IDENTIFICATION OF MOLECULAR MARKER ASSOCIATED WITH SPIKE BRANCHING TRAIT IN BLACK PEPPER (*Piper nigrum* L.)

by

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Identification of molecular marker associated with spike branching trait in black pepper (*Piper nigrum* L.)"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title of any other University or Society.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF PLATES CONTINUED

LIST OF APPENDICES

LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
A	Adenine
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
A_{595}	Absorbance at 595 nm wavelength
AMV-RT	*Avian myeloblastosis virus* reverse transcriptase
APS	Ammonium persulfate
bp	Base pair
BP	Brevipedicellus gene
bp	Brevipedicellus mutant
BP	Brevipedicellus protein
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
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
F	Forward primer
G	Guanine
g	gram

g	standard acceleration due to gravity at the earth's surface
h	Hour
ha	Hectare
kbp	Kilo basepair
kDa	Kilodalton
kg	Kilogram
LOG1	Lonely guy-1 gene
log1	Lonely guy-1 mutant
M	Molar
mg	Milligram
Mg^{2+}	Magnesium ion
Mn^{2+}	Manganese ion
min	Minute
ml	Millilitre
m*M*	Millimolar
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
ng	Nanogram
nm	Nanometre
°C	Degree celsius
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIN1	Pinformed-1 gene
pin1	Pinformed-1 mutant
PVP	Polyvinyl pyrrolidone
R	Reverse primer

RA2	Ramosa-2 gene
ra2	Ramosa-2 mutant
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
s	Second
SAM	Shoot apical meristem
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sp.	Species
spp.	Species (plural)
SSR	Simple sequence repeat
t	Tonne
T	Thymine
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N' –tetramethylethylenediamine
T_m	Melting temperature
Tris HCl	Tris (hydroxy methyl) aminomethane hydrochloride
U	Enzyme unit
V	Volt
v/v	volume/volume
w/v	weight/volume

INTRODUCTION

1. INTRODUCTION

Black pepper (*Piper nigrum* L.), often eulogized as the 'King of Spices' and also as the 'Black Gold' is a highly valued spice used throughout the world. The spice is also well recognized for its medicinal properties and has been a prized spice since the ancient times. Belonging to the family Piperaceae, the plant is a flowering vine native to humid, tropical evergreen forests of the Western Ghat in India. India being a renowned reservoir of its genotypes has produced many superior cultivars, and was the largest producer and exporter of the crop till 1990s. However, with a comparatively low productivity and decline in area, the current position of India has slipped down to second in terms of production and fifth in terms of export (Anon., 2013).

The spike, *i.e.* the inflorescence of black pepper is solitary, straight and non-branched which is produced at nodes opposite to the upper leaves of the fruiting branches. An exception to this is a type named 'Thekken', identified by Shri T.T. Thomas, a farmer residing at Kanchiyar, Kattappana, Idukki district of Kerala. He collected the type from the forest in Kanchiyar, Kattappana. The plant is characterized by profusely branched spikes (60-80 branches per spike) (Plate 1). This branching behavior of spike appears to be an economical trait giving rise to more area for floral meristems to originate, consequently bearing more number of berries. Each branched spike is reported to bear about 800-1000 berries as against 60-80 berries in normal varieties of black pepper (Farm innovators, 2010).

The inflorescence proliferation or branching of spike in Thekken is described as a consequence of mutation in the floral meristem of black pepper (Sasikumar *et al.*, 2007). The infield observation of the plant displayed that not all the spikes were producing the secondary branches, but few of the spikes were completely non-branched, identical to the spikes of other non-branching varieties. These non-branching spikes were distributed randomly, even in the fruiting branches bearing branched spikes.

In black pepper, the molecular work in the field of flowering and inflorescence development has not been reported yet. However, in model plants such as *Arabidopsis* and *Antirrhinum*, the genes regulating inflorescence architecture have been uncovered with complete knowledge of their interactions and functions. These genes are found to be highly conserved among several species of monocot and dicot plants. These genes may also share conserved and significant role in determining the fate of the inflorescence, whether to branch or not in black pepper plants. In this context, the present study was undertaken to examine the presence of inflorescence architectural genes, namely *BP, RA2, PIN1* and *LOG1* in black pepper in relation to their likely role in the branching trait of spikes.

This study also comprised RAPD and SSR marker analyses of branching and non-branching spikes of Thekken type in assessing the genetic similarity between the two types of spikes of Thekken plant, and revealing marker associated with the branching trait. Spike non-branching variety 'Karimunda' was used as a control in the experiment. In a similar fashion, protein profiling by SDS-PAGE method was also executed.

The alteration of flowering character by transfer of desired genes regulating flowering, or by over-expression or suppression of such genes through biotechnological methods has been found successful in many plants (Giovannini, 2006). Determination of the molecular pathways leading to spike branching can aid in the transfer of this trait to other superior varieties of black pepper and also to other plant species for improvement of the productivity.

1 2

Plate 1. Spikes of Thekken and Karimunda

(1) Profusely branched spike of Thekken type

(2) Spike of Karimunda variety

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Botanical family Piperaceae, belonging to the order *Piperales*, is a large pantropical family of subclass Dicotyledonae (Cronquist, 1978). The family shares close features to the monocotyledonous boundary (Metcalfe and Chalk, 1950) which makes their entry into 'paleoherbs'- an assemblage of dicots resembling monocot (Donoghue and Doyle, 1989). The family is represented by over 10 genera (Cronquist, 1981) with *Piper* (Linn.) and *Peperomia* (Ruiz and Pav.) being the major ones, each with a minimum of 1,200 species (Callejas, 2001).

The genus *Piper* is recorded with the greatest diversity in American tropics (700 spp.), followed by South Asia (300 spp.) where the economically important species, *Piper nigrum* L. (black pepper) and *P. betle* L. (betel leaf) are originated (Jaramillo and Manos, 2001). The Western Ghats of South India and the North-Eastern India are the two hotspots of diversity of the genus *Piper* in India, inhabiting about 110 species (Purseglove *et al.*, 1981; Parthasarathy *et al.*, 2006).

Black pepper is the most widely used spice in the world with the global production of 327,090 t in 2012. The productivity of black pepper in India is very low, producing 43,000 t from 182,000 ha area in 2012. Whereas, Vietnam the leading producer and exporter of black pepper produced 100,000 t of black pepper from 52,000 ha area in the same year (Joseph, 2012).

Black pepper is mostly dioecious in wild form undergoing cross pollination (Krishnamurthi, 1969). However, in cultivated types, the plants are mostly gynomonoecious (*i.e.* bearing female and bisexual flowers in the same plant) or trimonoecious (*i.e.* bearing female, male and bisexual flowers in the same plant), and are fertilized by self pollination (Nair *et al.*, 1993; George *et al.*, 2005; Thangaselvabal *et al.*, 2008).

The genus *Piper* generally bears terminal solitary type of inflorescence. Though, rare umbellate inflorescences were observed in *Macropiper* and *Pothomorphe* (Jaramillo and Manos, 2001), the branching of spike had remained

unfamiliar in *Piper* until the identification of black pepper type 'Thekken'. The uncovering of genetic pathways leading to spike branching, producing more number of berries per spike, can be a boon in crop improvement programme. This could be possible to some extent initially by identifying the molecular markers associated with spike branching trait and by applying the molecular studies in determining the genes regulating inflorescence architecture as carried out in this study.

In this chapter, the literature concerned with inflorescence architecture, the genetic pathways regulating them in some important plants like *Arabidopsis thaliana*, and the molecular techniques associated with identifying the genes or markers for desired traits have been presented.

2.1 GENERAL MORPHOLOGY OF BLACK PEPPER PLANT

Black pepper is a perennial climber, climbing on support trees with the help of aerial clinging roots. The climber 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. The nodes are swollen with 10-15 short adventitious roots and a leaf per each node. At the axil of each leaf of the orthotropic branches lies an axillary bud which develops into a plagiotropic branch. The plagiotropic branches are without aerial roots that grow laterally with sympodial growth habit, and produce flowers and fruits. As the shoot grows, the terminal bud gets modified into a spike, and the growth is continued by the axillary bud (Ravindran *et al.*, 2000).

The plant has a broad, shiny green, pointed, petiolate leaves alternately arranged on the stem. The inflorescence is catkin (spike), 7-10 cm long, clustered with sessile, small flowers. Between 50 to 150 whitish to yellow-green flowers are produced on a spike. The berry-like fruit is a drupe, each containing a single seed, and when dried, it is called a peppercorn (Chaveerach *et al.*, 2006).

2.2 INFLORESCENCE AND FLOWER DEVELOPMENT IN BLACK PEPPER

Detailed study on the development of inflorescence and flowers in black pepper has not been reported till date. The only available information is the studies on inflorescence and flower development by Tucker (1982) in three *Piper* spp., namely *P. aduncum, P. amalgo* and *P. marginatum*, and the process of development is believed to be conserved in all other species as well (Ravindran *et al.*, 2000).

According to the observations by Tucker (1982), initially the *Piper* inflorescence has a convex apical meristem, subtended by a vegetative leaf and a bract. The early apical meristem is zonate, usually with two tunica layers, a large central initial zone, a peripheral zone and a massive pith rib meristem. The apical meristem of the inflorescence grows extensively in length before any organs form, and as it grows in length its apical meristem diminishes. The bracts are initiated close to the apex by periclinal division in the second tunica layer on the flanks of the apical meristem. Each flower arises in the axil of a bract. Cells in the axils remain meristematic and cells in the outer layer divide anticlinally. The next two layers undergo intensive cell divisions, including periclinal divisions before the formation of protuberence. Cells elongate anticlinally in the outer two layers making the protuberance. This enlarges further and differentiates into a flower bud. As the primordium (protuberence) mentioned above grows, stamens are initiated from the two lateral sites. The carpels are initiated by periclinal divisions in the sub surface layer on the side of the small floral apex. Ovule initiation begins by a periclinal division in the second tunica layer at the centre of the floral apex.

2.3 THEKKEN: A SPIKE BRANCHING BLACK PEPPER TYPE

Pepper 'Thekken' is a unique type of black pepper. Among the entire plants in the genus *Piper*, Thekken is the only type that has been found to produce a profusely branched inflorescence (spike). The plant was identified by Shri T.T. Thomas, a farmer, from the forest of Kanchiyar, Idukki District, Kerala and has

been cultivated by him for the past 25 years in his farm at Kanchiyar, Kattapana, Idukki District. Thekken plant has been described as a natural mutant of *Piper nigrum* producing about 60-80 branches per spike. Each spike bears about 800-1000 berries as against 60-80 berries in normal pepper varieties. The normal pepper varieties grown in Idukki District yield about 3,000 kg/ha (dry weight) while Thekken yields about 8,600 kg/ha (dry weight). Thekken also possesses high tolerance to wilt disease. This plant type has been documented as an innovation by National Innovation Foundation (NIF), Gujarat, and the innovator, Shri T.T. Thomas has been honored with the certificate of honor from KVK, Kannur, Kattappana Block Panchayath, Kanchiyar Grama Panchayath, Bharathiya Janatha Karshaka Morcha, KVK Idukki, and Certificate of Merit from Society of Research and Initiatives for sustainable Technologies and Institutions, Amhedabad (Farm innovators, 2010).

Sasikumar *et al.* (2007) suggested that the profuse inflorescence proliferation in type 'Thekken' may be due to mutation in the floral meristem, and confirmed its vegetative progenies to be true to type. The proliferating spikes were observed to be of indeterminate growth habit with pronounced bracts. Individual flower buds were transforming into inflorescence branches of varying length. However, the transformation was not uniform and some of the flower buds remained untransformed and developed into flowers (Sasikumar *et al.*, 2007; Vimarsha, 2009).

2.4 INFLORESCENCE ARCHITECTURE

The aerial organs of the plants are derived from the shoot apical meristem (SAM) that produces leaves and shoots during the vegetative phase, and becomes an inflorescence meristem and flowers in the reproductive phase. Inflorescence meristem can either give rise to flowers, if taking floral meristem identity, or produce further lateral branches if taking branch meristem identity. Therefore, the architecture of the inflorescence depends on its branching pattern and the position

of the flowers which results from the pattern of changes in meristem identity (Weberling, 1989; Coen and Nugent, 1994; Benlloch *et al.*, 2007).

Inflorescence types have been classified as determinate or indeterminate depending upon whether the shoot apices end in terminal flowers or not. Inflorescences are also classified according to the complexity of their branching as simple and compound inflorescences. The flowers are directly formed from the main axis in former. While in latter, the flowers are formed from secondary or higher-order branches (Weberling, 1989).

The transition of vegetative to reproductive phase is regulated by a complex network of signal pathways that monitor the developmental state of the plant as well as environmental factors such as light and temperature (Giovannini *et al.*, 2006).

2.4.1 Genes regulating inflorescence architecture

The knowledge of molecular and genetic mechanisms leading to inflorescence and flower development is explored in much detail in *Arabidopsis thaliana* and *Antirrhinum majus* and also to some extent in many other plant species. Similar genetic interactions in the inflorescence and flower development has been found largely conserved among diverse angiosperms, with key differences often relating to the different inflorescence architecture of each species (Ambrose *et al.*, 2000; Ma and dePamphilis, 2000; Ng and Yanofsky, 2001; Benlloch *et al.*, 2007).

In *Arabidopsis*, the acquisition of floral meristem identity has been best analyzed by genes *LEAFY* (*LFY*), *APETALA1* (*AP1*) and *TERMINAL FLOWER1* (*TFL1*) (Weigel *et al.*, 1992; Bowman *et al.*, 1993; Schultz and Haughn, 1993). The homeobox gene called *BREVIPEDICELLUS (BP)* or *KNAT 1* is one of the key regulators of inflorescence architecture in *Arabidopsis* (Venglat *et al.*, 2002).

In maize and many other Gramineae, *RAMOSA2* (*RA2*) gene has been reported to regulate the inflorescence architecture (Vollbrecht *et al.*, 2005).

During inflorescence development, auxin and cytokinin have been found to play a crucial role in axillary meristem initiation and regulation of meristem size respectively (Barazesh and McSteen, 2008). The role of cytokinin in inflorescence development has been elicited by the analysis of genes such as *LONELY GUY* (*LOG*) (Kurakawa *et al.*, 2007) and *CYTOKININ OXIDASE* (*CKX*) (Ashikari *et al.*, 2005).

Studies on the genes involved in auxin synthesis and transport such as *PINFORMED* (*PIN*) (Benschop *et al.*, 2007), *BARREN STALK1* (*BA1*) (Ritter *et al.*, 2002) and *SPARSE INFLORESCENCE1* (*SPI1*) (Gallavotti *et al.*, 2008) revealed the role of auxin in shaping inflorescence architecture.

Some of these important genes involved in the development of inflorescence and flowers are briefly described below.

2.4.1.1 LEAFY (LFY) and APETALA1 (AP1)

LFY and its homolog *FLORICAULA* (*FLO*) of *Antirrhinum* encodes a transcription factor that is strongly expressed throughout the young floral meristems from the earliest stages of development (Weigel *et al.*, 1992). In *lfy* mutant, most of the lateral meristems that were fated to develop as flowers in a wild-type plant developed as inflorescence branches, where as a few developed as abnormal flowers (Huala and Sussex, 1992). Concordantly, its constitutive expression caused early flowering and the transformation of all shoots into flowers (Weigel and Nilsson, 1995). However, the studies on *LFY* homologs in monocots, *viz. ZFL* (in maize), *RFL* (in rice) and *WFL* (in wheat) showed that the gene is associated with spikelet formation rather than floral meristem identity in monocots (Kyozuka *et al.*, 1998; Bomblies *et al.*, 2003; Shitsukawa *et al.*, 2006).

Similar to *LFY*, *AP1* is expressed throughout young floral meristem shortly after the onset of *LFY* expression (Mandel *et al.*, 1992), showing shoot-to-flower

conversion phenotype in constitutively expressing *API* plant (Mandel and Yanofsky, 1995).

Homologues of *LFY* and *API* have been isolated and characterized from several model legume species with similar functions (Hecht *et al.*, 2005; Domoney *et al.*, 2006), which unlike *Arabidopsis*, have compound double racemes inflorescence (Weberling, 1989).

2.4.1.2 TERMINAL FLOWER1 (TFL1)

As opposed to *LFY* and *API*, *TFL1* specify shoot identity with its mutant showing early flowering, and the shoot meristems get converted into floral meristems and inflorescence shoots into terminal flowers (Shannon and Meeks-Wagner, 1991; Schultz and Haughn, 1993). Unlike *LFY* and *API* which encode a transcription factor, *TFL1* code for a protein homologous to phosphatidylethanolamine binding proteins (Bradley *et al.*, 1997; Ohshima *et al.*, 1997). Over-expression of *TFL1* resulted in an enlarged vegetative rosette with a high number of leaves, and a long inflorescence stem with many lateral branches, which eventually formed normal flowers (Ratcliffe *et al.*, 1998). Similar phenotype has also been observed in *TFL1*-related genes, named *ZEA CENTRORADIALIS* (*ZCN1* to *ZCN6*) in maize (Danilevskaya *et al.*, 2010).

The presence of *TFL1* gene was tested in eight black pepper varieties by Vimarsha (2009) with a primer pair designed using fourth exonic sequence of the gene from *Arabidopsis thaliana*. An amplicon of size approx. 700bp was observed in spike branching Thekken variety. However, similar band was also observed in two spike non-branching black pepper varieties, namely Vellamundi and Karimunda.

2.4.1.3 BREVIPEDICELLUS (BP)/ KNAT1

In *Arabidopsis thaliana*, the three amino acid loop extension (TALE) homeodomain superfamily plays a critical role in determining meristem function (Ragni *et al.*, 2008). This family comprises the KNAT (for KNOTTED like from

Arabidopsis thaliana) members and the BEL1-like (BELL) members that can form heterodimers to regulate plant development. KNAT class I contains four members: *SHOOT MERISTEMLESS (STM), BREVIPEDICELLUS (BP)/ KNAT1, KNAT2,* and *KNAT6,* of which *BP* contributes with *STM* to SAM maintenance and also regulate inflorescence development (Byrne *et al.*, 2003; Ragni *et al.*, 2008). *BP* encodes the homeodomain protein KNAT1 which plays a key regulatory role in important aspects of growth and cell differentiation of the inflorescence stem, pedicel and style in *Arabidopsis* (Venglat *et al.*, 2002; Mele *et al.*, 2003).

BP and its ortholog in rice, *OSH15* (*D6*), both are expressed in similar domains in the SAM (the peripheral zone) and in the cortical cell layers of the inflorescence stem (peduncle) and pedicel, but not in the leaves (Lincoln *et al.*, 1994; Sato *et al.*, 1999; Douglas *et al.*, 2002; Venglat *et al.*, 2002).

In mutant *bp* plants, the floral buds were arranged more compactly at the apex with shorter internodes, downward pointing siliques and an epidermal stripe of disorganized cells along the stem in *Arabidopsis.* The pedicels and internodes were compact because of fewer cell divisions (Douglas *et al.*, 2002; Venglat *et al.*, 2002).

A mutation in one of the Arabidopsis *BELL* genes, *PENNYWISE* (*PNY*), also appeared phenotypically similar to the *KNOX* mutant *brevipedicellus* (*bp*), inflorescence of both having randomly shorter internodes with a slight increase in the number of axillary branches. The double mutant showed a synergistic phenotype of extremely short internodes interspersed with long internodes and increased branching. The PNY and BP proteins are assumed to participate in a complex that regulates early patterning events in the inflorescence meristem (Smith and Hake, 2003).

In *Lactuca sativa*, inflorescence stem over-expressing *KNAT1* displayed loss of apical dominance with formation of several floral branches of different length with respect to the more regular architecture of the wild-type (Frugis *et al.*, 2001), and its over-expression was found to be associated with an overproduction of

specific types of cytokinin (Lincoln *et al.*, 1994; Ori *et al.*, 2000; Frugis *et al.*, 2001).

2.4.1.4 RAMOSA 2 (RA2)

The name '*ramose*' came from the Latin "ramus" meaning "branch" as *ra* mutant plants showed the phenotype of highly branched inflorescence (McSteen, 2006).

The three *RAMOSA* genes (*RA1-3*) regulate inflorescence branching in maize. *RA1* and *RA2* are transcription factors, while *RA3* encodes a phosphatase that is important in the biosynthesis of a sugar - trehalose, thought to be an important developmental signaling molecule. When these three genes were mutated, the mutant maize plants had more and longer branches, and produced smaller and deformed cobs (Vollbrecht *et al.*, 2005).

RA2 encodes a Lateral Organ Boundary (LOB) domain transcription factor transiently expressed in a group of cells that predicts the position of axillary meristem formation in inflorescence. It is expressed early in the inflorescence cells that predict the position of axillary meristem initiation. *RA2* is expressed upstream of other branch regulators and functions in the patterning of stem cells in axillary meristems (Bortiri *et al.*, 2006).

RA1 is likely to be expressed downstream of *RA2* based on maize data, and *RA3* may be involved in signalling the axillary meristems either to stop making branches or to continue growth (Vollbrecht *et al.*, 2005; Bortiri *et al.*, 2006).

Orthologs of *RA2* have been found in sorghum, rice and barley, and appeared to be conserved over many grass taxa (Vollbrecht *et al.*, 2005). *ASYMMETRIC LEAVES2-LIKE4* was found to be the closest homolog of *RA2* in *Arabidopsis*, but the C-terminus was completely different in these two genes (Bortiri *et al.*, 2006).

Normal maize ears are non-branched and tassels have long branches only at their base. However, in *ra2* mutants, short branches that normally make spikelet pairs were converted into longer branches that make multiple spikelets. Long branches at the base of the tassel increased secondary branching. In the ear, more than two spikelet meristems per row were produced causing disorganization of the rows. This mutant phenotype indicated that one function of *RA2* is to restrict branch growth and to establish determinacy on the spikelet pair meristems (Vollbrecht *et al.*, 2005; Bortiri *et al.*, 2006; McSteen, 2006).

2.4.1.5 LONELY GUY (LOG)

The phytohormone cytokinin plays a positive role in shoot meristem function. The increase in cytokinin level leads to an increase in meristem size and vice-versa in *Arabidopsis* (Helliwell *et al.*, 2001; Werner *et al.*, 2003; Ashikari *et al.*, 2005). Active cytokinin species are free-base forms, such as N^6- Δ^2-isopentenyl adenine (iP) and *trans*-zeatin (tZ), which are converted from cytokinin nucleotides in two-step reaction catalyzed by nucleotidase (Chen and Kristopeit, 1981a) and nucleosidase (Chen and Kristopeit, 1981b) encoded by unknown genes.

Through the analysis of rice (*Oryza sativa*) *lonely guy* (*log*) mutants, cytokinin riboside 5'-monophosphate phosphoribohydrolase, an important enzyme encoded by *LONELY GUY* (*LOG*) was identified. The enzyme directly converts the cytokinin nucleotides such as N^6- Δ^2-isopentenyl adenine riboside 5'-monophosphate (iPRMP) and *trans*-zeatin riboside 5'- monophosphate (tZRMP) to the free-base form in a single-step reaction (Kurakawa *et al.*, 2007). This gene is also reported to be involved in the direct activation pathway of cytokinins in *Arabidopsis* (Kuroha *et al.*, 2009).

Eleven and nine *LOG* genes have been identified in rice and *Arabidopsis* genomes, respectively (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009). A higher level of *LOG* expression has been repeatedly observed in all meristems of the

developing panicle, including panicle branch and floral meristems (Kurakawa *et al.*, 2007).

In rice *log* mutant, only the upper region of the flat floral meristem expressed *OSH1*, a meristem marker encoding a KNOTTED1-type homeobox (KNOX) protein. While a strong expression of *OSH1* was observed in wild-type floral meristem, presenting improper meristem activity in *log* mutants. The mutant *log* gene also caused the abortion of inflorescence meristems and panicle branch meristems soon after the production of a few lateral meristems. This led to the production of a small panicle with a reduced number of branches and flowers contrary to the wild-type inflorescence where a number of meristems that give rise to panicle branches and flowers were generated (Kurakawa *et al.*, 2007).

The number of floral organs was decreased in *log* mutants, and flowers often contained only one stamen but no pistil, thus given the term 'lonely guy' (Kurakawa *et al.*, 2007).

2.4.1.6 PIN-FORMED 1(PIN1)

Auxin determines the initiation of lateral organs and axillary meristems (Benjamins and Scheres, 2008). Cheng *et al.*, (2006) observed that the increase in branching of inflorescence meristems and/or floral meristems was correlated with the low auxin concentration.

Distribution of auxin is controlled by a combination of polar auxin transport (PAT) and localized auxin biosynthesis (Delker *et al.*, 2008). PAT requires polar localization of the PINFORMED (PIN) family of auxin efflux carriers which is regulated by serine/threonine protein kinase PINOID (PID) (Friml *et al.*, 2004; Zazimalova *et al.*, 2007; Michniewicz *et al.*, 2007).

PIN includes the member of genes that encodes for auxin efflux carrier transmembrane proteins. These proteins are asymmetrically localized in the plasma membranes of auxin-transporting cells consistent with the direction of auxin transport (Benjamins and Scheres, 2008). Altogether, *Arabidopsis* has eight

annotated *PIN* genes (*PIN1-PIN8*), of which the best characterized *PIN*s being *PIN1* and *PIN2* (Muller *et al.*, 1998; Krecek *et al.*, 2009).

PIN1 protein was found abundant in inflorescence apices (Reinhardt *et al.*, 2003; Heisler *et al.*, 2005), and has been reported to be involved in the early regulation of *LFY* and aerial organ development (Vernoux *et al.*, 2000; Tanaka *et al.*, 2006). Polar transport of the hormone auxin was severely reduced in *pin1* mutant of *Arabidopsis* (Okada *et al.*, 1991; Bennett *et al.*, 1995). Expression patterns and developmental roles analogous to *Arabidopsis* have also been observed in monocot counterpart rice (Xu *et al.*, 2005) and maize (Carraro *et al.*, 2006).

In *Arabidopsis, pin* mutants resulted in phenotypes consistent with altered auxin transport that failed to develop floral organs properly and generated naked, pin-like inflorescences, which gave the name *PIN-FORMED* (*PIN*) to the family (Zazimalova *et al.*, 2007; Benschop *et al.*, 2007).

The mutant phenotype was able to recover by the application of hormone at apex (Reinhardt *et al.*, 2000). In addition, inhibitors of auxin transport such as *N*-1-napthylphthalamic acid (NPA) mimicked the *pin1* mutant phenotype, resulting in the formation of naked inflorescence stems (Okada *et al.*, 1991).

2.5 IDENTIFICATION OF CANDIDATE GENE

Isolation of an unknown sequence related to known sequences is a powerful method for investigating biological function. PCR methods have succeeded in obtaining the genes that are unknown in one organism but may be homologous to the sequences of known genes from different organisms (Rose *et al.*, 1998).

The candidate genes for resistance in plants have been isolated successful by PCR using degenerate primers that are designed from the conserved motifs of the resistance genes of other plants (Aarts *et al.*, 1998; Shen *et al.*, 1998). Deng and Davis (2001) identified the gene associated with the color of strawberry using

degenerate primers made from the conserved regions of genomic DNA, cDNA and protein sequences of the candidate genes.

Shen *et al.* (1993) reported the identification of a cytochrome P450 related gene by RT-PCR using degenerate primers designed based on relatively conserved regions of the proteins of P450 gene families.

Many of the genes governing inflorescence architecture have been found to be conserved in plant species (Ambrose *et al.*, 2000; Ma and dePamphilis, 2000; Ng and Yanofsky, 2001; Benlloch *et al.*, 2007). Candidate gene approach could be advantageous in exploring the presence of these genes and their functions in different plant species using degenerate primers.

2.5.1 Degenerate primer

A nucleotide sequence is called degenerate if one or more of its positions can be occupied by one of several possible nucleotides. The degeneracy of a sequence is the number of different sequences that it represents (Kwok *et al.*, 1994).

Unlike non-degenerate primers which amplify the target region from different members only if the sequences of a pair of primers are strongly conserved in their genomes, the degenerate primers are useful in amplifying homologous genes with weakly conserved sequences among different organisms. These are designed based on multiple known sequence data of related and already sequenced gene homologs, and hence can be used to discover new homologous genes in other species (Aarts *et al.*, 1998; Shen *et al.*, 1998; Deng and Davis, 2001; Lang and Orgogozo, 2011). Although one can often do this manually for well conserved sequences, computational methods (e.g., HYDEN, SCPrimer, and iCODEHOP) are available to systematically look for conserved sections in the sequence and to design primers (Brand, 2011).

Highly degenerate primers are preferred to match a large number of known genes offering good chance to detect new related ones. However, primers with

low degeneracy avoid the probability of amplifying non-related sequences (Linhart and Shamir, 2005).

2.5.2 Primer designing

Primers function in pairs, the so-called forward primer and the so-called reverse primer. The primer pairs are chosen such that they will be extended towards each other to cover the given target region (Kampke *et al.*, 2001).

Proper primer designing is important for applications in PCR, DNA sequencing and hybridization. The specificity of primers to avoid mispriming, and the efficiency of primers to be able to amplify a product exponentially are the two main goals to be balanced while designing a primer (Dieffenbach *et al.*, 1993). Usually primer of 20-24 bases and GC content between 45-60 percent with T_m of 52-58 °C works best in most applications. Within a primer pair, the GC content and T_m should be well matched. An annealing temperature is generally calculated as 5 °C lower than the estimated T_m (Dieffenbach *et al.*, 1993; Abd-Elsalam, 2003).

To avoid mispriming, primers should not be very sticky (high G/C content) on their 3' ends. However, a "G" or "C" is desirable at the 3' end of primers since this will reduce "breathing" and thereby increase yield (Sheffield *et al.*, 1989; Abd-Elsalam, 2003).

When designing primers, it is important to have a minimum of intra-molecular or inter-molecular homology to inhibit the formation of either hairpins or primer dimerization (Abd-Elsalam, 2003).

The calculation of T_m can be performed in several ways. The one given by Suggs *et al.* (1981) as $T_m = 2\ (A+T) + 4\ (G + C)$ is popular for its simplicity and roughly accurate prediction of T_m. Whereas, T_m calculation based on nearest neighbor thermodynamic parameters appears to be slightly more accurate (Breslauer *et al.*, 1986; Freier *et al.*, 1986).

Generally the primers are designed to produce the PCR products of 150-750 bp (Dieffenbach *et al.*, 1993).

The use of degenerate oligonucleotide primers covering all possible combinations for the bases, as well as the use of inosine to replace the base corresponding to the third or variable position of certain amino acid codons has been successful for cDNA cloning and for detection of sequences with possible variations (Lin, 1992).

2.5.3 PCR for candidate gene identification

PCR is a scientific technique in molecular biology for exponential amplification of a particular fragment of DNA (Joshi and Deshpande, 2010). A specific region of the DNA can be picked up by using the primer pairs flanking the region of interest. Quality of DNA act as a main factor for the successful amplification of DNA fragments by PCR (Finkeldey *et al.*, 2010).

2.5.3.1 DNA isolation

Numerous DNA extraction methods in plants have been reported but none with universality (Varma *et al.*, 2007). This could be primarily due to the variations in the composition of the primary and secondary metabolites among the plants or even among the different tissues of the same plant, which makes the isolation process needful to be adjusted as plant specific or tissue specific (Sangwan *et al.*, 1998; Dhanya and Sasikumar, 2010).

DNA extraction is difficult in plants rich in polyphenols and polysaccharides which bind to nucleic acids during DNA isolation and interfere with the isolation process (Puchooa and Khoyratty, 2004; Mishra *et al.*, 2008). Polyphenol oxidation and co-precipitation causes the browning of the DNA (Varma *et al.*, 2007; Mishra *et al.*, 2008). The presence of polysaccharides in DNA will make it viscous causing difficulty in loading (Sharma *et al.*, 2000; Sablok *et al.*, 2009). These metabolites have also been reported to interfere with the activity of several biological enzymes like polymerases, ligases and restriction

endonucleases (Prittila *et al.*, 2001; Diadema *et al.*, 2003; Karaca *et al.*, 2005; Varma *et al.*; 2007; Moyo *et al.*, 2008; Singh and Kumar, 2010; Sahu *et al.*, 2012).

Increasing the concentrations of NaCl and CTAB can remove polysaccharides during DNA extraction (Syamkumar *et al.*, 2005; Sahu *et al.*, 2012). Adding high concentrations of PVP and ß-mercaptoethanol is helpful to remove tannins and other polyphenolics from the tissues (Warude *et al.*, 2003).

2.5.3.2 Polymerase chain reaction (PCR)

The PCR method involves enzymatic synthesis of a specific DNA segment, generating thousands to millions of copies (Joshi and Deshpande, 2010). In presence of dNTPs, a thermostable DNA polymerase uses each oligonucleotide primer to synthesize a copy of the adjacent DNA strand. Each newly synthesized strand then provides a new template for synthesis from the opposite primer (Fox and Parslow, 1988).

The three major steps, *viz.* denaturation, annealing and extension are involved in the PCR technique. DNA is initially denatured at high temperatures (from 90-97 °C). The annealing phase is the most important and occurs at 50-60 °C for 1-2 min. The extension of primers by DNA polymerase occurs at approx. 72 °C for 2-5 min. The time for last step depends both on the DNA polymerase itself and on the length of the DNA fragment to be amplified. As a rule of-thumb, 1 min is allowed for the synthesis of 1 kbp fragment. After the last cycle, samples are usually incubated at 72 °C for 5 min to fill in the protruding ends of newly synthesized PCR products. The three-stages are repeated 25–40 times in a typical PCR procedure (Joshi and Deshpande, 2010).

PCR is commonly carried out in a reaction volume of 10-200 µl, and the size of PCR products or amplicons ranges from 100–3000 bp in length (Cheng *et al.*, 1994). The method relies on the ability of DNA copying enzymes to remain stable at high temperatures. The most commonly used enzyme, Taq DNA polymerase, has a 5'-3' nuclease activity but lacks a 3'-5' proofreading

exonuclease activity. This makes its use less advisable when fidelity is the main consideration. Instead, the enzymes with proofreading exonucleases such as Vent or Pfu are favoured (Cline *et al.*, 1996). The K_m value of different enzymes varies, hence reaction conditions must be reoptimised every time a different enzyme is used (Bustin, 2000).

Mg^{2+} affects enzyme activity and increases T_m of double-stranded DNA. It also forms soluble complexes with dNTPs to produce the actual substrate that the polymerase recognizes. Therefore, a high concentration of dNTPs reduces free Mg^{2+}, consequently interfering with polymerase activity and affecting primer annealing (Eckert and Kunkel, 1991). Cobb and Clarkson (1994) described an easy and effective method to optimize the components of PCR for successful amplification. A master mix containing all of the reactants except the Taq polymerase can be made to minimize pipetting variables (Roux, 1995).

Various additives such as dimethyl sulfoxide (DMSO) (2-5%), polyethylene glycol (PEG 6000) (5-15%), glycerol (5-20%), non-ionic detergents and formamide (5%) can be incorporated into the reaction to increase the specificity of reaction (Pomp and Medrano, 1991; Newton and Graham, 1994)

2.5.4 RT-PCR for candidate gene identification

RT-PCR is an *in vitro* method for enzymatically amplifying defined sequences of RNA (Rappolee *et al.*, 1988). It is the most sensitive method for the detection of low-abundance mRNA, often obtained from limited tissue samples (Bustin, 2000).

RT-PCR can be used to compare the levels of mRNAs in different sample populations, to characterise patterns of mRNA expression, to discriminate between closely related mRNAs, and to analyse RNA structure (Bustin, 2000). It also circumvent time-consuming and technically demanding cloning steps, and generate reagents such as full-length complementary DNA (cDNA) inserts for

cloning (Borson *et al.*, 1992), or arbitrarily primed enhanced sequence tag cDNA libraries (Neto *et al.*, 1997).

However, this complex technique sometimes faces substantial problems (Wang and Brown 1999) and different factors must be well optimized. The RNA must be of the highest quality and free of DNA.

2.5.4.1 RNA isolation

RNA isolation requires special care and precautions as it is highly susceptible to degradation (Kojima and Ozawa, 2002; Buckingham and Flaws, 2007). The biggest problem encountered in RNA extraction usually originates from the initial sampling and extraction protocols, and from personal technique and care taken (MacRae, 2007).

Isolation of high quality RNA is difficult from tissues containing high amounts of polyphenols, polysaccharides and other secondary metabolites (Azevedo *et al.*, 2003; Mattheus *et al.*, 2003; Sharma *et al.*, 2003). These contaminants tend to co-precipitate with RNA in the presence of alcohol, leading to erroneous estimations of RNA quantity, and interfere with reverse transcription and PCR (Koonjul *et al.*, 1999; Salzman *et al.*, 1999; Singh *et al.*, 2003).

RNAse is heat-stable and refolds following heat denaturation. They are difficult to inactivate as they do not require cofactors. Strong denaturants has always been used in intact RNA isolation to inhibit endogenous RNases. The most common isolation methods can be divided into two classes, *viz.* utilizing guanidinium thiocyanate and utilizing phenol and SDS (Doyle, 1996).

A guanidinium-based salt is a strong protein denaturant and inhibitor of RNase. Therefore, it is an ingredient of choice in most of the RNA isolation systems. However, in some studies, the presence of secondary metabolites has been found to interfere with extraction of RNA when extracted with guanidinium salts (Bugos *et al.*, 1995; Ding *et al.*, 2008; Wang *et al.*, 2008; Ghawana *et al.*, 2011).

2.5.4.2 RT-PCR

As RNA cannot serve as a template for PCR, so the first step in an RT-PCR assay is the reverse transcription of RNA template into cDNA, followed by its exponential amplification in a PCR. The complete RT-PCR process involves the use of dedicated RNA- and DNA-dependent DNA polymerases, either in separate ('two-enzyme/two-tube') or, in single ('two-enzyme/one-tube') reactions. Separation of the RT and PCR steps has the advantage of generating a stable cDNA pool that can be stored virtually indefinitely. Alternatively, a single polymerase able to function both as an RNA and DNA-dependent DNA polymerase such as *Thermus thermophilus* (Tth) polymerase can be used in a 'one-enzyme/one-tube' reaction to minimise the risk of contamination (Myers and Gelfand, 1991; Bustin, 2000). The assay with Tth polymerase uses bicine buffers containing Mn^{2+} ions that are compatible with both RT and subsequent PCR (Chiocchia and Smith, 1997).

RT-PCR is a complex assay, and all physical and chemical components of the reaction are interdependent. They must be considered carefully when optimizing the specificity, sensitivity, reproducibility or fidelity of the reaction (Bustin, 2000). The secondary structures formed by RNA transcripts also affects the ability of the reverse transcriptase enzyme to generate cDNA (Buell *et al.*, 1978).

The two commonly used RTs are *Avian Myeloblastosis Virus* Reverse Transcriptase (AMV-RT) and *Moloney Murine Leukaemia Virus* Reverse Transcriptase (MMLV-RT). AMV-RT is more robust than MMLV-RT, retains significant polymerisation activity up to 55 °C, and can help eliminate problems associated with RNA secondary structure (Brooks *et al.*, 1995; Freeman *et al.*, 1996). In contrast, MMLV-RT has significantly less RNAse H activity than AMV-RT (Gerard *et al.*, 1997) which make it a better choice for the amplification of full-length cDNA molecules (Bustin, 2000).

The RT step can be primed using specific primers, random hexamers or oligo-dT primers. The use of mRNA-specific primers decreases background priming, whereas the use of random and oligo-dT primers maximises the number of mRNA molecules that can be analyzed from a small sample of RNA (Zhang and Byrne, 1999). cDNA synthesis using oligo-dT is more specific to mRNA than random priming, as it will not transcribe rRNA. However, since oligo-dT priming requires very high-quality RNA that is of full length, it is not a good choice for transcribing RNA that is likely to be fragmented (Bustin and Nolan, 2004).

2.6 MOLECULAR MARKER ANALYSES

Molecular markers are the powerful tools for identification of markers linked to important agronomic traits (Zhang and Stommel, 2001). They offer many advantages for the determination of genetic diversity and the identification of cultivars (such as not being influenced by the environment and detection directly at the DNA level) over the morphological traits which are governed by complex genetic interactions (Jiang and Liu, 2011). The genetic variation obtained through molecular marker is directly related to the number of polymorphisms detected and their reproducibility (Nguyen *et al.*, 2004).

Molecular markers linked to the important traits are of much interest, especially when traits are difficult and/or costly to be observed (Arens *et al.*, 2010). The usefulness of a DNA marker to select for a trait depends on the degree of linkage between the trait and the marker. If the marker is tightly linked to the trait, then selection for the marker also selects for the trait, which is equivalent to genotypic selection (Garcia *et al.*, 2008).

A variety of molecular markers such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and microsatellites or simple sequence repeats (SSRs), etc. have been developed and used for genetic analysis in several plant species (Philips and Vasil, 2001; Varshney *et al.*, 2004).

2.6.1 Random amplified polymorphic DNA (RAPD)

RAPD, a polymerase chain reaction (PCR) based marker technique described by Williams *et al.* (1990), is a commonly used molecular technique to develop DNA markers. It is a simple and low cost method producing a large number of genetic markers with small amount of DNA. In addition, the non-requirement of DNA sequence information (Kumar and Gurusubramanian, 2011) makes it most preferable in the initial studies in plants where the genetic information is lacking.

RAPD marker has been extensively used in identifying a marker linked to a desired trait. Ford *et al.* (1999) acquired 7 RAPD markers linked to the resistance against blight in lentil. Lin *et al.* (2006) identified 14 markers associated with heat-tolerance in tomato. The RAPD markers associated with distinct phenotypic traits have been reported in several species such as markers linked to dark green leaf, blue flower, silvery green pod, yellow wax pod and flat pod in common bean (Beltran *et al.*, 2002), bending stem and curling leaf in corkscrew willow (Lin *et al.*, 2007) and an erect glandular hair trait in alfalfa (Garcia *et al.*, 2008).

In genus *Piper*, the RAPD marker has been used widely and proved suitable for both inter-specific and intra-specific analysis. Sen *et al.* (2010) analyzed the genetic diversity of eight species of *Piper*, *viz. P. nigrum*, *P. longum*, *P. betle*, *P. chaba*, *P. argyrophyllum*, *P. trichostachyon*, *P. galeatum*, and *P. hymenophyllum* using RAPD markers. Their result showed high genetic variation producing 91.95 percent of the fragments polymorphic and also obtained species-specific bands in all eight species. The intra-specific analysis of thirteen land races and nine advanced cultivars of *P. nigrum* by Pradeepkumar *et al.* (2001) also highlighted RAPD as a worthy marker with a mean of 15.3 polymorphic bands per primer, producing cultivar-specific bands for all the released varieties except Panniyur-3. However, among the land races, specific bands were observed only for Cheriakaniakadan, Malligeswara and Karimunda. Sreedevi *et al.* (2005) identified six lines exhibiting unique bands out of seven high yielding new

promising lines of black pepper using RAPD. This implies the usefulness of RAPD markers in characterizing the varieties of black pepper.

Similarly, the existence of wide genetic diversity by RAPD analysis was observed in 22 cultivars of *Piper nigrum* and one accession each of *P. longum* and *P. colubrinum* (Pradeepkumar *et al.*, 2003), where twenty-four primers generated 372 RAPD markers of which 367 were polymorphic.

RAPD screening of spike branching type 'Thekken' and seven spike non-branching black pepper varieties has been done previously by Vimarsha (2009). He reported a specific band in spike branching type with primer OPA-10 and indicated the highest genetic similarity (74%) of cultivar Vellamundi with type Thekken.

2.6.1.1 RAPD marker in mutation analysis

RAPD marker technique has been reported suitable to detect different types of DNA mutation. Danylchenko and Sorschinsky (2005) reported the applicability of RAPD marker for detecting the mutations in plant DNA after the tissue from alfalfa seedlings were irradiated by high doses of UV-B and γ–ray (LD_{50}). They concluded that the sensitivity of the RAPD assay depends on the mutation level which affects the number of binding sites for Taq polymerase. In supplement to this, Singh and Roy (1999) emphasized the necessity to optimize RAPD reaction conditions by varying magnesium chloride and polymerase concentration with different amounts of DNA templates, and also using different polymerases while examining the mutations by RAPD. Their work revealed a gain or loss of amplified fragments in tumor cells compared to the controls. Atak *et al.* (2004) also reported the polymorphism detected by RAPD marker between γ -irradiated mutants and controls in three soybean varieties.

A morphological variant of neem considered as mutant produced distinct variation in DNA pattern from the normal plant when subjected to RAPD analysis (Bhatt *et al.*, 2011). Even the chimera producing two different colored petals in

the flower of hybrid plant (white flowered *Brassica carinata* and yellow flowered *B. rapa*) was confirmed using RAPD (Liu *et al.*, 2009).

RAPD has been utilized in genetic fidelity test of the micro-propagated plants of *Piper longum* L. and black pepper cv. Subhakara and Aimpiriyan (Parani *et al.*, 1997; Babu *et al.*, 2003). Polymorphism was detected by RAPD assay in somatic embryogenesis derived plants and the mother plant in coffee by Rani *et al.* (2000).

In black pepper, while analyzing the hybrid using RAPD marker, George *et al.* (2005) observed non-parental bands in some progenies and predicted them as a result of DNA recombination or mutation. Bhasi (2008) obtained 66.3 percent intraclonal variability in forty plants of Panniyur-1by RAPD analysis.

RAPD assay predominantly screens GC-rich regions which often contain fast mutating sites (Backeljau *et al.*, 1995). The genomic alterations were found to be picked up by RAPD assay only if the altered cells account for two percent of the cells in a culture (Jones and Kortenkamp, 2000).

2.6.2 Simple sequence repeats (SSRs)

Microsatellite or SSR is a tandemly repeated motif of 1-6 bases widely distributed throughout the eukaryotic genomes, generating a high degree of polymorphism. Its codominant, reproducible, multiallelic with Mendelian inheritance characters make it superior over many other markers (Powell *et al.*, 1996).

SSR markers have been widely used and proved to be efficient in the assessment of genetic diversity in several crops such as *Oryza sativa* (Cho *et al.*, 2000), *Triticum aestivum* (Eujayl *et al.*, 2001), *Solanum tuberosum* (Provan *et al.*, 1996), *Brassica napus* (Qu *et al.*, 2012) and cultivated *Helianthus annuus* (Dehmer and Friedt, 1998). Cholastova and Knotova (2012) reported a reliable estimation of genetic diversity in 98 alfalfa germplasm accessions using only three polymorphic SSR primers which generated 67 polymorphic bands with very

high average polymorphic information content (PIC) (> 0.90). High polymorphism (89.72%) was obtained in lowland rice cultivars and shallow water rice cultivars using microsatellite markers (Joshi *et al.*, 2010). The highly informative and polymorphic SSR markers were able to distinguish the soybean genotypes which were otherwise reported to be indistinguishable using RFLP probes (Diwan and Cregan, 1997).

In case of marker analysis for a peculiar trait, Lanteri *et al.* (2006) reported two SSR markers linked to spiny bracts in globe artichoke. Li *et al.* (2010) used SSR markers in identifying the marker linked to the genes for purple grain color in wheat and two markers Xgwm47 and Xgwm155 is found linked with two purple pigment genes. Madhav *et al.* (2010) identified the tightly linked SSR marker with a primer ARSSR-3 for aroma trait in rice.

SSR primers have shown transferability in different species within the same genus and also in closely related genus (Matsuoka *et al.*, 2002; Singh *et al.*, 2007), but cross-species amplification outside of the genus were much lower (Peakall *et al.*, 1998). Researchers have attempted to use SSR primers between different species or genera to reduce the expenses (Stafne *et al.*, 2005).

A report on SSR marker in black pepper is rare. Menezes *et al.* (2009) developed nine microsatellite markers (PN A5, PN B5, PN B9, PN E3, PN F1, PN G11, PN H4, PN H8a, PN D10) from an enriched library of *Piper nigrum* L.. They analyzed twenty varietal clones from the Brazilian germplasm collection and observed the mean heterozygosity value of 0.624. The nine microsatellites were also tested in four distinct species of the genus *Piper, viz. P. attenuatum, P. hispidinervium, P. tuberculatum and P. colubrinum*, and concluded that these SSR primers could support the characterization of the *Piper nigrum* L. germplasm. Similarly, Joy *et al.* (2011) conducted genotyping of forty popular genotypes of black pepper and four different species of *Piper* using SSR markers. They found that the majority of the genotypes were precisely discriminated with an average PIC value of 0.85.

Recently, Gordo *et al.* (2012) reported the identification of simple-sequence repeats in black pepper which are expected to be useful in the analysis of genetic diversity.

2.6.2.1 SSR marker in mutation analysis

SSR marker can be used to precisely analyze the chimera (Zhang *et al.*, 2007). Occurrence of allele(s) in the progeny that was absent in either parents were reported by Diwan and Cregan (1997) in the SSR marker profile of soyabean pedigrees which were regarded as a result of mutation. Detection and quantification of *in vitro* culture induced chimerism in *Theobroma cacao* (L.) was found successful using SSR marker (Lopez *et al.*, 2004).

SSR marker has been used to confirm periclinal chimera producing more than two alleles at a locus in some individuals of grapevine (Frank *et al.*, 2002). Harris-Shultz *et al.* (2011) reported a similar finding where in an additional allele was obtained in the shoot tissue that was missing in the root tissue of 'TifEagle' and 'Tifdwarf', a bermudagrass cultivars, suggesting the two cultivars as somatic chimeras.

2.7 SDS-PAGE PROTEIN ASSAY

Among biochemical techniques, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is considered to be a simple and reliable method for describing genetic structure of crop germplasm, separating proteins by its size (Ghafoor *et al.*, 2002; Javid *et al.*, 2004; Iqbal *et al.*, 2005). SDS denatures polypeptide chains (as well as separate protein subunits in oligomers) and then surrounds individual polypeptide chains, giving each chain the same overall surface charge (Murphy *et al.*, 1990).

Proteins unique to a particular condition are potentially useful in ultimately understanding the cellular events that are occurring during that time. Distinct polymorphism in electrophoretic banding patterns of seed storage proteins in different cultivars of crops such as rice, mustard, chickpea, lima bean and

common bean have been reported (Lioi *et al.*, 1999; Ferreira *et al.*, 2000; Nasir *et al.*, 2007; Sadia *et al.*, 2009; Galani *et al.*, 2011).

Wu and Cao (2008) characterized the protein profiles of root, leaf, and internode of vine and bush plants of *Cucurbita moschata* plants. Although they did not obtain any difference in the banding pattern of leaf proteins between the two phenotypically different plants, specific bands were observed in the protein profile from root samples. In contradictory, a low level of genetic variation has been disclosed by SDS-PAGE in lentil germplasm (Sultana and Ghafoor, 2009). Similar result was obtained by De Vries (1996) in *Lactuca sativa* revealing cultivar identification impossible with SDS-PAGE method.

Shim *et al.* (2010) reported the extraction of total cellular proteins from the leaves of *Piper sarmentosum*, a medicinal herb. Upon SDS-PAGE separation, most of the extracted proteins were of a low molecular weight ranging from 25 kDa to 75 kDa. Only few protein bands were detected in the higher molecular weight region.

Nazeem *et al.* (2008) evaluated protein profiles of relatively tolerant and susceptible black pepper varieties along with that of a resistant wild species (*Piper colubrinum*) to detect variations in the defense related protein/enzyme expression in response to *Phytophthora* infection. Variety specific proteins of molecular weight 90 and 5.5 kDa were expressed in 'Panniyur-1' while 'Kalluvally' had unique protein bands of 14.3, 8.8 and 7.0 kDa. The identification of tolerant and susceptible varieties were possible, however *P. colubrinum* had an altogether different banding pattern.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Identification of molecular marker associated with spike branching trait in black pepper (*Piper nigrum* L.)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2011-2013. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 PLANT SAMPLE COLLECTION

Black pepper samples (spikes) were collected from farmer's field in Idukki. The samples were collected from three Thekken plants (noted as Thekken A, B and C), and three Karimunda plants (noted as Karimunda A, B and C) which were used as control. Samples at two different developmental stages, *viz.* immature stage (spikes of length <3 cm) and mature stage (spikes of length >3 cm) were collected. From Thekken type, the branching spikes and non-branching spikes were collected separately. However, the identification of branching and non-branching spikes at immature stage was indistinguishable, so collected as a single lot. Each sample was labeled appropriately, packed in a polypropylene bag and kept in ice box to transport to the laboratory. In laboratory, the samples were frozen in liquid nitrogen and stored at -80° C (Sanyo ultra low deep freezer, USA) for downstream analysis.

3.2 IDENTIFICATION OF INFLORESCENCE ARCHITECTURAL GENES

For identification of genes associated with spike branching in black pepper, the degenerate primers were synthesized based on the sequences of genes reported to regulate inflorescence architecture in several other plant species. These primers were analyzed for amplification by PCR and RT-PCR in genomic DNA and mRNA respectively of Thekken type and Karimunda variety.

3.2.1 Degenerate primer designing

Degenerate primers were designed for four genes, namely *BREVIPEDICELLUS (BP), RAMOSA2 (RA2), PINFORMED1 (PIN1)* and *LONELY GUY1 (LOG1)* which are reported to function in inflorescence architecture maintenance in several plant species. Nucleotide sequences of these genes in different plant species were searched in NCBI GenBank. From the NCBI nucleotide database, available nucleotide sequences of the respective genes were downloaded in FASTA format.

Nucleotide sequences of *BP* were obtained from *Arabidopsis thaliana, Brassica rapa, Brassica napus, Brassica oleracea* and *Cardamine hirsute* available in the NCBI nucleotide database (Fig 1). Sequences of *RA2* were obtained from *Zea mays, Phacelurus digitatus, Andropterum stolzii, Sorghum bicolor, Chrysopogon gryllus, Andropogon hallii, Cymbopogon flexuosus, Loudetia* sp., *Schizachyrium sanguineum, Oryza sativa, Triticum turgidum, Triticum aestivum* and *Hordeum vulgare* (Fig 2).

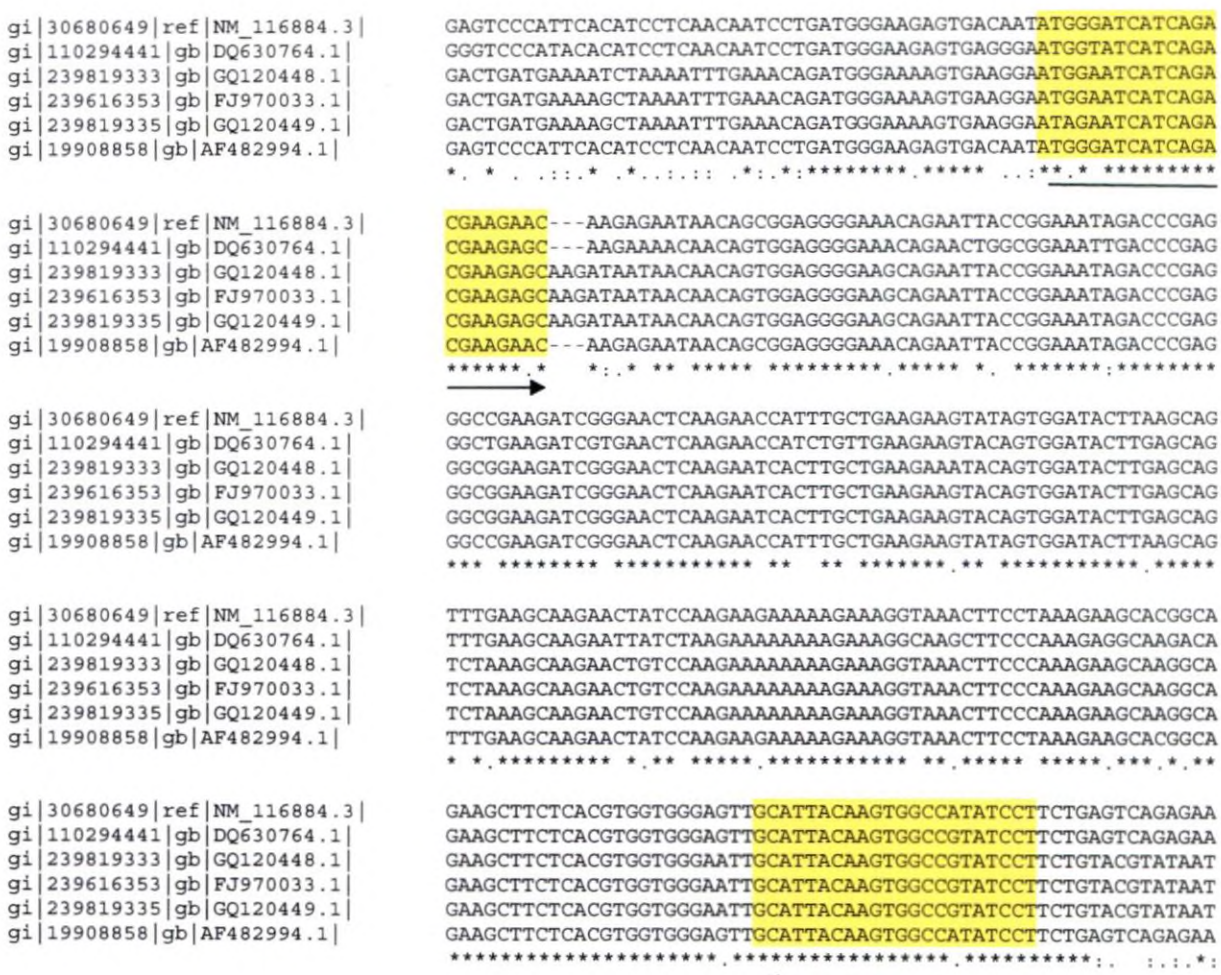
Similarly, *PIN1*sequences from *Arabidopsis thaliana, Nicotiana tabacum, Malus domestica, Medicago truncatula, Lupinus albus* and *Populus* sp. were used for *PIN1* primers designing (Fig 3). And *LOG1*sequences were obtained from *Arabidopsis thaliana, Eleusine coracana, Cucumis sativus, Cicer arietinum, Fragaria vesca* and *Cucumis sativus* (Fig 4).

The collection of FASTA sequences of each gene were then subjected to multiple sequence alignment using clustal omega program. The best two conserved regions in the multiple alignments were identified and the primers were designed according to the sequences of those conserved regions

3.2.1.1 Primer analysis

The designed set of primers prior to their synthesis were checked for several factors such as primer length, length of the PCR product, low degeneracy, maximum specificity at the 3' end, etc.. The properties such as feasible annealing

Fig. 1. Multiple sequence alignment of *BP*



(*) : conserved region

(⟶) : region used for forward primer

(⟵) : region used for reverse primer

Accession No.		**Plant sp.**
gi|30680649|ref|NM_116884.3	:	*Arabidopsis thaliana* (cDNA)
gi|110294441|gb|DQ630764.1	:	*Cardamine hirsuta* (cDNA)
gi|239819333|gb|GQ120448.1	:	*Brassica rapa* (gene)
gi|239616353|gb|FJ970033.1	:	*Brassica napus* (gene)
gi|239819335|gb|GQ120449.1	:	*Brassica oleracea* (gene)
gi|19908858|gb|AF482994.1	:	*Arabidopsis thaliana* Landsberg erecta (cDNA)

Fig. 2. Multiple sequence alignment of *RA2*

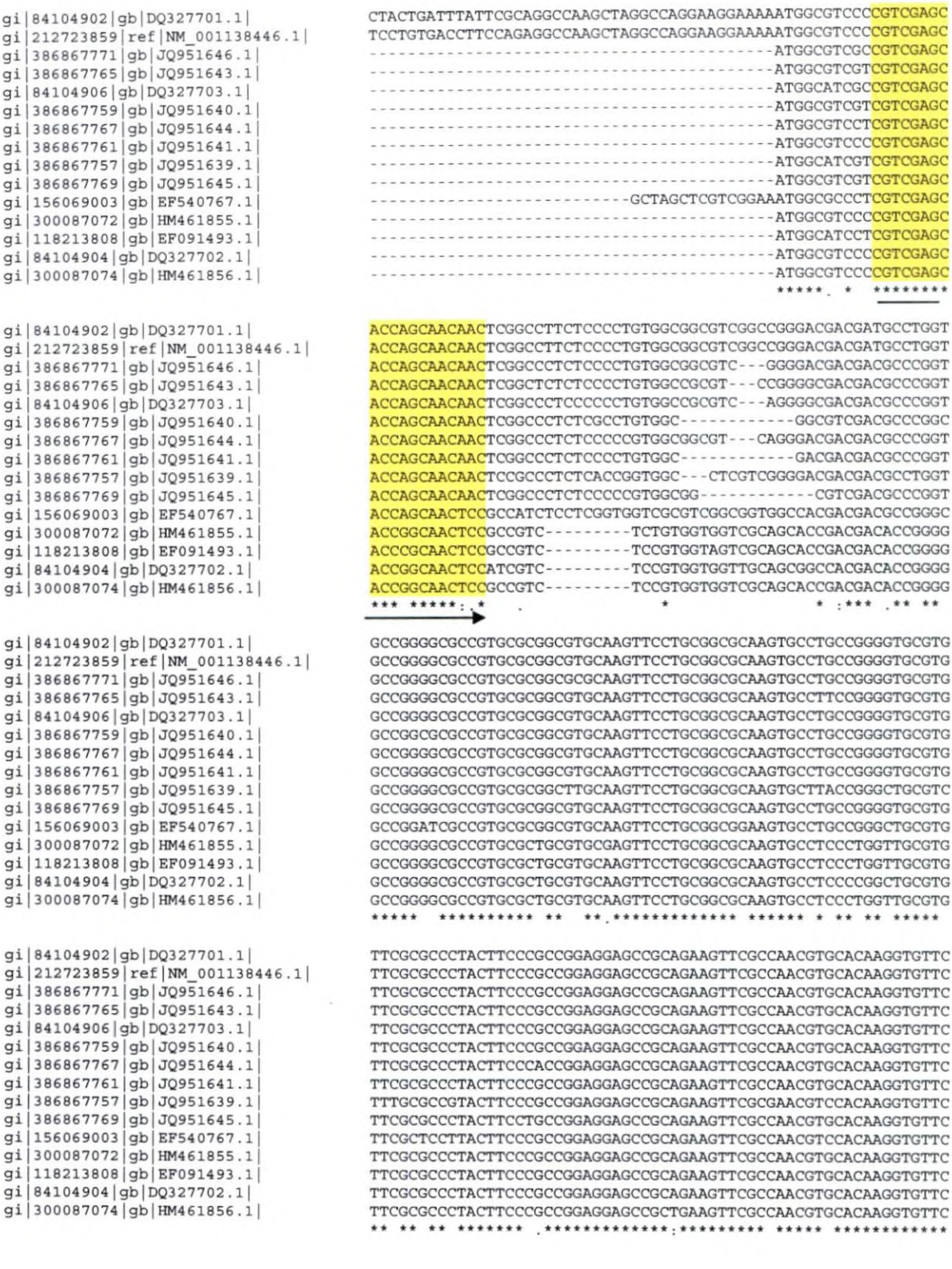
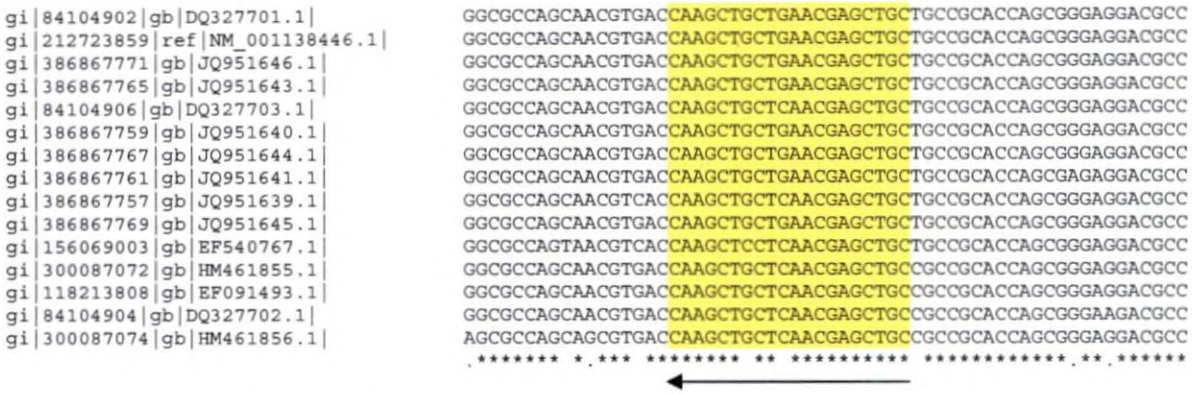


Fig. 2. continued



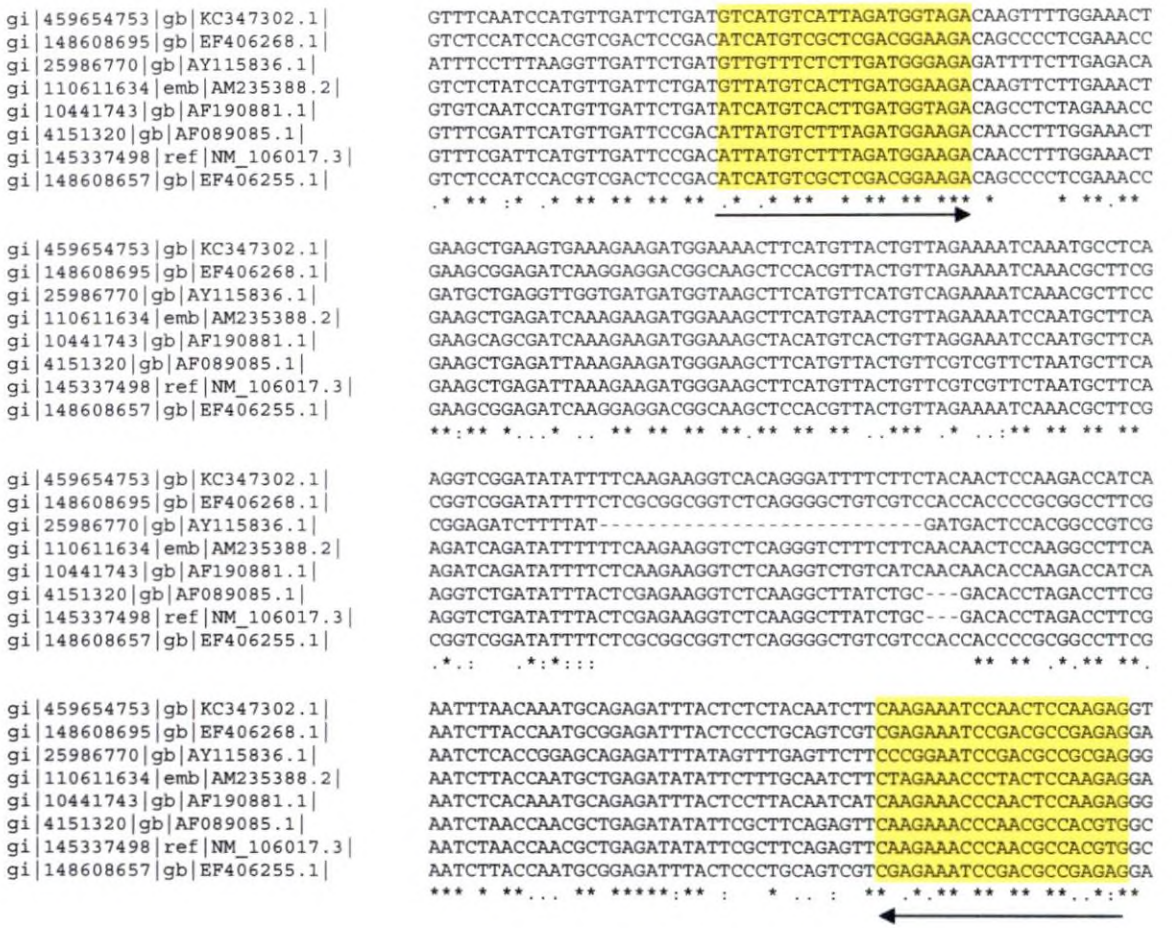
(*) : conserved region

(⟶) : region used for forward primer

(⟵) : region used for reverse primer

Accession No.		**Plant sp.**
gi|84104902|gb|DQ327701.1|	:	*Zea mays* (gene)
gi|212723859|ref|NM_001138446.1|	:	*Zea mays* (cDNA)
gi|386867771|gb|JQ951646.1|	:	*Phacelurus digitatus* (gene)
gi|386867765|gb|JQ951643.1|	:	*Andropterum stolzii* (gene)
gi|84104906|gb|DQ327703.1|	:	*Sorghum bicolor* (gene)
gi|386867759|gb|JQ951640.1|	:	*Chrysopogon gryllus* (gene)
gi|386867767|gb|JQ951644.1|	:	*Andropogon hallii* (gene)
gi|386867761|gb|JQ951641.1|	:	*Cymbopogon flexuosus* (gene)
gi|386867757|gb|JQ951639.1|	:	*Loudetia sp.* (gene)
gi|386867769|gb|JQ951645.1|	:	*Schizachyrium sanguineum* var. hirtiflorum (gene)
gi|156069003|gb|EF540767.1|	:	*Oryza sativa* Japonica (cDNA)
gi|300087072|gb|HM461855.1|	:	*Triticum turgidum* subsp. *durum* (cDNA)
gi|118213808|gb|EF091493.1|	:	*Triticum aestivum* (cDNA)
gi|84104904|gb|DQ327702.1|	:	*Hordeum vulgare* (gene)
gi|300087074|gb|HM461856.1|	:	*Triticum turgidum* subsp. *durum* (cDNA)

Fig. 3. Multiple sequence alignment of *PIN1*



(*) : conserved region

(⟶) : region used for forward primer

(⟵) : region used for reverse primer

Accession No.		**Plant sp.**
gi|459654753|gb|KC347302.1|	:	*Nicotiana tabacum* cultivar Bright Yellow 2 (gene)
gi|148608695|gb|EF406268.1|	:	*Malus domestica* (gene)
gi|25986770|gb|AY115836.1|	:	*Medicago truncatula* (gene)
gi|110611634|emb|AM235388.2|	:	*Lupinus albus* (gene)
gi|10441743|gb|AF190881.1|	:	*Populus tremula* x *Populus tremuloides* (cDNA)
gi|4151320|gb|AF089085.1|	:	*Arabidopsis thaliana* (gene)
gi|145337498|ref|NM_106017.3|	:	*Arabidopsis thaliana* (cDNA)
gi|148608657|gb|EF406255.1|	:	*Malus domestica* (cDNA)

Fig. 4. Multiple sequence alignment of *LOG1*

(*) : conserved region

(⟶) : region used for forward primer

(⟵) : region used for reverse primer

Accession No.		**Plant sp.**
ENA|HE800186|HE800186.1	:	*Eleusine coracana* (cDNA)
gi|30683837|ref|NM_128389.2|	:	*Arabidopsis thaliana* (cDNA)
gi|449524369|ref|XM_004169148.1|	:	*Cucumis sativus* (cDNA)
gi|502122290|ref|XM_004497616.1|	:	*Cicer arietinum* (cDNA)
gi|470110911|ref|XM_004291655.1|	:	*Fragaria vesca* subsp. *Vesca* (cDNA)
gi|449456019|ref|XM_004145700.1|	:	*Cucumis sativus* (cDNA)

temperature, an appropriate range of GC-content, potential hairpin formation and 3' complementarity were analyzed by using Oligo Calc programme (http://simgene.com/OligoCalc).

The sequences of the resultant primers were given to 'Genei, Bangalore' for synthesis.

3.2.2 Isolation of genomic DNA

C-TAB method of DNA extraction (Doyle and Doyle, 1990) with slight modifications was used for genomic DNA isolation.

β-mercaptoethanol and polyvinylpyrrolidone (PVP) were added fresh to the CTAB extraction buffer (Appendix I) to give a final concentration of 0.2 percent (v/v) and 4 percent (w/v) respectively. The solution was heated to 65 °C in water bath (ROTEK, India). The samples were chilled and pulverized to a fine powder in liquid nitrogen using a sterile mortar and pestle, and transferred 100 mg of powder into a sterile 2 ml centrifuge tubes containing 1 ml of freshly prepared warm extraction buffer. The content was homogenized by gentle inversion. The samples were incubated at 65 °C in water bath for 1 h with intermittent shaking. The homogenate was then extracted with an equal volume of 24: 1 (v/v) chloroform/isoamyl alcohol and mixed well by inversion for 5-10 min. The homogenate was centrifuged (Eppendorf centrifuge 5430 R, Germany) at 7500 x *g* for 10 min at 25 °C. The upper phase was transferred to a sterile centrifuge tube and the extraction process with chloroform/isoamyl alcohol was repeated twice. To the aqueous phase, 0.5 ml of 5 *M* NaCl was added and mixed properly by gentle inversion. After that, 0.6 volume of chilled isopropanol was added to the mixture and mixed by inversion. The mixture was then incubated at 4 °C overnight to precipitate the nucleic acid. After incubation, the precipitated DNA was pelletized by centrifugation at 11,000 x *g* for 10 min at 25 °C. The supernatant was decanted and the pellet was washed in 0.5 ml ethanol (80 %) twice, each time centrifuging at 5000 x *g* for 7 min at 25 °C and discarding the supernatant. The pellet was air dried for 30-40 min and dissolved in 40 µl of TE

buffer (Appendix II). The extracted DNA samples were then stored at -20 °C (Lab-Line Low Temperature Cabinet, India).

3.2.2.1 Agarose gel electrophoresis

The most common method to assess the integrity of genomic DNA is to run an aliquot of the DNA sample on agarose gel. The gel was run using horizontal gel electrophoresis unit (Genei, Bangalore). Aliquot of DNA sample (5 µl) was loaded on agarose gel (0.8 %) made of 0.5 x TBE buffer (Appendix III). The gel was run at 5 Vcm^{-1} until the dyes migrated $3/4^{th}$ of the distance through the gel. The gel was visualized under the gel documentation system (BIORAD, USA) using 'Quantity One Software'.

3.2.2.2 Spectrophotometer analysis

The absorbance of the DNA samples was recorded to determine the quantity and quality of DNA. T60 UV- Visible Spectrophotometer (Oasis Scientific, USA) was used for measuring optical density (O.D.) of the sample. Spectrophotometer was calibrated to blank (zero absorbance) at 260 nm and 280 nm wavelength with 3 ml TE buffer and absorbance of 5 µl DNA sample dissolved in 3 ml of TE buffer at respective wavelengths were recorded.

Since an absorbance value of 1.0 at 260 nm indicates the presence of 50 $ng\mu l^{-1}$ of double stranded DNA, the concentration of DNA in the extracted sample was estimated by employing the following formula:

$$\text{Amount of DNA } (ng\mu l^{-1}) = A_{260} \times 50 \times \text{dilution factor}$$

(Where A_{260} is absorbance reading at 260 nm)

DNA quality was determined by the ratio taken between A_{260}/A_{280} readings.

3.2.2.3 PCR amplification of genomic DNA with degenerate primers

The genomic DNA of both branched and non-branched spikes of Thekken as well as spikes of Karimunda were amplified using the designed degenerate

primers for the genes *BP, RA2, LOG1* and *PIN1*. The components of the mixture were optimized as listed below:

Water	:	12.9 µl
10x Taq buffer A (Tris with 15 m*M* $MgCl_2$)	:	2 µl
dNTPS (2.5 m*M* each)	:	1.6 µl
Forward primer (10 µ*M*)	:	1 µl
Reverse primer (10 µ*M*)	:	1 µl
Template DNA (50 $ng\mu l^{-1}$)	:	1 µl
Taq polymerase (3 $U\mu l^{-1}$)	:	0.5 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 30 s and extension at 72 °C for 45 s. Final extension was done at 72 °C for 5 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with Quantum PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1.5 %). The gel was viewed under gel documentation system (BIORAD, USA).

3.2.3 RNA Isolation

Trizol reagent (Invitrogen, USA) was used for extraction of total RNA. The samples included immature spikes from Thekken and Karimunda plants. All the materials used for RNA extraction were treated in 3 percent hydrogen peroxide overnight and autoclaved twice for sterilization. The double distilled water was also autoclaved twice for the same purpose.

Frozen samples (100 mg) were ground into a fine powder in liquid nitrogen using mortar and pestle. Immediately the powdered samples were transferred into the micro centrifuge tube containing 1 ml of Trizol Reagent. The content was mixed gently to homogenize and then incubated for 5 min at room temperature to

permit complete dissociation of the nucleoprotein complex. Chloroform (0.2 ml) was added and the tubes were shaken vigorously for 15 s. The tubes were incubated for 2-3 min at room temperature. The samples were centrifuged at 12,000 x *g* for 15 min at 4 °C. The aqueous phase of the sample was transferred into a fresh tube. 0.5 ml of chilled 100 percent isopropanol was added to the aqueous phase and incubated at room temperature for 10 min. The sample was centrifuged at 12,000 x *g* for 10 min at 4 °C. The supernatant was removed from the tube leaving only RNA pellet. The pellet was washed with 1 ml of 75 percent ethanol. The sample was briefly vortexed and centrifuged at 7500 x *g* for 5 min at 4 °C. The wash was discarded and the RNA pellet was air dried for 30-40 min. The RNA pellet was resuspended in 30 µl RNase free sterile water followed by incubation in a water bath at 55-60 °C for 10 min.

The integrity of the total RNA was determined by running 5 µl aliquot of RNA on agarose gel (1.5 %) as described in section 3.2.2.1.

The absorbance reading of extracted RNA using spectrophometer was determined as described in the section 3.2.2.2. Since an absorbance value of 1.0 at 260 nm indicates the concentration of 40 ngµl^{-1} of RNA, the concentration of RNA present in an aliquot was estimated by employing the following formula:

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

(Where A_{260} is absorbance reading at 260 nm)

RNA purity was determined by the ratio taken between A_{260} and A_{280}.

3.2.3.1 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is the most sensitive technique of mRNA detection and quantification among currently available techniques such as Northern blot analysis and RNase protection assay. RT-PCR can be used to quantify mRNA levels from much smaller samples.

RT-PCR was carried out using AMV Reverse Transcription Kit (Genei, Bangalore). RT-PCR is essentially a two step process. The first step involves the usage of reverse transcriptase to synthesize cDNA from single stranded mRNA. The second step is amplification of the cDNA by PCR. To avoid any RNase contamination, the sterile working environment was maintained. All the materials used were pre-treated with 3 percent hydrogen peroxide overnight and autoclaved twice.

3.2.3.1.1 First strand cDNA synthesis

To a sterile RNase free micro-centrifuge tube, 5 µl RNA and 1 µl oligo $(dT)_{18}$ primer were added, and made up the volume to 10 µl with nuclease free water. The tubes were warmed at 65 °C for 10 min and then kept at room temperature for 2 min to remove any secondary structures. The tubes were spun briefly and the components were added in the order given below:

RNasin (10 $U\mu l^{-1}$)	:	1 µl
100 m*M* DTT	:	1 µl
5 X Assay Buffer	:	4 µl
30 m*M* dNTP mix	:	2 µl
AMV Reverse Transcriptase	:	0.5 µl
Nuclease Free Water	:	1 µl
Total Volume	:	20 µl

The contents were mixed well and incubated at 42 °C for 1 h. The tubes were then incubated at 92 °C for 2 min and quickly placed the tubes in ice and spun briefly (this step denatures RNA-cDNA hybrids). The cDNA samples were stored at -20 °C (Lab-Line Low Temperature Cabinet, India) until the PCR amplification.

3.2.3.1.2 PCR amplification of cDNA with degenerate primers

The cDNA samples were subjected to PCR analysis with four degenerate primers afore mentioned in section 3.2.1. The PCR mixture of the total volume of

20 µl was prepared similarly as described in the section 3.2.2.3 except that 3 µl of cDNA was used as template instead of 1 µl of genomic DNA.

The PCR programme was set with initial denaturation temperature of 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing (different temperatures screened) for 30 s and extension at 72 °C for 45 s. The final extension was set at 72 °C for 5 min. The PCR products were separated on agarose gel (1.5 %) and the gel was observed under gel documentation system.

3.3 MOLECULAR MARKER ANALYSES

A total of six genomic DNA samples which included two from branched spikes of Thekken A and B, two from non-branched spikes of Thekken A and B, and two from spikes of Karimunda A and B were subjected to RAPD and SSR marker analyses.

3.3.1 RAPD Analysis

The genomic DNA samples of Thekken and Karimunda were amplified with twelve RPI decamer primers supplied by 'Genei, Bangalore'. The components of the reaction mixture were optimized as described in the section 3.2.2.3.

PCR programme was set with initial denaturation at 94 °C for 4 min followed by 35 repeated cycles of denaturation at 94 °C for 1 min, annealing at 42 °C for 1 min and extension at 72 °C for 2 min. Final extension was carried out at 72 °C for 5 min.

The PCR products were loaded onto agarose gel (1.5 %) and the gel was viewed using gel doc system.

The PCR products were scored for the presence (+) or absence (-) of bands. The numbers of monomorphic and polymorphic bands were recorded.

3.3.2 SSR Analysis

SSR markers are reported to be used between different species or genera (Stafne *et al.*, 2005). Hence, the SSR primers that were already available in the laboratory were used in the present study to reduce the expenses.

Similar to RAPD analysis, the genomic DNA samples were screened with thirteen SSR primers (RM primers) out of which two primers were amplifiable and selected for the analysis. The PCR mixture was prepared in the same way as mentioned in section 3.2.2.3.

PCR amplification programme involved initial denaturation of DNA template at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. Final extension was set at 72 °C for 5 min. The PCR products were separated on agarose gel (1.5 %) and visualized under UV light in gel documentation system. The PCR products were scored for the presence (+) or absence (-) of bands.

3.4 PROTEIN ANALYSIS

The total soluble protein was extracted from six samples that included branching spikes and non-branching spikes each from three Thekken plants (Thekken A, B and C). The total protein was estimated and separated by SDS-PAGE to determine the differences in protein expression pattern and expression level between the samples.

3.4.1 Protein extraction

Extraction of soluble proteins from the spikes of black pepper was carried out following the procedure described by Laemelli (1970).

One gram of sample was ground in liquid nitrogen and homogenized in 1 ml of cold denaturing solution (Appendix IV). The extract was centrifuged at 5000 rpm for 15 min at 4 °C. The supernatant was transferred into a fresh centrifuge

tube and mixed with chilled acetone in the ratio 1:1. The protein was allowed to precipitate by keeping the mixture at 4 °C for 30 min. The sample was centrifuged at 5000 rpm for 15 min at 4 °C. The supernatant was removed and the pellet was resuspended in 50 µl of denaturing solution and vortexed. The homogenate was centrifuged at 5000 rpm for 15 min at 4 °C. The supernatant was stored at 4 °C for later spectrophotometer analysis and SDS-PAGE.

3.4.2 Protein estimation

Total soluble protein content of the extracted samples was estimated by using procedure described by Bradford (1976). Bovine Serum Albumin working standard [BSA 100µg ml^{-1} of Phosphate Buffered Saline (PBS) (Appendix V)] 0.0, 0.2, 0.4, 0.6, 0.8 and 1ml, and 0.01ml of the extracted protein sample were pipetted out in to a series of labeled test tubes. The volume was made up to 1 ml in all the test tubes with PBS. A tube containing neither working standard nor sample, but only PBS served as blank. Five ml of diluted dye solution (Appendix VI) was added to all the test tubes. Mixed the contents of the tubes by vortexing and allowed the colour to develop for at least 15-20 min. The spectrophotometer was calibrated to zero absorbance at 595 nm wavelength with 3 ml of blank solution. The absorbance of different working standard solutions of known protein concentrations and the samples of unknown protein concentrations were recorded at 595 nm against blank. The concentration of standard protein samples was plotted against the corresponding absorbance resulting in a standard curve that was used to determine the protein content in samples.

3.4.3 Electrophoretic analysis of proteins using SDS PAGE

Electrophoretic separation of soluble protein was carried out as per the procedure described by Sambrook and Russell (2001).

The protein sample was mixed with equal volume of 1 X SDS gel loading buffer (Appendix VII) and kept in boiling water bath for 3 min. Resolving gel (10%) (Appendix VIII) was first casted in the glass plate leaving sufficient space

for stacking gel. Carefully overlaid the isobutanol. After the polymerization was completed (about 30 min), poured off the overlay and washed the top of the gel with deionized water to remove any unpolymerized acrylamide and drained the fluid. The stacking gel (Appendix IX) was poured onto the surface of polymerized resolving gel. A clean comb was immediately inserted.

After polymerization, the comb was removed. The wells were washed with deionized water immediately. The gel was mounted in electrophoresis unit. Tris-glycine buffer (Appendix X) was added to the top and bottom reservoirs. Sample proteins mixed with SDS gel loading buffer (1:1), having about 100 µg equivalent proteins were loaded onto the wells. Marker with known molecular weight was also loaded on one of the wells. A voltage of 80V ($8Vcm^{-1}$) was applied to the gel. After the dye front moved into the resolving gel, the voltage was increased to 150 V ($15\ Vcm^{-1}$) and continued till the dye reached the bottom of the resolving gel. Immediately after electrophoresis, the gel was immersed in 5 volumes of staining solution (Appendix XI) and placed on a slowly rotating shaker for overnight at room temperature, which was later transferred to the destaining solution (Appendix XII). The gel was destained changing the destaining solution 3-4 times until all the free dyes were removed from the gel leaving behind the protein bound dyes which appeared as a distinct band in the gel. The gel was photographed after placing on a transilluminator. The protein banding profiles were analyzed for the presence of polymorphism.

3.5 MICROSCOPIC ANALYSIS

The spike samples from both Thekken and Karimunda stored at -80 °C were transferred into the test tubes containing chilled Carnoy's fluid (Appendix XIII) that had been pre-stored at -20 °C for temperature stabilization overnight. The samples were kept at -20 °C overnight. Next day, the spikes were viewed directly under the stereomicroscope (Leica ez4hd, Germany).

The longitudinal cross sections of the spikes were also prepared by using a sharp blade. The sections were then observed under the microscope.

RESULTS

4. RESULTS

The results of the study entitled "Identification of molecular marker associated with spike branching trait in black pepper (*Piper nigrum* L.)" carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani, during 2011-2013 are presented in this chapter.

4.1 IDENTIFICATION OF INFLORESCENCE ARCHITECTURAL GENES

4.1.1 Degenerate primer designing

Forward (F) and reverse (R) degenerate primers for the genes governing inflorescence architecture, *viz. BP*, *RA2*, *PIN1* and *LOG1* were designed based on the most favorable combination of conserved regions in the multiple aligned nucleotide sequences and named as (BP-F, R), (RA2-F, R), (PIN1-F, R) and (LOG1-F, R) respectively. The details of the primers designed are presented in Table 1.

4.1.1.1 Primer analysis

The analysis of primers using Oligo Calc programme revealed fair GC content and annealing temperature, and also none of the designed primers exhibited hairpin formation and 3' complementarity.

4.1.2 DNA isolation

Branched and non-branched spikes of pepper type Thekken as well as spikes of Karimunda variety were used for genomic DNA extraction. The agarose gel electrophoresis (0.8%) of the extracted genomic DNA showed the presence of good quality unsheared DNA bands on the gel (Plate 2). Further absorbance reading of the extracted genomic DNA by using spectrophotometric method revealed good quality and quantity of DNA (Table 2).

Table 1. Sequences of designed degenerate primers

Sl. No.	Target gene	Primer name	Primer sequence (5' to 3')	No. of bases	GC content (%)	T_m (°C)
1	*BP*	BP- F	ATRGDATCATCAGACGAAGARC	22	36-50	49-55
		BP- R	AGGATAYGGCCACTTGTAATGC	22	45-50	53-55
2	*RA2*	RA2- F	CGTCGAGCACCVGCAACWMC	20	60-70	56-60
		RA2- R	GCAGCTCGTTSAGSAGCTTG	20	60	56
3	*PIN1*	PIN1- F	RTYRTKTCDYTHGAYGGDAGA	21	24-67	43-60
		PIN1- R	CWCKYGGMGTHGGRTTYCKNG	21	43-81	50-66
4	*LOG1*	LOG1- F	GHCAYGTYVTYGGDGTYATC	20	35-70	46-60
		LOG1- R	TGTARWAKCCDTCCACRTTC	20	35-55	46-54

Degenerate code		Bases
M	:	AC
R	:	AG
W	:	AT
S	:	CG
Y	:	CT
K	:	GT
V	:	ACG
H	:	ACT
D	:	AGT
N	:	ACGT

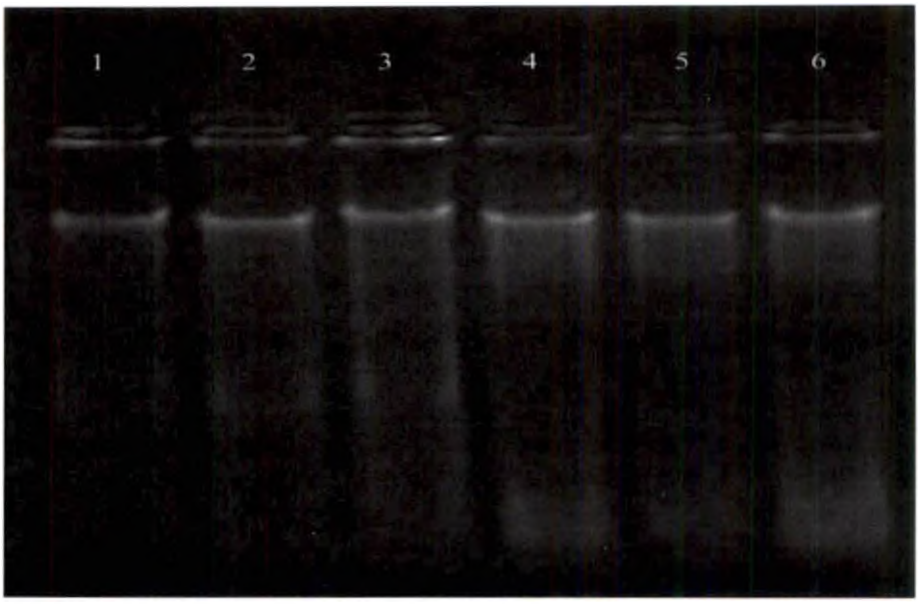


Plate 2. Agarose gel electrophoresis of black pepper genomic DNA

Lane No.	Sample
1 and 2	Thekken (branched spikes)
3 and 4	Thekken (non-branched spikes)
5 and 6	Karimunda spikes

Table 2. Quality and quantity of isolated genomic DNA

Sl. No.	Sample	Absorbance (A_{260} nm)	Absorbance (A_{280} nm)	A_{260}/A_{280}	DNA Yield ($ng\mu l^{-1}$)
1	Branched spikes (Thekken A)	0.012	0.007	1.71	360
2	Branched spikes (Thekken B)	0.014	0.009	1.55	420
3	Non branched spikes (Thekken A)	0.015	0.008	1.87	450
4	Non branched spikes (Thekken B)	0.009	0.005	1.80	270
5	Spikes (Karimunda A)	0.020	0.013	1.53	600
6	Spikes (Karimunda B)	0.017	0.011	1.54	510

4.1.2.1 PCR analysis of genomic DNA with degenerate primers

Out of four pairs of degenerate primers, three primer pairs, i.e., (RA2-F, R), (PIN1-F, R) and (LOG1-F, R) produced the amplification in the genomic DNA of both Thekken type and Karimunda variety of black pepper, while primer (BP-F, R) produced an amplicon only in Karimunda variety (Plate 3, 4 and 5). The banding patterns by all the primers were same for both branched and non-branched spikes of Thekken.

In Thekken, primers (RA2-F, R), (PIN1-F, R) and (LOG1-F, R) amplified a band of size ~500, ~1800 and ~500 bp respectively. Equal sized amplicons were also produced by all the respective primers in Karimunda sample. However, additional bands of size ~200, ~900 and ~300 were also produced by the primers (RA2-F, R), (PIN1-F, R) and (LOG1-F, R) respectively in Karimunda sample. The primer (BP-F, R) produced an amplicon of size ~600 bp only in Karimunda variety.

4.1.3 RNA isolation

Immature spikes from Thekken type and Karimunda variety were used for the extraction of total RNA. A distinct intact rRNA bands with no apparent RNA degradation and no genomic DNA contamination were observed on agarose gel showing good quality RNA extraction (Plate 6).

Further absorbance reading of the extracted RNA by using spectrophotometric method revealed good quality and quantity of RNA (Table 3).

4.1.3.1 RT-PCR with degenerate primers

The reverse transcription of the extracted RNA was carried out shortly to synthesize the first strand cDNA followed by PCR with the designed degenerate primers. However, the reaction did not produce any amplicon on both Thekken and Karimunda samples. Although different PCR conditions were tried, none of the primers showed the amplification.

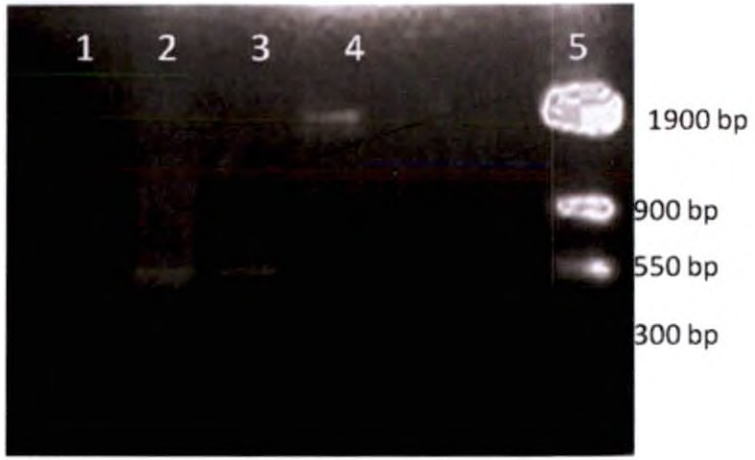


Plate 3. PCR with degenerate primers in branched spikes of Thekken

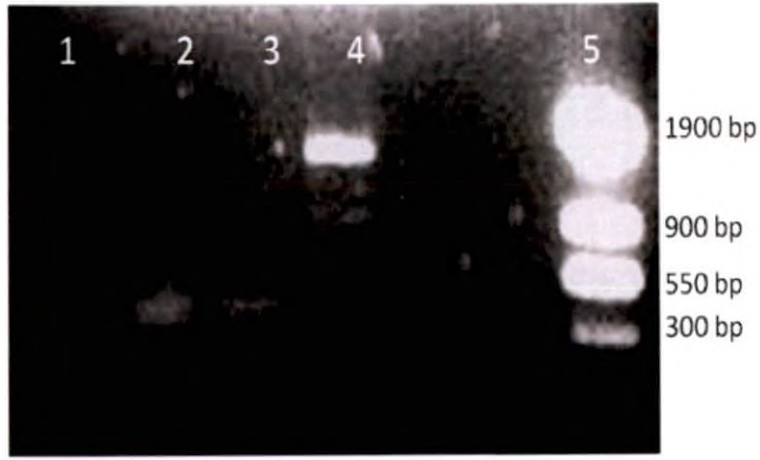


Plate 4. PCR with degenerate primers in non-branched spikes of Thekken

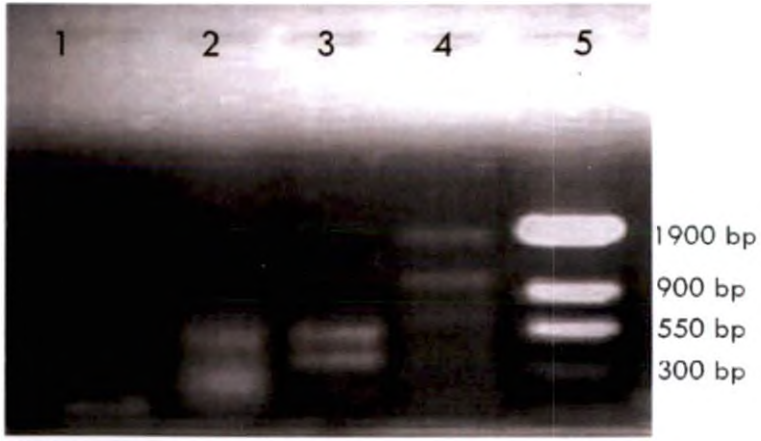


Plate 5. PCR with degenerate primers in Karimunda

Lane No.	Primer
1:	Primer (BP-F, R)
2:	Primer (RA2-F, R)
3:	Primer (LOG1-F, R)
4:	Primer (PIN1-F, R)
5:	DNA marker

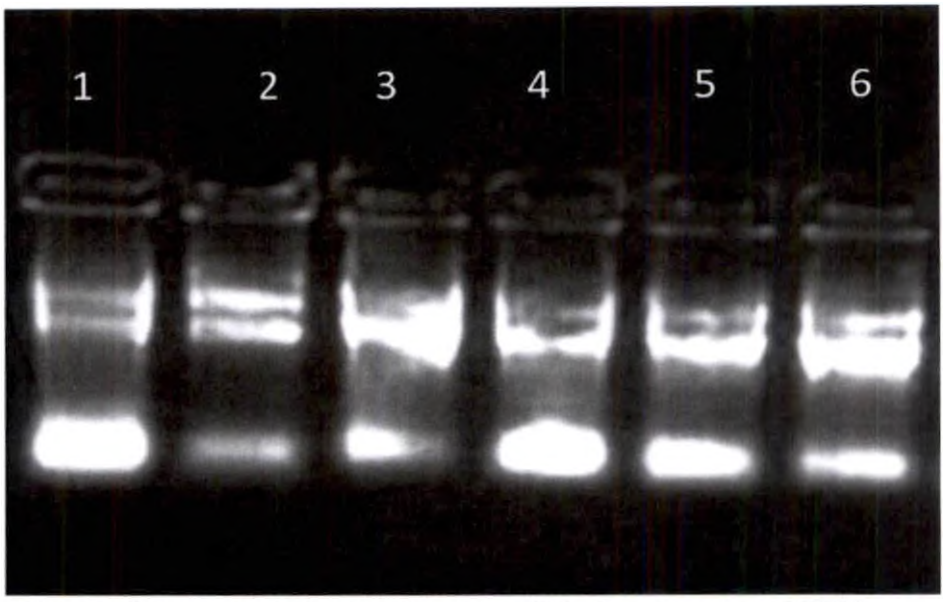


Plate 6. Agarose gel electrophoresis of black pepper total RNA

Lane No.	Sample
1,2,3	Thekken spikes
4,5,6	Karimunda spikes

Table 3. Quality and quantity of isolated total RNA

Sl. No.	Sample	Absorbance (A 260 nm)	Absorbance (A 280 nm)	A_{260}/A_{280}	RNA Yield ($ng\mu l^{-1}$)
1	Immature spikes (Thekken A)	0.050	0.028	1.78	1200
2	Immature spikes (Thekken B)	0.042	0.023	1.82	1008
3	Immature spikes (Karimunda A)	0.055	0.028	1.96	1320
4	Immature spikes (Karimunda B)	0.064	0.034	1.88	1536

4.2 MOLECULAR MARKER ANALYSES

4.2.1 RAPD analysis

Twelve primers which produced consistent amplification pattern were selected for RAPD analysis (Table 4). The primers altogether generated 70 scorable bands. Primer RPI 8 produced the maximum number of 9 scorable bands while primer RPI 5 produced the minimum of 3 bands. But none of the primers displayed polymorphism between the branched and non-branched spikes of the pepper Thekken.

Table 4. Sequences of primers used for RAPD analysis

Sl. No.	Primer	Primer sequence (5' to 3')	GC content (%)
1	RPI 1	AAAGCTGCGG	60
2	RPI 2	AACGCGTCGG	70
3	RPI 3	AAGCGACCTG	60
4	RPI 4	AATCGCGCTG	60
5	RPI 5	AATCGGGCTG	60
6	RPI 6	ACACACGCTG	60
7	RPI 8	ACCACCCACC	70
8	RPI 11	ACGGAAGTGG	60
9	RPI 12	ACGGCAACCT	60
10	RPI 13	ACGGCAAGGA	60
11	RPI 14	ACTTCGCCAC	60
12	RPI 15	AGCCTGAGCC	70

However, in RAPD analysis, 16 bands comprising 22.8 percent of the total 70 scorable bands were found to be polymorphic between Thekken type and Karimunda variety (Table 5). The maximum polymorphic bands were observed in primer RPI 4, whereas no polymorphism was shown by the two primers, *viz.* RPI 3 and RPI 13 between Thekken and Karimunda. The highest percentage of polymorphism (50% each) was obtained by primers RPI 11 and RPI 14, both amplifying 2 polymorphic bands out of 4 total bands produced.

Primer RPI 1 yielded a total of 4 scorable bands out of which one was polymorphic (Plate 7). Similarly, primer RPI 2 procured 1 polymorphic band out of 5 scorable bands produced (Plate 8).

The primer RPI 3 did not produce any polymorphic band out of 5 scorable bands (Plate 9). Eight scorable bands were obtained by primer RPI 4 with 3 bands showing polymorphism (Plate 10). Primer RPI 5 could amplify only 3 bands of which one was polymorphic (Plate 11).

Primer RPI 6 amplified 8 total scorable bands of which 7 were monomorphic and only one showed polymorphism (Plate 12). Primer RPI 8 produced the highest number of scorable bands, 8 bands with 2 showing polymorphism (Plate 13). Primer RPI 11 produced only 4 scorable bands, however 2 of these bands showed polymorphism (Plate14). RPI 12 displayed 7 scorable bands of which 2 were polymorphic (Plate 15).

RPI 13 amplified a total of 7 bands. None of these 7 bands produced polymorphism between Thekken and Karimunda (Plate 16). Primer RPI 14 exhibited 50 percent polymorphism by producing 2 polymorphic bands out of 4 scorable bands (Plate 17). RPI 15 showed one polymorphic band among 5 scorable bands produced (Plate 18).

Table 5. Polymorphism exhibited by RAPD between Thekken and Karimunda

Primer	Total No. of bands	No. of monomorphic bands	No. of polymorphic bands	Polymorphism (%)
RPI 1	4	3	1	25
RPI 2	6	5	1	16.6
RPI 3	5	5	-	-
RPI 4	8	5	3	37.5
RPI 5	3	2	1	33.3
RPI 6	8	7	1	12.5
RPI 8	9	7	2	22.2
RPI 11	4	2	2	50
RPI 12	7	5	2	28.5
RPI 13	7	7	-	-
RPI 14	4	2	2	50
RPI 15	5	4	1	20

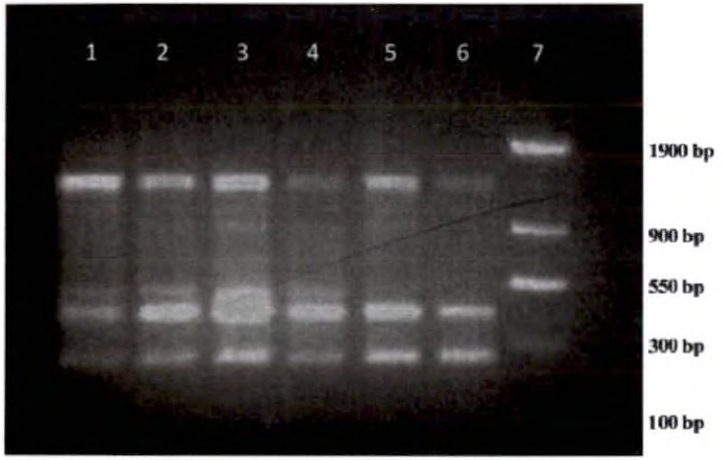


Plate 7. RAPD profile obtained with primer RPI 1

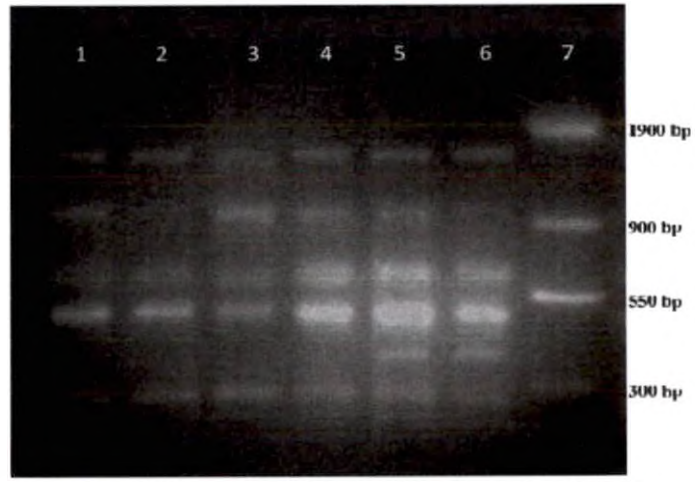


Plate 8. RAPD profile obtained with primer RPI 2

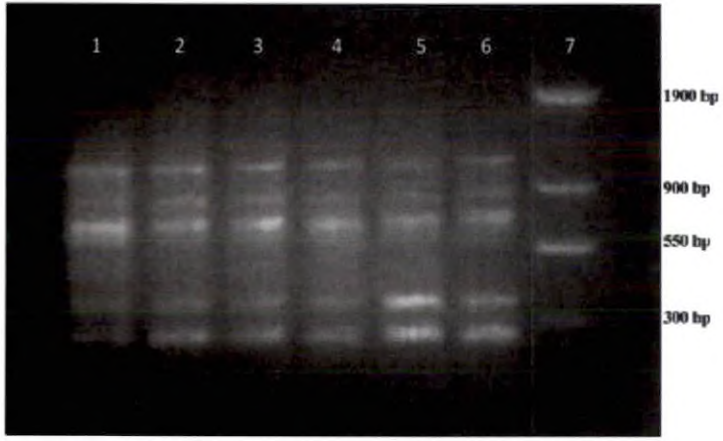


Plate 9. RAPD profile obtained with primer RPI 3

Lane No.		Sample
1	:	Branched spike (Thekken plant A)
2	:	Branched spike (Thekken plant B)
3	:	Non-branched spike (Thekken plant A)
4	:	Non-branched spike (Thekken plant B)
5	:	Spike (Karimunda plant A)
6	:	Spike (Karimunda plant B)
7	:	DNA marker

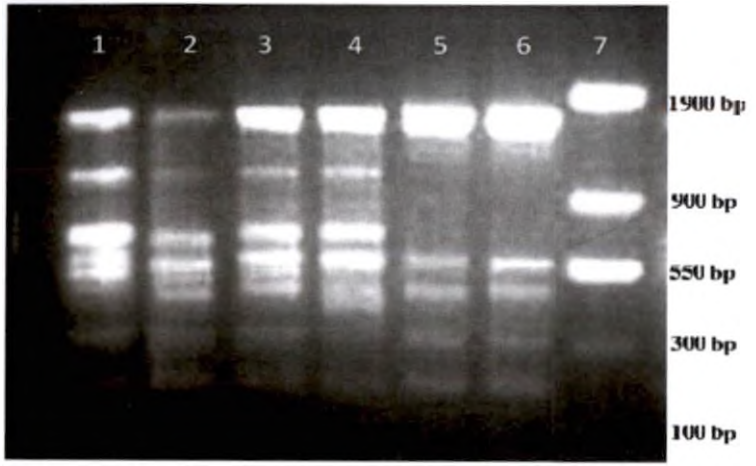


Plate 10. RAPD profile obtained with primer RPI 4

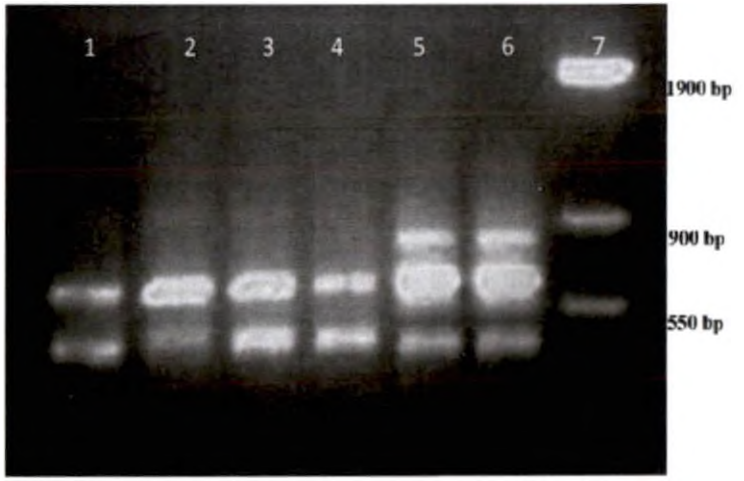


Plate 11. RAPD profile obtained with primer RPI 5

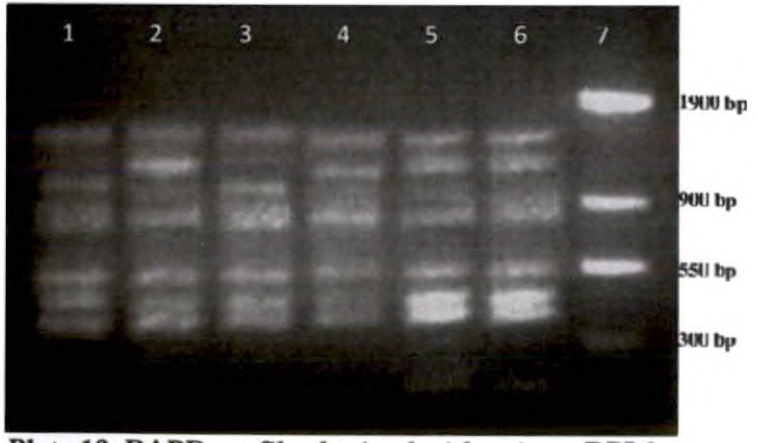


Plate 12. RAPD profile obtained with primer RPI 6

Lane No.		Sample
1	:	Branched spike (Thekken plant A)
2	:	Branched spike (Thekken plant B)
3	:	Non-branched spike (Thekken plant A)
4	:	Non-branched spike (Thekken plant B)
5	:	Spike (Karimunda plant A)
6	:	Spike (Karimunda plant B)
7	:	DNA marker

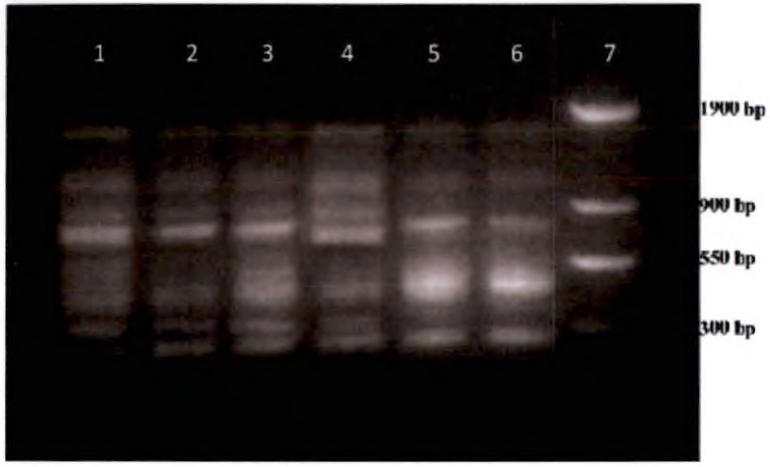


Plate 13. RAPD profile obtained with primer RPI 8

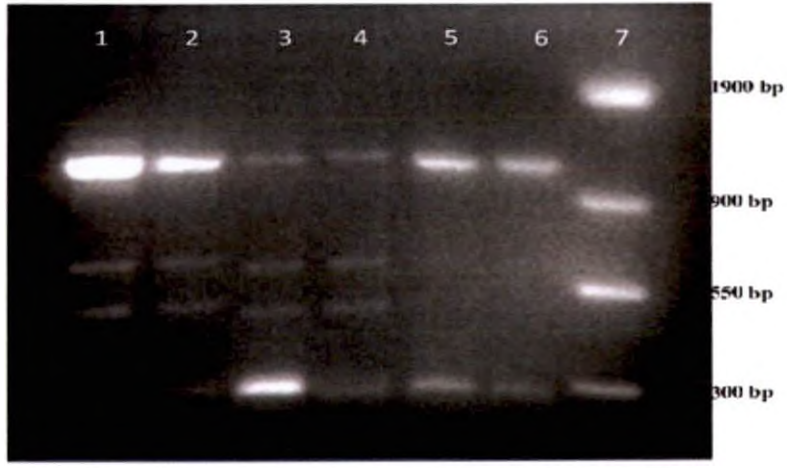


Plate 14. RAPD profile obtained with primer RPI 11

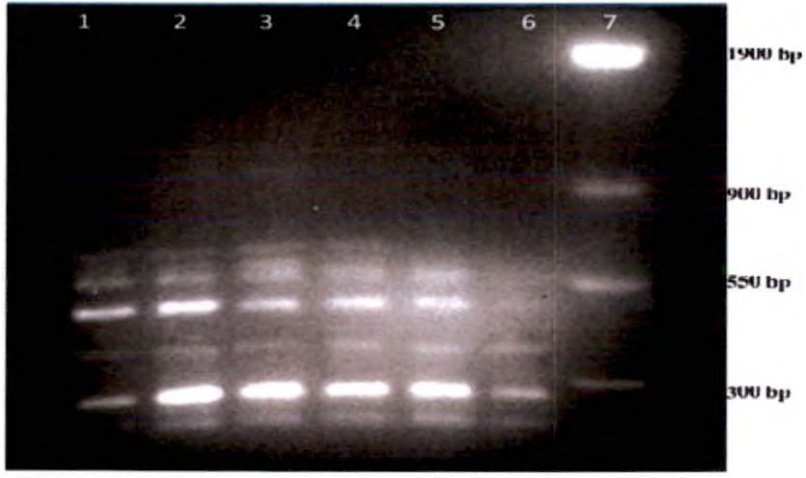


Plate 15. RAPD profile obtained with primer RPI 12

Lane No.		Sample
1	:	Branched spike (Thekken plant A)
2	:	Branched spike (Thekken plant B)
3	:	Non-branched spike (Thekken plant A)
4	:	Non-branched spike (Thekken plant B)
5	:	Spike (Karimunda plant A)
6	:	Spike (Karimunda plant B)
7	:	DNA marker

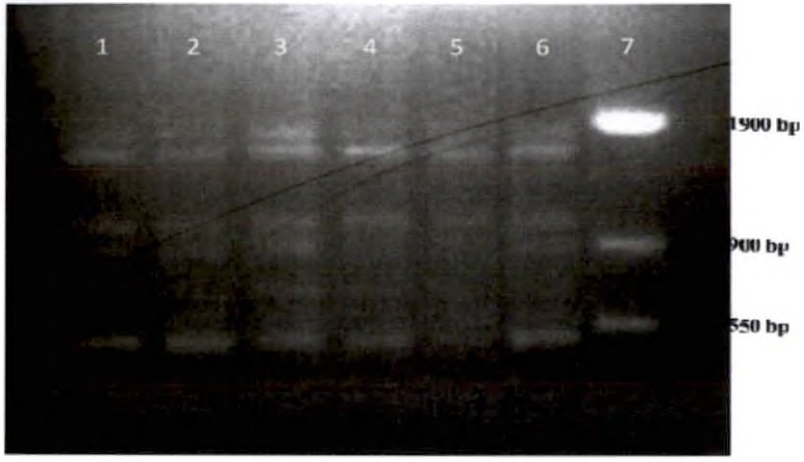


Plate 16. RAPD profile obtained with primer RPI 13

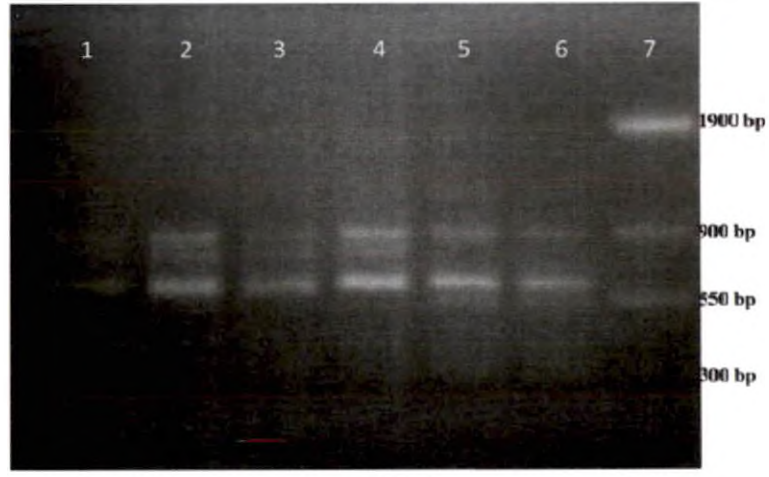


Plate 17. RAPD profile obtained with primer RPI 14

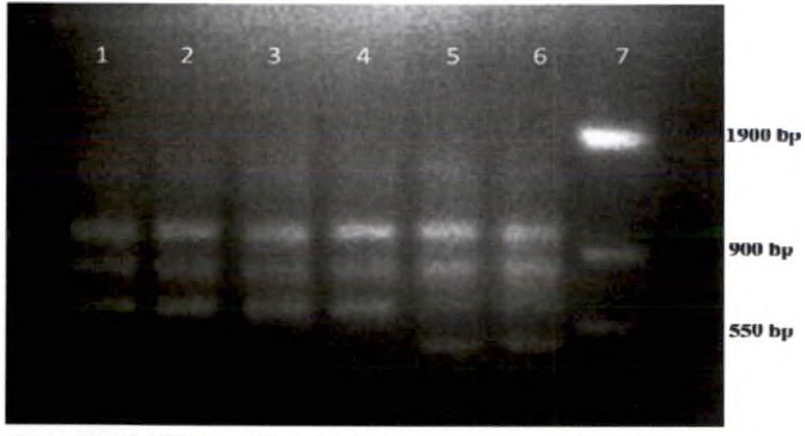


Plate 18. RAPD profile obtained with primer RPI 15

Lane No.		Sample
1	:	Branched spike (Thekken plant A)
2	:	Branched spike (Thekken plant B)
3	:	Non-branched spike (Thekken plant A)
4	:	Non-branched spike (Thekken plant B)
5	:	Spike (Karimunda plant A)
6	:	Spike (Karimunda plant B)
7	:	DNA marker

4.2.2 SSR analysis

SSR marker analysis was conducted in a similar way to RAPD analysis between the branched and non-branched spikes of Thekken and the spikes of Karimunda.

Two SSR primers (RM 125 and RM 214) were found to yield amplicons in the genomic DNA of black pepper. The sequences of primers are presented in Table 6. Primer RM 125 produced 3 scorable bands (Plate 19) and primer RM 214 yielded one scorable band (Plate 20). However, none of them exhibited polymorphism between the branched and non-branched spikes of Thekken as well as between the Thekken type and Karimunda variety.

Table 6. Sequences of primers used for SSR analysis

Sl. No.	Primer name	Primer sequence (5' to 3')	T_m (°C)	GC content (%)
1	RM 125 (F)	ATCAGCAGCCATGGCAGCGACC	65.8	63.6
	RM 125 (R)	AGGGGATCATGTGCCGAAGGCC	65.8	63.6
2	RM 214 (F)	CTGATGATAGAAACCTCTTCTC	56.5	40.9
	RM 214 (R)	AAGAACAGCTGACTTCACAA	53.2	40.0

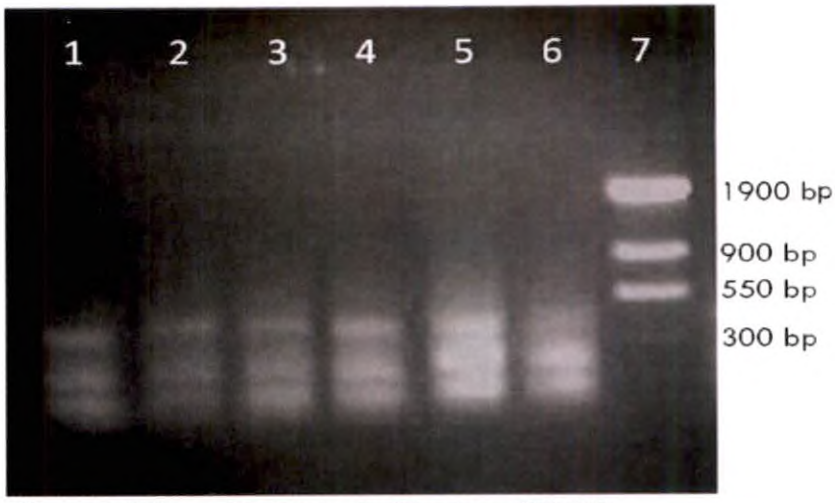


Plate 19. SSR profile obtained with primer RM 125

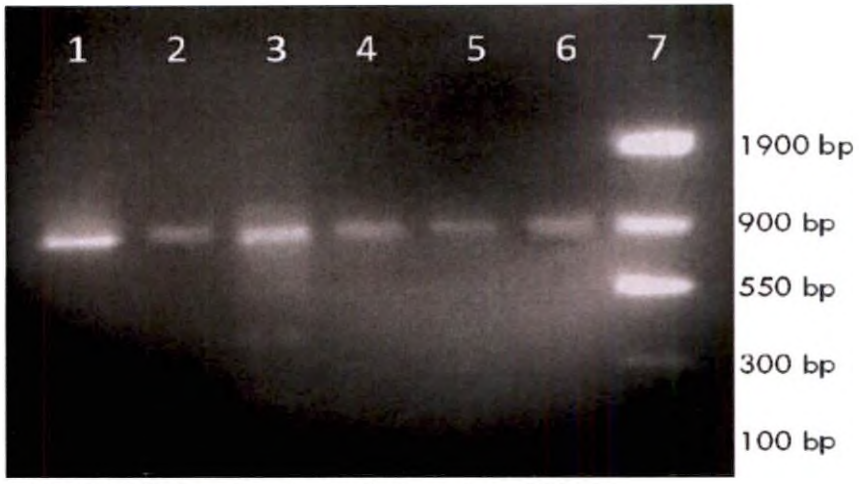


Plate 20. SSR profile obtained with primer RM 214

Lane No.		Sample
1	:	Branched spike (Thekken plant A)
2	:	Branched spike (Thekken plant B)
3	:	Non-branched spike (Thekken plant A)
4	:	Non-branched spike (Thekken plant B)
5	:	Spike (Karimunda plant A)
6	:	Spike (Karimunda plant B)
7	:	DNA marker

4.3 PROTEIN ANALYSIS

4.3.1 Estimation of protein content

The absorbance reading of the extracted protein samples at wavelength 595 nm using spectrophotometric method ranged from 0.198 to 0.312 (Table 7).

Standard curve obtained after plotting the concentration of protein standards against the corresponding absorbance provided the concentration of samples which ranged from 1.2 to 2.0 $\mu g \mu l^{-1}$.

Table 7. Spectrophotometric absorbance reading of protein samples

Sl. No.	Sample	Absorbance (A_{595} nm)	Conc. of Protein ($\mu g \mu l^{-1}$)
1	Thekken A (branched spikes)	0.228	1.2
2	Thekken B (branched spikes)	0.241	1.4
3	Thekken C (branched spikes)	0.210	1.2
4	Thekken A (non-branched spikes)	0.312	2.0
5	Thekken B (non-branched spikes)	0.219	1.2
6	Thekken C (non-branched spikes)	0.198	1.2

4.3.2 SDS-PAGE

The protein profiling of branched spikes and non-branched spikes of Thekken plants by SDS-PAGE obtained a total of seven prominent bands of which most of the bands were located between the molecular weight of 20 to 30kDa (Plate 21). The protein size was estimated by comparison with a low range protein molecular marker (PMWM). However, the protein banding profile on the gel showed that all the bands were monomorphic.

4.4 MICROSCOPIC ANALYSIS

The microscopic images of the spikes of type Thekken showed the presence of distinct, large and prominent bracts on the axil of the main inflorescence. In contrast, the spikes of Karimunda variety appeared to have comparatively small bracts (Plate 22).

The dissected spikes of type Thekken clearly displayed the initial development of the secondary inflorescence from the main inflorescence stem instead of floral organ formation as in the case of spikes of Karimunda variety (Plate 23). Nevertheless, some of the meristems on main inflorescence of the branched spike were also seen to be transformed into floral buds in Thekken (Plate 24).

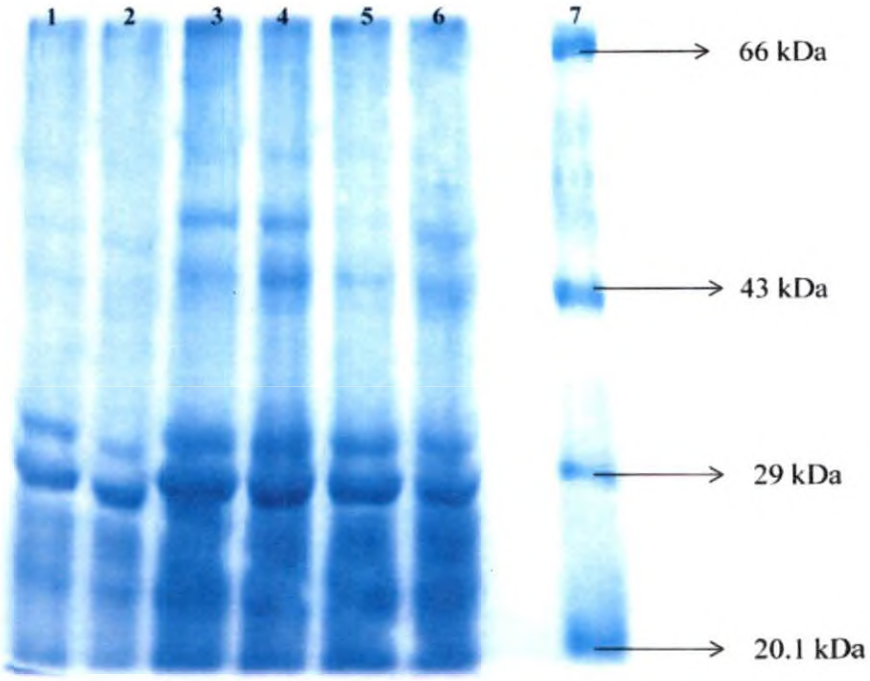


Plate 21. SDS-PAGE of black pepper total protein

Lane No.	Sample
1	Branched spikes (Thekken - A)
2	Branched spikes (Thekken - B)
3	Branched spikes (Thekken - C)
4	Non-branched spikes (Thekken - A)
5	Non-branched spikes (Thekken - B)
6	Non-branched spikes (Thekken - C)
7	Protein marker

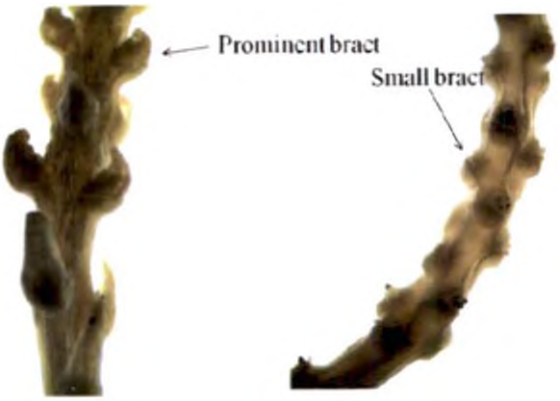


Plate 22. Microscopic image of spikes

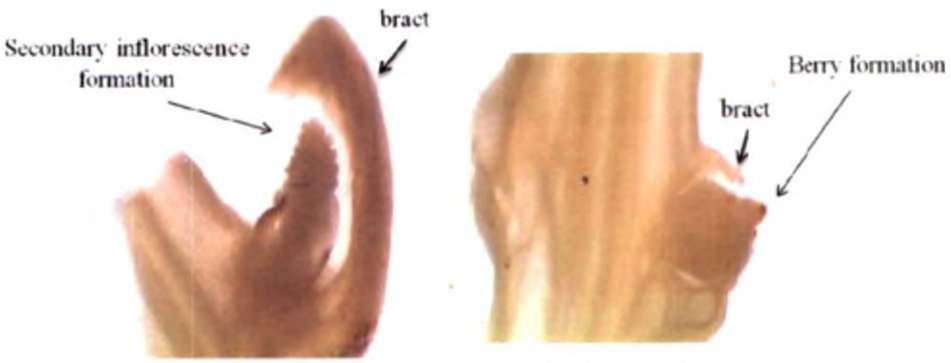


Plate 23. Microscopic image of sectioned spikes

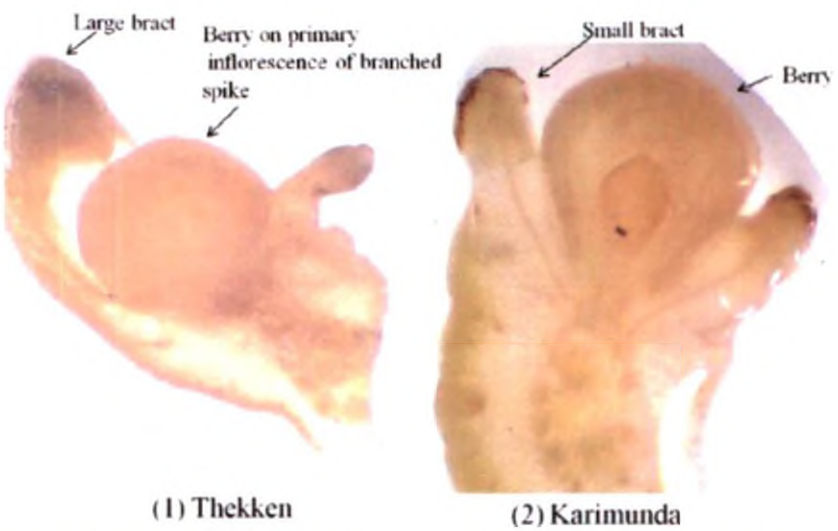
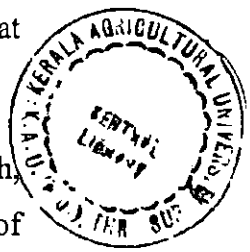


Plate 24. Microscopic image of sectioned spikes showing berry development

DISCUSSION

5. DISCUSSION

The Western Ghat region of India serves as the centre of origin of the black pepper (*Piper nigrum*), the most important commercial spice crop of the world. The region harbors the collection of useful genotypes of the crop, and Kerala state is privileged with the richest cultivar diversity. Surveys conducted by the scientists of Indian Institute of Spice Research, Kozhikode in some areas of Western Ghats in Idukki district, Kerala has led to the discovery of intermediate populations, apparently composed of hybrids between *P. nigrum, P. sugandhi, P. trichostachyon* and *P. galeatum* and their segregating progenies, supporting that the species are still in evolution in this region (Ravindran *et al.*, 2000).



A considerable variation in the morphological features such as leaf length, spike length, floral composition, etc. has been reported in the diverse cultivars of black pepper (Ravindran *et al.*, 2000). Nevertheless, the branching trait of the spike had remained unveiled until a farmer Shri T.T. Thomas identified and collected Thekken type from the forest of Idukki district, Kerala. The gain of knowledge on the genes involved in spike branching, an economic trait, would greatly benefit the improvement programmes in both black pepper and other crop species. Hence, the present study was undertaken as a preliminary approach in understanding the spike branching trait in black pepper type Thekken.

The infield observation of Thekken plants in this study identified the existence of few non-branched spikes borne arbitrarily in the plagiotropic branches of the plant. This behavior appeared as a chimera, which in botany is a plant or plant part that is a mixture of two or more genetically different types of cells. These genetically different cells exist in the shoot apical meristems which give rise to the cells that form the organs (Liu *et al.*, 2009). Though most chimeras have originated spontaneously due to mutation of somatic cells in the apical meristem (Burge *et al.*, 2002), chromosome deletion or the chromosome-bridge formation resulting from interspecific hybridization has been the main reason for the production of flower chimeras (Quiros and Douches, 1988). Flower

chimeras have been observed in interspecific hybrids, for e.g., Zhao and Zhang (1992) found yellow-salmon pink and red-yellow petal chimeras in the hybrids of *Chrysanthemum* species. Huang *et al.* (2002) and Yu *et al.* (2004) found some hybrid plants with yellow-white petals in *Brassica* species.

In this study, RAPD and SSR marker analyses were carried out to determine the genetic similarity between branched and non-branched spikes of Thekken type, and subsequently to find out the molecular marker linked with spike branching trait. A sample from Karimunda variety was used as a control for non-branching plants in the present work. The spikes of Thekken as well as Karimunda were also analyzed for the genes reported to regulate inflorescence branching in many other plant species using degenerate primers designed based on sequences of these genes. SDS-PAGE was conducted in branched and non-branched spikes of Thekken to determine the difference at protein level.

5.1 MOLECULAR ANALYSES

5.1.1 Identification of inflorescence gene

Recently, many genes have been identified to have a role in an inflorescence development in different plants, though no such study in black pepper is reported till date. Most of these genes are found to be conserved among diverse plant species (Benlloch *et al.*, 2007). Based on the nucleotide sequences of four different genes, *viz. BP, RA2, PIN1* and *LOG1* involved in regulating inflorescence branching in many other plant species such as *Arabidopsis*, the degenerate primers were designed. The degenerate primers are preferred over the specific primers since the former can amplify the weakly conserved sequences of the homologous genes in different organisms (Shen *et al.*, 1998). These genes were analyzed by PCR and RT-PCR techniques in genomic DNA and mRNA samples respectively.

Obtaining a good quality DNA is a prerequisite for a reliable PCR reaction. In black pepper, the isolation of DNA was affected by high level of polysaccharides and polyphenols. CTAB method of DNA isolation (Doyle and

Doyle, 1990) has been mostly accepted in plant species and was used in this study. Nonetheless, the method needed few modifications in order to procure a good quality and quantity of DNA from black pepper samples. CTAB is reported to reduce the visible amount of polysaccharides in DNA samples. An effective removal of polysaccharides was obtained by increasing the concentration of CTAB along with NaCl in the extraction buffer (Syamkumar *et al.*, 2005; Abdellaoui *et al.*, 2011; Sahu *et al.*, 2012). Relevant result came by in the present study when the CTAB concentration was increased to 2.5 percent along with increase in the concentration of NaCl and EDTA to 1.5 *M* and 25 m*M* respectively. An increase in the volume of extraction buffer per sample tissue to 1 ml per 100 mg of tissue also resulted to be effective as reported by Dhanya *et al.* (2007) and Abdellaoui *et al.* (2011).

Since black pepper is rich in secondary metabolites, a very careful and quick processing of the sample mainly at the time of grinding was required. The inclusion of PVP and β-mercaptoethanol in extraction buffer which are known to prevent the oxidation of secondary metabolites in the disrupted plant material (Prittila *et al.*, 2001; Warude *et al.*, 2003) avoided the brown pigmentation in the sample, increasing the yield and quality of DNA.

Phenol: chloroform: isoamyl alcohol (25:24:1) has been reported to give high purity DNA in many plant species (Aras *et al.*, 2003; Sablok *et al.*, 2009; Lele, 2011). Despite that, the use of phenol hindered the isolation process in this study. A better result was obtained when 5 *M* NaCl was added before precipitation step which is identified to facilitate the removal of polysaccharides by increasing their solubility in ethanol (Fang *et al.*, 1992; Arif *et al.*, 2010). Ansari and Khan (2012) reported ethanol precipitation to be better than isopropanol. However, this study revealed isopropanol precipitation to produce superior result. Alike the report of Buragohain and Konwar (2008), DNA degradation and contaminations were avoided by carrying out all the steps at 25 °C (room temperature), except the isopropanol precipitation step which gave good result at 4 °C in the present work.

The absorbance ratio (A_{260}/A_{280}) in the range of 1.8 to 2.0 indicates a high level of purity of DNA (Weising *et al.*, 2005). The isolated genomic DNA from black pepper spikes confirmed high purity with (A_{260}/A_{280}) value ranging from 1.7-2.2.

The PCR amplification of the genomic DNA was tried using the set of designed degenerate primers. The primers (BP-F, R) produced an amplicon of ~600 bp only in Karimunda sample. Absence of (BP-F, R) amplicon may be related to the spike branching trait in Thekken, since *bp* mutant in *Arabidopsis* showed the branching phenotype of inflorescence (Smith and Hake, 2003). Primers (RA2-F, R), (PIN1-F, R) and (LOG1-F, R) amplified the bands of ~500, ~1800 and ~500 bp respectively in both branched and non-branched spike samples of type Thekken as well as in Karimunda sample. In addition, the bands of ~200, ~900 and ~300 bp were also shown by the primers (RA2-F, R), (PIN1-F, R) and (LOG1-F, R) respectively in Karimunda sample.

Amplification might have resulted due to the conserved sequences of the respective genes in black pepper. The amplified bands need to be analyzed in detail for the verification of the presence of the full length gene. Also the additional bands produced by primers (RA2-F, R), (PIN1-F, R) and (LOG1-F, R) and the band formed by (BP-F, R) in Karimunda needs to be analyzed further to relate the role of these genes with the branching trait of spikes in Thekken.

These genes regulating inflorescence (*BP*, *RA2*, *PIN1* and *LOG1*) have been reported to express during the initial stages of inflorescence development (Vernoux *et* al., 2000; Bortiri *et* al., 2006; Dumonceaux *et al.*, 2009; Kuroha *et al.*, 2009). Therefore, the isolation of RNA was carried out from the immature spikes of Thekken and Karimunda. The branching and non-branching spikes of the pepper type Thekken were unable to distinguish in their immature stage; hence these characters were ignored during RNA expression assay.

The success of RNA extraction is governed by the quality, quantity and integrity of the RNA recovered. The trizol reagent was used for the isolation of

RNA from spikes of black pepper in this study. The maintenance of strictly aseptic condition and the use of sterile materials to inhibit the RNA degradation by RNase are the most important factors determining the quality of the isolated RNA (MacRae, 2007). The bands in agarose gel showed clear and discrete ribosomal RNAs confirming good quality. The spectrophotometric absorbance ratio (A_{260}/A_{280}) ranging from 1.7 to 2.0 is regarded to have little or no protein contamination (Accerbi *et al.*, 2010). The ratio of 1.78-1.96 obtained in this study unveiled that the extracted RNA was of good quality.

The extracted RNA was used for RT-PCR with the designed degenerate primers at varied annealing temperatures. However, no amplification was detected with any of the primers. The possible reason may be the degradation of cellular functional mRNA facing stress while transporting the samples overnight from Idukki to laboratory at Thiruvananthapuram.

5.1.2 Molecular marker analyses

The molecular markers revealing polymorphism at DNA level has been mostly used for the analysis of genetic studies since the last few decades. The simplicity, rapidity, low amount of genomic DNA requirement and non-necessity of prior genetic information make the RAPD technique advantageous over the other markers (Kumar and Gurusubhramanian, 2011). Bhasi (2008) obtained the intraclonal variability in Panniyur-1 var. of black pepper by RAPD. The RAPD marker has also been used to detect somaclonal variation among micro-propagated plants of *Piper longum* L. and black pepper cv. Subhakara and Aimpiriyan (Parani *et al.*, 1997; Babu *et al.*, 2003), *Picea glauca* (Isabel *et al.*, 1996), peach (Hashmi *et al.*, 1997), sugarcane (Taylor *et al.*, 1995), and coffee (Rani *et al.*, 2000). Liu *et al.* (2009) reported the identification of chimera in the hybrid of *Brassica carinata* and *B. rapa* producing two different coloured petals using RAPD. Contrary to these, RAPD analysis carried out in branched and non-branched spikes of the black pepper type Thekken, hypothesized to be due to chimera in the present study did not produce any difference in the RAPD banding

pattern. This indicates lack of genetic dissimilarity between the branched and non-branched spike of type Thekken. Thus, RAPD analysis using the 12 primers that were screened in Thekken could not discriminate chimerism. However, out of 12 RAPD primers tested, 10 primers produced polymorphic bands in Thekken type and Karimunda variety. The marker produced 22.8 percent of total scorable bands polymorphic between Thekken and Karimunda, revealing the potential of RAPD markers in varietal assessment of black pepper. The suitability of RAPD markers has also been reported previously in genetic diversity assessment of black pepper varieties producing varietal specific bands and high degree of polymorphism (Pradeepkumar *et al.*, 2001; Sreedevi *et al.*, 2005).

The SSR markers which possess the characteristics such as high levels of polymorphism, codominance and Mendelian segregation (Menenzes *et al.*, 2009) were also applied in this study in a similar way to RAPD. These markers have been reported to be effective in analyzing the genetic diversity in many crop lines (Cho *et* al., 2000; Eujayl *et al.*, 2001; Qu *et al.*, 2012). SSR markers are reported to be used between different species or genera (Stafne *et al.*, 2005). Hence, the SSR primers that were already available in the laboratory were used in the present study to reduce the expenses. Lin *et al.* (2011) achieved 53.3 percent of cross-species transferability of SSR markers in six related species of Poaceae. Cross-transferability of as high as 75 percent has been reported in cross-species amplification of *Shorea* microsatellite DNA markers in *Parashorea malaanonan* (Abasolo *et al.*, 2009). Peakall *et al.* (1998) obtained 65 percent marker transferability on using microsatellite markers among different *Glycine* species, while the efficiency dropped to 13 percent when analyses were conducted among species within different genera. In case of distantly related crops, Yasodha *et al.* (2005) obtained 30 percent success in cross amplification of *Eucalyptus* SSRs to Casuarinaceae, but no amplification products were observed in *Allocasuarina*. Stafne *et al.* (2005) reported the low percentage success of strawberry-derived SSR markers for the raspberries and blackberries, underscoring that the cross-generic use of SSR markers may be limited. Similarly, a very low cross-

transferability was observed using SSR markers belonging to rice in the analysis of black pepper in this research. Only two primers, *viz.* RM 125 and RM 214 could produce PCR amplification out of 13 primers screened. This clearly showed the incompatibility of the primers in cross-amplification in distant plants.

The two SSR primers used in this study generated only monomorphic bands in both branched and non-branched spike of Thekken. The same monomorphic bands were also seen in Karimunda variety. Similarly, Yasodha et *al.* (2005) obtained only monomorphic bands when SSR primers were used for cross amplification within the species of distant plants. Therefore, the SSR marker in this study was not successful in determining the genetic difference if any that exist between branched and non-branched spikes of Thekken as well as between Thekken and Karimunda.

The cross between Thekken and any other spike non-branching plant, and subsequent screening of the segregating progenies with molecular markers, though time consuming, may be useful in identifying the markers linked with spike branching trait.

5.2 PROTEIN ANALYSIS

Among biochemical techniques, SDS-PAGE is widely preferred due to its validity and simplicity for analysis of genetic structure of germplasm (Javid *et al.*, 2004; Iqbal *et al.*, 2005). The method has been a successful tool in the identification of genetic diversity mostly by sorting out the seed storage proteins which are highly independent of the environmental factors (Iqbal *et al.*, 2005; Nasir *et al.*, 2007; Ehsanpour *et al.*, 2010; Galani *et al.*, 2011).

The protein analysis between the branched and non-branched spikes of Thekken was performed in this study. The concentration of extracted proteins by Laemelli's method (1970) ranged from 1.2 to 2.0 $\mu g \mu l^{-1}$.

Total seven scorable protein bands were visible on the gel, but no difference in the banding profile between branched and non-branched spikes was observed.

This result showed that there is no difference in the protein expression level in branched and non-branched spikes of Thekken. The inability of SDS-PAGE in generating the less or no polymorphism has also been reported in many other studies (De Vries, 1996; Sultana and Ghafoor, 2009; Sarkar *et al.*, 2012). Nasir *et al.* (2006) reported that SDS-PAGE was unable to detect the linkage between protein banding and disease status.

More sensitive methods may be required to find out the differences at protein level between branched and non-branched spikes of Thekken.

5.3 MICROSCOPIC ANALYSIS

The analysis of inflorescence structure and development in black pepper has not been reported to date. Tucker (1982) did the study on inflorescence and flower development in three *Piper* spp., namely *P. aduncum, P. amalgo* and *P. marginatum* which are believed to be conserved in other species as well (Ravindran, 2000). Sokoloff *et al.* (2006) carried out the study of terminal flower-like structures (TFLS) in the inflorescence of *Piper arboretum* using light microscope and scanning electron microscope.

Sokoloff *et al.* (2006) reported the fixing of samples in either formalin-aceto-alcohol (FAA) or 70 percent ethanol for light microscopic study. However, Carnoy's fluid which has greater penetrating power than FAA is remarked as better fixative for plants with high phenolics (Sreekantan, 2002). In this study, the spikes stored at -80 °C when directly used or fixed in Carnoy's fluid stored at room temperature soon turned brown and were unfit for the study. On the other hand, fixing of the samples overnight in Carnoy's fluid pre-stored at -20 °C inhibited the browning of the sample as reported by Sreekantan (2002).

In Thekken spikes, the bracts were reported to be distinctly prominent in comparison to the spikes of other black pepper varieties (Sasikumar *et al.*, 2007; Vimarsha, 2009). The presence of prominent bracts in the spikes of Thekken was also observed in this study.

The development of the secondary inflorescence on the main spike of Thekken was clearly visible in the sectioned samples under microscope. However, the formation of secondary inflorescence was not uniform in the branched spike, as few berries were found to form directly from the axils of main inflorescence. Sasikumar *et al.* (2007) and Vimarsha (2009) also reported that some of the flower buds remained untransformed into secondary inflorescence branches and developed into flowers in the branched spikes of Thekken.

As a conclusion, the differences observed in the PCR amplicon profiles of Thekken and Karimunda by two designed primers, *viz.* (BP-F, R) and (RA2-F, R) suggest the possible role of transcription factors in the spike branching trait in black pepper in the present study. Likewise, the difference in PCR amplicons exhibited by primers (PIN1-F, R) and (LOG1-F, R) in Thekken and Karimunda proposes the possible difference in the level of auxin and cytokinin respectively which may affect the branching of spike. The study on branched and non-branched spikes of Thekken by molecular markers and designed degenerate primers revealed that the two forms of spikes of Thekken are similar at genetic level and/or does not favor the existence of chimera.

SUMMARY

6. SUMMARY

The study entitled "Identification of molecular marker associated with spike branching trait in black pepper (*Piper nigrum* L.)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2011-2013. The objective of the study was to screen the candidate genes regulating inflorescence branching, and to identify the molecular marker associated with spike branching trait in black pepper type 'Thekken'. Branched and non-branched spikes of black pepper type 'Thekken' along with the spikes of Karimunda variety as a control were collected from the farmer's plot in Idukki, and was frozen in liquid nitrogen and stored at -80°C for the analysis.

PCR and RT-PCR based approach was used to screen the inflorescence architectural genes at genomic and transcriptomic levels. Four pairs of degenerate primers (BP-F, R), (RA2-F, R), (PIN1-F, R) and (LOG1-F, R) were designed based on the nucleotide sequences of genes, namely *BP*, *RA2*, *PIN1* and *LOG1* respectively, which are reported to determine the inflorescence branching in several other plant species.

The presence of higher amount of secondary metabolites was the major hindrance in the isolation of genomic DNA. Extraction of genomic DNA by increasing the concentration of CTAB, EDTA, NaCl and PVP in extraction buffer, and addition of NaCl before precipitation with alcohol in CTAB method (Doyle and Doyle, 1990) was found to yield better quality and quantity of DNA.

PCR with primers (BP-F, R) gave an amplicon of ~600 bp only in Karimunda variety, while there was no amplification in both branched and non-branched spikes of Thekken. Primers (RA2-F, R), (PIN1-F, R) and (LOG1-F, R) produced the amplicons of approx. 500 bp, 1800 bp and 500 bp respectively in both branched and non-branched spikes of Thekken type as well as in Kamunda variety. Additional amplicons of approx. 200 bp, 900 bp and 300 bp were yielded

by the primers (RA2-F, R), (PIN1-F, R) and (LOG1-F, R) respectively in Karimunda variety.

Total RNA was extracted using Trizol reagent from the immature spikes of Thekken and Karimunda since the candidate genes screened are reported to express in the initial developmental stage of inflorescence. Although varied annealing temperatures were programmed, RT-PCR did not yield any amplicon by all four pairs of primers in both Thekken and Karimunda samples.

RAPD and SSR marker analyses were carried out in branched and non-branched spikes of Thekken and spikes of Karimunda to determine the genetic similarity in branched and non-branched spikes of Thekken type and find the marker associated with spike branching trait. Twelve RAPD primers were used, which produced a total of seventy scorable bands. Polymorphism (22.8%) was obtained by RAPD between Thekken type and Karimunda variety, and ten of twelve primers showed polymorphism. However, all 12 primers yielded monomorphic bands between branched and non-branched spikes of Thekken.

SSR marker analysis was conducted using two SSR primers. Neither of the primers yielded polymorphic band between both branched and non-branched spikes of Thekken as well as between Thekken type and Karimunda variety of black pepper.

SDS-PAGE of the protein samples from branched and non-branched spikes of Thekken was conducted for protein analysis. Laemelli's method (1970) was used for the isolation of total protein. Seven prominent bands were seen on the gel and all the bands were monomorphic between branched and non-branched spikes of Thekken.

Observation of the samples under light stereo microscope showed the presence of distinct prominent large bracts on spikes of Thekken in comparison to Karimunda spikes. The development of secondary inflorescence on the spikes of Thekken was clearly visible. However, the formation of secondary inflorescence on branched spikes of Thekken was non-uniform as few of the meristems were

observed to transform into floral meristems consequently producing berries on the primary inflorescence instead.

The difference in amplicons produced by the primers (BP-F, R), (RA2-F, R), (PIN1-F, R) and (LOG1-F, R) between Thekken and Karimunda suggests the involvement of transcription factors and phytohormones (auxin and cytokinmin) in governing the branching of spikes. These can be characterized by sequencing and can be used for designing more specific primers for further analysis. The study also suggests that there is no genetic difference between branched and non-branched spikes of Thekken, narrowing down the chances of chimera in a plant. Differences in the hormonal level can be analyzed. More sensitive molecular studies at transcriptomic and proteomic level may be helpful in revealing the molecular pathways leading to spike branching trait in black pepper type Thekken.

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7. REFERENCES

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http://simgene.com/OligoCalc

APPENDICES

APPENDIX I

CTAB Extraction Buffer

C-TAB	2.5 %	
Tris- HCl (pH 8.0)	100 m*M*	
EDTA	25 m*M*	
NaCl	1.5 *M*	
β-mercaptoethanol	0.2 % (v/v)	freshly added prior to DNA
PVP	4 % (w/v)	extraction

APPENDIX II

TE buffer

Tris- HCl (pH 8.0)	10 m*M*
EDTA	1 m*M*

APPENDIX III

TBE Buffer (5x) for 1 liter solution

Tris base	54 g	(0.445*M*)
Boric acid	27.5 g	(0.445*M*)
0.5 *M* EDTA (pH 8.0)	20 ml	(0.01*M*)

APPENDIX IV

Protein denaturing solution

10 *M* Urea	80 ml
1 *M* $NaH_2PO_4.2H_2O$ (pH 8)	5 ml
1 *M* Tris (pH 8)	1 ml
5 *M* Sodium chloride	2 ml

Make up the volume to 100 ml by adding 12 ml of distilled water.

APPENDIX V

Phosphate buffered saline (PBS)

NaCl	137 m*M*
KCl	2.7 m*M*
Na_2HPO_4	10 m*M*
KH_2PO_4	2 m*M*

Adjust pH to 7.4 with HCl

APPENDIX VI

Dye Concentrate

Dissolve 100 mg of coomassie brilliant blue G250 in 50 ml of 95 % ethanol.

Add 100 ml of concentrated orthophosphoric acid.

Add distilled water to a final volume of 200 ml. Store refrigerated in amber bottles.

Mix 1 volume of concentrated dye solution with 4 volumes of distilled water for use. Filter with Whatman No. 1 paper.

APPENDIX VII

1 X SDS gel loading buffer

Tris-Cl (pH 6.8)	50 m*M*
DTT	100 m*M* (added freshly)
SDS	2 % (w/v)
Bromophenol Blue	0.1 % (w/v)
Glycerol	10 % (v/v)

Add DTT from a 1*M* stock just before the buffer is used.

APPENDIX VIII

10% resolving gel (35ml)

Water	13.9 ml
30% acrylamide mix	11.6 ml
1.5*M* Tris (pH 8.8)	8.8 ml
10% SDS	0.35 ml
10% APS	0.35 ml
TEMED	0.014 ml

APPENDIX IX

5% stacking gel (10 ml)

Water	6.8 ml
30% acrylamide mix	1.7 ml
1.0*M* Tris (pH 6.8)	1.25 ml
10% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.01 ml

APPENDIX X

1 X Tris Glycine Electrophoresis Buffer

Tris	25 m*M*
Glycine (pH 8.3)	250 m*M*
SDS	0.1 % (w/v)

Prepare a 5 X stock of electrophoresis buffer by dissolving 15.1 g of Tris base and 94 g of glycine in 900 ml of deionized water. Then add 500 ml of a 10% (w/v) stock solution of electrophoresis grade SDS and adjust the volume to 1000 ml with water.

APPENDIX XI

Staining solution (Coomassie Brilliant Blue)

Add 0.25 g Coomassie Brilliant Blue R-250 per 100 ml of methanol: acetic acid solution.

Filter through Whatman No. 1 filter paper.

Methanol: Acetic Acid solution (100 ml)

Methanol	50 ml
Water	40 ml
Glacial Acetic Acid	10 ml

APPENDIX XII

Destaining solution

Methanol : Acetic Acid solution without the dye.

Methanol	50 ml
Water	40 ml
Glacial Acetic Acid	10 ml

APPENDIX XIII

Carnoy's fluid

Ethyl alcohol (100 %)	60 ml
Glacial acetic acid	10 ml
Chloroform	30 ml

ABSTRACT

IDENTIFICATION OF MOLECULAR MARKER ASSOCIATED WITH SPIKE BRANCHING TRAIT IN BLACK PEPPER (*Piper nigrum* L.)

ASHISH SUBBA

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Abstract of the

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ABSTRACT

The study entitled "Identification of molecular marker associated with spike branching trait in black pepper (*Piper nigrum* L.)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2011-2013. The objective of the study was to screen the candidate genes regulating inflorescence branching and to identify the molecular marker associated with spike branching trait in black pepper type 'Thekken' which shows a profuse branching in majority of the spikes (60-80 branches per spike) leading to higher berry yield. Genetic knowledge on this economically relevant trait could be useful in crop improvement programmes.

Spikes of Thekken and Karimunda variety (as control) were collected from the farmer's plot in Idukki and investigated at the molecular, protein and microscopic level.

In molecular analysis, the presence of candidate genes known to regulate inflorescence branching, *viz. BP*, *RA2*, *PIN1* and *LOG1* were screened at the genome and transcriptome level by using degenerate primers. DNA was isolated using CTAB method with modifications such as increasing the concentrations of CTAB, NaCl and EDTA and including PVP and β-mercaptoethanol in the extraction buffer.

There was difference in the amplicons between Thekken type and Karimunda variety at the genomic level. Additional amplicons were seen in Karimunda variety for *RA2* (200 bp), *PIN1* (900 bp) and *LOG1* (300 bp) gene primers. Amplicons of approx. 500 bp, 1800 bp and 500 bp were seen in both varieties with primers for *RA2*, *PIN1* and *LOG1* respectively. *BP* primers gave amplification (600 bp) only in Karimunda.

Good quality RNA was isolated using Trizol reagent. However, RT-PCR did not give any amplification in both Thekken and Karimunda samples.

Molecular profiles using twelve RAPD primers and two SSR primers showed no polymorphism between branched and non-branched spikes of Thekken. However, 22.8% polymorphism was exhibited between Thekken and Karimunda by RAPD.

In protein analysis using SDS-PAGE in branched and non-branched spikes of Thekken, seven prominent bands were observed, and all were monomorphic. At the microscopic level, prominent bract was a notable feature in the spikes of Thekken.

In the present study, a protocol for isolation of genomic DNA from the mature spikes of black pepper could be standardized. The study reveals that there is no difference between branched and non branched spikes of Thekken type, indicating lesser chances of Thekken being a chimera. Difference in the amplified products of *PIN1* and *LOG1* in Thekken and Karimunda suggest a difference in phytohormone levels (auxin and cytokinin respectively). Similarly, absence of amplification of *BP* in Thekken type and difference in the amplified band of *RA2* in Thekken and Karimunda suggest a possible role of transcription factors in spike branching in Thekken.