IN VITRO SYNTHESIS OF GINGEROL AND ANALYSIS OF EXPRESSED SEQUENCE TAGS FOR GINGEROL PRODUCTION IN GINGER

(*Zingiber officinale* Rosc.)

by

Manjusha Rani

(2015-21-027)

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KERALA, INDIA

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THESIS

Submitted in partial fulfillment of the requirements for the degree of

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2020

DECLARATION

I, hereby declare that this thesis entitled **"*In vitro* synthesis of gingerol and analysis of Expressed Sequence Tags for gingerol production in ginger (*Zingiber officinale* Rosc.)**" is a bonafide record of research work done by me during the course of research and that this thesis has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title of any other University or Society.

Vellanikkara
Date: 29/05/2020

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CERTIFICATE

Certified that this thesis entitled **"*In vitro* synthesis of gingerol and analysis of Expressed Sequence Tags for gingerol production in ginger (*Zingiber officinale* Rosc)**" is a record of research work done independently by Mrs. Manjusha Rani under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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ABBREVIATIONS

%	Percentage
@	At the rate
>	Greater than
ºC	Degree Celsius
ΔCt	Threshold cycle
µg	Microgram
µl	Microlitre
µM	Micromole
mgL^{-1}	Milligram per litre
ACAA1	Acetyl CoA Acyl Transferase 1
BioEdit	Biological sequence alignment Editor
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CA	Coumaric acid
cDNA	complementary DNA
CHS	Chalcone Synthases
CI	Callus inducing medium
CPBMB	Centre for Plant Biotechnology and Molecular Biology
DEPC	Diethyl pyrocarbonate
DIC	Distributed Information Centre
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphates
DW	Dry weight
EDTA	Ethylene Diamine Tetra Acetic acid
ESTs	Expressed Sequence Tags

EtBr	Ethidium Bromide
FA	Ferrulic acid
FW	Fresh weight
g	Gram
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IAA	Indole-3- acetic acid
KAAS	KEGG Automatic Annotation Server
KAU	Kerala Agricultural University
kb	Kilobase
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	Litre
LB	Luria Bertani
M	Molar
MAI	Month after induction
MCI	Modified callus inducing medium
mg	Milligram
min	Minutes
MJ	Methyl Jasmonate
ml	Millilitre
mm	Millimetre
mM	Millimole
MOPS	3-(N-Morpholino)-propanesulfonic acid
MS	Murashige and Skoog medium
mRNA	messenger RNA
NCBI	National Centre for Biotechnology Information
ng	Nanogram

nm	Nanometre
NS	Not significant
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PAL	Phenylalanine ammonia lyase
pH	Hydrogen ion Concentration
PKS	Polyketide Synthases
ppm	parts per million
PVP	Poly Vinyl Pyrrolidone
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	ribosomal RNA
RT	Room Temperature
s	Seconds
SA	Salicylic Acid
SNP	Single Nucleotide polymorphism
SSH	Suppression Subtractive Hybridization
t/ha	tonnes/hectare
TAE	Tris Acetate EDTA
Taq	*Thermus aquaticus*
TDZ	Thidiazuron
TE	Tris EDTA
TLC	Thin layer chromatography
tRNA	transfer RNA
UV	Ultra Violet
Viz.	Namely

Introduction

1. Introduction

Ginger (*Zingibe*r *officinale* Rosc.) belonging to family Zingeberaceae is one of the most commonly consumed spices. Ginger is valued as carmative and for its medicinal use such as proper digestion, treatment of stomach upset, cough, diarrhoea *etc*. In the nutraceutical and health food industries, ginger is important due to its health beneficial effects like its anti-hypercholesterolemic, anti-oxidant, anti-inflammatory and anti-diabetic properties (Shylaja and Peter, 2007). The spice contributes to world market as fresh/ dried rhizomes, oils and oleoresins, curry powders and encapsulated spices. As per the 2017-18 statistics of Spices Board of India, ginger is cultivated in an area of 1,68,989 ha with a production of 17,94,560 t/ha. In India, the state of Madhya Pradesh is leading in ginger cultivation with an area of 23,431 ha and production of 3, 77,470 t/ha. In Kerala, ginger is cultivated in an area of 4,370 ha with a production of 86,270 t/ha (www.indianspices.com).

The pharmacological properties of ginger are mainly attributed to the bioactive principles present in the ginger oleoresin, particularly gingerols and shogaols. Through molecular docking and cell cultures studies conducted at Centre for Plant Biotechnology and Molecular Biology (CPBMB), 6-gingerol was reported as a promising anti-cancerous compound superior to other ligands of ginger such as 8, 10 and 12 gingerol and eleven approved drugs for cancer (Kumara *et al*., 2017). Even though gingerols have many pharmacological properties, elaborate studies on gingerol biosynthesis are lacking. Some initial investigations on gingerol biosynthesis were published by Denniff and Whiting (1976), Denniff *et al.* (1980) and Ahumada *et al.* (2006). Marfori and Jane (2018) reported production of 6-gingerol in the *in vitro* produced ginger plantlets grown in high sucrose medium.

Ginger genome is little exploited and reports on genes involved in gingerol biosynthesis are scanty. But many ginger ESTs related to ginger genome have been deposited in public domain. The bioactive gingerols are derived from intermediates in phenylpropanoid pathway. Phenylalanine ammonia lyase is the starting enzyme of the phenylpropanoid pathway. Chalcone synthase belonging to the family of polyketide synthases are specifically involved in the synthesis of secondary

metabolite gingerols and shogaols (Schroder, 1977 and Dennif *et al*., 1980). Ahumada *et al*. (2006) investigated the potential role of specific phenylpropanoid pathway enzymes for the production of curcuminoids and gingerols and opined that a genomic based approach would be efficient to identify the genes responsible for the direct formation of curcuminoids and gingerols. The importance of Chalcone synthase in the production of gingerols is explained by Gosh and Mandi (2015). The metabolite profiling and gene expression analysis in high and low gingerol producing somaclones of ginger was carried out by Sreeja (2017) at CPBMB and she observed high *Chalcone synthase* activity in high gingerol producing somaclone of ginger.

Ginger is propagated vegetatively through rhizome bits. Crop improvement through hybridization in ginger is not possible due to poor flowering and lack of seed set. Hence somaclonal variation induced in plant cell and tissue culture is an important source of variability in ginger. By exploiting somaclonal variation, three high yielding high quality varieties *viz.* Athira, Karthika and Aswathy were released from Kerala Agricultural University (Shylaja *et al*., 2010 and Shylaja *et al*., 2014).

Studies on *in vitro* induced microrhizomes in ginger for production of disease free planting materials were initiated in CPBMB during 2014. Conventional tissue culture plants are not preferred in ginger cultivation as they take more time for rhizome production. But, if *in vitro* induced microrhizomes are used as planting materials the rhizome can be harvested in the same season as that of conventional seed rhizome bits. Also, year round availability of planting material can be ensured through microrhizome technology. Production of *in vitro* microrhizomes and standardization of the production technology for growing ginger microrhizomes in high tech polyhouses were undertaken at CPBMB (Shylaja *et al*., 2016 and Shylaja *et al.*, 2018). Currently, commercial production of ginger microrhizomes using temporary immersion bioreactor is also in progress at CPBMB for production of disease free planting materials.

Microrhizomes induced *in vitro* could be used for studying the synthesis of secondary metabolites. Ginger cell culture was investigated by Sakamura and Suga (1989) for production of secondary metabolites. Ilahi and Jabeen (1992) also reported

preliminary studies on alkaloid biosynthesis in callus cultures of ginger. Babu (1997) reported successful establishment of cell suspension cultures of ginger and reported presence of oil bearing cells in calli and emphasized the need of further work for the commercial exploitation of secondary metabolites in ginger. The effects of light on production of gingerol and zingiberene in callus cultures of ginger were studied by Ahasori and Asghari (2009). They evaluated the production of metabolites by TLC and concluded that the production of metabolites were high in cells with some sort of differentiation.

Nutrient stress was found to influence the accumulation of secondary metabolites in several plants. Jacob and Malpathak (2005) reported that half strength of $MgSO_4$ and $CaCl_2$ in MS medium increased solasodine production in *Solanum khasianum*. Naik *et al.* (2011) studied the effects of NH_4NO_3, KNO_3, $CaCl_2$, $MgSO_4$ and KH_2PO_4 in the MS medium at 0.05x, 1.0x, 1.5x and 2.0x each for the shoot cultures of *Bacopa monneri* and reported the highest production of bacoside A (17.9 mg/l DW) at 2x strength of NH_4NO_3. They also studied the effect of ammoniacal and nitrate nitrogen ratios and reported that bacoside A was optimum when NO_3^- concentration was higher than that of NH_4^+. But the reduced levels of NH_4^+ and increased level of NO_3^- promoted the production of withanolide in *Withania somineferum* (Praveen *et al.*, 2013).

Biotic and abiotic elicitors are found to enhance the *in vitro* accumulation of secondary metabolites. Johnson *et al.* (1991) reported increase in capsaicin content by elicitation with microbial polysaccharides like curldan and xanthan. Elicitation with methyl jasmonate and salicylic acid in *Capsicum chinense* (Molina *et al.*, 2010), jasmonic acid, copper sulphate and salicylic acid in *Bacopa monnieri* (Sharma *et al*., 2014), methyl jasmonate, chitosan and yeast extract in *Cleome rosea* (Rocha *et al.*, 2015) and fungal cultures of *Fusarium oxysporum, Phoma exigua* and *Botrytis cineria* in *Hypericum perforatum* (Simic *et al.*, 2015) were found to enhance the production of secondary metabolites. Salicylic acid induced enhanced production of beta carotene in *Capsicum* calli (Pooja, 2018) and gingerol production in ginger (Archita, 2019). Presently field trials using microrhizomes with high gingerol content are in progress

at CPBMB to assess the gingerol content in rhizomes derived from microrhizomes with high gingerol content.

Precursor feeding using the substrates of the biosynthetic pathway is another approach which affects the metabolite production with the basis of knowledge of the biosynthetic pathways (Namdeo *et al*., 2007). Production of rosmarinic acid was stimulated by the addition of phenylalanine to *Salvia officinalis* cell suspension cultures (Ellis and Towers, 1970). Cousins *et al.* (2011) attempted various methods to upregulate secondary metabolism in turmeric microrhizomes such as precursor feeding of phenylalanine, addition of proline, methyl jasmonate and induction of stress.

Ultra violet (UV) and gamma (γ) radiation induce accumulation of secondary metabolites in plants. The ionizing radiation induces stress in plants and as an adaptive response, oxidative stress protectors and antioxidant enzymes are produced which increase the production of secondary metabolites (Horling *et al*., 2003; Kim *et al*., 2004; El-Beltagi, 2011). Irradiation of gamma rays of 20 Gy in *Plumbago indica* root cultures increased plumbagin production (Jaisi *et al*., 2013).

ESTs are useful for the discovery of novel genes, identifying genes of unknown function and for comparative genomic studies. Analysis of ESTs in apple for flavour biosynthesis was reported by Newcomb *et al.* (2006). After studying the number of protein families they reported that many genes were involved in biosynthesis of flavour in apple. The higher frequency of SNPs in rhizomes of ginger as compared to leaves and roots were reported by Chandrasekar *et al*. (2009). Ghosh and Mandi (2015) investigated SNPs in *Chalcone synthase* gene in ginger and concluded that Chalcone synthase is the rate limiting enzyme in 6-gingerol biosynthesis pathway. The computational functional annotation of the available ESTs in ginger deposited in NCBI and identification of genes responsible for gingerol biosynthesis was done by James *et al*. (2015). Sreeja (2017) observed high *Chalcone synthase* activity in high gingerol yielding somaclones and she also identified the role of *3-ketoacyl CoA thiolase* (ACAA1) gene in gingerol production when she did

suppression subtractive hybridization using high and low gingerol yielding somaclones.

Validation of identified ESTs was attempted by Gohain *et al.* (2010) when they studied Darjeeling tea flavour on molecular basis. The quantitative real time PCR analysis helped in the identification of transcripts related to aroma and flavour formation in tea.

The present study on '*In vitro* synthesis of gingerol and analysis of Expressed Sequence Tags for gingerol production in ginger (*Zingiber officinale* Rosc) was carried out at the CPBMB and Distributed Information Centre (DIC), College of Horticulture, Kerala Agricultural University, Thrissur. The objectives of the study were to analyse the synthesis of gingerol under *in vitro* conditions, to characterize the ESTs related to gingerol synthesis and to validate the identified ESTs for high gingerol production in ginger.

Review of literature

2. Review of Literature

Ginger (*Zingiber officinale* Rosc.) is an important spice consumed worldwide. The plant is bestowed with unique medicinal properties attributed by a series of components, the majority being gingerols which is the pungent principle present in the rhizomes of ginger. The gingerols are thermally labile and easily gets converted to shogaols through dehydration reactions which imparts characteristic pungent taste to the dried ginger. Both the gingerols and the shogaols are biologically active. Of the different gingerols, the most potent and pharmacologically bioactive gingerol is 6-gingerol which is now a target for drug development. Even though gingerols have many pharmacological properties, elaborate studies on gingerol biosynthesis are very few and ginger genome is little exploited. Considering the importance of gingerols, the objectives of the present study focused to analyse the synthesis of gingerol under *in vitro* conditions, to characterize the ESTs related to gingerol synthesis and validation of the identified ESTs for high gingerol production in ginger.

The various aspects reviewed in this chapter includes, ginger genotypes used for the study, medicinal properties and health benefits of ginger, chemical composition of ginger, biosynthesis of gingerols in ginger, production of microrhizomes for secondary metabolite production, induction of calli for secondary metabolite production, scaling up of secondary metabolite production in microrhizome and calli, chromatographic separation of gingerols, gene expression studies, EST analysis and validation of differentially expressed genes obtained through suppression subtractive hybridization.

2.1 Ginger genome

Ginger is diploid, with somatic chromosome number: 2n = 22. Ginger genome is large with a genome size of 23,618 Mb (Wahyuni *et al*., 2003). The most recent estimate in Kew-C-Value database shows the genome size of ginger as 3164 Mb (Leitch, 2019). The total chromosome length ranged from 64.80 μm to 98.12 μm, with a total chromosome volume of 84.35 μm^3 to 1126.36 μm^3 (Ravindran and Babu, 2004). The length of the longest chromosome varied between 2.8μM (in cv. Jorhat)

and 4.8µM (in cv. China). The length of shortest chromosome ranged between 1.2µM (in cv. Rio-de-Janeiro) to 2.2µM (in cv. China).

The complete chloroplast genome of ginger was introduced by Cui *et al*. (2019). The chloroplast genome size was reported to be 162,621 bp. The protein coding region includes 37.1 per cent with 79 protein coding genes. Apart from the 79 protein coding genes, out of 113 unique gene identified in chloroplast genome, 30 tRNA genes and four r RNA genes were reported.

A total number of 38,262 ESTs related to *Zingiber officinale* is available in the public domain as on 20th May 2020.

2.2 Ginger genotypes used in the study

2.2.1 Athira

Athira is a high yielding variety of ginger which is the single plant selection from somaclones of the cultivar Maran suited for both fresh and dry ginger. An average yield of 22.6 t ha^{-1} with a dry recovery percentage of 22.6 per cent is recorded by the variety. The variety has bold rhizomes with a low crude fibre content of 3.4 per cent. The variety recorded a volatile oil content of 3.1 per cent and an oleoresin recovery of 6.8 per cent. The gingerols constitutes 16.5 per cent of oleoresin (Shylaja *et al.,* 2010).

2.2.2 Karthika

Karthika is also a single plant selection from somaclones of cultivar Maran which is highly suitable for extraction of oleoresin. It is a high yielding variety with an average yield of 19 t ha^{-1} which is used both for green and dry ginger purpose. The dry recovery percentage of Karthika is 21.6 per cent. High oleoresin recovery of 7.2 per cent and the volatile oil of 3.2 per cent attributes to its quality. The 21.3 per cent of oleoresin constitutes gingerol. The main components of volatile oil include 22.87 per cent of zingiberene and 4.03 per cent citral. The variety is more resistant to soft rot and bacterial wilt disease (Shylaja *et al.,* 2010).

2.2.3 Aswathy

Aswathy is single plant selection from somaclones of the parent cultivar Rio-de-Janeiro which is mainly used for green ginger purpose. An average yield of 23 t ha^{-1} is produced, with a dry recovery of 19.7 per cent. Volatile oil of 3.32 per cent and oleoresin of 7.45 per cent are recovered from the variety. Oleoresin combined 16.7 per cent gingerols and 2.10 per cent shogaols (Shylaja *et al.*, 2014).

2.2.4 Maran

Maran is the cultivar from Assam which was introduced to Kerala. The cultivar is suitable both as green and dry ginger. An average yield of 20 t ha^{-1} is produced by the cultivar (Paulose, 1973) with a dry ginger yield of 3.27 t ha^{-1} (Muralidharan, 1973). Nybe *et al*. (1982) reported the cultivar Maran produce 10 per cent oleoresin. The cultivar shows tolerance to leaf spot, but is infected by rhizome rot (Nybe and Nair, 1979)

2.2.5 Rio-de-Janeiro

Rio-de-Janeiro is an exotic variety of ginger used for green ginger purpose. An average yield of 21 t ha^{-1} is produced by the cultivar (Paulose, 1973) with a dry ginger yield of 3.27 t ha^{-1} (Muralidharan, 1973). Nybe *et al*. (1982) reported the cultivar Rio-de-Janeiro produce 10.5 per cent oleoresin. An essential oil of 2.0-2.8 per cent is produced by the cultivar (AICRPS, 1999). The cultivar is tolerant to shoot borer (Nybe *et al.*, 1982).

2.3 Medicinal properties and Health benefits

The medicinal use of ginger has a long history dating back 2500 years. Traditionally ginger has been used in different parts of globe for various purposes like proper digestion, treatment of stomach upset, cough, diarrhoea, *etc*. Ginger is a main ingredient in Ayurvedic medicines. The pungent and bioactive components of ginger have potent antioxidant and anti-inflammatory activities and some are anti-cancerous. Altmann and Marcussen (2001) have mentioned the use of ginger rhizome in Asian, Indian and Arabic herbal traditions. In western part of the world, its uses include

treatment of arthritis, rheumatic fever and muscular discomforts (Bordia *et al*., 1997). In addition to the medicinal properties ginger continued to be valued as an important cooking spice around the world. In the nutraceutical and health food industries, ginger plays an important role due to its health beneficial effects such as anti-hyperchlosterolemic, antioxidant, anti-inflammatory and anti-diabetic properties (Shylaja and Peter, 2007).

The protective influence of the ginger intake against the histological changes induced by cisplatin (a powerful chemotherapeutic agent) in cardiac muscle was studied by El-Hawwary and Omar (2019). Rats were given oral intake of ginger (500 mg/kg/day) along with cisplatin for 12 days. Cisplatin + ginger group showed improvement in cardiac histology and ultrastructure compared to disrupt and disorganized cardiac muscle fibers in control (cisplatin alone). They also reported the down regulation of P53 and TNF-α immune expressions and concluded ginger exhibits a protective effect to cisplatin cardiotoxicity through its anti-apoptotic, anti-oxidant and anti-inflammatory properties.

2.3.1 Anti-cancerous properties of ginger

Through molecular docking and cell culture studies, the potentiality of 6-gingerol as a promising anti-cancerous phytocompound was investigated by Manikeshkumar (2015). When the molecular docking was done with other ginger ligands (6, 8, 10 and 12 gingerols) and eleven approved drugs of cancer, the best result was obtained for 6-gingerol and the comparison of ADME/T properties also showed that 6-gingerol was superior to other ginger ligands and eleven approved drugs.

The anti-cancerous properties of ginger extract were investigated and evaluated by El-Ashmawy *et al*. (2017) in mice bearing Solid Ethrlich Carcinoma cells. They reported that the anti-cancerous effect of ginger extract was mediated through the down regulation of cycline D1 gene expression and activation of adenosine by monophosphate protein kinase (AMPK). They also described the suppressive effect of ginger extract on the nuclear factor kappa B content in tumor lines and its pro-apoptotic properties.

2.3.2 Antioxidant activity of ginger

Dugasani *et al*. (2010) reported the antioxidant activities of gingerols. They reported that 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol, expressed the anti-oxidant activities with IC50 values between 8.05 to 26.3 µM, 0.85–4.05 µM and 0.72–4.62 µM respectively for the DPPH radical, superoxide radical and hydroxyl radical.

Du *et al*. (2014) observed the antioxidant ability of 6-gingerol greater than 8-gingerol and 10-gingerol which is attributed by the short chains of the 6-gingerol.

The major pungent ingredient of ginger *ie* 6-gingerol was used to study the biochemical parameters and ovarian histological improvements in estradiol valerate (EV) induced PCOS rats. One group of female rats were given EV for 28 days and other group EV followed by 6-gingerol (200 µg/kg) for 14 days. The administration of EV increased body and ovarian weight abnormality, decreased antioxidant activity and increased CO-X-2 gene expression while 6-gingerol treatment reduced the CO-X-2 gene expression and increased antioxidant activity (Pournaderi *et al*., 2017).

2.4 Chemical composition of ginger

Analysis of the green ginger reported the presence of carbohydrates (12.3 %), protein (2.3 %), fat (0.9 %), moisture (80.9 %), fiber (2.4 %) and minerals (1.2 %) (Govardarajan, 1982). According to him, important vitamins like thiamine (0.06 mg), riboflavin (0.03 mg), niacin (0.6 mg) ascorbic acid (6.00 mg) were present in each 100 g of fresh ginger. Free sugars like glucose, fructose, and sucrose were also present in ginger rhizomes. He also reported that commercial dried ginger consisted of 1.5-3.0 per cent volatile oils and 3.5-10 per cent oleoresin. The two distinct groups of chemicals, the volatile oils and non volatile pungent principles, impart the sensory perception to fresh ginger. The sesquiterpene hydrocarbons mainly zingiberene (35%), curcumene (18%) and farnesene (10%) form the volatile oil components. The non volatile pungent principles of ginger are mainly constituted by gingerols, shogaols, paradols and zingiberene. The major active components of fresh ginger

were gingerols while dried ginger consisted of shogaols and dehydrated form of gingerols. Other constituents include oleoresin and the proteolytic enzyme zingibain.

Genotypic differences in oleoresin recovery from dry ginger were reported by Nybe *et al*. (1982) who evaluated 28 cultivars of ginger and concluded that the cultivars Rio-de-Janeiro and Maran had the highest oleoresin yield of 10.53 and 10.05 per cent respectively. Essential oil content varies from 1-4 per cent in dry ginger while the oleoresin, which imparts pungency to ginger varies from 4-8 per cent (Govindarajan, 1982). Gong *et al*. (2004) concluded that most of the composition in fresh and dried ginger was almost similar while differences were observed in the pharmacological properties.

Gingerols impart pungent taste to fresh ginger. Among the gingerols, the major compound responsible for pungency in ginger includes the 6-gingerols, while other gingerols like 4-, 8-, 10- and 12- gingerols are present in the rhizome at less concentration. These compounds were highly thermolabile and at high temperatures, transformed into shogaols which gives ginger a pungent and spicy fragrance (Wohlmuth *et al*., 2005).

The influence of genotype in essential oil composition was examined by Wohlmuth *et al*. (2006) in the diploid and tetraploid Australian ginger cultivars. Norajit *et al*. (2007) reported that the ginger oil is mainly constituted by the sesquiterpene hydrocarbons, zingiberene, ar-curcumene, beta-bisbabolene and beta-sesquiphellendrene.

Kiran *et al.* (2013a) investigated the variation in essential oil composition, oleoresin and 6-gingerol production in ten different fresh ginger cultivars from the North East region. The cultivars were harvested after six and nine months maturity separately. Genotypic difference was observed in essential oil composition of the fresh ginger. Oleoresin content decreased with maturity in all cultivars except Assam Fibreless and Manipur I. Highest oleoresin of 11.43 ± 0.58 per cent and 6-gingerol of 1.67 ±0.05 per cent were recorded in Tripura II and Nagaland Naida respectively.

Kiran *et al*. (2013b) studied the essential oil composition in seventeen cultivars of fresh ginger in the north east region. They reported that the major volatile constituents include camphene 8.49 ±0.41 per cent, neral 4.95 ±0.34 per cent and zingiberene 20.98 ±0.42 per cent.

Qualitative and quantitative determination of 6- gingerols were done in ginger *in vitro* cultures (calli, shoot and root) and *in vivo* rhizomes using TLC and HPLC, by Usama *et al*. (2013) who reported the presence of 6-gingerols in different plants parts derived from *in vitro* cultures and *in vivo* rhizomes. On analysis of 6-gingerols with HPLC, they also revealed that the amount of 6-gingerols in the *in vivo* rhizomes were higher than *in vitro* calli, shoots and roots.

Recovery of oleoresin in dry ginger was 6.8 and 7.2 per cent respectively in variety Athira and variety Karthika (Shylaja *et al*., 2010) while in the variety Aswathy an oleoresin recovery of 7.45 per cent was recorded (Shylaja *et al*., 2014).

Sreeja (2017) extracted oleoresin from the different ginger genotypes *viz.* somaclones B3 and 132M, the ginger variety Athira and the cultivar Maran which was control. The fresh and dry ginger at three different growth stages *ie* five, six and seven months after planting were subjected to extraction. She reported highest recovery of oleoresin yield of 0.86 per cent in the fresh ginger after six months of planting, irrespective of genotypes. The highest recovery of oleoresin was reported in the somaclone B3, irrespective of the growth stages. She also reported highest recovery of oleoresin of 5.31 per cent after seven months of planting in dry ginger, irrespective of the genotypes. The oleoresin recovery registered non significant among somaclone B3, variety Athira and cultivar Maran, irrespective of the growth stages.

The chemical constituents of essential oil were identified and biological assay was carried out by Bhattarai *et al*. (2018) in ginger collected from different regions of Nepal. Through the process of hydrodistillation ginger oils were extracted and components were determined using GC-MS. The major constituents determined were α- funebrene, α- farenesene, beta-phellandrene, sequisabiene, camphene, α- pinene,

linalool, neral, citronellol *etc*. They also revealed that the methanolic extraction of ginger contains biologically active glycosides, steroids, reducing sugars.

A study was designed to investigate the chemical profile of the essential oil extracted from the rhizomes of ginger by Teerarak and Laosinwattana (2019). They reported that the major identified components of essential oil of ginger include citral, zingiberene, α-farnesene, 8-cineole and β-sesquiphellandrene.

2.5 Chromatographic separation of pungency principles in ginger

Chromatography is the widely used technique for separation, identification and quantification of components from a mixture based on molecular structure and composition. Chromatography is based on the principle, wherein samples are injected to the stationary phase and the mobile phase is pumped to the stationary phase. The components in the samples are separated based on its migration rates to the stationary phase. The components having stronger interactions in stationary phase will move slowly through the column than components with weaker interactions and the components will be eluted at different times (Retention time) from the column, depending on the its partition behavior.

Metabolite profiling using GC-MS and LC-ESI-MS were carried out by Ma and Gang (2006) for determining the chemical differences which existed between greenhouse grown or *in vitro* micropropagation derived plant in three lines of ginger. They identified different constituents like gingerols, gingerol related compounds, diaryheptanoids and methyl ether derivatives, mono-sequiterpenoids. The analysis of principal component and hierarchical cluster analysis revealed chemical differences between the three lines of ginger (yellow ginger vs. white ginger and blue ring ginger) and tissues (rhizome, root, leaf and shoot).

Barman and Jha (2013) analyzed the essential oils from the normal and the organic ginger rhizomes using GC and GC-MS and reported zingiberene as the major constituents identified from both the samples. The 6-gingerol produced in rhizomes of conventionally grown plants and callus cultures produced plants were compared by Pawar *et al.* (2015), who reported that rhizome derived callus culture and

micropropagated plants produced the lowest amount of 6-gingerol compared to conventionally grown plants.

2.5.1 High performance liquid chromatography (HPLC) for separation of pungency principles in ginger

High performance liquid chromatography (HPLC) or High pressure liquid chromatography is one of the powerful techniques of chromatography for separation, identification and quantification of components.

Zick *et al.* (2010) determined the main pungent principles of ginger such as 6-gingerol, 8-gingerol and 6-shogaol by reverse phase C18 column of HPLC with electrochemical detection. The volatile oils of fresh and dry ginger rhizomes were analyzed by Sasidharan and Menon (2010), who reported Zingiberene as the major component in both the ginger rhizomes studied.

Rafi *et al*. (2013) developed reversed phase capillary liquid chromatography method for determining the pungent principles, 6-gingerol, 8- gingerol, 10- gingerol, and 6-shogaol. The separations of the mentioned compounds were done using C30 as stationary phase and 60 per cent acetonitrile as mobile phase. All the pungency principles separated within 25 minutes with good resolution.

A rapid method was developed and validated for the determination of 6, 8 and 10-gingerol from rhizomes of ginger by Ashraf *et al*. (2014). Isocratic separation of C18 column using 0.25 mL min^{-1} was carried out for chromatographic separation. The accumulation of 6-, 8- and 10- gingerols was recorded as 2.951, 2.727 and 3.013 ng mL^{-1} respectively.

Cafino *et al*. (2015) reported a simple HPLC method for separation of 6-gingerol in multiple shoot culture of ginger. Separation of 6-gingerol was carried out using ODS-3 column using 90:10 v/v methanol: water with a isocratic flow of 1 mL min^{-1}. They used the developed HPLC method for analysis of 6-gingerol production from multiple shoot cultures of ginger exposed to different light wavelengths. They concluded that light wavelengths influence production of 6-gingerol and exposure to red light yielded 191.07±1.25 µg/culture bottle.

Identification of 27 compounds was done from four types of ginger samples by Li *et al*. (2016). Five main constituents (zingerone, 6-gingerol, 8-gingerol, 6-shogaol and 10-gingerol) in the fresh ginger, dried ginger, stir-frying ginger and carbonized ginger sample extracts were simultaneously determined by Ultra-Performance Liquid Chromatography- Photo Diode Array (UPLC-PDA)

Sreeja (2017) studied the pungency principles in ginger at various growth stages using HPLC. The pungency principles were reported to accumulate in ginger rhizome at the rhizome formation stage, *ie* five months after planting and the highest total gingerols was observed seven months after planting in fresh ginger rhizomes. She also reported that the accumulation of total gingerols was highest at five months after planting in dry ginger.

2.6 Biosynthesis of gingerols in ginger

The bioactive gingerols are derived from the various intermediates of the phenylpropanoid pathway.

Dennif and Whiting (1976) proposed the biosynthesis of 6-gingerols in *Zingiber officinale* Rosc. The biosynthesis of 6-gingerol includes cinnamate acetate condensations through various routes involving many natural phenolics with related biogenesis. According to them, the phenylalanine is converted to dihydroferulate which then participates in the biological Claisen reaction with malonate and hexanoate to form a β-diketone called 6-dihydrogingedone, which is finally reduced to 6-gingerol.

The importance of dihydroferulic and heaxanoic acid in the biosynthesis of 6-gingerol were emphasized by Macleod and Whiting (1979). Further elucidation of complete route of biosynthesis of 6-gingerol with roles of dihydrofeluric acid and hexanoic acid was proposed by Denniff *et al*. (1980).

Ahumada *et al*. (2006) suggested an alternative pathway depicting the importance and key roles of the enzymes in the phenylpropanoid pathway that are involved in gingerol biosynthesis, such as phenylalnine ammonia lyase, polyketide

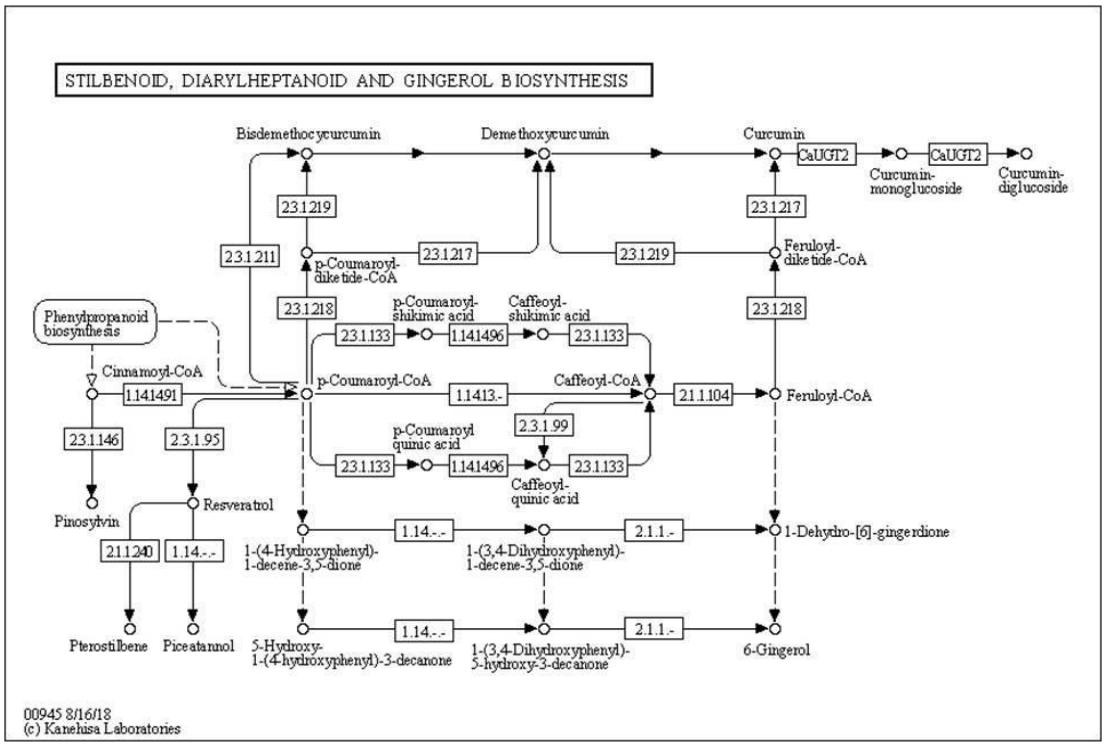


Figure 1:- Pathway map00945:- KEGG pathway map for gingerol synthesis

synthases, p-coumaroylquinate transferases, caffeic acid O- methyltransferase and caffeoyl-COA O-methyltransferase.

The KEGG pathway map00945 represents Stilbenoid, diarylheptanoid and gingerol biosynthesis (Figure 1:- Pathway map00945)

2.7 Induction of *in vitro* microrhizomes in crops

Miniature propagules can be induced using *in vitro* techniques under controlled conditions in those species normally producing organs as bulbs, corms and tubers. These micro structures produced *in vitro* from disease free stocks can be an ideal method of propagation for production of disease free planting material.

2.7.1 Induction of *in vitro* microrhizomes in ginger

Induction of microrhizomes in ginger has been reported by many researchers (Sakumura *et al*., 1989; Bhat *et al*., 1994; Sharma and Singh, 1995; Babu, 1997). The use of high sucrose concentrations of 9 to 12 per cent for induction of microrhizomes was reported by Bhat *et al*. (1994).

The MS medium supplemented with 75 gL^{-1} sucrose induced microrhizomes weighing 73 to 459 mg with four to five buds within 50-60 days of incubation (Sharma and Singh, 1995). Peter *et al*. (2002) tried different combinations of mannitol and sucrose for induction of microrhizomes in ginger. In the combination MS medium supplemented with one or 1.5 per cent each of sucrose and mannitol, smaller microrhizome weighing upto 0.05 to 1.2 g was produced after eight months of inoculation. When the sucrose content was increased to 9, 10 and 12 per cent, almost 80-100 per cent of the cultures produced microrhizomes. The field data analysis done on microrhizomes by Babu *et al*. (2003) reported that the microrhizomes were more stable than the micropropagated plants.

Zheng *et al.* (2008) examined the effect of kinetin, gibberelic acid and naphthalene acetic acid (NAA) on microrhizome production of ginger and opined that the effect of gibberelic acid on microrhizome induction was more than kinetin and NAA. They found that kinetin (0.49-066 g L^{-1}), gibberellic acid (1.33-2.35 g L^{-1})

and naphthalene acetic acid (0.62 g L^{-1}) produced microrhizomes weighing more than 0.25 g. They also reported that the media compositions and conditions for microrhizome induction were sucrose at 80 g L^{-1}, twice MS macro-elements, and MS micro-elements, with a photoperiod of 24 hours light without dark period.

An attempt was made to develop a convenient and effective protocol for high frequency microrhizome production *in vitro* in ginger by Singh *et al*. (2014). The two different ginger varieties used were Baishey and Naida. The role of growth regulators, sucrose and silver nitrate were explored for induction and production of microrhizome *in vitro*. They observed high frequency of microrhizome induction in medium with 6-8 per cent sucrose concentration. On testing the effect of silver nitrate on *in vitro* production, they found out that medium supplemented with silver nitrate produced more microrhizomes in comparison with medium devoid of silver nitrate.

Abbas *et al.* (2014) carried out an effective protocol for the *in vitro* production of ginger microrhizomes. The microrhizomes were induced using MS medium containing various concentrations of sucrose (30, 60 and 90 mg L^{-1}), BAP (3, 6, 9 mg L^{-1}) and growing under varying photoperiods. They concluded that MS medium supplemented with BAP 9 mg L^{-1} and 60 - 90 mg L^{-1} sucrose and incubation under 16 hour photoperiod within 10 weeks of cultivation were the best conditions for microrhizome induction.

Trials on microrhizome technology were initiated by Shylaja *et al*. (2016) in the varieties of ginger released by KAU *viz*. Athira, Karthika and Aswathy. High sucrose MS medium (60-80 g L^{-1} sucrose) was used for production of microrhizomes. The variety Athira was reported to produce more microrhizomes (273) within a period of six months from a single rhizome bud inoculated.

Swarnathilaka *et al*. (2016) developed an efficient protocol for production of microrhizomes in ginger using varying levels of growth hormones and sucrose and concluded that medium containing 4 mg L^{-1} BAP and 0.1 mg L^{-1} NAA produced the maximum number of microrhizomes within 60 days. They also concluded that sucrose at 90 mg L^{-1}concentration recorded highest fresh and dry weight of the microrhizomes.

The production technology of ginger microrhizomes in high tech polyhouses both in soil and soil-less media was standardized in the ginger varieties Athira, Karthika and Aswathy by Shylaja *et al*. (2018). They reported that the use of high-tech polyhouse cultivation for seed production and opined that *in vitro* induced microrhizomes could be included in the seed chain of ginger. They also concluded that the performance of microrhizomes of the three varieties Athira, Karthika and Aswathy in the polyhouse were on par.

2.7.2 Induction of *in vitro* microrhizomes in turmeric

An investigation on *in vitro* microrhizome production in turmeric was studied by Shirgurkar *et al*. (2001). They tried different strengths of MS basal medium (quarter, half and full) supplemented with sucrose at concentrations 2, 4, 6, 8 and 10 per cent for *in vitro* microrhizome induction. The sucrose concentration of eight per cent yielded bigger size but less number of microrhizomes. The higher sucrose concentration of ten per cent reduced the size and number of microrhizomes produced. They concluded that half strength MS basal medium containing eight per cent sucrose as an optimum medium for the production of microrhizomes in turmeric.

An efficient procedure for *in vitro* microrhizome induction was developed by Islam *et al*. (2005) in *Curcuma longa* L. They studied the effects of light, sucrose and growth regulators on *in vitro* microrhizome production. According to them nine per cent sucrose was most suitable for microrhizome induction on incubation in dark. On analyzing the various treatments with different concentrations of BA, KN and NAA for microrhizome induction, they reported BA (12.0 µM) and NAA (0.3 µM) suitable for the induction of microrhizomes.

Cousins and Adelberg (2008) developed microrhizome using MS liquid medium supplemented with six per cent sucrose in turmeric. The developed microrhizomes were observed after addition of methyl jasmonate and BA, wherein it was reported that methyl jasmonate reduced the leaf, root and rhizome biomass while BA increased biomass accumulation. The effects of light intensity and cytokinin on *in vitro* micropropagation and microrhizome induction were studied by Hashemy *et al*. (2009) in *Curcuma longa,* who reported that low light intensity favors

micropropagation and short day condition promotes well developed microrhizome induction.

Chougale *et al*. (2011) highlighted the development of a protocol for microrhizome production in turmeric including the influence of growth regulators, medium strength and photoperiod. The best response was obtained in medium containing BAP 1 mg L^{-1} with NAA 0.02 mg L^{-1}. They also concluded that increased number and size of the microrhizomes were obtained in MS medium supplemented with lower concentration of BAP.

2.7.3 Induction of *in vitro* microrhizomes in *Kaempferia* sp.

Chirangini *et al*. (2004) induced microrhizomes in *K. galanga* Linn. and *K. rotunda* Linn. The microshoots were regenerated from callus using 2.69 µM NAA and 2.22 µM BAP enriched medium and microrhizomes were induced from these microshoots using 6-9 per cent sucrose. In both the species they observed microrhizome production was observed in 6 per cent sucrose medium.

2.8 Induction of *in vitro* calli in crops

2.8.1 Induction of calli in ginger

Babu (1997) successfully induced ginger callus in vegetative bud, young leaf, ovary and anther tissues on MS medium supplemented with various levels of NAA and 2,4-D. Earlier reports on the induction of ginger callus were furnished by Pillai and Kumar (1982), Sakumara and Suga (1989), Ilahi and Jabeen (1992). The researchers also opined that reports on metabolite production using *in vitro* cell cultures are very preliminary and more work needs to be done before it can be used for commercial production of flavor constituents *in vitro*.

Induction of ginger callus was done by Resmi (2006) using various explants of ginger such as pseudostem base, leaves and shoot tip who reported shoot tip as the best explant among the explants studied. Different media composition such as ½ MS medium supplemented with 2, 4-D alone and in combination with BAP was tried for callus induction wherein ½ MS medium supplemented with 2, 4-D 3 mg L^{-1} and BAP

0.5 mg L^{-1} proved to be the best medium among the different media combination studied.

Jamil *et al*. (2007) initiated callus from shoot tip of young plants on MS media supplemented with NAA (0.1 mg L^{-1}) and Kinetin (1.0-2.0 mg L^{-1}), IAA (0.1 mg L^{-1}) and 6-BAP (1.0-2.0 mg L^{-1}). They reported production of callus within 3 weeks, with the highest frequency of callus induction of 98 per cent in MS media supplemented with IAA and BAP. The calli obtained were faster growing, delicate and white creamy in colour while the calli produced in the media supplemented with NAA and KN combination were compact with green colour.

Taha *et al*. (2013) initiated callus cultures from *in vitro* primordial leaves of about 8 weeks old plantlet cultures of ginger. They found that 2, 4-D at 3 mg L^{-1} resulted in the highest percentage of callus production. Young buds of *Zingiber officinale* were used for inducing callus using MS medium supplemented with various concentrations of NAA and BAP and reported that MS medium with 1 mg L^{-1} NAA was selected as best for production of calli (Shivakumar *et al*., 2014)

Ali *et al*. (2016) investigated on callus induction of ginger using MS medium supplemented with different concentrations and combinations of growth regulators. Calli were induced using shoot tips, *in vitro* leaf and root segments as explants in MS medium supplemented with various concentration of 2,4-D (0.5, 1.00, 2.00 and 3.00 mg L^{-1}) alone or in combination with BAP 0.5 mg L^{-1}. According to them, callus was induced only from shoot tip explants. They also reported that the highest fresh weight of callus of 1.302±0.09 g was obtained in the treatment MS medium supplemented 2, 4-D 1.00 mg L^{-1}.

2.8.2 Induction of *in vitro* calli in turmeric

Induction of callus was done by Zhang *et al*. (2011) in *Curcuma kwangsiensis*, an important medicinal and ornamental plant native to South China, using shoot base sections as explant excised from *in vitro* seedlings. It was reported that 91 per cent frequency of callus formation was observed in explants cultured on MS medium supplemented with 1.4 μM TDZ, 4.4 μM BA and 2.3 μM 2, 4-D. Kou

et al. (2013) reported the callus induction in *Curcuma attenuate* using anther as explants in MS medium supplemented with 13.6 µM 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 2.3 µM kinetin (KN) with highest percentage of callus induction within 30 days.

Induction of callus in *Curcuma caesia* was carried out by Abubaker and Pudake (2019) using leaf and rhizome explants in MS medium supplemented with various concentration of 2,4-D or IAA singly or combination with BAP or KN. They reported the callus response depended on the explant type and growth regulators. Percentage of callus induction was highest in the leaf explant and in the growth regulators combination 2, 4-D (0.5 mg L^{-1}) + BAP (0.1 mg L^{-1}).

2.9 Production of secondary metabolites using rhizomes, *in vitro* microrhizomes and calli

2.9.1 Production of secondary metabolites using rhizomes

Production of phenolics and flavonoids in relation with the photosynthetic rate was examined in two Malaysian ginger varieties (Ghasemzadeh *et al*., 2010). Four levels of glasshouse light intensity 310, 460, 630, 790 µmol m^{-2} s^{-1} were used. On HPLC analysis, it was observed that the plants grown under 310 µmol $m^{-2}s^{-1}$ produced high amount of secondary metabolites which include querctin, rutin, catechin. Flavonoid production increased with decreased light intensity and low photosynthetic rate. Higher production of querctin (1.12 mg g^{-1} DW) was recorded in the ginger variety Halia Bara leaves grown under 310 µmol m^{-2} s^{-1} .

Ghamsemzadeh *et al*. (2016) investigated the total flavonoids and phenolics from leaf, stem and rhizome of *Z. zerumbet* at different growth stages of three, six and nine months. Highest production of flavonoid quercetin (29.7 mg g^{-1} DW) and phenolic gallic acid (44.8 mg g^{-1} dry matter) were recorded in rhizome extracts of nine months old plants.

2.9.2 Production of secondary metabolites using *in vitro* microrhizomes

A study on production of curcumin, dimethoxy curcumin and bis- methoxy curcumin examined using LC-DAD-ESI-MS in the *in vitro* organs of *Curcuma longa* by Pistelli (2012). A significant production of curcumin was observed in the *in vitro* microrhizomes while production was comparatively less in the *in vitro* roots and shoots. On comparison with curcumin, the production of dimethoxy curcumin and bis- methoxy curcumin showed reduced amounts **.**

Effects of sucrose concentration, different levels of 6-BA and α-naphthalene acetic acid (NAA), and light quality on the production of curcumin and other curcuminoids in *Curcuma aromatica* were investigated by Wu *et al*. (2015). They concluded that microrhizomes grown on media containing three per cent sucrose produced more curcumin and other curcuminoids than those grown on higher concentrations. They also concluded that a five per cent sucrose medium supplemented with 3.0 mg L^{-1} of 6-BA and 0.5 mg L^{-1} of NAA enhanced the levels of curcumin and curcuminoids and the exposure to red light further increased its production.

Marfori and Jane (2018) studied the influence of sucrose on secondary metabolite production in the *in vitro* grown plantlets of ginger. The MS basal medium supplemented with varying amounts of sucrose at 30, 60, 90 and 120 g L^{-1} was employed. It was observed that the highest gingerol production was obtained in the sucrose concentration of 90 g L^{-1} and higher concentration of 120 g L^{-1} was found to be inhibitory.

2.9.3 Production of secondary metabolites using *in vitro* calli

Production of secondary metabolite in cultured cell is limited and the selection of *in vitro* system is very important in establishing plant cell cultures for secondary metabolite production. DiCosmo and Misawa (1995) reported that for the commercial exploitation, efforts should be focused on optimization of the cultural conditions and strategies like elicitation, precursor feeding, transformation and immobilization techniques.

Production of secondary metabolites in ginger cell culture was investigated by Sakamura and Suga (1989). Ilahi and Jabeen (1992) also reported preliminary studies on alkaloid biosynthesis in callus cultures of ginger. Babu (1997) reported the successful establishment of cell suspension cultures of ginger. He also reported the presence of oil bearing cells in calli and suggested the need of further studies for the commercial exploitation of secondary metabolites in ginger.

Dehghani *et al*. (2011) studied the production of gingerols and zingiberene in the *in vitro* and the *in vivo* ginger rhizomes and fresh calli. Fresh calli were induced from buds of ginger rhizomes. The fresh calli, *in vitro* and *in vivo* ginger rhizomes were extracted separately using dichloromethane (5 mL) and analyzed by TLC. They detected no gingerol or zingiberene spots on calli plates while *in vitro* and *in vivo* rhizomes of ginger produced gingerols and zingiberene.

2.10 Scaling up of secondary metabolite production by manipulation of cell cultures and culture conditions

2.10.1 Manipulation of macro and micro nutrients for scaling up of secondary metabolite production

In plant tissue culture the macro and micro nutrient composition have important role in primary and secondary metabolism of cells and the medium which limits early cessation of exponential growth, enhances secondary metabolite production (Dougall, 1980). The level of total nitrogen affects production of secondary metabolites. The lower levels of ammonia and higher levels of nitrate promote production of shikonin and betacyanins, while higher level of ammonia to nitrate ratio increased production of berberine and ubiquinone (Bohm and Rink, 1988; Nakagawa *et al*., 1984; Fujita *et al*., 1981; Ikeda *et al*., 1997). Reduced levels of total nitrogen also improved production of anthocyanins in *Vitis* sp. (Yamakawa *et al*., 1983).

Shikonin derivative was produced from cell suspension cultures of *Lithospermum erythrorhizon* using culture media with different nitrogen source by Fujita *et al*. (1981). Nitrogen source was found to influence the production of

shikonin and its derivatives. The yield of the shikonin derivatives increased with increase in concentration of nitrate below 6.7 mM but decreased above 10 mM. Stable production of shikonin derivatives were obtained by use of nitrate as the nitrogen source.

Hairy root cultures of *Solanum khasianum* Clarke were investigated for the growth and production of the secondary metabolite solasodine, by manipulation of major and micronutrients (Jacob and Malpathak, 2005). Manipulation of Fe-EDTA and vitamins of the MS medium were found affecting the growth phase of the roots. The total nitrogen present in the medium significantly affected the solasodine production. The ratio of ammoniacal and nitrate nitrogen was crucial for growth and production of the secondary metabolite. Half the concentration of $MgSO_4$ and $CaCl_2$ of MS medium increased growth and solasodine production in comparison with the control.

Naik *et al.* (2011) studied the effects of NH_4NO_3, KNO_3, $CaCl_2$, $MgSO_4$ and KH_2PO_4 in the MS medium at 0.05x, 1.0x, 1.5x, 2.0x each for the shoot cultures of *Bacopa monneri* and reported the highest production of bacoside A (17.9 mg L^{-1} DW) at 2x strength of NH_4NO_3. They also studied the effect of different ratios of ammoniacal and nitrate nitrogen and reported that bacoside A was optimum when NO_3^- concentration was higher than that of NH_4^+. But Praveen *et al.* (2013) reported the reduced levels of the NH_4^+ and increased level of NO_3^- promoted the production of withanolide in *Withania somnifera*.

Shehata *et al*. (2014) attempted to determine the phenolic profile of callus tissues of three date palm (*Phoenix dactylifera* L.) cultivars. The effect of different levels of ammonium nitrate on antioxidants activity and *in vitro* production of phenolic compounds was studied. They observed highest production of phenolic components in callus cultured in the medium composition MS with half concentration of ammoniacal nitrogen.

The B5 medium supplemented with enhanced and varying levels of total nitrogen by addition of diammonium hydrogen phosphate $(NH_4)_2HPO_4$ and ammonium dihydrogen orthophosphate was used to study the production of biomass

and alkaloids using suspension cultures of *Catharanthus roseus* (Mishra *et al*. 2019). The biomass and alkaloid production was found to be increasing with elevated levels of total nitrogen and phosphate along with optimum pH.

2.10.2 Elicitation using biotic and abiotic elicitors for scaling up of secondary metabolite production

Apart from the culture environment manipulation, other strategies can also be imposed based on the product needed, to enhance the secondary metabolite production. Generally, secondary metabolites are accumulated in the plants due to defense response against pathogenic attack, which is triggered by elicitors (Zhao *et al*., 2005). Elicitors are the environmental factors which can be microbial, physical or chemical that can enhance the production of secondary metabolites. Elicitors are classified as biotic and abiotic based on their nature (Namdeo, 2007a). The use of these elicitors has been a useful strategy for enhanced secondary metabolite production using organ/plant cell cultures. The frequently used elicitors in various studies include fungal carbohydrates, yeast extract, chitosan, methyl jasmonate and salicylic acid. Methyl jasmonate is the most effective elicitor for taxol production in *Taxus chinensis* Rosb. (Wu and Lin, 2003) and ginsenoside production in *Panax ginseng* C.A Meyer (Yu *et al*., 2000) using organ and cell cultures.

The effect of production of silymarin in *S. marianum* cultures were studied using biotic and abiotic elicitors like yeast extract, chitin and chitosan, methyl jasmonate by Sampedro *et al.* (2005). They noticed yeast extract treated cultures increased production of silymarin on comparison with chitin and chitosan treated cultures. They also reported that salicylic acid at 100 µM methyl jasmonate at 100 µM was observed to be optimum for the increased accumulation of silymarin, after 48 hours of treatment.

Chitin and chitosan induces phytoalexin accumulation in plant tissue. Orlita *et al*. (2008) applied chitin and chitosan to study the secondary metabolite accumulation in the *Ruta graveolens* shoots cultures. They observed significant increase of growth rate of shoots in the presence of either chitin or chitosan. Both the compounds also increased production of coumarins, pserolen, rutacultin, xanthotoxin and other

secondary metabolites. Chitin (0.01 %) and chitosan (0.1 %) increased production of coumarins like pinnarin (116.7 µg g^{-1} DW) and rutacultin (287.0 µg g^{-1} DW).

Baque *et al*. (2012) studied the effect of chitosan and pectin on the accumulation of anthraquinones (AQ), phenolics and flavonoids using adventitious root suspension cultures of *Morinda citrifolia*. They reported the optimum concentration of 0.2 mg mL^{-1} chitosan enhanced the production of AQ, phenolics and flavonoids. They also observed reduction of root growth by the same treatment of chitosan 0.2 mg mL^{-1}.

Sharma *et al.* (2014) investigated the effect of abiotic elicitors jasmonic acid, $CuSO_4$ and salicylic acid on stimulation of biomass and bacoside production in *Bacopa monnieri* shoot cultures. They reported that on eliciting the cultures with 45 mg L^{-1} of $CuSO_4$ exhibited highest bacoside content of 8.73 mg g^{-1} dry weight was observed which was 1.42 fold higher than the control cultures (6.14 mg g^{-1} dry weight).

Rocha *et al*. (2015) in their studies on production and optimization of cartenoid pigments through elicitation of the *in vitro* cultures of *Cleome rosea* reported a higher production of secondary metabolites and biomass from the cultures maintained under light. They noticed the callus cultures, on treatment with different concentrations of the elicitors such as chitosan, methyl jasmonate and yeast extract registered the highest carotenoid production in cultures treated with 60 mg L^{-1} methyl jasmonate with six fold increase compared to non-elicited callus cultures.

A rapid stimulation of secondary metabolite production and modification of cell redox state compared to control on elicitation of fungal elicitors were carried out by Simic *et al*. (2015) in *Hypericum perforatum* L. (St.John wort) cell cultures. The cell suspension cultures were taken for evaluation of phenylpropanoid and napthodiantrone production after treatment with fungal elicitors like *Fusarium oxysporum*, *Phomaexigia* and *Botrytis cinerea.* They reported a significant increase in the content of total phenolics, total flavonoids and total anthocyanins during entire period of culture while total flavonoids were observed to be enhanced at the end of post elicitation. The enzymatic activities of Phenyl ammonia lyase and Chalcone

synthase were remarkably elevated in elicited cells confirming the activation of phenylpropanoid and flavanoid pathway. They also concluded that the elicited cells represent a promising experimental system for scale up production of secondary metabolites.

Wee *et al*. (2015), studied the effects of elicitors using the *in vitro* cultures of *Sauropus androgynus* (sweet shoot). They reported that light induced callus cultures treated with 200 µM methyl jasmonate produced highest total phenolic and flavonoid contents with 2.10 fold increase compared to control, after three weeks of inoculation. They also reported the enhancement of antioxidant activity of the methyl jasmonate elicited cells and high production of naringin from salicylic acid elicited somatic embryos.

Callus induction and elicitation of the total phenolics using elicitors like jasmonic acid, salicylic acid and copper sulphate in cell suspension cultures of *Celastrus paniculatus* (black oil plant) were carried out by Anusha *et al*. (2016) who revealed that among the elicitors, jasmonic acid at higher concentration of 250 µM recorded maximum phenolics after 24 and 48 hrs treatments.

The effect of fungal cell wall elicitor extracted from *Piriformospora indica* on production of phenylpropanoid derivates was studied using hairy root cultures of *Linum album* by Tashackori *et al*. (2018). They reported a significant increase in accumulation of various metabolites like lignin, ligans, phenolic acids like cinnamic acid and flavonoids like diosmin, kaempferol in response to fungal elicitation. In addition to this, they also reported up-regulation of *PAL, CAD, CCR* and *PLR* genes involved in phenylpropanoid pathway.

A recent study conducted by Khan *et al.* (2019) on the effect of chitosan and salicylic acid on production of secondary metabolites in callus cultures of the thorny herb *Fagonia indica* revealed the enhanced accumulation of biomass and secondary metabolites. The phenols and flavonoids were observed to be increased in chitosan and salicylic acid treated callus cultures on comparison with that of the control.

A quite recent study by Hassani *et al*.(2019) on the effects of different concentrations of MJ and SA either individually or in combination in the quantitative changes in pigments, growth parameters, and volatile compounds in the shoot cultures of Z*iziphora persica* revealed that the combination treatments of MJ and SA produced highest amounts of chlorophyll *a* and carotenoids. The chlorophyll *b* content recorded the highest in the control treatment.

Kazmi *et al.* (2019) reported a rapid approach for *in vitro* adventitious root formation and production of steviol glycosides using methyl jasmonate and phenyl acetic acid as elicitor in *Stevia rebaudiana* Bertoni.

2.10.3 Precursor feeding using precursors of biosynthetic pathway for scaling up of secondary metabolite production

Exogenous supply of the biosynthetic precursors to the culture medium may also increase the yield of the desired compounds. The concept is that the addition of the compound (precursors) which is an intermediate in or at the beginning of the secondary metabolic route can increase the yield of final product. Addition of phenylalanine to cell suspension cultures of *Salvia officinalis* stimulated the Rosmarinic acid production (Ellis and Towers, 1970) and the taxol production in taxus cultures (Fett-neto *et al*., 1993).

Vanila planifolia callus cultures were treated with ferrulic acid and the concentration of vanillin produced was studied by Romagnoli and Knorr (1988). An increase of 1.7 fold was observed in the vanillin production after application of 1mM ferrulic acid solution in comparison with the non elicited callus cultures. They also reported that the higher concentration of 10 mM ferrulic acid solution decreased vanillin production.

Cousins *et al.* (2011) attempted various methods to up-regulate secondary metabolism, in turmeric microrhizomes. The clones were exposed to various treatments such as precursor feeding of phenylalanine, addition of proline, methyl jasmonate and induction of stress. They reported that precursor treatments lowered biomass accumulation and antioxidant capacity and the depression was dependent on

the genotype. They also reported a minute (0.6 %) increase in the phenolic content in microrhizomes exposed to nitrogen stress.

The influence precursors such as phenyalanine, mevalonic acid, leucine and valine and biotic elicitors such as yeast extract and *Aspergillus niger* on growth and production of active substances in *Zingiber officinale* callus cultures were studied by El-Nabarawy *et al*. (2015). They reported positive influence of mevalonic acid and yeast extract at lower concentration, on the production of 6-gingerol. The precursors and the biotic elicitors were not exerting considerable effect in the production of other gingerols (8-gingerol and 10-gingerol) and shogaol (6- shogaol). According to them, the precursors and the biotic elicitors studied were having a negative effect, in the fresh and dry weight of the callus production on comparison with the control.

El-Shennawy *et al*. (2017) reported the effect of phenylalanine (0, 0.5, 5, 10, and 15 mg L^{-1}) and NaCl on the secondary metabolite production using *in vitro* cultures of *Mentha longifolia*. The callus cultures used for the study were induced from shoot tip and leaf explants. They observed lower concentration of phenylalanine (5 mg L^{-1}) and NaCl enhanced the production of chlorophylls and other secondary metabolites.

The effect of cytokinin combined with elicitors L-Phenylalanine, salicylic acid and chitosan on *in vitro* propagation and secondary metabolite production in *Coleus aromaticus* was studied by Govindaraju and Arulselvi, (2018). They reported the increased production of various compounds like alkaloids (16 %), flavonoids (24 %) and phenolics (24.4 %) in elicited cultures compared to the control. They also reported the highest multiple shoot development was obtained in the media combination supplemented with 1.0 mg L^{-1} BAP and 40 mg L^{-1} chitosan.

2.10.4 Irradiation of calli for scaling up of secondary metabolites production

The ionizing radiations like UV rays and gamma radiation also influence the production of secondary metabolites. The *in vitro* mutagenesis by gamma radiation was reported in callus cultures of black pepper by Shylaja (1996). Chung *et al*. (2006) used relatively different doses of gamma radiation of 2, 16 and 32 Gy to study its

effect on production of shikonin in suspension cultures of *Lithospermum erythrorhizon* cells. They reported a significant increase in the shikonin yields of 400 per cent at 16 Gy and 240 per cent and 180 per cent at 2 and 32 Gy respectively. Callus cultures of ginger could with stand a gamma radiation dose of 20Gy (Resmi, 2006).

The enhancement of secondary metabolite production and antioxidant properties of rosemary callus a consequence of gamma radiation were studied by El-Betagi *et al*. (2011). They noticed the higher dose of gamma radiation enhanced the accumulation of total phenols and flavonoids in rosemary callus cultures. Jaisi (2012) studied the effects of gamma irradiation on secondary metabolite production of *Plumbago indica* root cultures and reported the highest increase in plumbagin production at an irradiation dose of 20Gy

The roles of UV radiation on the secondary metabolite production in callus cultures of *Vitis vinifera* were studied by Sema (2014). Calli were initiated from leaf petiole explants in B5 medium supplemented with BA. The callus tissues were exposed to UV radiation for 5-10 min at 10, 20, 30 cm distance from the UV source and the samples were collected for analysis after 0, 24 and 48 hrs. The highest total phenolic content was observed in calli exposed to UV radiation for 5 min from 30 cm distance and estimated after 24 hrs.

In a study by Fulzele *et al*. (2015) aimed to explore the effects of doses of gamma irradiation on improvement of camptothecin production by callus cultures of *Nothapodytes foetida*, different dosage of gamma irradiation ranging from 5-30 Gy were used. Gamma irradiation boosted the production of camptothecin by 20 fold at 20 Gy dosage. The dose of 20 Gy was selected as satisfactory for callus attenuation as irradiated cells above 25 Gy inhibited growth and productivity.

Khalil *et al*. (2015) studied the effect of different doses of gamma radiation (5, 10, 15 and 20 Gy) on 30 day old leaf derived callus cultures of *Stevia rebaudiana* Bertoni. They observed reduced callus proliferation by gamma radiation treatment as compared to the control. The dose of 15 Gy increased stevocide content (0.251 mg g^{-1} DW) over the control (0.232 mg g^{-1} DW).

The effect of gamma radiation on biomass formation and yield of pharmacologically relevant secondary metabolites in callus cultures of *Hypericum triquetrifolium* Turra were investigated by Azeez *et al*. (2017). Calli were induced from leaf, stem and root using MS medium supplemented with IAA and TDZ. The callus biomass was found to be higher in calli irradiated with 10 Gy dose. Chromatographic analyses of callus extracts revealed that elicitation with gamma rays at 10 Gy dose, higher amount of p-hydroxybenzoic acid was recorded in calli from leaf explant, while chlorogenic acid was more in calli from root explant on comparison with the control. The 10 Gy and 20 Gy irradiation doses stimulate epicatechin accumulation in calli from leaf and stem, compared to control.

Rambulana *et al*. (2017) investigated the effect of gamma radiation on induction of the anti- cancerous metabolite, glucomoringin and its derivatives in *Moringa oleifera*. Various gamma irradiation doses of (0.1 to 8 KGy) were tested and 2 KGy was noted to be the most potent with higher production of glucomoringin as compared to control plants.

2.11 Transcriptome analysis and gene expression studies

2.11.1 Transcriptome analysis for *Phenylalanine lyase* (*PAL*) gene expression

Facchini *et al*. (1996) reported the opium poppy cell cultures on treatment with an autoclaved mycelial homogenate of *Botryis* sp. resulted in the higher accumulation of the secondary metabolite sanguinarine while the elicitor preparation from *Pythium aphanidermatum* was less effective in accumulation of secondary metabolites. In contrast, both the elicitors increased the accumulation of *PAL* transcripts. The treatment with methyl jasmonate resulted in the production of less *PAL* mRNAs.

The accumulation of biomass and the secondary metabolite paclitaxel on modified MS medium treated with fungal elicitor on resistant and wild type cells of *Taxus* sp. were studied by Su *et al*. (2002). According to them the production of paclitaxel in resistant cells increased by 70 folds on treatment with the fungal elicitor.

They also noted evident difference in the activity of peroxidase and PAL between antifungal variant and wild type cells.

Khan *et al*. (2003) reported the enhanced activities of *PAL* gene and *tyrosine ammonia-lyase* (*TAL*) on treatment with chitin and chitosan oligomers in soyabean leaf tissues. The total phenolic content of soybean leaf tissues increased following the treatments with chitosan and chitin oligomer, which confirmed a positive correlation between PAL and TAL enzyme activities and total phenolic content. The addition of 50 and 300 µM SA increased the expression of *PAL* and *STS* (S*tilbene synthases*) gene expression in *Vitis amurensis* cell culture which encodes for the production of the phytoalexin reversertrol (Kiselev *et al*., 2009).

Bahabadi *et al*. (2012) investigated on fungal elicitor induced lignin synthesis and expression of the relevant genes in the cell cultures of *Linum album*. The anti-tumour compound podophyllotoxin and its related lignans were accumulated in *Linum album*. They examined the effects of five fungal extract on production of lignans and reported that *Fusarium graminearum* extract induced the highest accumulation of podophyllotoxin (Seven fold compared to control) while *Rhizopus stolonifer* extract enhanced larciresinol content (8.8 fold compared with the control). They also reported up-regulated expression of some the genes responsible for podophyllotoxin production including *PAL*.

2.11.1.1 Transcriptome analysis for *Phenylalanine lyase* (*PAL*) gene expression after elicitation with salicylic acid

Wen *et al*. (2006) studied the expression of *PAL* gene in grape berries on treatment with SA 150 µmol L^{-1}. The grape berries were sliced as small discs of 0.1 cm thickness and were treated with SA for 3 hours. The *PAL* activation by SA was observed by 0.5 hours of incubation and increased *PAL* activity of 1.87 fold was observed on comparison with the control.

Hao *et al.* (2014) reported the increased activity of *PAL* on treatment of the cell cultures of *Salvia miltiorrrhiza* with 22.5 mg L^{-1} SA after 20 min. The PAL

activity increased by 5.4 fold compared to that of the control. The accumulation of rosmarinic acid by 2.15 fold was also observed by the treatment.

In the study conducted by Jong *et al*. (2015) five phenylalanine lyase (*PAL*) were identified in *Salix viminalis* and characterized that encodes the enzymes that catalyses the deamination of L-phenylalanine to form transcinnamic acid. They also reported that the genes play wider roles in production of flavonoids, tannins and phenolic glycosides. Ejtahed *et al*. (2015) studied the *PAL* gene expression and accumulation of rosmarinic acid in *Salvia officinalis* and *Salvia virgata* shoots under elicitation of SA. On analysis using semi quantitative reverse transcription PCR, they noticed that exogenous application of SA led to up- regulation of *PAL* gene expression. In *S. virgate* higher concentration of SA accumulated higher amount of rosmarinic acid while in *S. officinalis* lower concentration of SA produced higher accumulation of rosmarinic acid. According to them, there is no positive correlation between the *PAL* gene expression and accumulation of rosmarinic acid.

The total phenylpropanoid accumulation and expression analysis of *PAL* genes from suspension cultures of *Osimum tenuiflorium* on elicitation with jasmonic acid, methyl jasmonate and the precursor L-phenylalanine were investigated by Vyas and Mukhopadhyay (2018). The addition of SA (1.5 µM) induced 61 fold and 4.5 fold increase of caffeic acid and *PAL* gene expression respectively. On treatment with MJ at 25 µM, an increased production of ferrulic acid by 62 fold with a *PAL* gene expression of seven fold on day 16 was reported.

2.11.1.2 Transcriptome analysis for *Phenylalanine lyase* (*PAL*) gene expression after elicitation with chitosan

Notsu *et al*. (1994) reported the activity of *PAL* in rice callus cultures stimulated by 2.0 fold increase within 24 hours of treatment of 0.25-1.5 per cent chitosan. They also reported the highest lignin production of 8.83±0.07 OD_{280}/g by fresh weight on treatment with chitosan.

Application of 100 µmol L^{-1} of elicitor solution of chitin and chitosan hexamers to soyabean leaves caused increased activity of PAL and TAL enzymes

(Khan *et al.,* 2003). The total phenolics accumulation increased by 37 per cent on treatment with the chitosan hexamer.

Treatment of the shoot cultures of *Melissa officinalis* by chitosan at different levels of 50, 100 and 150 mg L^{-1} and its effects on PAL activity and production of phenolics and rosmarinic acid were studied by Vanda *et al.,* (2019). They observed highest activity of *PAL* genes on treatment with 50 mg L^{-1} chitosan. They also reported highest production of phenolics at 100 mg L^{-1} chitosan treatment and non significant differences in production of rosmarinic acid at different levels of chitosan treatment. Three fold increase in rosmarinic acid on treatment with chitosan was observed after two weeks.

2.11.1.3 Transcriptome analysis for *Phenylalanine lyase* (*PAL*) gene expression after elicitation with ferulic acid

Politycka (1999) reported increased *PAL* activity and higher lignin content on treatment with 0.1 mM solution of ferrulic acid in seedlings of *Curcumis sativus* L cv. Wisconsin.

Foliar application of L-phenylalanine and ferrulic acid (50,100 and 150 ppm) was carried out in pea plants and the pea leaves were harvested after 48, 72 and 96 hours of treatment. The PAL activity increased with increased concentration of ferrulic acid. Higher production of the end product cinnamic acid (0.26 nM min^{-1} g^{-1} FW) was recorded in the treatment FA 100 ppm after 96 hours (Bahadur *et al*., 2012)

2.11.2 Transcriptome analysis for *P-hydroxylbenzoic acid* (*PHB*) gene expression

Chung *et al*. (2006) reported increase in secondary metabolite production in the suspension cultures of *Lithospermum erythrorhizon* cells on treatment with relatively different doses of gamma irradiation (2, 16, 32 Gy). Shikonin production was increased by gamma irradiation. They observed the PHB geranyl transferase, which is one of the key enzymes of the shikonin biosynthetic pathway, was stimulated by gamma irradiation. They noticed the PHB geranyl transferase was increased at gamma irradiation dosage of 2 and 16 Gy, while it is negligible at 32 Gy.

2.11.3 Transcriptome analysis for *Chalcone synthase gene expression* (*CHS*)

The *Chalcone synthase* (*CHS*) gene controls the first committed step in the flavonoid biosynthetic pathway. It is the regulatory focal point of substrate flow between flavonoid pathway and the phenylpropanoid pathway which leads to the production of many phenolic compounds like lignans and phytoalexins (Szalma *et al*., 2002). The genes encoding *CHS* constitutes a multigene family and varies in copy number among different plants which also varies functionally (Tuteja *et al*., 2004 and Yang *et al*., 2004). The *CHS* gene expression in fruit tissue is developmentally regulated (Aharoni and O' Conell, 2002) and single gene family encodes the *CHS* in some plants like soyabean and petunia (Koes *et al*., 1994).

Induction of the *Chalcone synthase* expression in white spruce by wounding and MJ application was reported by Richard *et al*. (2000). On examination of the gene expression of *CHS* gene family in spruce it was revealed that accumulation of *CHS* mRNA in needle tissue of white spruce following wounding. They also reported that the repeated application of MJ also enhanced the expression of *CHS* gene.

Ghasemzadeh *et al*, (2012) reported the effect of foliar application of SA (10^{-3} M and 10^{-5}M) on production of secondary metabolite and Chalcone synthase activity in two varieties of Malaysian ginger (Halia Bentong and Halia Bara). On HPLC analysis, they concluded that application of SA enhanced the accumulation of the secondary metabolites such as anthocyanin and fistein. They also reported the increased activity of *Chalcone synthase* gene on SA application.

The transcript expression of the *Chalcone synthase* gene, in different parts of physiologically mature grape berries and *in vitro* synchronized red cells of *Vitis rotundifolia* was carried out using real time PCR and bioinformatic approaches by Davis *et al*. (2012). According to them, a low level of *Chalcone synthase* gene expression was observed in berry flesh while elevated level of expression was noted in the berry skins and the *in vitro* synchronized red cells.

Feng *et al*. (2015) studied the molecular cloning and expression profiling of gene from *Lamiophlomis rotata*. On examination of *Chalcone synthase* gene expression of different tissues by realtime PCR using specific primers revealed the total flavonoid content and *CHS* gene expression exhibited a similar pattern during *L. rotata* organ development. They also reported its function as an elicitor responsive gene through experiments using MJ and UV light.

Ghosh and Mandi (2015) studied *Chalcone synthase* gene expression carried out in landraces of ginger revealed that the landraces with specific amino acids change from aspargine to serine caused low 6-gingerol content as asparagine is one of the critical amino acids of the catalytic triad of *Chalcone synthase* gene.

The cloning and expression analysis of *Chalcone synthase* gene in *Coleus forskholi* was studied by Aswathi *et al*. (2016). The full length of cDNA showing the homology with the *Chalcone synthase* gene was isolated from the leaves of *C. forskholi* (*CfCHS*). The expression of *CfCHS* from the different elicitor treatment revealed that MJ strongly induced its expression leading to increase of total flavonoids.

Zuk *et al*. (2016) reported the changes in wall synthesis and sensing genes, cell wall chemistry and stem morphology of Flax by the *Chalcone synthase* gene suppression. Sreeja, (2017) studied the *Chalcone synthase* gene expression in the ginger somaclone B3 using the cultivar Maran as the control. The somaclone B3 showed 54 per cent increase in the expression of *Chalcone synthase* gene as compared to that of Maran.

Wani *et al*. (2017) in their studies on *Chalcone synthase* gene expression in relation to flavonoid synthesis in *Grewia asiactica* L. revealed that the accumulation of flavonoids such as narringenin and quercetin was directly related with expression levels of the *Chalcone synthase* gene.

EL-Garhy *et al*. (2016) studied the effect of gamma radiation as a single elicitor or along with salinity on *Chalcone synthase* gene expression and the production of flavanol, lignans, silbyrin A+ B yield. They noticed that an increased

expression of *Chalcone synthase* gene was involved in the silbyrin biosynthetic pathway along with increased production of silbyrin.

Lee *et al*. (2017) analyzed transcript levels of seven genes such as *Phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate: coenzyme-A (CoA) ligase (4CL), chalcone synthase (CHS),* and *dihydroflavonol 4-reductase (DFR)* from *Ixeris dentata* var *albiflora* which are involved in phenylpropanoid biosynthesis and concluded that the expression of genes is highest in the leaf using quantitative real-time PCR. On analysis of the accumulation of phenylpropanoids such as catechin, chlorogenic acid, ferrulic acid in different organs of *I. dentata* using high-performance liquid chromatography, they reported that the phenylpropanoids were also accumulated highest in the leaf.

Wang *et al.* (2018) detected the expression of only three *Chalcone synthase* genes from the seventy-seven *Chalcone synthase* and *Chalcone synthase* like gene sequence studied in the citrus family which was involved in flavonoid production. The entire three identified *Chalcone synthase* gene (Cit*CHS1*, Cit*CHS*2, Cit*CHS3*) were highly conserved and were having different expression patterns in different tissues and developmental stages. These genes also presented different sensitivities to methyl jasmonate treatment and flavonoids production.

A recent study on the characterization and gene expression analysis of *Chalcone synthase* (*CHS*) and *Chalcone isomerase* (*CHI*) genes was carried out by Singh *et al.* (2019) in *Phyllanthus emblica*. The expression analysis at different developmental stages (leaf, flower and fruits) revealed the *CHS* gene expression is maximum in mature fruits while *CHI* gene expression is highest in the young leaves and the expression of *CHS* gene increases with the development of the fruit.

Archita (2019) studied on the effect of foliar application of SA 100 µM on *Chalcone synthase* activity and production of gingerols in the variety Karthika and reported the higher activity of the *CHS* gene along with higher production of gingerols as compared to that of control

2.12 Expressed Sequence Tags

Expressed Sequence Tags are small pieces of DNA sequence 200-500 nucleotide long generated by sequencing of wither one or both ends of the expressed genes. ESTs can be used as an inexpensive and quick method of discovering of new genes.

2.12.1 Identification and development of ESTs for secondary metabolite production

A total of 5548 ESTs from *Stevia rebandiana* leaf cDNA library were annotated using bioinformatics tools and the ESTs involved in diterpene synthesis were identified (Brandle *et al*., 2002). Jung *et al.* (2003) sequenced 11,636 ESTs from five ginseng libraries to create a gene resource for biosynthesis of gensenosides. Through Blastx analysis ESTs involved in gensenoside biosynthesis were identified.

The genes related to biosynthetic pathway of artemisinin were cloned by SSH and metabolite approach of related genes involved in blooming flowers and flower buds (Liu *et al.,* 2009). A total of 350 cDNA clone from subtractive cDNA library were randomly taken, sequenced and analysed from which 253 good quality sequences were obtained. Blastx analysis indicated that 9.9 per cent of the clones were involved in isoprenoid-artemisnin biosynthesis.

The cDNA library was generated from RNA isolated from the leaves of *Hydrangea serrata* (Luo *et al*., 2010). A total of 4012 clones were selected and 3451 ESTs were assembled to yield 1510 unique sequences. Through functional annotation, the biological processes, molecular function and biochemical pathways of the sequences were revealed. About 34 sequences were identified which were involved in secondary metabolite biosynthesis of alkaloids, terpenoids and flavones/flavonoids.

Expressed sequence tag dataset from the vegetative organs of *Glycyrrhiza uralensis* were carried out using 454 GS FLX platform. A total of 59,219 ESTs were generated and assembled to obtain 27,229 unique genes. The assembled genes

sequences were annotated. Genes encoding the enzymes of 18 total steps of glycyrrhizin synthesis pathway were found. Organ specific expression analysis using realtime PCR revealed three unigenes coding for cytochrome P450 and six unique genes most likely involved in glycyrrhin biosynthesis (Li *et al*., 2010).

Wenping *et al*. (2011) obtained 56,774 unigenes in the transcriptome of medicinal plant *Salvia miltiorrhiza* using Solexa deep sequencing and 1539 unigenes were identified as part of secondary metabolite pathway mainly involved in phenylpropanoid and terepenoid pathways.

Roslan *et al*. (2012) constructed standard cDNA library from *Polygonum minus* leaves, stem and root organs to create a gene resource for flavonoid biosynthetic pathway. They identified 4196 ESTs out of which eleven ESTs code for seven genes which were mapped to the flavonoid biosynthetic pathway. The expression of three flavonoid biosynthetic pathway-related ESTs *chalcone synthase, CHS* (JG745304), *flavonol synthase, FLS* (JG705819) and *leucoanthocyanidindi oxygenase, LDOX* (JG745247) were finally detected from leaf, stem and root by quantitative RT-PCR.

De novo transcriptome sequencing was carried out for the investigation of expression of genes associated with the biosynthesis of major compounds in matured ginger rhizome (MG), young ginger rhizome (YG), and fibrous roots of ginger (FR) (Jiang *et al*., 2017). They assembled 361,876 unigenes and genes involved in the biosynthetic pathway of major bioactive compounds differed between tissues. They also confirmed that most of the unigenes of the two pathways (terpenoid backbone biosynthesis and stilbenoid, diarylheptanoid and gingerol biosynthesis pathways), including *curcumin synthase, phenylpropanoylacetyl-CoA synthase, trans-cinnamate 4-monooxygenase,* and *4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase* were expressed at higher levels in FR than in YG or MG.

Jiang *et al.* (2018) investigated and quantified the concentrations of gingerols in different stages of rhizome development and in different tissues in ginger. They confirmed that the major source of gingerols is the rhizome and gingerol accumulation starts at early developmental stage. They also assembled a reference

ginger transcriptome having 219,479 unigenes with 330,568 transcripts. On analysis of 12,935 identified differentially expressed genes they concluded that five genes-*curcumin synthase* (*CURS*), *cinnamate 4-hydroxylase* (*CYP73A*), p-*coumaroylquinate/shikimate 3'-hydroxylase* (*CYP98A*), *caffeoyl-coenzyme A* O-*methyl transferase* (*CCoAOMT*), and *hydroxycinnamoyl-coenzyme A shikimate/ quinatehydroxy cinnamoyl transferase* (*HCT*) associated with gingerol synthesis and are expressed differentially in rhizome at early developmental stage and all of them are upregulated. They also concluded that, among the five differentially expressed genes, *CCoAOMT* and *HCT* may act as gatekeepers and rate-limiting enzymes in the gingerol biosynthesis pathway and plays a key role in regulating the biosynthesis of gingerol.

2.12.2 Analysis and validation of differentially expressed genes for secondary metabolite production

ESTs in strawberry fruit were analyzed by microarray technology by Aharoni and O'Connel (2002). A total of 1701 cDNA clones were obtained from strawberry ripe fruit cDNA library which was used for monitoring concurrent gene expression of metabolic pathways in receptacle and achene tissues and reported a four-fold difference in gene expression between the two tissue types.

Park *et al*. (2003) investigated on ESTs involved in the production of secondary metabolites in tea by suppression subtractive hybridization. They isolated 1000 cDNA clones of which 588 clones were sequenced and 508 showed high quality sequences. Around 8.7 per cent of the clones encoded enzymes involved in secondary metabolite biosynthesis in tea leaves. They isolated and characterized genes of tea flavonoid pathway such as *Chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), flavonoid 3'5'-hydroxylase (F3'5'H), flavonol synthase (FLS), dihydroflavonol 4-reductase (DFR)* and *leucoanthocyanidin reductase (LCR*) and reported the three genes highly expressed in young leaves were *F3H, DFR* and *LCR*, indicating the flavonoid biosynthesis genes in tea were differentially regulated in developmental stages.

Analysis of ESTs in apple for flavor biosynthesis was reported by Newcomb *et al.* (2006). On studying the number of protein families they reported that many genes were involved in biosynthesis of flavor in apple. Expressed Sequence Tags from different tissues of apple, especially the fruit tissues of the cultivar Royal Gala were studied. From 43 different cDNA libraries and 34 tissues, around 150,000 ESTs were collected and on analysis 42,938 non redundant sequences, 17,460 tentative contigs and 25,478 singletons were obtained after clustering. They noticed the presence of dinucleotide repeat in 4,018 nonredundant sequences mainly in the 5'-untranslated region along with commonly occurring trinucleotide repeats in the coding regions. Bi-allelic single-nucleotide polymorphisms were found abundantly on every 706 bp of transcribed DNA. The protein prediction indicated the presence of genes involved in disease resistance and the biosynthesis of flavor and health-associated compounds.

Kushwah (2008) and Vishvambhar (2010) analyzed ESTs for water stress in black pepper (*Piper nigrum*) and they observed good homology for their EST sequences with known sequences for drought.

Mondego *et al.* (2011) used differentially expressed transcripts for correlating gene expression profiles with developmental traits in coffee. The ESTs of *Coffea arabica* and *Coffea canephora* produced by the Brazilian Coffee Genome Project and the Nestle-Cornell Consortium were assembled which revealed 32,007 and 16,665 clusters of *C. arabica* and *C. canephora* respectively. Blast results showed similarities between coffee and grape genes. The protein domain and gene ontology in relation to complex sugar synthases and nucleotide binding proteins showed differences in *C. arabica* and *C. canephora* based on protein. The specific coffee protein family includes new cystatins, glycine-rich proteins and RALF-like peptides. The hierarchical clustering was done according to the expression data from EST libraries for *C. arabica* and *C. canephora*, resulting in identification of differentially expressed genes based on which plant defenses, abiotic stress and cup quality-related functional categories were conducted in the study.

The validation of identified ESTs was attempted by Gohain *et al.* (2012). They studied Darjeeling tea flavor on molecular basis. Identification of genes/ transcriptomes responsible for the typical flavor of Darjeeling tea was done along with studying the role of jassids and thrips in producing the best cup character and quality. Through quantitative real time PCR analysis, the transcripts related to aroma and flavor formation was identified based on SSH forward library of tea clone B157 infested with thrips. Some of the genes were expressed as a result of insect infestation such as leucine zipper, geraniol synthase, raffinose synthase, elicitor responsive proteins, trehalose synthase, amylase, farnesyl transferase, linalool synthase, peroxidases, linamarase, nerolidol linalool synthase 2, 12-oxophytodienoate reductase, glucosidase, MYB transcription factor and alcohol dehydrogenase which upregulated the quality of Darjeeling tea.

Miao *et al.* (2014) investigated the genes involved in biosynthesis of secondary metabolites in *Tripterygium wilfordii*. After evaluating subtraction efficiency of MJ elicitation through PCR and reverse northern hybridization, cDNA clones were randomly sequenced and it was concluded after BLAST and phylogenetic analysis that 11 genes were involved in biosynthesis of secondary metabolites in *Tripterygium wilfordii*.

Ghosh and Mandi (2015) investigated SNPs in *Chalcone synthase* gene in ginger and concluded that *Chalcone synthase* was the rate limiting enzyme in 6-gingerol biosynthesis pathway.

The computational functional annotation of the available ESTs in ginger deposited in NCBI and identification of genes responsible for gingerol biosynthesis was done by James *et al*. (2015). The clustered and assembled ESTs gave 8624 contigs and 8821 singletons. The data assembled was further subjected to functional annotation using various bioinformatics tool such as blast, gene ontology (go) analysis, and pathway elucidation by Kyoto Encyclopedia of Genes and Genomes and Interproscan where in, around 409 simple sequence repeats from the contigs were identified. They also reported the presence of single hypothetical miRNA, which plays an important role in controlling the genes involved in gingerol production, from

the rhizome tissue. The result was also integrated into web-based Ginger EST database and made available in the public domain.

A molecular approach combining SSH and next generation sequencing were used to identify the genes regulating developmental stages from fruit set to full ripening in strawberry (Baldi *et al*., 2018). They observed coordinated regulation of several metabolic processes such as flavonoid pathway, phenylpropanoid pathway and sugar metabolism. They also noticed the genes that were involved in the flavonoid pathway activated twice at distinct phases *viz,* very early stage of fruit development and during ripening.

Sreeja, (2017) performed SSH to identify the differentially expressed genes in somaclone B3 and the parent cultivar Maran. A total of 25 and 19 Expressed Sequence Tags (ESTs) could be identified from the rhizome and leaf. On analysis of the ESTs, she reported that no genes involved in the production of gingerol were present in rhizome ESTs, while in the leaf ESTs, when analyzed for differentially expressed genes, *3-ketoacyl CoA thiolase* (ACAA1) gene was found involved in the gingerol biosynthetic pathway, which was differentially expressed in somaclone B3.

The 3-ketoacetyl CoA *Thiolase* (EC 2.3.1.16) was reported a differentially expressed gene for gingerol production in the somaclone B3 (Sreeja, 2017). Acetyl CoA is produced from the benzoate degradation pathway which in turn acts as the molecule for plant metabolic pathways (Fatland *et al*., 2000). In plants, the acetyl CoA takes part in fatty acid biosynthesis in plastids and also for biosynthesis of secondary metabolites like flavonoids, stilbenoids and isoprenoids. The initial substrate for flavonoid synthesis is cytosolic acetyl CoA (Fatland *et al*., 2000).

Fonseca *et al*. (2004) monitored gene expression in pear fruit development, ripening and senescence. The 3-ketoaceyl CoA *Thiolase* gene was observed activated from day 12 until day 15. The gene encoding for jasmonic acid protein (JA2) also observed to show similar expression profile that of *Thiolase* gene. Jasmonic acid reported triggering defense responses which further trigger the secondary metabolite related pathways which leads to increased production of metabolites.

Moerkercke *et al*. (2009) reported the involvement of *Thiolase* gene in benzoic acid synthesis in *Petunia hybrida*. They reported on silencing of *Petunia*

hybrida 3-ketoacyl CoA Thiolase gene resulted in reduction of benzoic acid formation. They also reported that the other phenylpropanoid related volatiles were not affected by this.

Materials and Methods

3. Materials and Methods

The research work entitled "*In vitro* synthesis of gingerol and analysis of expressed sequence tags for gingerol production in ginger (*Zingiber officinale* Rosc.) was undertaken at the CPBMB, Distributed Information Centre (DIC) and Radio Tracer Lab (RTL), College of Horticulture, Kerala Agricultural University during 2015-2019. The present study was done to analyze the synthesis of gingerol under *in vitro* conditions, to characterize the ESTs related to gingerol synthesis and to validate the identified ESTs for high gingerol production in ginger. The main aspects covered in the study were *in vitro* gingerol synthesis in microrhizomes and calli, manipulation of macro and micro nutrients on gingerol synthesis, elicitation of biotic and abiotic elicitors on gingerol synthesis, precursor feeding on gingerol synthesis, gamma irradiation on gingerol synthesis, gene expression studies using Real time PCR for the identified best treatments, analysis of ESTs using bioinformatics tools and validation of identified ESTs. The materials and methods followed in the study are described in this chapter.

3.1 Materials

3.1.1 Experimental Materials

Three ginger varieties released from Kerala Agricultural University *viz.* Athira, Karthika and Aswathy were used as the experimental materials for the study. For comparison, the parent cultivar of varieties Athira and Karthika and the variety Aswathy were also used in the study. The varieties Athira and Karthika are single plant selection from the somaclones of cultivar Maran and the variety Aswathy is single plant selection from the somaclones of cultivar Rio-de-Janeiro. The seed rhizomes obtained from the demonstration plot of CPBMB were sown in sterile sand and were maintained in CPBMB for collection of explants (Plate 1). The differentially expressed ESTs for gingerol production in the ginger somaclone B3 obtained through SSH available at CPBMB (Sreeja, 2017) were used for characterization and validation for high gingerol production.

3.1.2 Chemicals, glasswares and plasticwares

Chemicals for preparation stock solutions of MS medium (Murashige and Skoog, 1962) used for the i*n vitro* induction of microrhizomes and calli were procured from Himedia Laboratories Pvt. Ltd (Mumbai) and SRL Pvt. Ltd (Chennai).Standards for HPLC analysis such as 6-,8-,10- gingerols and 6-shogaol and elicitors like methyl jasmonate, salicylic acid, chitin and chitosan and precursors for gingerol synthesis like phenylalanine, ferrulic acid and coumaric acid were obtained from Sigma Aldrich (Banglore). Molecular and gene expression studies were carried out using chemicals from Invitrogen (USA), Thermo Fisher Scientific (Bangalore) and Applied Biosystems (USA). Trizol was procured from Invitrogen, RNase and Nuclease-free water were supplied by Ambion (USA). Chemicals for chromatography were purchased from Merck Ltd. (Mumbai). All the glasswares used were procured from Borosil (Chennai) and Vensil (Bangalore). The plasticwares were procured from Tarsons India Ltd. (New Delhi). The syringe filters (size 0.22 µm and 13 mm diameter) and membrane filters (size 0.45 µm and 47 mm diameter) for chromatography were procured from Prama Instruments Private Limited (Mumbai) and Spincotech Pvt. Ltd. (Chennai) respectively. .

3.1.3 Laboratory equipment and facilities

The equipment facilities of CPBMB and RTL, KAU were utilized for the study. The *in vitro* induction of microrhizomes and calli were carried out using the facilities of the Tissue culture Laboratory of CPBMB. The facility of DIC of KAU was used for the *in silico* analysis. Experiments on gamma irradiation were done at Radio Tracer Lab, KAU. Metabolomics laboratory of CPBMB was used for estimation of gingerols and shogaol.

The major instruments like HPLC system (Promenience *i*, LC-2030C 3D), Soxhlet apparatus, Real Time PCR machine (Applied Biosystems 7300), Refrigerated Centrifuge (KUBOTA, Model: 3500), Themocycler machine (Applied Biosystems), Gel documentation unit (Gel DoC Tm XR+, Model: Universal hood II by BioRAD), Nanospectrophotometer (NanoDrop ®, Model ND-1000), Digital dry bath (ACCUBLOCK TM, Model: D1100 by lab net inter. Inc),Ultrasound Bath Sonicator

(Pci Analatics) of CPBMB were used the study. For studies on gamma radiation the Gamma Chamber 5000 (BRIT, Mumbai) of RTL, KAU was used.

The *in silico* tools like Vecscreen, Bioedit, BLASTx, BLASTn, Interproscan, ORF finder and Blast2GO were used for analysis and functional annotation of ESTs.

3.2 Methods

3.2.1 Investigations on gingerol synthesis *in vitro*

The synthesis of gingerol *in vitro* was studied using microrhizomes and calli induced from three KAU released varieties (Athira, Karthika and Aswathy) and two parent cultivars (Maran and Rio-de-Janeiro) of ginger. High throughput analytical platform, HPLC was used for analyzing gingerols and shogaol.

3.2.1.1 *In vitro* induction of microrhizomes

3.2.1.1.1 Culture establishment and induction of multiple shoots

Establishment of *in vitro* cultures and induction of multiple shoots were done in five ginger genotypes as per the protocol developed by Paul and Shylaja (2012). The sprouting buds were used as explants (Plate 2). The bud cultures were incubated at a temperature of 24± 2^{o}C with a relative humidity of 60-80 per cent under dark conditions for two weeks. After two weeks, the cultures were incubated in culture room with 24± 2^{o}C temperature and 60-80 per cent relative humidity. A photoperiod of 16 hours light (1000 Lux) and 8 hours darkness was maintained. The multiple shoot cultures were subcultured every 21st day to fresh medium of the same composition. Observations on establishment (%), sprouting (%) and shoot proliferation were recorded.

3.2.1.1.2 Induction of microrhizomes

Microrhizomes were induced in the ginger genotypes as per the protocol developed by Shylaja *et al.* (2016) in MS medium supplemented with sucrose 80 mgL^{-1} using the established multiple shoot cultures. The cultures were incubated in culture room with 24± 2^{o}C temperature, 60-80 per cent relative humidity and a

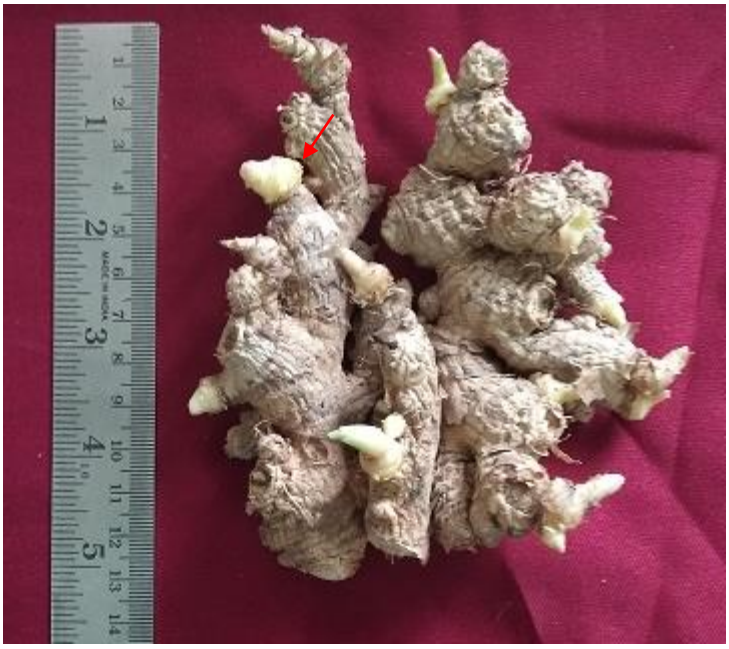


Plate 1: Seed rhizome with rhizome buds

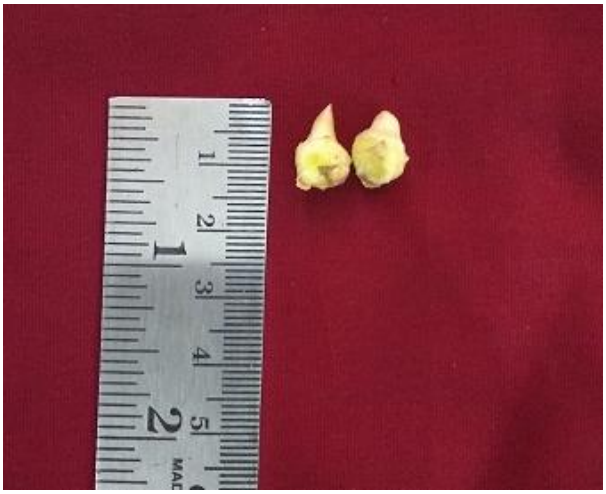


Plate 2: Rhizome buds collected as explants

photoperiod of 16 hours light (1000 Lux) and 8 hours darkness. Observations on initiation and production of microrhizomes were recorded. The number of microrhizomes/culture, fresh weight of microrhizome (g), dry weight of microrhizome (g), driage (%) and production of gingerols and shogaol in the microrhizomes were recorded for one, two and three months after induction of microrhizomes in all the five genotypes.

Further the production of microrhizomes and production of gingerols and shogaol were also observed for four and five months after induction.

Dry weight of the microrhizome was noted after drying two gram weight of the microrhizome in hot air oven at 40^{o}C for three hours after one, two and three months induction period.

3.2.1.2 Induction of Calli

Calli were induced from all the genotypes of ginger studied as per the protocol developed by Resmi *et al.* (2006) using *in vitro* shoot tip as explant in ½ MS medium supplemented with 2,4-D 3 mgL^{-1} and BA 0.5 mgL^{-1} (Callus Induction medium- CI). The cultures were initially incubated in dark with 24± 2^{o}C and 60-80 per cent relative humidity for one month. Observations on callus initiation and growth were recorded. Callusing and callus index were worked out after one month of callusing. The calli developed from the base of the shoot tips were subcultured in the medium of same composition for further proliferation and transferred to culture room with 24± 2^{o}C temperature, 60-80 per cent relative humidity and a photoperiod of 16 hours light (1000 Lux) and 8 hours darkness. The time taken for root and shoot morphogenesis was also observed. The fresh weight (g), dry weight (g), driage (%) of the calli and production of gingerols and shogaol in calli were recorded for one, two and three months induction periods.

Dry weight of the calli was noted after drying two gram weight of the calli in hot air oven at 40^{o}C for four hours.

3.2.1.3 Extraction of oleoresin

The extraction of oleoresin was carried out in Soxhlet apparatus using the solvent acetone as per the protocol reported by AOAC, (1980).

3.2.1.3.1 Extraction of oleoresin from microrhizomes

The microrhizomes one, two and three months after induction were taken for studying synthesis of gingerol. Further microrhizomes induced after four and five months after induction were also studied. Individual shoots with microrhizomes was taken out and washed properly to remove the media adhering to it. Observations on number of microrhizomes per culture were noted. The shoots and the roots were completely removed and fresh microrhizomes (3g) were taken, cut into small bits using sterile blade and crushed using mortar and pestle. The crushed samples were made to a thimble and subjected to Soxhlet extraction using acetone. The extract was later transferred to beakers of known weight. Acetone was evaporated completely and the weight of the beaker with extract was noted to calculate the recovery of oleoresin in percentage.

3.2.1.3.2 Extraction of oleoresin from calli

Calli induced one month, two months and three months after induction were taken for studying synthesis of gingerol. The fresh calli (3 g) were crushed using mortar and pestle. The crushed sample was subjected to Soxhlet extraction using acetone. The extract was transferred to beakers of known weight. Acetone was evaporated completely and further the weight of the beaker was taken to calculate the recovery of oleoresin in percentage.

3.2.1.4 Quantification of gingerols and shogaol

The quantification of gingerols and shogaol in the extracted oleoresin from microrhizomes and calli was carried out using HPLC analytical platform. The analysis was performed using Shimadzu HPLC Prominence *i* coupled with C18 column of 5µm particle size with dimensions 250 mm* 4.6 mm, autosampler, a photodiode array detection system (DAD) with the LC solutions software package.

3.2.1.4.1 Preparation of gingerol and shogaol standards and samples for HPLC analysis

The standards 10 mg each of 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol were dissolved in10 mL of methanol to prepare standard stock solutions of 1000 mg/mL each. From the standard stocks different aliquots were prepared to get concentrations of 500, 250, 200, 100 and 50 mg mL^{-1}. Calibration curves were constructed for 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol by plotting peak area versus concentration.

Methanolic extract of the samples (10 mg/mL) was prepared and filtered using syringe filter and 20µLof the sample was subjected to HPLC analysis for the separation, identification and quantification of the compounds.

3.2.1.4.2HPLC conditions

HPLC conditions for estimation of gingerols and shogaol were followed as reported by Shylaja (2017). The mobile phase solvents consisted of acetonitrile, orthophosporic acid (0.1 %), and methanol in the ratio of 55:44:1 v/v and the washing solvent consists of acetonitrile and water in equal proportions. All the solvents used were of HPLC grade and solvents were filtered using membrane filters. The solvents were properly mixed and degassed using ultrasound bath sonicator prior to use. The flow rate was maintained at 1.3 mL min^{-1} with an injection volume of 20µL. The detection was performed using a photodiode array detection (DAD) system at 282 nm.

Gingerol synthesis *in vitro* at different induction periods of microrhizome and calli of the five different genotypes of ginger was studied and the best genotype and the best induction period of microrhizome and calli were selected for imposing further treatments.

3.2.1.5 Manipulation of macro and micro nutrients for *in vitro* gingerol synthesis using microrhizomes

Microrhizomes were induced using the multiple shoot cultures of the selected genotype in MS medium supplemented with 80 gL^{-1} of sucrose with different levels of macro and micro nutrients as shown below in a and b. Two clumps were inoculated per culture for inducing microrhizomes in each jam bottle containing 50 mL medium. The inoculated cultures were incubated in culture rooms and kept undisturbed for three months.

The treatment details were as shown below:-

a. Different levels of macro and micro nutrients in MS medium supplemented with 80 gL^{-1} of sucrose

1. T1-MS medium (control)
2. T2-MS medium with twice macro nutrients
3. T3-MS medium with twice micro nutrients
4. T4-MS medium with half macro nutrients
5. T5-MS medium with half micro nutrients

b. Different levels of ammoniacal and nitrate nitrogen in MS medium supplemented with 80 gL^{-1} of sucrose

1. T1- MS medium (control)
2. T2- MS medium with twice ammoniacal nitrogen
3. T3- MS medium with twice nitrate nitrogen
4. T4- MS medium with half ammoniacal nitrogen
5. T5- MS medium with half nitrate nitrogen
6. T6- MS medium with twice NH_4^+/twice NO_3^- (T2/T3)
7. T7- MS medium with half NH_4^+/half NO_3^- (T4/T5)

The best treatment identified from the experiment on different levels of macro and micro nutrients and ammoniacal and nitrate nitrogen in MS medium was selected and the combination was designated as MMS medium and further experiments.

3.2.1.6 Effect of elicitors on *in vitro* gingerol synthesis

Different abiotic (Methyl Jasmonate and Salicylic acid) and biotic elicitors (autoclaved fungal mycelium of *Pythium aphanidermatum*, chitosan and chitin) were supplemented to culture media and the effect of elicitation on *in vitro* gingerol synthesis was studied.

3.2.1.6.1 Elicitation using different abiotic elicitors

For studying the influence of abiotic elicitation on *in vitro* synthesis of gingerols, two different abiotic elicitors namely MJ and SA were evaluated at different concentrations *viz* 5, 10 and 15 mgL^{-1}.

3.2.1.6.1.1 Preparation of stock solutions of abiotic elicitors

The stock solution of SA (1 mg mL^{-1}) was prepared. Salicylic acid (0.1g) was first dissolved with few drops of ethanol and the volume was made up to 100mL using distilled water and it was filter sterilized before adding to autoclaved media. Methyl jasmonate was dissolved in few drops of ethanol. The amount of MJ to be added to the media was calculated and added to media directly after filter sterilization.

3.2.1.6.1.2 Effect of abiotic elicitors on *in vitro* gingerol synthesis in ginger microrhizomes

Two studies were conducted simultaneously, in order to compare the accumulation of gingerols in microrhizomes of three months old cultures which was further maintained in MMS liquid medium supplemented with abiotic elicitors (MJ and SA) for a short period of time one week and in microrhizomes induced using MMS semisolid medium supplemented with the above mentioned abiotic elicitors incubated for three months..

Five individual shoots per culture were inoculated from three month old microrhizome cultures in MMS liquid medium supplemented with different abiotic elicitors as mentioned below, without disturbing the shoots and the roots to avoid stress due to injury. The inoculated cultures were then incubated in shaker at 80rpm

with a temperature of 24ºC and maintained for one week. After one week the microrhizomes were taken out from cultures and washed thoroughly, the oleoresin was extracted using acetone and gingerols and shogaol were analyzed using HPLC.

The treatments details were as shown below:

1. T1-MMS liquid medium (control)
2. T2-MMS liquid medium with methyl jasmonate 5 mg L^{-1}
3. T3- MMS liquid medium with methyl jasmonate 10 mg L^{-1}
4. T4- MMS liquid medium with methyl jasmonate 15 mg L^{-1}
5. T5- MMS liquid medium with salicylic acid 5 mg L^{-1}
6. T6- MMS liquid medium with salicylic acid 10mg L^{-1}
7. T7- MMS liquid medium with salicylic acid 15 mg L^{-1}

In the other experiment using MMS semisolid medium supplemented with abiotic elicitors at varying concentrations were studied. Two clumps per culture were inoculated in MMS semisolid medium supplemented with abiotic elicitors as mentioned below to induce microrhizomes. The microrhizomes were collected after three months, extracted using acetone and analyzed for gingerols and shogaol using HPLC as described in section 3.2.1.4.

The treatments details were as shown below:

1. T1-MMS semisolid medium (control)
2. T2-MMS semisolid medium with methyl jasmonate 5 mg L^{-1}
3. T3- MMS semisolid medium with methyl jasmonate 10 mg L^{-1}
4. T4- MMS semisolid medium with methyl jasmonate 15 mg L^{-1}
5. T5- MMS semisolid medium with salicylic acid 5 mg L^{-1}
6. T6- MMS semisolid medium with salicylic acid 10mg L^{-1}
7. T7- MMS semisolid medium with salicylic acid 15 mg L^{-1}

The best medium (MMS liquid or MMS semisolid medium) based on gingerols and shogaol production was identified and used for imposing further treatments.

3.2.1.6.1.3 Effect of abiotic elicitors on *in vitro* gingerol synthesis in ginger calli

The best abiotic elicitor identified from the experiments conducted using microrhizomes was used for the study. Calli were induced in the CI and Modified CI-MCI media (½ MMS medium with 2,4-D 3mgL^{-1} and BA 0.5mgL^{-1}) supplemented with the best identified abiotic elicitor. Three months old calli were extracted with acetone for recovery of oleoresin and the methanolic extract of the samples were analyzed for the estimation of gingerols and shogaol using HPLC as mentioned in section 3.2.1.4.

3.2.1.6.2 Elicitation using different biotic elicitors

The biotic elicitors used for the study included autoclaved fungal mycelium at concentrations 5 and 10 mg L^{-1}, chitosan and chitin each at concentration 50 mg L^{-1} and 100 mg L^{-1}.The autoclaved fungal mycelium of *Pythium aphanidermatum* was used for the study. The culture of *P. aphanidermatum* was obtained from Dept. of Plant Pathology, CoH, KAU. The culture was subcultured in Potato Dextrose Agar (PDA) medium in sterile petriplates. The permanent slide of *P. aphanidermatum* culture was prepared, morphological characters were studied and confirmed. Seven days old culture disc of size 5 mm was transferred to a conical flask containing 100 mL PDA liquid medium. The mycelium was allowed to grow for 15 days. After 15 days the conical flask containing the mycelial growth was autoclaved. The mycelial mat was crushed finely and added to MMS medium and autoclaved.

3.2.1.6.2.1 Preparation of stock solutions of biotic elicitors

Stock solutions of chitin and chitosan (1 mg mL^{-1}) were prepared. Chitin and chitosan 0.1 g each was first dissolved with few drops of two per cent trichloroacetic acid and the volume was made up to 100 mL using distilled water and it was filter sterilized before adding to autoclaved medium.

3.2.1.6.2.2 Effect of biotic elicitors on *in vitro* gingerol synthesis in ginger microrhizomes

Modified MS media with autoclaved fungal mycelium at concentrations 5, 10 mg L^{-1}, chitin and chitosan each at 50 and 100 mg L^{-1} were prepared and used for induction of microrhizomes as shown below. The analysis of gingerols and shogaol from induced microrhizomes were carried out.

The treatments details were as follows:

1. T1-MMS medium (control)
2. T2- MMS medium with autoclaved fungal mycelium-5.0g L^{-1}
3. T3-MMS medium with autoclaved fungal mycelium -10 g L^{-1}
4. T4- MMS medium with chitosan 50 mg L^{-1}
5. T5- MMS medium with chitosan 100 mg L^{-1}
6. T6- MMS medium with chitin 50 mg L^{-1}
7. T7- MMS medium with chitin 100 mg L^{-1}

3.2.1.6.2.3 Effect of biotic elicitors on *in vitro* gingerol synthesis in ginger calli

The best biotic elicitor identified for experiments with microrhizomes was used for studying the effect of biotic elicitors on calli. Calli were induced in CI and MCI media supplemented with the best identified biotic elicitor and incubated for three months. Three months old calli were extracted with acetone for recovery of oleoresin and the methanolic extract of the samples were analyzed for the estimation of gingerols and shogaol as mentioned in section 3.2.1.4.

3.2.1.7 Effect of precursor feeding on *in vitro* gingerol synthesis

The precursors in the biosynthetic pathway of gingerol such as phenylalanine, ferrulic acid and coumaric acid at 30, 60 and 90 mg L^{-1} were tried to study their effects on *in vitro* gingerol synthesis.

3.2.1.7.1 Preparation of stock solutions

Stock solutions of the three precursors were prepared. Phenylalanine was dissolved in few drops of formic acid while coumaric acid and ferrulic acid were dissolved in few drops of ethanol and stock solutions (1 mg mL^{-1}) were prepared using distilled water and filter sterilized before adding to the medium.

3.2.1.7.2 Effect of precursor feeding on *in vitro* gingerol synthesis using ginger microrhizomes

Modified MS medium supplemented with phenylalanine, ferrulic acid and coumaric acid at 30, 60 and 90 m gL^{-1} were prepared as mentioned below and used for induction of microrhizomes for a period of three months. The analysis of gingerols and shogaol from induced microrhizomes were carried out.

The treatments details were as follows:

1. T1-MMS medium (control)
2. T2- MMS medium with phenylalanine – 30 mg L^{-1}
3. T3- MMS medium with phenylalanine – 60 mg L^{-1}
4. T4-MMS medium with phenylalanine – 90 mg L^{-1}
5. T5- MMS medium with ferrulic acid- 30 mg L^{-1}
6. T6- MMS medium with ferrulic acid – 60 mg L^{-1}
7. T7- MMS medium with ferrulic acid – 90 mg L^{-1}
8. T5- MMS medium with coumaric acid- 30 mg L^{-1}
9. T6- MMS medium with coumaric acid – 60 mg L^{-1}
10. T7- MMS medium with coumaric acid – 90 mg L^{-1}

3.2.1.7.3 Effect of precursor feeding on *in vitro* gingerol synthesis in ginger calli

The best precursor identified for experiments with microrhizomes was used for studying the effect of precursor feeding on calli. Calli were induced in the CI and MCI media supplemented with the best identified precursor and incubated for three

months. Three months old calli were extracted with acetone for recovery of oleoresin and the methanolic extract of the samples were analyzed for the estimation of gingerols and shogaol as mentioned in section 3.2.1.4.

3.2.1.8 Effect of gamma radiation on *in vitro* gingerol synthesis

Different doses of gamma radiation 20 Gy and 30 Gy were used to study the effect of gamma radiation on *in vitro* gingerol synthesis.

3.2.1.8.1 Effect of gamma radiation on *in vitro* gingerol synthesis in microrhizomes

Two month old microrhizomes were subjected to different doses of gamma radiation (20 and 30 Gy) and the effect of gamma irradiation on *in vitro* gingerol synthesis was studied. Two month old microrhizomes after removal of shoots and roots were inoculated in MMS medium in the test tube and incubated in culture room for two weeks. After two weeks the microrhizomes were subjected to gamma irradiation. The irradiated microrhizomes were then transferred to fresh medium as mentioned below of the same composition immediately. The cultures were incubated in culture room for one month and observations were taken on shoot morphogenesis. After one month the cultures were grouped into two based on shoot morphogenesis, the irradiated microrhizomes with shoot morphogenesis and the irradiated microrhizomes without shoot morphogenesis. The oleoresin was extracted from each group of fresh microrhizomes and gingerols and shogaol were analyzed using HPLC as described in section 3.2.1.4.

The treatments details were as follows:

1. T1- Control without irradiation
2. T2- Irradiation with gamma rays- 20 Gy
3. T3- Irradiation with gamma rays-30 Gy

3.2.1.8.2 Effect of gamma radiation on *in vitro* gingerol synthesis in calli

The best dose of gamma radiation identified for experiments with microrhizomes was used for studying the effect of various dose of gamma radiation

on calli. Two month old calli induced in CI and MCI media were irradiated with identified dose of gamma radiation. The irradiated cultures were transferred to fresh media of same composition and kept undisturbed for one month. After one month the calli were taken out and oleoresin was extracted with acetone and analyzed for the content of gingerols and shogaol.

3.2.1.9 Effect of MS media supplemented with the identified best elicitor, precursor and dose of gamma radiation on *in vitro* gingerol synthesis in ginger microrhizomes

The best abiotic, biotic elicitor, precursor and dose of gamma radiation identified from the investigations of gingerol synthesis by microrhizomes were used for the study. The MS medium supplemented with the best identified elicitor, precursor and gamma irradiation was used for the investigation for induction of microrhizomes and incubated for three months. The MS medium and MMS medium were used as the control. The analyses of gingerols from induced microrhizomes were carried out.

3.2.2 Elucidation of the expression of gingerol pathway genes (*Chalcone synthase and Phenylalanine lyase* genes) in treatments with highest gingerol content

Real Time PCR assay was carried out to study the gene expression levels of *PAL* and C*HS* genes in the treatments with highest gingerol content identified in experiments with microrhizomes elicited with biotic elicitors, abiotic elicitors and precursors.

3.2.2.1 Isolation and analysis of total RNA

Total RNA was isolated after three months of inoculation from the leaves of the *in vitro* microrhizomes cultures of ginger with highest gingerol accumulation on treatment with highest gingerol content identified in experiments with microrhizomes elicited with biotic elicitors, abiotic elicitors and precursors.

3.2.2.1.1 Sample collection

The leaves were collected from the above mentioned treatments of *in vitro* microrhizome culture. The leaves were cleaned and wiped with 70 per cent ethanol followed by RNaseZap and the sample was flash frozen. These flash frozen samples were stored at -80 ^{0}C deep freezer until isolation of RNA.

3.2.2.1.2 Isolation of total RNA

The total RNA was isolated from the leaves as per the protocol given by Sreeja (2017).

Reagents

- Liquid nitrogen
- TRI reagent (Sigma-Aldrich)
- Chloroform
- Isopropanol
- 3 M sodium acetate
- DEPC ethanol (75%)
- Nuclease free water

Procedure

1. The leaf tissue (100 mg) was ground to a fine powder in a pestle and mortar after addition of liquid nitrogen
2. A pinch of PVP was added to the powdered sample and mixed thoroughly.
3. Trizol (1 mL) was added to the sample after mixing thoroughly with PVP. The sample was transferred then to a 1.5 ml microcentrifuge tube and the homogenate was then vortexed thoroughly for about 15 seconds and incubated at room temperature for three minutes.
   (Incubate the tube by laying it horizontally so as to enhance the surface area.)
4. Chloroform (200 µL) was added to tube and the contents were mixed by inversion for 15 seconds.
5. The tube with the sample was then centrifuged at 12,000 x g for 10 minutes at 4^{0}C.

6. The supernatant was pipetted out and transferred to a new 1.5 ml microcentrifuge tube. To this, again 200 µL of chloroform was added and the contents were mixed by inversion for 15 seconds.
7. The contents were then centrifuged at 12,000 x g for 15 minutes at 4 ^{0}C.
8. The supernatant was taken and transferred to a 1.5 ml microcentrifuge tube. To this 1/10th volume of 3 M sodium acetate and an equal volume of ice cold isopropanol was added and incubated in freezer for one hour.
9. The contents were then centrifuged at 12,000 x g for 10 minutes at 4^{0}C.
10. The supernatant was discarded and the pellet was washed with one milliliter of ice cold 75 per cent DEPC ethanol at 7500 x g for three minutes at 4^{0}C.
11. The ethanol was removed using a pipette and the pellet was dried at room temperature for about two to three minutes until the region just above the pellet becomes free of water.
12. The RNA pellet was then resuspended in 20µL nuclease free water and stored in -80^{0}C for prolonged use.

3.2.2.1.3 Quantity analysis of the isolated total RNA

The quantity of the isolated total RNA was spectrophotometrically analyzed using NanoDrop®ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). One microliter of nuclease free water was used to set the blank to zero before the sample readings were taken. One microliter of the sample was pipetted to quantify and the readings were measured in ng µl^{-1}. The absorbance of nucleic acids was measured at wavelengths of 260 nm and 280 nm. The A_{260}/A_{280} ratio of 1.8-2.0 and A_{260}/A_{230} ratio above one indicated the purity of RNA samples.

3.2.2.1.4 Quality analysis of the total RNA by agarose gel electrophoresis

The quality of the isolated total RNA was analyzed by agarose gel electrophoresis.

Materials required

Agarose (GeNei)
10X MOPS buffer (pH 7.0)
Formaldehyde (37%)
6X Loading/Tracking dye
Ethidium bromide solution (0.5µg/ml)
Electrophoresis unit (Biorad)
Power pack (Biorad)
Casting tray and comb (Biorad)
Gel documentation and analysis system (Biorad)/UV transilluminator (Herolab®)

Procedure

1. One litre of 1X MOPS buffer was prepared by diluting 10X stock solution of MOPS with autoclaved DEPC water. The electrophoresis tank was filled with MOPS buffer.

2. The casting tray and comb was wiped with 70 per cent ethanol and the free ends of the tray were sealed with cello tape. The tray was placed on a horizontal platform and the comb was placed at one end properly.

3. One per cent agarose was prepared for quality analysis. One gram of agarose was dissolved properly in 1X MOPS buffer by boiling in a microwave oven.

4. When solution was cooled to lukewarm temperature, formaldehyde (37 per cent) and ethidium bromide was added. The solution was then mixed properly.

5. The solution was poured into the casting tray and allowed to stay undisturbed for 30 min at room temperature to cool down and solidify.

6. Once the gel was solidified, the comb and the cellotape was removed from both sides.

7. The gel was placed in the electrophoresis tank containing 1X MOPS buffer (with the wells at the cathode end) such that the wells fully submerge in it.

8. Around 2-3 µl of RNA (3 µg total concentration) was loaded into the wells using a micropipette.

9. The cathode and anode ends were connected to the power pack and the gel was run at a steady voltage of 40V.

10. Once the tracking dye reaches 2/3rd of the gel, the power was turned off and bands were visualized.

11. The bands were visualized under UV light and were documented using the gel documentation unit.

3.2.2.2 Reverse transcription Polymerase chain reaction for the synthesis of cDNA

Total RNA consists of rRNA, mRNA, and tRNA. The effective isolation of non-degraded mRNA was very crucial for the first strand synthesis and subsequent amplifications by PCR. The total RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Thermoscientific), as per the guidelines of the manufacturer. The total RNA was incubated with reverse transcriptase enzyme and oligodT primer along with RevertAid.

3.2.2.2.1 First strand cDNA synthesis

The total RNA isolated as mentioned in section 3.2.2.1 were used for first strand cDNA synthesis. The synthesized single stranded cDNA was used for quantitative real time PCR.

Materials required

- Total RNA sample
- Oligo $(dT)_{18}$ primer (100 µM**)**
- Nuclease free water
- 5X reaction buffer
- RiboLockRNase inhibitor (20 U/µl)
- 10 mMdNTP mix
- RevertAid M-MuLV RT (200 U/µl)

Procedure

The following reagents were added to a 0.2ml microcentrifuge tube on ice.

Preparation of first strand cDNA synthesis incubation mix

Component	Volume per reaction
Total RNA	2 µg
Oligo $(dT)_{18}$ primer	1 µl
Nuclease free water	to 12 µl
Total	**20 µl**

1. The contents were gently mixed, briefly centrifuged and incubated at 65^0C for five minutes.
2. The mixture was chilled on ice immediately, spun down in a spinner and again kept on ice.
3. The following components were added

Preparation of first strand cDNA synthesis reaction mix

Component	Volume per reaction (µl)
5X Reaction buffer	4 µl
RiboLockRNase Inhibitor(20 U μl^{-1})	1 µl
10 mMdNTP Mix	2 µl
RevertAid M-MuL RT (200 U μl^{-1})	1 µl
Total	**20 µl**

4. The tube along with the contents were mixed gently and briefly centrifuged in a spinner.

5. The reaction tube was incubated in a thermocycler machine (ProFlex PCR System by Life Technologies) for 60 minutes at 42^0C and the reaction was then terminated by heating at 70^0 C for five minutes.

3.2.2.2.1 Confirmation of the synthesis of first strand cDNA

The first strand cDNA for the selected treatments were amplified by normal PCR using endogenous gene *Actin, PAL* and *CHS* gene specific primers.

Details of endogenous control *Actin* gene primers

Orientation	**Name of primers**	**Sequence (5' to 3')**	**No. of bases**
Forward	*Zo Actin* forward	GTGTGAGCCACACTGTGCCTAT	22
Reverse	*Zo Actin* reverse	CAGCAGTGGTGGTGAACGAAT	21

Amplicon size- 138 bp

Details of *Phenylalanine lyase* gene specific primers

Orientation	**Name of primers**	**Sequence (5' to 3')**	**No. of bases**
Forward	*Zo PAL* forward	CAATCGCAGCCATCGGAAAG	20
Reverse	*Zo PAL* reverse	CTGGAGCTCGGAGCAATAGG	20

Amplicon size- 167 bp

Details of *Chalcone synthase* gene specific primers

Orientation	**Name of primers**	**Sequence (5' to 3')**	**No. of bases**
Forward	*Zo CHS* forward	GGGACACCTGAGGGAAATCG	20
Reverse	*Zo CHS* reverse	CCACCCAAAACACGTCGTTC	20

Amplicon size- 141 bp

Procedure

The Master Mix for the PCR reaction was prepared as mentioned below. The reagents were gently spun and properly mixed. The cDNA was added separately and aliquots of the PCR reaction mix were added to 0.2 ml PCR tubes and the reaction mix was gently spun for proper mixing.

Preparation of PCR Master Mix for first strand cDNA amplification

Components	**Volume per reaction (µl)**
cDNA	2
10X PCR buffer	5
10 mM dNTP Mix	1
25 mM $MgCl_2$	2
Forward primer	1
Reverse primer	1
*Taq*DNA polymerase (3 U μl^{-1})	1
Nuclease free water	37
Total volume	**50**

The PCR reaction was carried out with the following thermal profile

Thermal profile for first strand cDNA amplification

S.No.	Steps	Temperature	Time	No. of cycles
1.	Initial denaturation	95°C	5 minutes	1
2.	Denaturation	95°C	30 seconds	35
3.	Annealing	60°C	30 seconds	
4.	Extension	72°C	30 seconds	
5.	Final extension	72°C	10 minutes	1

The PCR products were stored at -20 ^{0}C.

3.5.1.2.3 Quality analysis of the amplified cDNA by agarose gel electrophoresis

Materials required

- Agarose (HIMEDIA)
- 50x TAE buffer (pH 8.0)
- 6X Loading/Tracking dye
- Ethidium bromide solution (0.5 µg/ml)
- Electrophoresis unit (BioRad)
- Power pack (BioRad)
- Casting tray and comb (BioRad)
- Gel documentation and analysis system (BioRad/UV transilluminator)

Procedure

1. One litre of 1X TAE buffer was prepared by diluting 50 X TAE stock solution with double autoclaved distilled water which was used to fill the electrophoresis tank and to prepare the gel.
2. The casting dam, casting tray and comb was wiped with 70 per cent ethanol. The casting tray was mounted horizontally over the casting dam such that the

free ends were tightened and the comb was placed on the gel tray at one end of the tray at about one centimeter from the ends of the tray.

3. Agarose gel 1.8 per cent (1.8 g in 100 ml) was prepared in a conical flask with 100 ml 1X TAE buffer by boiling in a microwave oven to dissolve the agarose completely.
4. The solution was then cooled to lukewarm temperature, ethidium bromide was added and the solution was mixed properly.
5. The solution was then poured on the gel casting tray. The gel was left undisturbed so as to solidify for about 30 min.
6. The gel tray containing the gel and the comb was removed from the casting dam once the gel got solidified.
7. The gel was then placed in the electrophoresis tank containing 1X TAE buffer (with the wells at the cathode end) such that the wells are completely submerged in the buffer.
8. Around 20 µl of the PCR product mixed with 2 µl 6X loading dye were loaded into the wells with the help of a micropipette.
9. The cathode and the anode ends were connected to the power pack and the gel was run at a steady voltage of 80 V.
10. The power was turned off when the tracking dye reached about 2/3rd of the gel.
11. The gel was placed on a gel documentation unit. The bands in the gel were then visualized under UV light and documented.

3.2.2.3 Real time PCR assay for gene expression analysis

The gene expression analysis of *Phenylalanine lyase* (*PAL*) and *Chalcone synthase* (*CHS)* was performed using Real time PCR with the first strand cDNA synthesized as mentioned in 3.2.2.2. The first strand cDNA was synthesized from the total RNA isolated after three months of inoculation from the leaves of the *in vitro* microrhizomes cultures of ginger with highest gingerol accumulation on treatment with biotic and abiotic elicitors and precursors. *Actin* gene was used as endogenous (control) gene.

Materials required

- SYBR® Premix Ex Taq™(TliRNaseH Plus) (2X)
- ROX Reference Dye (50X)
- cDNA template
- Forward and reverse primers
- Nuclease-Free water
- 96 well plates and the seals for the plate

3.2.2.2.1 General precautions followed when performing Real-Time PCR assay

- The contents of SYBR® Premix Ex Taq™ (TliRNaseH Plus) 2X and ROX Reference Dye (50 X) was briefly centrifuged before use to ensure adequate reactivity
- The cDNA samples and primers were gradually thawed down and vortexed gently for proper mixing
- Exposure of SYBR Green Master Mix to direct light was avoided as it is sensitive to light
- In order to avoid contamination between the samples fresh disposable tips were used

3.2.2.2.2 Baseline and threshold value setting

The initial cycle of the PCR reaction which shows a slight change in the fluorescent signal is generally regarded as the baseline. A numerical value was assigned for each run. This corresponds to a statistically significant point over the calculated baseline and the value is considered as the threshold value. The point of measurement at the baseline and threshold level should be exactly determined as this corresponds to the amount of the target gene with in a reaction. By default the Applied Biosystems 7300 sequence detection software system was set from three to fifteen (3-15) cycles.

3.2.2.2.3 Real-time PCR reaction mix and temperature profile for the reaction

The Real Time PCR reactions for the test genes (*PAL* and *CHS*) and the endogenous housekeeping gene (*Actin*) were carried out using the first strand cDNA obtained from the RNAs isolated from the leaves of various treatments of *in vitro*

microrhizome cultures. The master mix was prepared and spun down properly to ensure proper mixing of the components. The cDNA samples were loaded carefully on a 96 well plate and the master mix was added separately and mixed gently by pipetting.

Preparation of PCR Master Mix for Real Time PCR

Components	**Volume (µl)**
SYBR® Premix Ex Taq^{TM} (2 X)	10.0
PCR Forward Primer (10 µM)	0.4
PCR Reverse Primer (10 µM)	0.4
ROX Reference Dye (50 X)	0.4
Template (<100 ng)	2.0
Sterile distilled water	6.8
Total	**20.0**

Thermal profile setting for Real Time PCR to study the gene expression

Stage: 1 Initial denaturation **Repeat: 1**

95^0 C for 30 seconds

Stage: 2 PCR cycle **Repeat: 40**

95^0 C for 5 seconds

60^0C for 31 seconds

Stage: 3 For melting profile cycle **Repeat: 1**

95^0 C for 15 second

60^0C for 1 minute

95^0 C for 15 seconds

Stage: 4 Storage

12^0 C for 10 minutes

3.2.2.2.4 Relative quantification analysis

Generally two methods are used for the analysis of Real Time PCR data - absolute quantification and relative quantification. In absolute quantification, the C_T of the unknown samples is generally compared against a standard curve and with known copy numbers. However in relative quantification the target is compared either by using the standard curve or by comparative C_T method with an internal standard. The fluorescence signals were recorded at each cycle in order to monitor the amplified product generated. The difference in efficiency of amplification was taken into account for the calculation of initial target levels. To calculate the fold change expression the relative quantification with PCR efficiency correction was adopted. The relative expression ratio of the gene was determined by using comparative C_T method as stated by Livak and Schmittgen, (2001).

Relative quantification method was employed in this experiment to compare the expression levels of *PAL* and *CHS* genes in various treatments of *in vitro* microrhizome cultures and *Actin* was used as the endogenous house-keeping gene.

3.2.3 Analysis of available ESTs and validation of the identified ESTs for high gingerol production using real time PCR

Suppression subtractive hybridization was done at CPBMB by Sreeja (2017) to identify differentially expressed genes in the somaclone B3 and control Maran. Eleven rhizome ESTs and four leaf ESTs were available. EST library was prepared, clones were sequenced and the sequence data were analyzed using bioinformatic tools.

3.2.3.1 Preparation of EST library

Procedure

1. Fresh grid plates of the clones were prepared by subculturing from the master grid plate and were incubated overnight at 37^0C.

2. Fifty milliliters of LB medium with ampicillin (10 %) was prepared. About 1.5 mL was poured into individual sterile vials.

3. Each clone was picked from the fresh grid plates using a sterile loop and was stabbed deep down into the soft agar several times.

4. The vials were incubated overnight at 37 ^{0}C for 8-12 hours leaving the cap slightly loose.

5. The vials were sealed tightly and stored in the dark at 4^{0}C

3.2.3.2 Sequencing of EST clones

The clones were sent for Sanger sequencing (forward and reverse) to DNA sequencing facility of Scigenom, Cochin. The primers used for sequencing were universal M13 forward and reverse primers. A total of 15 clones for ESTs from rhizomes and leaves were sent for sequencing.

3.2.3.3 Analysis of EST sequences using various bioinformatic tools

Various online bioinformatic tools were used for EST sequence analysis.

1. Screening and removal of vector and adaptor sequences

The region covering vector and adaptor sequences were detected using the VecScreen tool (www.ncbi.nim.nih.gov/tools/vecscreen) provided by NCBI. For removing the vector and adaptor sequences, Bioedit (Biological Sequence Alignment tool) was used. Screening and removal of vector sequences was done for both forward and reverse sequences.

2. Assembly of forward and reverse sequences

The edited forward and reverse sequences were assembled using CAP3 Sequence Assembly Program (http://doua.prabi.fr/software/cap3) provided by PRABI Doua.

3. Homology search for assembled sequences

All the assembled sequences were compared with the sequences in the NCBI database using the BLAST tool (www.ncbi.nim.nih.gov/blast) provided by NCBI. Blastn and Blastx tool were used for Nucleotide-Nucleotide and Nucleotide-Protein comparison respectively. The best sequence alignment data were recorded.

4. Detection of Open Reading Frame (ORF)

The program 'ORF finder'(https://www.ncbi.nim.nih.gov/orffinder) by NCBI was used to find the open reading frames present in the nucleotide sequence. The program displayed all the ORFs as well as the amino acid sequence for the longest ORF present in the query sequence and the details were saved.

5. Analysis of amino acid sequence

The longest amino acid sequence analysis using the program InterProScan (http://www.ebi.uk/interpro/search/sequence-search). The program provided the details of amino acid length, protein family, domain and repeats, conserved domains, biological process, molecular function and cellular components.

6. Functional annotation.

The Functional annotation for the gene of the interest was done using the program KAAS-KEGG Automatic Annotation Server (http//www.genome.jp'tools'kaas'). Blast comparisons were done against KEGG GENES database and the KEGG orthology and pathways were obtained.

7. Blast2GO annotation

Blast2GO was carried out with both rhizome and leaf ESTs for high throughput functional annotation .The functional annotation was represented through the Gene ontology. The software was downloaded (http.//www.blast2go.com), installed and run, which was manufactured by BioBam_Bioinformatics.

3.2.3.4 Validation of identified ESTs for high gingerol using Real time PCR

The EST sequence obtained through the process of SSH by Sreeja (2017) was validated by using real time PCR. Blastx results done by her showed homology of the

EST sequence with 3-ketoacyl CoA thiolase. Thiolase was reported to be an important enzyme involved in beta oxidation cycle which in turn is crucial for plant germination and sustainability. For different cellular functions, the final step of breakage of two carbon unit was performed by 3-ketoacyl-CoA thiolase with release of shortened fatty acids (Pye *et al.,* 2010). The thiolases are also known as acetyl-coenzyme A acetyltransferases (ACAT). Through a thioester-dependent Claisen-condensation-reaction mechanism the thiolase superfamily enzymes catalyse the C-C bond formation. The thiolases and Acetyl-CoA-acetyltransferase are involved in the benzoate degradation pathway and these enzymes are also involved in valine, leucine, and isoleucine degradation pathway. Both the pathways produced acetyl CoA which is the central molecule involved in plant metabolic pathways along with interconnecting different biochemical pathways.

3.2.3.4.1 Isolation and analysis of total RNA

Total RNA was isolated from leaves of field grown ginger genotypes Maran, B3, Athira, Karthika and Aswathy. The RNA was isolated from the samples as per the procedure discussed in section 3.2.2.1.2.The quantity and quality analyses were carried out as per the procedure explained in section 3.2.2.1.3 and 3.2.2.1.4 respectively. First strand cDNA was synthesized using the RNA isolated from the samples as per the section 3.2.2.2.1.

3.2.3.4.2 Confirmation of the synthesis of first strand cDNA

The first strand cDNA synthesis was amplified by normal PCR using endogenous gene *Actin* and *Thiolase* gene specific primers as per the procedure explained in section 3.2.2.2.2.

Details of *Thiolase* gene specific primers

Orientation	Name of primers	Sequence (5' to 3')	No. of bases
Forward	*Zo Thiolase* forward	ACTGGATCCTCTTGGCATTG	20
Reverse	*Zo Thiolase* reverse	ATGGAGGTGCTCTTGCTCTC	20

Amplicon size- 204 bp

3.2.3.4.3 Real time assay

The gene expression analysis of *Thiolase* using the first strand cDNA from the ginger genotypes was performed using real time PCR. The mastermix was prepared and the thermal profile was set as per the section 3.2.2.2.3 and relative quantification was done to find out the fold change in gene expression.

3.3 Statistical analysis

The experiments were conducted in Completely Randomized Design. All the experiments were done using three biological and two technical replicates. The data were subjected to statistical analysis, employing one factor analysis of variance (Snedecor and Cochran, 1989) using the software OP stat.

Results

4. RESULTS

The results obtained from the present study entitled "*In vitro* synthesis of gingerol and analysis of expressed sequence tags for gingerol production in ginger (*Zingiber officinale* Rosc.) are presented in this chapter.

4.1 Investigations on gingerol synthesis *in vitro*

4.1.1 Gingerol synthesis in ginger microrhizomes induced *in vitro*

4.1.1.1Culture establishment and shoot proliferation in different genotypes of ginger

Cultures were initiated from the different genotypes of ginger (varieties Athira, Karthika and their parent cultivar Maran and the variety Aswathy and its parent cultivar Rio-de-Janeiro) as per the protocol reported by Paul and Shylaja (2012). Observations on culture establishment, sprouting of buds and number of shoots proliferated/culture one, two and three months after inoculation were recorded and presented in Table 1.

Culture establishment varied from 68.44 to 76.81 per cent. The highest establishment of culture was observed in the cultivar Rio-de-Janeiro (76.81 %) which was significantly superior over other genotypes. Next to the cultivar Rio-de-Janeiro, the variety Athira recorded higher culture establishment of 74.65 per cent. The lowest culture establishment was recorded in the variety Karthika (68.44 %). Sprouting of buds was observed in more than 60-70 percent of the established cultures in different ginger genotypes. Sprouting of buds varied from 60.09 to 75.37 per cent. The cultivar Rio-de-Janeiro recorded the highest sprouting (75.37 %) and the variety Karthika recorded the lowest (60.09 %).

The sprouted cultures were multiplied and the number of shoots proliferated/culture was recorded one, two and three months after inoculation. No significant difference was observed between the genotypes with respect to shoot proliferation one, two and three months after inoculation.

Table 1:-Establishment of rhizome bud explants and shoot proliferation in different ginger genotypes

Genotypes	Establishment (%)	Sprouting (%)	Number of shoots proliferated/culture		
			1 MAI	2 MAI	3 MAI
Athira	74.65	70.27	1.50	4.50	11.50
Karthika	68.44	60.09	0.75	3.25	8.00
Aswathy	72.26	67.79	1.25	4.00	9.50
Maran	70.81	64.96	1.25	3.25	8.75
Rio-de-Janeiro	76.81	75.37	1.25	5.00	12.25
CD(0.05)	0.85	0.83	NS	NS	NS
SE(m)±	0.28	0.27	0.37	0.46	1.08

MAI- Months after inoculation NS-Not significant

Data represent the mean of four replications with ten cultures/ replication

4.1.1.2 Effect of culture period on microrhizome production in different ginger genotypes

Induction of microrhizomes was done from the multiple shoot cultures of all the ginger genotypes using MS medium supplemented with 80 g L^{-1} sucrose. Different microrhizome induction periods *viz*, one, two, three, four and five months were studied. The days taken for initiation of microrhizomes, number of microrhizomes/culture and fresh weight, dry weight and driage of microrhizomes for the different genotypes were recorded for different induction periods. The data are presented in Tables 2, 3 and 4.

Initiation of microrhizomes started as bulging at the base of the shoots, after one and a half to three weeks of inoculation. An average of 14.50 days was taken by the genotypes for the initiation of microrhizomes. Varietal difference was observed in the initiation of microrhizomes. The cultivars Maran (19.25 days) and Rio-de-Janeiro (15.75 days) recorded more days for initiation compared to the varieties Athira, Karthika and Aswathy. The variety Aswathy responded within 9.25 days of inoculation followed by variety Karthika (14.00 days) and Athira (14.25 days).

The number of microrhizomes/ culture increased with increase in induction periods and the highest number was recorded three months after induction. An average of 6.30, 11.30 and 17.56 microrhizomes was produced respectively at one, two and three months of induction by the ginger genotypes.

At one month of induction, the parent cultivar Rio-de-Janeiro recorded the highest number of microrhizomes/culture (12.00) followed by variety Athira recording 9.50 microrhizomes. No significant difference was observed between the varieties Karthika (2.50), Aswathy (4.50) and the cultivar Maran (3.00) with respect to the number of microrhizomes/ culture. The highest number of microrhizomes/ culture after two months of induction was recorded for the cultivar Rio-de-Janeiro (16.00) which was on par with the variety Athira (13.50) while the varieties Aswathy (10.00), Karthika (8.33) and the cultivar Maran (8.67) were on par with respect to number of microrhizomes/ culture .In three months induction period (Plates 3 and 4) among the parent cultivars, the cultivar Rio-de-Janeiro (26.83) and among the three

varieties, the variety Athira (20.16) recorded the highest number of microrhizomes/culture. The variety Aswathy recorded 16.33 microrhizomes while the lowest was recorded by variety Karthika (12.50) and its parent cultivar Maran (12.00) which was on par with respect to number of microrhizomes.

Further, the production of microrhizomes and accumulation of gingerols and shogaol were studied for four and five months after induction. Highest production of microrhizomes/ culture was recorded in the cultivar Rio-de-Janeiro, four and five months after induction, recording 28.75 and 29.50 microrhizomes (Plate 5). The lowest number of microrhizomes/ culture was recorded by variety Karthika. In all the genotypes, the increase in the number of microrhizomes was less for the fourth and fifth months, compared to the second and third month.

The trend in the production of microrhizomes at different induction periods is presented in Figure 1. The number of microrhizomes was more in the variety of Karthika and its parent cultivar Maran, in the second month. In the varieties Athira, Aswathy and the cultivar Rio-de-Janeiro, the increase in the number of microrhizomes observed was more in the third month. The variety Aswathy produced more number of microrhizomes in fourth month while in the fifth month, the variety Karthika and its parent cultivar recorded more number of microrhizomes.

The fresh weight of the microrhizomes was found to increase with the increase in induction periods in all the five genotypes of the ginger which recorded the highest at five months of induction. The fresh weight of the microrhizomes increased almost three-fold by third month in all the genotypes, after which the increase was not substantial. The ginger genotypes produced an average fresh weight of 0.153 g, 0.192 g and 0.474 g at one, two, and three months of induction respectively.

At one month after induction, no significant difference was observed in the fresh weight of the microrhizomes among the genotypes. In two months induction period, the fresh weight of the microrhizomes in the varieties Athira (0.242 g), Aswathy (0.233 g) and the cultivar Rio-de-Janeiro (0.192 g) was on par. The lowest fresh weight of the microrhizome was recorded by the variety Karthika (0.147 g) which was on par with the cultivar Maran (0.170 g), at two months of induction. For

three months induction period, no significant difference was observed in the fresh weight of microrhizome in the different genotypes of ginger studied. Further on four and five month induction periods with respect to fresh weight, the variety Athira recorded the highest fresh weight, which was statistically on par with the variety Aswathy.

The trend in the fresh weight of the microrhizomes observed the highest in the variety Aswathy and the lowest in the variety Karthika for the second month. Even though the increase in the fresh weight of the microrhizomes was lowest in variety Karthika for the second month, it was the highest for the third month. The increase in fresh weight of microrhizomes was more in varieties as compared to the parent cultivars in the third month induction period. The increase in fresh weight in all the genotypes observed was less for the fourth and fifth month. The cultivar Rio-de-Janeiro recorded the highest increase in fresh weight for fourth and fifth month after induction (Figure 2).

The dry weight of the microrhizomes was recorded after drying an equal quantity of fresh microrhizomes (2 g) in a hot air oven. The dry weight of the microrhizome increased with increase of induction periods from one month to third month depending on the genotype. The highest dry weight was recorded three months after induction. An average of 0.027 g, 0.037 g and 0.138 g dry weight was recorded respectively at one, two and three months after induction irrespective of the genotypes. Even though the fresh weight of the microrhizome was less in the variety Karthika, the dry weight recorded was the highest for the three different induction periods.

At one month of induction, the varieties Athira (0.027g) and Aswathy (0.029 g) were on par with respect to the dry weight. The dry weight of 0.020 g recorded for the parent cultivars Maran and Rio- de-Janeiro at one month after induction. The same trend was followed for the two months induction period with the variety Karthika significantly superior to other genotypes with respect to dry weight recording 0.052 g followed by varieties Athira (0.040 g) and Aswathy (0.036 g) which were on par. For the third month after induction, the highest dry weight of the

microrhizome was recorded in variety Karthika (0.074 g) while all the other genotypes were on par with each other. In the variety Karthika, the increase in dry weight recorded more for the second month and the third month compared to all other genotypes of ginger.

The driage of the microrhizomes was calculated for one, two and three months after induction. The driage was found increased from one month to three months after induction and recorded the highest at three months of induction. Irrespective of the genotypes studied, an average of 1.38, 1.83, and 2.80 per cent driage was recorded at one, two and three months of induction. The driage recorded for the varieties were more compared to the cultivars studied for all the three different induction periods. The variety Karthika recorded the highest driage for all the consecutive three months studied.

The variety Karthika recorded the highest driage of 2.05 per cent one month after induction, 2.60 per cent two months after induction and 3.01 per cent three months after induction. The lowest driage of 1.00 per cent was recorded in the cultivar Maran which was on par with the cultivar Rio-de-Janeiro (1.03 %) at one month of induction. At two months of induction, the variety Athira (1.99%) and variety Aswathy (1.82 %) were on par with respect to driage. The lowest driage of 1.31 per cent and 2.23 per cent was recorded by the cultivar Maran respectively after two and three months of induction.

Table 2:- Production and growth of microrhizomes in different ginger genotypes one month after induction

Genotypes	Days taken for initiation of microrhizomes	Number of microrhizomes /culture	Fresh weight of microrhizome (g)	Driage of microrhizomes (%)		
				Initial fresh weight (g)	Dry weight (g)	Driage (%)
Athira	14.25	9.50	0.206	2.0	0.027	1.39
Karthika	14.00	2.50	0.134	2.0	0.041	2.05
Aswathy	9.25	4.50	0.136	2.0	0.029	1.42
Maran	19.25	3.00	0.129	2.0	0.020	1.00
Rio de Janeiro	15.75	12.00	0.162	2.0	0.020	1.03
Mean	14.50	6.30	0.153	-	0.027	1.38
CD(0.05)	1.30	1.56	NS	-	0.005	0.06
SE(m)±	0.43	0.32	0.029		0.002	0.02

NS-Not Significant

Data represent the mean of four replications with eight cultures/ replication

Table 3:- Production and growth of microrhizomes in different ginger genotypes two months after induction

Genotypes	Number of microrhizomes /culture	Fresh weight of microrhizome (g)	Driage of microrhizomes (%)		
			Initial fresh weight (g)	Dry weight (g)	Driage (%)
Athira	13.50	0.242	2.0	0.040	1.99
Karthika	8.33	0.147	2.0	0.052	2.60
Aswathy	10.00	0.233	2.0	0.036	1.82
Maran	8.67	0.170	2.0	0.026	1.31
Rio-de-Janeiro	16.00	0.192	2.0	0.029	1.43
Mean	11.30	0.192	-	0.037	1.83
CD (0.05)	2.77	0.062	-	0.004	0.05
SE(m)	0.95	0.021	-	0.001	0.02

Data represent the mean of four replications with eight cultures/ replication

Table 4:- Production and growth of microrhizomes in different ginger genotypes three months after induction

Genotypes	Number of microrhizomes/ culture	Fresh weight of microrhizome (g)	Driage of microrhizomes (%)		
			Initial fresh weight(g)	Dry weight (g)	Driage (%)
Athira	20.16	0.612	2.0	0.060	3.01
Karthika	12.50	0.381	2.0	0.074	3.50
Aswathy	16.33	0.569	2.0	0.056	2.80
Maran	12.00	0.373	2.0	0.045	2.23
Rio-de-Janeiro	26.83	0.434	2.0	0.050	2.45
Mean	17.56	0.474	-	0.138	2.80
CD (0.05)	3.69	NS	-	0.004	0.13
SE(m)±	0.79	0.032	-	0.001	0.03

Data represent the mean of four replications with eight cultures/ replication

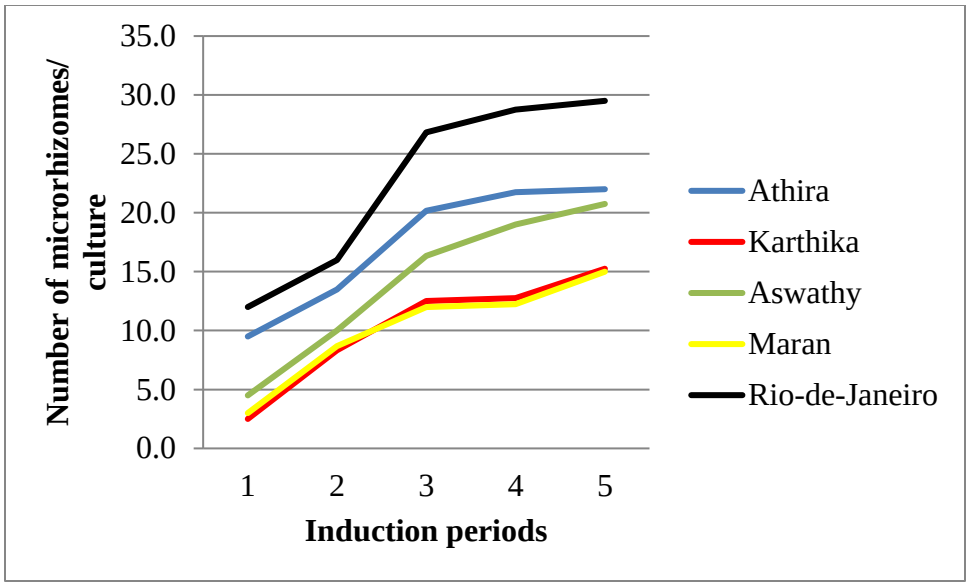


Figure 1:- Trend in production of microrhizomes /culture in different genotypes of ginger for different induction periods

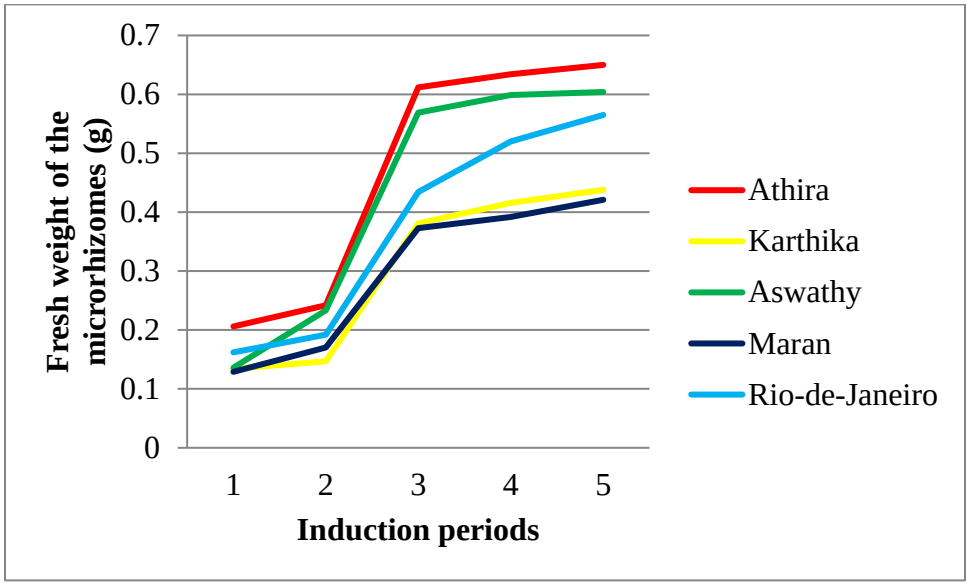
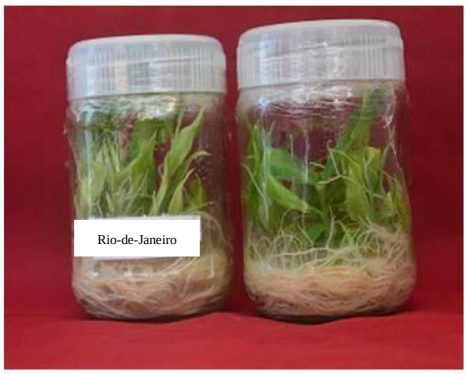


Figure 2:- Trend in fresh weight of microrhizome in different genotypes of ginger for different induction periods



Maran

Plate 3:-Microrhizomes of different ginger genotypes in three month old cultures

Plate 4:- Microrhizomes of different ginger genotypes in three month old cultures

Variety Aswathy 4 Month after induction

Variety Aswathy 5 Month after induction

Plate 5:-Microrhizomes of the variety Aswathy in four and five month old cultures

4.1.1.3 Effect culture period of microrhizome on *in vitro* synthesis of gingerols and shogaol in different ginger genotypes (fresh weight basis)

Oleoresin was extracted from fresh microrhizomes of all the five genotypes of ginger at different induction periods using Soxhlet apparatus with acetone as solvent. The oleoresin was subjected to HPLC analysis to separate the major pungency principles such as gingerols and shogaol. The data on oleoresin recovery (%), gingerols (%) and shogaol (%) content in microrhizomes for five different induction periods are presented in the Tables 5, 6,7, 8, 9, and 10.

The recovery of oleoresin was found increased with increase of induction periods and recorded the highest after five months induction period. An average oleoresin recovery of 1.67, 2.11 and 2.94 per cent was recorded respectively for one, two and three months induction periods irrespective of the genotypes studied. The recovery of oleoresin was observed the highest in the variety Aswathy for all the consecutive induction periods studied.

Oleoresin recovery recorded the highest in the variety Aswathy (1.99%) one month after induction. Next to the variety Aswathy, higher oleoresin recovery was noted in the variety Athira (1.84 %) which was statistically insignificant with the cultivar Rio-de-Janeiro (1.80 %). The variety Karthika recorded an oleoresin recovery of 1.59 per cent and the lowest was recorded by cultivar Maran (1.15 %), one month after induction. After two months induction period, no significant difference was observed between the variety Aswathy (2.63 %) which recorded the highest and its parent cultivar Rio-de-Janeiro (2.50 %) in the oleoresin recovery. The varieties Athira and Karthika recorded an oleoresin recovery of 2.12 per cent and 1.83 percent respectively after two month of induction and the lowest oleoresin recovery was noted in the cultivar Maran (1.46 %). After three month of induction, the highest oleoresin recovery was recorded by the variety Aswathy (3.38 %) followed by the variety Athira (3.07 %) which was on par with the cultivar Rio-de-Janeiro (3.04 %).The variety Karthika recorded an oleoresin recovery of 2.74 per cent and the lowest was recorded by its parent cultivar Maran (2.46 %) after three month induction period.

Further, in four and five month induction periods, the highest oleoresin recovery of 3.70 and 3.94 per cent respectively were recorded by the variety Aswathy. The lowest was recorded by the cultivar Maran for both the induction periods of four and five months. The variety Aswathy and its parent cultivar Rio-de-Janeiro recorded the highest increase in oleoresin recovery for the second month while the varieties Athira, Karthika and the cultivar Maran recorded a maximum increase for oleoresin recovery in the third month. The increase of oleoresin recovery was observed the lowest for the fourth and fifth month. The cultivar Rio-de-Janeiro recorded the highest increase in oleoresin recovery for the fourth month while in the fifth month, the variety Karthika recorded the highest (Figure 3).

In the microrhizomes, irrespective of growth stages the major components separated were 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol. The gingerols and shogaol increased with increase in induction period and recorded the highest four and five months after induction respectively. A decline in gingerol accumulation was observed after five months induction period. Irrespective of the genotypes studied, an average gingerol content of 0.016, 0.178, 0.545, 1.287 and 1.135 per cent recorded respectively after one, two, three, four and five months induction periods. Highest shogaol accumulation was observed after five months induction period recording an average of 0.087 per cent.

The gingerols and the shogaol content were observed almost zero in the initial growth stage of one month induction period. The highest accumulation of total gingerols (sum total of 6, 8, and 10- gingerols) were recorded in the variety Aswathy in all the five induction periods. For one month induction period, the accumulation of total gingerols was observed only in the varieties Aswathy (0.060 %) and Athira (0.020 %).After two month of induction, the highest total gingerol was recorded in the variety Aswathy (0.295 %). Next to the variety Aswathy, the higher total gingerol was recorded in the variety Athira (0.195 %) followed by the variety Karthika (0.145 %) and the cultivar Rio-de-Janeiro (0.145 %).The variety Aswathy recorded the highest total gingerol of 0.850,1.615 and 1.550 per cent after three, four and five months of induction respectively. The varieties recorded higher increase in total gingerol as

compared to the cultivars for all the induction periods and among the varieties, the variety Aswathy recorded the highest increase in total gingerols (Figure 4).

The production of 6-gingerols increased with increase in induction periods, and recorded the highest at five month induction period. Irrespective of the genotypes studied, an average of 0.016, 0.171, 0.486, 1.031 and 0.926 per cent 6-gingerols accumulated after one, two, three, four and five months of induction periods. The highest 6-gingerol production was observed in the ginger varieties compared to the cultivars, the highest accumulation was recorded in variety Aswathy for all the five different induction periods.

Only in the varieties Aswathy (0.060%) and Athira (0.020%), 6-gingerol was accumulated after one month of induction. At two months induction period, the accumulation of 6-gingerol recorded highest in the variety Aswathy (0.290 %) followed by variety Athira (0.185 %). The variety Karthika recorded 0.145 per cent 6-gingerol which was on par with the cultivar Rio-de-Janeiro at two months induction period. The lowest 6- gingerol was produced by the cultivar Maran (0.100%). The same trend observed for two months induction period was followed for the three months induction period with respect to 6-gingerol. The variety Aswathy recorded the highest accumulation of 6-gingerol (0.720 %) followed by variety Athira (0.570 %). There were no significant differences observed between the variety Karthika (0.475 %) and the cultivar Rio-de-Janeiro (0.455 %). The lowest 6- gingerol accumulation was noticed in the cultivar Maran (0.210 %)after three months induction period.

Further, at four and five months induction periods, the variety Aswathy recorded the highest accumulation of 6-gingerols while the lowest being recorded in the cultivar Maran. Among the varieties studied, variety Aswathy showed highest increase in accumulation of 6-gingerols for all the induction periods studied.

Accumulation of 8-gingerol was not observed for one month and two months induction period in any of the ginger genotypes studied. The production of 8-gingerols increased from three months induction period and recorded the highest after five months induction periods in all the genotypes. After three month of induction,

Table 5:- Recovery of oleoresin from one, two, three, four and five month old microrhizome in different ginger genotypes

Genotypes	Oleoresin recovery (%)				
	1MAI	2 MAI	3 MAI	4MAI	5MAI
Athira	1.84	2.12	3.07	3.29	3.39
Karthika	1.59	1.83	2.74	2.91	3.03
Aswathy	1.99	2.63	3.38	3.70	3.94
Maran	1.15	1.46	2.46	2.55	2.69
Rio-de-Janeiro	1.80	2.50	3.04	3.50	3.76
Mean	1.67	2.11	2.94	3.19	3.36
CD (0.05)	0.13	0.14	0.17	0.05	0.11
SE(m)±	0.05	0.06	0.03	0.07	0.06

MAI-Months after induction

Data represent the mean of three biological and two technical replications

Table 6:- Gingerols and shogaol content in one month old microrhizomes of different ginger genotypes (fresh weight basis)

Genotypes	Pungency principles in one month old ginger microrhizomes				
	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Total gingerol (%)	6-shogaol (%)
Athira	0.020	0.000	0.000	0.020	0.000
Karthika	0.000	0.000	0.000	0.000	0.000
Aswathy	0.060	0.000	0.000	0.060	0.000
Maran	0.000	0.000	0.000	0.000	0.000
Rio- de-Janeiro	0.000	0.000	0.000	0.000	0.000
Mean	0.016	0.000	0.000	0.016	0.000
CD (0.05)	NS	NS	NS	NS	NS
SE(m)±	0.002	0.000	0.000	0.002	0.000

Data represent the mean of three biological and two technical replications

Table 7:- Gingerols and shogaol content in two month old microrhizomes of different ginger genotypes (fresh weight basis)

Genotypes	Pungency principles in two month old ginger microrhizomes				
	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Total gingerol (%)	6-shogaol (%)
Athira	0.185	0.000	0.010	0.195	0.030
Karthika	0.145	0.000	0.010	0.145	0.035
Aswathy	0.290	0.000	0.010	0.295	0.035
Maran	0.100	0.000	0.010	0.110	0.010
Rio- de-Janeiro	0.135	0.000	0.010	0.145	0.030
Mean	0.171	0.000	0.010	0.178	0.028
CD(0.05)	0.022	NS	NS	0.024	0.012
SE(m)±	0.006	0.000	0.000	0.005	0.004

NS-Not Significant

Data represent the mean of three biological and two technical replications

Table 8:- Gingerols and shogaol content in three month old microrhizomes of different ginger genotypes (fresh weight basis)

Genotypes	Pungency principles in three month old ginger microrhizomes				
	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Total gingerol (%)	6-shogaol (%)
Athira	0.570	0.010	0.045	0.615	0.025
Karthika	0.475	0.010	0.060	0.540	0.070
Aswathy	0.720	0.010	0.110	0.850	0.050
Maran	0.210	0.000	0.010	0.220	0.020
Rio-de-Janerio	0.455	0.000	0.040	0.500	0.040
Mean	0.486	0.006	0.053	0.545	0.041
CD(0.05)	0.084	NS	0.030	0.091	0.008
SE(m)±	0.008	0.000	0.008	0.025	0.002

Data represent the mean of three biological and two technical replications

Table 9:- Gingerols and shogaol content in four month old microrhizomes of different ginger genotypes (fresh weight basis)

Genotypes	Pungency principles in four month old ginger microrhizomes				
	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Total gingerol (%)	6-shogaol (%)
Athira	1.123	0.020	0.210	1.375	0.062
Karthika	1.053	0.020	0.189	1.245	0.126
Aswathy	1.273	0.021	0.310	1.615	0.105
Maran	0.710	0.000	0.115	1.085	0.035
Rio-de-Janerio	0.998	0.008	0.155	1.115	0.050
Mean	1.031	0.014	0.196	1.287	0.076
CD(0.05)	0.039	0.003	0.012	0.020	0.013
SE(m)±	0.013	0.001	0.004	0.006	0.004

Data represent the mean of three biological and two technical replications

Table 10:- Gingerols and shogaol content in five month old microrhizomes of different ginger genotypes (fresh weight basis)

Genotypes	Pungency principles in five month old ginger microrhizomes				
	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Total gingerol (%)	6-shogaol (%)
Athira	1.038	0.022	0.218	1.260	0.072
Karthika	0.810	0.022	0.198	1.088	0.150
Aswathy	1.165	0.023	0.338	1.550	0.118
Maran	0.708	0.010	0.115	0.840	0.048
Rio-de-Janerio	0.793	0.010	0.175	0.938	0.045
Mean	0.926	0.016	0.209	1.135	0.087
CD(0.05)	0.023	0.002	0.011	0.042	0.012
SE(m)±	0.008	0.001	0.004	0.014	0.004

Data represent the mean of three biological and two technical replications

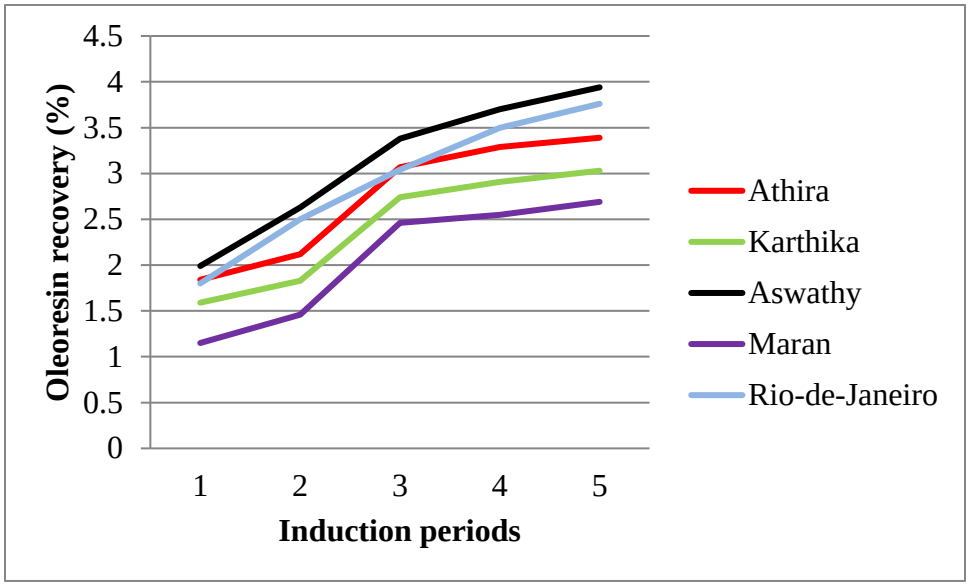


Figure 3:- Trend in oleoresin recovery from microrhizomes in different genotypes of ginger for different induction periods

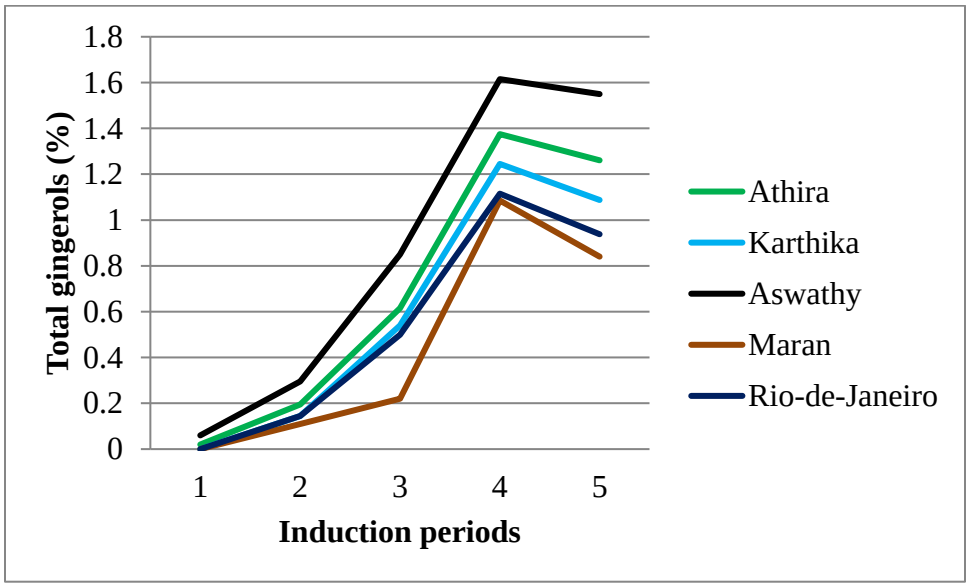


Figure 4:- Trend in accumulation of total gingerols in microrhizomes of different genotypes for different induction periods

the varieties accumulated 8-gingerol of 0.010 per cent but accumulation of 8-gingerol was not observed in the cultivars. The 8-gingerol content increased in all the genotypes in the four months induction period and revealed an almost steady trend for five months induction period.

Similar to 8-gingerol, 10-gingerol was also not accumulated in the initial growth stage of one month after induction. The 10-gingerol content increased with increase in induction periods from two month onwards and recorded the highest after five months induction period. Irrespective of the genotypes, an average of 0.010, 0.053, 0.196 and 0.209 per cent 10-gingerols accumulated respectively after two, three, four and five months induction periods. An accumulation of 0.010 per cent of 10-gingerol was observed in all the genotypes, after two months induction period. For the three month induction period, the accumulation of 10- gingerol recorded the highest in the variety Aswathy (0.110 %). The varieties Karthika (0.060 %), Athira (0.045 %) and the cultivar Rio-de-janeiro (0.040 %) were on par with respect to 10-gingerol production. The lowest 10-gingerol content was recorded in the cultivar Maran (0.010 %). For the four and five months induction period, the highest and lowest accumulation of 10-gingerols recorded respectively in the variety Aswathy and the cultivar Maran.

The 6-shogaol content was not accumulated at the initial growth stage of one month after induction. No significant difference was observed between the three varieties and the cultivar Rio-de-Janeiro for the two months after induction while the lowest accumulation of 6-shogaol was observed in the cultivar Maran. Three months after induction, it was observed that the variety Karthika recorded the highest 6-shogaol content (0.070 %) followed by the variety Aswathy (0.050 %). The cultivar Rio-de-Janeiro recorded a 6-shogaol of 0.040 per cent. The lowest accumulation of 6-shogaol was observed in the cultivar Maran (0.020 %) which was on par with the variety Athira (0.025 %). At four months of induction period no significant difference was observed between the varieties Karthika and Aswathy with respect to 6-shogaol production while the variety Karthika recorded the highest at five months induction period.

From the experiment on gingerol synthesis using microrhizomes in five ginger genotypes, the variety Aswathy was selected as the best genotype with maximum gingerol production for all the five induction periods studied. Eventhough the number of microrhizomes and fresh weight of the microrhizomes showed slight increase at four and five months of induction, due to exhaustion of media components most of the microrhizomes were not of good quality and exhibited shrinkage. Hence three months induction period was fixed for microrhizome induction.

Further treatments were imposed using the variety Aswathy with three month induction period.

4.1.2 Gingerol synthesis in the *in vitro* induced ginger calli

4.1.2.1 Induction of calli in different ginger genotypes

Calli were initiated from all the genotypes of ginger using *in vitro* shoot tip as explant, in callus inducing medium (CI). Bulging was seen at the base of the inoculated shoot tip after 10 days of inoculation. This was followed by initiation of callus at the cut ends of the shoot tip, one to one and half months after inoculation depending on the genotypes. Observations on days taken for callusing, callusing (%) and callus index (callusing percentage× visual growth score of callus) were recorded and the data is presented in Table 11. The initiated calli were subcultured to fresh medium of same composition and observations were taken on fresh and dry weight (g), driage (%) and accumulation of gingerols and shogaol in one, two and three months of inoculation.

The calli from shoot tip were friable, creamy initially and turned brown, after three months of induction (Plate 6). Shoot or root morphogenesis was observed in the calli after one and a half months of calli initiation in all the ginger genotypes. The varieties studied recorded less number of days for callusing as compared to the cultivars. Among the varieties, the variety Athira recorded less number of days for callusing (31.25 days) followed by the variety Aswathy (33.75 days). The variety Karthika recorded 37.75 days which was on par with the cultivar Rio-de-Janeiro with respect to number of days taken for callusing. The cultivar Maran recorded more

number of days (39.25) for callusing. In general, callusing and callus growth were observed poor in shoot tip explants of ginger.

The variety Athira recorded highest callusing of 53.23 per cent one month after inoculation with a callus index of 68.24 per cent. All the other genotypes were on par with respect to callusing. The varieties Aswathy and Karthika recorded callusing of 43.62 and 46.42 per cent respectively, while the cultivars Maran and Rio-de-Janeiro recorded 42.89 per cent and 43.37 per cent. No significant difference was observed among the genotypes, with respect to callus index.

Different calli induction periods *viz* one, two and three months were studied. The fresh weight, dry weight and driage of the calli were recorded for one, two and three months after induction, in all the five genotypes of ginger and the data are presented in Table 12 and 13. The fresh yield of the calli increased from the one month of induction to three months induction period in all the genotypes. An average of 0.101, 0.472 and 0.764 g of fresh weight was recorded respectively after one, two and three months induction period, irrespective of the genotypes.

At one month after induction, the highest yield of fresh calli (0.148 g) was recorded for the variety Athira. Next to variety Athira, variety Aswathy (0.101 g) recorded higher yield of callus, which was on par with variety Karthika (0.083 g) and cultivars Rio-de-Janeiro (0.099 g) and Maran (0.076 g). At two months after induction, no significant difference was observed between the genotypes with respect to fresh weight of the microrhizomes. The varieties Athira, Karthika and Aswathy and the cultivars Maran and Rio-de-Janeiro recorded fresh weight of 0.530 g, 0.435 g, 0.575 g, 0.356 g and 0.465 g respectively after two months induction period. After three months induction period, the fresh weight of calli recorded, was on par among the cultivar Rio-de-Janeiro (2.118 g) and the varieties Aswathy (2.005 g) and Athira (1.763 g).The variety Karthika recorded a fresh calli weight of 1.505 g which was on par with its parent cultivar Maran (1.428 g).

The trend in production and growth of calli at different periods is presented in Figure 5. The varieties recorded higher increase in fresh weight of the calli as compared to the cultivars for the second month. Among the varieties, the variety

Aswathy recorded more increase in the fresh weight of the calli for the second month. The cultivar Rio-de-Janeiro recorded the maximum increase of fresh weight for the third month. Hence three months induction period was observed best for the production and growth of the calli.

Even though the variety Karthika yielded the lowest calli fresh weight for one, two and three months after induction, the dry weight and driage recorded were highest in the variety for all the three induction periods studied. One month after induction, a dry weight of 0.013 g was recorded for variety Karthika which was on par with the other genotypes of ginger studied. At two months induction period, the variety Karthika recorded the highest dry weight of 0.133 g. The variety Aswathy recorded a dry weight of 0.094 g which was on par with the variety Athira (0.071 g) and the cultivar Rio-de-Janeiro (0.082 g). The lowest dry weight was recorded by the cultivar Maran (0.066 g) two months after induction. After three months induction period, the varieties recorded higher dry weight as compared to the cultivars of ginger. The highest dry weight was recorded by the variety Karthika (0.518 g) followed by variety Aswathy (0.293 g).The variety Athira recorded a dry weight of 0.255 g followed by the cultivars Maran (0.175 g) and Rio-de-janeiro (0.153 g) which were on par. The trend in production of dry weight was higher in the varieties as compared to the cultivars for both the induction periods of second and third months. The variety Karthika recorded the highest trend in increase of the dry weight of the calli for both the induction periods of two and three months induction period.

The highest driage of 0.64 per cent, 6.63 per cent and 25.90 per cent respectively was recorded for one, two and three months after induction in the variety Karthika. The lowest driage of 0.33 per cent and 3.35 per cent was observed in the cultivar Maran after one and two month of induction respectively, while for the three months after induction, the cultivar Rio-de-Janeiro recorded the lowest driage of 7.43 per cent. The trend in production of driage was also more in the variety Karthika for both the induction periods of second and third months (Figure 6).

Table 11:- Growth of ginger calli one month after inoculation in different ginger genotypes

Genotypes	Days taken for callusing	Callusing (%)	Callus index (%)
		1MAI	1MAI
Athira	31.25	53.23	68.24
Karthika	37.75	46.42	58.92
Aswathy	33.75	43.62	53.91
Maran	39.25	42.89	47.29
Rio-de-Janeiro	37.75	43.37	52.89
CD (0.05)	2.48	5.78	NS
SE(m)±	0.71	0.84	0.88

MAI-Month after inoculation

Data represent mean of four replications with ten cultures/ replication

Table 12: Fresh weight of calli in different ginger genotypes at different induction periods

Genotypes	Fresh weight (g)		
	1 MAI	2MAI	3MAI
Athira	0.148	0.530	1.763
Karthika	0.083	0.435	1.505
Aswathy	0.101	0.575	2.005
Maran	0.076	0.356	1.428
Rio-de-Janeiro	0.099	0.465	2.118
Mean	0.101	0.472	0.764
CD(0.05)	0.041	NS	0.356
SE(m)±	0.013	0.065	0.821

MAI-Month after induction

Data represent mean of four replications with ten cultures/ replication

Table 13: Driage of calli in different ginger genotypes at different induction periods

Genotypes	Driage of calli (%)								
	1 MAI			2 MAI			3 MAI		
	Initial fresh weight (g)	Dry weight (g)	Driage (%)	Initial fresh weight (g)	Dry weight (g)	Driage (%)	Initial fresh weight (g)	Dry weight (g)	Driage (%)
Athira	2.0	0.010	0.50	2.0	0.071	3.47	2.0	0.255	12.75
Karthika	2.0	0.013	0.64	2.0	0.133	6.63	2.0	0.518	25.90
Aswathy	2.0	0.008	0.42	2.0	0.094	4.75	2.0	0.293	14.65
Maran	2.0	0.006	0.33	2.0	0.066	3.35	2.0	0.175	8.56
Rio-de-Janeiro	2.0	0.010	0.50	2.0	0.082	4.25	2.0	0.153	7.43
CD(0.05)	-	NS	0.08	-	0.036	0.21	-	0.020	0.36
SE(m)±	-	0.005	0.03	-	0.008	0.07	-	0.031	0.11

MAI-Month after induction NS-not significant

Data represent mean of four replications with ten cultures/ replication

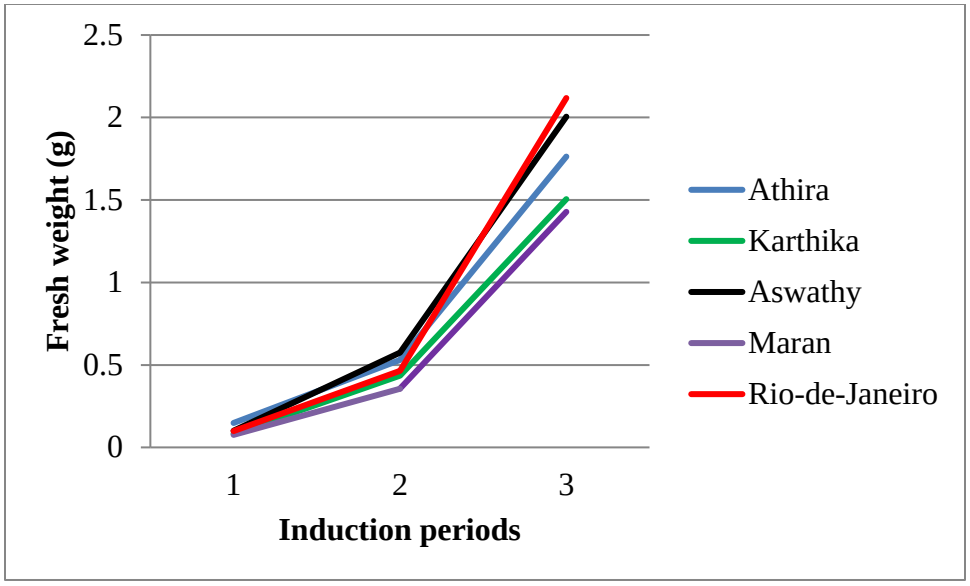


Figure 5:- Trend in fresh yield of calli in different genotypes for different induction periods

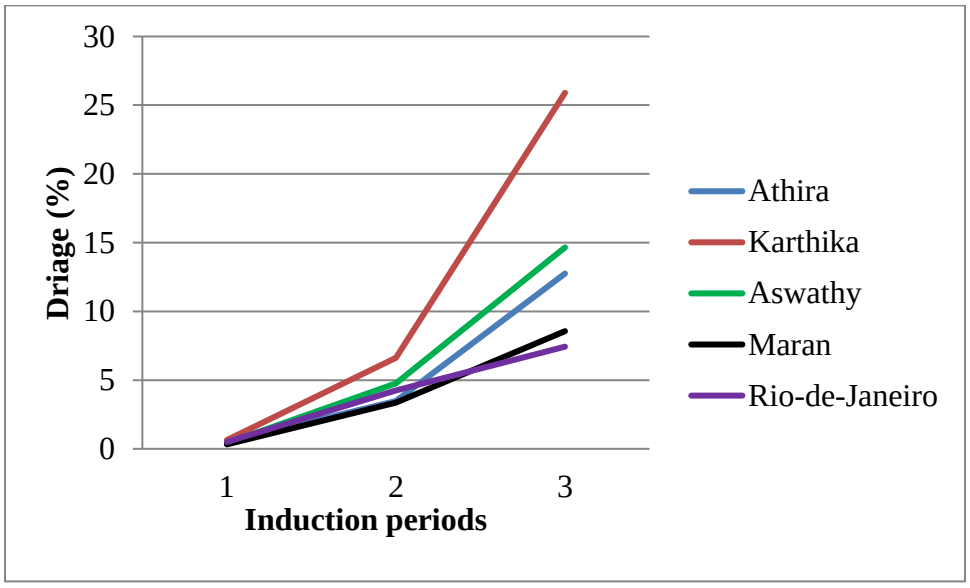
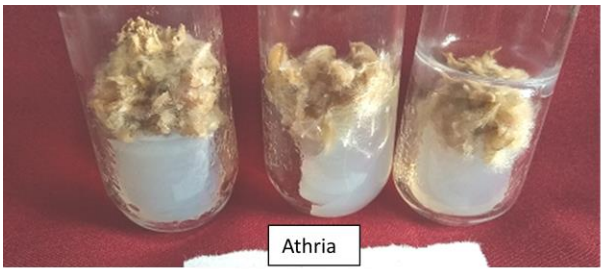
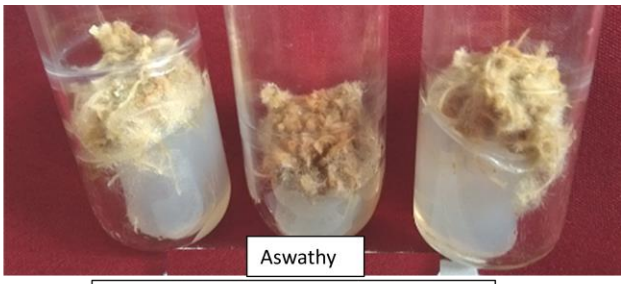


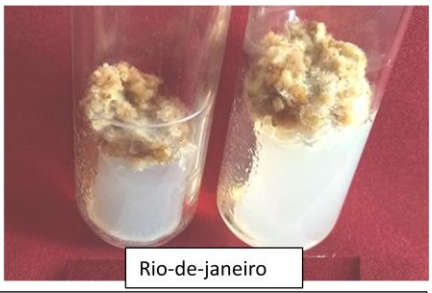
Figure 6:- Trend in driage of calli in different ginger genotypes for different induction periods



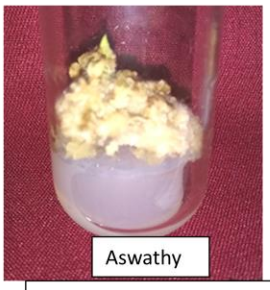
Variety Athira with root morphogenesis



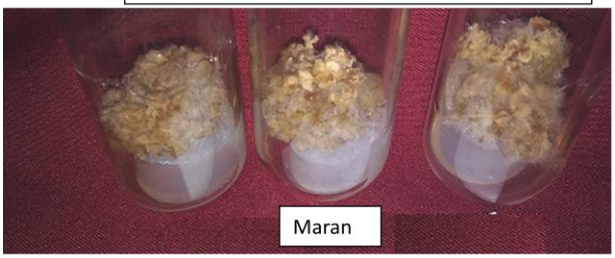
Variety Aswathy with root morphogenesis



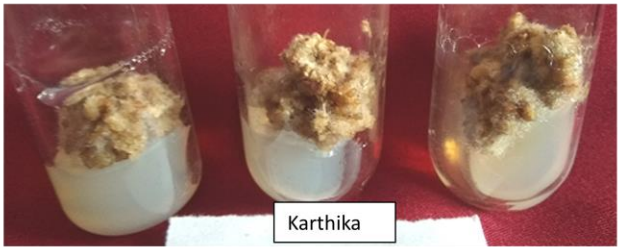
Cultivar Rio-de-Janeiro with root morphogenesis



Variety Aswathy with shoot morphogenesis



Cultivar Maran with root morphogenesis



Variety Athira with root morphogenesis

Plate 6:- Calli of three months old cultures in five different genotypes of ginger

4.1.2.2 Effect of culture period on gingerols and shogaol production in calli of different ginger genotypes (fresh weight basis)

Fresh calli from all the five genotypes were extracted and analyzed for gingerols and shogaol after one, two and three months induction periods. Root morphogenesis was observed in the calli of all the genotypes while 20 per cent of calli of the variety Aswathy showed shoot morphogenesis after one and a half months of induction. The calli at the shoot/root morphogenesis stage was also analyzed for oleoresin recovery, gingerols and shogaol content. Observations on oleoresin recovery (%), gingerols and shogaol content (%) at different growth stages *viz* one, two and three months were recorded and presented in Table 14, 15 and 16.

Recovery of oleoresin increased with increase in induction period and the highest recovery was after three months induction period. Irrespective of the genotypes studied, an average oleoresin recovery of 1.62, 2.28, and 2.87 per cent was recorded after one, two and three months induction period.

Atone month after induction, no significant difference was observed among the genotypes Karthika (1.71 %), Athira (1.70 %), Aswathy (1.67 %) and Rio-de-Janeiro (1.57 %) with respect to oleoresin recovery. The lowest oleoresin recovery was recorded by cultivar Maran (1.44 %). Root morphogenesis was observed in the calli from all the genotypes of ginger after two months induction period. The variety Aswathy recorded the highest oleoresin recovery of 2.43 per cent after two months of induction which was on par with the genotypes Athira (2.35 %) and Rio-de-Janeiro (2.32 %). The cultivar Maran recorded an oleoresin recovery of 2.07 per cent which was on par with the variety Karthika (2.25 %) after two months of induction. Calli with root/shoot morphogenesis were analyzed after three month of induction. The calli with root morphogenesis recorded higher oleoresin recovery compared to calli with shoot morphogenesis. Recovery of oleoresin was the highest (3.15 %) in calli from the variety Aswathy with root morphogenesis while the calli with shoot morphogenesis recorded an oleoresin recovery of 2.73 per cent. Next to the variety Aswathy, the higher recovery of oleoresin was observed in the variety Athira (3.00 %) with root morphogenesis. The variety Karthika with root morphogenesis recorded an

oleoresin recovery of 2.82 per cent which was on par with that of cultivar Rio-de Janeiro (2.83 %). The lowest oleoresin recovery was recorded by cultivar Maran with root morphogenesis (2.71 %) which was on par with the variety Aswathy with shoot morphogenesis (2.73 %).

The trend in recovery of oleoresin from calli at different induction periods is presented in Figure 7. The variety Aswathy recorded more increase in oleoresin recovery at both second and third month of induction.

Irrespective of the genotypes, increased contents of gingerols and shogaol was observed with an increase in induction periods, wherein, the highest content was recorded three months after induction (Figure 8). The accumulation of gingerols and shogaol were not observed in any of the genotypes one month after induction. The calli from the variety Aswathy with root morphogenesis recorded highest total gingerols (sum total of 6, 8, and 10-gingerol) of 0.030 per cent on analysis of calli at two months induction period followed by the cultivar Rio-de-Janeiro (0.003 %). The variety Athira with root morphogenesis recorded a total gingerol of 0.002 per cent which was on par with the cultivar Maran with root morphogenesis (0.002 %). After the analysis of the three month old calli, it was observed that the highest total gingerols were recorded in the varieties as compared to the cultivars. The highest total gingerol was recorded by variety Aswathy with root morphogenesis (0.090 %). No significant difference was observed between the varieties Athira and Karthika with root morphogenesis recording 0.025 and 0.020 per cent respectively. The variety Aswathy with shoot morphogenesis recorded a total gingerol of 0.010 per cent which was on par with the cultivars Maran and Rio-de-Janeiro with root morphogenesis.

It was noticed that 8, 10 gingerol and 6- shogaol were not accumulated for one and two months induction periods while after three months of induction the accumulation of 8-gingerol was observed non-significant among the genotypes. The accumulation of 10- gingerol was also the highest in the variety Aswathy with root morphogenesis (0.030 %) after three months of induction. The varieties Athira and Karthika with root morphogenesis recorded 0.010 per cent accumulation of 10-

Table 14:- Recovery of oleoresin, gingerol and shogaol content in one month old calli in different ginger genotypes (fresh weight basis)

Genotypes	Oleoresin and pungency principles in one month old ginger calli					
	Oleoresin (%)	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Tot gingerol (%)	6-shogaol (%)
Athira	1.70	0.000	0.000	0.000	0.000	0.000
Karthika	1.71	0.000	0.000	0.000	0.000	0.000
Aswathy	1.67	0.000	0.000	0.000	0.000	0.000
Maran	1.44	0.000	0.000	0.000	0.000	0.000
Rio-de-Janerio	1.57	0.000	0.000	0.000	0.000	0.000
Mean	1.62	0.000	0.000	0.000	0.000	0.000
CD(0.05)	0.16	NS	NS	NS	NS	NS
SE(m)±	0.05	0.000	0.000	0.000	0.000	0.000

NS-not significant

Data represent the mean of three biological and two technical replications

Table 15:- Recovery of oleoresin, gingerol and shogaol content in two months old calli in different ginger genotypes (fresh weight basis)

Genotypes	Oleoresin and pungency principles in two month old ginger calli					
	Oleoresin (%)	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Tot gingerol (%)	6-shogaol (%)
Athira (R)	2.35	0.002	0.000	0.000	0.002	0.000
Karthika(R)	2.25	0.000	0.000	0.000	0.000	0.000
Aswathy (R)	2.43	0.030	0.000	0.000	0.030	0.000
Maran (R)	2.07	0.002	0.000	0.000	0.002	0.000
Rio-de-Janerio(R)	2.32	0.003	0.000	0.000	0.003	0.000
Mean	2.28	0.010	0.000	0.000	0.010	0.000
CD(0.05)	0.18	0.002	NS	NS	0.002	NS
SE(m)±	0.06	0.001	0.000	0.000	0.001	0.000

R- Root morphogenesis NS-not significant

Data represent the mean of three biological and two technical replications

Table 16: Recovery of oleoresin, gingerol and shogaol content in three months old calli in different ginger genotypes (fresh weight basis)

Genotypes	Oleoresin and pungency principles in three month old ginger calli					
	Oleoresin (%)	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Total gingerol (%)	6-shogaol (%)
Athira(R)	3.00	0.015	0.001	0.010	0.025	0.002
Karthika(R)	2.82	0.010	0.001	0.010	0.020	0.005
Aswathy (R)	3.15	0.060	0.001	0.030	0.090	0.001
Aswathy(S)	2.73	0.010	0.001	0.000	0.010	0.002
Maran(R)	2.71	0.010	0.001	0.000	0.010	0.004
Rio- de-Janerio(R)	2.83	0.010	0.001	0.000	0.010	0.002
Mean	2.87	0.019	0.001	0.008	0.028	0.003
CD(0.05)	0.10	0.012	NS	0.005	0.008	NS
SE(m)±	0.03	0.003	0.000	0.002	0.002	0.001

R- Root morphogenesis S- Shoot morphogenesis

Data represent the mean of three biological and two technical replications

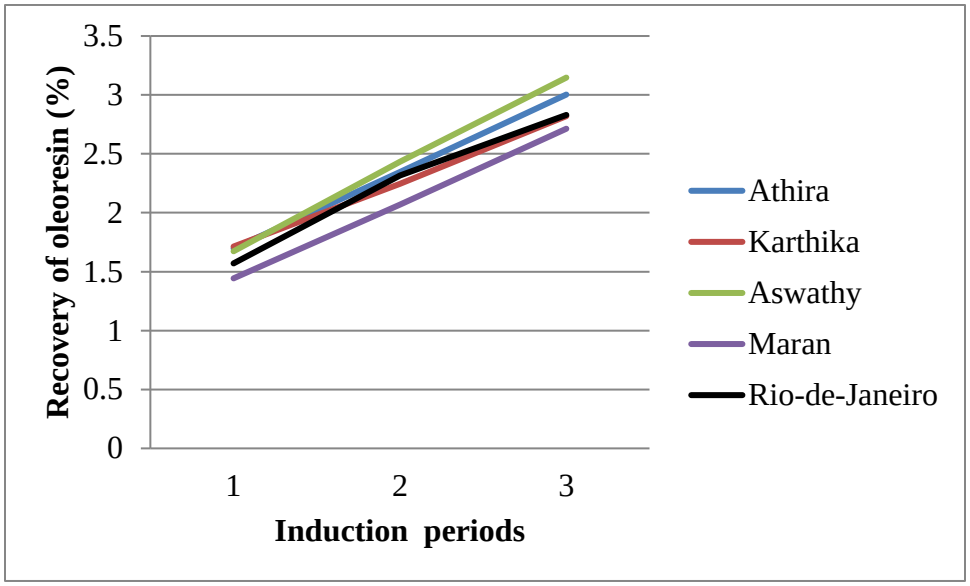


Figure 7:- Trend in recovery of oleoresin from calli in different ginger genotypes for different induction periods

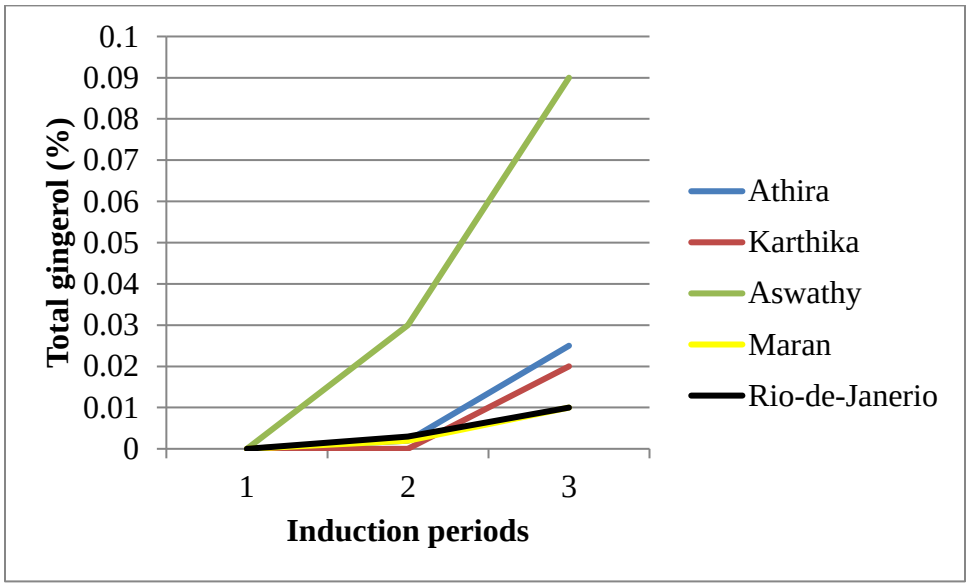


Figure 8:- Trend in total gingerol production in calli of different ginger genotypes for different induction periods

gingerol. There was no significant difference among the accumulations of 6-shogaol content for three months old calli in the genotypes studied.

The variety Aswathy recorded maximum total gingerol content from calli and hence for further experiments the variety Aswathy was used. The production and growth of calli and the highest accumulation of gingerols were observed in the calli after three months induction period hence three month old calli were selected for imposing further treatments.

4.1.3 Comparison of gingerol and shogaol production in ginger microrhizomes and calli induced *in vitro*

Production of total gingerols and shogaol was found increased with an increase of induction periods both in the *in vitro* microrhizomes and calli and the highest production of total gingerols and shogaol was recorded after three months induction period. The highest production of gingerols was observed in the *in vitro* microrhizomes as compared to *in vitro* calli in all the genotypes of ginger studied (Figure 9). The variety Aswathy recorded highest gingerol production in both in microrhizomes and calli.

Total gingerol accumulation in microrhizomes of ginger genotypes studied ranged from 9.4 - 50 times over that of the calli. The accumulation of total gingerols in the *in vitro* microrhizomes was 50 times higher over the *in vitro* calli in the cultivar Rio-de-Janeiro. In the varieties Athira and Karthika and in the cultivar Maran, the accumulation of total gingerol in the *in vitro* microrhizome recorded respectively 24.6, 27 and 22 times over that of the total gingerol production in calli. In the variety Aswathy, however 9.4 times increase in total gingerols in microrhizomes over that of calli was recorded indicating better accumulation of gingerols in calli of the variety Aswathy.

The increase in accumulation of 6-shogaol in microrhizomes of ginger genotypes studied, ranged from 4-50 times over that of the calli. In the microrhizomes of the variety Aswathy, the accumulation of 6- shogaol recorded 50 times higher than

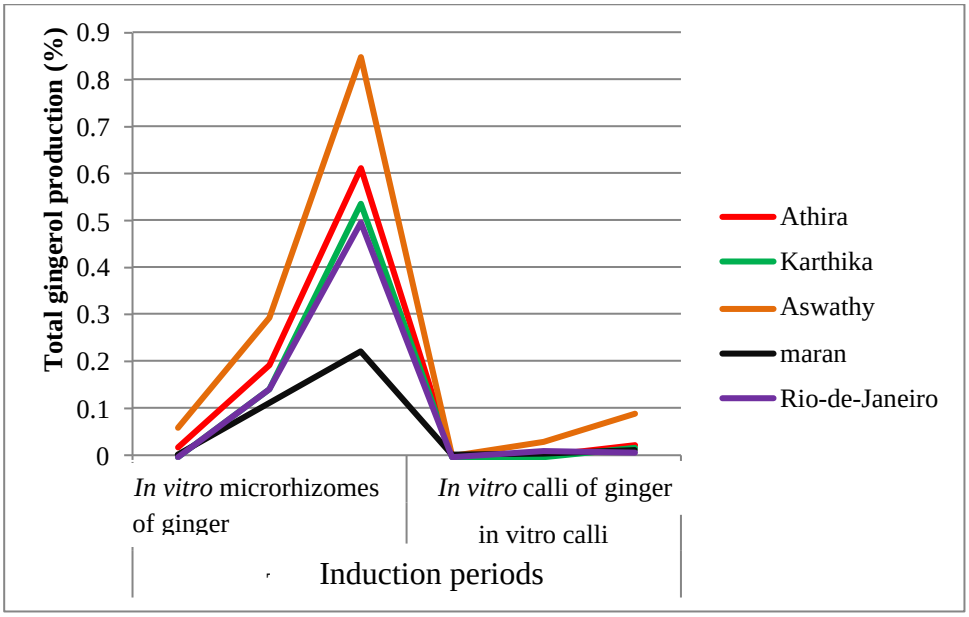


Figure 9:-Comparison of gingerol production in *in vitro* microrhizomes and in *vitro* calli

that of calli. In the microrhizomes of varieties Athira and Karthika, 12.5 and 14 times higher accumulation of 6-shogaol were recorded respectively over the calli.

Hence, *in vitro* induced microrhizomes observed very good experimental material to study the synthesis and accumulation of gingerols and shogaol.

4.1.4 Effect of manipulation of macro and micro nutrients in MS medium on *in vitro* gingerol synthesis

4.1.4.1 Effect of manipulation of macro and micro nutrients in MS medium on *in vitro* gingerol synthesis in microrhizomes of variety Aswathy

The effect of manipulation of macro and micro nutrients on production of microrhizomes and accumulation of gingerols and shogaol were studied. Different levels of macro and micro nutrients such as MS medium with twice and half the concentration of macro and micronutrients in MS medium were studied. Similarly different levels of ammoniacal and nitrate nitrogen such as twice and half the concentration of ammoniacal and nitrate nitrogen, ratio of twice and half ammoniacal to nitrate nitrogen in MS medium were also studied. The MS medium was used as the control.

4.1.4.1.1 Effect of different levels of macro and micro nutrients in MS medium on production and growth of microrhizomes of variety Aswathy

Microrhizomes were induced in MS medium with different levels of macro and micro nutrients supplemented with 80 g L^{-1} sucrose. Observations on production of microrhizomes/ culture, fresh weight and dry weight of microrhizome (g), driage (%) were recorded after three months of induction (Plate 7). The MS medium supplemented with 80 gL^{-1} sucrose was used as the control. The number of microrhizomes/ culture was not significantly different among the treatments (Table 17). Stunted growth of shoots and brittle roots were observed in cultures grown in MS medium with half macro nutrients. The fresh weight of the microrhizome yielded the highest in MS control medium (0.606 g) followed by MS medium with twice micro nutrients (0.508 g). The fresh weight recorded was the lowest with half micro and macro nutrients. The highest dry weight of microrhizome was recorded in the control

(0.056 g) followed by MS medium with twice micro nutrients (0.051 g). The highest driage was recorded by MS medium (2.70 %) which was on par with the MS medium with twice micro nutrients (2.53 %). The MS medium supplemented with twice macro nutrients recorded a driage of 1.65 per cent. The lowest dry weight and driage of the microrhizome were recorded for the treatments with half micro and macro nutrients. The production and growth of microrhizomes was observed best in the treatments MS medium and MS medium supplemented with twice micronutrients.

4.1.4.1.2 Effect of different levels of macro and micro nutrients in MS medium on accumulation of gingerols and shogaol in microrhizomes of the ginger variety Aswathy (fresh weight basis)

Fresh microrhizomes were collected from each treatment after three months of induction. The microrhizomes were subjected to extraction and the methanolic extract of the oleoresin at a concentration of 10 mg mL^{-1} was injected to HPLC system for analysis of gingerols and shogaol content. The highest recovery of oleoresin was observed in the microrhizomes induced in MS medium supplemented with twice micro nutrients (3.70 %) (Table 18) which was statistically on par with the MS control (3.31 %). The MS medium with half micro nutrients recorded an oleoresin recovery of 2.98 per cent. The lowest recovery of oleoresin was recorded in MS medium with twice macro nutrients (2.17 %).

The highest production of total gingerols (0.970 %) was recorded in the microrhizomes induced in MS medium with twice micronutrients followed by microrhizomes induced in MS medium (0.858 %). The MS medium with modifications in macro nutrients recorded lowest total gingerols. The MS medium with twice macro nutrients recorded a total gingerols of 0.093per cent while MS medium with half macro nutrients recorded 0.090 per cent.

The same trend observed in total gingerols was observed with respect to 6-gingerol production also. Highest production of 6- gingerol (0.877 %) was recorded in the microrhizomes induced in MS medium with twice micronutrients followed by MS medium (0.723 %). The MS medium with manipulations in macro nutrients recorded low accumulation of 6- gingerol.

Table 17: - Effect of different levels of macro and micro nutrients in MS medium on production and growth of microrhizomes in the ginger variety Aswathy

Sl. No.	Treatments	Number of microrhizomes/ culture	Fresh weight of microrhizome (g)	Driage (%)		
				Initial fresh weight(g)	Dry weight (g)	Driage (%)
1	MS+2Macro	16.00	0.400	2.0	0.038	1.65
2	MS+2Micro	13.00	0.508	2.0	0.051	2.53
3	MS+½Macro	13.66	0.238	2.0	0.022	1.09
4	MS+½Micro	12.33	0.237	2.0	0.022	1.09
5	MS Medium (control)	16.33	0.606	2.0	0.056	2.70
	CD (0.05)	NS	0.035	-	0.004	0.25
	SE(m)±	1.37	0.011	-	0.001	0.08

NS- Not Significant

Data represent mean of four replications with eight cultures/ replication

Table 18: - Effect of different levels of macro and micro nutrients in MS medium on accumulation of gingerols and shogaol in microrhizomes of the ginger variety Aswathy

Treatments	Oleoresin and pungency principles of ginger					
	Oleoresin (%)	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Total gingerol (%)	6-shogaol (%)
MS+2Macro	2.17	0.065	0.000	0.017	0.093	0.027
MS+2Micro	3.70	0.877	0.015	0.077	0.970	0.057
MS+½Macro	2.70	0.085	0.002	0.000	0.090	0.020
MS+½Micro	2.98	0.105	0.015	0.003	0.115	0.010
MS Medium (control)	3.31	0.723	0.015	0.115	0.858	0.045
C.D (0.05)	0.17	0.010	0.006	0.006	0.013	0.005
SE(m)±	0.06	0.003	0.002	0.002	0.004	0.002

Data represent mean of three biological and two technical replications

Plate 7:-Microrhizomes induced in MS medium supplemented with different levels of macro and micro nutrients in three month old cultures

The accumulation of 8 and 10– gingerol was more in the MS medium with modifications of micro nutrients as compared to the MS medium with modifications in macro nutrients. The accumulation of 8-gingerol was not significantly different between the control MS and MS medium with twice and half micro nutrients. The accumulation of 10-gingerol was recorded the highest in the MS control (0.115%) followed by MS medium with twice micronutrients (0.077 %).

From the study on the effect of macro and micro nutrients on gingerol production, the MS medium with twice micronutrients was selected as the best treatment with highest production of total gingerols.

4.1.4.2 Effect of different levels of ammoniacal and nitrate nitrogen in MS medium for production and growth of microrhizomes of the ginger variety Aswathy

The effect of different levels of ammoniacal and nitrate nitrogen was studied with microrhizomes induced in MS medium supplemented with different combinations of ammoniacal and nitrate nitrogen after three months of induction (Plate 8). The MS medium was employed as the control. The production of microrhizomes/ culture was statistically on par in control (16.33) and the MS medium with the ratio of half ammoniacal nitrogen to nitrate nitrogen (13.67) (Table 19). Stunting of shoots with pale leaves and browning of microrhizomes were seen in the cultures induced in MS medium with twice nitrate nitrogen which recorded the lowest production of microrhizomes/ culture with 5.68 microrhizomes.

Fresh yield of the microrhizomes was higher in treatments with half the concentrations of ammoniacal and nitrate nitrogen as compared to treatments with twice the concentrations of ammoniacal and nitrate nitrogen. Fresh weight of the microrhizome recorded the highest in the control (0.606 g) followed by MS medium along with half ammoniacal nitrogen (0.524 g). The lowest fresh weight was recorded in MS with twice nitrate nitrogen which was on par with MS medium with twice the ratio of ammoniacal to nitrate nitrogen.

Table 19: - Effect of different levels of ammoniacal and nitrate nitrogen on production and growth of microrhizomes in the ginger variety Aswathy

Sl. No.	Treatments	Number of microrhizomes /culture	Fresh Weight of microrhizome (g)	Driage of microrhizome (%)		
				Initial fresh weight (g)	Dry weight (g)	Driage (%)
1	MS+2 AN	11.00	0.322	2.0	0.035	1.70
2	MS+2 NN	5.68	0.134	2.0	0.028	1.51
3	MS+½AN	12.67	0.524	2.0	0.054	2.67
4	MS +½NN	12.00	0.372	2.0	0.030	1.65
5	MS+ 2AN/2NN	9.33	0.155	2.0	0.029	1.49
6	MS+½AN/ ½NN	13.67	0.337	2.0	0.038	1.92
7	MS Medium (control)	16.33	0.606	2.0	0.056	2.70
	CD(0.05)	3.11	0.046	-	0.003	0.15
	SE(m)	1.02	0.015	-	0.001	0.05

AN- Ammoniacal nitrogen NN- Nitrate nitrogen

Data represent mean of four replications with eight cultures/ replication

Plate 8:-Microrhizomes induced in MS medium supplemented with different levels of ammoniacal and nitrate nitrogen in three month old cultures

Note: MS + 2 AN/ 2NN- Represents MS medium supplemented with ratio of twice ammoniacal nitrogen to twice nitrate nitrogen

MS + ½ AN/ ½ NN- Represents MS medium supplemented with ratio of half ammoniacal nitrogen to half nitrate nitrogen

There was no significant difference between the dry weight and driage of the microrhizome in MS medium along with half ammoniacal nitrogen and the control. The MS medium with twice nitrate nitrogen produced the lowest dry weight and driage.

4.1.4.3 Effect of different levels of ammoniacal and nitrate nitrogen in MS medium on accumulation of gingerols and shogaol in microrhizomes of the ginger variety Aswathy (fresh weight basis)

Accumulation of gingerols and shogaol in microrhizomes induced in different levels of ammoniacal and nitrate nitrogen was compared and presented in Table 20. Recovery of oleoresin and accumulation of gingerols were more in the microrhizomes induced in MS medium with half the concentration of ammoniacal and nitrate nitrogen while it was observed less in the microrhizomes induced in MS medium with twice the concentration of ammoniacal and nitrate nitrogen. It was found that the microrhizomes induced in MS medium supplemented with half ammoniacal nitrogen recorded the highest recovery of oleoresin (3.69 %) followed by microrhizomes induced in MS medium with half nitrate nitrogen (3.40 %) and the control (3.29 %) which were on par. The lowest recovery of oleoresin was recorded in MS medium with twice nitrate-nitrogen (2.37 %) which was on par with MS medium with twice the ratio of ammoniacal nitrogen to nitrate nitrogen (2.40 %).

Accumulation of total gingerols was observed more in MS medium with half the concentrations of ammoniacal and nitrate nitrogen than MS medium with twice the concentration (Table 20). The highest accumulation of total gingerols (1.102 %) was observed in MS medium supplemented with half ammoniacal nitrogen followed by the treatment MS medium supplemented with half nitrate nitrogen (0.895 %). The lowest accumulation of total gingerols was observed in MS medium with twice the ratio of ammoniacal to nitrate nitrogen which was on par with MS medium with twice ammoniacal nitrogen and MS medium with twice nitrate nitrogen. The accumulation of 6- gingerol (0.978 %) and 10- gingerol (0.107 %) were also recorded the highest in the same treatment of MS medium with half ammoniacal nitrogen. No significant difference was observed between the control (MS medium), MS medium with half

Table 20:- Effect of different levels of ammoniacal and nitrate nitrogen in MS medium on accumulation of gingerols and shogaol in microrhizomes of the ginger variety Aswathy

Treatments	Oleoresin and pungency principles of ginger					
	Oleoresin (%)	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Total gingerol (%)	6-shogaol (%)
MS+2 AN	2.52	0.082	0.002	0.003	0.090	0.000
MS+2 NN	2.37	0.043	0.002	0.005	0.050	0.013
MS+½AN	3.69	0.978	0.015	0.107	1.102	0.052
MS +½NN	3.40	0.818	0.013	0.078	0.895	0.063
MS + 2AN/2NN	2.40	0.043	0.000	0.012	0.047	0.013
MS+½ AN/ ½ NN	2.98	0.512	0.010	0.047	0.563	0.038
MS Medium (control)	3.29	0.723	0.015	0.115	0.858	0.045
CD (0.05)	0.12	0.083	0.005	0.011	0.082	0.010
SE(m)±	0.04	0.029	0.002	0.004	0.028	0.003

AN- Ammoniacal nitrogen NN- Nitrate nitrogen

Data represent the mean of three biological replications and two technical replications.

ammoniacal nitrogen, MS medium with half nitrate nitrogen and MS medium with half ammoniacal nitrogen to nitrate nitrogen with respect to 8-gingerol content. The highest accumulation of 6-shogaol content was noted in MS medium with half nitrate-nitrogen (0.063 %) followed by MS medium with half nitrate-nitrogen (0.052 %).

Microrhizomes induced in MS medium with half ammoniacal nitrogen recorded higher yield of microrhizomes and accumulated more total gingerols compared to all other treatment hence was selected as the best treatment.

4.1.5 Effect of MMS medium on production and growth of microrhizomes and accumulation of gingerols and shogaol in the ginger variety Aswathy

The MS medium with twice micronutrients was selected as the best treatment from the experiment on different levels of macro and micro nutrients. And from the experiments on different levels of ammoniacal and nitrate nitrogen, the MS medium with half ammoniacal nitrogen was selected best which recorded the highest gingerol production and the combination of the selected treatments (MS+ twice micronutrients+ half ammoniacal nitrogen) was designated as MMS medium. The selected treatments and the MMS medium were tried to study their effects on production of microrhizomes and accumulation of gingerols and shogaol. The MS medium was used as the control.

4.1.5.1 Effect of MMS medium on production and growth of microrhizomes in the ginger variety Aswathy

The MMS medium was observed the best in production of more number of microrhizomes/culture (20.00) (Table 21 and Plate 9). The control MS medium recorded 15.25microrhizomes/ culture which were on par with the MS medium with half ammoniacal nitrogen (12.00) and MS medium with twice micro nutrients (12.25).No significant difference was observed in the fresh weight of the microrhizome in the treatments MMS medium (0.497g) and the control (0.569g) while the dry weight (0.061 g) and driage (3.10%) recorded was the highest in MMS medium.

4.1.5.2 Effect of MMS medium on accumulation of gingerols and shogaol in microrhizomes of the ginger variety Aswathy (fresh weight basis)

Oleoresin was extracted from the fresh microrhizomes induced in different treatments after three months. The MS medium was employed as the control. The highest recovery of oleoresin of 3.45 per cent was recorded in MMS medium followed by the control (3.24 %) and the recovery of oleoresin was the lowest in MS medium with half ammoniacal nitrogen recording 2.80 per cent (Table 22).

On analysis of gingerols and shogaol by HPLC, it was observed that total gingerols accumulated the highest in MMS medium recording 1.305 per cent (Figure 10) followed by MS medium with half ammoniacal nitrogen (1.097 %). The lowest total gingerol content was recorded in the control (0.838 %). The 6-gingerol and 10-gingerol of 1.173 per cent 0.122 per cent respectively was the highest in MMS medium. Next to the treatment MMS medium, 6-gingerol content recorded higher in the treatment MS medium with twice micronutrients (0.873 %).The 6-gingerol content recorded in MS medium (0.713 %) was on par with the treatment MS medium with half ammoniacal nitrogen (0.790 %). The 10-gingerol content in the treatments MS medium and MS medium with half ammoniacal nitrogen registered the same value (0.107 %). No significant difference was found in 8-gingerol content among the treatments.

Hence MMS medium was found the best with respect to production of microrhizomes, recovery of oleoresin and accumulation of gingerols and was used for further experiments.

Table 21:- Effect of MMS medium production and growth of microrhizomes in the ginger variety Aswathy

Sl. No.	Treatments	Number of microrhizomes/culture	Fresh weight of microrhizome (g)	Driage (%)		
				Initial fresh weight (g)	Dry weight (g)	Driage (%)
1	MS+2 Micro	12.25	0.413	2.0	0.051	2.50
2	MS+½ AN	12.00	0.227	2.0	0.054	2.76
3	MS+2Micro+½ AN (MMS)	20.00	0.497	2.0	0.061	3.10
4	MS Medium (control)	15.25	0.569	2.0	0.056	2.70
	CD(0.05)	4.25	0.099	-	0.004	0.17
	SE(m)±	1.37	0.032	-	0.001	0.05

AN- Ammoniacal nitrogen MMS- Modified MS medium

Data represent mean of four replications with eight cultures/ replication

Table 22:- Effect of MMS medium on accumulation of gingerols and shogaol using microrhizomes of the ginger variety Aswathy

Treatments	Oleoresin and pungency principles in ginger					
	Oleoresin (%)	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Total gingerol (%)	6-shogaol (%)
MS+2 Micro	2.99	0.873	0.010	0.077	0.967	0.057
MS+½ AN	2.80	0.790	0.010	0.107	1.097	0.048
MS+2Micro +½AN(MMS)	3.45	1.173	0.010	0.122	1.305	0.043
MS Medium (control)	3.24	0.713	0.010	0.107	0.838	0.043
CD (0.05)	0.12	0.076	NS	0.009	0.080	0.006
SE(m)±	0.04	0.053	0.000	0.003	0.027	0.002

AN - Ammoniacal nitrogen MMS- Modified MS medium

Data represent the mean of three biological replications and two technical replications

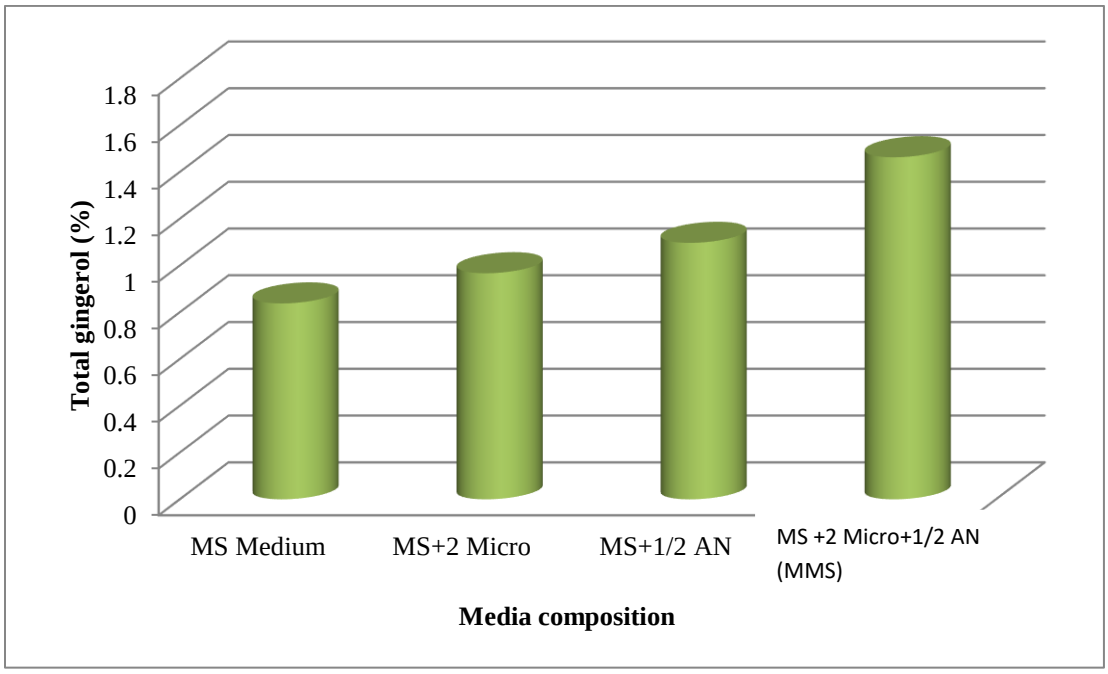
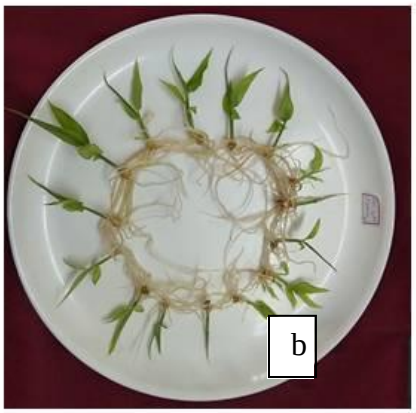


Figure 10:- Comparison of media identified for high gingerol production

MS + 2 Micro + ½ AN



MS + 2 Micro + ½ AN

Plate 9 (a) Three month old microrhizomes induced in MMS medium (MS + twice micro nutrients + Half ammoniacal nitrogen)

(b) Microrhizome induced in MMS medium after three months of induction

4.1.6 Effect of elicitors on *in vitro* gingerol production using microrhizomes and calli of the variety Aswathy

4.1.6.1 Effect of abiotic and biotic elicitors in MMS medium on production, growth and accumulation of gingerols and shogaol in microrhizomes of the variety Aswathy

Different levels of biotic elicitors, including autoclaved fungal mycelium (5, 10 mg L^{-1}), chitin (50 and 100 mg L^{-1}), chitosan (50 and 100 mg L^{-1}) were supplemented to MMS medium to study their effects on production of microrhizomes and accumulation of gingerols and shogaol in ginger microrhizomes. Abiotic elicitors such as MJ and SA at different levels 5, 10, 15 mg L^{-1} were also used for the study. The MMS medium without elicitors was used as the control.

4.1.6.1.1 Effect of abiotic elicitors in MMS medium on production and growth of microrhizomes

Two clumps were inoculated per culture in MMS medium supplemented with MJ and SA at different concentrations of 5, 10, 15 mg L^{-1} and the microrhizomes produced were extracted and analyzed after three months of induction (Plate 10). The MMS medium supplemented without elicitors was used as the control. The multiplication rate was reduced with increase in concentration of abiotic elicitors (Table 23). The highest number of microrhizomes/culture (15.00) was recorded in the control followed by the treatment MMS medium supplemented with SA 5 mg L^{-1} recording 12.00 microrhizomes. The fresh weight of the microrhizome was recorded the highest in the control (0.536 g) followed by MMS medium supplemented with SA 5 mg L^{-1} (0.394 g). There was no significant difference observed among the treatments with respect to the dry weight of the microrhizome. Highest percentage of driage was recorded in MMS medium (2.56 %) followed by MMS supplemented with SA 5 mgL^{-1} (2.09 %). The driage decreased with increased concentration of elicitors.

Table 23:- Effect of abiotic elicitors in MMS medium on production and growth of microrhizomes in the ginger variety Aswathy

Sl. No.	Treatments	Number of microrhizomes /culture	Fresh weight of microrhizome (g)	Driage (%)		
				Initial fresh weight (g)	Dry weight (g)	Driage (%)
1	MMS+MJ 5 mg L^{-1}	10.66	0.342	2.0	0.033	1.65
2	MMS+MJ 10 mg L^{-1}	8.33	0.282	2.0	0.027	1.13
3	MMS+MJ 15 mg L^{-1}	6.00	0.265	2.0	0.021	1.08
4	MMS+SA 5 mg L^{-1}	12.00	0.394	2.0	0.042	2.09
5	MMS+SA 10 mg L^{-1}	10.33	0.326	2.0	0.033	1.64
6	MMS+SA 15 mg L^{-1}	7.67	0.284	2.0	0.019	0.98
7	MMS medium (control)	15.00	0.536	2.0	0.051	2.56
	CD(0.05)	1.39	0.120	-	NS	0.17
	SE(m)±	0.94	0.039	-	0.015	0.06

Data represent the mean of four replications with eight cultures/ replication

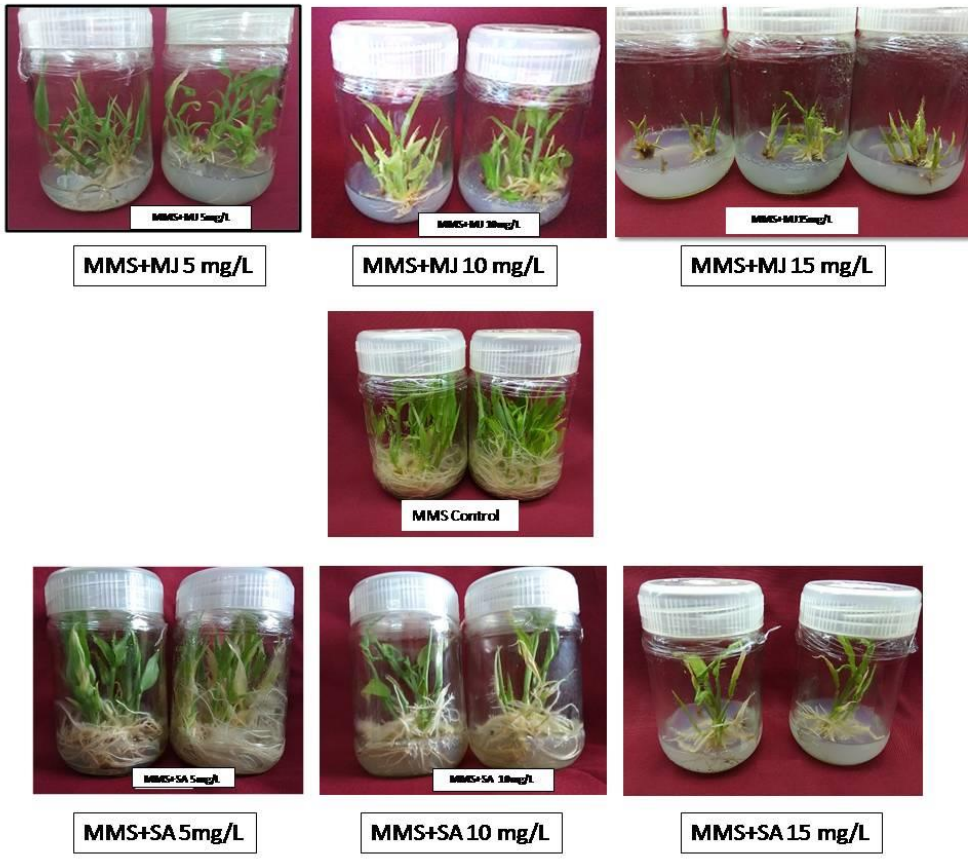


Plate 10:- Microrhizome induced in MMS semisolid medium supplemented with different levels of abiotic elicitors in three month old cultures

4.1.6.1.2 Effect of different levels of abiotic elicitors in MMS medium on accumulation of gingerols and shogaol in microrhizomes of the ginger variety Aswathy (fresh weight basis)

Two studies were conducted simultaneously to fix the duration period of the elicitation and to assess the influence of elicitors on production of gingerols and shogaol. In one study, three months old microrhizomes induced in MS medium were inoculated to MMS liquid medium supplemented with different levels of MJ and SA and incubated for one week. In the other study, the microrhizomes induced in MMS semisolid medium supplemented with different levels of MJ and SA were used for analysis of gingerols and shogaol at three months of induction.

From the study using liquid medium, the microrhizomes were subjected to extraction and HPLC analysis after one week. The MMS liquid medium supplemented with 80 g L^{-1} sucrose was used as the control. Oleoresin recovery was found decreased with increase in elicitor concentration (Table 24 and Plate 11). The highest recovery of oleoresin (2.86 %) was recorded in the treatment MMS liquid medium supplemented with SA 5 mg L^{-1}. The MMS liquid medium supplemented with SA 10 mg L^{-1} (2.30 %), MMS liquid medium supplemented with MJ 5 mg L^{-1} (2.47 %) and MMS liquid medium (2.29 %) were on par with one other with respect to oleoresin recovery. The lowest oleoresin recovery was observed in MMS liquid medium supplemented with SA 15 mg L^{-1} (1.84 %).

Accumulation of total gingerol decreased with increased concentration of elicitors. The highest total gingerol (1.035 %) production was observed in MMS liquid medium supplemented with SA 5 mg L^{-1} (Figure 11 and 12) followed by MMS liquid medium supplemented with MJ 5 mg L^{-1} (0.623 %). The lowest total gingerol accumulation was observed in the treatment MMS liquid medium supplemented with SA 15 mg L^{-1} (0.163 %). The highest accumulation of 6-gingerol (0.890 %), and 8-gingerol (0.058 %) were recorded in MMS liquid medium supplemented with SA 5 mg L^{-1}. The 10-gingerol content (0.078 %) observed in MMS liquid medium supplemented with SA 5 mg $L^{-1}$was on par with that of control (0.070 %). The 6-shogaol did not register significant

difference in the treatments MMS liquid medium supplemented with SA 15 mg L^{-1} (0.015 %), MMS liquid medium supplemented with SA 10 mg L^{-1} (0.020 %) and MMS liquid medium supplemented with MJ 10 mg L^{-1} (0.015 %).

From the study using MMS semisolid medium, the microrhizomes were extracted after three months of induction and analyzed for gingerols and shogaol. The MMS semisolid medium without elicitors was used as the control. As observed in experiment with MMS liquid medium, the recovery of oleoresin decreased with increased concentration of elicitors (Table 25). The highest oleoresin recovery was recorded in MMS semisolid medium supplemented with SA 5 mg L^{-1} (3.64 %). Next to the best medium, higher recovery of oleoresin was observed in MMS semisolid medium supplemented with MJ 10 mg L^{-1} (3.30 %) which was on par with MMS semisolid medium supplemented with MJ 5 mg L^{-1} (3.36%)and MMS semisolid medium (3.26 %). The lowest recovery of oleoresin was observed in MMS semisolid medium supplemented with SA 15 mg L^{-1} (2.78 %).

The same trend of accumulation of gingerols observed in MMS liquid medium was found in MMS semisolid medium. The highest accumulation (Table 25 and Figure 13) of total gingerols was observed in the MMS semisolid medium supplemented with SA 5 mg L^{-1} (Figure 14). The total gingerols was found decreased with increased concentration of abiotic elicitors. The highest accumulation of total gingerols of 1.702 percent recorded in the treatment MMS semisolid medium supplemented with SA 5 mg L^{-1}. The same treatment, recorded the highest 6, 8 and 10 gingerols of 1.398 per cent, 0.117 per cent, and 0.182 per cent respectively. The MMS medium supplemented with MJ at a concentration 5 mg L^{-1} recorded 1.297 per cent 6-gingerol, 0.080 per cent 8- gingerol and 1.392 per cent total gingerols production which was next to the best treatment.

Irrespective of the concentration of the elicitor, among the two abiotic elicitors, salicylic acid was observed the best elicitor with respect to production of microrhizomes/ culture, fresh weight of the microrhizomes, recovery of oleoresin and production of gingerols. The abiotic elicitor SA at a lower concentration of 5 mg L^{-1} was effective in production of gingerols. Gingerol production in the two experiments

Table 24:- Effect of different levels of abiotic elicitors on accumulation of gingerols and shogaol in microrhizomes of the ginger variety Aswathy in MMS liquid medium

Treatments	Oleoresin and pungency principles of ginger					
	Oleoresin (%)	**6-gingerol (%)**	**8-gingerol (%)**	**10-gingerol (%)**	**Total gingerol (%)**	**6-shogaol (%)**
Liquid MMS+MJ 5 mg L^{-1}	2.47	0.550	0.043	0.065	0.623	0.010
Liquid MMS+MJ 10 mg L^{-1}	2.19	0.388	0.033	0.058	0.523	0.015
Liquid MMS+MJ 15 mg L^{-1}	2.11	0.255	0.013	0.023	0.288	0.010
Liquid MMS+SA 5 mg L^{-1}	2.86	0.890	0.058	0.078	1.035	0.010
Liquid MMS+SA 10 mg L^{-1}	2.30	0.395	0.020	0.060	0.483	0.020
Liquid MMS+SA 15 mg L^{-1}	1.84	0.153	0.013	0.018	0.163	0.015
Liquid MMS Medium (control)	2.29	0.280	0.030	0.070	0.392	0.010
CD (0.05)	0.28	0.043	0.009	0.011	0.058	0.005
SE(m)±	0.09	0.015	0.003	0.004	0.016	0.002

Data represent the mean of three biological and two technical replications

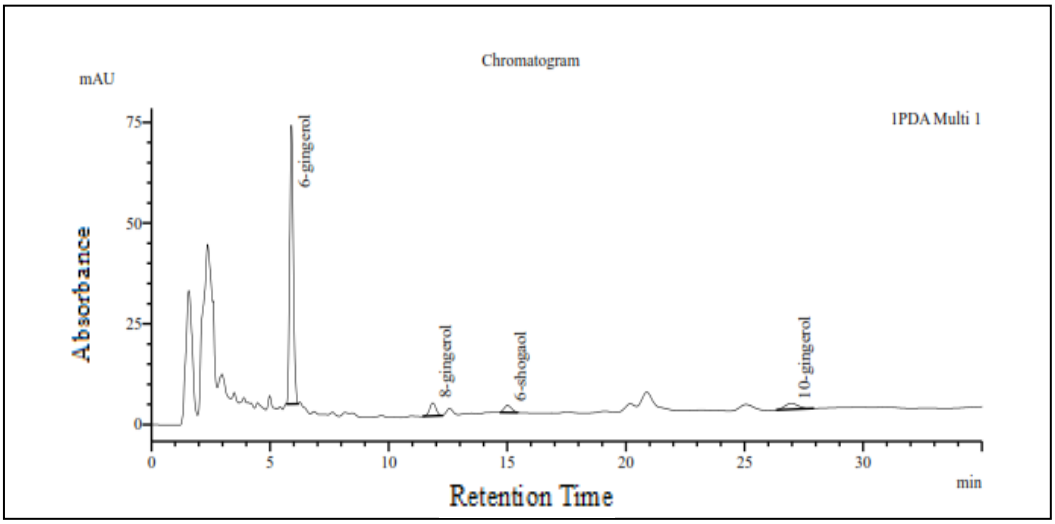


Figure 11: HPLC chromatogram for gingerols and shogaol in microrhizomes induced in MMS liquid medium elicited with SA 5 mgL^{-1}

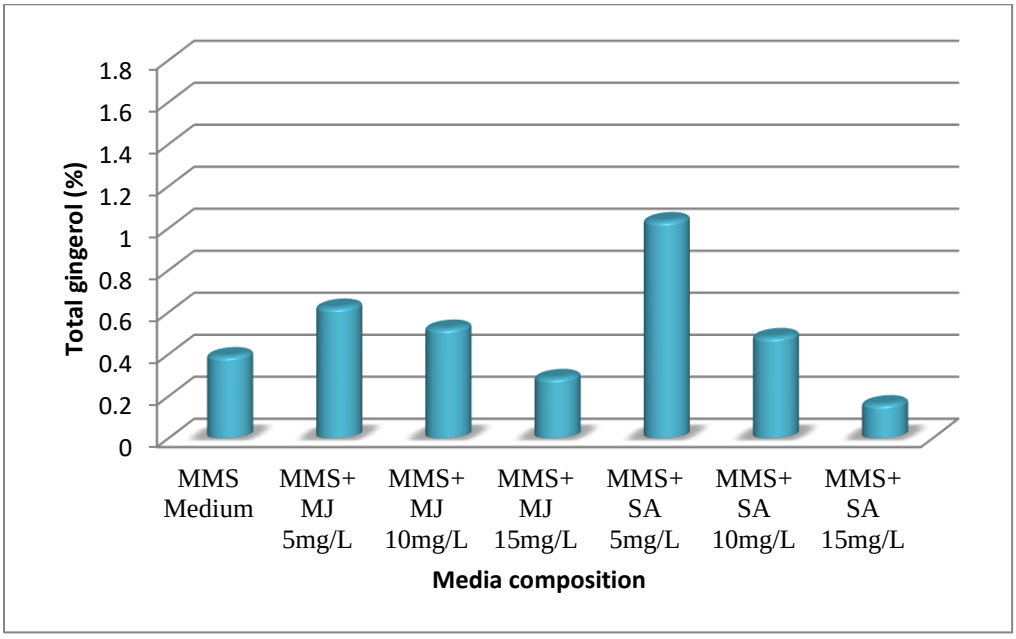


Figure 12:-Total gingerols (%) in microrhizomes in MMS liquid medium supplemented with different levels of abiotic elicitors

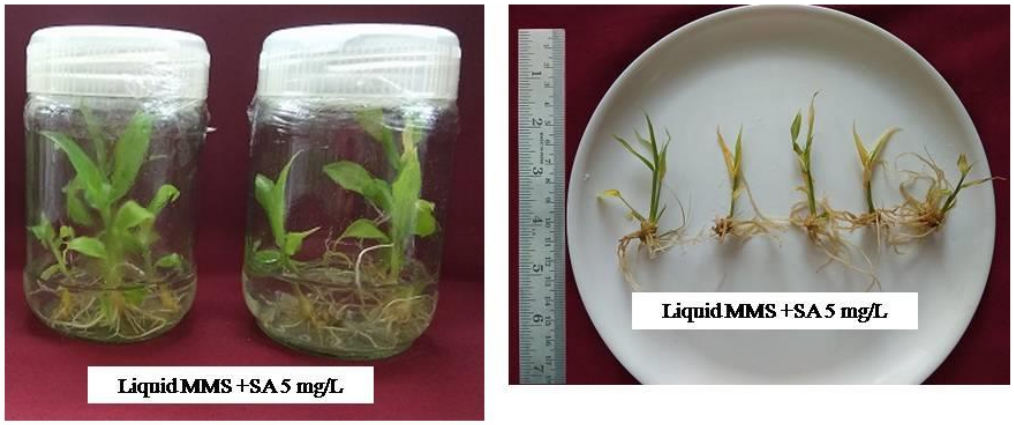


Plate 11: Microrhizome induced in MMS liquid medium supplemented with abiotic elicitor (SA and MJ) in one week old cultures

with MMS liquid medium and MMS semisolid medium supplemented with abiotic elicitors were compared. The highest accumulation of total gingerols was observed in MMS semisolid medium supplemented with abiotic elicitors with microrhizomes induced at three month induction period. Hence, further experiments were conducted using MMS semisolid medium with microrhizomes induced for three month induction period.

4.1.6.1.3 Effect of biotic elicitors in MMS medium on production and growth of microrhizomes and accumulation of gingerols and shogaol in the ginger variety Aswathy

The MMS medium supplemented with biotic elicitors such as autoclaved fungal mycelium at concentrations 5 and 10 mg L^{-1}, chitosan and chitin each at concentration 50 mg L^{-1} and 100 mg L^{-1}were used for the study.

4.1.6.1.3.1 Effect of biotic elicitors on production and growth of microrhizomes in the ginger variety Aswathy

Autoclaved fungal mycelium of *Pythium aphanidermatum* was used as biotic elicitor along with the other two biotic elicitors chitin and chitosan. The permanent slide of *P. aphanidermatum* was prepared and the morphological characters were confirmed. The aseptate hyphae with irregular spores and finger like sporangiospore of *P. aphanidermatum* were observed and confirmed (Plate 12). The fungal culture was prepared and the autoclaved crushed fungal mat (Plate 13) was used as biotic elicitor.

Two clumps were inoculated per culture in MMS medium containing 80 g L^{-1} sucrose supplemented with biotic elicitors and incubated for three months. The MMS medium was used as the control. The production of microrhizomes/ culture, fresh weight, dry weight and driage was found increased with increase in concentration of biotic elicitors (Table 26). Chitosan 100 mg L^{-1} was the best biotic elicitor. Lower production of microrhizomes/culture was observed in MMS medium supplemented with AFM 5 mg L^{-1} (7.68 microrhizomes) while increasing the concentration to 10 mgL^{-1} increased the production of microrhizomes/ culture (12.00 microrhizomes).

Table 25: Effect of different levels of abiotic elicitors on accumulation of gingerols and shogaol in microrhizomes ginger of the ginger variety Aswathy in MMS semisolid medium (on fresh weight basis)

Treatments	Oleoresin and pungency principles of ginger					
	Oleoresin (%)	**6–gingerol (%)**	**8-gingerol (%)**	**10-gingerol (%)**	**Total gingerol (%)**	**6-shogaol (%)**
MMS+ MJ 5 mg L^{-1}	3.36	1.297	0.080	0.065	1.392	0.020
MMS+ MJ 10 mg L^{-1}	3.30	1.215	0.040	0.050	1.303	0.030
MMS+ MJ 15 mg L^{-1}	2.92	0.775	0.050	0.063	0.888	0.020
MMS+ SA 5 mg L^{-1}	3.64	1.398	0.117	0.182	1.702	0.017
MMS+ SA 10 mg L^{-1}	3.12	1.198	0.038	0.060	1.305	0.035
MMS+ SA 15 mg L^{-1}	2.78	0.393	0.020	0.035	0.452	0.020
MMS Medium (control)	3.26	1.173	0.010	0.120	1.307	0.043
CD(0.05)	0.10	0.033	0.005	0.008	0.062	0.005
SE(m)±	0.03	0.011	0.002	0.003	0.021	0.002

Data represent the mean of three biological and two technical replications

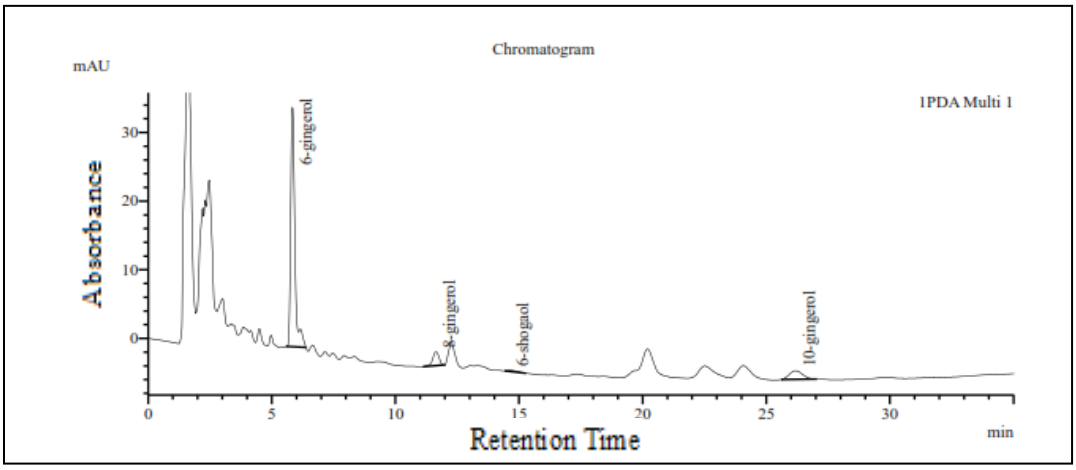


Figure 13:- HPLC Chromatogram for gingerols and shogaol in microrhizomes induced in MMS semisolid medium elicited with SA 5 mgL^{-1}

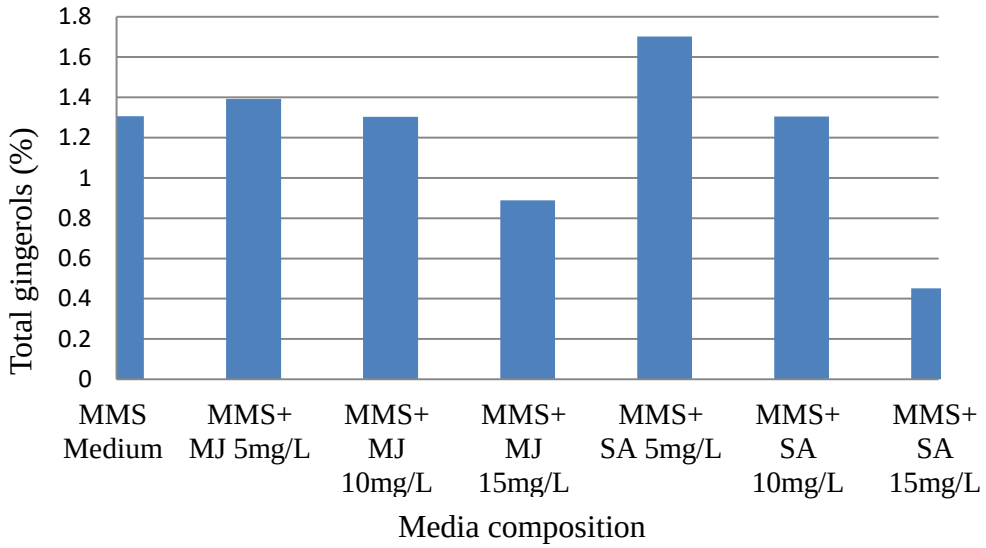


Figure 14:- Total gingerols (%) in microrhizomes induced in MMS semisolid medium supplemented with different levels of abiotic elicitors

Similar observation of increased production of number of microrhizomes was found with increased concentration of chitin and chitosan. The production of microrhizomes/culture was on par in the treatments MMS medium supplemented with chitosan 100 mg L^{-1} (15.68) and MMS medium (15.00). The lowest number of microrhizomes/ culture was recorded in MMS medium supplemented with chitin 50 mg L^{-1} (7.00) which was on par with MMS medium supplemented with AFM 5 mg L^{-1} (7.68).

No significant difference in fresh weight of microrhizomes was noticed between the treatments MMS control medium (0.536 g) and MMS supplemented with chitosan 100 mg L^{-1} (0.511 g).The lowest fresh weight was recorded in MMS medium elicited with autoclaved fungal mycelium 5 mg L^{-1} (0.229 g) and 10 mg L^{-1} (0.282 g). No significant difference was observed in dry weight of microrhizome between MMS medium (0.061 g) and MMS supplemented with chitosan 100 mg L^{-1} (0.057 g). The driage recorded was highest in MMS medium (3.0 3%) followed by the treatment MMS supplemented with chitosan 100 mg L^{-1} (2.78%). The lowest driage was recorded in the treatment MMS medium elicited with autoclaved fungal mycelium 5 mg L^{-1} (1.09 %).

4.1.6.1.3.2 Effect of biotic elicitors in MMS medium on gingerols and shogaol production in microrhizomes of the ginger variety Aswathy (fresh weight basis)

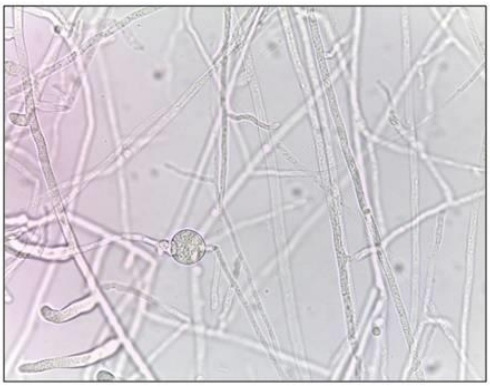
Microrhizomes induced in MMS medium supplemented with different concentrations of biotic elicitors were extracted and analyzed after three months induction period (Plate 14). The MMS medium containing 80 g L^{-1}sucrose without elicitors was used as the control. The recovery of oleoresin and gingerol accumulation increased with increase in the concentration of elicitor and recorded the highest with chitosan 100 mg L^{-1} (Table 27). The highest oleoresin recovery was recorded for the MMS medium supplemented with chitosan 100 mg L^{-1} (3.80 %) followed by the treatment MMS medium (3.55 %). The lowest recovery of oleoresin was recorded in MMS medium supplemented with AFM 5 mg L^{-1} (2.58 %).

Table 26: Effect of biotic elicitors in MMS medium on production and growth of microrhizomes in the ginger variety Aswathy

Sl. No.	Treatments	Number of microrhizomes/ culture	Fresh weight of microrhizome (g)	Driage (%)		
				Initial fresh weight (g)	Dry weight (g)	Driage (%)
1	MMS+ AFM 5 mg L^{-1}	7.68	0.229	2.0	0.020	1.09
2	MMS+AFM 10 mg L^{-1}	12.00	0.282	2.0	0.031	1.46
3	MMS+ Chitosan 50 mg L^{-1}	11.33	0.422	2.0	0.042	2.02
4	MMS+ Chitosan 100 mg L^{-1}	15.68	0.511	2.0	0.057	2.78
5	MMS+ Chitin 50 mg L^{-1}	7.00	0.300	2.0	0.037	1.77
6	MMS+ Chitin 100 mg L^{-1}	11.00	0.337	2.0	0.038	1.85
7	MMS Medium (control)	15.00	0.536	2.0	0.061	3.03
	CD(0.05)	2.22	0.100	-	0.004	0.22
	SE(m)±	0.72	0.033	-	0.001	0.07

Data represent the mean of four replications with eight cultures/ replication

Aseptate hyphae with irregular spores



Finger like sporangiospore of *Pythium aphanidermatum*

Plate 12:-Morphological characters of *Pythium aphanidermatum*

Seven day old cultures of *Pythium aphanidermatum*

Fifteen day old cultures of *Pythium aphanidermatum* in PDA medium

Fifteen days old mycelial mat of *Pythium aphanidermatum* after autoclaving

Plate 13:- Establishment of *Pythium aphanidermatum* cultures and mycellial mat

The highest accumulation of total gingerols (1.463 %) was recorded in MMS medium supplemented with chitosan 100 mgL^{-1} followed by MMS medium (1.300 %). The lowest accumulation of total gingerols was observed in MMS medium supplemented with AFM 5 mg L^{-1} (0.033 %) which was on par with MMS medium supplemented with chitin 50 mg L^{-1} (0.045 %). The accumulations of 6- gingerol (1.228 %), 8- gingerol (0.070 %) and 10- gingerol (0.148 %) were also recorded the highest in the same treatment, MMS medium supplemented with chitosan 100 mg L^{-1}. The 6-shogaol content recorded the highest in the treatment MMS supplemented with chitin 100 mg L^{-1}.

4.1.6.2 Effect of elicitors on *in vitro* gingerol production in calli of the ginger variety Aswathy

The best elicitation treatment identified in microrhizomes was tried in calli. The best biotic elicitor and abiotic elicitor identified were chitosan 100 mg L^{-1} and SA 5 mg L^{-1} respectively. The control used for the study were ½ MS medium supplemented with 2, 4-D 3 mg L^{-1} and BA 0.5 mg L^{-1} (callus inducing medium- CI) and ½ MMS medium supplemented with 2,4-D 3 mg L^{-1} and BA 0.5 mg L^{-1} (Modified callus inducing medium -MCI).

4.1.6.2.1 Effect of abiotic elicitor (SA 5 mg L^{-1}) on callus growth and accumulation of gingerol and shogaol in the ginger variety Aswathy

The calli induced in CI and MCI media supplemented with SA 5 mg L^{-1} were extracted and analyzed after three months of incubation (Plate 15). The calli maintained in CI and MCI media were used as the control. The fresh and dry weight of the calli was recorded (Table 28). The fresh weight recorded was highest in calli induced in CI medium as compared to the MCI medium while the dry weight and driage recorded the highest in MCI medium. The fresh weight of the calli induced in CI medium supplemented with SA 5 mg L^{-1} was 1.315 g which was on par with the CI medium which yielded the fresh weight of calli of 1.258 g. Dry weight of calli was found non significant under the different treatments. The dry weight of 0.451 g and 0.439 g was recorded for MCI medium without elicitation and MCI medium

Table 27:- Effect of biotic elicitors in MMS medium on accumulation of gingerols and shogaol in microrhizomes of the ginger variety Aswathy (on fresh weight basis)

Treatments	Oleoresin and pungency principles of ginger					
	Oleoresin (%)	6–gingerol (%)	8-gingerol (%)	10-gingerol (%)	Total gingerol (%)	6-shogaol (%)
MMS+ AFM 5 mg L^{-1}	2.58	0.033	0.000	0.000	0.033	0.000
MMS+AFM 10 mg L^{-1}	2.98	0.113	0.010	0.020	0.133	0.010
MMS+ Chitosan 50 mg L^{-1}	3.38	0.538	0.035	0.085	0.653	0.038
MMS+ Chitosan 100 mg L^{-1}	3.80	1.228	0.070	0.148	1.463	0.038
MMS+ Chitin 50 mg L^{-1}	3.07	0.045	0.000	0.000	0.045	0.044
MMS+ Chitin 100 mg L^{-1}	3.28	0.135	0.010	0.010	0.163	0.005
MMS Medium (control)	3.55	1.148	0.010	0.118	1.300	0.045
CD (0.05)	0.05	0.015	0.003	0.005	0.017	0.007
SE(m)±	0.02	0.005	0.001	0.002	0.004	0.002

Data represent mean of three biological and two technical replications

Plate 14: Microrhizome induced in MMS medium supplemented with various levels of biotic elicitors in three month old cultures

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supplemented with SA 5 mg L^{-1} respectively. The driage recorded the highest in MCI medium without elicitation (21.89 %) which was on par with MCI medium supplemented with SA5 mg L^{-1} (21.30 %).

Analyses of gingerols and shogaol were carried out from the calli induced in CI and MCI media supplemented with abiotic elicitors after three months. The calli induced were creamish brown with root morphogenesis. The calli induced in CI and MCI media without elicitation were used as the control. The oleoresin was extracted from different treatments and analyzed using HPLC. The highest recovery of oleoresin and accumulation of gingerols were recorded from the calli induced in CI medium compared to MCI medium (Table 29). The recovery of oleoresin observed in CI medium (3.09 %) was on par with the oleoresin recovery in CI medium supplemented with SA 5 mg L^{-1} (3.06 %).

The highest accumulation of total gingerols (0.128 %) was recorded in the calli induced in CI medium supplemented with SA 5mg L^{-1} followed by CI medium (0.105 %) (Table 29 and Figure 15). The lowest total gingerol was recorded in MCI medium. The highest accumulation of 6-gingerol was also recorded in the calli induced in CI medium supplemented with SA 5mg L^{-1} (0.078 %). No significant difference was observed between the CI medium and CI medium supplemented with SA 5 mg L^{-1} with respect to 8-gingerol production recording 0.018 per cent each. The accumulation of 10-gingerol and 6-shogaol recorded the highest in CI medium.

4.1.6.2.3 Effect of biotic elicitor (chitosan 100 mg L^{-1}) on callus growth and accumulation of gingerol and shogaol in the ginger variety Aswathy (fresh weight basis)

The calli induced in CI and MCI media supplemented with chitosan 100 mg L^{-1}were extracted and analyzed for gingerols and shogaol after three months of induction. The calli induced in CI and MCI media were used as the control. The calli induced were friable and creamish brown with root morphogenesis (Plate 16). The fresh weight and dry weight of the calli induced were recorded (Table 30). There

Table 28:- Effect of abiotic elicitor (SA 5 mg L^{-1}) on fresh weight and dry weight of calli in the ginger variety Aswathy

Treatments	Fresh weight (g)	Driage of calli (%)		
		Initial fresh weight (g)	Dry weight (g)	Driage (%)
CI + SA 5 mg L^{-1}	1.315	2.0	0.370	18.29
CI(control)	1.258	2.0	0.358	17.10
MCI + SA 5 mg L^{-1}	0.547	2.0	0.439	21.30
MCI (control)	0.859	2.0	0.451	21.89
CD(0.05)	0.473	-	NS	0.91
SE(m)±	0.071	-	0.037	0.03

Data represent the mean of four replications with five cultures/ replication

Table 29:- Effect of abiotic elicitor (SA 5 mgL^{-1}) on accumulation of gingerols and shogaol in calli of the ginger variety Aswathy

Treatments	Oleoresin and pungency principles of ginger					
	Oleoresin (%)	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Tot gingerols (%)	6-shogaol (%)
CI + SA 5 mgL^{-1}	3.06	0.078	0.018	0.030	0.128	0.038
CI control	3.09	0.055	0.018	0.035	0.105	0.063
MCI + SA 5 mgL^{-1}	3.01	0.063	0.013	0.013	0.083	0.018
MCI control	2.91	0.030	0.003	0.025	0.063	0.013
CD(0.05)	0.12	0.010	0.008	0.007	0.010	0.008
SE(m)±	0.04	0.003	0.003	0.002	0.003	0.002

Data represent mean of three biological and two technical replications

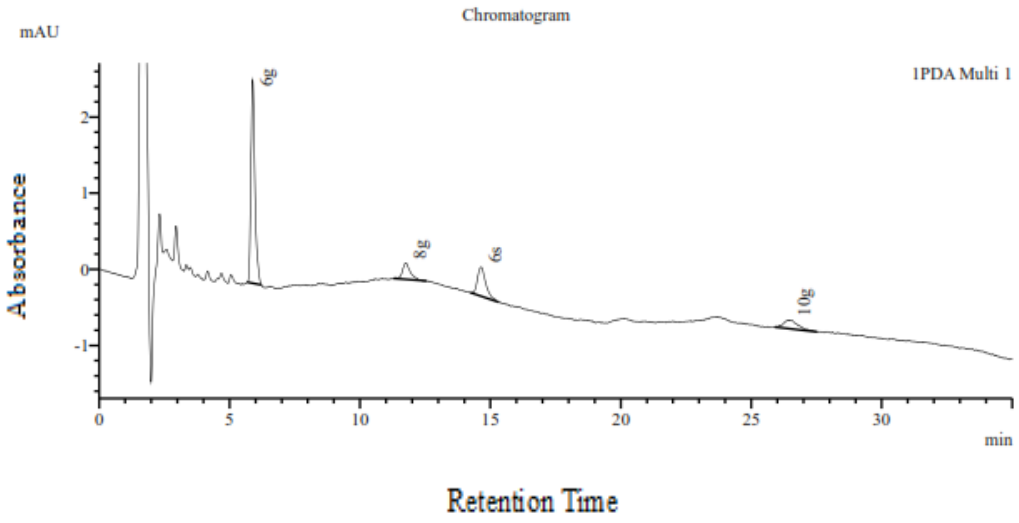


Figure 15:- HPLC chromatogram of gingerols and shogaol in three months old calli induced in MCI medium supplemented with SA 5 mgL^{-1}

was no significant difference in the fresh weight and dry weight of the calli among the treatments, while the driage recorded the highest in the treatment MCI medium (21.89 %).

On analysis, the oleoresin recovery recorded the highest in the treatment CI medium (3.09) and the lowest in the treatment MCI medium supplemented with chitosan 100 mg L^{-1} (2.79 %) (Table 31). The highest content of total gingerols was recorded in the treatment CI medium supplemented with chitosan 100 mg L^{-1} (0.124 %) followed by CI medium (0.105 %). The MCI medium supplemented with chitosan 100 mg L^{-1} recorded a total gingerol of 0.085 per cent. Accumulation of 6- gingerol of 0.088 per cent and 8-gingerol of 0.013 per cent recorded in the calli induced in CI medium supplemented with chitosan 100 mg L^{-1}.

4.1.7 Effect of precursor feeding in MMS medium on *in vitro* gingerol production in microrhizomes and calli of the ginger variety Aswathy

The precursors of the gingerol biosynthetic pathway like phenylalanine (PAL), coumaric acid (CA) and ferrulic acid (FA) at different concentrations of 30, 60 and 90 mg L^{-1} were supplemented to MMS medium and incubated for three months to study their effect on growth and production of microrhizomes and accumulation of gingerols and shogaol (Plate 17). The MMS medium containing 80 g L^{-1} sucrose without precursors was used as the control.

4.1.7.1 Effect of precursor feeding in MMS medium on growth production of microrhizomes and accumulation of gingerols and shogaol in the ginger variety Aswathy

4.1.7.1.1 Effect of precursor feeding in MMS medium on growth and production of microrhizomes in the ginger variety Aswathy

An average production of microrhizomes/culture of9.44, 9.64 and 9.22 microrhizomes was recorded respectively by treatments supplemented with FA, PAL and CA, irrespective of the concentration. An average fresh weight of microrhizome of 0.385 g with an average dry weight of 0.037 g and average driage of 1.85 per cent was

Table 30: Effect of biotic elicitor (chitosan 100 mg L^{-1}) on fresh weight and dry weight of calli in the ginger variety Aswathy

Treatments	Fresh weight (g)	Driage (%)		
		Initial fresh weight (g)	Dry weight (g)	Driage (%)
CI + Chitosan 100 mg L^{-1}	1.205	2.0	0.362	18.01
CI control	1.258	2.0	0.358	17.10
MCI + Chitosan 100 mg L^{-1}	0.947	2.0	0.415	20.59
MCI control	0.859	2.0	0.451	21.89
CD(0.05)	NS	-	NS	0.63
SE(m)±	0.230	-	0.027	0.29

NS- Not Significant

Data represent the mean of four replications with five cultures/ replication

Table 31:- Effect of biotic elicitor (chitosan 100 mg L^{-1}) on accumulation of gingerols and shogaol in calli of the ginger variety Aswathy

Treatments	Oleoresin and pungency principles in ginger					
	Oleoresin (%)	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Tot gingerols (%)	6-shogaol (%)
CI + Chitosan 100 mg L^{-1}	2.82	0.088	0.013	0.023	0.124	0.028
CI control	3.09	0.055	0.018	0.035	0.105	0.063
MCI +Chitosan 100 mg L^{-1}	2.79	0.068	0.003	0.013	0.085	0.055
MCI control	2.91	0.030	0.003	0.025	0.063	0.013
CD(0.05)	0.12	0.008	0.006	0.008	0.007	0.008
SE(m)±	0.04	0.003	0.003	0.003	0.002	0.002

Data represent the mean of three biological and two technical replications

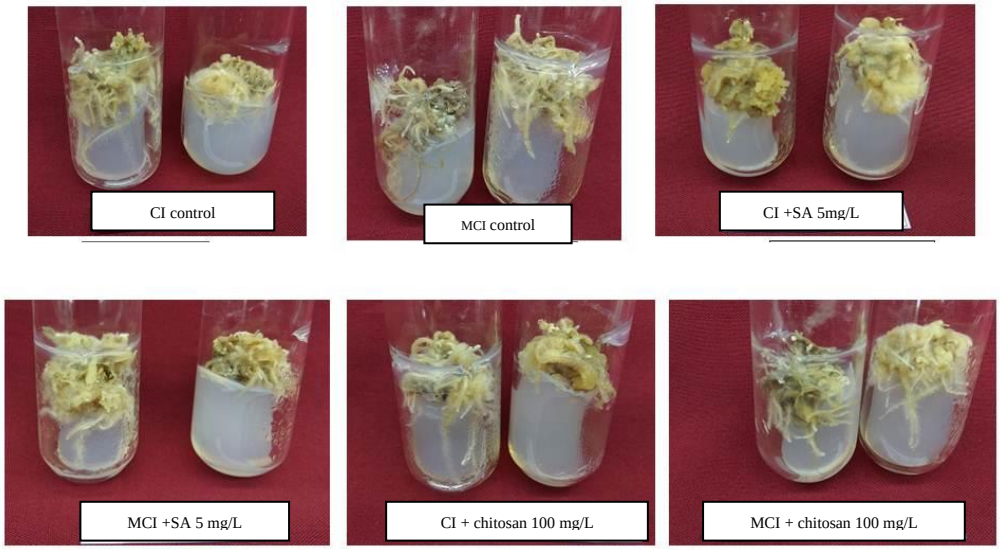


Plate 15: Calli of three month old cultures in CI and MCI media supplemented with biotic (Chitosan 100 mgL^{-1}) and abiotic (SA 5 $mgL^{-}1$) elicitors

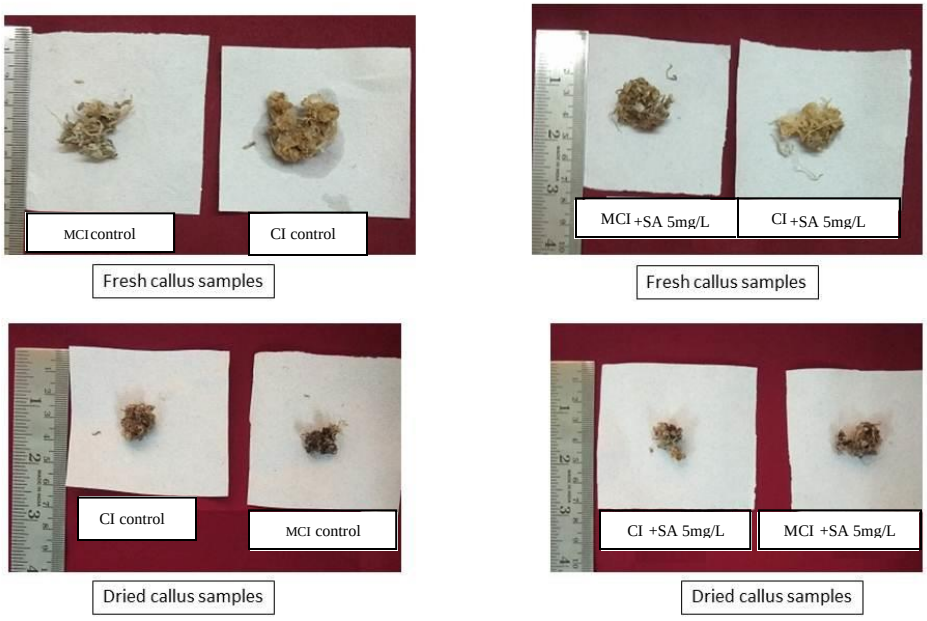


Plate 16 : Fresh and dry calli from three months old cultures induced in CI and MCI media supplemented with SA 5 mgL^{-1}

recorded by the treatments supplemented with FA, irrespective of concentration. Reduction in production of microrhizomes/culture, fresh weight, dry weight and driage was observed with increasing concentrations of the precursors (Table 32). Ferrulic acid 30 mg L^{-1} was selected as the best precursor among the three precursors studied. No significant difference was observed between the treatments MMS medium supplemented with CA 30 mg L^{-1}, MMS medium supplemented with FA 30 and 60 mg L^{-1}, MMS medium supplemented with PAL 30 and 60 mg L^{-1} and MMS medium with respect to production of microrhizomes/ culture after three months of induction (Table 32). There was decrease in weight of the microrhizomes with increase in concentration of precursors. But the difference was not statistically significant. The dry weight recorded in the MMS medium (0.061 %) was on par with the treatment MMS medium supplemented with FA 30 mg L^{-1} (0.057 %). The lowest dry weight was recorded in MMS medium supplemented with FA 90 mg L^{-1}. The driage recorded highest in the treatment MMS medium (2.98 %) followed by the treatment MMS medium supplemented with FA 30 mgL^{-1} (2.82 %).

4.1.7.1.2 Effect of precursor feeding in MMS medium on gingerols and shogaol production in microrhizomes of the ginger variety Aswathy (fresh weight basis)

The highest average recovery of oleoresin recorded highest in MMS control (3.55 %) which was on par with MMS medium supplemented with FA (Table 33). The accumulation of gingerols was observed highest in MMS medium supplemented with FA. The oleoresin recovery of 3.50, 2.71 and 3.29 per cent and total gingerols of 0.937, 0.402 and 0.494 per cent was recorded by the treatments supplemented with FA, PAL and CA respectively, irrespective of the concentration. The recovery of oleoresin decreased with increase in concentration of precursors. The MMS medium supplemented with FA 30 mg L^{-1} recorded the highest oleoresin yield of 3.83 per cent followed by MMS medium with oleoresin recovery of 3.55 per cent. The lowest recovery of oleoresin (2.49 %) was recorded in the treatment MMS medium supplemented with PAL 90 mg L^{-1}.The accumulation of total gingerols was observed decreased with increase in concentration of precursors. The highest total gingerols of 1.453 percent were recorded in MMS medium supplemented with FA

Table 32: Effect of precursor feeding in MMS medium on production of microrhizomes in the ginger variety Aswathy

Treatments	Number of microrhizomes/ culture	Fresh weight of microrhizome (g)	Driage (%)		
			Initial fresh weight (g)	Dry weight (g)	Driage (%)
MMS+FA30 mg L^{-1}	11.00	0.487	2.0	0.057	2.82
MMS+FA60 mg L^{-1}	10.00	0.376	2.0	0.034	1.77
MMS+FA90 mg L^{-1}	7.33	0.292	2.0	0.019	0.96
Mean	9.44	0.385	-	0.037	1.85
MMS+PAL30mg L^{-1}	11.68	0.335	2.0	0.034	1.60
MMS+PAL60mg L^{-1}	10.00	0.345	2.0	0.031	1.55
MMS+PAL90mg L^{-1}	7.25	0.329	2.0	0.025	1.20
Mean	9.64	0.336	-	0.030	1.45
MMS+CA30 mg L^{-1}	12.00	0.386	2.0	0.033	1.63
MMS+CA60 mg L^{-1}	9.00	0.343	2.0	0.031	1.61
MMS+CA90 mg L^{-1}	6.66	0.251	2.0	0.024	1.23
Mean	9.22	0.327	-	0.029	1.49
MMS Medium (control)	14.33	0.526	2.0	0.061	2.98
CD(0.05)	2.01	NS	-	0.003	0.14

FA-Ferrulic acid PAL- Phenylalanine CA- Coumaric acid NS-Not Significant

Data represent the mean of four replications with eight cultures/ replication

30 mg L^{-1} followed by MMS medium (1.300 %) (Table 33 and Figure 16 and 17).The lowest total gingerols of 0.210 per cent was recorded in the treatment MMS medium supplemented with PAL 90 mg L^{-1}. The accumulation of 6- gingerol also recorded the highest in MMS medium supplemented with FA 30 mg L^{-1} (1.363 %) followed by MMS medium (1.170 %). The 8-gingerolrecorded the highest in the MMS supplemented with PAL 30 mg L^{-1} (0.073 %) while the 10-gingerol recorded the highest in MMS medium (0.113 %).

4.1.7.1.3 Effect of precursor feeding on callus growth and accumulation of gingerol and shogaol in the ginger variety Aswathy

The best treatment identified from precursor feeding in microrhizomes was ferrulic acid 30 mg L^{-1}. This treatment was tried in calli to study the effect on gingerol production. Hence for the study effect of precursor feeding on calli CI and MCI medium supplemented with ferrulic acid 30 mg L^{-1} was used. The calli induced in CI and MCI media without elicitation were used as the control. The data on growth of calli, recovery of oleoresin and accumulation of gingerols and shogaol is given in Table 34 and 35.

Calli induced in CI and MCI medium supplemented with FA 30 mgL^{-1} after three months of induction were used for analysis (Plate 18). The callus was friable creamish brown with root morphogenesis. There was no significant difference among the treatments with respect to the fresh weight and dry weight of the induced calli (Table 34). The driage recorded the highest in the treatment MCI medium supplemented with FA 30 mg L^{-1}.The fresh callus induced in CI and MCI medium supplemented with FA 30 mg L^{-1}were extracted with acetone and analyzed for gingerols and shogaol using HPLC.

The highest recovery of oleoresin was recorded in the treatment CI control medium (3.09 %) which was on par with the treatment CI medium supplemented with FA 30 mg L^{-1} (2.99 %) (Table 35). The production of total gingerols recorded the higher in CI medium in comparison with MCI medium. No significant difference was observed between the treatments CI medium supplemented

Table 33:- Effect of precursor feeding in MMS medium on accumulation of gingerols and shogaol in microrhizomes of the ginger variety Aswathy (fresh weight basis)

Treatments	Oleoresin and pungency principles of ginger					
	Oleoresin (%)	**6-gingerol (%)**	**8-gingerol (%)**	**10-gingerol (%)**	**Total gingerol (%)**	**6-shogaol (%)**
MMS+FA 30 mg L^{-1}	3.83	1.363	0.050	0.060	1.453	0.023
MMS+FA 60 mg L^{-1}	3.39	0.777	0.010	0.010	0.830	0.010
MMS+FA 90 mg L^{-1}	3.28	0.457	0.010	0.017	0.527	0.010
Mean	3.50	0.866	0.023	0.029	0.937	0.014
MMS+PAL 30 mg L^{-1}	2.94	0.257	0.073	0.073	0.593	0.020
MMS+PAL 60 mg L^{-1}	2.70	0.377	0.010	0.017	0.403	0.010
MMS+PAL 90 mg L^{-1}	2.49	0.160	0.040	0.010	0.210	0.040
Mean	2.71	0.265	0.041	0.033	0.402	0.023
MMS+CA 30 mg L^{-1}	3.37	0.547	0.050	0.050	0.727	0.037
MMS+CA 60 mg L^{-1}	3.30	0.400	0.043	0.010	0.480	0.043
MMS+CA 90 mg L^{-1}	3.20	0.147	0.010	0.010	0.277	0.010
Mean	3.29	0.365	0.034	0.023	0.494	0.030
MMS Medium (control)	3.55	1.170	0.010	0.113	1.300	0.010
CD(0.05)	0.08	0.094	0.009	0.008	0.057	0.005
SE(m)±	0.03	0.020	0.003	0.005	0.006	0.002

Data represent the mean of three biological and two technical replications

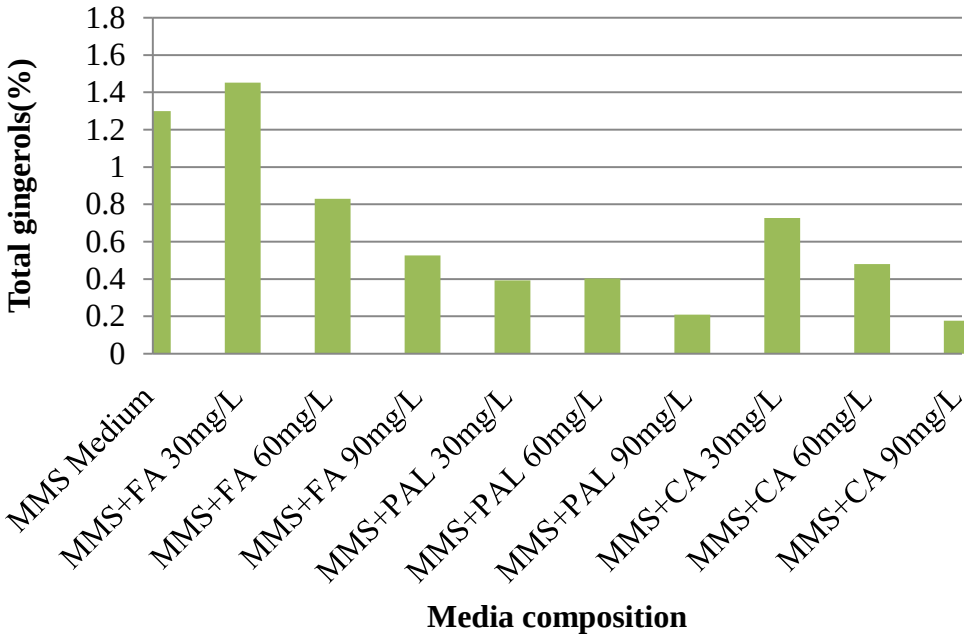


Figure 16: Effect of precursor feeding in MMS medium on total gingerols in ginger microrhizomes

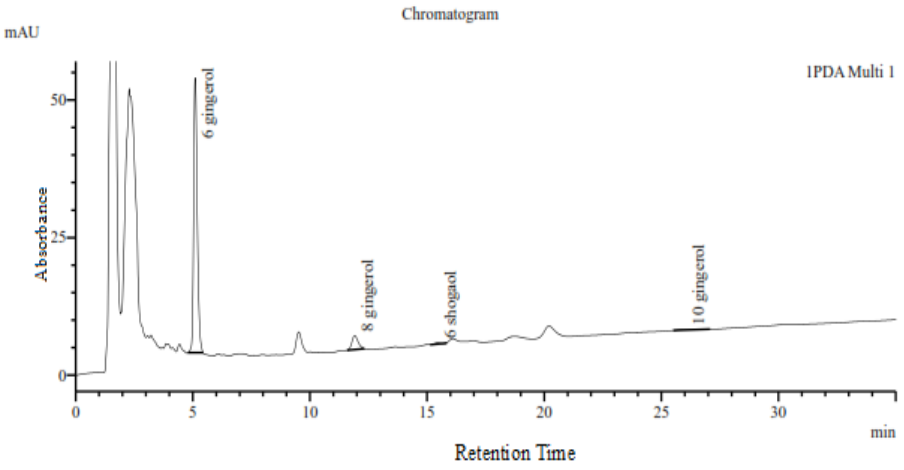


Figure 17:- HPLC chromatogram for gingerols and shogaol from microrhizomes induced in MMS medium supplemented with FA 30 mgL^{-1}

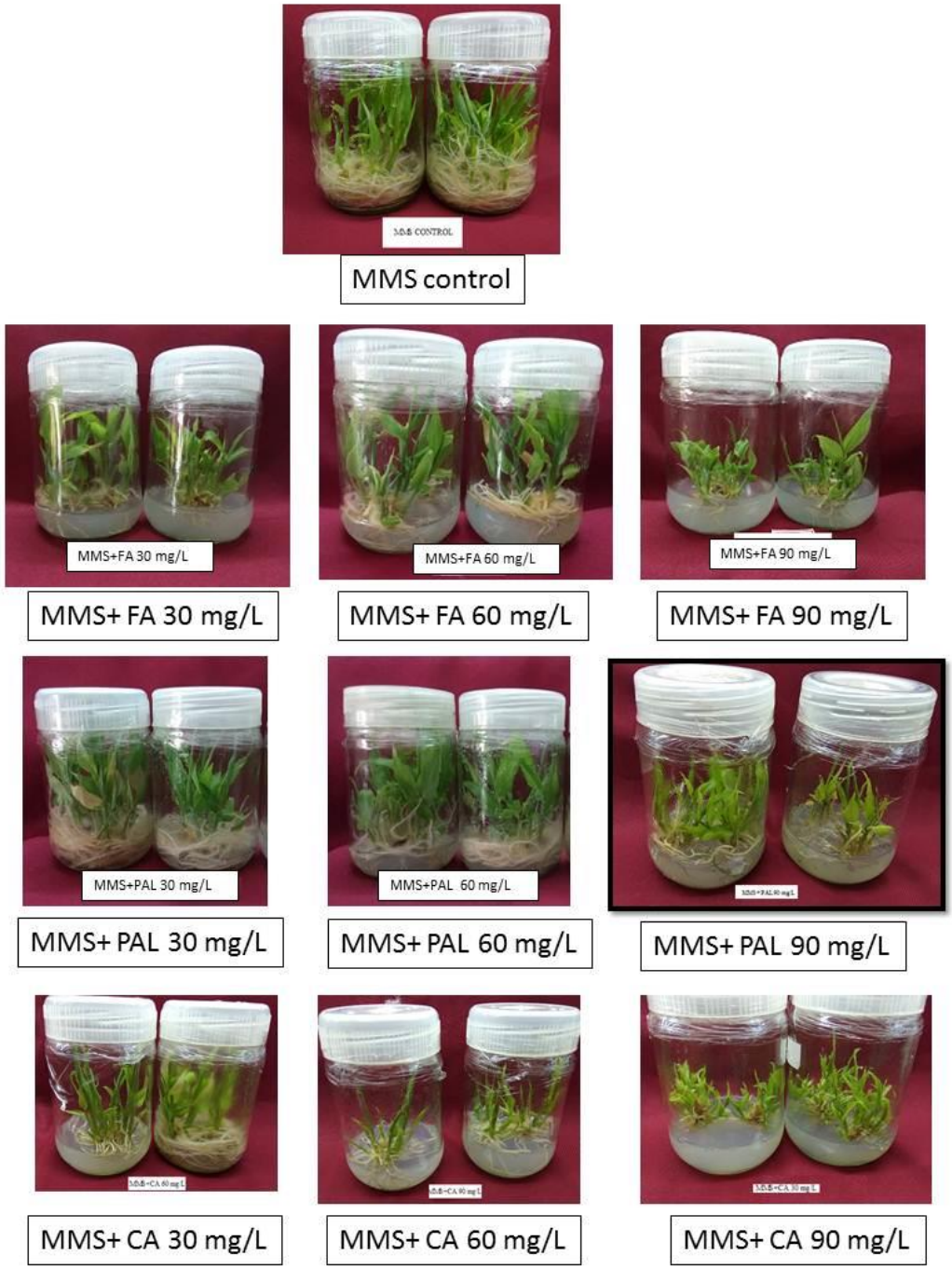


Plate 17:-Microrhizome induced in MMS medium supplemented with different levels of precursors

Table 34:- Effect of precursor feeding (FA 30 mgL^{-1}) on fresh weight and dry weight of calli in the ginger variety Aswathy

Treatments	Fresh weight (g)	Driage (%)		
		Initial fresh weight (g)	Dry weight (g)	Driage (%)
CI + FA 30 mg L^{-1}	1.264	2.0	0.431	20.80
CI control	1.258	2.0	0.358	17.10
MCI + FA 30 mg L^{-1}	1.000	2.0	0.479	22.48
MCI control	0.859	2.0	0.451	21.89
CD(0.05)	NS	-	NS	1.09

NS- Non significant

Data represent the mean of four replications with five cultures/ replication

Table 35:- Effect of precursor feeding (FA 30 mgL^{-1}) on accumulation of gingerols and shogaol content in calli of the ginger variety Aswathy (fresh weight basis)

Treatments	Oleoresin and pungency principles of ginger					
	Oleoresin (%)	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Tot gingerols (%)	6-shogaol (%)
CI + FA 30 mg L^{-1}	2.99	0.072	0.021	0.018	0.111	0.018
CI control	3.09	0.055	0.018	0.035	0.105	0.043
MCI + FA 30 mg L^{-1}	2.68	0.064	0.013	0.013	0.048	0.063
MCI control	2.91	0.030	0.003	0.025	0.063	0.013
CD(0.05)	0.12	0.008	0.008	0.008	0.010	0.008
SE(m)±	0.04	0.002	0.002	0.002	0.003	0.002

Data represent the mean of three biological and two technical replications

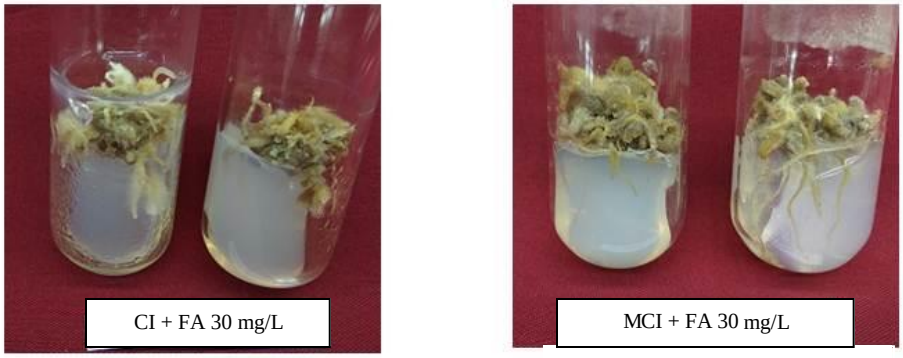


Plate 18: -Calli induced in CI and MCI media supplemented with FA 30 mgL^{-1}

with FA 30 mg L^{-1} (0.111 %) and CI medium (0.105 %) with respect to the production of total gingerols. The highest accumulation of 6- gingerol was recorded in CI medium supplemented with FA 30 mgL^{-1}(0.072 %) which was on par with the treatment MCI medium supplemented with FA 30 mgL^{-1} (0.064 %).The accumulation of8-gingerol was observed on par in the treatments CI medium supplemented with FA 30 mg L^{-1} (0.021 %) and CI medium (0.018 %).

4.1.8 Effect of gamma irradiation on *in vitro* gingerol production in microrhizomes and calli of the ginger variety Aswathy

4.1.8.1 Effect of gamma irradiation on accumulation of gingerols and shogaol in the ginger variety Aswathy

Two month old microrhizomes in MS and MMS medium were exposed to gamma radiation of two different dose 20 and 30 Gy and its effect on the accumulation of gingerols and shogaol was studied after one month of gamma irradiation. The observations on survival (%), shoot morphogenesis, gingerols and shogaol contents accumulated were recorded one month after the irradiation.

4.1.8.1.1 Effect of gamma irradiation on survival and shoot morphogenesis in microrhizomes of the ginger variety Aswathy

Irradiation of microrhizomes with gamma rays at doses of 20 Gy and 30 Gy was done. The microrhizomes induced in MS and MMS medium containing 80 mg L^{-1} sucrose without gamma irradiation were used as control. Browning to complete drying of microrhizomes was observed after two and half weeks of irradiation depending on the dose of gamma irradiation in 10-20 per cent of the irradiated microrhizomes. Survival percentage and shoot morphogenesis were observed non significant among the treatments. It was observed that with increase in dose of radiation, the survival percentage decreased (Table 36).

Table 36:- Response of gamma irradiation on survival and shoot morphogenesis in microrhizomes of the ginger variety Aswathy

Treatments	Survival percent (%)	Shoot morphogenesis in one month
MS + 20 Gy	86.66	3.80
MMS + 20 Gy	86.66	3.00
MMS + 30 Gy	73.33	2.00
MMS control	100	4.80
CD (0.05)	NS	NS
SE(m)±	0.07	0.02

NS- Not Significant

Data represent the mean of four replications with five cultures/ replication

4.1.8.1.2 Effect of gamma irradiation on accumulation of gingerols and shogaol content in microrhizomes of the ginger variety Aswathy (fresh weight basis)

The gamma irradiated microrhizomes under each treatment were grouped based on shoot morphogenesis. Extraction of oleoresin and analysis of gingerols and shogaol were done for the two groups of irradiated microrhizomes with shoot and without shoot morphogenesis and are presented in the Table 37 and Plate 19. The dose of gamma irradiation 20 Gy was selected as the best for higher accumulation of gingerols. Higher oleoresin recovery and accumulation of gingerols were observed in the microrhizomes irradiated with 20 Gy as compared to 30 Gy. The microrhizomes in MMS medium recorded higher oleoresin recovery and accumulation of gingerols as compared to that of the microrhizomes in MS medium. It was found that, the irradiated microrhizomes at shoot morphogenetic stage accumulated more oleoresin and gingerols as compared to the irradiated microrhizomes without shoot morphogenesis.

The highest oleoresin recovery was obtained from the microrhizomes with shoot morphogenesis maintained in MMS medium irradiated with 20 Gy (3.74%). The MMS medium irradiated with 20 Gy (without shoot morphogenesis) and MMS medium irradiated with 30 Gy (with shoot morphogenesis) were on par with respect to oleoresin recovery recording 3.46 per cent and 3.34 per cent respectively. The lowest recovery of oleoresin was recorded in the treatment MS medium irradiated with 20 Gy (without shoot morphogenesis) which recorded 2.61 per cent.

The highest accumulation of total gingerols (1.110 %) was observed in the microrhizomes with shoot morphogenesis maintained in MMS medium irradiated with 20 Gy (Table 37 and Figure 18). Next to the best treatment, MMS medium irradiated with 20 Gy (without shoot morphogenesis) recorded total gingerol of 0.790 per cent. The lowest total gingerols of 0.167 per cent were recorded in the MMS medium irradiated with 30 Gy (without shoot morphogenesis).

The highest accumulation of 6- gingerol (0.943 %), and 10 gingerol (0.113 %) was also recorded in the treatment, MMS medium irradiated with 20 Gy (with shoot morphogenesis). The accumulation of 8-gingerol content was on par in

Table 37: Effect of gamma irradiation on recovery of oleoresin, accumulation of gingerols and shogaol in microrhizomes of the ginger variety Aswathy (fresh weight basis)

Treatments	Oleoresin and pungency principles in ginger					
	Oleoresin (%)	**6–gingerol (%)**	**8-gingerol (%)**	**10-gingerol (%)**	**Total gingerol (%)**	**6-shogaol (%)**
MMS+ 20Gy (without shoot morphogenesis)	3.46	0.653	0.040	0.093	0.790	0.017
MMS+ 20Gy (with shoot morphogenesis)	3.74	0.943	0.053	0.113	1.110	0.047
MS+ 20Gy (without shoot morphogenesis)	2.61	0.260	0.023	0.033	0.317	0.007
MS+20Gy (with shoot morphogenesis)	3.10	0.353	0.013	0.060	0.443	0.020
MMS+ 30Gy (without shoot morphogenesis)	2.77	0.143	0.010	0.017	0.167	0.003
MMS+30Gy (with shoot morphogenesis)	3.34	0.530	0.050	0.010	0.587	0.010
MMS control (without shoot morphogenesis)	2.98	0.320	0.017	0.030	0.363	0.010
MMS control (with shoot morphogenesis)	3.21	0.430	0.027	0.050	0.507	0.013
CD(0.05)	0.13	0.036	0.010	0.014	0.028	0.008
SE(m)±	0.04	0.009	0.003	0.004	0.006	0.002

Data represent the mean of three biological and two technical replications

MMS medium irradiated with 20 Gy (with shoot morphogenesis) and MMS medium irradiated with 30 Gy (with shoot morphogenesis) recording 0.053 per cent and 0.050 per cent respectively. The 6-shogaol content was on par in MMS medium irradiated with 20 Gy (with and without shoot morphogenesis), MS medium irradiated with 20 Gy (with shoot morphogenesis) and MMS medium (with shoot morphogenesis).

4.1.8.2 Effect of gamma irradiation of calli in the ginger variety Aswathy on gingerol production

The best dose of gamma irradiation in microrhizome was 20 Gy. This was tried in calli to study the effect of gamma irradiation using CI and MCI media. The calli induced in CI and MCI media without irradiation were used as the control. Two month old calli induced in CI and MCI medium were irradiated with 20Gy and incubated for one month after transferring to fresh medium of same composition. Observations on survival (%), fresh and dry weight (g), driage (%) and accumulation of gingerols and shogaol were recorded and presented in Table 38 and 39.

4.1.8.2.1 Effect of gamma irradiation on growth and production of gingerols and shogaol in calli of the ginger variety Aswathy(fresh weight basis)

Browning and complete drying of calli was observed in about ten per cent of the irradiated calli. The survival (%) of irradiated calli in various treatments was not significant. The calli was friable and pale creamy initially and turned to creamish brown after one month of irradiation with root morphogenesis (Plate 20). There was no significant difference among the treatments with respect to fresh weight of the calli. Dry weight of the calli was on par in the treatments MCI control medium (0.451 g), CI medium irradiated with 20 Gy (0.409 g) and MCI medium irradiated with 20 Gy (0.498 g).The driage recorded the highest in the treatment MCI medium irradiated with 20 Gy (23.33 per cent).

Oleoresin was extracted from fresh calli and analysis of gingerols and shogaol was carried out. The calli in CI and MCI medium without gamma irradiation were used as the control. The accumulation of gingerols recorded the highest in CI medium as compared to MCI medium (Table 39). The oleoresin recovery was

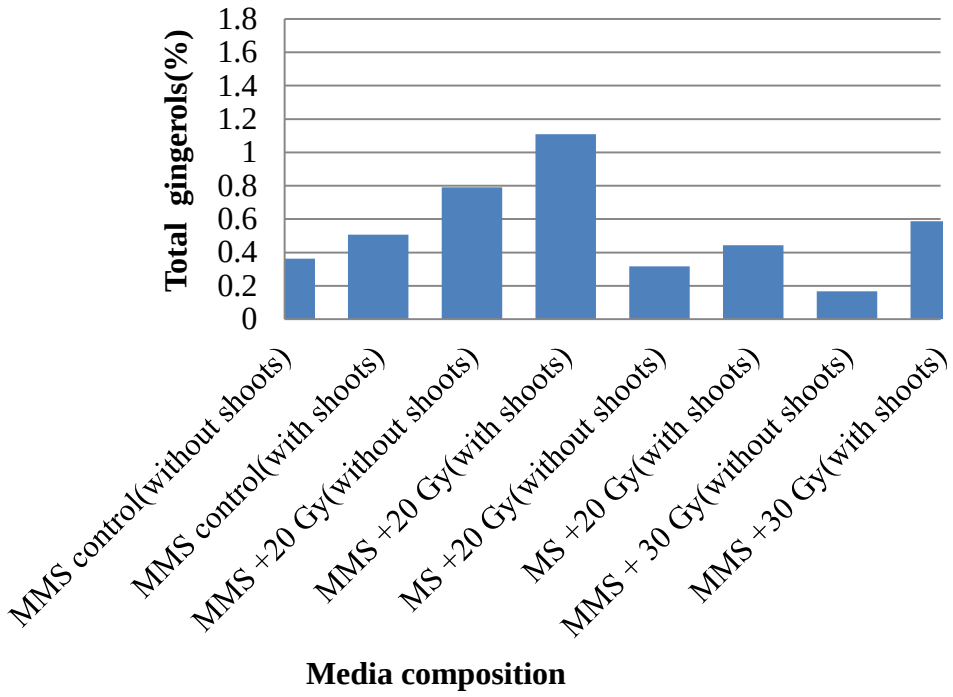


Figure 18: Total gingerols in microrhizomes irradiated with gamma rays

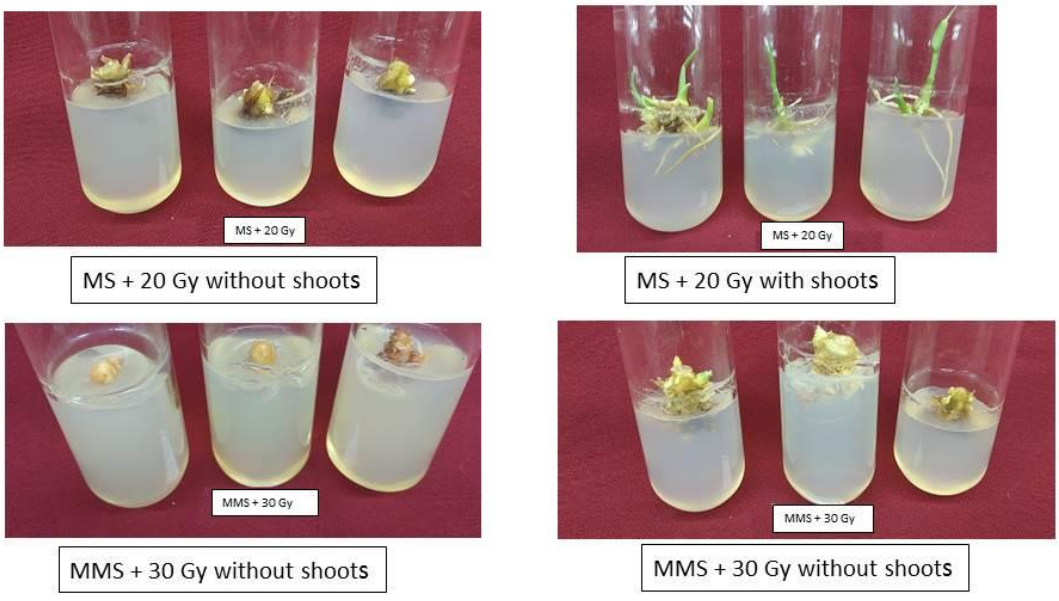


Plate 19:- Irradiated microrhizomes in MS and MMS media after one month of radiation

Table 38:- Response of gamma irradiation on fresh weight and dry weight of calli in the ginger variety Aswathy

Treatments	Survival (%)	Fresh weight (g)	Driage (%)		
			Initial fresh weight (g)	Dry weight (g)	Driage (%)
CI + 20 Gy	90.23	0.875	2.0	0.409	19.97
CI control	100	1.258	2.0	0.358	17.10
MCI+20 Gy	91.41	0.813	2.0	0.498	23.33
MCI control	100	0.859	2.0	0.451	21.89
CD(0.05)	NS	NS	-	0.090	1.11
SE(m)±	0.08	0.031	-	0.009	0.36

NS-Not Significant

Data represent the mean of four replication with five cultures/ replication

Table 39:- Response of gamma irradiation on production of gingerols and shogaol in calli of the ginger variety Aswathy

Treatments	Oleoresin and pungency principles in ginger					
	Oleoresin (%)	6-gingerol (%)	8-gingerol (%)	10-gingerol (%)	Tot gingerols (%)	6-shogaol (%)
CI + 20 Gy	2.77	0.073	0.023	0.035	0.115	0.023
CI control	3.09	0.055	0.018	0.035	0.105	0.063
MCI+20 Gy	2.72	0.058	0.003	0.035	0.105	0.028
MCI control	2.91	0.030	0.003	0.025	0.063	0.013
CD(0.05)	0.13	0.008	0.008	NS	0.009	0.008
SE(m)±	0.04	0.002	0.002	0.004	0.003	0.002

NS-Not Significant

Data represent the mean of three biological and two technical replications

CI + 20 Gy

MCI + 20 Gy

Plate 20:–Irradiated calli with 20 Gy dose of gamma rays in CI and MCI media after one month

recorded the highest in the CI medium (3.09 %) followed by MCI medium (2.91 %). Highest total gingerols was observed in the CI medium irradiated with 20 Gy (0.115 %). The highest accumulation of 6-gingerol (0.073 %) also recorded the highest in CI medium irradiated with 20 Gy. The 8-gingerol accumulation was observed to be on par in the CI medium irradiated with 20 Gy(0.023 %) and CI medium (0.018 %). No significant difference was observed among the treatments with respect to the accumulation of 10-gingerol. The 6-shogaol content recorded the highest in CI medium (0.063 %).

4.1.9 Comparison of different treatments using microrhizomes accumulating high gingerol

From the experiments of *in vitro* gingerol production in induced microrhizomes using MMS medium, chitosan 100 mg L^{-1} and SA 5 mg L^{-1} were selected as the best biotic and abiotic elicitor respectively registering the highest production of total gingerols. Similarly, the best precursor and the best dose of gamma irradiation were selected as FA 30 mg L^{-1} and 20 Gy respectively. To compare effect of these factors in MS medium, microrhizomes were induced in MS medium supplemented with chitosan 100 mg L^{-1}, SA 5 mg L^{-1} and FA 30 mg $L^{-1}$for three months (Plate 21). The MS and MMS media containing 80 mg L^{-1} sucrose were used as the controls. Two month old microrhizomes irradiated with 20 Gy gamma irradiation were taken after one month of incubation and the effect of irradiation on gingerol synthesis was studied. The oleoresin was extracted from the three month old microrhizome employing acetone and analyses of gingerols and shogaol was carried out. The oleoresin recovery was observed on par between the treatments MMS medium (3.55 %) and MS medium supplemented with SA 5 mg L^{-1}(Table 40). The lowest oleoresin recovery of 2.61 per cent was recorded in the treatment MS medium irradiated with 20 Gy (without shoot morphogenesis).

Total gingerol production was observed highest in MMS medium (1.300 %) followed by the treatment MS medium supplemented with the abiotic elicitor SA 5 mg L^{-1} (1.170 %). The lowest total gingerols of 0.317 per cent were recorded in the treatment MS medium irradiated with 20 Gy (without shoot

Table 40:- Effect of MS media supplemented with the identified best elicitor, precursor and dose of gamma radiation on *in vitro* gingerol synthesis in microrhizomes of the ginger variety Aswathy (fresh weight basis)

Treatments	Oleoresin and pungency principles of ginger					
	Oleoresin (%)	**6-gingerol (%)**	**8-gingerol (%)**	**10-gingerol (%)**	**Total gingerol (%)**	**6-shogaol (%)**
MS+SA 5 mg L^{-1}	3.57	0.953	0.090	0.123	1.170	0.047
MS+ FA 30 mg L^{-1}	3.46	0.733	0.033	0.058	0.823	0.040
MS+ Chitosan 100 mg L^{-1}	3.22	0.670	0.010	0.110	0.787	0.040
MS+20Gy (with shoot morphogenesis)	3.10	0.353	0.013	0.060	0.443	0.020
MS+ 20Gy (without shoot morphogenesis)	2.61	0.260	0.023	0.033	0.317	0.007
MS Medium (control)	3.16	0.627	0.010	0.110	0.747	0.040
MMS Medium (control)	3.55	1.148	0.010	0.118	1.300	0.045
CD (0.05)	0.10	0.013	0.010	0.007	0.019	0.006
SE(m)±	0.004	0.004	0.003	0.002	0.006	0.002

Data represent the mean of three biological and two technical replications

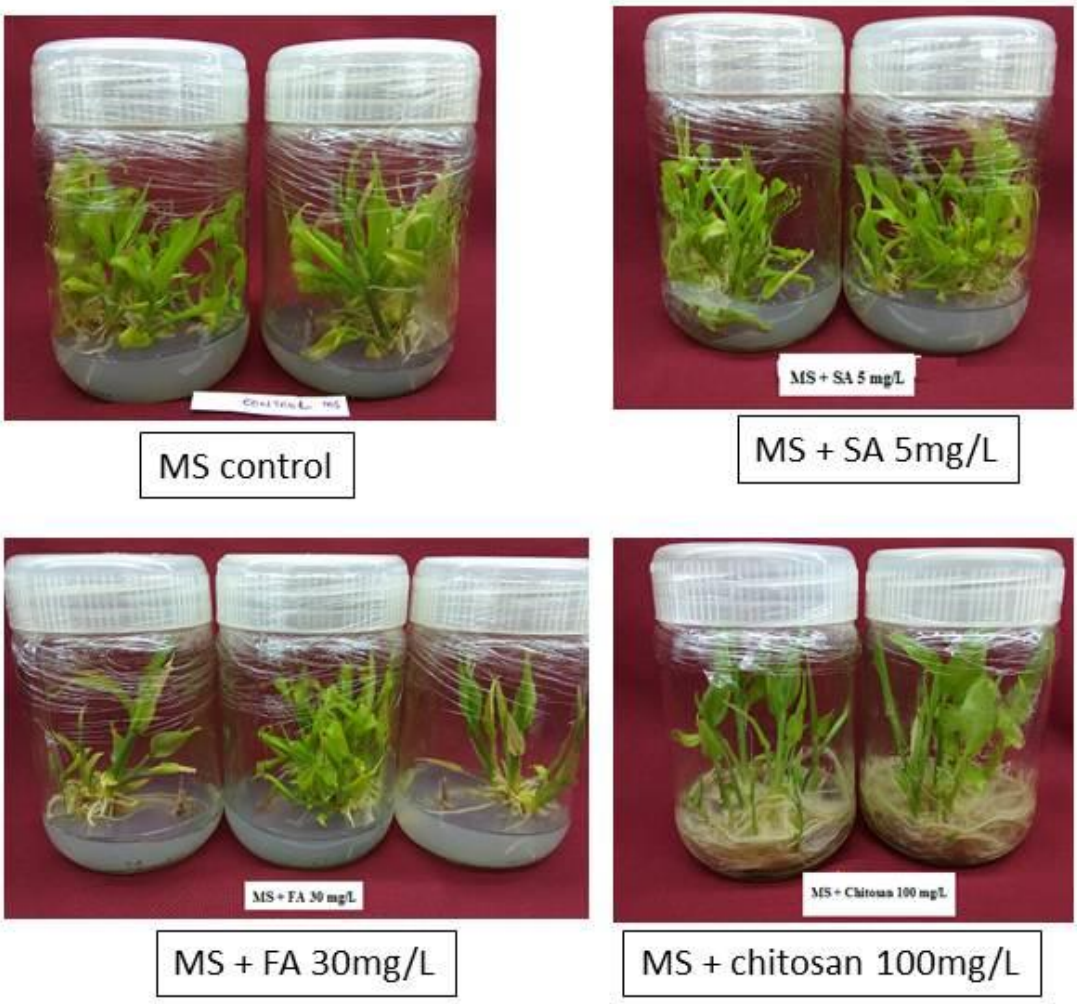


Plate 21:- Microrhizome cultures treated with elicitors/precursors

morphogenesis). Total gingerols of 0.823 per cent was recorded by MS medium supplemented with FA 30 mg L^{-1}. On analysis of total gingerols from irradiated samples, total gingerols of 0.443 per cent were noted in the microrhizomes (with shoot morphogenesis) in MS medium irradiated with 20 Gy dose of gamma irradiation. The accumulation of 6-gingerol was also recorded the highest in MMS medium (1.148 %) while the 8- gingerol recorded the highest in MS medium supplemented with SA 5 mg L^{-1} (0.090 %).

The data on MS and MMS medium with selected elicitor, precursor and dose of irradiation on total gingerol production were compared and presented in Table 41. The MMS medium supplemented with SA 5 mg L^{-1} was found significantly superior over other treatments with respect to production of total gingerols recording 1.702 per cent. Next to the best treatment, MMS medium supplemented with chitosan 100 mg L^{-1} (1.463 %) produced highest gingerol followed by MMS medium supplemented with FA 30 mg L^{-1} (1.453 %). The total gingerols accumulated higher in MMS medium compared to MS medium. Even with simple manipulation of macro and micro nutrients in MMS medium (MS medium with twice micronutrients and half ammoniacal nitrogen), the accumulation of gingerols enhanced in microrhizomes by 53.53 per cent over the MS medium. The MMS medium supplemented with SA 5 mgL^{-1} recorded increase in total gingerols of 100.23 per cent over MS control. The MMS medium supplemented with FA 30 mg L^{-1} recorded increase in total gingerols of 70.94 per cent over the treatment MS medium. The MMS medium supplemented with chitosan 100 mg L^{-1} recorded an increase in total gingerols of 72.12 per cent over the treatment MS medium.

4.2 Expression analysis of *Phenylalnine ammonia lyase* and*Chalcone synthase* gene in ginger microrhizomes with high gingerol content

The gene expression studies on *Phenylalanine ammonia lyase* (*PAL*) and *Chalcone synthase* (*CHS*) genes were done using quantitative real-time PCR. The housekeeping gene used for the study was *Actin*.

Table 41:- Comparison of different treatments using microrhizomes accumulating high gingerol

Treatments	Total gingerols (%)	Per cent increase over MS medium
MMS medium+ SA 5 mg L^{-1}	**1.702**	**100.23**
MMS medium+ chitosan 100 mg L^{-1}	1.463	72.12
MMS medium+ FA 30 mg L^{-1}	1.453	70.94
MMS medium +20 Gy (with shoot morphogenesis)	1.110	
MMS medium	1.305	53.53
MS medium	0.850	
MS medium+ SA 5 mg L^{-1}	1.070	
MS medium+ chitosan 100 mg L^{-1}	0.787	
MS medium+ FA 30 mg L^{-1}	0.823	
MS medium +20 Gy (with shoot morphogenesis)	0.443	
MS medium	0.747	

4.2.1 Isolation and analysis of total RNA

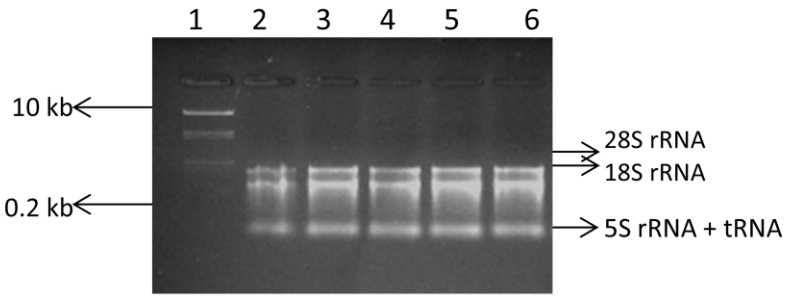
Total RNA was isolated from the leaves of the three months old *in vitro* induced microrhizomes from the treatments showing the highest production of total gingerols in each set of experiments. The quality and quantity of the isolated RNA were analyzed.

4.2.2 Quality and quantity analysis of isolated total RNA

In order to check the quality of total RNA isolated, the samples were subjected to gel electrophoresis using one percent agarose (formaldehyde) gel in 1X MOPS buffer. Good quality total RNA with three intact bands corresponding to 28S, 18S and 5S rRNA was observed (Plate 22). Quantification of the RNA was done using NanoDrop ® ND-1000 spectrophotometer by determining the absorbance of nucleic acids at a wavelength 260 nm and 280 nm. The concentration of total RNA isolated from the leaves of *in vitro* cultures of microrhizomes established in MS medium, MMS medium, MMS medium supplemented with SA 5 mg L^{-1}, MMS medium supplemented with FA 30 mg L^{-1}, MMS medium supplemented with chitosan 100 mgL^{-1} were estimated 1.05, 1.20, 1.63, 1.52 and 2.05 µg $µL^{-1}$ respectively. The A_{260}/A_{280} ratio was greater than 1.8, indicating good quality RNA, free from protein contamination. The A_{260}/A_{230} was more than 1.0 indicating the sample to be free from carbohydrate and protein contamination.

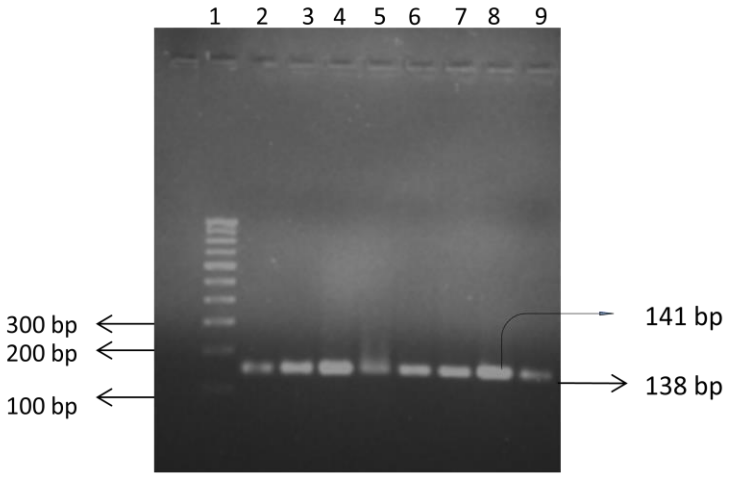
4.2.3 First strand cDNA synthesis and confirmation of cDNA using primers

Reverse transcription of the isolated RNA was carried out using Revert Aid First strand cDNA synthesis kit as per the manufacturer's protocol. The cDNA synthesis was confirmed by amplification of the *PAL, CHS* and *Actin* gene specific primers (Plate 23). The PCR product was run in 1.8 per cent agarose gel and the band size of 167bp, 141bp and 138 bp corresponding to *PAL, CHS* and *Actin* gene were obtained confirming synthesis of cDNA.



1: Transcript RNA marker (0.2kb-10 kb)
2: MS
3: MMS
4: MMS + SA 5mg/L
5: MMS + FA 30 mg/L
6: MMS + Chitosan 100 mg/L

Plate 22:- Total RNA isolated from the leaves of *in vitro* cultures of microrhizomes induced in the medium with highest gingerol production



1: Low Range DNA Ruler Plus (100bp to 3000 bp)
2 & 3:MMS
4 & 5: MMS + SA 5mg/L
6 & 7: MMS + FA 30 mg/L
8 & 9: MMS + Chitosan 100 mg/L

Plate 23:- Amplification of first strand cDNA synthesis using gene specific primers of *Actin* and *CHS* gene

Amplicon size of *Actin* gene is 138 bp and *CHS* gene is 141 bp

4.2.4 Gene expression of *PAL* and *CHS* gene using Real time PCR assay

The gene expression of the *PAL* and *CHS* gene was analyzed by real time PCR by determining the relative gene expression of *PAL* and *CHS* gene over housekeeping gene *Actin* comparative ΔC_T method.

4.2.5 Threshold cycle values

The real time PCR data are represented as the cycle number necessary to achieve a threshold cycle value. The reference gene used for expression analysis was *Actin*. No significant difference was found between the expression analyses of *PAL* gene of various treatments such as MS medium, MMS medium, MMS medium supplemented with each of SA 5 mg L^{-1}, chitosan 100 mg L^{-1}, FA 30 mg L^{-1} (Table 42). The expression analysis of *CHS* gene reveals a two fold increase of the gene expression in the treatment MMS medium supplemented with SA 5 mg L^{-1} while the gene expression in the other treatments such as MMS medium, MMS medium supplemented with FA 30 mg L^{-1} and MMS supplemented with chitosan 100 mg L^{-1} were on par with each other.

4.2.6 Amplification plots

Amplification plots were visualized, which showed the results of Real-time PCR with the number of cycles corresponding to the X-axis and fluorescence corresponding to the Y-axis (Rn). The threshold cycle is the point of interaction between the amplification curve and the threshold line.

4.2.7 Relative quantification analysis

The relative gene expressions of *PAL* and *CHS* were estimated as per the comparative ΔC_T method as stated by Livak and Schmittigen (2001). The relative expression of *PAL* and *CHS* genes ranged from 1.000 to 1.342 and 1.001 to 2.005 respectively in various treatments, when normalized with the endogenous gene, *Actin* gene. The highest expression of *PAL* and *CHS* gene expression in various treatments is represented as average $2^{-\Delta\Delta Ct}$ values.

Table 42:- Relative quantification of *PAL* and *CHS* gene expression using comparative ΔC_T method

Treatments	*PAL* gene expression (fold change)	*CHS* gene expression (fold change)
MS	1.000	1.001
MMS	1.137	1.392
MMS+SA 5 mg L^{-1}	1.342	2.005
MMS+ Chitosan 100 mg L^{-1}	1.187	1.468
MMS + FA 30 mg L^{-1}	1.242	1.510
CD(0.05)	NS	0.172
SE(m)±	0.129	0.056

NS-Not significant

Data represent the mean of three biological replicates

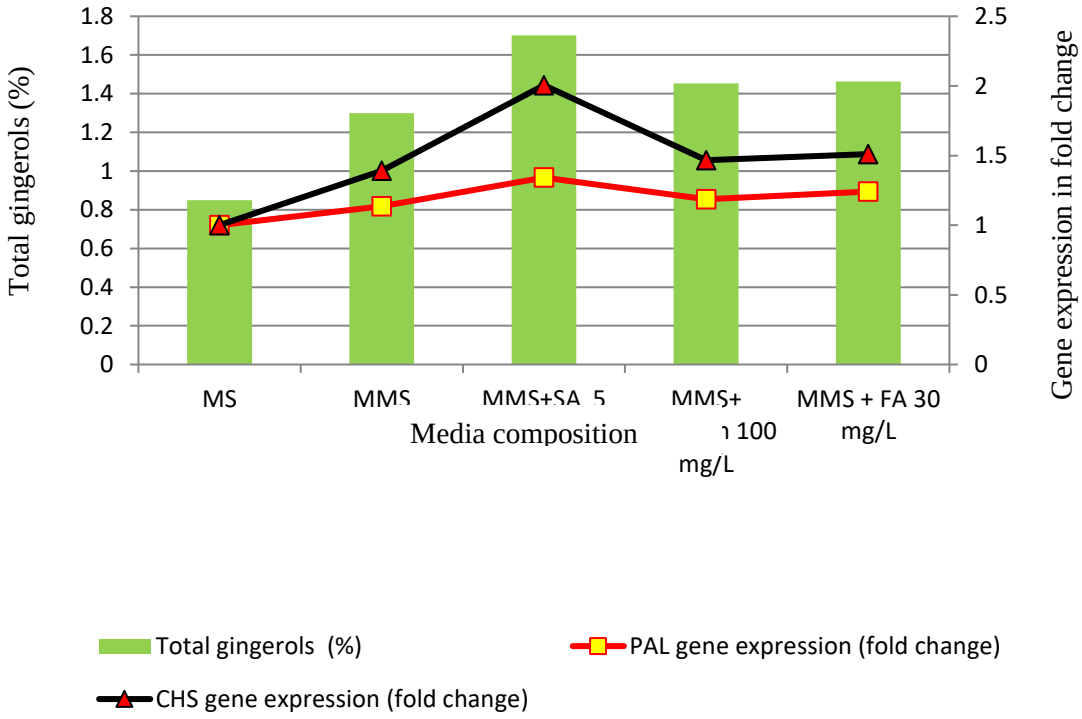


Figure 19:- Comparison of *PAL* and *CHS* gene expression with total gingerol content

4.3 Comparison of gene expressions of *PAL* and *CHS* genes with highest total gingerol production

Comparison of gene expressions of *PAL* and *CHS* genes *vs* the best treatments identified with highest total gingerol production from the experiments of *in vitro* synthesis using microrhizomes revealed that the *PAL* and *CHS* gene expressions and the gingerol content followed the same trend in the treatment, wherein, as the gene expression increased, the accumulation of the total gingerol content also increased.

In the treatment MMS supplemented with SA 5 mg L^{-1},1.342 fold and 2.005 fold increases of the *PAL* and *CHS* gene expressions respectively compared to the control was observed and in the same treatment the highest total gingerol content of 1.702 per cent was recorded depicting the correlation of gene expression with the total gingerol production. The details are represented graphically in Figure 19.

4.4 Sequencing, sequence analysis and validation of available ESTs

4.4.1 Sequencing of subtracted rhizome and leaf ESTs

The ginger ESTs available in the Department from the previous doctoral research obtained through the process of SSH done by Sreeja (2017) were sequenced by Sanger sequencing at Scigenom, Cochin. Eleven quality sequences from rhizomes and four from leaf were obtained after sequencing in the form of electropherogram and nucleotide sequence in fasta format.

4.4.2 Analysis of ESTs using various bioinformatic tools

4.4.2.1 Sequence data analysis of rhizome ESTs

Rhizome EST 1

The length of the forward and reverse sequence obtained from the Rhizome EST 1 was of 954 bp and 1241 bp respectively. In the forward sequence, 1-72 bp and 512 to 1100 bp indicated the vector/adaptor sequences. In the reverse sequence, 468-954 bp indicated the vector/adaptor sequence. The sequence details of EST 1 are given in Table 43. Bioediting of the vector/adaptor sequence was done and

the resulting forward and reverse sequences were joined to get a final sequence of 580bp which is given below:-

Vector/adaptor edited sequence of Rhizome EST 1

5'CTTTCTATACCACGAGTATATTGGTCTTCACAGAGAGCGGAATAACAAA
TGAATGTTTCAAACTACATTGGGGTCGCGCCACATTCCGAAAGTGCACCT
GACGCGCCCTGTAGGCGGGGCATAGCGCGCGATGTGTGTACGTAGCGTGG
TCGCGGCCGAGGTGCCTGCTGAGTGTTGGACTCACCCTGCCTCCATTGTTG
TTATATTTCAGGATGATGCTGTCAGGAGAGAGTTCCAGTTGCTAGTCCCCT
GCAGACCACCAGTTGTAGTTGTCTTTTGGTTTTCTTTTGTTTCTTATTTGAC
TATGTTTGGACTTAGTCTTGTGTTGGTGTTACTTATGGATTTTGTATGGATT
CTTATCGTTGATGGTTTTCGTATGATTTGGTTTTATTCTACTACATACCTGC
CCGGGCGGTCGAGCGGCCGCCCGGGCAGGTACAAATGTGAGAAGCACAT
CCGATATCTAATACTCATGATGAAGAAATTGATAGATTGACTTCTATAACA
TATATACCTTAAGTGGAAGTCTCACTTCTCTTCTTCTTAAGATCTTCTAGGT
ACCTCGGCCGCGACCACGCTA**3'**

Blastx results analysis showed that the EST was having homology with Putative RNA-directed DNA polymerase (*Medicago trunculata*) and Hypothetical protein CCACVL1 04694 (*Corchoru scapsularis*). Blastn results analysis showed EST was having similarity with *Zingiber officiale*cDNA clone ZO CPBMB B3 R203' mRNA sequence and *Garcina mangostana* cDNA clone p4e12 mRNA sequence. The open reading frame analysis showed the ORF length of 366 bp.

Rhizome EST 2

The length of the forward and reverse sequence obtained from the Rhizome EST 2 was 1267 bp and 954 bp respectively. In the forward sequence, 239-1133 bp indicated the vector/adaptor sequences. In the reverse sequence 1-74 bp and 285-954 bp indicated the vector/adaptor sequence. The sequence details of EST 2 are given in Table 44. Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 282 bp which is given below:-

Table 43:- *In silico* analysis of Rhizome EST 1

(1) Details of sequence length for Rhizome EST 1

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	954
2	Initial sequence length (reverse)	1241
3	Vector/adaptor sequence length (forward)	29-72,512-1100 Strong 14-28 –Moderate 1-13 –Suspect origin
4	Vector/adaptor sequence length (reverse)	468-952 Strong 953-954–Suspect origin
5	Final length of coding sequence	580

(2) Blastx result analysis of Rhizome EST 1

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
RHN45312.1	Putative RNA-directed DNA polymerase (*Medicago trunculata*)	29%	9e-08	52.54
OMO97034.1	Hypothetical protein CCACVL1 04694 (*Corchorus capsularis*)	26%	2e-07	64.15

(3) Blastn result analysis of Rhizome EST 1

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
JZ979593.1	*Zingiber officiale* cDNA clone ZO CPBMB B3 R203' mRNA sequence	44	7e-44	80.59
FG618486.1	*Garcina mangostana* cDNA clone p4e12 mRNA sequence	22	2e-34	87.69

(4) Open Reading Frame analysis of rhizome EST 1

ORF location	ORF length (bp)	Frame
131-496	366	+2

Table 44:- *In silico* analysis of Rhizome EST 2

(1) Details of sequence length for Rhizome EST 2

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1267
2	Initial sequence length (reverse)	954
3	Vector/adaptor sequence length (forward)	239-1133- Strong
4	Vector/adaptor sequence length (reverse)	12-74,285-954- Strong 1-11 –Suspect origin
5	Final length of coding sequence	282

(2)Blastx result analysis of Rhizome EST 2

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
DY375681.1	*Zingiber officinale* cDNA clone ZO Eq0001C17 3’ mRNA sequence	60	2e-62	92

(3)Blastn result analysis of Rhizome EST 2

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
JK543849.1	*Musa accuminata* AAA group cDNA clone MACVLIMFLS004D Co6.b 038 5’ mRNA sequence	45	1e-119	88
JK543848.1	*Musa accuminata* AAA group cDNA clone MACVLIMFLS004A Co6.b 038 5’ mRNA sequence	45	6e-118	86

(4)Open Reading Frame analysis of rhizome EST 2

ORF location	ORF length (bp)	Frame
217->2	216	-3

Vector/adaptor edited sequence of Rhizome EST 2

5’CGTATATACACCCGCGCGCAGGGTGATGTATTGTGGCCGGAATCTTCGA
GCGGCCGCCCGGGCAGGTACATAGCATGGCGTACATGATCGATCCTATGG
CTGAGGCATAAGGGATCTGATCCATGCGGTCTCTCTCCTCTCTAGAAGAG
GGACCTTGAGTCTTCGAAAGACTCATGCCATGTGACATCGGCAGAAATCC
CTTCTTGGAGTTCTGCATGGCAAACCGAAGGAGTACCTCGGCCGCGACCA
CGCTAATCTAGATGCATCGTCGAGTCAGACCGG**3’**

The Blastx analysis of the EST 2 showed 92 per cent homology with the *Zingiber officinale* cDNA clone ZO Eq0001C17 3’ mRNA sequence and on Blastn analysis, the EST showed maximum similarity with *Musa accuminata* AAA group cDNA clone MACVLIMFLS004D Co6.b 038 5’ mRNA sequence and Hypothetical protein CCACVL1 04694 (*Corchorus capsularis*). An ORF length of 216 bp was showed by the EST on open reading frame analysis.

Rhizome EST 3

The length of the forward and reverse sequence obtained from the Rhizome EST 3 was of 1232 bp and 1249 bp respectively. In the forward sequence, 163- 1110 bp indicated the vector/adaptor sequences. In the reverse sequence, 1-76 bp and 210-1135 bp indicated the vector/adaptor sequence. The sequence details of EST 3 are given in Table 45. Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 243bp which is given below:-

Vector/adaptor edited sequence of Rhizome EST 3

5’CAACCCGAAGGTGCTCTCTCTGGACCGTCAGATCTACGCTAGCGTGGTC
GAATTGACCATCCTGATGTCTCTGGGTTGAGTAATACCCGTCCAAGGGTAC
CTGCCCGGGCGGCCGCTCGAAGCAGTACTTCGCAAAAGAGTTGGTAGCTC
TGATCGCACACCACCCGCTGTACGATGTTTTTGTTGCAGCACGATACGCGC
AGAAAGGATCTCAGAGACCCCTTGAACTTCTTTTTCCTCAAC**3’**

Table 45:- *In silico* analysis of Rhizome EST 3

(1) Details of sequence length for Rhizome EST 3

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1232
2	Initial sequence length (reverse)	1249
3	Vector/adaptor sequence length (forward)	239-1133- Strong
4	Vector/adaptor sequence length (reverse)	32-76,210-1135 Strong 15-31-weak 1-14 -Suspect origin
5	Final length of coding sequence	243

(2)Blastn result analysis of Rhizome EST 3

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
CO494671.1	Sw04061 *Gossypium hirsutum* cDNA, mRNA sequence	14	1e-07	100
CO499420.1	Sw04061 *Gossypium hirsutum* cDNA, mRNA sequence	13	5e-07	100

(3)Open Reading Frame analysis of rhizome EST 3

ORF location	ORF length (bp)	Frame
175->243	69	+1

On Blastx analysis, no similarity was observed with any of the protein sequences in the database while Blastn analysis showed similarity with *Gossypium hirsutum* cDNA, mRNA sequence. An ORF length of 69 bp was shown by the EST on open reading frame analysis.

Rhizome EST 4

The length of the forward and reverse sequence obtained from the Rhizome EST 4 was of 1247 bp and 1254 bp respectively. In the forward sequence 391-1103 bp indicated the vector/adaptor sequences. In the reverse sequence 1-73 bp and 435-1169 bp indicated the vector/adaptor sequence. The sequence details of EST 4 are given in Table 46. Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 590 bp which is given below:-

Vector/adaptor edited sequence of Rhizome EST 4

5'CAAAAAATGGGGAATATTACGGGCGGACACCGGAATGTGATATCAATG
CTCTCTGTCCTTTTGATTTAATGAAGCATTATCAGGCTAGCGTGGTCGCGG
CCGAGGTACTTACTAGGGATAACTTCCCTAGATACCTTCCTAGTGACCTTG
TTGGGCTTCTTAGAAGCCTTGGTCAACCTTTCTAGGTCAACTCTAGGGATA
GCCTCCCTTGTGACCTTGTTTGTGACTTTCTTAGACTTCTTAGAAGTCTTAG
TCACATTTATTGCAAAAATACTCTTAGGGATGACTTCCCTAGTATTTTTGG
CTTGACCACTAGACCTAGGGTTTGTTCCATAACTATATGGAACTCTATGGT
AAGAGGGCACATCCTTCTTAGCCTTTGGTTTGTATCCCAAACCTCTATGGC
CATTGGATGACTTTTGTACCTGCCCGGGCGGCCGCTCGAATCGTCGCTTCA
GCTGGGCTGGTGTGCACCGAACCCCCGATCAGTCGACGCTGCGCCTTATC
GTACTATCGTCTTGGAGCCCAATCGGTAGACCGACTATCGGCCATTGCAA
GCAGTCACCGTGTTACACAGGAATATTATGTGC**3'**

The Blastn analysis showed similarity of the EST 4 with *Boechera divaricarpa* GSS, clone B62-01-F_017 and *Boechera divaricarpa* GSS, clone B62-01-F_H16.

Table 46:- *In silico* analysis of rhizome EST 4

(1) Details of sequence length for Rhizome EST 4

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1247
2	Initial sequence length (reverse)	1254
3	Vector/adaptor sequence length (forward)	391-1103- Strong
4	Vector/adaptor sequence length (reverse)	29-73 Strong 435-1169-weak 1-28 -Suspect origin
5	Final length of coding sequence	590

(2) Blastn result analysis of Rhizome EST 4

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
HF951497	*Boechera divaricarpa* G SS, clone B62-01-F_017	21.4	1.6e-18	82.5
HF951328	*Boechera divaricarpa* GSS, clone B62-01-F_H16	20.08	5.8e-18	82.9

Rhizome EST 5

The length of the forward and reverse sequence obtained from the Rhizome EST 5 was of 1235 bp and 1223 bp respectively. In the forward sequence, 391-1103 bp indicated the vector/adaptor sequences. In the reverse sequence, 1-74 bp and 557-1061bpindicated the vector/adaptor sequence. The sequence details of EST 5 are given in Table 47. Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 776 bp which is given below:-

Vector/adaptor edited sequence of Rhizome EST 5

5'CGATCAGCGGCGGATACATATGGAAGTGTATTGAACTACATGTTCGGCA
ATCCGAAGTCACTGACGGTCGTACGCGCATAGCCGCATTGTAGTACGGGC
AGCGTGACGTACCCTTGCAGCTCTAGCGCCGTCCTTCGCTCTGCACTTCTC
GCACGTTCGCGCTAGCGTGGTCGCGGCCGAGGTACCCCACTCGGCTTGCTT
GGGATAGTAAAAGTAGTGAAATATTTTTGGGTCCAAGGGAATGTTGTTCA
ACTTGAACAAAACTATCACTCCGCTCAGCAGCTTAATAGAGTTGGGAACT
ACCTGCCCGGGCGGCCGCTCGACGCCCGGGCAGGTACTACTCTTGGAGCT
AGATATTAATTAAGTGAGTCGTCAGTAACTTACTTAATTAGTGGACATTTG
TTATCTTAAACACAGGGAGACTAACACACTCATGATAAGAAGGAGCCCAA
AATGTAATTTGGGATTGGTGCGGTAGTTCAATAATAGTTCTCTAGTGGAAT
GAATTATTATTGATAAAATTAAGTTGTGTGTTCGGGGCGAGCACGGGATG
CTTAATTTTATCGGGAGACCAAAACCAATTCCTCCTCTCGGTCCCTATCGT
AGTCGGCCGCGACCACGCTATGTCGACCCTGCGGCTACGAATACTGTCGC
TTCTCCCTCGGAGCGTGCGCTTTCTCAAGCTCAGCTGTAGGTATCACAGTC
GGTTAGTCGTCGATCAGCCTGGCCTTGGTGCCACGACCCCCCGTTCAGGCG
3'

The Blastn analysis results showed that the EST 5 was having similarity with *Boechera divaricarpa* GSS, clone B53-01-F_ B14 and *Iris fulva.*

Table 47:-*In silico* analysis of rhizome EST 5

(1) Details of sequence length for Rhizome EST 5

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1235
2	Initial sequence length (reverse)	1223
3	Vector/adaptor sequence length (forward)	509-1103- Strong
4	Vector/adaptor sequence length (reverse)	12-74 Strong 557-1061-weak 1-11 -Suspect origin
5	Final length of coding sequence	776

(2)Blastn result analysis of Rhizome EST 5

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
HF950413	*Boechera divaricarpa* GSS ,clone B53-01-F_ B14	15.6	1.5e-20	86.0
AY245310	*Iris fulva*	32.2	1.15e16	69.2

Rhizome EST 6

The length of the forward and reverse sequences obtained from the Rhizome EST 6 was 1224bp and 1225bp respectively. In the forward sequence, 585-1105bp indicated the vector/adaptor sequences. In the reverse sequence 1-75 bp and 632-1089bp indicated the vector/adaptor sequence. The detail of the results is given in Table 48. Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 811bp which is given below:-

Vector/adaptor edited sequence of Rhizome EST 6

5'TCTGTATACGCGCGGCATATAACGCGCGCGTTGGTTTACGGAGTGAACG
TACTGGAAGCCCTAGCCCGTCGTCCTTTTCACTTTCGGCACGTGCCGGCTT
GCCGTCAAGCTTAATCGGAGCTCCCTTAGGGTCCGATAGCGTGGTCGCGG
CCGAGGTACAACAAGGAAAGTCCAAATGTGATCTTGGCAAAGGTAAAAG
TCTAAGTGAGATCTTGGTAAAAGGAAAAGTCTAAGGGTGAGTCTTGGCGG
TGTAAGTCCAAGTATATAGTCTTGGCAACGTAAGTCCAAGTGTGACTTGG
CAAGGTTGAAATCCAGGAGCGAGGATCTCTTGGCAATGGAAGACCCGACA
ATAAGGACAAGGCCGATAGAAGCTCCTGATGGCAAAGCGTGAAGGATGG
GGAGGCATCTGAGGGACGCGAGGCCAATAGAGGAGGCTAGAAGGCTAGT
CTAGGTTGGTCGGGTAAGGACGAGTGCTGAGTGAACGTACTCAGAGGTTA
AATCCTAGGATTATGATTTACTGTAGCGTTACTGTAGCGTTACTATAGCAG
TATTGTAGCGTTACTGTAACAGTACTGTAGCAACCGACTGATATTTTCATC
AGTCGACTAGTAGCCGACCGTCGGCATGTAATGGTCGAATTTTACTTCGA
ACCAATCAACTGATGTCGAGTGCGAATCCCGAAAGGACTATCACGATCAG
CGTTCCCCTGAGCTTCCTCTGCCTCTCCTGTTCGACCTGACCTACCGATCCT
TTCGCATTCCCCTTCGGAAGCGGTGGCGCGCGGTTGTACCTGCCCGGGCG
GCCGCTCGAA**3'**

The Blastn analysis showed similarity of the EST 6 with that of *Boechera divaricarpa* GSS, clone B25-01-F_D10 and *Boechera divaricarpa* GSS,

Table 48:-*In silico* analysis of rhizome EST 6

(1) Details of sequence length for Rhizome EST 6

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1224
2	Initial sequence length (reverse)	1225
3	Vector/adaptor sequence length (forward)	585-1105- Strong
4	Vector/adaptor sequence length (reverse)	15-75,632-1089 Strong 1-14 -Suspect origin
5	Final length of coding sequence	811

(2)Blastn result analysis of Rhizome EST 6

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
HF948922.1	*Boechera divaricarpa* GSS ,clone B25-01-F_D10	14.4	5.16e-14	82.1
HF949948	*Boechera divaricarpa* GSS ,clone B59-01-F_o04	15.3	2.6e-12	80.6

(3)Open Reading Frame analysis of rhizome EST 6

ORF location	ORF length (bp)	Frame
406-230	177	-1

Clone B59-01-F_o04. An ORF length of 177 bp was shown by the EST on open reading frame analysis.

Rhizome EST 7

The length of the forward and reverse sequences obtained from the Rhizome EST 7 was 1251bp and 1249 bp respectively. In the forward sequence, 205-1082bp indicated the vector/adaptor sequences. In the reverse sequence 1-75 bp and 252-1140bp indicated the vector/adaptor sequence. The sequence details of the EST 7 are given in Table 49. Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 297bp which is given below:-

Vector/adaptor edited sequence of Rhizome EST 7

5'TATAAAGTGTGTTCTATCTACATGGAAGCGCGGTGCAGCAGTAGCGTGG
TCGCGGCCGAGGTACTTGAGCATCTCACCAACACCCTAGGTCTACCTTGCT
TGTGATTGACAAACATAGAAAGGGATGAGATGCATAAGCCAGTTGCCTGG
ACTTAAGATGTTTATGTCAGTTAGATTTGACTTTGACTCAATGTACCTGCC
CGGGCGGCCGCTCGAAGAGTCTGACTGGTGCCTCAACACCGCTGTACGTG
ATCTTTGTGCAGCACCGATACGCGCGCGAAAAGGAGTCTCTCACCG**3'**

The blasts and blastn analysis showed that the EST 7 was not having similarity with any of the sequences in the database. An ORF length of 171 bp was shown by the EST on open reading frame analysis.

Rhizome EST 8

The length of the forward and reverse sequence obtained from the Rhizome EST 8 was 1244 bp and 1226bp respectively. In the forward sequence, 806-1143bp indicated the vector/adaptor sequences. In the reverse sequence 1-75 bp and 853-1056bp indicated the vector/adaptor sequence. The sequence details of the EST 8 are given in Table 50. Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 1048bp which is given below:-

Table 49:-*In silico* analysis of rhizome EST 7

(1) Details of sequence length for Rhizome EST 7

Sl.no	**Particulars**	**Sequence length (bp)**
1	Initial sequence length (forward)	1251
2	Initial sequence length (reverse)	1249
3	Vector/adaptor sequence length (forward)	205-1082- Strong
4	Vector/adaptor sequence length (reverse)	31-75,250-1140 Strong 1-30 -Suspect origin
5	Final length of coding sequence	297

(2) Open Reading Frame analysis of rhizome EST 7

ORF location	ORF length (bp)	Frame
125 ->295	171	+2

Table 50:-*In silico* analysis of rhizome EST 8

(1) Details of sequence length for Rhizome EST 8

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1244
2	Initial sequence length (reverse)	1226
3	Vector/adaptor sequence length (forward)	806-1143- Strong
4	Vector/adaptor sequence length (reverse)	13-75,853-1056 Strong 1-12 -Suspect origin
5	Final length of coding sequence	1048

(2)Blastx result analysis of Rhizome EST 8

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
OIT21703.1	PREDICTED: uncharacterized protein LOC1082027215 (*Daucus carota* subsp. *sativis*)	40	1e-14	46
XP 017233167	Hypothetical protein VITISV 032735 *Vitis venifera*	30	9e-13	34

(3)Blastn result analysis of Rhizome EST 8

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
KJ938508	*Elettaria cardamomum* clone SCGG contig2 microsatellite sequence	25.4	1.92e-52	80.5
AM453271	*Vitis venifera* contig VV8X205608.11 whole genome shotgun sequence	57.2	2.6e-25	66.9

(4)Open Reading Frame analysis of rhizome EST 8

ORF location	ORF length (bp)	Frame
831-1004	174	+3

Vector/adaptor edited sequence of Rhizome EST 8

5’ATTGGCCCTTGATAGACCGATTTTCGCCTGACGTTGAGTTCCAGGTCTAT
ATGACTCTTGTTGCACTGACACACTCAGCCTATTCTCGTCTATCCTTGATTT
TAAGGGATTTGCGATTCCGCTATGCAAAAATGAGCTGATTACAAAATTTA
CGCGATTTTACAAAAATATAGCGTGGTCGCGGCCGAGGTACCTTAGAGGC
ACTAGAGATTATATGCTAGCTTACAAGGCAGTTAATTTGGTCTCTGTGGGT
TGCACGGATTTTGACTTCCAGTCGAATAGGGACAATAATAAGTCAACCTC
GGGGTTTTGTGTTTACTTTAGGAGGTAAAGTCATAACTATGGAAGAATGA
TAAGCATAGGTGTTTTTCTGGACTCCACCATAAAAGCTGAGTATATGGCG
AACCTCTGAGGTAGTCATAAAAGCTGAATGACTTGATAACCTCAAGATAG
ACTTAGATATGATTTCTGGTTTGTCCAAAGATTATTACAATTGTAATAATA
GTGGTGCAGTAGCAAACTCGAAGAAACCATGAGTCTATAAGGCAAGTAA
ACATAATAGAGCTCAAATACCACCCAATACGAGAAATCGTATAAATGAGG
AGAAGTTGTTGCTGCCTAGATTGTATCAGATAATGACCTATAGATCTTTTC
ACTAAGCCCTTAAGGAAAGAGCTTTTGATGGGCATGTTGAAGGGTTGGGA
TTCAGATGTATAGCAGCAGATATGGCAGCTTAGTTTTTAGTATAAGTGGG
AGATTGTTAGGATGTATACTAAAAGCCTAGCTTTTGGTATAAACATTTATT
TAGAAATAAGAATCACATTGGTCAATGTCTACATTTATGATAAATGAAGT
TGTTCAATTAATTTATATTGTAGATAACATGGTGTGGTGTCACACACAGAG
GATCATGTTATCAGTACCTGCCCGGGCGGCCGCTCGAATCATCGCTGGCG
GGCACGTATTCAGCTCCACTCAAGGCCGTATACGGTTATCCATGATCAGG
AATTACGCCAGGAAAGAACACATGGTGTGGAGCTCGAAG**3’**

The blastx analysis showed that the EST 8 was having maximum similarity of 46 per cent with PREDICTED: uncharacterized protein LOC1082027215 (*Daucus carota* subsp. *sativis*). The blastn analysis showed the similarity with *Elettaria cardamomum* clone SCGG contig2 microsatellite sequence. An ORF length of 174 bp was shown by the EST on open reading frame analysis.

Rhizome EST 9

The length of the forward and reverse sequence obtained from the Rhizome EST 9 was 1254 bp and 1227bp respectively. In the forward sequence, 361-1208 bp and 1209-1254 bp indicated the vector/adaptor sequences. In the reverse sequence, 1-74 bp and 407- 1149bp indicated the vector/adaptor sequence. The sequence details of EST 9 are given in Table 51. Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 378 bp which is given below:-

Vector/adaptor edited sequence of Rhizome EST 9

5'GGGTGAGGCAAAACCGAGCAATGCGCCAAAGATTAGCGACCGAAATGT
GATATCCATACTCTCATTTACTACTCTTGGACTAAGATATTAATTAAATGA
GTTGTCAGTAACTCACTTAATTAGTGGACATTCGATATCTTAAACACAGGG
AGACTAACACACTCATAATAAGAAGGAGCCCAAAAATGTAATTTGGGATT
GATGCGGTAGTTCAATAATAGTTCTCTAGTGGAATGAATTATTATTGATAA
AATTAAGTTGTGTGTTCGGGGCGAACACGGGATGCTTAATTTTATCGGGA
GACCAAAACCAATTCCTCCTCTCGATCCCTATCGTAGCCTCTTATTTATAG
AGTACCTGCCCGGGCGGCCGCTCGAA**3'**

On blastn analysis the EST 9 showed 70 per cent homology with *Iris fulva* clone FGC8 repetitive sequences. An ORF length of 71 bp was shown by the EST on open reading frame analysis.

Rhizome EST 10

The length of the forward and reverse sequence obtained from the Rhizome EST 10 was 1225bp and 1244bp respectively. In the forward sequence, 776-1105bp and 1106-1137 bp indicated the vector/adaptor sequences. In the reverse sequence, 1-72 bp and 821- 1072bp indicated the vector/adaptor sequence. The detail of results is given in Table 52. Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 1008bp which is given below:-

Table 51:-*In silico* analysis of rhizome EST 9

(1) Details of sequence length for Rhizome EST 9

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1254
2	Initial sequence length (reverse)	1227
3	Vector/adaptor sequence length (forward)	361-1208- Strong 1209-1254 -Suspect origin
4	Vector/adaptor sequence length (reverse)	15-29-Moderate 407-1149 Strong 1-14 -Suspect origin
5	Final length of coding sequence	378

(2)Blastn result analysis of Rhizome EST 9

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
AY245310	*Iris fulva* clone FGC8 repetitive sequences	64.8	1.25e-17	70.6
HQ883969	*Crocus sativus* clone cs1 RAPD marker genomic sequence	65.3	5.01e-10	69.2

(3)Open Reading Frame analysis of rhizome EST 9

ORF location	ORF length (bp)	Frame
273->378	71	+1

Table 52:-*In silico* analysis of rhizome EST 10

(1) Details of sequence length for Rhizome EST 10

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1225
2	Initial sequence length (reverse)	1244
3	Vector/adaptor sequence length (forward)	776-1105- Strong 1106-1137-Moderate
4	Vector/adaptor sequence length (reverse)	28-72, 821-1072 Strong 1-27 -Suspect origin
5	Final length of coding sequence	1008

(2)Blastn result analysis of Rhizome EST 10

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
MK270534	*Musa*AAB group eukaryotic translation initiation factor 4E-2 mRNA, complete *cds*	32.6	6.8e-96	84.5
MK270532	*Musa*AAB group eukaryotic translation initiation factor 4E-6 mRNA, complete *cds*	32.4	5.2e-91	83.2

Vector/adaptor edited sequence of Rhizome EST 10

5’GTCTAGAGGGACTATTGGTATCACGTACAGTGTGGCAATTCGGCCCGTA
TATCAGGACCGTTTGCCTTGACCGGGAGTTCACGGTCTTATAGTGGACCTT
GTTCAAACTGACCAAACATCACCCTTTTTTCGTCTATTCTTGATTATAGGG
ATTTGCCGATTCGGCTATTGTTTCGAGCGGCCGCCCGGGCAGGTACACTCA
GAGTGTATTGAGTAGGACCATTTGAGGTCGTTTCTTTTATACTGACTTTAT
AAAGAAACAAAGACCTCGGTTATTATGGAAGTGTGTGCTCTTAATCCTAA
TATAATAACAAGCACATATATTTGATATTAATTTCTTTAATTTATCAATGG
GTGAGATTTAGTTCGATAAATCAACAAGCCCGATAAATTGGGAAATGATA
TCACTTATAGTGTGTGTTGTTGATTATAGAAGGAAACTGTGTCCTAGAGAT
ACTAGGTTGATAATGTCCTCAAGAGGAGCTCATAAGGATTATCATGTTAA
ACCCTGCAGGTGGACTTAGTCCGACATGACGATAAGGTTGAGTGGTACCT
CGGCCGCGACCACGCTTGTCCCTCAGAGCCATCACAGCCTTTTGGTCAGAT
GCATTGCGATTCCAAACACTTAAGATGTCCTCGTTGAAACGAATGCTTAGC
ACAACACCGCAAACATTGTCTCCGAATTCTAGTTGGTCGCCGATCAATGA
CAATACCAAATCTTCCCAAAAACGACCTGAAATGACCTTTTTGAATCTCAA
TATCCACTTACCACCATTACAGTTTGCAGAATCCTCCCATAGGGGCCTAAT
GCCTTCTCTGAAAAGATGGATATCAGTTGGGCATGGCAGTGAGGAAGGAC
GTGCTATATGGCAATAGCAGACCCAGAAACCTTCAACCGTACCTCGGCCG
CGACCACGCTAGGCGTTATACGTATCCAGATCAGGAATAACGCCAGAAGA
CTGTGATCCAATGCTCAGCTAAGGTCAGTATCGTAAAGGCTCCGCGTTC**3’**

On blastn analysis the EST 10 showed sequence similarity with *Musa*AAB group eukaryotic translation initiation factor 4E-2 mRNA, complete *cds*.

Rhizome EST 11

The length of the forward and reverse sequences obtained from the Rhizome EST was 1247 bp and 1235bp respectively. In the forward sequence 340-1129bp, 1232-1247 bp and 1194-1131 bp indicated the vector/adaptor sequences. In the reverse sequence, 1-72 bp and 385-1133 bp indicated the vector/adaptor sequence. The sequence details of EST 11are given in Table 53. Bioediting of the vector/adaptor

sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 429bp which is given below:-

Vector/adaptor edited sequence of Rhizome EST 11

5'CCTAACAAACAGCCTTCGGGTGAGCCAAATCGGAGGCAATTGGCGCCA
AAAGTGATTAGTGCGTACTACTGGATGTTGAATACTCCAACTCTCTTTTCA
ATCTAGCGTGGTCGCGGCCGAGGTACATGCAGCAGCATGTGGTGCTGCCG
GCGCAGGTGTAGTAGTTATCGCAAGCGGTAAGAGACTCGACGAGGGAAG
GCACAGAGGAAGAAGAGGTAGTAGGGTTGCGCAGGTTAGTAGCCCCTTCT
TTTATGGGGTAAGAGGGGCTGATTGCAATGCCACATTTGCCAGAAGATTC
TGCTATGTTTCGTTCCATCAGTATGTAGCCAGAATCACCCCAGCTCTCACC
CCATGAGTTCTTCACAATCCAGTAGTCGTTGCCATTTACAGTACCTGCCCG
GGCGGCCGCTCGAAATCCAGCTGGTTGTA**3'**

The blastx analysis showed 100 per cent of homology of EST 11 with Cysteine protease gp 3b (*Zingiber officinale).* The blastn analysis showed similarity with Ea *Curcuma longa* clone cDNA clone CL Ea0002D14 3' mRNA sequence and Ea *Zingiber officinale* cDNA clone ZO Ec0003K11' mRNA sequence.

4.4.2.2 Blast2Go results of rhizome ESTs

The Blast2Go analysis of the eleven rhizome ESTs showed maximum similarity hits with *Boechera divaricarpa* (Figure 20). The length of the rhizome EST sequence ranged between 243 and 1047 bp. Four rhizome ESTs were showing similarity with *Boechera divaricarpa* (Figure 21).

The gene ontology distribution of rhizome ESTs showed that the biological processes which ESTs involved were metabolic and cellular processes. Out of the eleven rhizome ESTs, four EST sequences were involved in metabolic process and two EST sequences involved in cellular process. The major molecular function involved is binding followed by catalytic activity. The ESTs also represents the cellular components (Figure 22).

Table 53:-*In silico* analysis of rhizome EST 11

(1) Details of sequence length for Rhizome EST 11

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1247
2	Initial sequence length (reverse)	1235
3	Vector/adaptor sequence length (forward)	340-1129- Strong 1194-1231-Moderate 1232-1247-Suspect origin
4	Vector/adaptor sequence length (reverse)	30-72, 385-1133 Strong 1-29-Suspect origin
5	Final length of coding sequence	429

(2)Blastx result analysis of Rhizome EST 11

Accession No.	Description	Q.coverage (%)	E value	Homology (%)
AAW34137.1	Cysteine protease gp 3b (*Zingiber officinale)*	63	5e-43	100
AAW34136.1	Cysteine protease gp 3a (*Zingiber officinale*)	63	9e-39	93

(3)Blastn result analysis of Rhizome EST 11

Accession No.	Description	Q.coverage (%)	E value	Homology (%)
DY383514.1	Ea *Curcuma longa* clone cDNA clone CL Ea0002D14 3' mRNA sequence	63	4e-57	82
DY352129.1	Ea *Zingiber officinale* cDNA clone ZO Ec0003K11' mRNA sequence	62	3e-51	81

(4)Open Reading Frame analysis of rhizome EST 11

ORF location	ORF length (bp)	Frame
56-316	216	-3

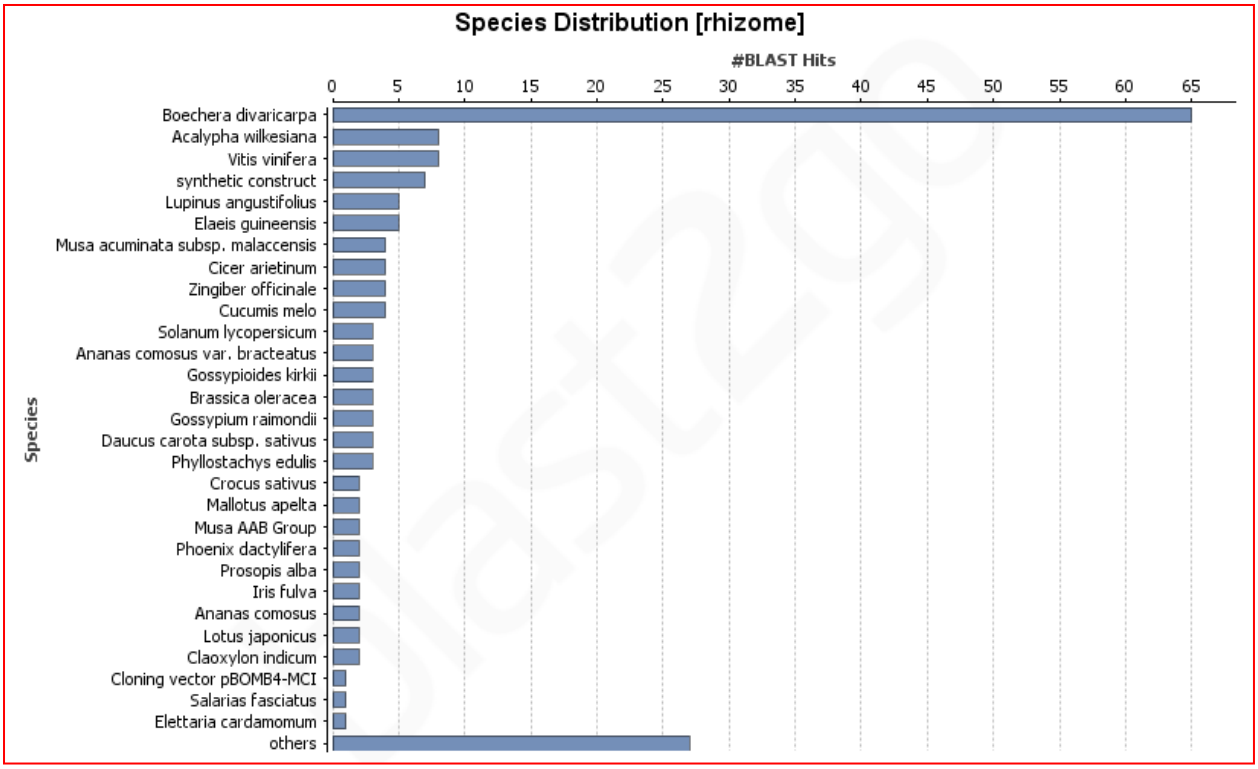


Figure 20:- Species distribution chart of rhizome ESTs

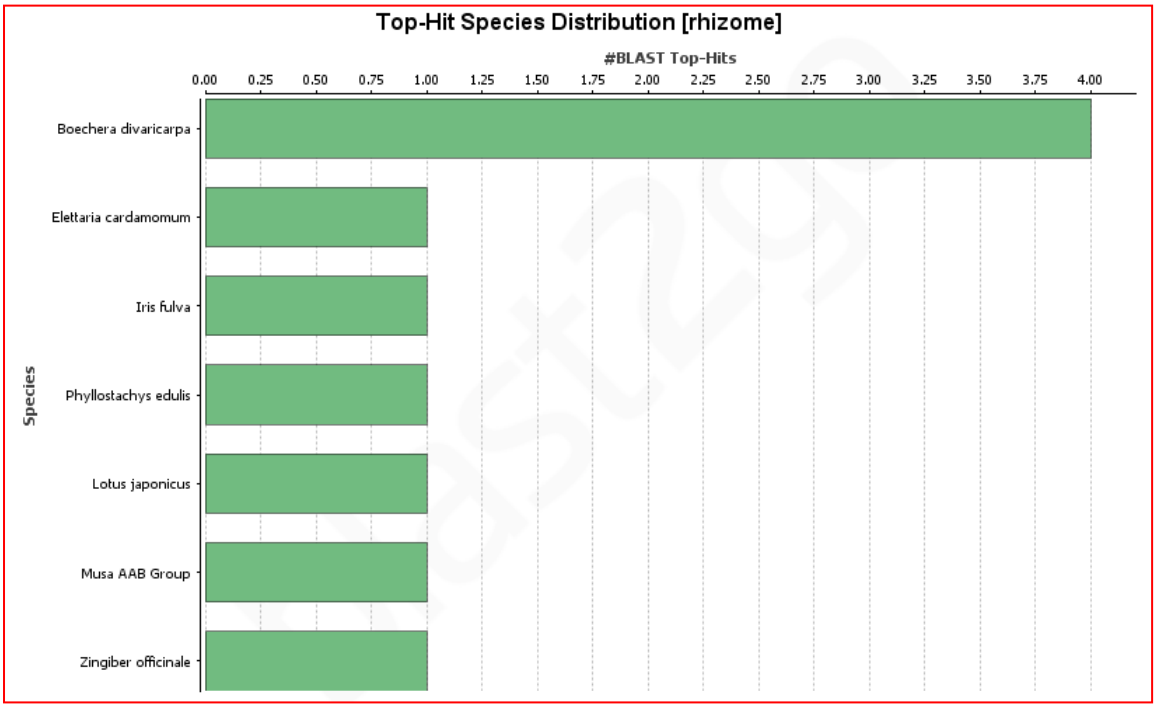


Figure 21:- Top hits-Species distribution chart of rhizome ESTs

4.4.2.3 Sequence data analysis of leaf ESTs

Leaf EST 1

The length of the forward and reverse sequences obtained from the leaf EST 1 was 1230bp and 1214bp respectively. In the forward sequence 1-30 bp and 691-1121bp indicated the vector/adaptor sequences. In the reverse sequence 1-78bp and 739-1089 bp indicated the vector/adaptor sequence. The sequence details of EST 11 are given in Table 54.Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 594bp which is given below:-

Vector/adaptor edited sequence of leaf EST 1

5'ATCAGGGGACTCCTTAAGGATCCGATAGGCTTAACGCACCTTGAACCAA
AAACTTGAATAAGGTGATGTTCACGTAGTGGCCATGCCTGATAGACGTTT
CGCCCTTGACGTTGAGGTCACGTCGTTAGCGTGGTCGCGGCCGAGGTACA
TCCTCTCTGAATCTGTGATGTATATTTACATCCATAAACAATTGCACAATC
TAGGAATAATGATACCCCCTTTTGTAATGTTTACCATCGATAAGTCGATGT
TTCATGAGTAGGATCTTATCAATCGGAAGCTCTGAAACGATACCCTAAAG
AGATTGGCAAAGAAAAGACTAACAGTGCTTACAACTTCGGGAGGATTCGA
AGGCTGCAGTGTGTTCTGCTTCGCGGTTAGGTTTGTTAGGCCAAGTTGTCT
GCGTATCAAATCAATTTTATTAGGATCCACAGGACTGCCTGAGGTTTCAGA
AACATAGAAGTTGTCCGCTACTTGAGCTCCTTCTCGCGAGATCTCTGCTCT
TCGGATCGTCAATCCATTCTCACAGAACACTCTCGTGATTTCTGGGAGTGA
AAATTTTCCATCTTCTCTCAATTCC**3**ATGCTAGACCCTCTGAGGATCTTCTC
TCTATAGCTGCCTCAAGGCACTGAATCAGGTGTCGTTGTTCGACCTCTTCA
CTTACGGGAAGGCCATCGGCATTTCTGATATAGTATTCCTGATAGGCTTCA
TCCTCATCTGAACTAACGGTGCCATGATACACGACATATTGCATATCGGTG
AGCGTACCTGCCCGGGCGGCCGCTAGGCAGCAAAGCCAGACGTAAGGCC
GCGTGCTGGCGTTTCATAGCTCGCCCCTTGACGAGCACGAAAATCGACGC
CTCAAGTCAAGTGTTGTGATCTCGCAGGGGAACCT **3'**

Table 54:-*In silico* analysis of leaf EST 1

(1) Details of sequence length for leaf EST 1

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1230
2	Initial sequence length (reverse)	1214
3	Vector/adaptor sequence length (forward)	691-1121- Strong 12-30-Moderate 1-11-Suspect origin
4	Vector/adaptor sequence length (reverse)	34-78, 739-1089 Strong 8-33- Moderate 1-7-Suspect origin
5	Final length of coding sequence	594

(2)Blastx analysis of leaf EST 1

Accession No.	Description	Q. coverage(%)	E value	Homology (%)
XM 009385129.2	PREDICTED:*Musa accuminata* subsp. *malaccensis* ACT domain containing protein ACR6 (LOC103971170) transcript X2 mRNA	57	5e-63	76
XM 018821049.1	PREDICTED: *Musa accuminata* subsp.*malaccensis* ACT domain containing protein ACR6 (LOC103971170) transcript X1 mRNA	57	3e-56	75

(3)Blastn result analysis of leaf EST 1

Accession No.	Description	Q. coverage(%)	E value	Homology (%)
DY385989.1	*Curcuma longa* cDNA clone Ea0006C03 3’ mRNA sequence	69	0.0	99
DY385988.1	*Curcuma longa* cDNA clone Ea0006C03 5’ mRNA sequence	10	5e-42	100

(4)Open Reading Frame analysis of leaf EST1

ORF location	ORF length (bp)	Frame
253-753	501	-1

On blastn analysis of leaf EST 1, 76 per cent homology was shown with PREDICTED: *Musa accuminata* subsp. *malaccensis*A CT domain containing protein ACR6 (LOC103971170) transcript X2 mRNA. The blastn analysis showed 99 per cent similarity to that of *Curcuma longa* cDNA clone Ea0006C03 3' mRNA sequence. An ORF length of 501 bp was showed by the EST on open reading frame analysis.

Leaf EST 2

The length of the forward and reverse sequence obtained from the leaf EST 2 was 1314 bp and 1253 bp respectively. In the forward sequence, 1147-1178 bp and 697-1095 bp indicated the vector/adaptor sequences. In the reverse sequence, 1-81 bp and 742-1150 bp indicated the vector/adaptor sequence. The sequence details of the leaf EST 2 are given in Table 55. Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 898 bp which is given below:-

Vector/adaptor edited sequence of leaf EST2

5'CGTTACCAGGGCTTAATTCCGGGTCCCTTAGGATTCCAGGTAGGTCCAC
ACTAGCGTGGTCGCGGCCGAGGTACATCCTCTCTGAATCTGTGATGTATAT
TTACATCCATAAACAATTGCACAATCTAGGAATAATGATACCCCCTTTTGT
AATGTTTACCATCGATAAGTCGATGTTTCATGAGTAGGATCTTATCAATCG
GAAGCTCTGAAACGATACCCTAAAGAGATTAGCAAAGAAAAGACTAACA
GTGCTTACAACTTCGGGAGGATTCGAAGGCTGCAGTGTGTTCTGCTTCGCG
GTTAGGTTTGTTAGGCCAAGTTGTCTGCGTATCAAATCAATTTTATTAGGA
TCCACAGGACTGCCTGAGGTTTCAGAAACATAGAAGTTGTCCGCTACTTG
AGCTCCTTCTCGCGAGATCTCTGCTCTTCGGATCGTCAATCCATTCTCACA
GAACACTCTCGTGATTTCTGGGAGTGAAAATTTTCCATCTTCTCTCAATTC
CAATGCTAGACCCTCTGAGGATCTTCTCTCTATAGCTGCCTCAAGGCACTG
AATCAGGTGTCGTTGTTCGACCTCTTCACTTACGGGAAGGCCATCGGCATT
TCTGATATAGTATTCCTGATAGGCTTCATCCTCATCTGAACTAACGGTGCC
ATGATACACGACATATTGCATATCGGTGAGCGTACCTGCCCGGGCGGCCG
CTACCAAAATTCAGGGGAATAACCGCAGGAAAAGAACCTGGTGAACCAA

ATGGCCCGCGTTGTCCTGGCGTTTTTTTCACTATAGACTTCTCCGCCCCTCA
CTTACGGAACATTCACAAAAATCTGACGCGCTCACTAGTTCTAAAAGTGT
GTGTGCAAGAAACACCCCTCGCATGCGAGGGAGTACATG**3'**

The blastx analysis of leaf EST 2 showed 72 per cent homology with PREDICTED: *Musa accuminata* subsp. *malaccensis* ACT domain containing protein ACR4 like isoform X2. The blastn analysis showed 100 per cent similarity with *Curcuma longa* cDNA clone Ea0006C03 3' mRNA sequence. An open reading frame of 501bp was obtained on open reading frame analysis

Leaf EST 3

The length of the forward and reverse sequence obtained from the leaf EST 3 was of 1005 bp and 1214 bp respectively. In the forward sequence, 313-1005bp indicated the vector/adaptor sequences. In the reverse sequence, 1-85 bp and 364-1050bpindicated the vector/adaptor sequence. The sequence details of leaf EST 3 are given in Table 56. Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 407 bp which is given below:-

Vector/adaptor edited sequence of leaf EST 3

5'AGGATTTTTACCTTACCAGGTTCGGGGAGCATTCAGAGGCAAATTCGCG
CAAAAAGGAAAATAGGGGCACGGGAAGTCAGAATATTCCATACTTTTCAT
TTTCCATTTTAGTGGAAGCATTTTTCAGGGTAATGTTTTCCTGAGCGGATC
CATTTTGAACTTATTAGAAAATTCGAGCGGCCGCCCGGGCAGGTACGAGA
GACCGGCGCCGTGGAACGAATAGTTGAGCCAATAACTGGTGGCCGGCCGA
TCGGTGGTTGATCGGCAGCTAATCCATAATTAAGTGTTTATGTTCTCTTGT
GCTATCTATCTAGCTATCTGGCTTGGTTATGGTGATCTAGAATAAAGCATC
CCACCTATCCTCTTTAGTACCTCGGCCGCGACCACGCTAAAGAAAGGGAA
ATTTA**3'**

Table 55:-*In silico* analysis of leaf EST 2

(1) Details of sequence length for leaf EST 2

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1314
2	Initial sequence length (reverse)	1253
3	Vector/adaptor sequence length (forward)	697-1095- Strong 1147-1178-Moderate
4	Vector/adaptor sequence length (reverse)	742-1150 Strong 37-81- Moderate 1-36-Suspect origin
5	Final length of coding sequence	898

(2)Blastx result analysis of leaf EST 2

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
XP 018683776.1	PREDICTED: *Musa accuminata* subsp. *malaccensis* ACT domain containing protein ACR4 like isoform X2	56	6e-74	72
XP 009404247.1	PREDICTED: *Musa accuminata* subsp. *malaccensis* ACT domain containing protein ACR6-like isoform X1	56	2e-73	72

(3)Blastn result analysis of leaf EST 2

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
DY385989.1	*Curcuma longa* cDNA clone Ea0006C03 3’ mRNA sequence	69	0.0	99
DY385988.1	*Curcuma longa* cDNA clone Ea0006C03 5’ mRNA sequence	10	5e-42	100

(3)Open Reading Frame analysis of leaf EST2

ORF location	ORF length (bp)	Frame
179-679	501	-1

Table 56:-*In silico* analysis of leaf EST 3

(1) Details of sequence length for leaf EST 3

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1005
2	Initial sequence length (reverse)	1222
3	Vector/adaptor sequence length (forward)	313-1005- Strong
4	Vector/adaptor sequence length (reverse)	41-85,364-1050 Strong 24-40- Moderate 1-23-Suspect origin
5	Final length of coding sequence	407

(2)Blastn result analysis of leaf EST 3

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
HF950864	*Boechera divaricarpa* GSS, clone B62-01-R_E08	43.5	2.03e-15	75.7
sHF950056	*Boechera divaricarpa* GSS, clone B62-01-R_O24	44.5	1.05e-12	75.1

(3)Open Reading Frame analysis of leaf EST3

ORF location	ORF length (bp)	Frame
13-120	108	-3

On blastn analysis of leaf EST 3 showed 75 per cent similarity with *Boechera divaricarpa* GSS, clone B62-01-R_E08 and on open reading frame analysis and ORF length of 108 bp was observed

Leaf EST 4

The length of the forward and reverse sequence obtained from the leaf EST was of 1265bp and 1274 bp respectively. In the forward sequence, 718-1001 bp indicated the vector/adaptor sequences. In the reverse sequence, 1-78bp and 762-1054bp indicated the vector/adaptor sequence. The sequence details of leaf EST 4 are given in given in Table 57. Bioediting of the vector/adaptor sequence was done and the resulting forward and reverse sequences were joined to get a final sequence of 896 bp which is given below:-

Vector/adaptor edited sequence of leaf EST 4

5'AGGTGGGGGGCGGGAACCCTTATGGGAAAACGCCAAGGCAAAACGCGG
CCATTTTTACCGTTTCCCGGCCTTTGGCTGGAGCCCTTTGACTCACACATAT
CTCTTCCCGGCGTATATCCCCCAGGATTCTGTGAGTAAGCCGTTATTAACC
CCCAATTGAGGGAGCGTAGATAACACTCTTCGAGCGGCCGCCCGGGCAGG
TACCTGAAGGAGACCTTGTAAAAGATCAATAACATCGCCAGCAGAGTGAT
CGACATGCTGCATAACAATATTCTGCAGACGAAGTAGTTTTTGAACAGCTT
TAATACTCTCCCTGGATGTAGCACCTGCTGGCCAGTTCAGCCGACCTTTTC
TAATGTATTTCTCAGCGTGCCTATCAGCTCTCTTCAGCATATGCTGGGGAA
AGGGACCCAACACTCTCTCCATCATGGCCATGTGTTCTAAATTTTCATGGG
TCTGGAACAAGGCCTCCCCCGAGCAAAGTTCTACGATGATACAACCAATG
CTCCATATATCACATGGATAACTCCATCCAAGCCCCAAGATAACCTCTGGT
GCTCGATAATGCCTGGTCGAGACTATATAAGTATGGTCCTTGCGGTCATAG
GTTGCGCTACCAAAATCAATTAACTTGATGGCACTAGATTTTGGAAGCCTC
TTGGAGCATGAACCATCCTTATATTCAGAGACCTTGATATACTCAGGGGA
CACAAAAAGAATGTTCTCAGGTTTTAAGTCTGTATGAATGAGATGCAAAT
CTGCATAACTGCAACAAAAGAAAAGGCAGTCCATCATGAACAAGAAGCA
ATAC

Table 57:-*In silico* analysis of leaf EST 4

(1) Details of sequence length for leaf EST 4

Sl.no	Particulars	Sequence length (bp)
1	Initial sequence length (forward)	1265
2	Initial sequence length (reverse)	1274
3	Vector/adaptor sequence length (forward)	718-1001- Strong
4	Vector/adaptor sequence length (reverse)	34-78,762-1054- Strong 1-33-Suspect origin
5	Final length of coding sequence	896

(2)Blastx result analysis of leaf EST 4

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
P51567.1	RecName: Full Serine/threonine – protein kinase AFC2	69	2e-109	71
P51566.2	RecName: Full Serine/threonine – protein kinase AFC1	67	1e-103	72

(3)Blastn result analysis of leaf EST 4

Accession No.	Description	Q. coverage (%)	E value	Homology (%)
JK543849.1	*Musa accuminata* AAA group cDNA clone MACVLIMFLS004D Co6.b 038 5’ mRNA sequence	45	1e-119	88
JK543848.1	*Musa accuminata* AAA group cDNA clone MACVLIMFLS004A Co6.b 038 5’ mRNA sequence	45	6e-118	86

(4)Interproscan analysis of EST4

Data entry	Description
Length	224 amino acids
IPR011009	**Domain-**Protein kinase like domain,
IPR000719,IPR000271	**Signature matches-** Protein kinase, Serine/threonine-protein kinase,active site
GO:0006466	**Biological process**-protein phosphorylation
GO:0004672,GO:0005524	**Molecular Function-**Protein kinase activity,ATP binding

(5)Open Reading Frame analysis of leaf EST 4

ORF location	ORF length (bp)	Frame
91-765	675	-3

ATGCCCAGAAAACCACTATTATTTAAGGTACCTCGGCCGCGACCACGCTA
ATCTAGATGCAACGCGATTAAAAGGCCGCGCCCT**3'**

The blastx analysis of the leaf EST 4 showed 71 per cent similarity with that of RecName: Full Serine/threonine –protein kinase AFC2 and blastn analysis showed 88 per cent similarity with *Musa accuminata* AAA group cDNA clone MACVLIMFLS004D Co6.b 038 5' mRNA sequence. The interproscan analysis showed that the leaf EST 4 had protein kinase like domain, with protein phosphorylation as the biological process and ATP binding was the molecular function of protein kinase activity. The EST showed ORF length of 675 bp on open reading frame analysis.

4.4.2.4 Blast2GO analysis of leaf ESTs

The Blast2GO analysis of leaf ESTs showed that three out of four EST sequences showed similarity to *Musa accuminata* subsp. *malaccensis* and one EST sequence to *Boechera divaricarpa* (Figure 23).

4.5 Validation of identified ESTs for high gingerol production using Real time PCR

The gene expression of *Thiolase* was quantified using real-time PCR assay. The thiolases and Acetyl-CoA-acetyltransferase are involved in the benzoate degradation pathway and these enzymes are also involved in valine, leucine, and isoleucine degradation pathway. Theacetyl CoA produced through both the pathways are the central molecule involved in plant metabolic pathways along with interconnecting different biochemical pathways.

4.5.1 Isolation and analysis of total RNA

Total RNA was isolated from the leaves of genotypes of ginger *viz* Maran, B_3, Athira, Karthika and Aswathy.

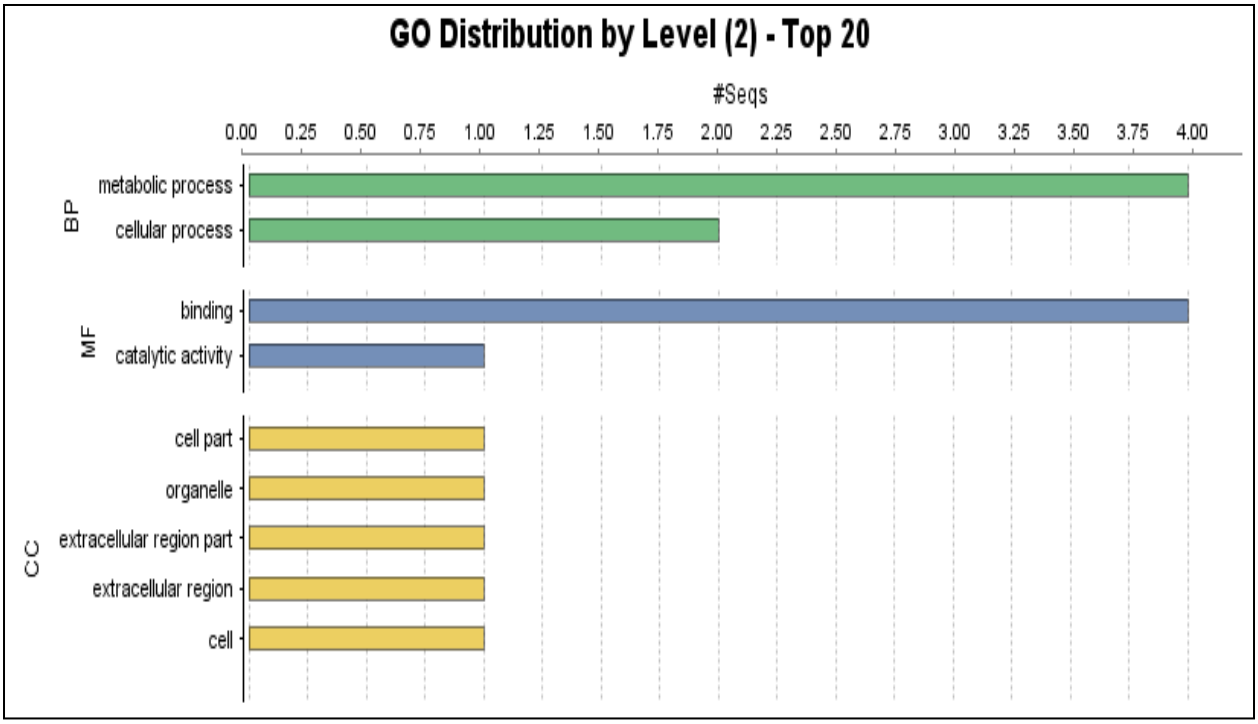


Figure 22:- Gene ontology (GO) count for biological process, molecular function and cellular components in rhizome ESTs.

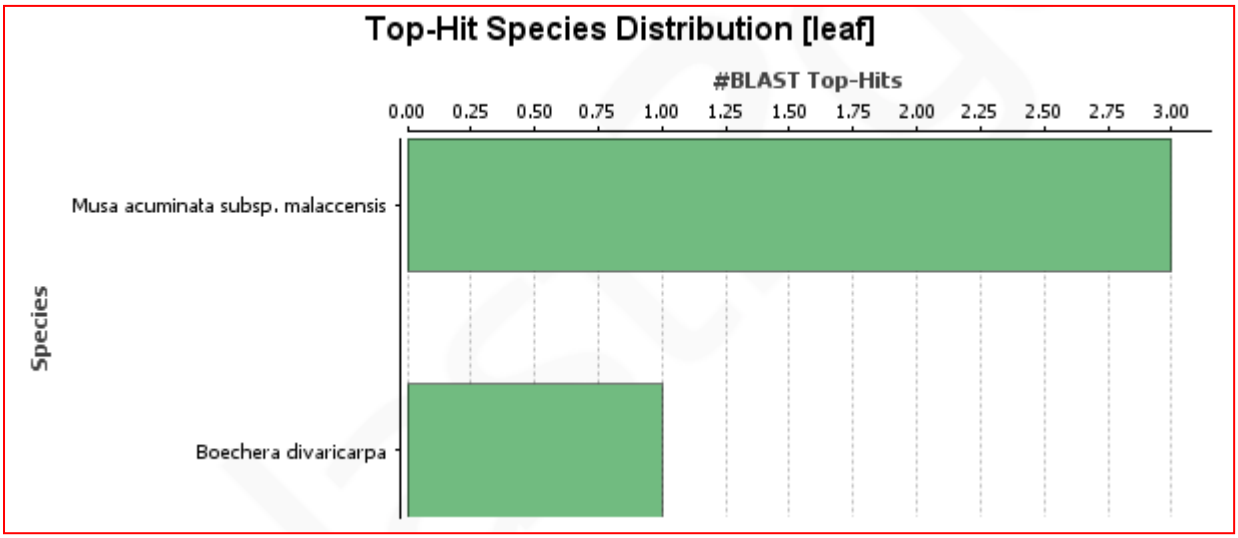


Figure 23:- Species distribution chart of leaf ESTs

4.5.2 Quality and quantity analysis of isolated total RNA

In order to check the quality of total RNA isolated, the samples were subjected to gel electrophoresis using one percent agarose (formaldehyde) gel in 1X MOPS buffer. Three intact bands corresponding to 28S, 18S and 5S rRNA observed under gel documentation unit showed good quality total RNA (Plate 24). Quantification of the RNA was done using NanoDrop ® ND-1000 spectrophotometer by determining absorbance of nucleic acids at wavelength 260nm and 280nm. The concentration of total RNA in Athira, Aswathy, Karthika, B3 and Maran were 0.80, 1.10, 0.98, 1.35 and 1.40 µg μl^{-1} respectively. The A_{260}/A_{280} ratio was greater than 1.8 indicating good quality RNA free from protein contamination. The A_{260}/A_{230} was more than 1.0 indicating the sample to be free from carbohydrate and protein contamination.

4.5.3 First strand cDNA synthesis and confirmation of cDNA using primers

Reverse transcription of the isolated RNA was carried out using Revert Aid First strand cDNA synthesis kit as per the manufacturer's protocol. The cDNA synthesis was confirmed by amplification of the *Thiolase* (Plate 25) and *Actin* gene specific primers. The PCR product was run in 1.8 per cent agarose gel and the band size of 204 bp and 138 bp corresponding to *Thiolase* and *Actin* gene was obtained confirming synthesis of cDNA.

4.5.4 Gene expression of *Thiolase* gene using Realtime PCR assays

Quantification of the *Thiolase* gene expression was performed using real-time PCR by determining the relative gene expression of *Thiolase* gene using the C_T values by comparative ΔC_T method with *Actin* as the housekeeping gene.

4.5.5 Threshold cycle values

The realtime PCR data are represented as the cycle number necessary to achieve a threshold cycle value. The reference gene used for expression analysis was *Actin*. The represented C_T value for *Actin* gene was the mean of three biological replicates. Significant fold change in the *Thiolase* gene expression was found among the genotypes studied as compared to the control.

4.5.6 Relative quantification analysis

The relative gene expression of *Thiolase* was estimated as per the comparative ΔC_T method as stated by Livak and Schmittigen (2001). The relative expression of *Thiolase* gene ranged from 1.00 to2.737 fold among the genotypes studied, when normalized with the endogenous *Actin* gene. The gene expression *Thiolase* gene in various genotypes is represented as average $2^{-\Delta\Delta Ct}$ values in the Table 58. There was no significant difference between the varieties Athira, Karthika and Aswathy and the somaclone B3 with respect to the gene expression of *Thiolase* gene. In comparison with the cultivar Maran, the variety Athira showed 2.737 fold, variety Karthika 2.150 fold, variety Aswathy2.018 fold and somaclone B3 1.992 fold increased gene expression of *Thiolase* gene (Figure 24).

Table 58:- Relative gene expression of the *Thiolase* gene in different ginger genotypes

Genotypes	*Thiolase* gene expression as $Av2^{-\Delta\Delta Ct}$ (fold change)
Maran	1.000
Athira	2.737
Karthika	2.150
Aswathy	2.018
B3	1.992
CD(0.05)	0.763
SE(m)±	0.239

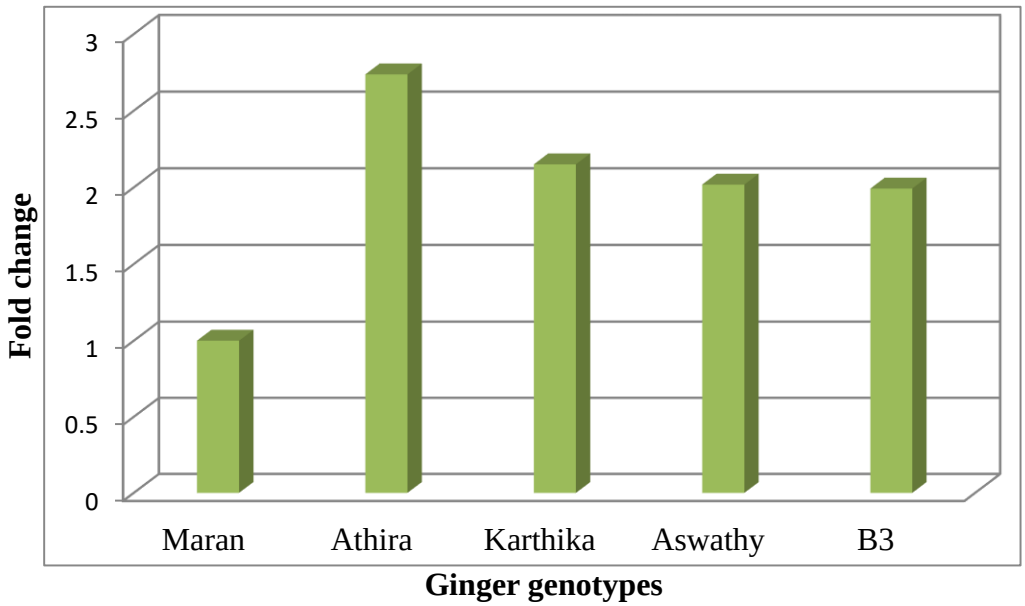
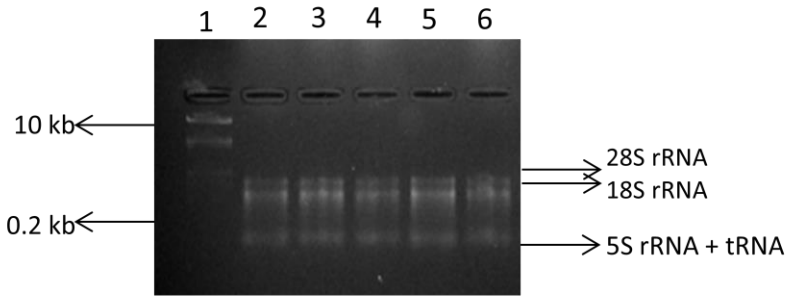
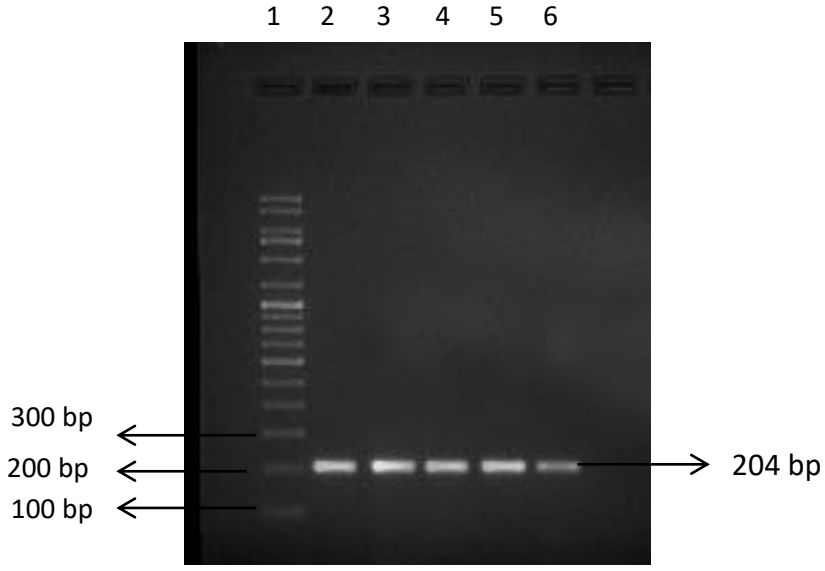


Figure 24:- Relative gene expression of the *Thiolase* gene in different ginger genotypes



1: Transcript RNA marker (0.2kb-10 kb)
2: Variety Athira
3: Variety Karthika
4: Variety Aswathy
5: SomacloneB3
6: Cultivar Maran

Plate 24:- Total RNA isolated from the leaves of different ginger genotypes



1: Low Range DNA Ruler Plus (100bp to 3000 bp)
2: Variety Athira
3: Variety Karthika
4: Variety Aswathy
5: Somaclone B3
6: Cultivar Maran

Plate 25:- Amplification of first strand cDNA synthesis using gene specific primers of *thiolase* gene

Amplicon size of *thiolase* gene is 204 bp

Discussion

5. Discussion

The nutritional, health and medicinal properties of ginger have been associated with the bioactive gingerols. Gingerols exhibit a broad spectrum of biological activities ranging from anti-allergic, anti-microbial and anti-cancerous to anti-hypercholesterolemic. In traditional ayurvedic system, ginger has been used for hundreds of years for the treatment of stomach problems, digestion, diarrhoea cough etc. Ginger also plays an important role in the nutraceutical and health food industries. Currently, many ginger and gingerol products are available in the markets which are used for medicinal purposes. Among the different gingerols, 6-gingerol is reported to be a highly potent anti-cancerous phytocompound. The gingerols are produced through the phenylpropanoid biosynthetic pathway. Extraction of gingerols from ginger requires large quantities of rhizomes and solvents, and also a vast area of agricultural land for its cultivation (Stoilova *et al*., 2007).Even though gingerols have much importance, the biosynthetic pathway of gingerols is not well studied and the ginger genome is also exploited only to a limited extent.

Plants are the remarkable sources of secondary metabolites. The secondary metabolites are currently targeted for drug development in the health industry. In the developed world, most of the clinically used drugs originate from natural resources and for the growing population, plants play the dominant role in primary health care. In the tropics, people mainly depend on plants as the medicinal source. The limited supply of the plants may get exhausted and there are lots of problems with the conventional methods of propagation. The secondary metabolites that are used as drugs are extracted from the plant materials procured from commercial cultivators. The main disadvantage of such secondary metabolites is the presence of pesticide residues. The removal of pesticide residues from the metabolites is either difficult or very costly. These calls for adopting plant cell and tissue culture for clean secondary metabolite production. Biotechnological techniques are environmental friendly approaches for the production of the metabolites even when the natural supply is limited. Plant cell and tissue culture are suggested as alternatives for year round production of secondary metabolites, with the added potential of increasing yields by manipulation of culture and culture environments. The *in vitro* grown plant cells and

tissues have been extensively used for the production of secondary metabolites. The compounds accumulated through *in vitro* cultures are identical to the compounds which are produced inside the plants. Hence, the biotechnological approach of production using plant cultures can be an alternative for enhanced secondary metabolite production, but to date, there are only limited commercial successes reported because of lack of understanding of the process of complete biosynthesis of the secondary metabolites production inside the plants.

In the present investigation, attempts were made to induce microrhizomes and calli in the five genotypes of ginger and the production of gingerols and shogaol were studied in these genotypes at different induction periods. Additionally, studies on scaling up of production of secondary metabolites (gingerols and shogaol) were taken up by manipulation of macro and micronutrients of the MS medium, elicitation of the secondary metabolite production by biotic and abiotic elicitors, feeding of the precursors/substrates of the biosynthetic pathway of gingerols and irradiation with lower doses of gamma rays. The quantification of the gene expression using real time PCR was carried out for two important candidate genes involved in the biosynthetic pathway of gingerols from the identified treatments with higher gingerol production using microrhizomes. Analyses and validation of available ESTs for high gingerol production were also carried out.

The results of the present investigations are discussed in this chapter under the headings:

(i) Investigations on gingerol production *in vitro*

(ii) Manipulation of macro and micro nutrients on gingerol production

(iii) Elicitation using biotic and abiotic elicitors for gingerol production

(iv)Effect of precursor feeding on gingerol production

(v) Effect of gamma irradiation on gingerol production

(vi) Gene expression studies using Real time PCR

(vii) Analysis of available ESTs using bioinformatics tools

(viii)Validation of identified ESTs for high gingerol production

5.1 Investigations on gingerol production *in vitro*

5.1.1 Gingerol production in ginger microrhizomes induced *in vitro*

Induction of microrhizomes was done in the present investigation from the induced multiple shoot cultures of all the five ginger genotypes using MS medium supplemented with high content of sucrose of 80 g L^{-1}. Initially, cultures were initiated using rhizome buds as explants in MS medium supplemented with the cytokinin BAP 3mgL^{-1} in all the five genotypes of ginger. The culture establishment and sprouting of buds varied in different ginger genotypes. Highest culture establishment was observed in the cultivar Rio-de-Janeiro (76.81 %) with sprouting of 75.37 per cent while the variety Karthika recorded the lowest. Shoot proliferation in the different genotypes was observed non significant after one, two and three months of inoculation. The number of shoots proliferated from 1.50 (1MAI) to 11.50 (3MAI) in the variety Athira was higher among the three varieties of ginger studied. Incidence of the adventitious shoot formation is influenced by the growth regulators used. The cytokinins, besides suppressing apical dominance, initiate adventitious buds into shoots which produce a mass of ramified shoots (Stefaans and Debergh, 1990). Generally, cytokinins are known to stimulate cell division and axillary bud proliferation (Kyte and Kleyn, 1996).

Apart from growth regulators, genotype of a species is also the most important factor that influences the morphogenic responses of *in vitro* culture. In fact, the different types of responses *in vitro* like multiplication and other morphogenic responses including shoot proliferation, organogenesis, rooting etc are strongly determined by the genetic factors of an explant (Bhau and Wakhlu, 2001; Gahan and George, 2008 and Gomes *et al*., 2010). The cytoplasmic compositions of cells are dependent on the genotypes and hence the *in vitro* response of an explant may differ (Ma *et al*., 1987). In addition, the interaction between the genotype and the media may also vary. The conditions which are conducive for the tissue culture responses of

one genotype are not usually conducive for another genotype of the same species (Mahmood *et al*., 2012) due to which, the different genotypes respond differently *in vitro*.

Multiple shoot induction in ginger using *in vitro* techniques has been attempted extensively using shoot tip and rhizome bud as explant (Smith and Hamill, 1996; Khatun *et al*., 2003; Hamirah *et al*., 2007 and Kambaska and Santilata, 2009). Genotypic variations of *in vitro* cultures were reported in crops like ginger (Paul and Shylaja, 2010 and Shylaja *et al*., 2016) and turmeric (Tyagi *et al*., 2004).The studies on plant regeneration through *in vitro* protocols in wild species of turmeric suggest that both phenomena depend on genotype (Tyagi *et al*., 2004). Indirect organogenesis in two cultivars of ginger, Rio-de-Janeiro and Maran was done by Paul and Shylaja (2010) who observed the highest response in the cultivar Rio-de-Janeiro with respect to callusing, callus growth, shoot morphogenesis and shoot proliferation. In another study, the highest number of shoots/ explant was reported in ginger variety Athira when compared to varieties Karthika and Aswathy (Shylaja *et al*., 2016) which is in harmony with our findings

Microrhizomes, the miniature propagules can be induced *in vitro* using rhizomes. Plant hormones, culture environment and sucrose concentration influence the microrhizome induction (Bhat*et al*., 1994; Kambaska and Santilata, 2009). In the study, the initiation of microrhizome started as swelling of shoot bases after one to one and a half weeks of inoculation. The days taken for initiation varied between 9.25 to 19.75 days and were found high for the cultivars. The variety Athira recorded the least number of 9.25days, while the cultivar Maran recorded more number(19.75 days) for the initiation of microrhizomes. This agrees with the findings of Sharma and Singh (1995) who reported 18-20 days of incubation for initiation of microrhizomes in the ginger cultivar Himachal local. On contrary, a longer duration of 35-45 days for induction of microrhizomes was reported by Singh *et al*. (2014) in ginger varieties Baishey and Naida induced in MS liquid medium supplemented with sucrose (8%), 1 mg L^{-1} NAA, 2 mg L^{-1} BAP and 11 µM silver nitrate.

Higher concentration of sucrose promotes the formation of storage organs such as rhizomes, bulbs, corms and tubers (Abbott and Belcher, 1986; Arora *et al*., 1996, Dantu and Bhojwani, 1987; Grewal, 1996). Assimilate partitioning from the source of production to the sink where it is stored is essential for the harvestable component of the economically important plants (Gifford and Evans, 1981). The fixation of carbondioxide followed by subsequent allocation of fixed assimilates into economical yield component results in harvestable yield. In ginger, the rhizomes are the harvestable yields where the assimilates are unloaded from the source of production. The sucrose provided to the culture medium may be transported to the base of the stem for initiation of microrhizomes during *in vitro* microrhizome induction and enhanced production of microrhizomes may be attributed to the high sucrose content.

The use of high sucrose medium for induction of microrhizome*in vitro* was reported by Bhat *et al.* (1994) and Sharma and Singh (1995) in ginger, Cousins and Adelberg (2008) in turmeric and Chirangini *et al.* (2004) in *Kaempferia.* Optimal production of microrhizomes was obtained in ginger with full strength MS medium supplemented with 7.5 per cent sucrose (Sharma and Singh, 1995). In potato, the full strength MS medium supplemented with 8 per cent sucrose was found better as compared to MS medium supplemented with 4 and 12 per cent sucrose (Garner and Blake, 1989). In turmeric, Shirgurkar *et al*. (2001) found half strength MS medium supplemented with 80 g L^{-1} sucrose optimal for the production of microrhizomes.

The number of microrhizomes produced in the study increased with an increase in the induction periods. Irrespective of the genotypes, an average of 6.30, 11.30, 17.56 microrhizomes/culture were produced in MS medium supplemented with 80 g L^{-1} sucrose without any growth regulators after one, two and three months induction period. Abbas *et al.* (2011) reported production of 14.00 microrhizomes in MS medium supplemented with 90 g L^{-1} sucrose and 3 mg L^{-1} BAP, after 10 weeks of incubation, which is comparatively less, considering our results. The highest number of microrhizomes/culture were recorded in the cultivar Rio-de-Janeiro among the two cultivars and in the variety Athira, among the three varieties studied, for all the five induction periods. Genotypic variations affect the production and growth of

microrhizomes. In the present investigation, varietal difference was observed in the initiation, formation, fresh weight, dry weight and driage of microrhizomes. A great variation in the number of rhizome/ culture in 33 genotypes of ginger was reported by Tyagi *et al.* (2004). An average number of three rhizomes/ culture to 15 rhizomes/ culture was recorded with 12 months of induction in the 33 genotypes of ginger studied. In another study, the higher response of ginger variety Rejatha as compared to other varieties Mahima and Varadha with respect to the number of shoots with microrhizomes was reported by Archana *et al*. (2013). The report of highest production of microrhizomes/ explants after incubation of three months in the variety Athira was reported by Shylaja *et al*. (2016) which is in agreement with our results.

The absorption of water and other compounds from the medium attributes an increase of fresh weight (Ozgen and Yildiz, 2004). The photosynthate translocation to microtuber which acts as the sink accounts for the increase in the fresh weight of the microtubers (Slimmon *et al*., 1989). In the current study, the fresh weight, dry weight and driage increased with the increasing induction period. Irrespective of the genotypes studied, an average of 0.153 g, 0.192 g and 0.474 g were recorded respectively at one, two and three months induction periods. Sharma and Singh (1995) reported an average microrhizome weight of 73.8 mg after incubation of 50-60 days which is two times lesser than the average fresh weight we have obtained even after the lesser induction period of one month. The variety Athira recorded the highest fresh weight of microrhizome for all the induction periods studied, recording the highest of 0.650 g after five months after induction. The variety Karthika recorded the highest dry weight and driage for all the three induction periods studied. A similar observation of increased fresh weight with increasing incubation period was observed by Sharma and Singh, (1995) in ginger microrhizomes. Abbas *et al*. (2011) reported rhizome fresh yield of average 5.8 (g/jar) obtained in ginger cultures (14.00 rhizomes and 51.33 buds) in MS medium supplemented with 90 g L^{-1} sucrose and 3 mg L^{-1} BAP incubated under light condition 16-h/day after 10 weeks.

Genetic factors are found to influence the growth parameters of miniature propagules. Genetic factors were observed to affect the yield of micro tubers in potato (Hossain and sultana, 1998). The variety Patrones of potato produced significantly

heavier microtubers compared to Lalsheel and Chamak variety (Hossain and sultana, 1998). Sucrose concentration has a direct correlation with dry weight and dry matter (%).The increase in sucrose concentration of 90 g L^{-1} increased dry weight to 0.95 g (Abbas *et al*., 2011). The dry matter content of *Jerusalem artichoke* microtubers was observed to be unusually high when the sucrose concentration increased to 10 per cent (Gamburg *et al.*, 1998).

The microtubers, microrhizomes and adventitious tuberous roots can be better alternatives for the production of secondary metabolites on large scale (Jean and Cappadocia, 1991; Xie *et al*., 2000). The quality analysis of the *in vitro* developed miniature propagules of rhizomes indicated the presence of the same constituents as that of the original rhizomes with differences in its quantity (George, 1993). Microrhizomes induced in turmeric were found to produce the secondary metabolites with antioxidant activities equaling or even surpassing dried powdered rhizomes preparations from commercial field grown plants (Cousins *et al*., 2007).Ginger microrhizomes induced *in vitro* can be an alternative for the production of gingerols as compared to field grown rhizomes as the latter requires larger area and are affected by seasonal variations and are easily exposed to pests and diseases which alters its medicinal qualities. But for the microrhizomes to be a feasible source for gingerol production, the biosynthesis of gingerols along with the factors which can improve the gingerol production under controlled conditions should be identified.

In the present study, oleoresin extracted in different ginger genotypes at various induction periods of one, two, three, four and five months was analyzed. Irrespective of the genotypes studied, recovery of oleoresin was observed increased with an increase in induction periods. The recovery of oleoresin and accumulation of the gingerols and shogaol was observed highest in the varieties as compared to the cultivars. The recovery of oleoresin was observed the highest in the variety Aswathy for all the five consecutive months with an average of 3.13 per cent after five months induction period. Genotypic difference in oleoresin recovery from fresh ginger was reported by Kiran *et al*. (2013b). They extracted oleoresin from fresh ginger rhizomes of ten ginger cultivars after six and nine months maturity and reported the highest oleoresin yield from the cultivar Tripura II after nine months maturity. Sreeja (2017)

reported that the oleoresin extracted from the fresh ginger of somaclone B3 recovered the highest oleoresin of 1.18 percent after seven months of planting as compared to other genotypes, which is comparatively less with the results we obtained even after one month induction period. Studies were also reported on oleoresin recovery from dry ginger. Genotypic differences in oleoresin recovery from dry ginger were reported by Nybe *et al*. (1982) who evaluated 28 cultivars of ginger and concluded the cultivars Rio-de-Janeiro and Maran had the highest oleoresin yield of 10.53 and 10.05 per cent respectively. Recovery of oleoresin was 6.8 and 7.2 percent respectively from the dry ginger of variety Athira and variety Karthika respectively (Shylaja *et al*. 2010) while in the variety Aswathy an oleoresin recovery of 7.45 per cent was observed (Shylaja *et al*. 2014).

The components of the oleoresin, 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol were analyzed in the present investigation. The accumulation of the total gingerols (sum total of 6, 8, and 10- gingerol) increased with an increase in the induction period and recorded the highest, four months after induction. A decline in the accumulation of total gingerols was observed at five months induction period while the shogaol content increased. The accumulation of 6-gingerol increased with the induction period and was also observed the highest in the variety Aswathy for all the five induction periods studied. The production of 8-gingerols was not observed in the initial induction period of one and two months and increased from three months induction period, which recorded the highest after five months of induction periods, in all the genotypes. Irrespective of the induction periods, the variety Karthika recorded the highest 6-shogaol content recording an average of 0.076 per cent after five months of induction. In ginger, 6-gingerol was the abundant pungency principle present compared to other homologues (Purseglove *et al*., 1981). Pawar *et al*. (2015) and Salmon *et al.* (2012) reported significant variation in 6, 8, 10 gingerol content in various genotypes. Similar observation of an increase of 6, 8, 10 and total gingerol from fresh ginger rhizome was reported by Sreeja (2017) in the genotypes of ginger Athira and Maran after five, six and seven months of planting. In another study, the variation of secondary metabolite production was studied at different growth stages in *Zingiber zerumbet* and reported the total flavonoid content and total phenolics were

detected higher in rhizome extracts of the nine month old plants than 3-8 month old plants (Ghasemzadeh *et al*., 2016). They also reported that when the plant matured from 3 to 9 months, the total flavonoid content and total phenolic content was observed decreased in the leaf, but increased significantly in the rhizomes.

The production of 6- gingerol in the *in vitro* ginger plantlets with microrhizomes established in MS medium supplemented with varying levels of sucrose was reported by Marfori and Jane (2018). They observed the highest production of 6-gingerol (6418.61 µg mL^{-1}) after three months of incubation in MS medium supplemented with 90 mgL^{-1} sucrose. This is in agreement with our findings of high gingerol production of 0.850 per cent in the variety Aswathy from microrhizomes induced in MS medium supplemented with high sucrose of 80 mg L^{-1} after three months induction period. Sucrose is the carbohydrate and the carbon energy source in medium. The growth and metabolism of the cultures are influenced by the carbohydrate source. The enhanced accumulation of 6-gingerol is contributed by increased microrhizome formation (Marfori and Jane, 2018).

5.1.2 Gingerol production in the ginger calli induced *in vitro*

In the present investigation, calli were initiated in the five genotypes of ginger using *in vitro* shoot tip as explants using CI medium. Days taken for callusing, callusing percentage, and callus index were recorded after one month. Further the calli were subcultured and growth and production of calli along with accumulation of gingerols and shogaol were observed after different induction periods.

Cell division is regulated by joint action of auxins and cytokinins, each of which influences different phases of the cell cycle. Auxins exert an effect on cell replication while cytokinins exert some control over the events leading to mitosis (Jouanneau, 1971).Callus induction is promoted by auxin along with cytokinin (Skoog and Miller (1957) and MS medium supplemented with 2, 4-D is the optimum condition for callus induction in ginger(Ibrahim *et al.,* 2015; El-Nabarawy *et al*., 2015). The RNA and protein syntheses needed for callus induction are regulated by 2, 4-D and the role of 2, 4- D in DNA replication and mitosis might be responsible for inducing callus (Sen *et al*., 2014) from different explants. Calli was induced

successfully in ginger earlier by Pillai and Kumar (1982); Sakumara and Suga (1989); Ilahi and Jabeen (1992); Babu (1997), who opined that secondary metabolite production using *in vitro* cell cultures are preliminary and more work needs to be done before commercialization of secondary metabolite production *in vitro.*

In the present study, CI medium constitutes the combination of with 2, 4-D 3 mg L^{-1} and 0.5 mg L^{-1} BAP in ½ MS medium. Initiation of calli was observed at the cut ends of the shoot tips one to one and half months after inoculation depending on the genotypes. In general, low callusing and callus index were observed in the study. Seyyedyousefi *et al*. (2013) reported that callus induction is time consuming and very difficult in monocotyledons. Taha *et al*. (2013) reported an average callugenesis of less than 50 per cent in ginger incubated in MS medium supplemented with 2, 4-D alone and in combination of BAP. Similar observation of callus initiation at the cut ends of the shoot tip explants of the ginger after 30 days of inoculation in MS medium with 2, 4-D and BAP were made by El-Nabarawy *et al*. (2015).

Callusing and calli growth were genotype dependent. The days taken for callusing recorded less in the varieties studied as compared to the cultivars. In the variety Athira callusing was observed 53.23 per cent of cultures in 31.25 days after incubation expressing a callus index of 68.24 per cent. The lowest callusing of 42.89 per cent was recorded in the cultivar Maran in 39.25 days after inoculation, with a callus index of 47.29 per cent. Similar genotype dependent response of *in vitro* cultures in callusing and callus growth was observed in several spice crops like ginger (Paul and Shylaja,2010), black pepper (Shylaja, 1996), garlic (Barandiaran *et al*., 1999). In another study on the five cultivars of black pepper, a significant difference was observed with respect to callusing and callus growth. The cultivar Kalluvally registered highest callusing percentage and callus growth index (Shylaja, 1996). The genotypic differences of endogenous auxin and cytokinin balances (Looney *et al*., 1988) along with the differences in uptake of exogenous cytokinins by different genotypes (Marino, 1988) might have resulted in the difference in response of callusing.

The fresh weight of the calli increased with an increase in induction periods and recorded the highest three months after induction irrespective of the genotypes used in the study. At three month induction period, the variety Aswathy recorded a callus fresh weight of 2.005g, with a dry weight of 0.293g and driage of 14.65 per cent. The lowest fresh weight of 1.428g was recorded by the cultivar Maran. Eventhough among the three varieties, the variety Karthika recorded the lowest fresh weight of 1.505g, the dry weight and driage was recorded highest in the variety after three months induction period. The quantity and quality analyses of callus induced in three ginger varieties were carried out by Arijanti and Suryaningsih (2019) who reported that the variety Gajah produced the highest quantity of callus as compared to the varieties of ginger Emprit and Merah.Shylaja *et al.* (2010) observed the highest dry recovery of 21.6 per cent in variety Karthikaas compared to the other varieties Aswathy and Athira

Oleoresin was extracted from ginger calli and the pungency principles separated were 6, 8, 10 gingerols and 6-shogaol. The accumulation of total gingerols and shogaol increased with increase in induction period, which recorded the highest three months after induction. The varieties accumulated higher gingerols as compared to the cultivars. Based on morphogenetic response, it was observed that the calli from the variety Aswathy at root morphogenic stage produced more gingerol content (3.15 %) as compared to the calli with shoot morphogenesis. The variety Aswathy recorded highest total gingerols (0.090 %), 6-gingerol (0.060 %) and 10-gingerol (0.030%). The result is in harmony with the findings of Arijanti and Suryaningsih (2019) who reported genotypic differences in accumulation of gingerol, shogaol and zingerone in calli of three ginger varieties Gajah, Emprit and Merah. They concluded that the variety Emprit showed high gingerol (1.181 %), shogaol (0.118 %) and zingerone (0.098 %) content. The variation in production of secondary metabolites with the age of the callus was observed in *Morinda citrifolia* (Hagendoorn *et al*., 1997) and in *Salvia multiorrhza* (Tsay and Agarwal, 2005). The callus from the ninth subculture of *Salvia santolinifolia* recorded more secondary metabolites than the eighth subculture (Jan *et al*., 2015).Contrary to our results, Zarate and Yeoman, (1994) reported that due to lack of morphological differentiation, accumulation of gingerols and shogaol was

not detected in ginger callus. The effect of light and dedifferentiation of secondary metabolite production of ginger calli was studied by Ahasori and Asghari (2009). They reported the absence of gingerols and zingiberene in dedifferentiated callus while in callus with some sort of differentiation showed the presence of gingerols and zingiberene. The accumulation of gingerols and shogaol may be due to differentiation of callus. No accumulation of gingerols was observed from the ginger calli induced in 2, 4-D along with BA (El-Nabarawy *et al*. (2015). The 6-gingerol produced in rhizomes of conventionally grown plants and callus cultures produced plants were compared by Pawar *et al*. (2015),who reported that rhizome derived callus culture and micropropagated plants produced the lowest amount of 6-gingerol compared to conventionally grown plants.

In plant cells, the sites for production and storage of secondary metabolites are often located in individual compartments such as specialized cells with secretory and accumulatory elements or oil glands. But in calli, as it is an undifferentiated mass of cells the storage cells will be missing and probably due to the missing of sites in calli, either very low or no detectable quantity of secondary metabolites are observed (Dorenburg and Knorr,1995). However in the present study production of gingerols were observed in the calli induced from all the genotypes of ginger after three month of induction.

5.1.3 Comparison of gingerol production in ginger microrhizomes and calli induced *in vitro*

The cultured plants cells sometimes produce reduced quantities of secondary metabolite than intact plant (Whitaker, 1986). In the study, the gingerols and shogaol content were observed to be highest in the *in vitro* microrhizomes than the *in vitro* calli for all the three induction periods. Total gingerol accumulation in microrhizomes of ginger genotypes studied ranged from 9.4 - 50 times over that of the calli. The accumulation of 6-shogaol in microrhizomes of ginger genotypes studied ranged from 4-50 times over that of the calli. The cultivar Rio-de Janeiro was showing 50 times increase in accumulation of total gingerols in microrhizomes over the calli. The variety Aswathy was showing 9.4 times increase in total gingerols in microrhizomes as compared to the calli of the variety indicating better accumulation of total gingerols

in calli of the variety Aswathy. The results harmonized with the findings of Janarthanam *et al*. (2010).They reported the stevioside content in leaf extract of *Stevia rebaudiana* Bertoni (19.25 g kg^{-1}) was higher than the callus extracts (17.63 g kg^{-1}).

5.2 Manipulation of macro and micro nutrients on gingerol production

Macro and micro nutrients in definite concentration influences growth and morphogenesis in tissue culture. Murashige and Skoog (1962) suggested that increasing micronutrients increases cell growth and morphogenesis. Minor elements are involved as cofactors for enzymes in many plant metabolic processes and hence are critical for growth and development. Doubling of micronutrients increased embryogenesis in *Hevea brasillensis* anthers (Wang *et al*., 1981). In plant tissue culture, macro and micro nutrient composition play important role in secondary metabolism of cells and the medium which limits early cessation of exponential growth enhances secondary metabolite production (Dougall, 1980).

Nitrogen source along with its concentration, influence the growth and metabolite accumulation in cell and organ cultures. Tissue culture media like MS contain both ammonium and nitrate as the nitrogen source. The nitrogen concentration regulated the expression of specific proteins through transcription and mRNA stability (Sugiharto and Sugiyama, 1992). In general, nitrate is the most essential form of nitrogen for culturing tissues. However, the culturing of tissue in nitrate alone as the source of nitrogen may not be successful (Halperin and Wetherell, 1965; Selby and Harvey, 1990; Smith and Krikorian, 1990). Certain studies suggested requirement of ammonium for optimal use of nitrate by cultures. Culturing in medium with ammonium alone or along with the presence of citrate, malate and pyruvate, facilitates tobacco cells growth (Behrend and Mateles, 1975; Gamborg, 1970). Dougall and Verma (1978) demonstrated ammonium as a sole nitrogen source for suspension cultures of carrot. However, a combination of nitrate and ammonium as an ideal source of nitrogen in tissue culture was suggested by Kirby *et al*. (1987).

In the investigation, the effect of manipulation of macro and micro nutrients on the induction of microrhizomes and accumulation of gingerols and shogaol were studied. Even though the number of microrhizomes/ culture was found non

significant among the treatments, the highest number of microrhizomes/ culture of 16.33 microrhizomes were observed in the control MS medium. It was observed that twice the concentration of macro and micro nutrients produced more number of microrhizomes/ culture compared to the half concentrations. In contrary to our results, Zheng e*t al*., (2008) suggested that the optimal conditions for the production of ginger microrhizomes were sucrose concentration of $80gL^{-1}$, twice the normal concentrations of macro and micro elements with a photoperiod of 24hrs light.

In microprogated hazelnuts the shoot growth and development were observed affected due to minor nutrients (Hand *et al*., 2014). The positive correlation of number of shoots produced in *Typhonium flagelliforme* in MS full strength medium compared to half and quarter strength MS media was reported by Rezali *et al*. (2017).The increase in number of shoots with increase of micronutrients (3xMS) was reported in the *in vitro* propagation of *Stevia rebaudiana* Bertoni (Poothong *et al*., 2018).

The fresh weight of the microrhizomes recorded was the lowest for the treatments with half micro and macro nutrients. The highest fresh weight (0.606g), dry weight (0.056g) and driage (2.70 %) of the microrhizome was registered in MS control medium followed by MS medium with twice micronutrients. Manipulation of macro and micro nutrients may alter the nutrient components and this may also affect the plant organogenesis *in vitro*. In a study on the effect of fresh weight and dry weight of *Typhonium flagelliforme* cultured in full strength, half strength and quarter strength MS medium, the full strength MS medium was observed best (Rezali *et al*., 2017).

Among the different treatments, the highest recovery of oleoresin was observed in the microrhizomes induced in MS medium supplemented with twice micro nutrients (3.70%), with the highest total gingerols of 0.970 per cent and 6-gingerol of 0.877 per cent. The MS control recorded the highest 10-gingerol of 0.115 per cent, while 8- gingerol content was highest in MS control and MS media with manipulations in micronutrients. Doubling of calcium concentration in SH medium increased indirubine content by 140 per cent in the cell suspension culture of *Polygonum tinctorium* (Marero *et al*., 1997) which is in harmony with our study. But,

varying the minor salts of MS medium decreased solasodine production in hairy root cultures of *Solanum khasianum* while normal concentration of MS minor salts increased solasodine production (Jacob and Malpathak,2005).

Apart from the concentration, the relative proportions of nitrate/ammonium balance are important in tissue culture. Grimes and Hodges (1990) explained the importance of NO_3^+ to NH_4^- ratio for plant regeneration from embryo callus in rice and concluded that small alterations of NO_3^+ to NH_4^- ratio affects differentiation and growth. In many plants, an unsuitable balance in nitrate and ammonium ratio results in abnormal growth. Abnormal leaves were observed in *Adiantum capillus- veneris* (Pais and Casal, 1987) and abnormal thickened roots were observed in *Carica papaya* due to absence of ammonium in medium. Increased number of shoots was observed with reduced total nitrogen in *S. hybidus* (Gertsson, 1988).

In the study, the effect of different levels of ammoniacal and nitrate nitrogen was studied on the production of microrhizomes. The MS control medium produced highest number of microrhizomes/culture (16.33) with a fresh weight of 0.606 g microrhizome. A dry weight of microrhizome of 0.056g and a driage of 2.70 per cent were recorded in the same treatment in MS control. The treatment MS medium with half the concentration of ammoniacal nitrogen produced microrhizome with a fresh weight of 0.524 g with a dry weight of 0.054g and a driage of 2.67 per cent. The highest oleoresin recovery of 3.69 per cent with the highest total gingerols of 1.102 per cent was recorded in the MS medium with half the concentration of ammoniacal nitrogen.

The effect of NH_4NO_3, KNO_3, $CaCl_2$, $MgSO_4$ and KH_2PO_4at varying levels of 0.05, 1.0, 1.5 and 2.0 strengths respectively, was tested in shoot cultures of *Bacopa monneri* by Naik *et al*. (2011) who observed the MS medium 2.0 strength of NH_4NO_3 produced the optimum number of shoots /explants (99.33 shoots/explants) and the highest production of Bacoside A (17.9 mg g^{-1} DW) which was in contrary to our observations. Another report suggests that lower concentration of NH_4^+ and increased concentration of NO_3^- increased accumulation of gymnemic acid (Praveen *et al*., 2011) and withanolide A (Praveen and Murthy, 2013). The production of capsaicin in

*Capsicum frutescent s*and antraquinones in *Morinda citrifolia* improved by reduced levels of total nitrogen (Ravishankar *et al*.,1988; Zenk *et al*., 1975). However, two fold increase in pyrethrin accumulation in the second phase of culture was noticed in *Chrysanthemum cinerariifolium* by complete elimination of nitrate in cultures (Rajashekeran *et al*., 1991).In a recent study conducted by Archita (2019) on production of gingerol in the ginger variety Karthika inside poly house and open condition under different treatments, it was reported that the production of total gingerol recorded the highest in the treatment with soil test based nutrient management.

From our study, the MMS medium which was the combination of MS medium with twice the concentration of micronutrients and half the concentration of ammoniacal nitrogen was selected the best with respect to production of microrhizomes/culture (20.00). A fresh weight of 0.497 g with a dry weight of 0.061 g and a driage of 3.10 per cent were recorded in the microrhizomes induced in MMS medium. Highest oleoresin recovery of 3.45 per cent with the highest total gingerol, 6-gingerol and 10-gingerol of 1.305, 1.173 and 0.122 per cent respectively, were observed in the same medium.

Stress is produced in the *in vitro* cultures as a result of limiting factors of nutrition, light and carbon dioxide concentration. These stresses suppress plant growth. Stunted and abnormal growth of the plants is observed due to non-optimal mineral nutrients as a result of which higher amounts of reactive oxygen species or production of metabolites occurs in plants (Poothong *et al*., 2017; Reed *et al*., 2013). Environmental stresses of pathogen attack, light, wounding, UV-radiation, nutrient deficiencies, temperature, herbicide and pesticide application increases the accumulation of phenylpropanoids (Dixon and Paiva, 1995). When plants are stressed, the growth may be inhibited more than photoproduction and the secondary metabolite production may be increased because the carbon fixed is predominantly allocated to the production of secondary metabolites (Seigler, 1998). In *Daucus carota* callus, due to phosphate stress 7.2 per cent dry weight of anthocyanin is produced against 5.4 per cent dry weight in the control (Rajendran *et al*., 1992). In tomato, a three-fold increase in anthocyanidin content and accumulation of quercetin-

3-O-glucoside occurs under nutrient stress (Bartelsman and Phillips,1995).Zeid (2009) reported an increase in putrescine contents in *Phaseolus vulgaris* cell suspensions due to increased urea concentration in the nutrient solution. The anthocyanin production in *Vitis vinifera* cultures was increased due to osmotic stress created by sucrose and other osmotic agents (Tuteja and Mahajan, 2007).

In the study, the nutritional stress induced might have resulted in reduced production and growth of microrhizomes while it increased the production of gingerol. The enhanced production of total gingerols on manipulation of macro and micro nutrients observed in the study is a significant finding. The effect of altering the concentrations of macro and micro nutrients and different levels and ratio of ammoniacal and nitrate nitrogen on total gingerol production were confirmed from our results. Supplementation of MS medium with twice the concentration of micronutrients increased the total gingerols production by 13.05 per cent over control MS medium. Similarly, reducing the ammoniacal nitrogen by half the concentration enhanced the total gingerols production by 28.43 per cent over control MS medium. When the combination of twice micro nutrients and half ammoniacal nitrogen was supplemented in MS medium, the production of total gingerols increased by 53.53 per cent over the control MS medium. This indicates that by manipulating the nutrient composition of MS media, secondary metabolite production could be enhanced easily which is a simple strategy that can be adopted for scaling up of gingerol production than elicitation and precursor feeding which are costlier.

5.4. Effect of elicitation on *in vitro* gingerol production

Elicitation is the potential strategy for enhancing the metabolites of cultured plant cells with respect to the highest efficiency, lowest cost and ease in operation (Murthy *et al*., 2014; Xu *et al*., 2016). Elicitation includes biological, chemical and physical. The biological elicitation in plant tissue culture is achieved on the inoculation of microorganisms that stimulate the biosynthetic pathway as a result of microbial attack, improving the secondary metabolite produces and in some cases enhancing the biomass production (Verpoote *et al*., 1991; Al-Amier *et al*., 1999; Mewis *et al*., 2011; Baenas *et al*., 2014). The biotic elicitors are substances generated

from living organisms either from plants or which activates or inactivates the enzymes or ion channels of biosynthetic pathways as pathogens (Patel and Krishnamurthy, 2013). Biotic elicitors include microorganisms, fungi, bacteria, plant cell wall fragments and chemicals released at the attack site in plants upon pathogen attack (Namdeo, 2007a), carbohydrates like chitin, chitosan etc. Chitin, the fungal cell wall component is a potent elicitor (Shibuya and Minami, 2001).

Elicitors induced stress stimulates any type of plant defense, promoting secondary metabolism to protect the cell and the whole plant (Klarzynski and Friting, 2001). The biotic elicitors recognize specific receptors bound to cell membrane, these signals will be transferred to signal transduction system which ultimately leads to phytoalexins (Baenas *et al*. 2014). The plant innate immunity is triggered by microbe-associated molecular patterns or exogenous elicitors. This triggers the release of plant exogenous molecules. Parallel cross linking pathways were also activated and many compounds are released. When exogenously applied, MJ regulates defense genes (Farmer *et al.*, 1992) and stimulate secondary biosynthetic pathways (Uppalapati *et al*., 2005; Rischer *et al*., 2006; Wasternack and Hause, 2013). Salicylic acid induces systemic acquired resistance (Hayat *et al*., 2010) which further leads to hypersensitive responses (Wasternack and Hause, 2013) including production of secondary metabolites.

Chemical elicitation is achieved by signaling molecule (abiotic elicitors), plant growth regulators and addition of precursors. Elicitation usually increases the secondary metabolite production *in vitro* by triggering the morphological and physiological responses. This stimulation is a response to stress stimulus of signal compounds that activate the protective systems in the plants (Rea *et al*., 2011).

Commonly used abiotic elicitors include SA and MJ. In plants, the defense gene expressions are controlled by the key signal molecule SA which is involved in the systemic acquired resistance against pathogen (Ryals *et al*., 1996) and elicits the production of secondary metabolites (Hayat *et al*., 2010) while jasmonic acid provides resistance to plants against insects by producing various proteins (Farmer *et al*., 1992). Methyl Jasmonate is a cyclopentane compound that stimulates the biosynthesis

of secondary metabolites (Tamogami *et al.,* 1997). Salicylic acid and methyl jasmonate can be used as synthetic mimics when added exogenously to plants induce the resistance as induced by pathogens and insects (Walling, 2000).

The effect of abiotic elicitors on growth and production of microrhizomes and calli and production of gingerols and shogaol were studied. The highest number of microrhizomes /culture (15.00) and fresh weight (0.536 g) were recorded in MS control medium compared to the elicitated medium. The negative impact of methyl jasmonate on fresh weight and dry weight of leaf, root and plant biomass was reported by Cousins and Adelberg (2008) on turmeric microrhizomes which agree with our observation of decrease of fresh weight in elicitated treatments compared to control.

Both liquid and semisolid MMS medium elicitated with elicitors were used for the study on the production of gingerols and shogaol. Salicylic acid 5 mg L^{-1} was selected as the best abiotic elicitor both in solid and liquid MMS medium. An increase in the concentration of elicitors decreased the accumulation of gingerols. The MMS medium supplemented with SA 5 mg L^{-1}recorded oleoresin recovery of 2.86 and3.64 per cent respectively in the liquid and solid medium. Highest total gingerols of 1.035 and 1.702 per cent were recorded respectively in the liquid and solid medium MMS medium supplemented with SA 5 mg L^{-1}. Similar observation on highest and stable production of anthocyanin in the solid medium than liquid medium was reported by Narayan *et al*. (2005) in cell lines of *Daucus carota*. Many researchers reported the enhancement of secondary metabolites on elicitation with SA. Similar to our observations, a higher percentage of alkanes and fatty acids were observed in SA elicitated cell cultures of *Jatropha curcas* by Mahalakshmi *et al*. (2013). Salicylic acid elicitation improved ginsenoside production in *Panax ginseng* adventitious roots (Tewari *et al*., 2011) and hairy roots (Jeong *et al*., 2005).In a study, two fold increases in xanthonescandensin G and paxanthone production in hairy root cultures and cell suspension of *Hypericum* sp. were observed by Zubricka *et al*. (2015). Increased production of colchicine by eight fold was observed in SA treated suspension cultures of *Gloriosa superba* (Mahendran *et al*., 2018).

In the present study, calli induced in CI medium supplemented with the abiotic elicitor SA 5 mg L^{-1}recorded the highest fresh weight (1.315 g) while the dry weight (0.451 g) and driage (21.89 %) recorded highest in MCI medium supplemented with SA 5 mg L^{-1}. The highest oleoresin yield of 3.06 per cent and the highest total gingerols of 0.128 per cent and 6-gingerols of 0.078 per cent were recorded in the treatment CI medium supplemented with SA 5 mg L^{-1}.The use of SA to enhance the secondary metabolite accumulation in callus cultures and suspension cultures of many plants have been reported such as *Withania somnifera* (Sivanandhan *et al*., 2013), *Rosa hybrida* (Ram *et al.*, 2013), *Gymnema silvestre* (Chodisetti *et al*., 2015)and *Calendula officinalis* L (Ibrahim *et al*., 2019). Application of SA 20 mgL^{-1} enhanced the paclitaxel production in *Taxus chinensis* cell suspension (Wang *et al*., 2007). Successful enhancement of anticancer compound in *Corylus avellana* cell cultures by SA elicitation was reported by Rezaei *et al*. (2011). In another study, the elicitation of callus cultures of *Capsicum annum* with SA 20 mg L^{-1}increased the beta carotene content (Pooja, 2018). Archita (2019) reported enhanced production of gingerols in ginger variety Karthika on treatment with SA (100 µM spray).

The biotic elicitor chitosan induces stress which elicits the hydrogen peroxide production and increases transcription/translation of plant defense genes and phytoalexins (Loschke *et al*., 1983; Hadwiger, 1999) and stimulates the enhanced activity of *PAL* gene. Chitosan also elicits the liberation of linolenic acid from membranes, which are transformed into signaling molecules like jasmonic acid (Hadwiger, 1999). Jasmonic acid and methyl jasmonates are the signal stress molecules in plants produced as a response to biotic and abiotic stress, which in turn enhances the secondary metabolite production as a defense mechanism.

In the study, among the biotic elicitors studied, chitosan 100 mg $L^{-1}$was selected as the best elicitor recording highest number of microrhizomes/ culture (15.68) with a fresh weight of microrhizome of 0.511 g. A dry weight of 0.057 g with a driage of 2.78 per cent was recorded in the microrhizome induced in MMS medium supplemented with chitosan 100 mg L^{-1}.However, the number of microrhizomes/ culture and fresh weight were on par with the MS control medium recording 15.00 microrhizomes and 0.536 g fresh weight respectively. Abraham *et al*. (2011) reported

that the addition of biotic elicitors like yeast extract did not influence the fresh and dry biomass of *in vitro* shoot cultures of *Curcuma amada*, which is in agreement with our observation.

In the same medium of MMS supplemented with chitosan 100 mg L^{-1} recorded the highest recovery of oleoresin (3.80 %) and total gingerols (1.463 %). Production of 6-gingerol (1.228 %) and 10-gingerol (0.148 %) also recorded the highest in the MMS medium supplemented with chitosan 100 mg L^{-1}. Increased production of coumarins, rutacultin and other secondary metabolites in *in vitro* culture of *Ruta graveolens* shoots was observed in the presence of chitosan (Orlita *et al*., 2008). In another study, chitosan 0.2 mg ml^{-1} enhanced the production of anthraquinones, phenols and flavonoids in the adventitious root suspension culture of *Morinda citrifolia*. Vanda *et al*. (2019) reported the highest production of phenolics in shoot cultures of *Melissa officinalis* induced in ½ MS medium supplemented with chitosan 100 mg L^{-1} which totally agrees with our results.

Therefore, our result suggests the biotic elicitor enhanced the production of gingerols in an optimum amount. The biotic elicitors like yeast extract (Sanchz-Sampedro *et al*., 2005) and chitosan (Doares *et al*., 1995; Rakwal *et al*., 2002) were reported not to directly influence the biosynthetic pathway of secondary metabolites in plants. However, it triggers the production of jasmonic acid and/or methyl jasmonate endogenously, which enhances the production of secondary metabolites.

The calli induced in CI medium supplemented with the biotic elicitor chitosan100 mgL^{-1} recorded the highest fresh weight of 1.205 g as compared to the MCI medium. This is in agreement with the findings of Drewes and Staden (1995) who reported lower nitrogen reduced callus growth in callus cultures of *Solanum mauritianum* Scop. The highest total gingerols were recorded in the CI medium supplemented with chitosan 100 mg L^{-1} (0.124 %) as compared to the MCI medium supplemented with chitosan. Drewes and Staden (1995) reported similar findings of reduced solasodine production in callus cultures of *S. mauritianum* Scop. with altered ammonium and potassium nitrate.

Different events of calcium flux, ROS burst and MAPK phosphorylation are the initial steps triggered by the elicitor plant cell interactions (Seybold *et al*., 2014). Consequently, events like activation of signaling pathways and activation of transcription factors enhance the plant secondary metabolism (Maeda *et al*., 2005; Naoumkina *et al*., 2008; Schluttenhofer *et al*., 2015).

5.5 Effect of precursor feeding on *in vitro* gingerol production

Precursor feeding is an important and popular approach for enhancing secondary metabolite production in plant cell cultures with the basis of knowledge on biosynthetic pathways (Namdeo *et al*., 2007b). The fact that any compound which is the intermediate or beginning of the biosynthetic pathway of secondary metabolites stands a good chance for enhancing the yield of the final compound (Rao and Ravishankar, 2002). Earlier attempts of precursor feeding showed favorable effects on secondary metabolite production. Production of rosmarinic acid was stimulated by the addition of phenylalanine to *Salvia officinalis* cell suspension cultures (Ellis and Towers, 1970).

Phenylalanine and tyrosine produce two major phenolic acids, one with three carbon side chains including caffeic acid, ferulic acid, coumaric acid and sinapic acids. The other is with six carbon chain compounds like vanilic acid, gallic acid (Pereira *et al*., 2009).

Among the three precursors studied ferulic acid was selected as the best precursor with a maximum production of gingerols as compared with coumaric acid and phenylalanine. The findings of Dennif and Whiting (1976) support our results. Labelled precursors of gingerol biosynthetic pathway such as phenylalanine, ferulic acid and coumaric acid were fed to shoots during the period of rhizome extension. The 6- gingerol was isolated, purified and degraded to establish labeled atom distribution. Through the degradation study, they reported coumaric acid and ferulic acid are better precursor than phenylalanine, with respect to gingerol production.

Number of microrhizomes/culture recorded highest in MS control medium (14.33) compared to the elicited medium. The highest fresh weight of 0.526 g was

recorded by the microrhizomes induced in the control with the highest dry weight of 0.061 g and a driage of 2.98 per cent. Application of precursors reduced the fresh weight, dry weight and driage. Increasing the levels of precursors decreased the fresh weight, dry weight and driage. Similar results of the negative effect of precursors such as mevalonic acid, phenylalanine, leucine and valine on callus growth fresh weight and dry weight were reported by El-Nabarway *et al*. (2015) in *Zingiber officinale* callus cultures. They also reported increasing the precursor levels of phenylalanine, leucine and valine from 50 mg L^{-1} to 150 mg L^{-1} reduced callus growth. Contrary to our study, Klerk *et al*. (2011) observed increased production of the adventitious root in apple variety Jork 9 on the addition of 300 µM ferulic acid to rooting media during the initial five days. But they also observed a reduction of adventitious roots after five days of treatment with ferulic acid.

Oleoresin recovery and gingerol production recorded the highest in the MS medium supplemented with FA 30mgL^{-1} recording 3.83 and 1.453 per cent respectively. The increased level of precursor decreased gingerol production. Application of 1 mM ferulic acid increased vanillin production in callus cultures of *Vanila planifolia* by 1.7 fold, which agrees with our observation (Romagnoli and Knorr (1988). Similar to our results they also observed decreased vanillin production with an increase of ferulic acid level to 10 mM.

The calli induced in CI medium supplemented with FA 30 mg L^{-1} recorded the highest fresh weight as compared to MCI medium with a dry weight of 0.431 per cent. The driage recorded highest in MCI medium supplemented with FA 30 mg L^{-1}. Production of total gingerols recorded the highest in CI medium supplemented with FA 30 mg L^{-1}.

In the biosynthetic pathway of gingerols (Phenylpropanoid pathway) ferrulic acid is nearer to our target compound gingerol. Phenylalanine is the entry point in the pathway. So addition of phenylalanine may results in enhanced production of any of the compounds in the pathway. Even in our study, the oleoresin extract from the microrhizomes induced in MMS medium supplemented with phenylalanine at different levels were observed green in colour (normally light brown colour) (Plate

26). This may be attributed to the role of aminoacid phenyl alanine in the biosynthesis of green pigments. Hence addition of phenylalanine might have contributed to the production of chlorophyll. Similar findings were reported by El- Shennaway *et al*. (2017) on their study on effects of NaCl and phenylalanine on production of secondary metabolites in *in vitro* cultures of *Mentha longifolia*. They observed enhanced production of chlorophyll (a, b and total) in the used explants on addition of phenylalanine.

5.5 Effect of gamma irradiation on *in vitro* gingerol synthesis

Ionizing radiations induce free radical formation. Hence in plants, to confront the induced oxidative stress, antioxidant enzymes and secondary metabolites are produced. So irradiation can be a strategy to increase the production of secondary metabolites in plants. However, a higher dose damages the cells, which depend on the nature of tissues. The optimum dose of irradiation increases the secondary metabolite production in plants by stimulating stress response. The type of stress along with its magnitude affects the production of secondary metabolites (Eilert, 1987)

A lower dose of gamma irradiation accelerates cell growth, enzyme activity, cell proliferation, germinate rate, stress resistance and crop yields (Davies, 1970; Sparrow *et al*., 1971). Relative low doses of gamma irradiation have almost similar effects on cell cultures *in vitro* and also enhance secondary metabolites. Gamma irradiation enhances ROS production in cells resulting in oxidative stress (Azzam *et al*., 2012; Kebeish *et al*., 2015) which damages the cellular components (Nocto r*et al*., 2002). As a consequence, the plants produce adaptive responses like the production of oxidative stress protectors and the accumulation of protective solutes (Horling *et al*., 2003). Plants also produce antioxidant enzymes that produce secondary metabolites which in turn reduces the effect of induced oxidative stress (Kim *et al*., 2004; El-Beltagi, 2011).

Microrhizomes were irradiated with 20 Gy and 30 Gy dose of gamma rays and their effects were studied. The increase of gamma irradiation dose from 20 Gy to 30 Gy decreased the survival of microrhizomes from 86.66 to 73.33 per cent. Shoot morphogenesis also decreased from 3.4 to 2.0 per cent. Fulzele *et al*. (2015) on their

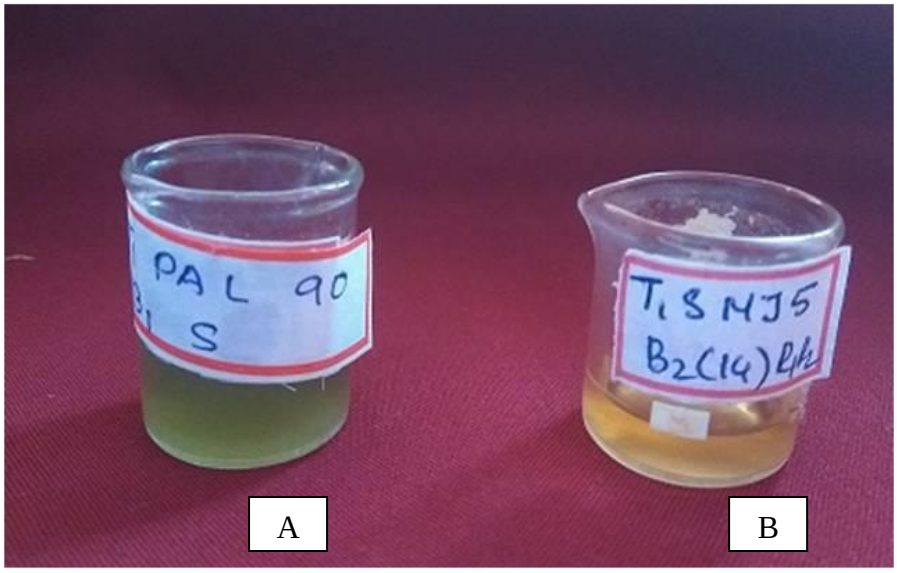


Plate 26:- (A) Green colour oleoresin extract from microrhizomes treated with precursor phenylalanine

(B) Light brown colour oleoresin extract from microrhizomes treated with abiotic elicitor methyl jasmonate

study on callus cultures of *Nothapodytes foetida* reported the dose of 20 Gy gamma irradiation was selected as satisfactory for callus attenuation as irradiated cells above 25 Gy inhibited growth and productivity.

Higher oleoresin recovery and production of total gingerols recorded the highest in the 20 Gy dose of gamma irradiation as compared to 30 Gy. The irradiated microrhizome with shoot morphogenesis recorded the highest total gingerols (1.110 %). A similar observation of highest plumbagin production at irradiation dose of 20 Gy was reported in *Plumbago indica* root cultures (Jaisi, 2013). Fulzele *et al*. (2015) reported low doses of gamma irradiation improved camptothecin production in callus cultures of *Nothapodytes foetida* which is in support of our results. They also reported the gamma irradiation boosted the production of camptothecin by 20 fold at 20 Gy dosage. Another study by Azeez *et al*. (2017) reported the 10 Gy and 20 Gy dose of gamma irradiation stimulate epicatechin accumulation in calli from leaf and stem of *Hypericum triquetrifolium* Turra.

In calli, no significant difference was noted with respect to fresh weight in irradiated microrhizomes at 20 Gy and30 Gy dose of gamma rays. Dry weight recorded the highest in MCI medium irradiated with 20 Gy dose. The highest recovery of oleoresin and total gingerols recorded the highest in the calli irradiated with 20 Gy dose of gamma irradiation. Irradiation dose of 20 Gy was observed optimum for callus cultures of ginger (Resmi, 2006) and in black pepper (Shylaja, 1996). Enhanced secondary metabolite production was noticed in rosemary callus as an effect of gamma irradiation (El-Betagi *et al*., 2011). Gamma irradiation with different doses of 0, 5, 10, 15 and 20 Gy were tried, wherein it was reported that irradiation dose of 20 Gy increased the total phenolics (4.38 mg g^{-1}) and total flavonoids (3.35 mg g^{-1}).

5.6 Gene expression studies for *PAL* and *CHS* genes involved in gingerol biosynthetic pathway

Plant secondary metabolites are detected in cells of the whole plant but, site of biosynthesis, in most of the cases, is restricted to a particular organ/organs and finally the assimilates will be transported to the site of storage mainly to the

economically harvestable portion through vascular tissues or symplastic and apoplastic transport, depending on polarity of the metabolite (Acamovic and Brooker, 2005; Rai *etal*., 2017).

In the study the accumulation of gingerols increased with increase of induction periods, number of leaves and maturation of microrhizomes. Sreeja (2017) reported the differential expression of genes in leaf samples of the somaclone B3. She also reported that leaves are the synthesizing areas for production of the secondary metabolites like volatile oil and oleoresin. The metabolite synthesis occurs during the active growth phase of ginger which spans up to four months after planting and then the synthesized metabolites are accumulated in rhizomes when tuberization starts that is five months after planting and further distributed in the growing rhizomes. Hence in the study RNA was isolated from the leaves of *in vitro* cultures with highest gingerol production.

5.6.1 Isolation of total RNA

The total RNA was isolated from the leaves of the three months *old in vitro* microrhizomes from the treatment yielded highest total gingerols in each set of experiments using TRIzol reagent as per the procedure given by Sreeja (2017). The leaf samples collected were crushed homogenously using liquid nitrogen for cell breakage and better nucleic acid recovery. TRIzol reagent is a monophasic solution consisting of phenol and guanidium isothiocynate that simultaneously denatures proteins and solubilizes biological materials. The guanidium salts present in the TRIzol reagent facilitates removal of the effects of the nucleases while the phenol aids the phase separation. The homogenate was then incubated at room temperature by laying down the tube horizontally in order to enhance the area of contact for adequate nucleoprotein complex dissociation. Addition of chloroform causes phase separation in the presence of phenol where proteins impurities like chlorophyll and other pigments denature and separates to the organic phase, DNA resolves in the interphase and RNA remains in the aqueous phase. The phenol-chloroform combination reduces the partitioning of poly (A) + mRNA into the organic phase and thus reduces the formation of RNA-protein complexes at the interphase (Perry *et al*., 1972).

Chloroform extraction was repeated to completely remove the impurities and to enhance phase separation. The RNA is precipitated by the addition of sodium acetate and ice cold isopropanol. The positively charged sodium ions of sodium acetate neutralize the negative charge of the sugar phosphate backbone of RNA which makes the molecule less hydrophilic and hence less soluble in water. The lower concentration of isopropanol precipitates the large nucleic acid. The sample was incubated in the freezer and finally, the pellet precipitated was washed with 75 per cent DEPC ethanol. The white pellet obtained was dried and was dissolved in the required amount of nuclease free water. Modified protocol for high quality total RNA isolation from rhizomes of ginger was reported by Sreeja *et al*., (2018).

Gel electrophoresis analysis was done and three distinct RNA bands corresponding to 28SrRNA, 18SrRNA and 5SrRNA + tRNA were obtained indicating high quality RNA from all the leaf samples. On spectrophotometric analysis, the absorbance ratio A_{260}/A_{280}recorded was greater than 1.8 and absorbance ratio A_{260}/A_{230} was more than 1.0 for all the leaf samples indicating the total RNA isolated were free from contamination of carbohydrate, protein and polyphenols. The absorbance ratio of $A_{260}/A_{280}$of 1.8 indicates the contamination of proteins and value more than 2.0 indicates the contamination of polysaccharides and polyphenols (Sambrook and Russel, 2001).

5.6.2 First strand cDNA synthesis

The isolated total RNA was used to synthesize cDNA using First strand cDNA production kit. Reverse Aid Reverse Transcriptase with lower RNase H has a consistent activity at a temperature of 42 to 50 ^{o}C which is suitable for cDNA production up to 13 kb. The degradation of RNA is prevented by the addition of Ribolock RNase inhibitor at 55 ^{o}C. The poly A tail of mRNA was annealed using the oligo$(dT)_{18}$ primer. The cDNA is synthesized using RNA as template and cDNA synthesized was directly employed as template for PCR and Real Time PCR.

5.6.3 Confirmation of production of First strand cDNA using primers

Primers were designed for the three genes *Actin* (reference gene), *PAL* and *CHS* (target genes). The PCR reaction was carried out using the synthesized cDNA and designed primers. The desired band size of 167, 141 and 138 bp was observed for *PAL*, *CHS* and *Actin* gene specific primers on gel electrophoresis using 1.8 per cent agarose gel.

5.6.4 Real Time PCR

In Real Time PCR, a reference gene was used as the internal control in Real Time PCR which different from the target. The reference gene is usually the housekeeping genes. A consistent reference gene should not be affected by experimental factors and should show minimal variability in its expression between the tissue and physiological states of the organism (Chervoheva *et al*., 2010). A suitable reference gene should have similar application efficiency, must be stably expressed between different experimental groups and should be abundant in gene of interests (Guitierrez *et al*., 2008). Commonly used reference genes or the internal controls in Real Time PCR includes the housekeeping genes like *Actin, ubiquitin-conjugating enzyme, glyceraldehyde-3-phosphate dehydrogenase (GADPH), β-tubulin (β-TUB) etc.*

For our study using Real Time PCR, *Actin* gene of *Zingiber officinale* was selected as the internal control. The *Actin* gene is responsible for plant cell cytoplasmic streaming, cell shape determination, cell division and organelle movement (Staiger and Schliwa, 1987). The expression of two target genes *PAL* and *CHS* were studied.

The gene expression can be well quantified by Real Time PCR. In Real Time PCR, the amount of DNA complied is measured at the end of each cycle by the use of fluorescent markers that are incorporated in the PCR product. The increase of the fluorescent signal is directly proportional to amplicons generated in the exponential phase of the reaction. The change in fluorescence over course of the reaction is measured by an instrument that combines the thermal cycling with scanning capability and expressing the accumulation of product over duration of entire PCR

reaction by plotting fluorescence against the cycle number. The read out is given as C_T value. The C_T value is fixed in the exponential phase of the PCR. The fluorescent dye used in Real Time PCR such as SYBR Green binds to the double stranded DNA and is observed by 1000-fold increase in fluorescence intensity (Hugget *et al*., 2005). In the present study, SYBR Green dye from Applied Biosystems was used.

The C_T or threshold cycle is the cycle at which the fluorescence emitted by the target is above the threshold and the baseline and is within the exponential region of the amplification curve. The lower amount of target DNA lower will be the increase in fluorescence and higher will be the C_T value and vice versa.

5.6.5 Relative gene expression analysis

Two methods for the analysis of the amount of gene transcript are absolute and relative quantification.

In the absolute quantification, a standard curve for each gene of interest is constructed and the quantification cycle (Cq) values are plotted against log (quantity) of a dilution series of the known gene of interest amount. The standard curve generated once is used repeatedly to quantify samples over a period of time. Ferre (1992) explained the complexity and accuracy of absolute quantification.

The relative quantification is simple and cheap to perform and uses intercalating dyes. It uses one or more reference genes against which the genes of interest are normalized (Hugget *et al*., 2005). The choice of the reference genes with an optimal number is necessary and there are several computational methods for this (Vandesompele *et al*., 2002; Ptaffi *et al*., 2002). In the relative method of quantification one sample is used as the calibrator that is the sample against to which the change is given (Tyburski *et al*., 2008). The relative quantification was done in the present study using $2^{-\Delta\Delta Ct}$ method by Livak and Schmittigen (2001). For each sample difference between ΔC_T of target gene and control gene is first calculated, then subtracted between ΔC_T of the sample with unknown concentration and ΔC_T of the calibrator.

The endogenous control in the study was *Actin*, the C_T values gave nearly the same values in the control and all the treatments indicating the cDNA samples from all the treatments were normalized. In the study relative quantification was used to study the gene expression pattern under various treatments.

5.6.6 Relative gene analysis of *PAL* and *CHS* gene

Phenylalanine ammonia lyase is the key enzyme at the entry point of phenylpropanoid pathway. In plants, the *PAL* links the metabolism of aromatic acids to that of secondary metabolic products (Koukal and Conn, 1961). Biosynthesis of phenylpropanoid compounds are controlled and regulated by *PAL*. The phenylalanine is deaminated to transcinnamic acid and ammonia in the presence of PAL enzyme and transcinnamic acid, in turn, is the precursor of lignin and flavonoid biosynthesis pathway (Ritter and Schulz, 2004). Enhanced activity of *PAL* enhances phenylpropanoid products (Ozeki and Komamine, 1985) and the activities of *PAL* vary with different growth stages of cell differentiation and exposure to stress (Jones, 1984; Shufflebottom *et al*., 1993). The enzyme PAL is stimulated by wounding, drought stress, temperature change and UV radiation and fungal elicitation (Edward *et al*., 1985; Camphell and Ellis, 1992). In a study by Singh *et al*. (2016), the micro RNA miR5015 was reported to regulate gingerol biosynthesis by inhibiting the precursor enzyme phenylalanine ammonia lyase.

The upregulation of *PAL* genes was observed in all the treatments studied compared to the control. The highest expression of *PAL* gene was observed in the MMS medium supplemented with SA 5mgL^{-1} (1.342 fold) with higher gingerol production of 1.702 per cent. Similar observation of enhanced expression of *PAL* gene by treatment with SA 150 µmol/L after 0.5 hours of incubation in grape berries was reported by Wen *et al*. (2005). They also reported the increase of *PAL* mRNA transcripts. In another study, the addition of 50 and 300 µM SA increased the expression of *PAL* and *STS* (S*tilbene synthases*) gene expression in *Vitis amurensis* cell culture which encodes for the production of the phytoalexin reversertrol (Kiselev *et al*., 2010). The activity of *PAL* gene increased from 20 min after the treatment of 22.5 mg/L SA and the activity reached 5.46 folds that of control by 16 hours in *Salvia*

miltiorrhiza cell cultures which is inconsistent with our results (Ejtahed *et al*.,2015). Increased *PAL* activity increased the production of rosmarinic acid by 2.15 folds (Hao *et al*., 2014). The SA induced stress may be attributed for increasing the gene expression level of *PAL*.

Higher expression level of 1.187 fold of *PAL* gene was noted in MMS medium supplemented with chitosan 100 mg L^{-1} as compared to the control with an increased gingerol production of 1.463 per cent. In a study, two fold increases of PAL activity and enhanced production of lignin was noticed in rice callus by chitosan treatment (Notsu *et al*., 1994). Similar findings of increased expression of PAL and TAL enzymes along with enhanced production of total phenolics in soybean leaf tissues on the application of chitin and chitosan were reported by Khan *et al*. (2003). In another study by Vanda *et al*. (2019), chitosan treatment resulted in increased PAL activity and increased production of rosmarinic acid and other phenolics in *Melissa officinalis.*

Increased PAL activity by 1.242 fold was observed in MMS medium supplemented with FA 30 mg L^{-1} with an increased gingerol production of 1.453 per cent. Application of 0.1 mM solutions of ferulic acid and coumaric acid in seven day old seedlings of *Cucumis sativus* L.stimulated the PAL activity and beta –glucosidase activity (Politycka, 1999) which is in harmony with our observations. They also reported that the cucumber root growth was slightly inhibited. In another study, treatment of L-phenylalanine and ferulic acid increased the PAL activity in pea leaves. Application of FA 50 ppm initially reduced PAL activity while higher levels of 100 and 150ppm FA increased the PAL activity as compared to control and increased the production of the end product cinnamic acid (Bahadur *et al*., 2012).

Chalcone synthase is the first committed enzyme in flavonoid synthesis. It channels the flow of phenylpropanoid pathway towards flavonoid pathway. The enzyme has essential role in many physiological processes such as fruit/seed development, floral pigmentation, pollination resistance to abiotic and biotic stress in plants (Dao *et al*., 2011). The CHS enzyme belongs to type III polyketide synthase (PKS) and is structurally and mechanistically the simplest PKS (Schroder, 1997). The

enzyme has two independent sites that catalyze a series ofcyclization, condensation and decarboxylation reactions (Tropf *et al*., 1994). The CHS catalyze the condensation of one molecule of p-coumaroyl-CoA and three malonyl-CoA for the production of chalcone further through clasein condensation to form other intermediates.

The *CHS* activity is enhanced in plants under different forms of biotic and abiotic stresses like UV rays, wounding, microbial pathogens, light, temperature etc. Elicitors such as jasmonic acid and MJ activate *CHS* gene in soybean (Creelman *et al*., 1992) and result in the production of phyoalexins and other secondary metabolites which takes part in the defense mechanism.

In a study conducted by Ghosh and Mandi (2015) on *Chalcone synthase* gene expression in landraces of ginger, it was revealed that the landraces with specific amino acids change from aspargine to serine caused low 6-gingerol content as asparagine is one of the critical amino acids of the catalytic triad of *Chalcone synthase* gene.

Increased expression of *CHS* gene (2.005 fold) was observed in the MMS medium supplemented by SA 5 mg L^{-1} as compared to the control. Similar observation of enhanced *CHS* gene expression on application of 20 µM SA to *Saussurea medusa* cell cultures was reported by Yu *et al*., (2006). In another study on the effect of foliar application of SA in two varieties of malayasian ginger, Ghasemzadeh *et al*. (2012) reported the enhanced activity of *CHS* gene. They also reported the increased production of anthocyanin and fistein in both the varieties. The enhanced expression of *CHS* gene along with increased production of gingerols was reported in the ginger variety Karthika on foliar application of SA 100µM (Archita, 2019).

Higher expression of 1.468 fold increase of *CHS* gene was recorded in MMS medium supplemented with chitosan 100 mg L^{-1} as compared to the control with higher gingerol production of 1.463 per cent. Our result is in harmony with the report of enhanced *CHS* and flavonoid 3′-hydroxylase activity and increased production of total flavonoids by 7.08 fold in hairy root cultures of *Isatis tinctoria* L.(Jiao *et al*.,

2018). In another study, Gai *et al*., (2019) reported the enhanced transcript levels of *CHS* genes along with other genes on elicitation with chitosan (100 and 150mgL$^{-1)}$. They reported chitosan elicitation promoted the production of formononetin in *Astragalus membranaceus* hairy root cultures.

5.7 Analysis of available ESTs using bioinformatics tools

ESTs provide remarkable information about the gene expression pattern of an organism at different stages. ESTs have been used for gene discovery (Lee*et al*., 2005). The ESTs deposited in dbEST provides an opportunity to explore the prokaryotic and eukaryotic diversity using only a few fully sequenced genomes. ESTs provide the snapshot of gene expression and can be used to study the expression pattern of genes at different stages or different environmental conditions (Parkison, 2009)

Through suppression subtractive hybridization, differential genes from the rhizomes and leaves of somaclone B3 were isolated by Sreeja (2017). Eleven rhizome ESTs and four leaf ESTs obtained through the study were analyzed using different bioinformatic tools.

5.7.1 Analysis of Rhizome ESTs

Rhizome EST 1

The Blastn results showed 44 per cent of the rhizome EST1 having 80 per cent similarity with *Zingiber officinale* cDNA clone and 22 per cent of the EST sequence having homology with *Garcina mangostena* cDNA clone. The Blastx homology revealed similarity with putative RNA – directed DNA polymerase of *Medicago trunculata* and hypothetical protein of *Corchorus capsularis*.

Rhizome EST 2

The Blastn homology revealed the EST sequence having similarity with *Musa accuminata* cDNA clone. The Blastx homology revealed 60 per cent of the EST sequence having 92 per cent similarity with *Zingiber officinale* cDNA. The longest ORF of 216 bp was obtained on open reading frame analysis.

Rhizome EST 3

The Blastn results showed 13-14 percent of the EST sequence having 100 per cent homology with *Gossypium hirsutum* cDNA. No results were observed on Blastx analysis. The open reading frame analysis revealed the longest ORF length of 69 bp.

Rhizome EST 4

The Rhizome EST 4 on Blastn analysis revealed 20-21 per cent of the EST having 82 per cent similarity with *Boechera divaricarpa* GSS clone**.**

Rhizome EST 5

The Blastn homology revealed fifteen per cent of the EST having 86 per cent similarity with *Boechera divaricarpa* GSS clone and 32 per cent of EST having 69 per cent similarity with *Iris fulva.*

Rhizome EST 6

The Blastn analysis showed 14-15 per cent of the EST sequence having similarity with different *Boechera divaricarpa* clones. No result was obtained in blastx analysis. Longest ORF of 177 bp located at 406-230 bp region was obtained after open reading frame analysis.

Rhizome EST 7

NoBlastx or Blastn results were obtained for rhizome EST 7 indicating it may be a novel EST sequence.

Rhizome EST 8

On Blastn analysis 25 per cent of the EST sequence was showing 80 per cent similarity with *Elettaria cardamomum* clone. Fifty seven per cent of the EST sequence showed 66 per cent similarity with *Vitis vitifera*

Rhizome EST 9

The Blastn homology showed that 64 percent of the EST sequence had70 per cent similarity with *Iris fulva* clone and 69 per cent similarity with *Crocus sativus*. No Blastx result was obtained.

Rhizome EST 10

The Blastn homology showed 32 per cent of the SET sequence having 83-84 per cent similarity with *Musa accuminata* AAB group initiation factors.

Rhizome EST 11

The Blastn analysis of rhizome EST 11 showed 82 per cent similarity of 63 per cent of the sequence with *Curcuma longa* cDNA clone. Sixty two per cent of the EST sequence showed similarity with *Zingiber officinale* cDNA clone. The Blastx analysis showed 100 per cent similarity of 63 per cent of the EST sequence with cysteine protease of *Zingiber officinale.*

KEGG orthology search showed the involvement of the enzyme in linolenic pathway.

Cysteine protease is a proteolytic enzyme with multiple functions of extracellular matrix turnover, antigen presentation, digestion, processing of surface proteins etc. Cysteine protease contains a Cys-His-Asn triad at the active site. The histdine residue act as proton donor. It attacks the carbon of reactive peptide bond producing first tetrahedral thioester intermediate with the release of amine group of the substrate (Coulombe *et al*., 1996).

5.7.2 Blast2Go analysis of rhizome ESTs

The Blast2Go analysis of assembled ESTs enabled the identification of GO term on the three categories such as molecular function, biological processes and cellular location. Out of the eleven rhizome ESTs analyzed, four EST sequences were involved in the metabolic process and two EST sequences involved in the cellular process.

5.7.3 Analysis of Leaf ESTs

Leaf EST 1

The Blastn analysis revealed that 69 per cent of the EST sequence having 99 per cent homology with *Curcuma longa* cDNA clone. The Blastx analysis showed 76 per cent of homology of the 57 per cent of the EST sequence with Predicted *Musa accuminata* subsp. *malanccensis* ACT domain containing protein ACR6.

The ACT domain is an aminoacid binding domain is a regulatory domain involved in aminoacid and purine metabolism (Aravind and Koonin, 1999). The ACR protein is located in chloroplast and its main molecular function is aminoacid binding and regulates the amino acid metabolic enzymes in plants (Hsieh, 2002)

Leaf EST 2

The Blastn analysis showed 99 per cent similarity of 69 per cent of EST sequences with *Curcuma longa* cDNA clone. The Blastx homology showed 72 per cent similarity of 56 per cent of EST sequences with predicted *Musa accuminata* subsp. *malancensis* ACT domain containing protein ACR4.

The molecular function of the ACR4 protein includes ATP binding, protein homodimerization activity, protein serine/threonine kinase activity and transmembrane receptor protein kinase activity (Tanaka *et al*., 2002). The biological processes include embryo developments, flower morphogenesis, cell differentiation, cell division and root development (Tanaka *et al*., 2002, Yue *et al*., 2016)

Leaf EST 3

The Blastn analysis of Leaf EST 3 showed 75 per cent similarity of 43 per cent of EST sequence with *Boechera divaricarpa.*

Leaf EST 4

The Blastn analysis of leaf EST 3 showed 45 per cent of EST sequence having similarity of 88 per cent with *Musa accuminata* AAA group cDNA. The Blastx analysis showed 71-72 per cent similarity of 67-69 per cent of the EST sequence with

full serine/threonine protein kinase AFC1 and AFC2. Interproscan result showed a length of 224 amino acids in the EST with protein kinase like domain. The main biological process includes protein phosphorylation while the molecular function includes protein kinase activity and ATP binding.

5.7.4 Blast2Go analysis of leaf EST

The Blast2GO analysis of leaf ESTs showed that three out of four EST sequences showed similarity to *Musa accuminata* subsp. *malancensis* which is also from the same order Zingiberales of *Zingiber officinale* and one EST sequence to *Boechera divaricarpa.*

The genus *Boechera* comprises mainly North American species of biennial and perennial herbaceous crucifers, characterized by a base chromosome number of $n = 7$. Previously, these species were included in the genus *Arabis* L., from which they were excluded based on the difference in the base chromosome number which is $n = 8$ in *Arabis* spp (Love and Love, 1976).

5.8 Validation of identified ESTs for high gingerol production

The 3-ketoacetyl CoA *Thiolase* (EC 2.3.1.16) was reported a differentially expressed gene for gingerol production in the somaclone B3 (Sreeja, 2017). The enzyme involved in benzoate degradation pathway. Acetyl CoA is produced from the benzoate degradation pathway which in turn acts as the molecule for plant metabolic pathways (Fatland *et al*., 2000). In plants, the acetyl CoA takes part in fatty acid biosynthesis in plastids and also for biosynthesis of secondary metabolites like flavonoids, stilbenoids and isoprenoids. The initial substrate for flavonoid synthesis is cytosolic acetyl CoA (Fatland *et al*., 2000).

The thiolase enzyme was validated using Real Time PCR.

5.8.1 Isolation of total RNA

Total RNA was isolated from immature leaves of ginger genotypes Athira, Karthika, Aswathy, Maran and B3 using TRIzol reagent.

Gel electrophoresis analysis was carried out and three RNA bands corresponding to 28SrRNA, 18SrRNA and 5SrRNA +tRNA were obtained indicating high quality RNA. Spectrophotometric analysis confirmed that the RNA isolated was free from carbohydrate and protein contamination.

5.8.2 First strand cDNA synthesis

The isolated RNA was used to synthesize first strand cDNA which was used as template for PCR and Real Time PCR.

5.8.3 Confirmation of synthesis of First strand cDNA using primers

Primers were designed for reference gene *Actin* and the target gene *Thiolase.* The PCR reaction was carried out using the designed primers and synthesized cDNA. The desired band size of 204 bp and 138 bp corresponding to the *Thiolase* gene and the *Actin* gene were confirmed using 1.8 per cent agarose gel.

5.8.4 Real Time PCR and Relative gene expression analysis of *3-ketoaceyl CoA Thiolase* gene

Relative quantification was carried for relative gene expression study using the using $2^{-\Delta\Delta Ct}$ method.

In the present study, the cultivar Maran was used as the control. Enhanced expression of *3-ketoaceyl CoA Thiolase* gene was observed in all the other genotypes studied. The *Thiolase* gene expression was observed non significant among the varieties Athira, Karthika, Aswathy and somaclone B3. The variety Athira showed 2.737 fold increase over the cultivar Maran. Fonseca *et al*. (2004) monitored gene expression in pear fruit development, ripening and senescence. The *3-ketoaceyl CoA Thiolase* gene was observed activated from day 12 until day 15. The gene encoding for jasmonic acid protein (JA2) also observed to show similar expression profile that of *Thiolase* gene. Jasmonic acid reported triggering defense responses which further trigger the secondary metabolite related pathways which leads to increased production of metabolites.

The expression of *Thiolase* gene was studied using total RNA isolated from one month old leaf of varieties Athira, Karthika and Aswathy and the cultivar Maran. From our results it is evident that the *Thiolase* gene expression was higher in the varieties Athira, Karthika and Aswathy as compared to the cultivar Maran. Higher productions of total gingerols were also observed in these varieties which emphasize the correlation of *Thiolase* gene in enhanced gingerol production.

The significant achievement from the present investigations is the suitability of *in vitro* induced microrhizomes for studying the gingerol synthesis *in vitro*. Microrhizomes recorded 26.2 times higher accumulation of total gingerols over calli. Scaling up of gingerol production using the identified treatments can be hence taken up using *in vitro* induced microrhizomes. Another salient finding in the present investigation the scaling up of gingerol production by simple manipulation of macro and micro nutrients. Modified MS medium with twice macro and micro nutrients and half ammoniacal nitrogen increased the gingerol content to the tune of 53.53 per cent over MS medium.

Elicitation, precursor feeding and gamma irradiation are also effective in scaling up of gingerol production. Elicitation with SA 5 mg L^{-1} in MMS medium further increased the gingerol content to the tune of 100.23 per cent over MS medium. Precursor feeding with FA 30 mg L^{-1} and gamma irradiation with 20 Gy also improved the gingerol production.

The variety Aswathy recorded the higher gingerol content on fresh weight basis while the variety Karthika recorded high dry weight and higher gingerol content on dry weight basis as compared to other genotypes. The higher expression of *Thiolase, PAL* and *CHS* in genotypes/ treatments with high gingerol gave an insight on the gingerol metabolic pathway, further research will lead to metabolite pathway engineering for high gingerol production. Cultivation of microrhizomes with high gingerol content and assessing the gingerol content in rhizomes derived from high gingerol microrhizomes need to be investigated further.

Summary

6. Summary

Investigations on "*In vitro* synthesis of gingerol and analysis of expressed sequence tags for gingerol production in ginger (*Zingiber officinale* Rosc.)" were carried out at Centre for Plant Biotechnology and Molecular Biology (CPBMB), Distributed Information Centre (DIC) and RadioTracer Lab, College of Horticulture, Kerala Agricultural University. The objectives of the study were to analyse the synthesis of gingerol under *in vitro* conditions, to characterize the ESTs related to gingerol synthesis and to validate the identified ESTs for high gingerol production in ginger.

The salient findings of the study are summarized below:-

6.1 Investigations of gingerol synthesis *in vitro*

- Microrhizomes induced *in vitro* were identified as the best experimental material for studying the *in vitro* synthesis of gingerol as compared to callus cultures.

6.1.1 Gingerol synthesis using *in vitro* induced microrhizomes

- The number of microrhizomes produced, growth of microrhizomes and accumulation of gingerols and shogaol increased with increase in duration of induction periods of microrhizomes.
- At five months induction period, irrespective of genotypes studied 20.5 microrhizomes were produced with a fresh weight of 0.599 g. But due to exhaustion of media components, drying and shrinkage of microrhizomes were observed when induction periods prolong beyond three months.
- Based on number of microrhizomes produced, fresh weight of microrhizomes and gingerol content, three months induction period was selected as the best induction period for induction of microrhizomes *in vitro*.
- At three months induction period, in the three released varieties studied (Athira, Karthika and Aswathy), the number of microrhizomes produced ranged from 12.50 to 20.18, fresh weight from 0.381 g to 0.612 g, dry

weight from 0.056 g to 0.070 g, oleoresin from 2.74 to 3.38 per cent and total gingerols from 0.540 to 0.615 per cent.

- At three months induction period, in the two cultivars studied (Maran and Rio-de-Janeiro), the number of microrhizomes produced ranged from 12.00 to 26.83, fresh weight from 0.373 g to 0.434 g, dry weight from 0.045 g to 0.050 g, oleoresin from 2.46 to 3.04 per cent and total gingerols from 0.220 to 0.500 per cent.
- The variety Aswathy was selected as the best among all the genotypes studied with respect to oleoresin (3.38 %), total gingerols (0.615 %), 6-gingerols (0.720 %) and 10- gingerols (0.110 %) at three months induction period.

6.1.2 Gingerol synthesis using *in vitro* induced calli

- The growth of calli and accumulation of gingerols and shogaol increased with increase in induction periods.
- Based on fresh weight of calli and gingerol content, three months induction period was selected as the best induction period.
- Shoot or root morphogenesis was observed in the calli after one and a half month of initiation of calli in all the ginger genotypes.
- Early callusing was observed in the varieties Athira, Karthika and Aswathy as compared to cultivars Maran and Rio-de-Janeiro.
- At one month induction period, in the varieties Athira, Karthika and Aswathy, the callusing ranged from 43.62 to 53.23 per cent and the callus index from 53.91 to 68.28 per cent.
- At one month induction period, in the cultivars Maran and Rio-de-Janeiro, the callusing ranged from 42.89 to 43.37 per cent and callus index from 47.29 to 52.89 per cent.
- At three months induction period, in the three released varieties (Athira, Karthika and Aswathy), the fresh weight of calli ranged from 1.505 g to 2.005 g, dry weight from 0.255 to 0.518 g, oleoresin from 2.73 to 3.15 per cent and total gingerols from 0.020 to 0.090 per cent.

- At three months induction period, in the two cultivars studies (Maran and Rio-de-Janeiro), fresh weight of the calli ranged from 1.428 g to 2.118 g, dry weight from 0.153 g to 0.175 g, oleoresin from 2.71 to 2.83 per cent and total gingerols of 0.010 per cent in each cultivar.
- The variety Aswathy was selected as the best among all the genotypes studied with respect to highest fresh weight (2.005 g), highest oleoresin (3.15 %), highest total gingerols (0.090 %), highest 6-gingerol (0.060 %) and highest 10-gingerol (0.030 %) at three months induction period.
- The variety Karthika recorded highest dry weight in all induction periods/treatments.

6.1.3 Comparison of gingerol synthesis in microrhizome and calli induced *in vitro*

- Microrhizomes induced *in vitro* accumulated higher gingerol content as compared to *in vitro* induced calli.
- Irrespective of the genotypes studied *in vitro* induced microrhizomes recorded 26.2 times higher accumulation of total gingerols than the calli.
- At three months induction period, the microrhizomes of varieties Athira, Karthika and Aswathy accumulated total gingerols 9.4 to 27 times over calli of same induction period.
- At three months induction period, the microrhizomes of cultivars Maran and Rio-de-Janeiro accumulated total gingerols 22 to 50 times over calli of same induction period.
- In the cultivar Rio-de-Janeiro, microrhizomes recorded 50 times higher accumulation of total gingerols as compared to the calli.
- In the variety Aswathy, calli also accumulated higher gingerol and microrhizomes recorded only 9.4 times higher accumulation as compared to calli.

6.2 Effect of manipulation of macro and micro nutrients on gingerol production

6.2.1 Manipulation of macro and micro nutrients using *in vitro* microrhizomes of variety Aswathy

- Different levels of macro and micro nutrients in MS medium and different levels of ammoniacal and nitrate nitrogen in MS medium had no favorable influence on production and growth of microrhizomes but the nutrient manipulation influenced gingerol content.
- The MS medium with twice micro nutrients, recorded oleoresin recovery of 3.70 per cent and total gingerols of 0.970 per cent.
- The MS medium with half ammoniacal nitrogen, recorded oleoresin recovery of 3.69 per cent and total gingerols 1.102 per cent.
- Combined effect of twice micronutrients and half ammoniacal nitrogen in MS medium, recorded higher oleoresin recovery of 3.45 per cent and total gingerols of 1.305 per cent. It also recorded higher number of microrhizomes/ culture (20.00 microrhizomes), fresh weight (0.497 g), dry weight (0.061 g) in microrhizomes.
- The MS medium with twice micronutrients and half ammoniacal nitrogen was designated as modified MS (MMS) medium.
- Modified MS medium recorded 6.48 and 53.53 per cent increase in oleoresin recovery and total gingerols respectively as compared to MS medium.

6.3 Elicitation of *in vitro* induced microrhizomes and calli using biotic and abiotic elicitors

6.3.1 Elicitation of *in vitro* induced microrhizomes of variety Aswathy using biotic elicitors

- Chitosan was the observed as the best biotic elicitor among the three biotic elicitors studied *viz.* autoclaved fungal mycelium of *Pythium aphanidermatum*, chitin and chitosan with respect to gingerol production.
- Chitosan 100 mg L^{-1} was found more effective with respect to oleoresin recovery and total gingerol production than chitosan 50 mg L^{-1}.
- Chitosan 100 mg L^{-1} when supplemented in MMS medium, recorded oleoresin recovery of 3.80 per cent and total gingerols of 1.463 per cent.
- Chitosan 100 mg L^{-1} when supplemented in MS medium, recorded oleoresin recovery of 3.22 per cent and total gingerols of 0.787 per cent.
- The MMS medium supplemented with chitosan 100 mg L^{-1} recorded an increase of 85.89 per cent in total gingerol content over MS medium supplemented with chitosan 100 mg L^{-1}.
- The MMS medium supplemented with chitosan 100 mg L^{-1} recorded an increase of 12.11 per cent in total gingerol production over MMS control

6.3.2 Elicitation of *in vitro* calli of variety Aswathy using biotic elicitors

- Callus induction (CI- ½ MS medium supplemented with 2, 4-D 3 mg L^{-1} and BA 0.5 mgL^{-1}) medium supplemented with chitosan was observed best with respect to gingerol production as compared to MCI (½ MMS medium supplemented with 2, 4-D 3mgL^{-1} and BA 0.5 mgL^{-1}) medium.

- Chitosan 100 mg L^{-1} in CI medium, recorded oleoresin recovery of 2.82 per cent and the total gingerols of 0.124 per cent in calli with root morphogenesis.

- Chitosan 100 mg L^{-1} in MCI medium, recorded oleoresin recovery of 2.79 per cent and the total gingerols of 0.085 per cent in calli with root morphogenesis.
- The CI medium supplemented with chitosan 100 mg L^{-1} recorded 45.88 per cent increase in total gingerols over the MCI medium supplemented with chitosan 100 mg L^{-1}

6.3.3 Elicitation of microrhizomes induced *in vitro* of the variety Aswathy using abiotic elicitors

- Methyl jasmonate (MJ) and salicylic acid (SA) when supplemented in MS/MMS media were found to improve gingerol production.
- Salicylic Acid and Methyl Jasmonate at lower concentration of 5 mg L^{-1} recorded higher gingerol production as compared to higher concentrations of 10 mg L^{-1} and 15 mg L^{-1}.
- The MMS medium supplemented with SA 5 mg L^{-1} was found significantly superior with respect to oleoresin recovery (3.64 %) and total gingerols (1.702 %).
- The MS medium supplemented with SA 5 m gL^{-1} recorded oleoresin recovery of 3.57 per cent and total gingerols of 1.035 per cent.
- The MMS medium supplemented with SA 5 mg L^{-1} recorded 59.07 per cent increase in total gingerols over the MS medium supplemented with SA 5 mg L^{-1}.

6.3.4 Elicitation of *in vitro* calli of variety Aswathy using abiotic elicitors

- Callus induction medium supplemented with SA was found best with respect to gingerol production as compared to MCI medium with SA.

- Callus induction medium supplemented with SA 5 mg L^{-1} recorded the oleoresin recovery of 3.06 per cent and total gingerols of 0.128 per cent in calli with root morphogenesis.

- Modified CI medium supplemented with SA 5 mgL^{-1} recorded oleoresin recovery of 3.01 per cent and total gingerols of 0.083 per cent in calli with root morphogenesis.

- The CI medium supplemented with SA 5 mgL^{-1} recorded 54.21 per cent increase in total gingerols over the MCI medium supplemented with SA 5 mgL^{-1}.

6.4 Effect of precursor feeding on *in vitro* gingerol production using microrhizomes and calli

6.4.1 Precursor feeding in microrhizomes induced *in vitro* of variety Aswathy

- Ferrulic acid was found as the best precursor among the three precursors *viz.* phenylalanine, coumaric acid and ferulic acid studied with respect to gingerol production.
- Ferrulic acid 30 mg L^{-1} produced higher gingerols (1.453 %) as compared to FA 60 mg L^{-1} (0.830 %) and 90 mg L^{-1} (0.527 %).
- Modified MS medium supplemented with FA 30 mg L^{-1} recorded oleoresin recovery of 3.83 per cent and total gingerols of 1.453 per cent.
- The MS medium supplemented with FA 30 mg L^{-1} recorded oleoresin recovery of 3.46 per cent and total gingerols of 0.823 per cent.
- The MMS medium supplemented with FA 30 mg L^{-1} recorded 76.54 per cent increase in total gingerols over MS medium supplemented with FA 30 mg L^{-1}.

6.4.2 Precursor feeding in calli of variety Aswathy

- The CI medium supplemented with FA 30 mgL^{-1} recorded oleoresin recovery of 2.99 per cent and total gingerols of 0.111 per cent in calli with root morphogenesis.

- Modified CI medium supplemented with FA 30 mg L^{-1} recorded oleoresin recovery of 2.68 per cent and total gingerols of 0.048 per cent in calli with root morphogenesis.

- The CI medium supplemented with FA 30 mg L^{-1} recorded 131.25 per cent increase in total gingerols over the MCI medium supplemented with FA 30 mg L^{-1}.

6.5 Effect of gamma irradiation on gingerol production using microrhizomes and calli

6.5.1 Gamma irradiation of *in vitro* induced microrhizomes of the variety Aswathy

- Gamma radiation of 20 Gy was selected the best dose for irradiation for *in vitro* induced microrhizomes recording survival of 86.6 per cent microrhizomes.
- In MMS medium, microrhizomes irradiated with 20 Gy recorded oleoresin recovery of 3.74 per cent and total gingerols of 1.110 per cent.
- In MS medium, microrhizomes irradiated with 20 Gy recorded oleoresin recovery of 3.10 per cent and total gingerols of 0.443 per cent.
- In MMS medium, microrhizomes irradiated with 20 Gy of gamma rays recorded 150.56 per cent increase in total gingerols as compared to microrhizomes induced in MS medium.

6.5.2 Gamma irradiation of *in vitro* calli of the variety Aswathy

- In CI medium, calli irradiated with 20 Gy of gamma rays recorded oleoresin recovery of 2.77 per cent and total gingerols of 0.115 per cent

- In MCI medium, calli irradiated with 20 Gy of gamma rays recorded oleoresin recovery of 2.72 per cent and total gingerols of 0.105 per cent

- In CI medium calli irradiated with 20 Gy of gamma rays recorded 9.5 per cent increase in total gingerols over the irradiated calli induced in MCI medium.

6.6 Expression of *PAL* and *CHS* gene in identified treatments with high gingerol production

- Highest expression of *PAL* and *CHS* gene were recorded in MMS medium supplemented with SA 5 mgL^{-1} recording 1.342 fold and 2.005 fold increase respectively over the control.
- Expression of *PAL* and *CHS* gene in MMS medium supplemented with chitosan 100 mg L^{-1} recorded 1.187 fold and 1.468 fold increase respectively over the control.
- Expression of *PAL* and *CHS* gene in MMS medium supplemented with FA 30 mg L^{-1} recorded 1.242 fold and 1.510 fold increase respectively over the control.

6.8 Analysis of available ESTs

- Blast2GO analysis of eleven rhizome ESTs showed maximum similarity hits with *Boechera divaricarpa*.
- Blast2GO analysis of four leaf ESTs showed similarity with *Musa accuminata* subsp *Maleccensis.*
- The gene ontology distribution of rhizome ESTs showed the involvement of ESTs in metabolic and cellular processes.
- Out of the eleven rhizome ESTs, four EST sequences were involved in metabolic process and two EST sequences involved in cellular process.
- The major molecular function of rhizome EST was binding followed by catalytic activity.

6.9 Validation of identified EST for high gingerol production

- The gene expression of *3-ketoacyl CoA thiolase* (ACAA1) was validated in somaclone B3, varieties Athira, Karthika and Aswathy using Real time PCR. The cultivar Maran was used as the control
- Higher expression of *3-ketoacyl CoA thiolase* (ACAA1) gene was recorded in Athira (2.737 fold) followed Karthika (2.150 fold), Aswathy (2.018 fold) and somaclone B3 (1.992 fold) over the control Maran.

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IN VITRO SYNTHESIS OF GINGEROL AND ANALYSIS OF EXPRESSED SEQUENCE TAGS FOR GINGEROL PRODUCTION IN GINGER (*Zingiber officinale* Rosc.)

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ABSTRACT OF THE THESIS

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Abstract

Ginger (*Zingiber officinale* Rosc.) is one of the important known spice crops, valued for its medicinal properties and health beneficial effects. The pharmacological properties of ginger are mainly attributed to the bioactive principles in rhizomes mainly gingerols. The present investigations on "*In vitro* synthesis of gingerol and analysis of expressed sequence tags for gingerol production in ginger (*Zingiber officinale* Rosc.)" were carried out at Centre for Plant Biotechnology and Molecular Biology (CPBMB), Distributed Information Centre (DIC) and RadioTracer Lab, College of Horticulture, Kerala Agricultural University from 2016-2019. The objectives of the study were to analyse the synthesis of gingerol under *in vitro* conditions, to characterize the ESTs related to gingerol synthesis and to validate the identified ESTs for high gingerol production in ginger.

Microrhizomes and calli were induced *in vitro* in three ginger varieties *viz.* Athira, Karthika, Aswathy and in two cultivars *viz.* Maran and Rio-de-Janeiro. The pungency principles synthesized *in vitro* in microrhizomes and calli were analyzed using High Performance Liquid Chromatography (HPLC). For scaling up of *in vitro* gingerol synthesis, nutrient manipulation, elicitation, precursor feeding and gamma irradiation were studied. Analysis of differentially expressed ESTs for gingerol production identified through Suppression Subtractive Hybridization (SSH) at CPBMB and validation of *3-ketoacyl CoA thiolase* gene and expression of *PAL* and *CHS* genes for gingerol production were also carried out in the present investigations.

Based on growth and gingerol content, three months culture period was selected as the best for *in vitro* induction of microrhizomes and calli. The variety Aswathy found to record the highest gingerol content in microrhizomes and calli among the five genotypes. The total gingerols accumulated in various treatments in microrhizomes ranged from 0.443 to 1.702 per cent and in calli from 0.111 to 0.128 per cent. Microrhizomes recorded 26.2 times higher accumulation of total gingerols over calli of same induction period.

Modified MS medium with twice micro nutrients and half ammoniacal nitrogen (MMS) recorded 53.53 per cent increase in total gingerols over the control MS medium in microrhizomes. The biotic elicitor chitosan 100 mg L^{-1} supplemented in MMS medium recorded 72.12 per cent increase in total gingerols over MS medium. The abiotic elicitor SA 5 mg L^{-1} supplemented in MMS medium recorded 100.23 per cent increase in total gingerols over the MS medium.

In calli the control MS medium at half strength supplemented with 2, 4-D 3 mg L^{-1} and BAP 0.5 mg L^{-1} (CI medium) was found to accumulate more gingerol and manipulation of macro and micronutrients was not found effective. Chitosan 100 mg L^{-1} supplemented in CI medium recorded 45.88 per cent increase and SA 5 mg L^{-1} recorded 54.21 per cent increase in total gingerols over MMS medium at half strength supplemented with 2, 4-D 3 mg L^{-1} and BAP 0.5 mg L^{-1} (MCI medium).

Precursor feeding improved gingerol production. Modified MS medium supplemented with FA 30 mg L^{-1} recorded 1.453 per cent total gingerols in microrhizomes and MS medium without FA 30 mg L^{-1} recorded 0.850 per cent total gingerols. In calli, CI medium supplemented with FA 30 mg L^{-1} recorded 0.111 per cent total gingerols while MCI medium recorded 0.048 per cent.

Microrhizomes irradiated with gamma rays of 20 Gy recorded higher survival (86.6 %) than microrhizomes irradiated with 30 Gy. In MMS medium, microrhizomes irradiated with 20 Gy recorded 1.110 per cent total gingerols and microrhizomes induced in MS medium recorded 0.443 per cent recording an increase of 150.56 per cent. Irradiated calli with 20 Gy of gamma rays recorded 0.115 per cent total gingerol in CI medium and 0.105per cent in MCI medium.

In treatments with high gingerol production, higher expressions of *PAL* and *CHS* genes were observed. Expression of *PAL* gene was recorded in MMS medium supplemented with SA 5 mg L^{-1} recording 1.342 fold increase in expression over the control. The *CHS* gene in MMS medium supplemented with SA 5 mgL^{-1} recorded 2.005 fold increase in gene expression over control.

Eleven rhizome ESTs and four leaf ESTs found differentially expressed for gingerol production were analyzed. The rhizome ESTs showed maximum similarity hits with *Boechera divaricarpa* while leaf ESTs showed similarity with *Musa accuminata* subsp. *Malaccensis*. The gene ontology distribution of rhizome ESTs showed its involvement in metabolic and cellular processes.

The gene *3-ketoacyl CoA thiolase* (ACAA1) identified to play a key role in gingerol synthesis was validated in four ginger genotypes using Real Time PCR. Higher expression of *Thiloase* gene was recorded in Athira (2.737 fold) followed by Karthika (2.150 fold), Aswathy (2.018 fold) and somaclone B3 (1.992 fold) over the control Maran.

In vitro synthesis of gingerol and analysis of expressed sequence tags for gingerol production in ginger were studied. Microrhizomes were identified as the best experimental material for studying gingerol synthesis *in vitro*. Scaling up of gingerol production in microrhizomes is possible through manipulation of micro and macro nutrients in MS medium and by using salicylic acid at 5 mg L^{-1}. The higher expression of *3-ketoacyl CoA thiolase, PAL* and *CHS* in genotypes/ treatments with high gingerol gave an insight on the gingerol metabolic pathway, further research will lead to metabolite pathway engineering for high gingerol production.