the present study shows that both the auxin biosynthetic genes *viz., TAA1* and *YUC2* were differentially regulated during different stages of

inflorescence development in different cultivars of black pepper *viz.*, Panniyur-1, Karimunda and Thekken. The expression levels of both the biosynthetic genes were least in the branching type Thekken compared to the non- branching type Panniyur-1 and Karimunda. The lower expression levels of these genes in Thekken may contribute towards the lower auxin accumulation in Thekken.

The results of the present study correlates with a previous work conducted in the Department of Plant Biotechnology, College of Agriculture, Vellayani by Bhasi *et al.* (2018). In the study, the differential auxin content in different black pepper cultivars was quantified by using HPLC. According to Bhasi *et al* (2018), the IAA content of Thekken was one-fourth compared to Panniyur-1 and one-third compared to Karimunda in the inflorescence development stage 3.

The differential expression of these auxin biosynthesis genes in the present study indicates that these genes may have a significant role in inducing spike branching in the black pepper type Thekken.

5.2 FUTURE LINE OF WORK

In addition to *TAA1* and *YUC2* genes analysed in the present study, more genes involved in auxin biosynthesis pathway are to be explored in different varieties of black pepper for a better understanding of the role of auxin in inducing spike branching in black pepper.

Summary

6. SUMMARY

The study entitled "Expression profiling of auxin biosynthesis genes during inflorescence development in black pepper (*Piper nigrum* L.)" was conducted during 2017-19 at Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram. The objective of the study was to compare the transcript profile of auxin biosynthesis genes such as *Flavin monooxygenase* and *Tryptophan aminotransferase* during inflorescence development in different cultivars of black pepper using Reverse Transcription quantitative PCR (RT- qPCR).

Spike samples of three cultivars of black pepper *viz.*, Panniyur, Karimunda and Thekken were used for the study. Samples were collected at three different developmental stages *viz.*, stage 1 (1-2cm; 12-15 days after bud emergence), stage 2 (6-8cm; 22-25 days after bud emergence) and stage 3 (9-12cm; 32-35 days after bud emergence) from two different plants of each cultivar. Genomic DNA was isolated from the collected spike samples by modified CTAB method.

Primers were designed for auxin biosynthesis genes such as *Tryptophan aminotransferase (TAA1)* and *Flavin monooxygenase (YUC2)* based on the sequence from the related species using "Primer Express" software and absence of secondary structure at the primer binding site was confirmed by "mfold" web server. The specificity of the designed gene specific primers was checked by PCR using genomic DNA. Amplification with *TAA1* and *YUC2* genes produced amplicons of expected sizes *viz.,* 266 bp and 228 bp respectively indicating specificity of the designed primers.

The amplification efficiency of the designed primers was determined by standard curve analysis. But, clustering of data was observed in standard curve analysis. Hence, amplification efficiency was determined by using "Lin Reg" software. All the primers exhibited cent per cent amplification efficiency.

Total RNA was isolated from the spike samples by Trizol method. RNA isolated from the spike samples was converted to cDNA by Thermo Scientific Verso cDNA synthesis Kit which contains DNase that degrades DNA ensuring that the PCR product is obtained from the cDNA not from DNA and the quality was confirmed by PCR using gene specific primer for *Actin* and agarose gel electrophoresis. Single amplicon of 190 bp obtained in all the samples during agarose gel electrophoresis indicates the quality of the synthesized cDNA. Specificity check of cDNA was also done 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 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 two biological replicates and three technical replicates for each sample. Amplification plot and Melt curve plot were generated by the software. The amplification plot generated shows the raw expression data. The Melt curve plot shows prominent peak for both the primers indicating the specificity of Real time PCR reaction. Cq values generated from real time PCR was used for further analysis.

Gene expression analysis was performed using "qbase plus" software. Analysis was done using *Actin* as the reference gene and two as taken as the amplification factor for all the genes. Raw Cq values were converted to relative expression value by qbase plus software.

The expression pattern of *TAA1* showed regulation during inflorescence development in all the three cultivars. Higher expression was noticed in all the three stages of Panniyur-1 compared to Karimunda and Thekken. In Karimunda and Thekken, the expression was low in the first stage and peaked at stage 2 and decreased in stage 3. The expression of *TAA1* was very less in stage 2 and 3 of Thekken.

The expression pattern of *YUC2* also showed differential regulation during inflorescence development in all the three cultivars. The expression pattern was similar in all the three cultivars with a peak at stage 2. The expression of *YUC2* was highest in Panniyur-1 and lowest in Thekken.

To conclude, the auxin biosynthetic genes such as *TAA1* and *YUC2* were differentially regulated during different stages of inflorescence development in all the three cultivars of black pepper. The expression levels of both the biosynthetic genes were least in the branching type Thekken. Low expression levels of these genes may contribute towards low auxin accumulation in Thekken.

However, the lower expression level of auxin biosynthesis genes *viz.*, *YUC2* and *TAA1* observed in Thekken compared to Karimunda and Panniyur-1 further may support the possible role of these genes in induction of the spike branching trait in black pepper. This result indirectly indicates that auxin may have a role in induction of branching pattern of spikes seen in Thekken.

Analysis of other genes involved in auxin signaling might help in a better understanding of inflorescence development in black pepper. In addition to *TAA1* and *YUC2* genes analysed in the present study, more genes involved in auxin biosynthesis pathway are to be explored in different varieties of black pepper for a better understanding of the role of auxin in inducing spike branching in black pepper.

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Appendices

APPENDIX I

CTAB Extraction Buffer

CTAB	2% (w/v)
Tris-HCl	100mM
EDTA	25mM
Nacl	2M
β-mercaptoethanol	0.2% (v/v)
PVP	4% (w/v)

APPENDIX II

TBE Buffer (5X) for 1 litre solution

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

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

by

ARATHY L. S.

(2017-11-106)

Abstract of the thesis
Submitted in partial fulfilment of the
requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695522

KERALA, INDIA

2019

9. ABSTRACT

The study entitled "Expression profiling of auxin biosynthesis genes during inflorescence development in black pepper (*Piper nigrum* L.)" was carried out during 2017-2019, in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective of the study was to compare the transcript profile of auxin biosynthesis genes such as *Flavin monooxygenase* and *Tryptophan aminotransferase* during inflorescence development in different cultivars of black pepper (*Piper nigrum* L.) using Reverse Transcription quantitative PCR (RT- qPCR).

Black pepper exhibits wide variability in inflorescence architecture. Auxin is a major hormone involved in patterning and formation of lateral organs and regulation of branching. Auxin biosynthesis in plants occurs mainly through tryptophan dependent and independent pathways. Among the different known pathways, the most significant and well studied is the Indole-3-Pyruvic Acid (IPA) pathway. *Tryptophan aminotransferase* (*TAA1*) and *Flavin monooxygenase* (*YUC2*) are the consecutive enzymes of the pathway converting tryptophan to IPA and subsequently to Indole Acetic Acid (IAA).

Spike samples of three cultivars of black pepper *viz.*, Panniyur, Karimunda and Thekken were used for the study. Samples were collected at three different developmental stages *viz.*, stage 1 (1-2cm), stage 2 (6-8cm) and stage 3 (9-12cm) from two different plants of each cultivar. Genomic DNA and RNA were extracted by modified Cetyl Trimethyl Ammonium Bromide (CTAB) method and Trizol method respectively.

Primers were designed for auxin biosynthesis genes such as *Tryptophan aminotransferase* and *Flavin monooxygenase* using "Primer Express" software and absence of secondary structure at the primer binding site was confirmed by "mfold" web server. Specificity of the designed gene specific primers was checked by PCR using genomic DNA. Single amplicon was obtained for all the primers indicating specificity of thegenomic DNA. Single amplicon was obtained for all the primers indicating specificity of the designed primers.

Amplification efficiency of the designed primers was determined by standard curve analysis and "Lin Reg" software. All the primers exhibited cent per cent amplification efficiency. RNA isolated from the spike samples was converted to cDNA and the quality was confirmed by PCR and agarose gel electrophoresis. cDNA was used for RT-qPCR using SYBR Green dye-based assay. Thermal conditions for RT-qPCR were 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 45 sec. RT-qPCR for each gene was performed with three technical replicates for each sample. Cq values obtained were used for further analysis.

Gene expression analysis was performed using "qbase plus" software using *Actin* as the reference gene and two as the amplification factor for all the genes. The expression pattern of *TAA1* showed regulation during inflorescence development in all the three cultivars. Higher expression was noticed in all the three stages of Panniyur-1 compared to Karimunda and Thekken. In Karimunda and Thekken, the expression was low in the first stage and peaked at stage 2 and decreased in stage 3. Expression of *TAA1* was very less in stage 2 and 3 of Thekken. The expression pattern of *YUC2* also showed differential regulation during inflorescence development in all the three cultivars. The expression pattern was similar in all the three cultivars with a peak at stage 2. The expression of *YUC2* was highest in Panniyur-1 and lowest in Thekken.

To conclude, auxin biosynthetic genes *TAA1* and *YUC2* were differentially regulated during different stages of inflorescence development in all the three cultivars of black pepper. The expression levels of both the biosynthetic genes were least in the branching type Thekken. Low expression levels of these genes may contribute towards low auxin accumulation in Thekken. Analysis of other genes involved in auxin signalling might help in a better understanding of inflorescence development in black pepper.