ELICITATION OF PHENYL PROPANOID PRODUCTION AND EXPRESSION PROFILING OF ACTEOSIDE BIOSYNTHETIC GENES IN *Artanema sesamoides* Benth (VATHOMVARETTI).

by

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(2017-11-150)

THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Elicitation of phenyl propanoid production and expression profiling of acteoside biosynthetic genes in *Artanema sesamoides* Benth (vathomvaretti)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations	**Full form**
ABA	Abscisic acid
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
A	Adenine
et al.	and others
bp	Base pair
BA	6- benzyl adenine
cm	Centimetre
cDNA	Complementary DNA
C	Cytosine
°C	Degree Celsius
DNA	Deoxy ribonucleic acid
DEPC	Diethyl pyrocarbonate
EDTA	Ethylene diamine tetra acetic acid
Fig.	Figure
FP	Forward Primer
g	Gram
G	Guanine
HPLC	High Pressure Liquid Chromatography
H	Hour
T_m	Melting temperature
Mg	Microgram
µL	Microlitre
µM	Micromolar

Mg	Milligram
Ml	Milliliter
Mm	Millimolar
Min	Minute
M	Molar
MJ	Methyl Jasmonate
MS	Murashige and Skoog, 1962
viz.	Namely
NAA	Naphthaleneacetic acid
NCBI	National Center for Biotechnology Information
ppm	Parts per million
%	per cent
PPGs	Phenyl propanoid glycocides
PGR	Plant growth regulator
PCR	Polymerase Chain Reaction
Ph	Potential of hydrogen
RP	Reverse primer
RT	Reverse transcriptase
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
rpm	Revolution per minute
RNase	Ribonuclease
RNA	Ribonucleic acid
s	Second
SA	Salicylic acid
Sl. No	Serial number
NaOH	Sodium hydroxide
Sp. or spp.	Species (Singular and plural)

i.e.	That is
T	Thymine
TBE	Tris-borate EDTA buffer
YE	Yeast extract

Introduction

1. INTRODUCTION

Medicinal herbs are used for curative purpose since prehistoric period. Approximately 80 per cent of the world population prefers traditional medicine for their health care needs. With the onset of research in medicine it was understood that plants contain active principles of pharmaceutical importance, which are responsible for the curative action of the herbs. On an average, about 85 per cent of the traditional medicines are derived from the plant extracts and the active ingredients of some clinically useful drugs are also derived from plants (Payne *et al.*, 1991). Presently medicinal plants are used in pharmaceutical, cosmetics, agricultural and food industries.

Artanema sesamoides Benth (family: Scrophulariaceae), is a commercially unexploited and lesser known medicinal plant, commonly called as 'vathomvaretti'. In Kerala, it is mainly seen in marshy localities (Aiyer and Kolammal, 1963). Main component present in *Artanema* is phenyl propanoid glycosides in which acteoside is found to be the prominent one (Joseph *et al.*, 2010). Phenyl propanoid glycosides (PPGs) have immunomodulatory effect and antimicrobial property. It also shows therapeutic properties, against hypertension and tumors (Pan *et al.*, 2003). Acteoside has antioxidant, hepato protective, anti-inflammatory, cell apoptosis regulation activities and various other pharmaceutical uses (Jiang *et al.*, 2011).

In *A. sesamoides*, phenyl propanoid glycosides content is less than 5% (w/w). The content of acteoside in leaves and dried root powder of *Artanema* is found to be 0.86 % and 0.80% respectively. The root extracts of *Artanema* has high antioxidant and anti-inflammatory property, because it contains more number of structurally related phenyl propanoids along with acteoside, which constitute 46.55% of total sequential methanolic extractives on dry weight basis (Joseph *et al.*, 2015).

Due to the low content of PPGs in plants, several synthetic routes have been developed for its production, which are time consuming. *In vitro* culture of medicinal plants has been found as an alternative route of secondary metabolite production. The advantages of *in vitro* system include large scale production, control over environment factors, ease of extraction and elicitation.

Recent study conducted on *in vitro* cultures of *Artanema* showed higher content of phenyl propanoid glycosides in callus than in the roots of field grown plants (Elizabeth, 2018). Elicitation of *in vitro* cultures is a promising technique where trace amount of abiotic or biotic stress factors are added to the cultures to induce plant stress response and enhance production of desired metabolites.

With this background, the present study entitled "Elicitation of phenyl propanoid production and expression profiling of acteoside biosynthetic genes in *Artanema sesamoides* Benth (vathomvaretti)" was proposed with the objective of enhancing the production of phenyl propanoid glycosides in *in vitro* cultures of *Artanema sesamoides* using elicitors and and to analyse the expression profile of key genes of acteoside biosynthesis pathway, such as *PAL* (phenylalanine ammonia-lyase), *HCT* (Shikimate O-hydroxycinnamoyl transferase) and *UGT* (UDP-glucose glucosyl transferase). The elicitors used are salicylic acid, methyl jasmonate, abscisic acid and yeast extract.

Review of Literature

2. REVIEW OF LITERATURE

Plants are the major source of traditional medicines as well as the active ingredient of some modern medicines, which are produced by secondary metabolism. Vincristine, reserpine, quinine, taxol, morphine etc. are examples of some plant based pharmaceuticals. Since artificial synthesis of these drugs is quite difficult, extraction from the plants is the best way. But commercialization of plant based extraction is uneconomical due to low yield, difficulty in field planting and its maintenance, incidence of pest and diseases and hiking labour charges. In this scenario, *in vitro* culture of medicinal plants was considered as an alternative route of secondary metabolite production. Several studies are going on, to develop techniques for the enhancement of secondary metabolites in *in vitro* cultures. The literature relating to these aspects of research is discussed in this chapter.

2.1 *Artanema sesamoides* Benth

Artanema sesamoides Benth belonging to the family Scrophulariaceae, and commonly known as "vathomvaretti", is a lesser known medicinal crop of Kerala (Syn: *Artanema longifolium* L., *Artanema longifolia* L.). It is also known as neermulli in Tamil, kokilaksha in Sanskrit, and vathomvaretti, kolivalen (kozhivalen) vayalchulii in Malayalam (Kirtikar *et al.*, 1987).

2.1.1 Botanical Description

A. sesamoides is a thick and erect grass of 60-90 cm in height. It is slightly branched, glabrous and often tinged with purple on the petioles of the leaves and stem and bears opposite leaves. The leaves are sessile or little petiolate with a width of 18-25 mm and a length of 10 cm. The flowers are large, bisexual and bilaterally symmetric with violet opaque corolla that appears as erect terminal clusters with pedicels of 6 to 16 mm in length and calyx of 8 to 11 mm in length. The sepals are

ovate, sharp, almost glabrous and imbricate. The globose and glabrous capsules have a length of 6 to 10 mm and the valves when mature are separated from the placentíferous axis of wide wings. The seeds of *Artanema* are small, truncated, papillose or rough (Aiyer and Kolammal, 1963).

2.1.2 Occurrence and Distribution

A. sesamoides Benth is widely seen in tropical Africa and Asia. The plant is located in Liberia to Cameroon, Congo, Uganda and Tanzania areas in tropical Africa. It is also seen in India and Southeast Asia. In Kerala, *Artanema* is commonly seen in the districts of Kollam, Idukki, Pathanamthitta, Kottayam, Palakkad, Kannur, Thiruvanthapuram, Thrissur and Malappuram. It favors the marshy localities as the margins of the lakes or lagoons and the low banks of the rivers for a better growth (Aiyer and Kolammal, 1963).

2.1.3 Medicinal Properties

The root and seeds of *A. sesamoides* Benth is reported to be medicinally significant in traditional system of medicine. Decoction of root is used to treat diarrhoea, rheumatism, stone, ophthalmia and syphilis (Kirtikar *et al.*, 1987). Chopra *et al.* (1986) reported that the seeds have ability to cure biliousness, to improve vitality and favours conception.

According to Rao (1987), leaves are used as leafy vegetable in Nigeria and Tanzania. Jansen (2004) reported that the aqueous extract of the plant can be used against inflammations of the skeleto- muscular system.

The methanolic extract of *A. sesamoides* was found to have antidiabetic activity. It increases the glycogen level in liver and reduces the cholesterol and triglycerides level significantly. It increases the anti-oxidant defense in liver and

kidney and reduces damages of the same (Selvan *et al.*, 2008). Analgesic and anti-inflammatory activities of the plant were reported by Gupta *et al.* (2008).

Joseph *et al.* (2010) evaluated the anti-inflammatory and anti-oxidant properties of the root extract. Ethanol, hexane, chloroform and water solvent extracts were tested in mouse erythema model in which ethanol extract showed higher anti-inflammatory activity. Single dose oral administration of root ethanol extract at 400 mg/kg and 200 mg/kg reduced the rat paw inflammation by 30.1% and 47.98% respectively. High antioxidant property was also shown by root ethanol extract.

Antioxidant property of *Artanema* reduced the free radicals in the human body and controlled ageing related problems (Mazumder, 2012).

Studies conducted by Hosseini *et al.* (2015) showed that, it reduces the necrosis and degeneration of islet cells of pancreas.

2.1.4 *In vitro* Propagation

Several studies were reported on *in vitro* propagation of different members of Scrophulariaceae family, commonly known as figwort family of flowering plants. Some of the economically important plants in this family are Foxglove (*Digitalis purpurea* L.), Snapdragon (*Antirrhinum majus* L.) and Mullein (*Verbascum thapsus* L.) (Encyclopedia Britannica, 2009).

Studies on *in vitro* propagation of several *Digitalis* sp. were mainly conducted for the enhanced production of cardiac glycosides like digitoxin, digoxin, digoxigenin etc. Hagimori *et al.* (1982) studied various cultures of *Digitalis purpurea* L. in order to compare the amount of digitoxin produced and found that the green shoot-forming cultures produced maximum amount of digitoxin. Verma *et al.* (2016) gave an updated review on *in vitro* culture of various *Digitalis* species and production of cardenolides. Their study reported that only for ten *Digitalis* species, regeneration

and propagation protocols have been developed so far. Among them microshoot propagation method is more suitable (than callus or plantlets) for triggering the digitoxin content and amongst all the known *Digitalis* species, *Digitalis lanata* is the prime source of cardiac glycosides for pharmaceutical industry.

Mullein (*Verbascum thapsus* L.), a medicinal herb in Scrophulariaceae family was cultured *in vitro* on MS medium with benzyladenine (BA) or kinetin for shoot proliferation using leaf discs and petioles as explants (Turker *et al.*, 2000). Improvement of phenols in Mullein using aminoacids was also studied (Al-Jibouri *et al.*, 2016).

A generalized protocol for tissue culture in snap dragon was developed by Hamza *et al.* (2013). *In vitro* seedlings of snapdragon (*Antirrhinum majus* L.) were successfully raised using sterilized seeds. Hypocotyl, cotyledon and cotyledonary node obtained from *in vitro* grown seedlings were used as explants. Multiple shoot formation, callus formation, indirect organogenesis and indirect embryogenesis of snapdragon were studied. In this study maximum shoot regeneration was obtained in MS medium supplemented with BAP at 0.50 $mgL^{-1}$and 1.00 mgL^{-1} and ninety percent of the microshoots were rooted on MS medium supplemented with 0.50 mgL^{-1} and 1.0 mgL^{-1} IBA.

A recent study on *in vitro* propagation of *Artanema sesamoides* Benth by Elizabeth (2018) in the Department of Plant Biotechnology reported that MS medium with 0.5 mgL^{-1} BA and 0.5 mgL^{-1} NAA is best for callus induction.

2.2 SECONDARY METABOLITES IN *Artanema sesamoides* Benth

In traditional medicine, *A. sesamoides* Benth was used for its anti inflammatory and antioxidant property. Bio-active molecules from *A. sesamoides* were isolated and chemically characterized. It was found to contain iridoid, flavonoids and phenyl propanoid glycosides. A considerable amount of pectin was also reported in roots

(3.19%) and leaves (5.69 %) of the plant which also contributes to anti inflammatory property. The bioactive compound responsible for high antioxidant activity was found to inhibit enzymes like 15-lipoxygenase and cyclooxygenase 2 in inflammatory pathways and its content was reported to be more in root extracts. All the compounds isolated from *A. sesamoides* were found to have molecular mass of 624.2-954.3 with similar structures and most of them could be grouped to a compound family. All the compounds contain the acteoside / verbascoside moiety (2-(3, 4-dihydroxyphenyl) ethyl 3-O-(6-deoxy-α-L-mannopyranosyl)-4-O-[(2E)-3-(3, 4-dihydroxyphenyl)-2-propenoyl]-β-D-glucopyranoside). Two compounds among them have an additional sugar moiety ($C_6H_{10}O_4$) (Joseph *et al.*, 2010).

2.2.1 Phenyl Propanoid Glycosides

Phenyl ethanoid glycosides are water soluble natural compounds with notable biological properties (Alipieva *et al.*, 2014).

Garcia *et al.* (1996) isolated irioid glucoside named harpagoside from *Scrophularia frutescens* L. According to Giner *et al.* (1998) *Scrophularia auriculata* ssp. *pseudo auriculata* contains iridoid glycosides, saponins, phenyl ethanoid glycosides etc. Some phenyl propanoid glycosides named ningposides A, sibirioside A, cistanoside D, angoroside C, acteoside, decaffeoyl acteoside and cistanoside F were obtained from the roots of *Scrophularia ningpoensis* (Li *et al.*, 2000). Monsef-Esfahani *et al.* (2010) isolated flavonoids, cinnamic acids and phenyl propanoids from aerial parts of *Scrophularia striata.*

2.2.2 Acteoside

Acteoside ($C_{29}H_{36}O_{15}$) is a type of phenyl propanoid glycoside, also called as kusagin or verbascoside. In 1963, Scarpati and Delle-Monache first isolated acteoside from scrophulariaceous medicinal plants. They studied the distribution of acteoside in various plants. According to He *et al.* (2011), acteoside showed bioactivities like

antioxidant, anti-inflammatory, antinephritis, hepatoprotective, immunoregulative, neuroprotective effects and also cell regulation.

Molnar *et al.* (1989) reported that antiplasmid function of the acteoside provides antibacterial property to the content.

Acteoside or verbascoside was first isolated from mullein and also reported in other plant species. Acteoside is hydrophilic in nature and possesses pharmacologically beneficial properties like antioxidant, anti-inflammatory and antineoplastic properties. It was also produced *in vitro* using genetically transformed roots / hairy roots from *Paulownia fortune* (Alipieva *et al.*, 2014).

2.2.3 Acteoside Pathway

The complete acteoside biosynthesis pathway was first studied in *Olea europaea* cell suspension cultures by isotope-labeled precursor feeding experiments. Two pathways *viz.*, the phenyl propanoid and tyrosine derived pathway for acteoside biosynthesis were identified. Acteoside was biosynthesized from tyrosine through dopamine, whereas caffeoyl moeity of acteoside was synthesized from phenylalanine *via* cinnamate pathway. In the phenyl propanoid pathway, phenylalanine is transformed into caffeic acid by *PAL* (phenylalanine ammonia-lyase), *C4H* (cinnamate-4-hydroxylase) *and C3H* (coumaroyl shikimate 3′-monooxygenase) genes. The synthesis of acteoside from an intermediate caffeoyl COA was regulated by *HCT* (Shikimate O hydroxycinnamoyl transferase) and *UGT* (UDP-glucosyl transferase) genes. In the tyrosine-derived pathway, *TyDC* (tyrosine decarboxylase), *CuAO* (copper-containing amine oxidase), *ALDH* (alcohol dehydrogenase gene) and *UGT* (UDP-glucosyl transferase) genes are involved for the synthesis of acteoside from tyrosine (Saimaru and Orihara, 2010; Alagna *et al.*, 2012).

2.3 *IN VITRO* PRODUCTION OF SECONDARY METABOLITES

Plant secondary metabolites are the most tremendous phytochemicals having huge significance in human health as well as in pharmaceutical industry. *In vitro* culture of medicinal plants has been found as an alternative route of secondary metabolite production due to the difficulties in synthetic production. An early study on *in vitro* production of secondary metabolites was started with taxol (paclitaxel), a complex diterpene alkaloid. It was found to be one of the most promising anticancereous drug because of unique mode of action on the microtubular cell system. Plant based extraction of taxol was done from the bark of the *Taxus* tree. But due to the scarcity of the *Taxus* tree and the costly synthetic process, *in vitro* techniques of taxol production were commercialised (Fett-Neto *et al.*, 1994).

Berberine is an antibacterial isoquinoline alkaloid present in the roots of *Coptis japonica*. It has been reported that *in vitro* production of berberine from cell cultures of *Coptis japonica* by optimizing the nutrients in the growth medium and the levels of phytohormones are more productive than any synthetic methods (Vanisree and Tsay, 2004).

In 2010, Zhao *et al.* also reported that in cell suspension cultures of *Vitis vinifera*, anthocyanin accumulation was more. A recent study conducted on *in vitro* cultures of *Artanema* showed higher content of phenyl propanoid glycosides in callus than in the roots of field grown plants (Elizabeth, 2018).

Various techniques are emerging for large scale synthesis of pharmaceutically important compounds from *in vitro* systems, such as improvement of strain, elicitation, precursor feeding, pathway engineering, biotransformation methods, bioreactor cultures, and micropropagation. Recent studies have reported that the production of secondary metabolites can be improved by elicitation (Hussain *et al.*, 2012).

2.3.1 Elicitors

Elicitors are chemical compounds that can induce various types of stress response in plants and, thereby improve synthesis of secondary metabolites. The key factors of elicitation that influence the production of the secondary metabolite are the type of elicitor, its concentration, the duration of exposure and the age of culture. So incorporation of elicitation techniques with *in vitro* production techniques and bioreactors can successfully enhance secondary metabolite accumulation (Naik and Al-Khayri, 2016).

Elicitors based on their source of origin can be classified into abiotic and biotic. Abiotic elicitors are grouped into physical, chemical, and hormonal factors. Biotic elicitors include polysaccharides derived from cell walls of plants (e.g. chitin, pectin, and cellulose) and microorganisms.

Sánchez-Sampedro *et al.* (2005) reported in *Silybum marianum* about three-fold production of silymarin occured by yeast extract treatment. Methyl jasmonate (200 µmol/L) showed a strong stimulating effect on accumulation of ginsenosides in suspension culture of *Panax ginseng* roots (Ali *et al.*, 2006). Sivanandhan *et al.* (2013) reported that in the hairy root culture of *Withania somnifera*, salicylic acid and methyl jasmonate elicited the production of withanolide A, withanone, and withaferin A. Farag *et al.* (2016) reported that in response to elicitation using methyl jasmonate, activation in lactonic alkaloid formation occurred in cell suspension cultures of *Erythrina lysistemon*. Sayed *et al.* (2017) reported that in the callus culture of *Rumex vesicarius* L., salicylic acid and chitosan elicited the production of flavonoids. A study conducted by Maqsood and Abdul (2017), where 22.74% vinblastine and 48.49% vincristine enrichment was noted in yeast extract (1.5 gL^{-1}) treated germinating embryos of *Catharanthus roseus*. Some of the secondary metabolites produced by application of different elicitors in *in vitro* cultures of various plant species are shown in the Tables 1 and 2.

Table 1. Secondary metabolites produced by treatment of different abiotic elicitors

Abiotic elicitor	Plant species	Nature of culture	Compounds	References
Sodium chloride	*Catharanthus roseus*	Embryogenic tissues	Vinblastine and vincristine	Fatima *et al.*, 2015
Sorbitol	*Perovskia abrotanoides*	Adventitious roots	Cryptotanshinone and tanshinone	Arehzoo *et al.*, 2015
Silver (Ag)	*Datura metel*	Hairy root	Atropine	Zahra *et al.*, 2015
Cobalt (Co)	*Vitis vinifera*	Cell suspension	Resveratrol	Cai *et al.*, 2013
Copper (Cu)	*Bacopa monnieri*	Shoot	Bacoside	Munish *et al.*, 2015
Light	*Zingiber officinale*	Callus	Gingerol and Zingiberene	Anasori and Asghari, 2008
UV	*Vitis vinifera*	Cell suspension	Stilbene	Xu A *et al.*, 2015
Proline and polyethylene glycol	Stevia rebaudiana	Callus & Cell suspension	Steviol glycosides	Pratibha *et al.*, 2015
Jasmonic acid	*Plumbago indica*	Hairy roots	Plumbagin	Gangopadhyay *et al.*, 2011
	Mentha piperita	Cell suspension	Rosmarinic acid	Krzyzanowska *et al.*, 2012
Methyl jasmonate	*Bacopa monnieri*	Shoot	Bacoside	Sharma *et al.*, 2013
	Andrographis paniculata	Cell suspension	Andrographolide	Sharma *et al.*, 2015

Methyl jasmonate	*Tacus canadensis*	Cell suspension	Paclitaxel	Ketchum *et al.*, 1999
Salicylic acid	*Salvia miltiorrhiza*	Hairy roots	Tashonines	Xiaolong *et al.*,2015
	Withania somnifera	Hairy roots	Withaferin-A Withanone Withanolide-A	Sivanandhan *et al.*, 2013
	Brugmansia candida	Hairy roots	Scopolamine	Pitta–Alvarez *et al.*, 2000

Table 2. Secondary metabolites produced by treatment of different biotic elicitors

Biotic elicitor	Plant species	Nature of culture	Compounds	References
Chitin, Pectin, Dextran	*Hypericum perforatum*	Shoot	Hypericin and pseudohypericin	Sonja *et al.*, 2014
Yeast extract	*Perovskia abrotanoides*	Adventitious roots	Cryptotanshinone and tanshinone	Arehzoo *et al.*, 2015
	Plumbago rosea	Cell suspension	Plumbagin	Silja *et al.*, 2014
	Silybum marianum	Cell suspension	Silymarin	Firouzi *et al.*, 2013
Claviceps purpurea	*Azadirachta indica*	Hairy root	Azadirachtin	Satdive *et al.*, 2007
Aspergillus niger, Saccharomyces cerevisiae, Agrobacterium Rhizogenes	*Gymnema sylvestre*	Cell suspension	Gymnemic acid	Chodisetti *et al.*, 2013

2.4 QUANTIFICATION OF PHENYL PROPANOID GLYCOSIDES

Quantitative and qualitative analysis of secondary metabolites in plants was done by High Performance Liquid Chromatography (HPLC). In a study done by Schafer *et al.* (2016) more than hundred primary and secondary metabolites were quantified by a single solid- phase extraction.

Sequential extraction method using ethanol and chloroform was performed for the extraction of Anthraquinone from *Ornithogalum umbellata*. For 1 g of dried hairy roots, 10 ml of solvent was used and finally ethanol extract was used for HPLC analysis (Krishnan and Siril, 2016).

In a study conducted by Balasubramanian *et al.* (2018), content of quercetin in hairy root cultures of radish was determined by HPLC analysis. Methanol was used as the solvent. Waters 2998 liquid chromatography system with the photodiode array detector (PDA) and C18 column was used for the detection of quercetin.

Detection of secondary metabolites from *Sclerotium rolfsii* (a microbe present in chickpea) was also done by HPLC technique (Amber *et al.*, 2012).

Phenyl propanoids like salidroside, verbascoside, iso-verbascoside, leucoseptoside A, jionoside D and martynoside present in *Pedicularis densispica* were determined by HPLC. Separation was perfomed with C18 column with step-wise gradient elution with water (A) - methanol (B) as mobile phase (Chu *et al.*, 2017).

Purification, identification and quantification of PPGs in *A. sesamoides* was done by HRMS, H NMR, C NMR and HPLC techniques (Joseph *et al.*, 2016, Personal communication; unreferenced). They identified and quantified six PPGs (acteoside, artanemoside A, isoacteoside, leucoseptoside A, plantainoside C, martynoside) from the plant.

In a recent study, quantification of PPGs in *in vitro* cultures of *A. sesamoides* was done by HPLC techniques. Five PPGs (acteoside, artanemoside A, isoacteoside, leucoseptoside A, martynoside) were identified and quantified from the callus and *in vitro* roots of the plant (Elizabeth, 2018).

2.5 EFFECT OF ELICITORS ON GENES OF PPG BIOSYNTHESIS PATHWAY

Elicitors may change enzymatic activity, regulate the expression of related genes, and enhance the accumulation of plant secondary metabolites. Early reports on the action of YE on alkaloid biosynthesis in cell culture of *Catharanthus roseus* showed that accumulation of alkaloids was associated with activation of terpenoid indole alkaloids (TIA) biosynthetic genes *STR* and *TDC* encoding strictosidine synthase and tryptophan decarboxylase (Pauw *et al.*, 2004).

mRNA levels of kaurene synthase-like (KSL) and copalyldiphosphate synthases (CPS) in *Salvia miltiorrhiza* hairy roots were improved by treatment of MJ and Ag^+ which in turn enhanced biosynthesis of tanshinone (Gao *et al.*, 2009).

MJ and Ag^+ could improve mRNA levels of kaurene synthase-like (KSL) and copalyl diphosphate synthases (CPS) in *Salvia miltiorrhiza* hairy roots. Transcriptome analysis of adventitious root cultures of *Panax quinquefolium* showed a significant upregulation of UDP-xylose synthases in response to MJ, corresponding to the increase in ginsenoside biosynthesis (Wang *et al.*, 2016).

Wang *et al.* (2017) reported that, in salicylic acid treated *Rehmannia glutinosa* hairy root cultures, 54 unigenes were upregulated in the acteoside pathway, out of 219 putative unigenes. They included genes such as phenylalanine ammonia-lyase (*PAL*) genes, copper-containing amine oxidase (*CuAO*) genes, cinnamate-4-hydroxylase (*C4H*), coumaroyl quinate (coumaroyl shikimate) 3′-monooxygenase (*C3H*), polyphenol oxidase (*PPO*) genes, UDP-glucosyl transferase (*UGT*) genes, and four shikimate *HCT* genes. Among these genes *PAL* was expressed after 12 h of

salicylic acid treatment whereas *HCT* and *UGT* significantly up-regulated at both 12 and 24 h time points.

2.6 TECHNIQUES AND TOOLS USED FOR EXPRESSION PROFILING OF GENES

Gene expression is directly related to its function. Reverse Transcription quantitative PCR (RT-qPCR) or Real Time PCR is a highly sensitive and specific technique for analysing the expression of target genes. The expression profiling of genes involves several steps *viz.*, RNA isolation, primer designing, cDNA synthesis, specificity check of primers using cDNA by PCR and Reverse Transcription quantitative PCR (RT-qPCR) or Real Time PCR.

2.6.1 Primer Designing

Primer designing is an important step that greatly affects the success of a polymerase chain reaction (PCR). In PCR, forward and reverse primer helps the amplification of specific region of DNA by annealing to the complimentary sequence (Beij *et al.*, 1991). Primer having short length can result in non-specific amplification and ideal length of primers should be between 18-24 bp (Lowe *et al.*, 1990). A primer is a short oligo nucleotide which is the reverse complement of a region of a DNA template and it would facilitate the amplification of the targeted DNA sequence (Garg *et al.*, 2008).

While designing the primer several parameters should be considered including the length of the primer, melting temperature, annealing temperature and GC content (Wu *et al.*, 1991; Rychlik *et al.*, 1999). The important criteria to be considered for primer designing are the specificity and the efficiency of primers (Dieffenbach *et al.*, 1993). The specificity implies that only the specific target sequence is amplified and the efficiency implies that more products are amplified in

less number of cycles. The specificity depends on length of primer and annealing temperature (Garg *et al.*, 2008).

The optimum G-C content of primer is between 40-60% (Lowe *et al.*, 1990). Dinucleotide repeats and continuous execution of same bases are to be avoided as it might result in non-specific binding. Inorder to avoid mispriming, the 3' end of the primer should contain G or C nucleotide for stronger binding (Dieffenbach *et al.*, 1993). The optimum annealing temperature for polymerase chain reaction is usually kept five degree celsius lower than the estimated melting temperature (Dieffenbach *et al.*, 1993; Rychlik *et al.*, 1999; Abd-Elsalam, 2003). Length of amplicon also has impact on efficiency of amplification (Rychlik *et al.*, 1999). Secondary structures, such as hairpin loops and primer dimers can also result in no amplification or less efficiency (Sahdev *et al.*, 2007).

2.6.2 RNA Isolation

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

The simple method for RNA isolation is the Trizol method. It uses Guanidinium thiocyanate, a chaotropic agent that allows the purification of RNA from cells (Cox, 1968; Chomczynski and Sacchi, 1987). Trizol reagent is a ready-to-use reagent for RNA extraction and it makes RNA isolation process easier and fast (Simms *et al.*, 1993). Trizol reagent disrupts cells and dissolves cell components and maintains the integrity of RNA. The mixture separates into an aqueous phase and an organic phase. RNA remains in the aqueous phase and can be precipitated with isopropanol (Chomczynski, 1993).

The secondary metabolites produced by certain plants may result in poor yield of RNA (Gasic *et al.*, 2004). Polyvinyl pyrrolidone (PVP) is used during RNA isolation to reduce phenolic compounds and polysaccharides (Liu *et al.*, 2018).

2.6.3 Quantification of Nucleic Acids

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

2.6.4 Polymerase Chain Reaction

Karry Mullis invented Polymerase chain reaction in 1983, which opened a new era in molecular biology. PCR mainly consists of three steps *viz.*, denaturation of DNA, annealing of primer to the template and extension by DNA polymerase. The forward and reverse primers hybridize with the complementary sequence in the target and initiate DNA synthesis with the help of the enzyme DNA polymerase and the process is repeated for several cycles. In each cycle, the DNA gets duplicated. After 20 to 40 such cycles, enough amplified product is generated and can be visualized on an agarose gel by using specific staining method (Saiki *et al.*, 1988). PCR became more efficient after the discovery of heat stable DNA polymerase, Taq polymerase from the bacteria *Thermus aquaticus* (Karcher, 1995). In PCR, amplification of a specific nucleotide sequence was done using highly specific techniques (Culley *et al.*, 2014; McCall *et al.*, 2015).

The basic components of PCR master mix include template, primers, magnesium ion, dNTPs, buffer for PCR reaction, and thermostable DNA polymerase enzyme (Mullis *et al.*, 1986). The template can be DNA, RNA or cDNA. The enzyme *Taq* DNA polymerase is the most commonly used thermostable DNA polymerase and it is suitable for routine amplifications. The magnesium ion acts as cofactor for the enzyme and it affects enzyme activity, primer annealing, melting temperature etc. (Zamft *et al.*, 2012).

2.6.5 RT-qPCR or Real Time PCR

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

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

The melting temperature of a DNA molecule depends on both its size and its nucleotide composition; hence GC-rich amplicons have a higher T_m than those having an abundance of AT base pairs (Nolan *et al.*, 2006). During melt curve analysis, the real-time machine continuously monitors the fluorescence of each sample as it is slowly heated from a user defined temperature below the T_m of the products to a temperature above their melting point. Fluorescent dye is released upon melting (denaturation) of the double-stranded DNA, providing accurate T_m data for every single amplified product. Melting peaks are calculated by taking the differential (the first negative derivative (–dF/dT) of the melt curve. These peaks are analogous to the bands on an electrophoresis gel and allow for the qualitative monitoring of products at the end of a run. Short primer dimers will melt at lower temperature than longer, target amplicon products (Armstrong and Schulz, 2015).

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Elicitation of phenyl propanoid production and expression profiling of acteoside biosynthetic genes in *Artanema sesamoides* Benth (vathomvaretti)" was carried out during 2017-2019, in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 *IN VITRO* CULTURE OF *Artanema sesamoides* Benth

3.1.1 Cleaning and Sterilization of Glasswares and Other Equipments

Borosilicate glassware, membrane filters (Sartorius, Germany), disposable sterile petridishes (Tarsons, India) were used for the study. Glassware required for media preparation were soaked overnight (16 h) in detergent solution and then washed using tap water. After washing, glassware were kept for 2 h of drying in hot air oven at a controlled temperature. Then all the utensils were wiped with alcohol and dried again for 15 min in hot air oven at a controlled temperature. Later glassware, forceps, scalpels, filtration units etc. were sterilized by autoclaving at 121^0 C, 100 Kpa for 45 min. Filter papers, micropipette tip boxes and distilled water needed for the aseptic works were also autoclaved before use.

A horizontal laminar flow cabinet with HEPA filter of 0.2 µ was used for the *in vitro* culture. The hood surface of laminar air flow chamber was cleaned using 70% ethanol and sterilized by germicidal ultraviolet light for at least 20 min prior to use. Scalpel and forceps were subjected to flame sterilization by dipping in 100 % ethanol before use.

3.1.2 Composition of Medium

Murashige and Skoog (1962) medium (MS) was used for the *in vitro* propagation of *A. sesamoides*. For callus induction, MS medium supplemented with 0.5 mgL^{-1} NAA and 0.5 mgL^{-1} BA was used (Elizabeth, 2018).

3.1.3 Preparation of Tissue Culture Medium

All the chemicals required for media preparation such as major and minor nutrients plant growth regulators, vitamins, aminoacids, antibiotics and elicitors were of analytical grade and procured from HIMEDIA, Sisco Research Laboratories (SRL), Merck India Ltd. and Sigma Chemicals, USA.

The plant tissue culture medium was prepared based on the standard procedures of Gamborg and Shyluk (1981). Stock solutions of major and minor nutrients were prepared and stored in autoclaved glass bottles under refrigerated conditions. Stock IV (Na_2EDTA and $FeSO_4.7H_2O$) was stored in amber coloured bottle to avoid the photochemical reaction of iron.

Medium was prepared in a clean beaker rinsed with distilled water. Required volumes of stock solutions were pipetted and added to the distilled water taken in the beaker. The required quantity of sucrose (30 gL^{-1}), myoinositol (100 mgL^{-1}) and MS supplement (3.55 gL^{-1}) were added and dissolved. The pH of the solution was adjusted to 5.7 with 0.1 N HCl/ 0.1 N NaOH. After pH adjustment, volume was made up to 1 litre. Agar (6.3 gL^{-1})/ Gelrite (4.5 gL^{-1}) was added and heated in microwave oven. After melting the solidifying agent, medium was poured into clean, autoclaved culture bottles. Similarly liquid MS was also prepared, without adding any solidifying agent. Culture bottles containing the media were sterilized by autoclaving at 121°C in 100 Kpa for 20 min and allowed to cool to room temperature. Inoculation was done only after checking media contamination.

3.1.4 Growth Regulators

Cytokinins and auxins were the major group of phytohormones used in the *in vitro* culture of *A. sesamoides*. Stock solutions of auxins (NAA) and cytokinins (BA) were prepared by dissolving in NaOH.

3.1.5 Callus Induction

MS medium supplemented with 0.5 mgL^{-1} NAA and 0.5 mgL^{-1} BA were used for callus induction. Leaf of *in vitro* raised *A. sesamoides* was used as explant. The culture bottles after inoculation were incubated at 25±2° C in dark condition. Humidity varied from 60-80 per cent according to the prevailing climate. Callus was kept for two months for complete establishment. Later two weeks old callus was established by further subculturing.

3.2 ELICITATION OF CULTURES

3.2.1 Elicitors

Stock solutions of elicitors *viz.*, salicylic acid, methyl jasmonate, abscisic acid and yeast extract were prepared by dissolving in suitable solvents. Salicylic acid, methyl jasmonate, abscisic acid were dissolved in a small quantity of ethanol and used water to make up required volume. Stock solution of yeast extract was prepared by dissolving in water. Stock solutions were filter sterilized using Randisc PVDF syringe filters with pore size 0.22 µ and stored in refrigerator.

3.2.2 Elicitation Studies

For elicitation studies, liquid MS supplemented with 0.5 mgL^{-1} NAA and 0.5 mgL^{-1} BA was used. Two weeks old callus was transferred to liquid cultures and stabilized for 10 days in orbital shaker at 120 rpm in dark condition and at 25±2° C temperature. Elicitors *viz.*, salicylic acid (SA; 40 and 100 µM), methyl jasmonate (MJ; 15 and 25 µM), abscisic acid (ABA; 20 and 50 µM) and yeast

extract (1 and 1.5 gL^{-1}) were added to the liquid cultures and incubated in orbital shaker at 25±2° C under dark condition at 120 rpm for 48 h.

3.3 BIOCHEMICAL ANALYSIS

3.3.1 Sequential Extraction of Phenyl Propanoid Glycosides from Samples

After elicitation, callus was harvested, dried and finely powdered. Sequential extraction using solvents like hexane, chloroform and methanol was done to extract the phenyl propanoid glycosides. Extraction was done three times using each of these solvents. For each 1 g of powdered sample, 10ml solvent was added and kept in orbital shaker at 110 rpm for 16 h for extraction. Methanol extract obtained after 3 times extraction, was centrifuged at 4500 rpm for 5 min and residue was removed. The extracts were evaporated, weighed and redissolved in 10 ml HPLC grade methanol. Methanol extract was filtered using Randisc PVDF syringe filters with pore size 0.22 µ. After filtration, sample was collected in HPLC vials of 2 ml capacity.

3.3.2 Details of HPLC Assay

Column: Merck LiCrospher 100 RP 18e (250 mm x 4.6 mm)

Mobile phase: Two solvents (A & B)

A: H_2O with 0.1 % ortho phosphoric acid (v/v)

B: 90% ACN and 10% A (w/v)

Programme: Multistep gradient

20 % B (0-20 min)

20-30% B (20-40 min)

30-50 % B (40-55 min)

50-60 % B (55-65 min)

60-70 % B (65-75 min)

Flow rate: 1 ml/ min

Injection volume: 20µl

Detector: Dionex Ultimate 3000 with ultimate 3000 PDA detector

Wavelength: 197 nm, 218 nm, 254 nm and 330 nm

Data obtained were analysed and compared with the reference.

3.4 GENE EXPRESSION ANALYSIS

3.4.1 Primer Designing

The nucleotide sequences of genes such as *PAL* (phenylalanine ammonia-lyase), *HCT* (Shikimate O-hydroxycinnamoyl transferase) and *UGT* (UDP-glucosyl transferase) of *A. sesamoides* were not available in NCBI database. In the case of *PAL* and *HCT* genes, degenerate primers were designed based on sequence similarity of closely related species of *Artanema* and for *UGT* gene, sequence from the related species was used for designing gene specific primers. The primer designing was done using "Primer Express software". The designed forward primers were subjected to "Oligos" for synthesising its reverse primer. Factors such as primer length, annealing temperature, GC content, potential hair pin formation and 3' complementarity were analysed by using Primer Express and mfold Server. The primers were synthesised at Xetra Bio solutions, Poojapura, Thiruvananthapuram.

3.4.2 RNA Isolation

Total RNA was isolated from the callus after 24 h and 48 h of elicitation using SA, MJ, ABA and yeast extract. Trizol method was used for RNA isolation. Mortar and pestle, microtips, microfuge tubes, forceps and reagents were sterilised by double autoclaving. DEPC (Di Ethyl Pyro Carbonate) treated water was used for reagent preparation.

RNase away (Thermofischer Scientific) and RNase Zap (Thermofischer Scientific) were used for removing RNase contamination. Chilled mortar and pestle wiped with RNase away was used for grinding the samples into a fine powder using liquid nitrogen. 1 mL of Trizol reagent was added to the powdered tissue in mortar and gently mixed to homogenize the mixture and incubated at room temperature for 5 min. The homogenate was transferred to a 2 ml pre chilled microfuge tube.

To remove protein contamination, 0.2 ml chloroform was added and shaked vigorously for 15 s and incubated at room temperature for 5 min. The sample was kept in ice for 10 min and then centrifuged at 12000g for 15 min at 4°C. The aqueous phase of the sample was transferred to a fresh tube. 0.5 ml ice cold isopropanol (100%) was added to each tube and incubated at room temperature for 10 min for RNA precipitation and after that mixed by inverting the tube slowly. The sample was centrifuged at 12000g for 10 min at 4°C.

The supernatant was removed and pellet washed with 1 ml of 75% alcohol (in DEPC treated water). Then sample was briefly vortexed and spun at 7500g for 5 min at 4°C and dried for 30-40 min in RNase free laminar air flow chamber. The RNA pellet was resuspended in 30 µl RNase free water and incubates at 55-60°C for 10 min and stored at -20^{0}C. The quality of the isolated RNA was estimated by agarose gel electrophoresis. RNA (5 µL) mixed with 6X gel loading dye (2 µL) and DEPC treated water (5 µL) was poured into wells using a micropipette on agarose gel (1.2 %) and 1 kb marker was added to know the size of different RNA bands.

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

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

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

3.4.3 Preparation of cDNA

From the isolated RNA, cDNA was synthesised using Verso cDNA synthesis kit (Thermo Fisher Scientific). It includes 5X cDNA buffer, dNTP mix, oligo dT primer, random hexamer, RT enhancer for removing DNA contamination and Verso enzyme mix.

The composition of the reaction mixture (20 µl) for cDNA synthesis was as follows

5X cDNA buffer	:	4 µl
dNTP mix (5 mM)	:	2 µl
Oligo dT (500 ngµL^{-1})	:	0.5 µl
Random Hexamer (400 ngµL^{-1})	:	0.5 µl
RT enhancer	:	1 µl
Verso enzyme mix (200 UµL^{-1})	:	1 µl
RNA (0.5 µg)	:	5 µl
Nuclease free water	:	6 µl
Total volume	:	20µl

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

3.4.4 Quality Analysis of cDNA

The quality of newly synthesized cDNA was confirmed by PCR using β-*ACTIN* gene specific primers. A standard PCR mix was prepared of 20 µl total volume containing 50 ng of template DNA, 0.2 mM dNTPs, and 400 nM each of primers, 1 unit of Taq polymerase and 1X PCR buffer. The amplified products were separated on agarose gel (1.2 %) and viewed under gel documentation system (BIORAD).

3.4.5 Specificity Check of Primers with cDNA

The cDNA synthesised was amplified using gene specific primers for *PAL* (phenylalanine ammonia-lyase), *HCT* (Shikimate O-hydroxycinnamoyl transferase) and *UGT* (UDP-glucosyl transferase). A standard PCR mix was prepared of 20µl total volume containing 50 ng of template DNA, 0.2 mM dNTPs, and 400 nM each of primers, 1 unit of Taq polymerase and 1X PCR buffer. The amplified products were separated on agarose gel (1.2 %) and viewed under gel documentation system (BIORAD).

3.4.6 Real-Time Quantitative PCR

Reverse Transcription quantitative PCR or Real Time PCR was done to analyse the expression profile of key genes of acteoside biosynthesis pathway. The house keeping gene ß-*ACTIN* was taken as the reference gene for RT-qPCR. SYBR green, an intercalating dye, was used for generating fluorescence. The reaction mixture (20 µl) was prepared as follows:

2X Real time PCR Smart Mix : 10 µl

Forward primer (10 pmol/µl) : 0.6 µl

Reverse primer (10 pmol/µl) : 0.6 µl

Template cDNA (50 ng) : 4 µl

Nuclease free water : 4.8 µl

Total volume : 20 µl

Table 3. Thermal profile for Real Time PCR programme

Step	Stage	Temperature (^{0}C)	Duration
1	Initial denaturation	95^{0}C	2 min
2	Denaturation	95^{0}C	15 s
3	Annealing	40^{0}C	15 s
4	Extension	72^{0}C	45 s
The steps 2-4 were allowed to repeat 40 times.			
5	Final extension	72^{0}C	5 min

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

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

Results

4. RESULTS

The results of the study on "Elicitation of phenyl propanoid production and expression profiling of acteoside biosynthetic genes in *Artanema sesamoides* Benth (vathomvaretti)" are presented in this chapter.

4.1 GROWTH RATE OF CALLUS

Two weeks old callus cultures were transferred to liquid MS medium with 0.5 mgL^{-1} NAA and 0.5 mgL^{-1} BA and stabilized for 10 days. Elicitors *viz*., salicylic acid (SA; 40 and 100µM), methyl jasmonate (MJ; 15 and 25µM), abscisic acid (ABA; 20 and 50µM) and yeast extract (1 and 1.5 gL^{-1}) were added to the cultures and incubated in orbital shaker at 24 °C under dark condition at 120 rpm for 48h. After 48h of elicitation, there was no significant change in the growth of callus (Plate 1).

a. SA (40 µM)

b. SA (100 µM)

c. MJ (15 µM)

d. MJ (25 µM)

e. ABA (20 µM)

f. ABA (50 µM)

g. Yeast (1 g l^{-1})

h. Yeast (1.5 g l^{-1})

i. Control

Plate.1 Callus of *A. sesamoides* grown in liquid medium

4.2 BIOCHEMICAL ANALYSIS

4.2.1 Sequential Extraction of Phenyl Propanoid Glycosides

Phenyl propanoid glycosides were extracted by sequential extraction using organic solvents namely hexane, chloroform and methanol in the increasing order of polarity. The sequential methanol extracts were pooled treatment wise, solvent removed by evaporation and dry yield was recorded. The yield of methanol soluble extracts indicated the PPG content present in the sample (Table 4). The total yield of dry extract rich in phenyl propanoid glycosides was maximum (4.160 mgg^{-1}) in callus treated with yeast extract (1.5 gL^{-1})

Table 4. Total yield of phenyl propanoid glycoside containing dry sequential methanol extracts obtained from the callus treated with different concentration of elicitors

Treatments	Total yield (mgg^{-1})
SA (40μM)	2.004
SA (100μM)	1.532
MJ (15 μM)	0.516
MJ (25μM)	1.283
ABA (20μM)	2.565
ABA (50 μM)	2.572
YE (1 gL^{-1})	3.035

YE (1.5 gL^{-1})	4.160
Control (callus)	2.692
Control (root of field grown plants of *A. sesamoides*)	0.732
CD(0.05)	0.469
SE	0.146

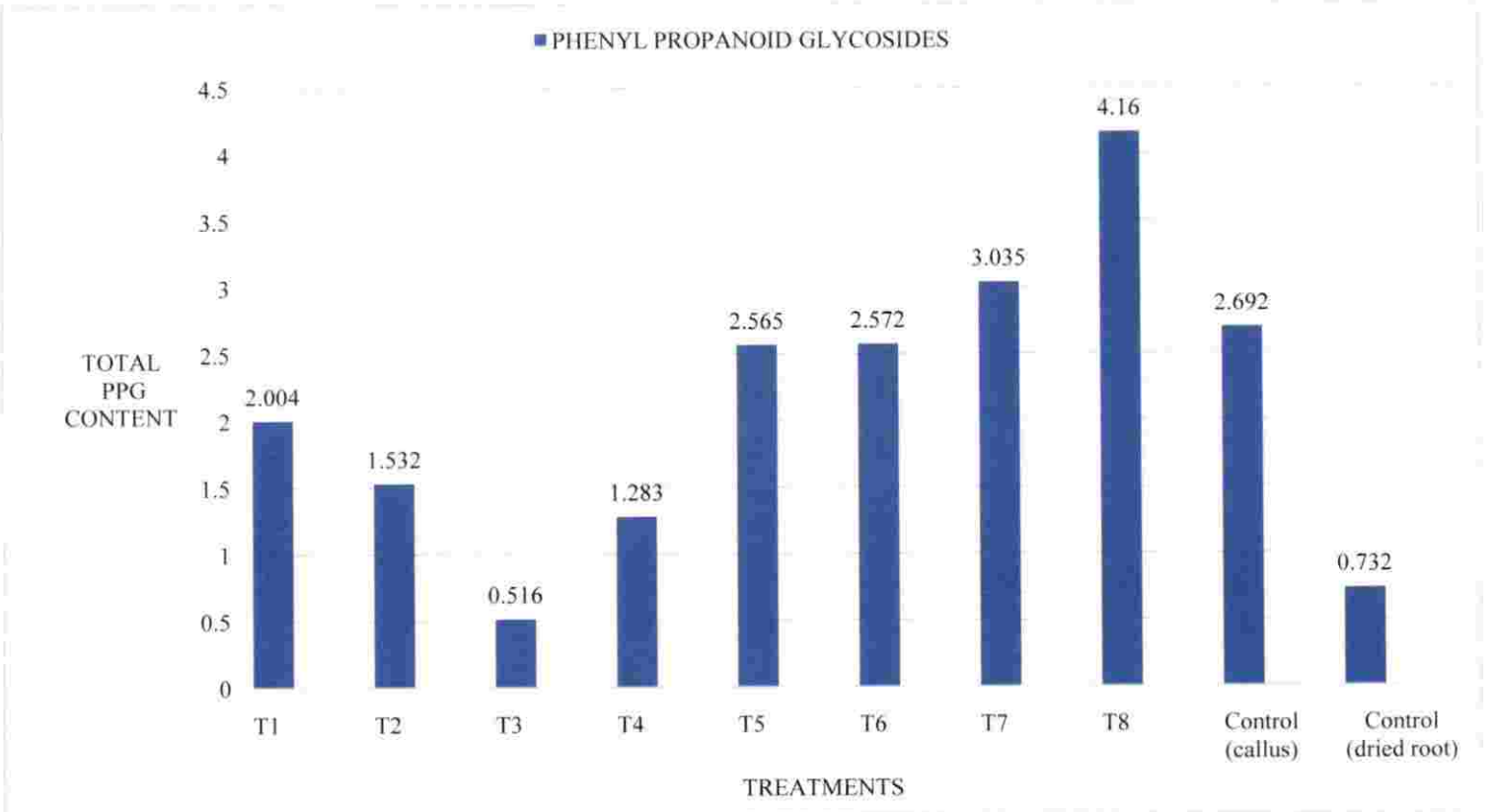


Fig.1 Total yield of phenyl propanoid glycosides containing dry sequential methanol extract (mg g^{-1}) with different concentration of elicitor

4.2.2 Biochemical Profiling of Phenyl Propanoid Glycosides by HPLC

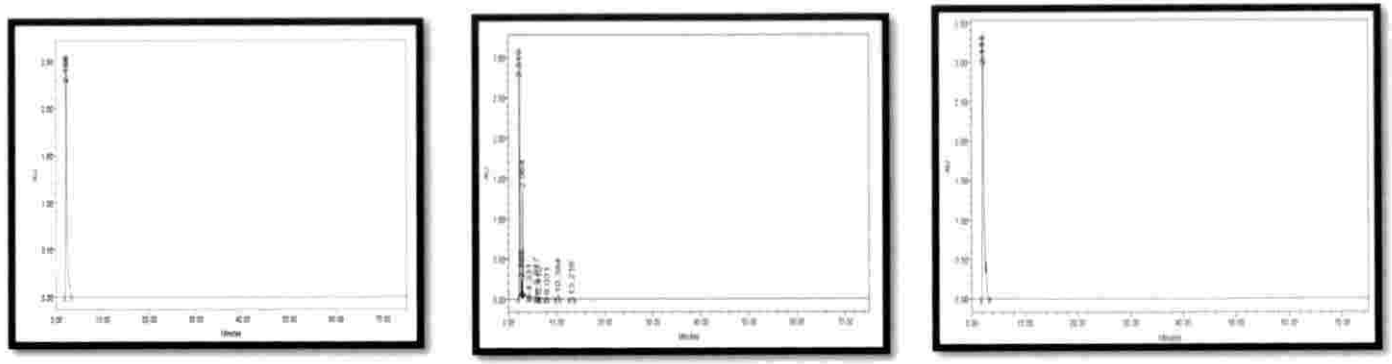
Biochemical profiling of PPGs in *A. sesamoides* was done with the help of analytical HPLC technique. Chromatogram were analysed with the help of class LC10 software (Shimadzu, Japan).

The initial chromatograms of all treated callus, untreated callus and dried roots of field grown plants developed are shown in fig.2. Among the treatments, calli treated with salicylic acid (100μM) and yeast extract (1 gL^{-1}) showed more number of peaks (7 and 6 respectively) as seen in dried roots of field grown plants (13 peaks). So chromatograms of most promising treatments were developed again at different wavelengths (197, 218, 254 and 330 nm) in terms of different phenyl propanoid glycosides present and its proportionate content in the sequential methanolic extract along with that of callus control (figs. 3- 22). PPGs showed maximum absorbance at 197 nm. Six important PPGs were identified from the methanol extract. The highest peak of HPLC chromatogram representing the most prominent antioxidant phenyl propanoid glycoside (acteoside) in the extract appeared within the retention time of 59 min. The peaks of other PPGs such as artanemoside, isoacteoside, leucosceptoside, martynoside and plantainoside appeared within the retention time of 61- 79 min. The proportionate content (in terms of peak area) of major PPGs present in the methanolic extract of callus treated with different elicitors is given in Table 5.

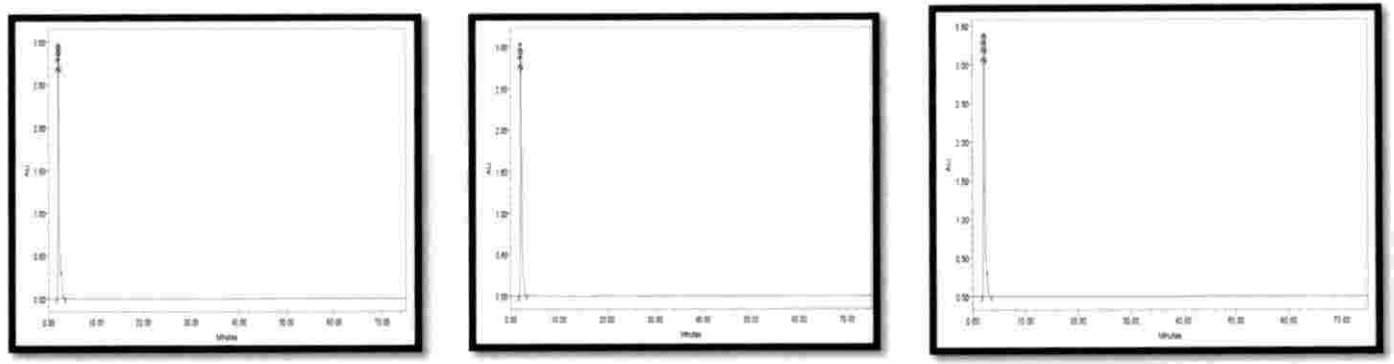
YE (1gL^{-1}) have significantly increased the content of all the major PPGs (Table 3). The maximum content of acteoside (36.951%) and isoacteoside (22.220%) was obtained in calli treated with yeast extract (1.5 gL^{-1}). Treatment with SA (100μM) has enhanced the content of acteoside (5.853%), isoacteoside (15.511%), martynoside (19.990%) and plantainoside (11.694%). Some of the PPGs like artanemoside (17.025%), martynoside (20.594%) and plantainoside (10.683%) were enhanced by SA (40μM) treatment.

Table 5. Percentage (in terms of peak area) of major PPGs present in the methanolic extract of callus treated with different elicitors

Phenyl propanoid glycosides	SA (40 µM) (%)	SA (100µM) (%)	YE ($1\ gL^{-1}$) (%)	YE ($1.5\ gL^{-1}$) (%)	Control callus (%)
Acteoside	3.357	5.853	24.073	36.951	3.851
Artanemoside	17.025	0.889	16.600	5.346	8.213
Isoacteoside	6.416	15.511	20.696	22.220	6.119
Leucosceptoside	0.057	0.308	10.350	2.185	2.583
Martynoside	20.594	19.990	14.842	18.826	15.305
Plantainoside	10.683	11.694	13.438	4.378	8.377

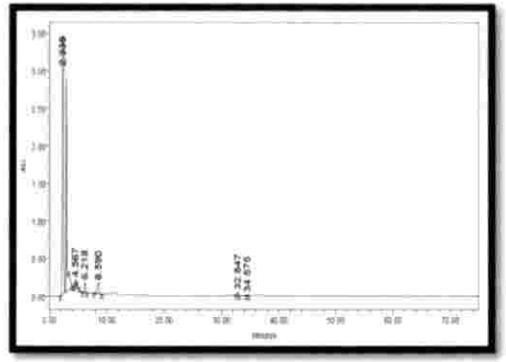


a. SA (40 µM) b. SA (100 µM) c. MJ (15 µM)

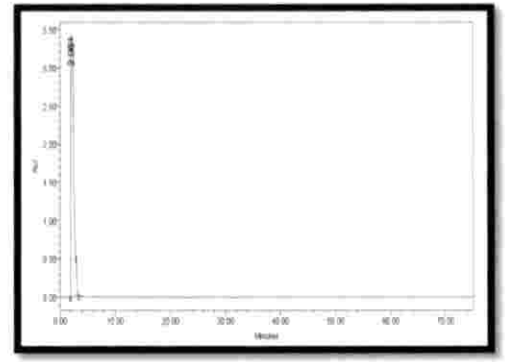


d. MJ (25 µM) e. ABA (20 µM) f. ABA (50 µM)

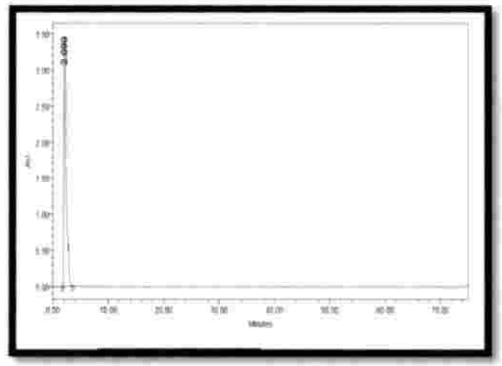
Fig.2 HPLC chromatograms of phenyl propanoid glycosides in callus treated with elicitors



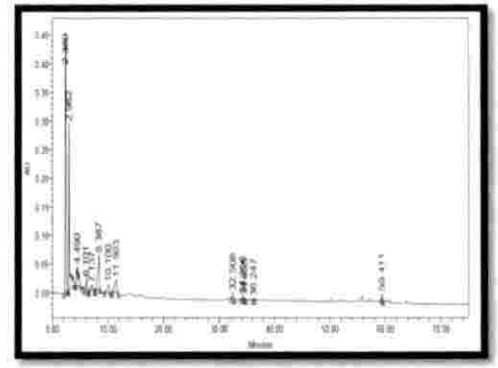
g. Yeast (1 g l^{-1})



h. Yeast (1.5 g l^{1})



i. Control (callus)



j. Control (dried root)

Fig.2 HPLC chromatograms of phenyl propanoid glycosides in different treatments

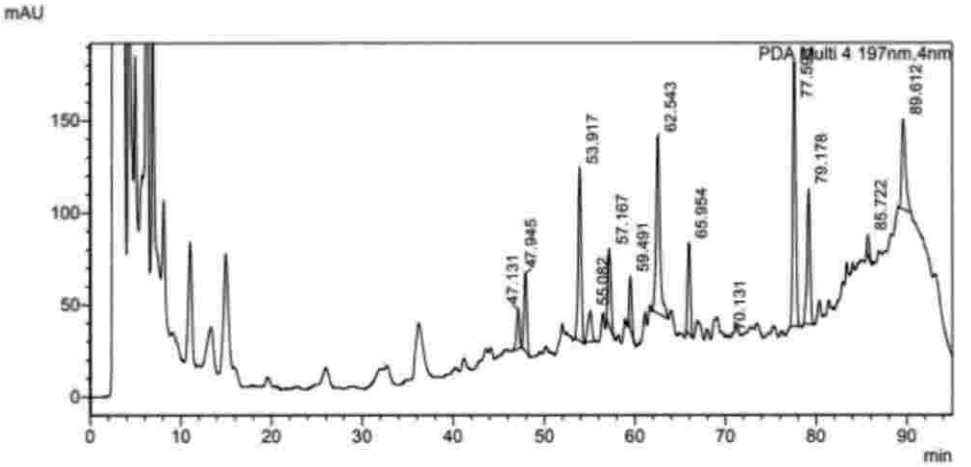


Fig.3 HPLC chromatogram of phenyl propanoid glycosides in callus treated with SA (40 µM) at 197 nm

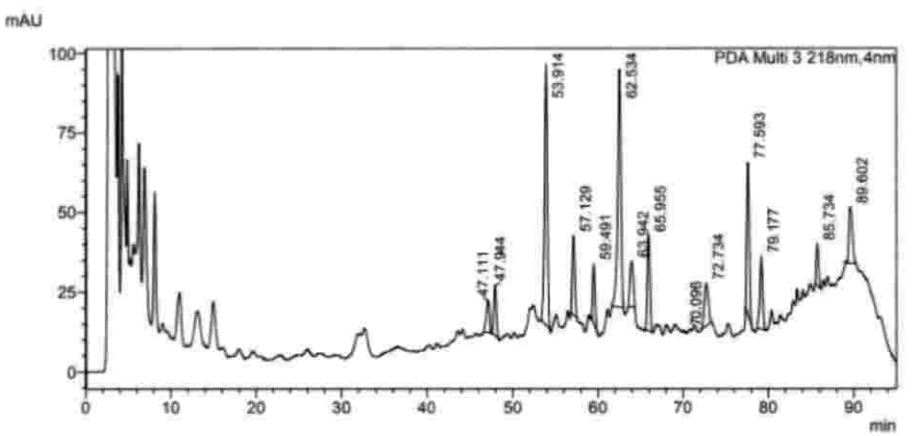


Fig.4 HPLC chromatogram of phenyl propanoid glycosides in callus treated with SA (40 µM) at 218 nm

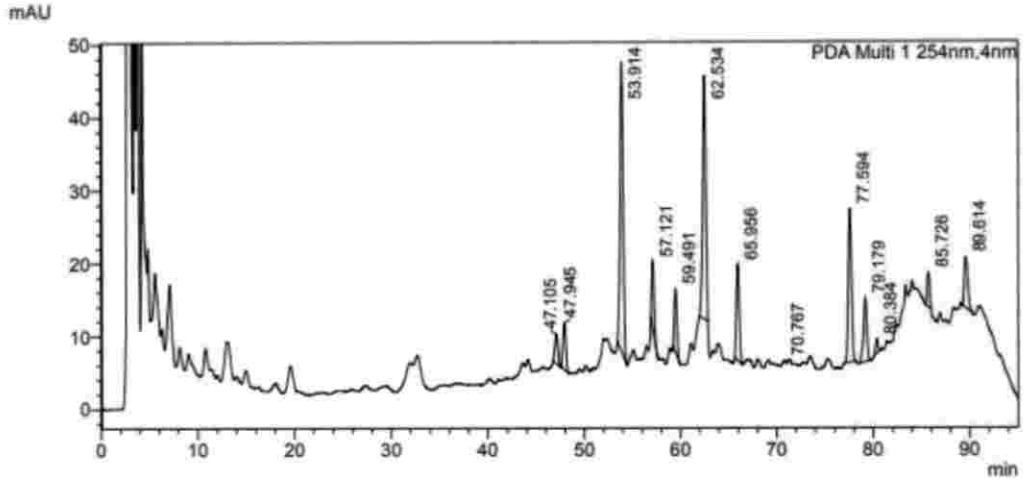


Fig.5 HPLC chromatogram of phenyl propanoid glycosides in callus treated with SA (40 µM) at 254 nm

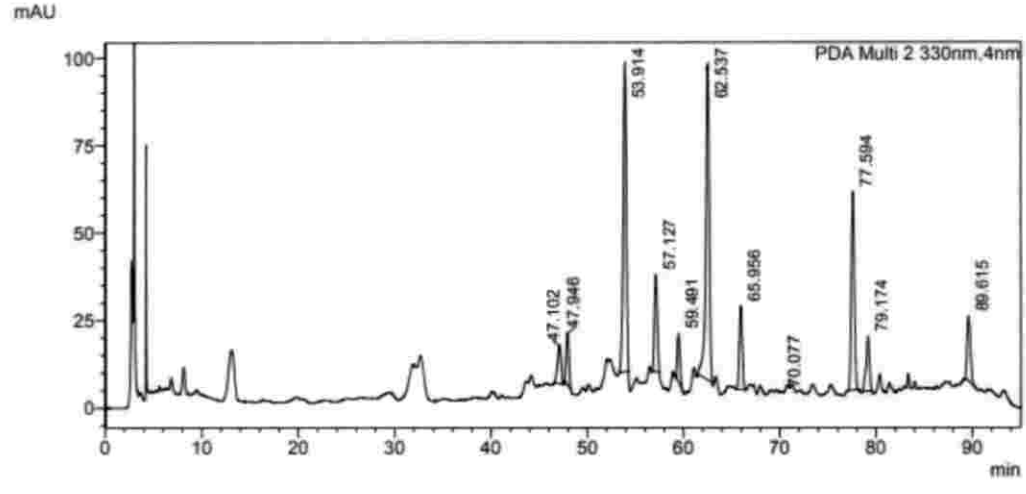


Fig.6 HPLC chromatogram of phenyl propanoid glycosides in callus treated with SA (40 µM) at 330 nm

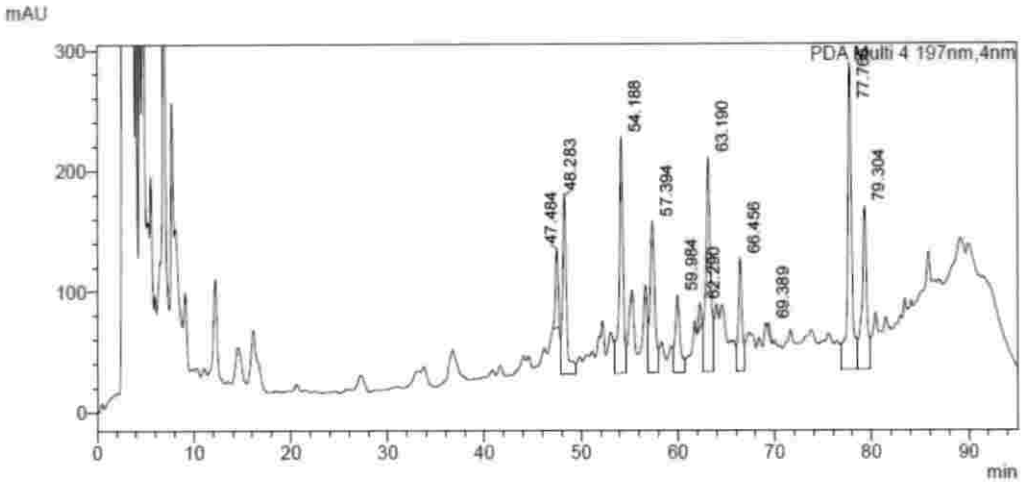


Fig.7 HPLC chromatogram of phenyl propanoid glycosides in callus treated with SA (100 µM) at 197 nm

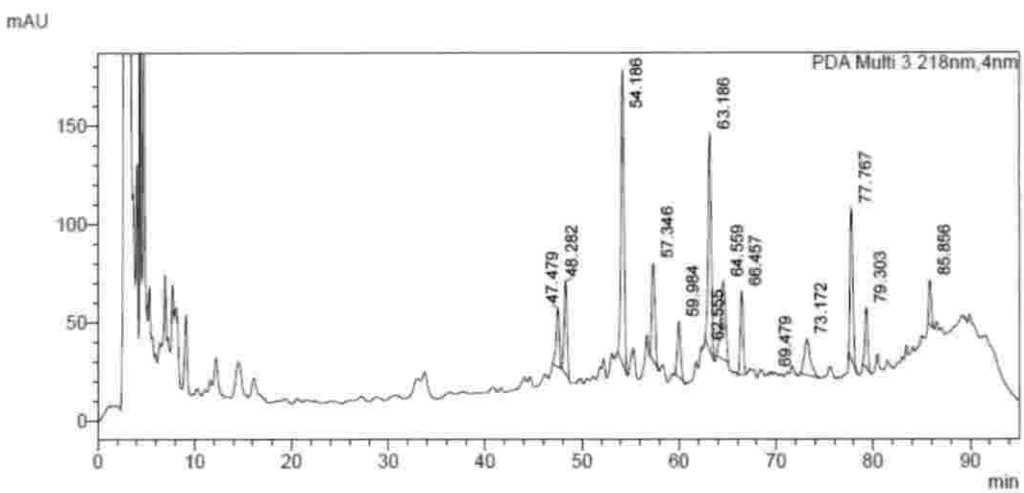


Fig.8 HPLC chromatogram of phenyl propanoid glycosides in callus treated with SA (100 µM) at 218 nm

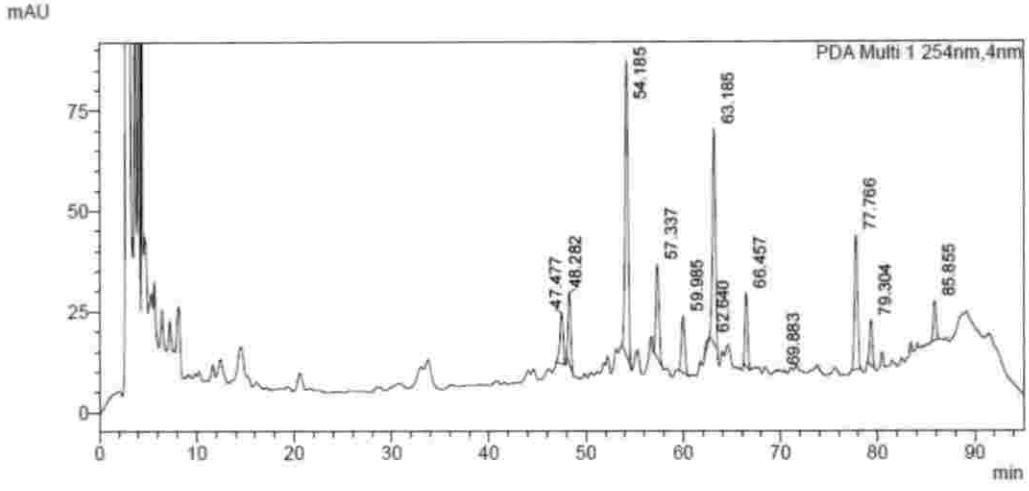


Fig.9 HPLC chromatogram of phenyl propanoid glycosides in callus treated with SA (100 µM) at 254 nm

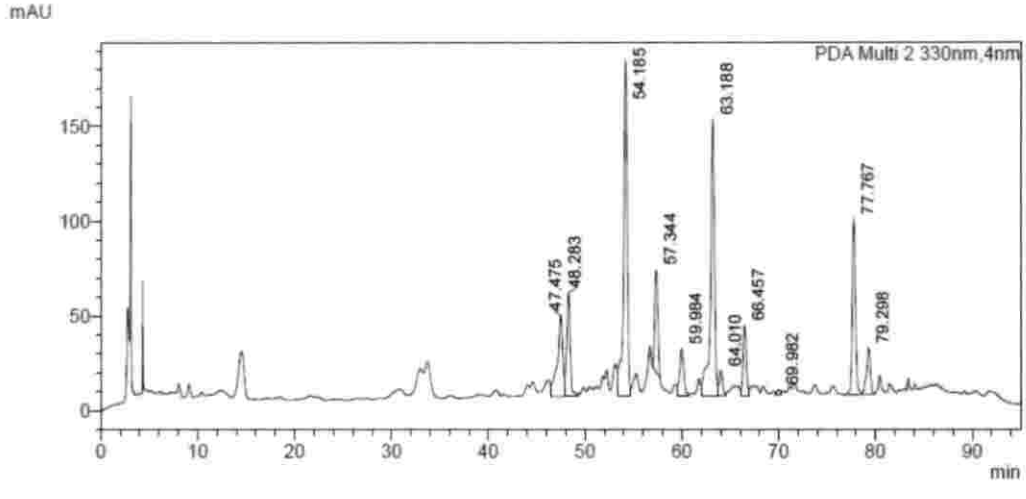


Fig.10 HPLC chromatogram of phenyl propanoid glycosides in callus treated with SA (100 µM) at 330 nm

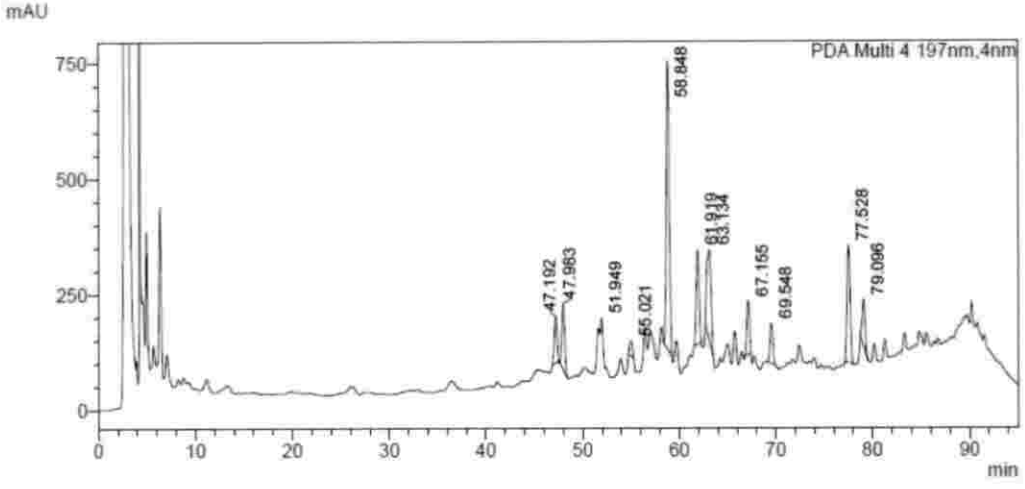


Fig.11 HPLC chromatogram of phenyl propanoid glycosides in callus treated with YE (1 gL^{-1}) at 197 nm

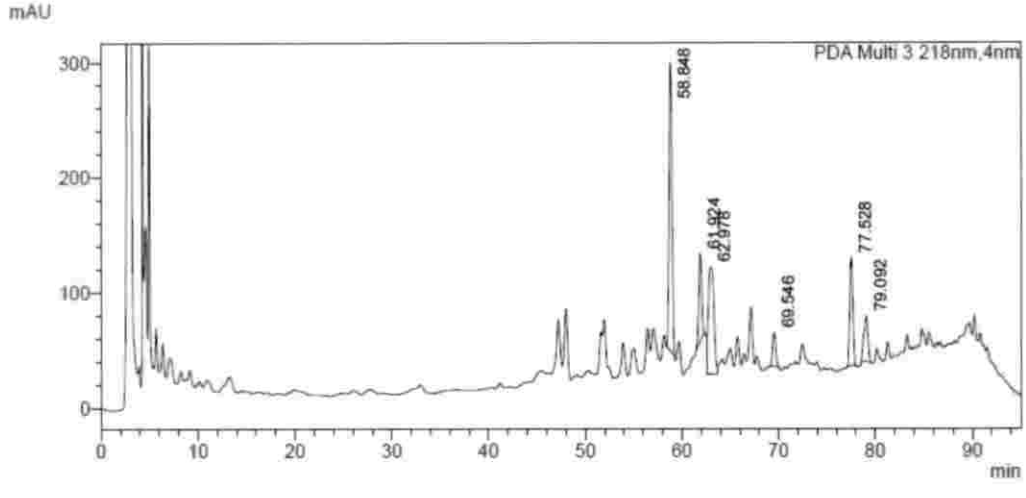


Fig.12 HPLC chromatogram of phenyl propanoid glycosides in callus treated with YE (1 gL^{-1}) at 218 nm

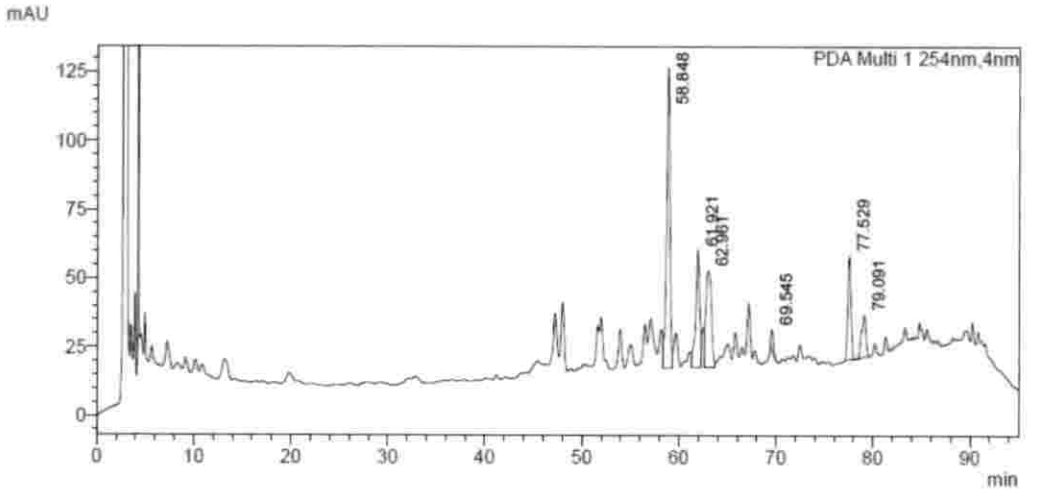


Fig.13 HPLC chromatogram of phenyl propanoid glycosides in callus treated with YE (1 gL^{-1}) at 254 nm

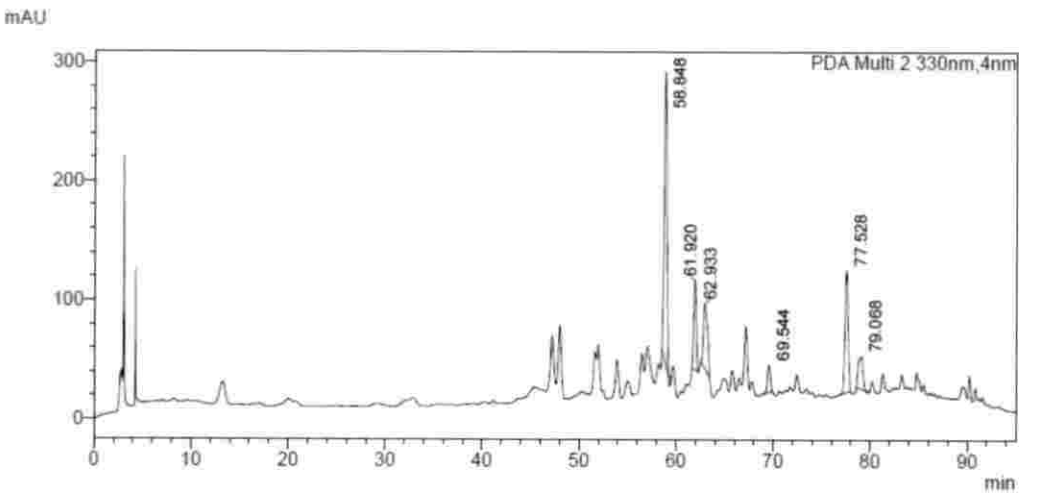


Fig.14 HPLC chromatogram of phenyl propanoid glycosides in callus treated with YE (1 gL^{-1}) at 330 nm

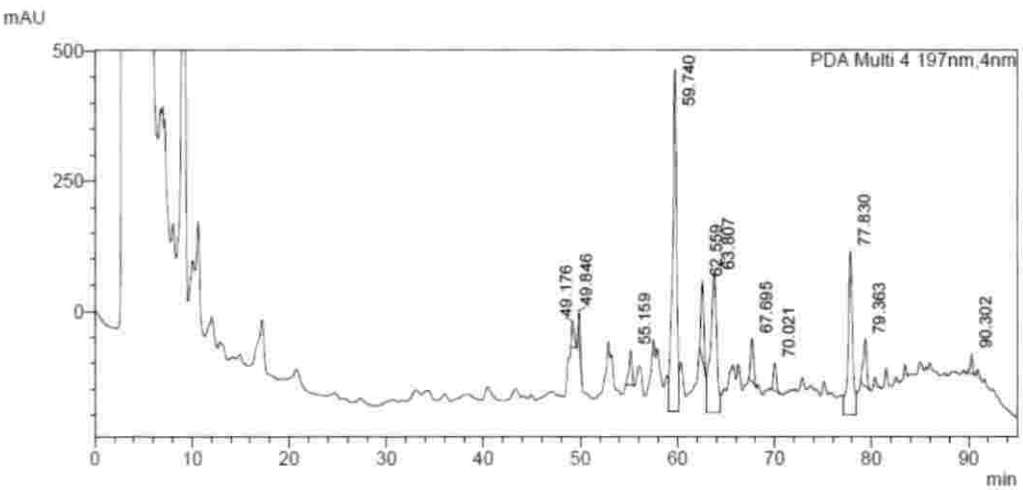


Fig.15 HPLC chromatogram of phenyl propanoid glycosides in callus treated with YE (1.5 gL^{-1}) at 197 nm

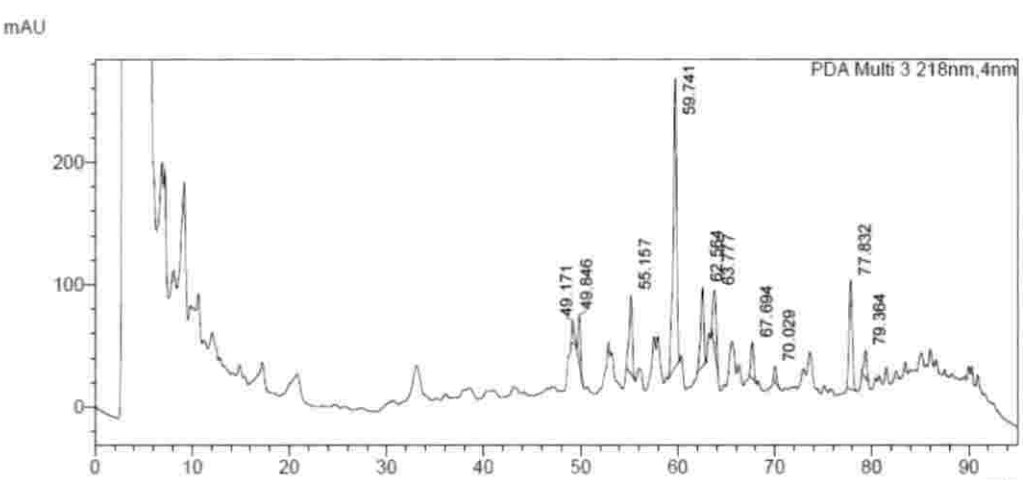


Fig.16 HPLC chromatogram of phenyl propanoid glycosides in callus treated with YE (1.5 gL^{-1}) at 218 nm

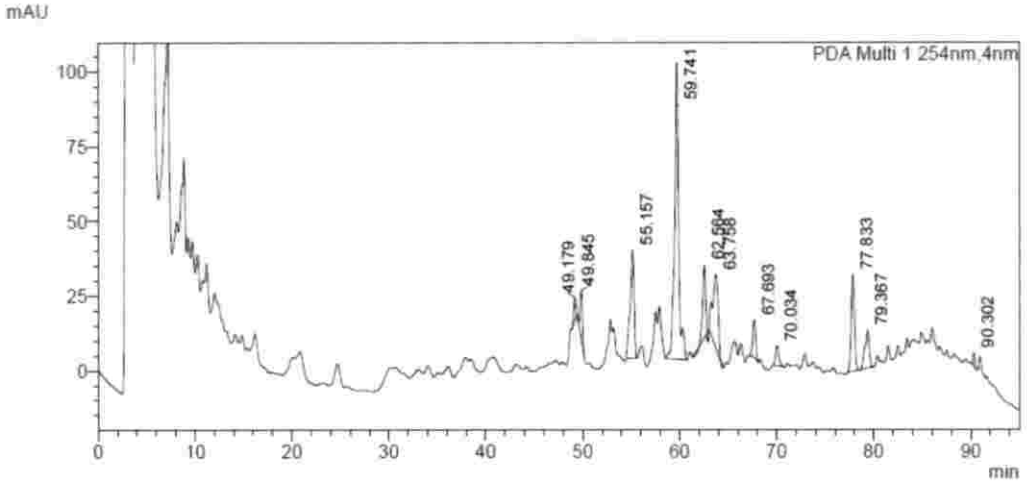


Fig.17 HPLC chromatogram of phenyl propanoid glycosides in callus treated with YE (1.5 gL^{-1}) at 254 nm

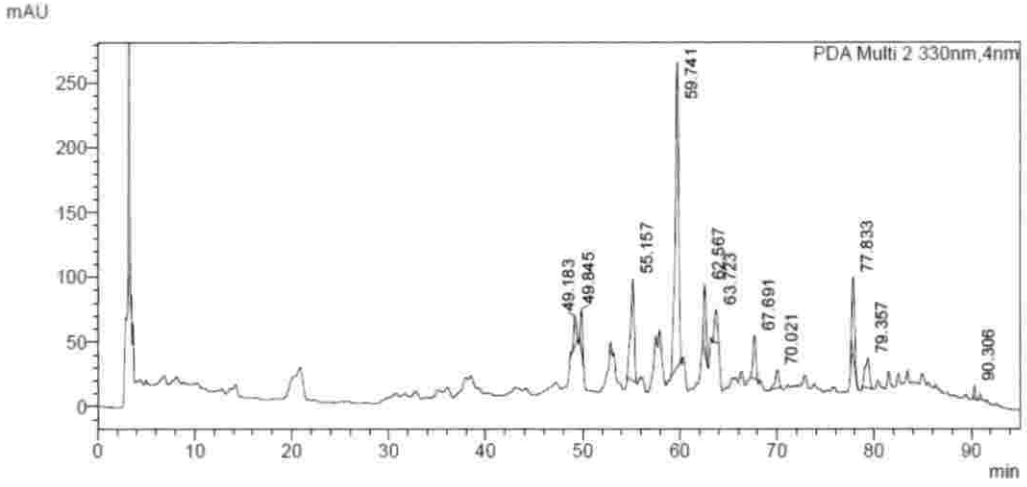


Fig.18 HPLC chromatogram of phenyl propanoid glycosides in callus treated with YE (1.5 gL^{-1}) at 330 nm

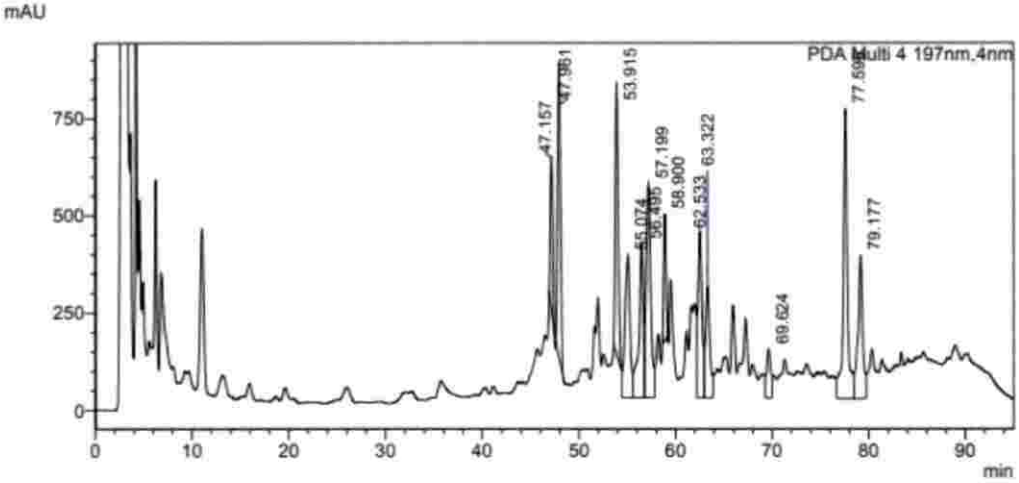


Fig.19 HPLC chromatogram of phenyl propanoid glycosides in control callus at 197 nm

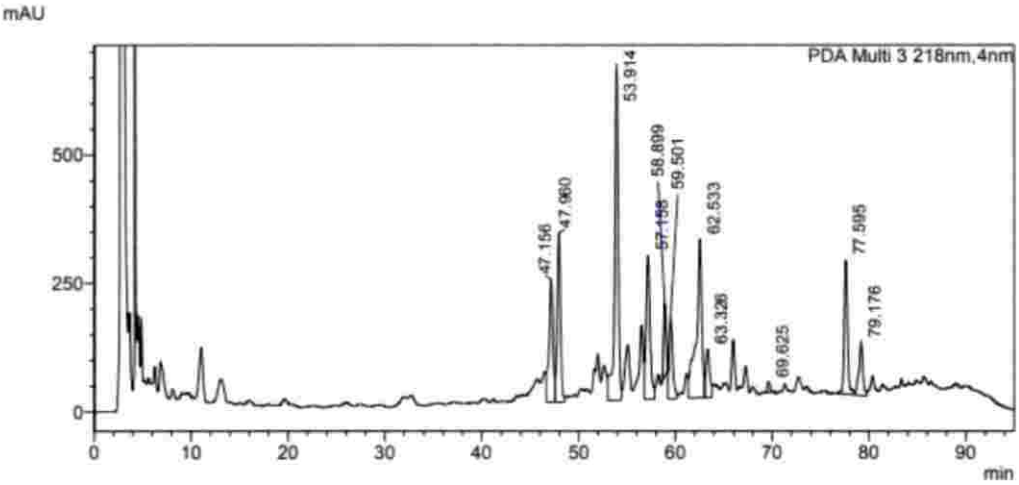


Fig.20 HPLC chromatogram of phenyl propanoid glycosides in control callus at 218 nm

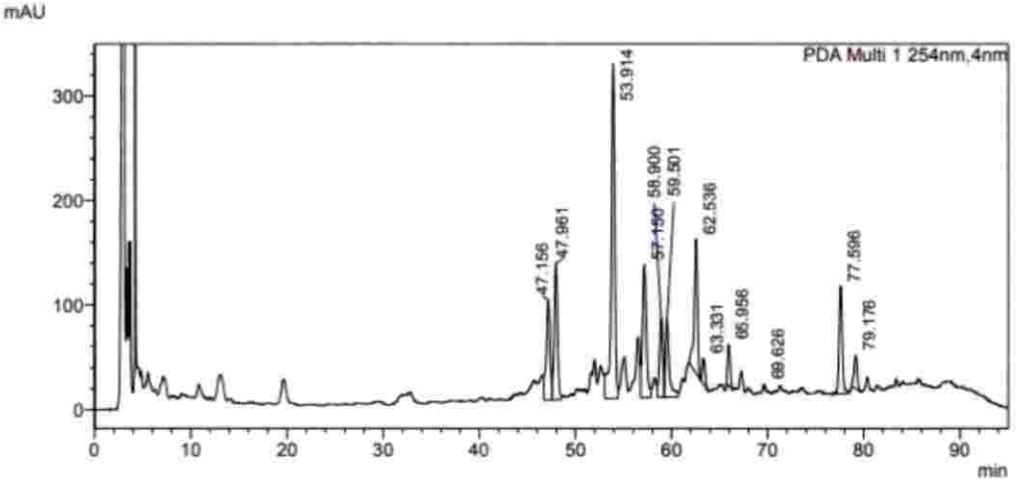


Fig.21 HPLC chromatogram of phenyl propanoid glycosides in control callus at 254 nm

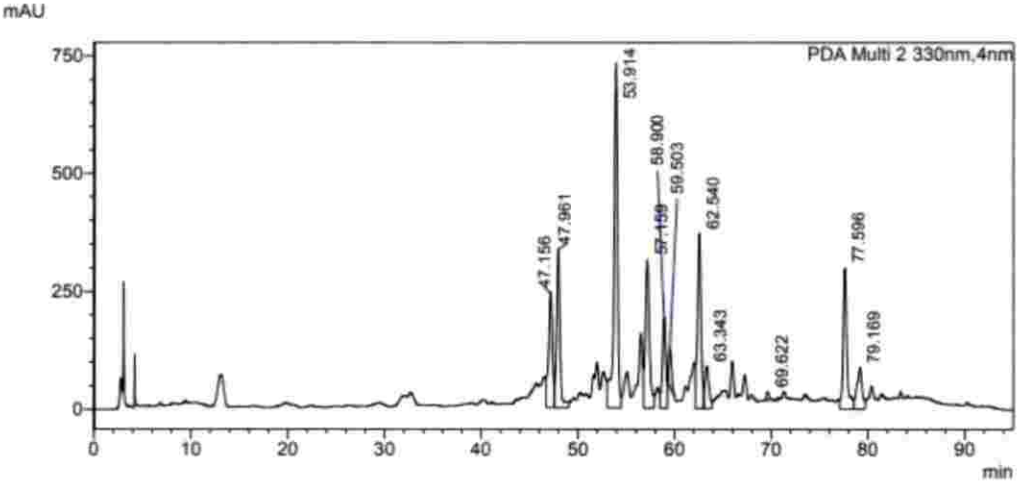


Fig.22 HPLC chromatogram of phenyl propanoid glycosides in control callus at 330 nm

Table 6. Details of chromatogram of extract of callus treated with SA (40 µM) obtained at 197 nm

Peak	Retention Time	Area	Area%	Height%
1	47.131	500171	3.049	3.231
2	47.945	931737	5.680	6.402
3	53.917	2229261	13.591	13.958
4	55.082	509175	3.104	2.488
5	57.167	1014707	6.186	6.322
6	59.491	550604	3.357	4.287
7	62.543	2792492	17.025	14.364
8	65.954	1052323	6.416	7.406
9	70.131	9330	0.057	0.118
10	77.593	3377958	20.594	21.481
11	79.178	1752307	10.683	10.991
12	85.722	196228	1.196	1.678
13	89.612	1486276	9.061	7.277
Total		16402569	100.000	100.000

Table 7. Details of chromatogram of extract of callus treated with SA (40 µM) obtained at 218 nm

Peak	Retention Time	Area	Area%	Height%
1	47.111	264084	2.841	2.607
2	47.944	330385	3.554	4.065
3	53.914	1946239	20.938	21.209
4	57.129	535935	5.766	6.447
5	59.491	381060	4.100	4.864
6	62.534	1971073	21.205	19.426
7	63.942	410810	4.420	3.750
8	65.955	667399	7.180	7.783
9	70.096	8021	0.086	0.133
10	72.734	454063	4.885	3.498
11	77.593	1014782	10.917	12.414

12	79.177	536164	5.768	5.840
13	85.734	257700	2.772	3.409
14	89.602	517484	5.567	4.556
Total		9295199	100.000	100.000

Table 8. Details of chromatogram of extract of callus treated with SA (40 µM) obtained at 254 nm

Peak	Retention Time	Area	Area%	Height%
1	47.105	89403	2.525	2.520
2	47.945	137199	3.876	4.042
3	53.914	922000	26.045	25.134
4	57.121	161209	4.554	5.789
5	59.491	170988	4.830	5.505
6	62.534	759883	21.466	21.074
7	65.956	283148	7.999	8.304
8	70.767	5360	0.151	0.220
9	77.594	492921	13.924	13.253
10	79.179	214280	6.053	5.468
11	80.384	33123	0.936	1.256
12	85.726	90109	2.545	3.023
13	89.614	180389	5.096	4.413
Total		3540011	100.000	100.000

Table 9. Details of chromatogram of extract of callus treated with SA (40 µM) obtained at 330 nm

Peak	Retention Time	Area	Area%	Height%
1	47.102	278203	3.252	3.041
2	47.946	297605	3.479	4.123
3	53.914	2049938	23.966	24.541
4	57.127	604742	7.07	7.654
5	59.491	272783	3.189	3.781
6	62.537	2345948	27.427	25.026
7	65.956	523750	6.123	6.638

8	70.077	2370	0.028	0.054
9	77.594	1326873	15.513	15.738
10	79.174	389573	4.555	4.308
11	89.615	461747	5.398	5.096
Total		8553532	100.000	100.000

Table 10. Details of chromatogram of extract of callus treated with SA (100 µM) obtained at 197 nm

Peak	Retention Time	Area	Area%	Height%
1	47.484	1298827	3.326	5.136
2	48.283	4218495	10.804	11.523
3	54.188	5701136	14.601	15.195
4	57.394	4152017	10.633	9.754
5	59.984	2285313	5.853	4.942
6	62.29	347208	0.889	1.555
7	63.19	6056433	15.511	13.817
8	66.456	2495812	6.392	7.244
9	69.389	120140	0.308	0.566
10	77.766	7805432	19.990	19.769
11	79.304	4566324	11.694	10.499
Total		39047138	100.000	100.000

Table 11. Details of chromatogram of extract of callus treated with SA (100 µM) obtained at 218 nm

Peak	Retention Time	Area	Area%	Height%
1	47.479	700568	4.642	4.627
2	48.282	997995	6.612	7.317
3	54.186	3291893	21.811	23.009
4	57.346	1131570	7.497	7.688
5	59.984	685603	4.543	4.396
6	62.555	17519	0.116	-0.008
7	63.186	2532924	16.782	17.058
8	64.559	1371915	9.090	6.119

9	66.457	887465	5.880	6.590
10	69.479	15464	0.102	0.128
11	73.172	813833	5.392	2.839
12	77.767	1575808	10.441	11.906
13	79.303	649718	4.305	4.790
14	85.856	420705	2.787	3.542
Total		15092978	100.000	100.000

Table 12. Details of chromatogram of extract of callus treated with SA (100 µM) obtained at 254 nm

Peak	Retention Time	Area	Area%	Height%
1	47.477	293745	5.004	4.730
2	48.282	345200	5.880	6.613
3	54.185	1607451	27.381	27.458
4	57.337	539224	9.185	8.790
5	59.985	349205	5.948	5.246
6	62.64	30655	0.522	-0.013
7	63.185	1184944	20.184	20.057
8	66.457	357794	6.095	6.836
9	69.883	-2094	-0.036	-0.008
10	77.766	739985	12.605	12.474
11	79.304	225385	3.839	4.158
12	85.855	199123	3.392	3.658
Total		5870618	100.000	100.000

Table 13. Details of chromatogram of extract of callus treated with SA (100 µM) obtained at 330 nm

Peak	Retention Time	Area	Area%	Height%
1	47.475	1619388	8.937	6.404
2	48.283	1427503	7.878	8.193
3	54.185	4733445	26.122	26.359
4	57.344	1231172	6.794	8.064
5	59.984	742692	4.099	3.705

6	63.188	4204434	23.203	21.803
7	64.01	334670	1.847	1.968
8	66.457	848663	4.683	5.551
9	69.982	89617	0.495	0.455
10	77.767	2217007	12.235	13.839
11	79.298	671898	3.708	3.659
Total		18120490	100.000	100.000

Table 14. Details of chromatogram of extract of callus treated with YE (1 gL^{-1}) obtained at 197 nm

Peak	Retention Time	Area	Area %	Height %
1	47.192	2061146	3.6454	5.702
2	47.983	2999161	5.3044	8.036
3	51.949	301690	0.5336	1.745
4	55.021	707292	1.2509	1.890
5	58.848	13611151	24.073	35.190
6	61.919	9385677	16.600	15.696
7	63.134	11701898	20.696	15.713
8	67.155	2203371	3.897	6.523
9	69.548	5852112	10.350	6.956
10	77.528	8392035	14.842	16.496
11	79.096	7597930	13.438	9.950
Total		56540803	100.000	100.000

Table 15. Details of chromatogram of extract of callus treated with YE (1 gL^{-1}) obtained at 218 nm

Peak	Retention Time	Area	Area %	Height %
1	58.848	5450760	36.826	43.129
2	61.924	1578313	10.663	12.977
3	62.978	3814435	25.771	15.981
4	69.546	651485	4.401	5.032

5	77.528	2147395	14.508	16.087
6	79.092	1159079	7.831	6.793
Total		14801467	100.000	100.000

Table 16. Details of chromatogram of extract of callus treated with YE (1 gL^{-1}) obtained at 254 nm

Peak	Retention Time	Area	Area %	Height %
1	58.848	2665067	38.975	44.317
2	61.921	1228755	17.970	17.078
3	62.961	1453052	21.250	14.170
4	69.545	127862	1.870	3.014
5	77.529	873064	12.768	15.260
6	79.091	490082	7.167	6.162
Total		6837883	100.000	100.000

Table 17. Details of chromatogram of extract of callus treated with YE (1 gL^{-1}) obtained at 330 nm

Peak	Retention Time	Area	Area %	Height %
1	58.848	4967816	41.948	46.363
2	61.92	1419359	11.985	14.218
3	62.933	1707006	14.414	10.396
4	69.544	497474	4.201	4.327
5	77.528	2362151	19.946	19.497
6	79.068	888959	7.506	5.198
Total		11842766	100.000	100.000

Table 18. Details of chromatogram of extract of callus treated with YE (1.5 gL^{-1}) obtained at 197 nm

Peak	Retention Time	Area	Area%	Height%
1	49.176	863101	1.602	2.720
2	49.846	714690	1.327	3.005
3	55.159	1521770	2.825	3.712
4	59.74	19902495	36.951	35.974
5	62.559	2879621	5.346	8.338
6	63.807	11968425	22.220	14.498
7	67.695	1688922	3.136	4.546
8	70.021	1177028	2.185	2.922
9	77.83	10139994	18.826	17.163
10	79.363	2358273	4.378	4.992
11	90.302	648081	1.203	2.129
Total		53862401	100.000	

Table 19. Details of chromatogram of extract of callus treated with YE (1.5 gL^{-1}) obtained at 218 nm

Peak	Retention Time	Area	Area%	Height%
1	49.171	297296	2.046	2.958
2	49.846	703282	4.840	6.480
3	55.157	1511115	10.400	10.338
4	59.741	5981764	41.170	37.673
5	62.564	1500818	10.329	10.399
6	63.777	1155069	7.950	7.460
7	67.694	598306	4.118	4.775
8	70.029	226098	1.556	2.035
9	77.832	2084880	14.349	14.208
10	79.364	470835	3.241	3.672
Total		14529464	100.000	100.000

Table 20. Details of chromatogram of extract of callus treated with YE (1.5 gL^{-1}) obtained at 254 nm

Peak	Retention time	Area	Area%	Height%
1	49.179	130191	1.729	2.845
2	49.845	283276	3.763	5.867
3	55.157	1016505	13.504	13.031
4	59.741	2982670	39.623	35.741
5	62.564	477753	6.347	8.769
6	63.758	971955	12.912	8.921
7	67.693	272448	3.619	4.576
8	70.034	159374	2.117	2.373
9	77.833	782738	10.398	11.652
10	79.367	393048	5.221	4.557
11	90.302	57755	0.767	1.669
Total		7527713	100.000	100.000

Table 21. Details of chromatogram of extract of callus treated with YE (1.5 gL^{-1}) obtained at 330 nm

Peak	Retention time	Area	Area%	Height%
1	49.183	457153	3.415	3.972
2	49.845	469825	3.509	5.542
3	55.157	2104429	15.719	13.397
4	59.741	5950010	44.443	39.994
5	62.567	885254	6.612	8.746
6	63.723	542189	4.050	4.322
7	67.691	680045	5.080	5.702
8	70.021	361769	2.702	2.388
9	77.833	1085153	8.106	10.270
10	79.357	741756	5.541	3.887
11	90.306	110231	0.823	1.780
Total		13387815	100.000	100.000

Table 22. Details of chromatogram of extract of control callus obtained at 197 nm

Peak	Retention Time	Area	Area %	Height %
1	47.157	6840097	4.555	7.222
2	47.961	16587010	11.046	14.036
3	53.915	14805361	9.859	12.815
4	55.074	13553879	9.026	6.727
5	56.495	13360187	8.897	7.249
6	57.199	18273126	12.169	10.129
7	58.9	5782821	3.851	5.971
8	62.533	12333566	8.213	7.831
9	63.322	9189347	6.119	5.238
10	69.624	3879471	2.583	2.298
11	77.595	22982289	15.305	13.686
12	79.177	12579093	8.377	6.798
Total		150166246	100.000	100.000

Table 23. Details of chromatogram of extract of control callus obtained at 218 nm

Peak	Retention Time	Area	Area %	Height %
1	47.156	6840923	9.457	9.254
2	47.96	8159497	11.280	12.719
3	53.914	17163076	23.727	25.290
4	57.158	8361765	11.560	10.822
5	58.899	2612221	3.611	5.320
6	59.501	4177101	5.775	6.141
7	62.533	12292337	16.993	11.954
8	63.326	2802992	3.875	3.635
9	69.625	439445	0.608	0.811
10	77.595	6452880	8.921	10.061
11	79.176	3034113	4.194	3.994
Total		72336351	100.000	100.000

Table 24. Details of chromatogram of extract of control callus obtained at 254 nm

Peak	Retention Time	Area	Area %	Height %
1	47.156	2687049	9.078	8.305
2	47.961	3248846	10.976	11.369
3	53.914	8310637	28.076	27.779
4	57.15	3677295	12.423	11.069
5	58.9	1812421	6.123	6.384
6	59.501	2165113	7.314	6.470
7	62.536	3169343	10.707	11.102
8	63.331	496932	1.679	2.107
9	65.956	740831	2.503	3.417
10	69.626	48874	0.165	0.315
11	77.596	2499748	8.445	8.915
12	79.176	743264	2.511	2.770
Total		29600352	100.000	100.000

Table 25. Details of chromatogram of extract of control callus obtained at 330 nm

Peak	Retention Time	Area	Area %	Height %
1	47.156	6964007	9.209	8.834
2	47.961	8846715	11.698	12.149
3	53.914	19360715	25.602	26.575
4	57.159	9425788	12.464	11.333
5	58.9	4774613	6.314	6.826
6	59.503	1682605	2.225	3.019
7	62.54	9934694	13.137	13.399
8	63.343	2687776	3.554	3.244
9	69.622	350904	0.464	0.639
10	77.596	8188335	10.828	10.795
11	79.169	3406732	4.505	3.188
Total		75622884	100.000	100.000

4.3 GENE EXPRESSION PROFILING

4.3.1 Primer Designing

The primers for three key genes of acteoside biosynthesis pathway, such as *PAL* (phenylalanine ammonia-lyase), *HCT* (Shikimate O-hydroxycinnamoyl transferase) and *UGT* (UDP-glucosyl transferase) were designed using Primer Express software based on the sequences of related species retrieved from NCBI database. In the case of *PAL* and *HCT* genes, degenerate primers were designed based on sequence similarity of closely related species of *Artanema* and for *UGT* gene, sequence from the related species was used for designing gene specific primers. The designed primers were analysed using mfold web server to confirm the absence of secondary structure formation at the primer binding site. The details of the primers designed are given in Table 26.

Table 26. Sequence of primers designed

Gene	Primer Sequence (5'...3')	Amplicon Size (bp)
PAL F *PAL* R	CTCGTYCCYCTCCTACAT ARATGRTCGGTGAACTC	220
HCT F *HCT* R	GTGCTSTTYGTGGGCGGA CRATGGTGYTGCATTCC	190
UGT F *UGT* R	CCCCAAATGGAAGAAATAGC GAATTCCAACCGCAATG	243

β-*ACTIN* F β-*ACTIN* R	GCCACAGGTGCCTATCTAT AAATGCGAGCTTCTCCTTCA	125

4.3.2 Isolation of RNA

From the treated calli, the total RNA was isolated using Trizol method after 24h and 48h of elicitation. The integrity of the isolated RNA was estimated by agarose gel electrophoresis (2%). Two intact bands of 28S and 18S obtained and their fluorescence indicated good quality of RNA isolated. The gel profile of RNA isolated after 24 h and 48 h of elicitation are shown in Plates 2 and 3.

4.3.3 Quantification of RNA Using Spectrophotometer

The quantity and quality of isolated RNA after 24 and 48 h of elicitation were estimated by measuring the absorbance values at wavelengths 260 nm and 280 nm using a UV spectrophotometer. The A_{260}/A_{280} value of the RNA isolated from the callus (elicited for 24 h and 48 h) ranged from 1.84 to 2.11 indicating the good quality of RNA. The details are presented in Tables 27 and 28.

Table 27. Quantity and quality of RNA isolated 24h after elicitation

Treatment	Concentration (μg ml^{-1})	A_{260}/A_{280}
SA (40 μM)	2237.8	1.93
SA (100 μM)	1798.6	1.96
MJ (15 μM)	1428.6	2.01
MJ (25 μM)	2398.5	1.99
ABA (20 μM)	1678.6	1.88

ABA (50 µM)	1988.3	1.97
YE (1 gL^{-1})	2152.3	2.01
YE (1.5 gL^{-1})	1797.3	2.03
Control (callus)	1432.8	1.89

Table 28. Quantity and quality of RNA isolated 48h after elicitation

Treatment	Concentration (µg ml^{-1})	A_{260}/A_{280}
SA (40 µM)	1861.5	1.84
SA (100 µM)	1795.1	1.93
MJ (15 µM)	1251.2	1.89
MJ (25 µM)	2094.3	1.86
ABA (20 µM)	2206.2	2.01
ABA (50 µM)	1918.7	1.98
YE (1 gL^{-1})	1637.2	2.09
YE (1.5 gL^{-1})	1867.5	2.11
Control (callus)	1387.5	1.82

4.3.4 cDNA Synthesis and Quality Analysis

RNA isolated was reverse transcribed to cDNA using Thermo scientific Verso cDNA synthesis kit. The quality check of the synthesized cDNA was done by PCR using gene specific primer for ß-*ACTIN*. Agarose gel electrophoresis of the amplicon obtained showed the expected size of 125 bp in all the samples indicating the good

quality of cDNA. Plate 4 shows gel picture of PCR amplification product of cDNA using the primer ß-*ACTIN*.

4.3.5 Specificity Check of Primers with cDNA

The cDNA synthesised was amplified using gene specific primers for *PAL* (phenylalanine ammonia-lyase), *HCT* (Shikimate O-hydroxycinnamoyl transferase) and *UGT* (UDP-glucosyl transferase). Agarose gel electrophoresis showed the presence of an amplicon of size 220bp (Plates 5 and 6) with *PAL* gene specific primers with an additional amplicon of 300bp, which may be due to the non specific amplification of degenerate primers. In the case of *UGT* genes, an amplicon of size 243bp (Plates 7 and 8) was obtained where as in case of *HCT* gene; no amplicons of expected size was obtained even after repeating the experiment with gradient PCR (Plate 9).

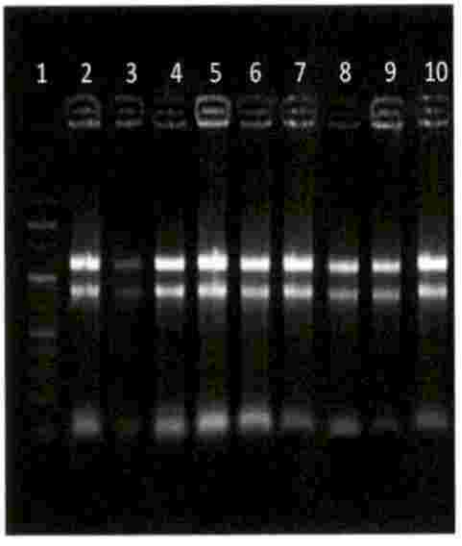


Plate.2 RNA isolated after 24 h of elicitation

Lane 1: 100bp ladder
Lane 2: Control
Lane 3: SA (40 µM)
Lane 4: SA (100 µM)
Lane 5: MJ (15 µM)

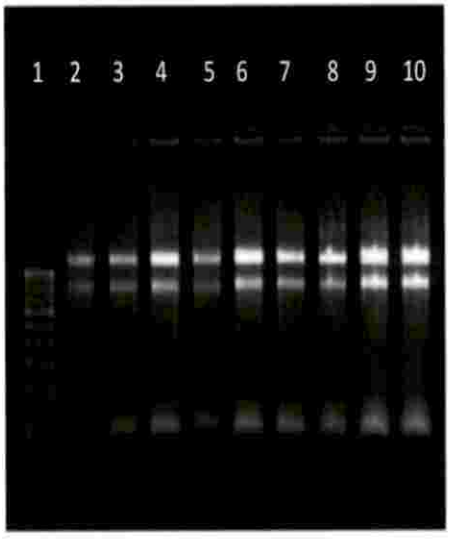


Plate.3 RNA isolated after 48 h of elicitation

Lane 6: MJ (25 µM)
Lane 7: ABA (20 µM)
Lane 8: ABA (50 µM)
Lane 9: YE (1 gL^{-1})
Lane 10:YE (1.5 gL^{-1})

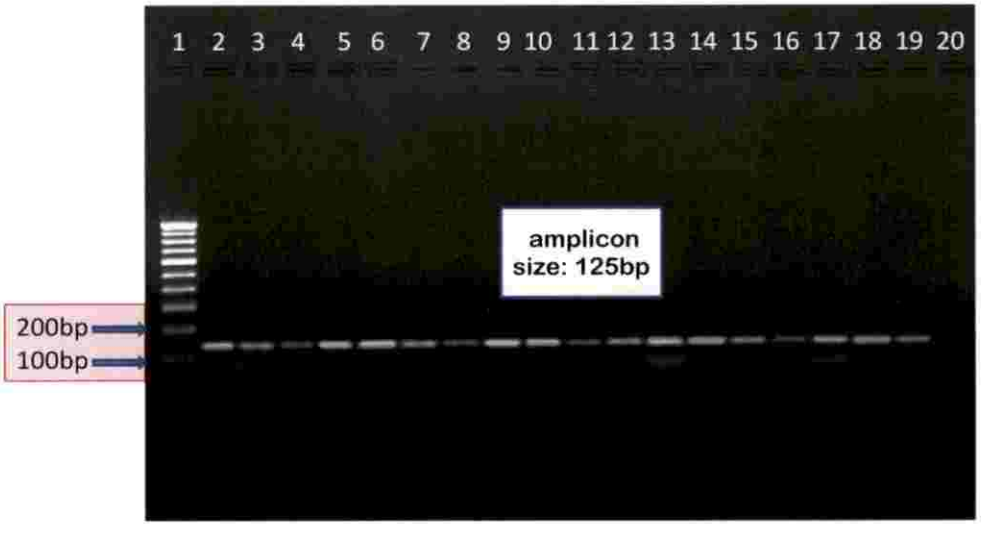


Plate.4 Amplicons obtained by PCR using β-*ACTIN* specific primers

Lane 1: 100bp ladder
Lane 2: Control (24 h)
Lane 3: SA (40 µM) (24 h)
Lane 4: SA (100 µM) (24 h)
Lane 5: MJ (15 µM) (24 h)
Lane 6: MJ (25 µM) (24 h)
Lane 7: ABA (20 µM) (24 h)
Lane 8: ABA (50 µM) (24 h)
Lane 9: YE (1 gL^{-1}) (24 h)
Lane 10: YE (1.5 gL^{-1})(24 h)
Lane 11: Control (48 h)
Lane 12: SA (40 µM) (48 h)
Lane 13: SA (100 µM) (48 h)
Lane 14: MJ (15 µM) (48 h)
Lane 15: MJ (25 µM) (48 h)
Lane 16: ABA (20 µM) (48 h)
Lane 17: ABA (50 µM) (48 h)
Lane 18: YE (1 gL^{-1}) (48 h)
Lane 19: YE (1.5 gL^{-1})(48 h)
Lane 20: Negative control

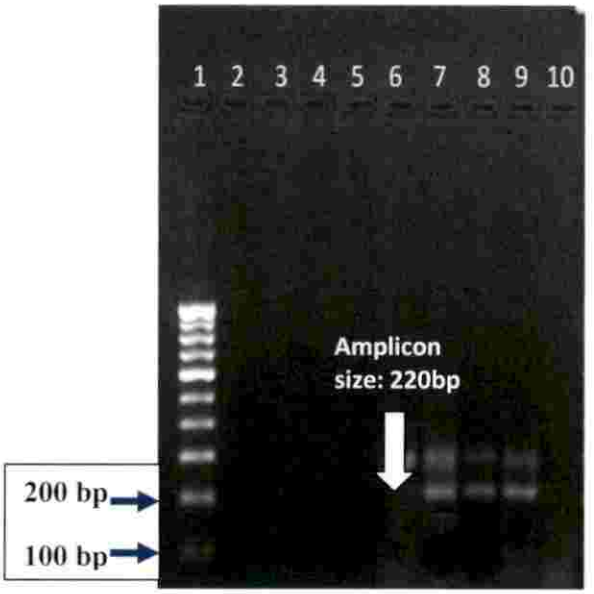


Plate.5 Amplicons obtained by PCR using *PAL* specific primers after 24 h of elicitation

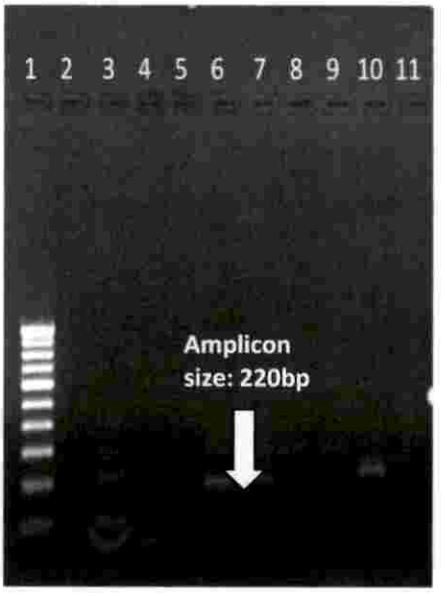


Plate.6 Amplicons obtained by PCR using *PAL* specific primers after 48 h of elicitation

Lane 1: 100bp ladder
Lane 2: Control
Lane 3: SA (40 µM)
Lane 4: SA (100 µM)
Lane 5: MJ (15 µM)
Lane 11: Negative Control

Lane 6: MJ (25 µM)
Lane 7: ABA (20 µM)
Lane 8: ABA (50 µM)
Lane 9: YE (1 gL^{-1})
Lane 10:YE (1.5 gL^{-1})

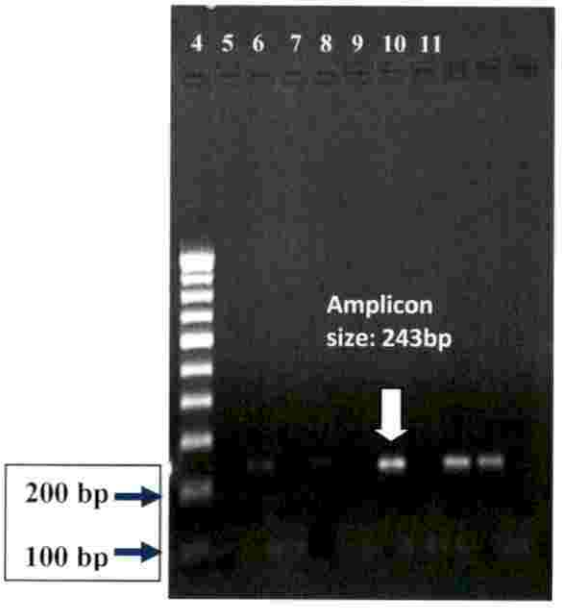


Plate.7 Amplicons obtained by PCR using *UGT* specific primers after 24 h of elicitation

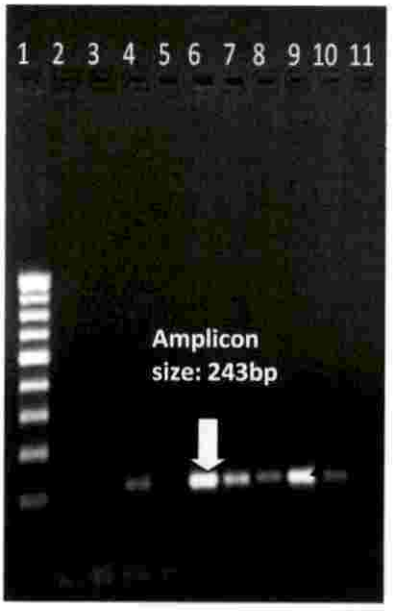


Plate.8 Amplicons obtained by PCR using *UGT* specific primers after 48 h of elicitation

Lane 1: 100bp ladder
Lane 2: Control
Lane 3: SA (40 µM)
Lane 4: SA (100 µM)
Lane 5: MJ (15 µM)
Lane 11: Negative Control

Lane 6: MJ (25 µM)
Lane 7: ABA (20 µM)
Lane 8: ABA (50 µM)
Lane 9: YE(1 gL^{-1})
Lane 10:YE (1.5 gL^{-1})

Plate.9 Amplicons obtained by PCR using *HCT* specific primers after 48h of elicitation

Lane 1: 100bp ladder
Lane 2: Control (24 h)
Lane 3: SA (40 µM) (24 h)
Lane 4: SA (100 µM) (24 h)
Lane 5: MJ (15 µM) (24 h)
Lane 6: MJ (25 µM) (24 h)
Lane 7: ABA (20 µM) (24 h)
Lane 8: ABA (50 µM) (24 h)
Lane 9: YE (1 gL^{-1}) (24 h)
Lane 10: Yeast extract (1.5 gL^{-1}) (24 h)
Lane 11: Control (48 h)
Lane 12: SA (40 µM) (48 h)
Lane 13: SA (100 µM) (48 h)
Lane 14: MJ (15 µM) (48 h)
Lane 15: MJ (25 µM) (48 h)
Lane 16: ABA (20 µM) (48 h)
Lane 17: ABA (50 µM) (48 h)
Lane 18: YE (1 gL^{-1}) (48 h)
Lane 19: YE (1.5 gL^{-1}) (48 h)
Lane 20: Negative control

4.3.5 Real Time PCR

Reverse Transcription quantitative PCR or Real Time PCR was done to analyse the expression profile of key genes of acteoside biosynthesis pathway namely *PAL* (phenylalanine ammonia-lyase), *HCT* (Shikimate O-hydroxycinnamoyl transferase) and *UGT* (UDP-glucosyl transferase). The house keeping gene ß-*ACTIN* was taken as the reference gene for RT-qPCR.

RT-qPCR for each gene was performed with two technical replicates for each sample. Amplification plot and Melt curve plot were generated by the software. The Cq values generated by RT-qPCR was used for further analysis. Figs. 23, 24 and 25 shows amplification plots generated for the primers.

Melt curve plot shows prominent peak for both the primers indicating the specificity of Real time PCR reaction. Figs. 26, 27 and 28 shows melt curve generated for the primers. The Cq values generated by RT-qPCR was used for further analysis. Cq values obtained are given in Tables 29 and 30.

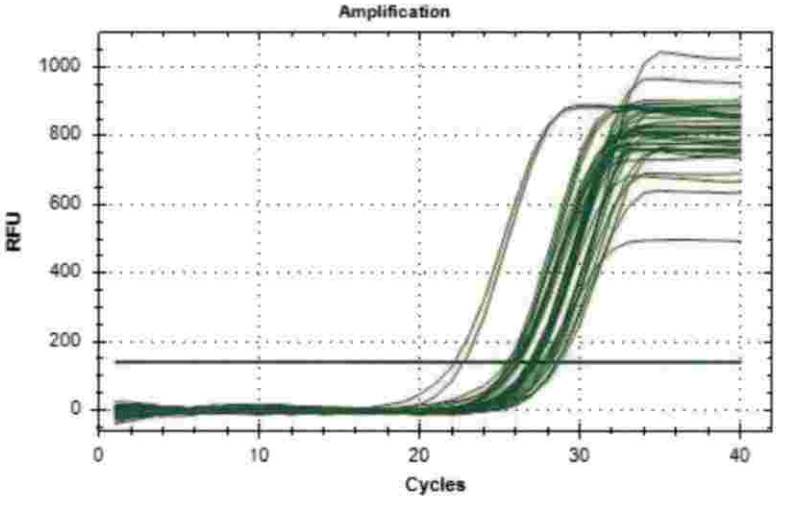


Fig.23 Amplification plot of β-*ACTIN* gene

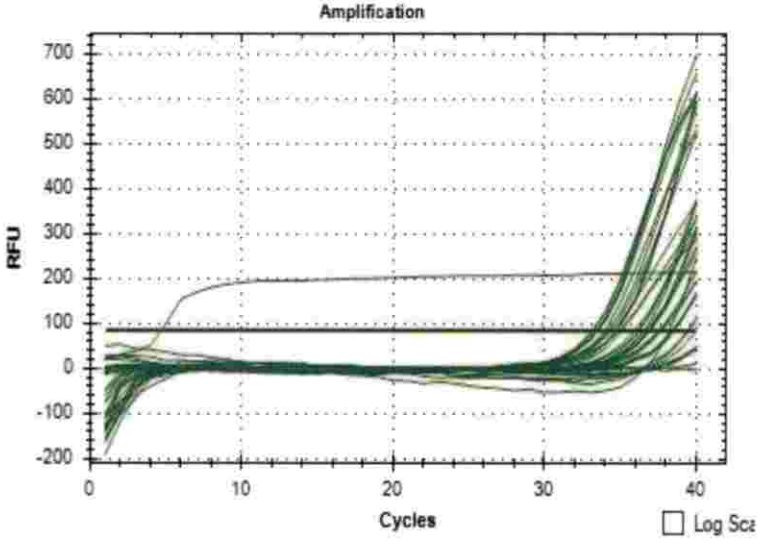


Fig.24 Amplification plot of *PAL* gene

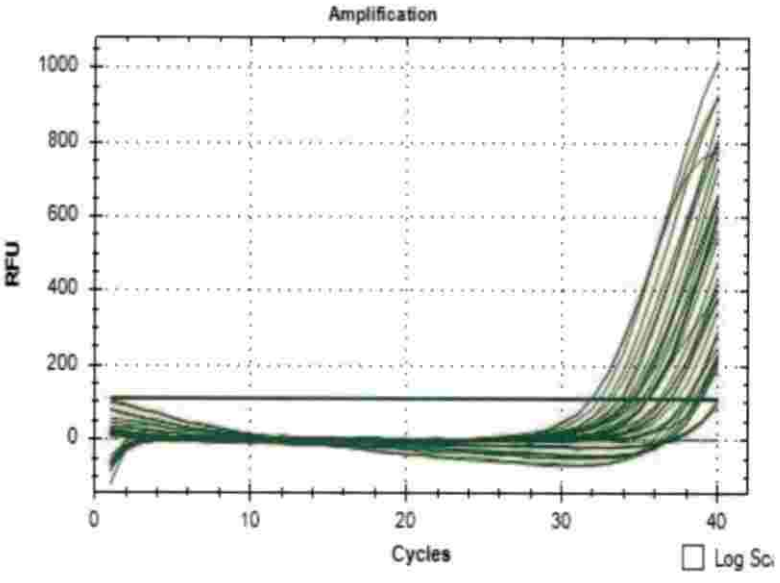


Fig.25 Amplification plot of *UDP-glucosyl transferase* gene

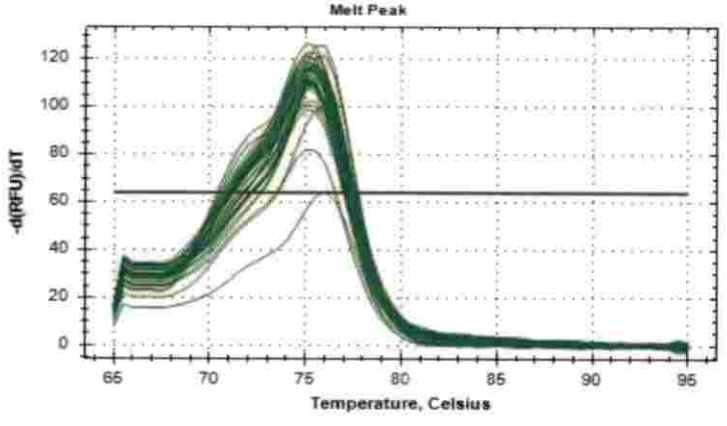


Fig.26 Melt curve of β-*ACTIN* gene

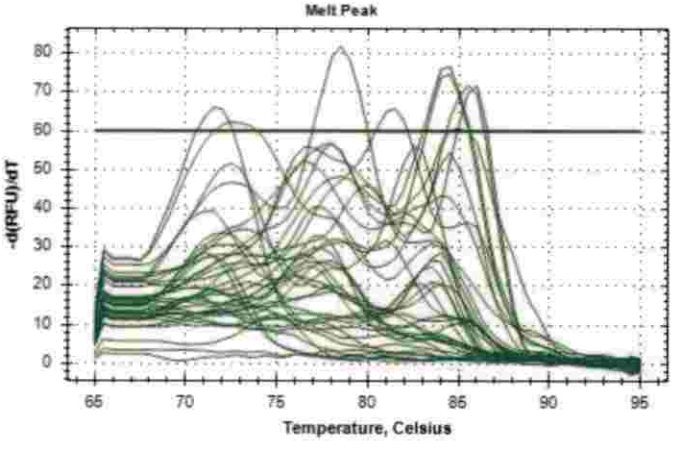


Fig.27 Melt curve of *Phenylalanine ammonia-lyase* gene

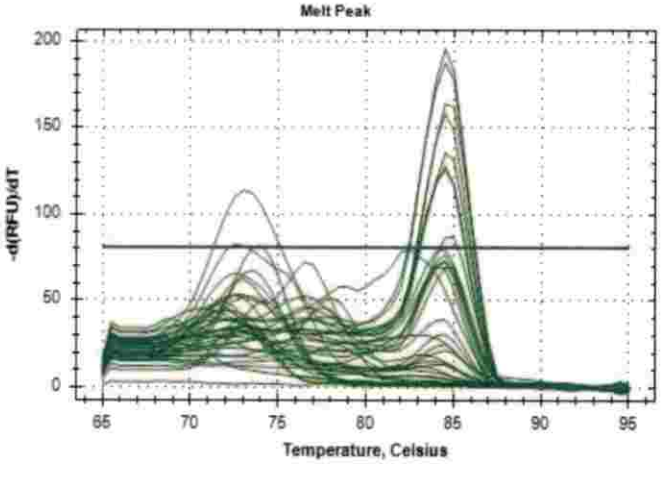


Fig.28 Melt curve of *UDP-glucosyl transferase* gene

Table 29. Cq values generated for each sample after 24 h of elicitation

Treatments (after 24 h elicitation)	ß-*ACTIN*	*PAL*	*UGT*
SA (40 µM)	28.71	33.17	39.04
SA (100 µM)	26.89	37.92	40.00
MJ (15 µM)	29.62	38.33	38.95
MJ (25 µM)	27.40	36.37	40.00
ABA (20 µM)	26.75	35.41	35.44
ABA (50 µM)	26.46	40.00	39.52
YE (1 gL^{-1})	26.67	36.16	37.97
YE (1.5 gL^{-1})	27.92	36.35	37.63
Control (callus)	22.79	38.56	34.64

Table 30. Cq values generated for each sample after 48 h of elicitation

Treatments (after 48 h elicitation)	ß-*ACTIN*	*PAL*	*UGT*
SA (40 µM)	26.26	40.00	40.00
SA (100 µM)	28.51	39.04	33.03
MJ (15 µM)	27.47	39.54	40.00
MJ (25 µM)	27.76	32.92	34.25
ABA (20 µM)	27.61	33.70	35.20
ABA (50 µM)	28.38	34.10	36.68
YE (1 gL^{-1})	27.46	33.34	33.03
YE (1.5 gL^{-1})	29.14	34.15	36.55
Control (callus)	28.27	40.00	40.00

4.4.1 Analysis Using qbase plus Software

Gene expression analysis was performed using "qbase plus" software using ß-*Actin* as the reference gene. Raw Cq values were converted to relative expression

value by qbase plus software and normalized with reference gene. The relative expression of the target genes after 24 and 48 h of elicitation are shown in the Tables 31 and 32.

Table 31. Relative expression values for target genes normalized with reference gene in callus of *A. sesamoides* after 24 h of elicitation

Treatments (after 24h elicitation)	PAL NRQ	UGT NRQ
SA (40 µM)	26.5746	0.74728
SA (100 µM)	0.28	0.10915
MJ (15 µM)	0.00	0.00
MJ (25 µM)	1.1645	0.15502
ABA (20 µM)	1.4514	2.33647
ABA (50 µM)	0.049	0.11311
YE (1 gL^{-1})	0.8157	0.38441
YE (1.5 gL^{-1})	1.6948	1.15003
Control (callus)	0.0105	0.26168

Table 32. Relative expression values for target genes normalized with reference gene in callus of *A. sesamoides* after 48 h of elicitation

Treatments (after 48 h elicitation)	PAL NRQ	UGT NRQ
SA (40µM)	0.0428	0.07049
SA (100µM)	0.3973	42.15599

MJ (15 µM)	0.1362	0.16316
MJ (25 µM)	16.3431	10.69301
ABA (20µM)	8.592	4.99173
ABA (50 µM)	11.0878	3.06016
YE (1 gL^{-1})	9.9225	20.23844
YE (1.5 gL^{-1})	18.151	5.66392
Control (callus)	0.1728	0.28487

After 24h of elicitation 26 fold increase in the expression of *PAL* gene, which acts in the upstream of acteoside biosynthetic pathway, was observed in callus treated with SA (40 µM). Expression of UGT was increased by 2.3 fold. After 48h of elicitation, *PAL* expression showed a drastic decrease in SA treated callus. Upregulation of this gene was noticed in callus treated with yeast extract (18 fold with 1.5 gL^{-1}), MJ (16 fold with 25 µM) and ABA (11 fold with 50 µM). All the elicitors enhanced the expression of *UGT* at 48 h of elicitation. The highest expression (42 fold) was with SA (100 µM), followed by yeast extract (20 fold with 1 gL^{-1}) and MJ (10 fold with 25 µM). A comparison of the PAL and UGT expression at 24 and 48h of elicitation is shown in fig. 29 and 30.

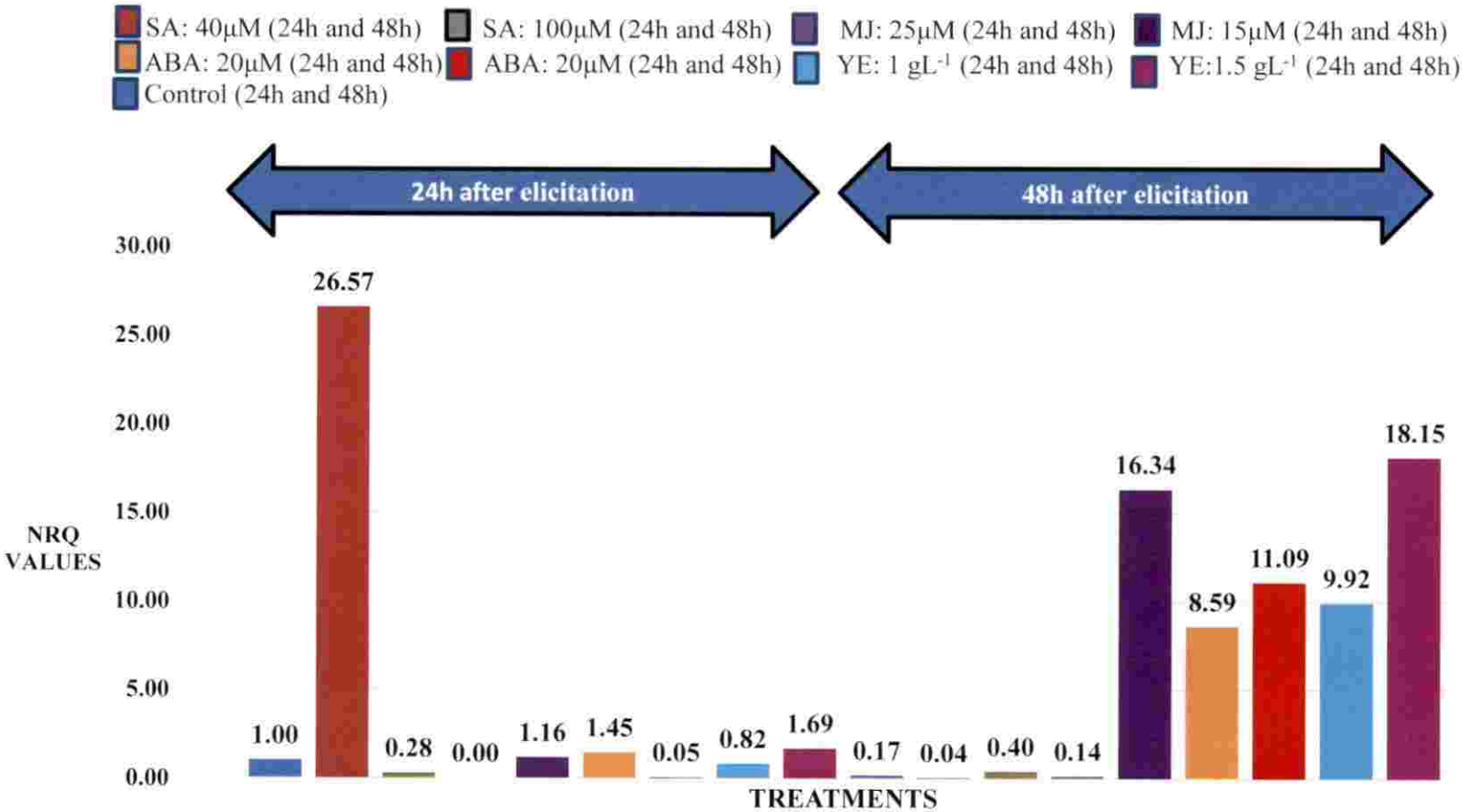


Fig.29 Expression of *PAL* in callus after elicitation

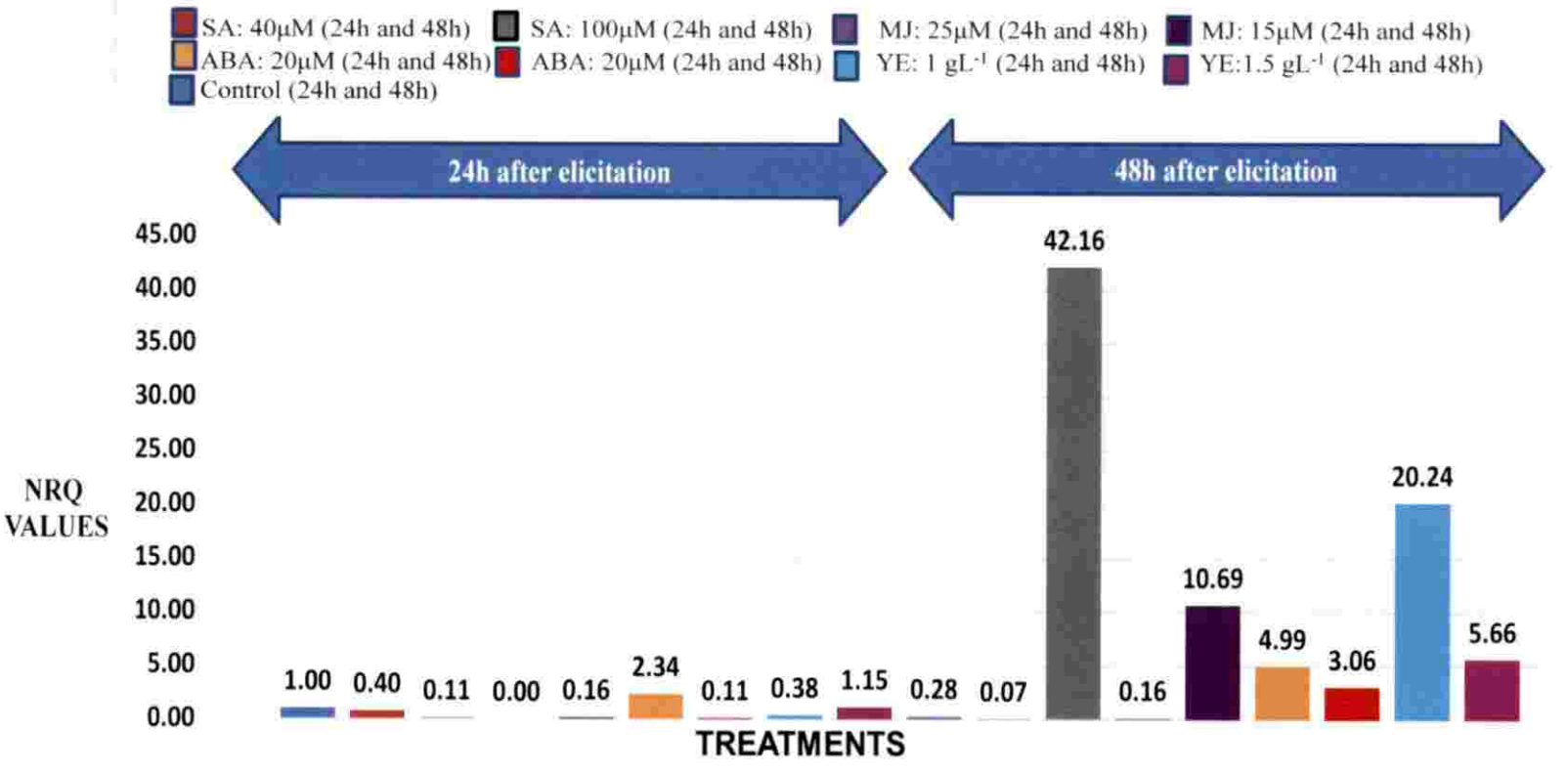


Fig.30 Expression of *UGT* in callus after elicitation

Discussion

5. DISCUSSION

India is a hotspot of various medicinal herbs which are part of our traditional medicine since ages. With the onset of research in medicine it was understood that plants contain active principles of pharmaceutical importance, which are responsible for the curative action of the herbs. Vincristine, reserpine, quinine, taxol, morphine etc. are examples of some plant based pharmaceuticals. But most of these medicinal plants remain unexploited till date.

Artanema sesamoides Benth (family: Scrophulariaceae), is such a commercially unexploited and lesser known medicinal plant, commonly called as 'vathomvaretti'. In Kerala, it is mainly seen in marshy localities (Aiyer and Kolammal, 1963). Main component present in *Artanema* is phenyl propanoid glycosides in which acteoside is found to be the prominent one (Joseph *et al.*, 2010). Acteoside, one of the important PPG has been used as an active ingredient in many pharmaceuticals.

In *A. sesamoides*, phenyl propanoid glycosides content is less than 5% (w/w) and the content of acteoside in leaves and dried root powder of *Artanema* is found to be 0.86 % and 0.80% respectively. The root extracts of *Artanema* has high antioxidant and anti-inflammatory property, because it contains more number of structurally related phenyl propanoids along with acteoside, which constitute 46.55% of total sequential methanolic extractives on dry weight basis (Joseph *et al.*, 2015). Due to the low content of PPGs in plants, several synthetic routes have been developed for its production, which are time consuming. So, *in vitro* culture of medicinal plants was considered as an alternative route of secondary metabolite production.

Many of the *in vitro* systems are incorporating elicitation techniques with bioreactors for enhancing secondary metabolite accumulation on a commercial scale (Naik and Al-Khayri, 2016). Elicitors are chemical compounds that can

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induce stress response in plants and, thereby improving the synthesis of secondary metabolites.

The present study was undertaken at the Department of Plant Biotechnology, College of Agriculture, Vellayani under the title "Elicitation of phenyl propanoid production and expression profiling of acteoside biosynthetic genes in *Artanema sesamoides* Benth (vathomvaretti)". The results obtained are discussed in this chapter.

In a recent study conducted on *in vitro* cultures of *A. sesamoides,* a higher phenyl propanoid glycosides content was observed in callus compared to the roots of field grown plants (Elizabeth, 2018). It suggested the uses of *in vitro* cultures of *A. sesamoides* as an alternative source of PPGs. In the present study elicitors like salicylic acid, methyl jasmonate, abscisic acid and yeast extract were tried for enhancing the *in vitro* production of PPGs.

MS medium supplemented with 0.5 mgL^{-1} BA and 0.5 mgL^{-1} NAA was used for callus induction, which was found best for callus induction by Elizabeth (2018). For elicitation studies, two week old calli were used.

Liquid cultures are ideal for elicitation studies because they help in uniform distribution of the elicitors to the cells in the culture. Here elicitation was done in liquid MS medium supplemented with 0.5 mgL^{-1} NAA and 0.5 mgL^{-1} BA. The liquid cultures were stabilized for 10 days before elicitation to the maintain cells in active state and elicited for 48 h. No significant change in growth of callus was observed during 48 h of elicitation. In different studies where elicitation was done for more than 7 days, significant change in callus growth and biomass accumulation have been reported (Omer and Bengi, 2013; Sonja *et al.*, 2015). To know the effect of elicitation on the synthesis of PPG, callus from each treatment was harvested and the PPGs were isolated by sequential extraction. The weight of methanol soluble phyto-constituents obtained from each treatment roughly indicates the content of PPGs synthesized in the callus, and here it was maximum (4.160 mg/g dry weight) in callus treated with yeast extract (1.5 gL^{-1}). Ogata *et*

al., 2005 reported a rapid and transient increase in production of rosmaric acid by yeast extract elicitation in suspension cultures of *Lithospermum erythrorhizon*. Yeast extract elicitation was found to be effective in accumulation of certain valuable secondary metabolites like azadirachtin, artemisinin and tanshinones (Putalun *et al.*, 2007; Prakash and Srivastava, 2008; Zhao *et al.*, 2010).

Analytical HPLC techniques is one of the accurate techniques for biochemical profiling of PPGs. HPLC analysis of methanolic extracts of callus treated with different elicitors generated chromatograms, with peaks representing distinct identified PPGs and their proportionate peak area is presented in the fig 3-22. Chromatograms of most promising treatments were developed at different wavelengths (197, 218, 254 and 330 nm). PPGs showed maximum absorbance at 197 nm. Six important PPGs were identified from the methanol extract. The highest peak of HPLC chromatogram representing the most prominent antioxidant phenyl propanoid glycoside (acteoside) in the extract appeared within the retention time of 59 min. The peaks of other PPGs such as artanemoside, isoacteoside, leucosceptoside, martynoside and plantainoside appeared within the retention time of 61- 79 min. In a similar study, purification, identification and quantification of PPGs in *A. sesamoides* was done by HRMS, H NMR, C NMR and HPLC techniques. They identified and quantified six PPGs (acteoside, artanemoside A, isoacteoside, leucoseptoside A, plantainoside C, martynoside) from the plant (Joseph *et al.*, 2016, Personal communication; unreferenced).

YE ($1gL^{-1}$) have significantly increased the content of all the major PPGs. The maximum content of acteoside (36.951%) and isoacteoside (22.220%) was obtained in calli treated with yeast extract ($1.5\ gL^{-1}$). The synthesis of rosmaric acid (RA) and phenolic compounds in *Salvia miltiorrhiza* hairy roots was similarly stimulated by yeast extract treatment (Qiong *et al.*, 2006). Sanchez-Sampedro *et al.* (2005) reported in *Silybum marianum* cell suspension culture about three-fold production of silymarin occured by yeast extract treatment. A study conducted by Maqsood and Abdul (2017), reported 22.74% vinblastine and

48.49% vincristine enrichment in yeast extract (1.5 gL^{-1}) treated *in vitro* germinating embryos of *Catharanthus roseus*.

Treatment with SA at a high concentration (100μM) has enhanced the content of acteoside (5.853%), isoacteoside (15.511%), martynoside (19.990%) and plantainoside (11.694%). SA at a low concentration (40μM) enhanced artanemoside (17.025%), martynoside (20.594%) and plantainoside (10.683%). Sivanandhan *et al.* (2013) reported that in the hairy root culture of *Withania somnifera*, salicylic acid and methyl jasmonate elicited the production of withanolide A, withanone and withaferin A. Salicylic acid is a great inducer of systematic resistance in plant-pathogen (Jian *et al.*, 2005), and mainly used as an elicitor for enhancement of secondary metabolism (Hao *et al.*, 2014; Saini *et al.*, 2014). Methyl jasmonate (200 μmol/L) showed a strong stimulating effect on accumulation of ginsenosides in suspension culture of *Panax ginseng* roots (Ali *et al.*, 2006). Farag *et al.* (2016) reported that in response to elicitation using methyl jasmonate, activation in lactonic alkaloid formation occurred in cell suspension cultures of *Erythrina lysistemon*. Sayed *et al.* (2017) reported that in the callus culture of *Rumex vesicarius* L., salicylic acid and chitosan elicited the production of flavonoids.

When plant cells are exposed to elicitor treatment, the elicitor induces plant defense response and enhances plant secondary metabolism by regulating key enzymes which catalyse the biosynthesis of target secondary metabolites (Zhao *et al.* 2005). Studies on gene-to-metabolite networks in *Catharanthus roseus* cells revealed that the gene expression was related to metabolite accumulation (Rischer *et al.*, 2006). Recently, transcriptome approaches have become powerful tools to screen candidate genes involved in the biosynthesis of secondary metabolites in plants, particularly in non-model species. Incorporation of elicitors with *in vitro* production techniques and bioreactors has been done successfully to enhance secondary metabolite accumulation (Naik and Al-Khayri, 2016). But for that, a better knowledge on different elicitors, its role in metabolite pathway and synthesis is pre requisite.

In a study conducted at AMPRS, Odakkali, Joseph *et al.* (2010) found that *Artanema sesamoides* Benth contains different PPGs, among them acteoside was found to be prominent one. It is an active ingredient of many pharmaceutical and has high antioxidant and anti-inflammatory properties. Hence, in the present study, the expression of key genes in the acteoside biosynthesis pathway was examined for a time period of 48 h after elicitation for a comprehensive understanding of mode of action of SA, MJ, ABA and YE in PPGs synthesis in the callus of *Artanema sesamoides*.

In the phenyl propanoid pathway, phenylalanine is transformed into caffeic acid by *PAL, C4H and C3H.* The synthesis of acteoside from an intermediate caffeoyl COA was regulated by *HCT* and *UGT* genes (Saimaru and Orihara, 2010; Alagna *et al.*, 2012). Elicitation for 24 h using SA, MJ, Ag^{+}, and putrescine in *Rehmmania glutinosa* hairy roots, resulted in higher expression levels of *PAL, C4H, C3H, PPO, HCT* and *UGT* genes with acteoside accumulation (Wang *et al.*, 2017). Therefore, from upstream of the pathway *PAL* gene (phenylalanine ammonia-lyase) and from downstream *HCT* (Shikimate O-hydroxycinnamoyl transferase) and *UGT* (UDP-glucosyl transferase) genes were selected for expression studies.

Trizol method was used to isolate RNA from the callus after 24h and 48h of elicitation using SA, MJ, ABA and yeast. George *et al.* (2005) reported that Trizol like strong denaturing buffer containing guanidinium thiocyanate produces a good quality RNA which can be used for reverse transcription experiments. Intact RNA bands without shearing were obtained on agarose gel electrophoresis which indicates the good quality of isolated RNA (Plate 2 and 3). The A_{260}/A_{280} value of the RNA isolated from the callus after 24 h and 48 h of elicitation ranges from 1.74 to 2.11 indicating the good quality of RNA isolated.

From the isolated RNA, cDNA was synthesized using Thermo scientific Verso cDNA synthesis kit. The quality of cDNA synthesised was assessed by PCR using gene specific primer for ß-*ACTIN* and agarose gel electrophoresis. An

amplicon of expected size of 125 bp in all the samples indicates the good quality of cDNA synthesized.

The primer designing is a crucial step for the proper estimation of transcript abundance of the genes. Sequence of selected genes from *Artanema sesamoides* was not available in NCBI; hence degenerate primers were designed based on sequence similarity of closely related species of *Artanema* for *PAL* and *HCT* genes. For *UGT* gene, sequence from the related species was used for designing gene specific primers.

For checking the specificity of the primers designed, PCR was performed with the cDNA using gene specific primers of *PAL* (phenylalanine ammonia-lyase), *HCT* (Shikimate O-hydroxycinnamoyl transferase) and *UGT* (UDP-glucosyl transferase). In agarose gel electrophoresis, an amplicon size of 220bp was obtained for *PAL* gene specific primers and an additional amplicon of 300bp was also appeared, may be because *PAL* gene comes under a multigene family (Li *et al.*, 2015). For *UGT* genes, an amplicon size of 243bp was obtained. Only in case of *HCT* gene; no amplicons of expected size was obtained even after repeating the experiment with gradient PCR.

Reverse Transcription quantitative PCR (RT- qPCR) or Real Time PCR was done for the expression profiling of acteoside biosynthesis genes. Real time PCR is considered as the one of the best techniques which is used for gene expression analysis using the phenomenon of fluorescence resonance energy transfer (Yilmaz *et al.*, 2012). It has been reported that SYBR Green dye is widely used in real time PCR because it exhibits fluorescence only when bound to a double stranded DNA molecule (Navarro *et al.*, 2015; Kuang *et al.*, 2018).

RT-qPCR for each gene was performed with two technical replicates for each sample. Amplification plot generated showed the raw expression data and the Melt curve plot showed a prominent peak indicating the specificity of Real time PCR reaction. The Cq values generated by RT-qPCR, was used for further analysis using "qbase plus" software. In RT-qPCR, normalization is very crucial

to obtain accurate results. Several strategies have been developed to normalize the data in RT-qPCR and one such method is normalization using stably expressed gene as reference (Hugget *et al.*, 2005). Maroufi *et al.* (2010) showed that the most stably expressed reference genes in chicory leaf and root tissue are *ACTIN* and *EF*. Jaiswal *et al.* (2019) reported that in *Cyamopsis tetragonoloba*, *ACTIN* was found to be the most stable gene during seed development and under abiotic stress conditions. In the present study, the house keeping gene ß-*ACTIN* was taken as the reference gene.

Comparison of relative expression of different treatments in this study showed that expression of *PAL* gene and *UGT* gene was upregulated by all the elicitors. Treatment with SA produced upregulation of *PAL* gene after 24 h of elicitation and upregulation of *UGT* gene after 48 h of elicitation. The highest elicitation (26 fold) of *PAL* gene was shown by SA (40 µM) whereas in case of *UGT* gene, the highest expression (42 fold) was with SA (100 µM). So among the elicitors tried, SA (100 µM) induced six different types of PPGs in callus and also, SA shown the maximum upregulation of the genes of acteoside pathway. A similar study by Wang *et al.* (2017) reported that, in salicylic acid treated *Rehmannia glutinosa* hairy root cultures 54 unigenes were upregulated in the acteoside pathway, out of 219 putative unigenes. Among these genes *PAL* was expressed after 12 h of salicylic acid treatment whereas HCT and UGT significantly up-regulated at both 12 and 24 h time points.

Except SA, all other elicitors upregulated *PAL* and *UGT* genes after 48 h of elicitation. YE produced 18 fold increase in *PAL* gene expression at higher concentration (1.5 gL^{-1}) and 20 fold increase in *UGT* gene expression at lower concentration (1 gL^{-1}). Early reports on the action of YE on alkaloid biosynthesis in cell culture of *Catharanthus roseus* showed that accumulation of alkaloids was associated with activation of terpenoid indole alkaloids (TIA) biosynthetic genes *STR* and *TDC* encoding strictosidine synthase and tryptophan decarboxylase (Pauw *et al.*, 2004).

MJ (25 μM) produced 16 fold upregulation of *PAL* gene and 10 fold upregulation of *UGT* gene. Transcriptomic analysis of adventitious root cultures of *Panax quinquefolium* showed that gene encoding UDP-xylose synthases showed a significant up-regulation in response to MJ treatment, corresponding to the increase in ginsenoside biosynthesis (Wang *et al.*, 2016). mRNA levels of kaurene synthase-like (KSL) and copalyldiphosphate synthases (CPS) in *Salvia miltiorrhiza* hairy roots was improved by treatment of MJ and Ag^{+} which in turn enhanced biosynthesis of tanshinone (Gao *et al.*, 2009).

In this study, the two key genes, *PAL* and *UGT*, involved in the acteoside biosynthetic pathway were upregulated by all the elicitors. But only yeast extract significantly enhanced the *in vitro* production of six major phenyl propanoid glycosides (artanemoside, isoacteoside, leucosceptoside, martynoside and plantainoside) in *A. sesamoides*. The study shows a possible use of yeast extract in the *in vitro* production of phenyl propanoid glycosides.

FUTURE LINE OF WORK

Studies including wider range of concentrations of elicitors especially yeast extract, for extended time period might help in optimising maximum production of phenyl propanoid glycosides *in vitro*.

Expression profiling of other major genes in PPG pathway might also help in understanding their role in acteoside pathway.

Summary

6. SUMMARY

The study entitled "Elicitation of phenyl propanoid production and expression profiling of acteoside biosynthetic genes in *Artanema sesamoides* Benth (vathomvaretti)" was carried out during 2017-2019, in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objectives were to study the effect of elicitors like salicylic acid, methyl jasmonate, abscisic acid and yeast extract on phenyl propanoid glycosides production in *A. sesamoides* and to analyse the expression profile of key genes of acteoside biosynthesis pathway, such as *PAL* (phenylalanine ammonia-lyase), *HCT* (Shikimate O hydroxycinnamoyl transferase) and *UGT* (UDP glucose glucosyl transferase).

Callus culture was established in MS medium supplemented with 0.5 mgL^{-1} BA and 0.5 mgL^{-1} NAA using *in vitro* plants of *A. sesamoides*. Two week old callus cultures were transferred to liquid MS medium with 0.5 mgL^{-1} NAA and 0.5 mgL^{-1} BA and stabilized for 10 days. Elicitors *viz.*, salicylic acid (SA; 40 and 100µM), methyl jasmonate (MJ; 15 and 25µM), abscisic acid (ABA; 20 and 50µM) and yeast extract (1 and 1.5 gL^{-1}) were added to the cultures and incubated in orbital shaker at 24 °C under dark condition at 120 rpm for 48h. After elicitation, callus was harvested, dried and finely powdered.

Phenyl propanoid glycosides were extracted by sequential extraction using solvents like hexane, chloroform and methanol. For each 1g of powdered sample, 10ml solvent was added and kept in orbital shaker at 110 rpm for 16h for extraction. Methanol extract obtained by extracting 3 times, centrifuged at 4500 rpm for 5 min and residue was removed. Methanol extracts were evaporated to get the residues. The total residue representing the content of phenyl propanoid glycosides was maximum (4.160 mg) in callus treated with yeast extract (1.5 gL^{-1}). In callus treated with ABA, content of phenyl propanoid glycosides residue was on par with untreated callus.

The residues were re-dissolved in HPLC grade methanol. HPLC analysis was done using Merck LiCrospher 100 RP 18e column (250 mm x 4.6 mm) and PDA detector at different wavelengths (197, 218, 254 & 330 nm). Two solvents were used as mobile phase; solvent A (H_2O with 0.1 % ortho phosphoric acid) and solvent B (90% ACN and 10% A). PPGs showed maximum absorbance at 197 nm. Six important PPGs *viz.*, acteoside, artanemoside, isoacteoside, leucosceptoside, martynoside and plantainoside were identified from the methanol extract. The highest peak of HPLC chromatogram representing the most prominent antioxidant phenyl propanoid glycoside (acteoside) in the extract appeared within the retention time of 59 min. YE ($1gL^{-1}$) has significantly increased the content of all the major PPGs. The maximum content of acteoside (36.951%) and isoacteoside (22.220%) was obtained in calli treated with yeast extract ($1.5\ gL^{-1}$).

The effect of elicitors on expression profile of acteoside biosynthesis genes such as *PAL*, *HCT* and *UGT* was analysed 24h and 48h after elicitation by real time PCR using SYBR® Green dye. Primers were designed using "Primer Express" software. RNA isolated from the callus after 24 and 48h of elicitation was converted to cDNA and the quality was confirmed by PCR using ß-*ACTIN* gene specific primers.

For checking the specificity of the primers designed, PCR was performed with the cDNA using target gene specific primers. In agarose gel electrophoresis, an amplicon size of 220bp was obtained for *PAL* gene specific primers. In case of *UGT* genes, an amplicon size of 243bp was obtained where as in case of *HCT* gene; no amplicons of expected size was obtained even after repeating the experiment with gradient PCR.

cDNA was used for RT-qPCR using SYBR® Green dye-based assay. Thermal conditions for RT-qPCR were 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 45°C for 15 sec and 72°C for 45 sec. RT- qPCR for each gene was

performed with two technical replicates for each sample. Amplification plot and Melt curve plot were generated by the software. The Melt curve plot showed prominent peaks for both the primers indicating the specificity of Real time PCR reaction. Cq values obtained in RT-qPCR for each gene was analysed using "qbase plus" software with ß- *ACTIN* as the reference gene.

Expression of *PAL* gene which acts in the upstream of acteoside biosynthetic pathway was found upregulated by all the elicitors. The highest elicitation (26 fold) was shown by SA (40µM), followed by yeast extract (18 fold with 1.5 gL^{-1}), MJ (16 fold with 25µM) and ABA (11 fold with 50µM). Treatment with SA led to upregulation of *PAL* at 24h, while all other elicitors enhanced its expression at 48h. All the elicitors enhanced the expression of *UGT* at 48h of elicitation. The highest expression (42 fold) was with SA (100µM), followed by yeast extract (20 fold with $1gL^{-1}$) and MJ (10 fold with 25µM).

In this study, the two key genes, *PAL* and *UGT*, involved in the acteoside biosynthetic pathway were upregulated by all the elicitors. But only yeast extract significantly enhanced the *in vitro* production of six major phenyl propanoid glycosides (artanemoside, isoacteoside, leucosceptoside, martynoside and plantainoside) in *A. sesamoides*. The study shows a possible use of yeast extract in the *in vitro* production of phenyl propanoid glycosides.

Studies including more concentrations of elicitors, especially yeast extract, for extended time period might help in optimising maximum production of phenyl propanoid glycosides *in vitro*.

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Abstract

ELICITATION OF PHENYL PROPANOID PRODUCTION AND EXPRESSION PROFILING OF ACTEOSIDE BIOSYNTHETIC GENES IN *Artanema sesamoides* Benth (VATHOMVARETTI).

by

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ABSTRACT

The study entitled "Elicitation of phenyl propanoid production and expression profiling of acteoside biosynthetic genes in *Artanema sesamoides* Benth (vathomvaretti)" was carried out during 2017-2019, in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objectives were to study the effect of elicitors like salicylic acid, methyl jasmonate, abscisic acid and yeast extract on phenyl propanoid glycosides production in *A. sesamoides* and to analyse the expression profile of key genes of acteoside biosynthesis pathway, such as *PAL* (phenylalanine ammonia-lyase), *HCT* (Shikimate O hydroxycinnamoyl transferase) and *UGT* (UDP glucose glucosyl transferase).

Callus culture was established in MS medium supplemented with 0.5 mgL^{-1} BA and 0.5 mgL^{-1} NAA using *in vitro* plants of *A. sesamoides*. Two week old callus cultures were transferred to liquid MS medium with 0.5 mgL^{-1} NAA and 0.5 mgL^{-1} BA and stabilized for 10 days. Elicitors *viz.*, salicylic acid (SA; 40 and 100µM), methyl jasmonate (MJ; 15 and 25µM), abscisic acid (ABA; 20 and 50µM) and yeast extract (1 and 1.5 gL^{-1}) were added to the cultures and incubated in orbital shaker (120 rpm) at 24 °C under dark condition for 48h. After elicitation, callus was harvested, dried and finely powdered. Phenyl propanoid glycosides were extracted by sequential extraction using solvents like hexane, chloroform and methanol. Methanol extracts were collected, purified and evaporated to get the residues. The total content of phenyl propanoid glycosides was maximum (4.160 mg/g dry weight) in callus treated with yeast extract (1.5 gL^{-1}).

The residues re-dissolved in HPLC grade methanol were analysed by HPLC using PDA detector at different wavelengths (197, 218, 254 & 330 nm). Six important PPGs *viz.*, acteoside, artanemoside, isoacteoside, leucosceptoside, martynoside and plantainoside were identified from the methanol extract. The highest

peak of HPLC chromatogram representing the most prominent antioxidant phenyl propanoid glycoside (acteoside) in the extract appeared within the retention time of 59 min. YE ($1gL^{-1}$) has significantly increased the content of all the major PPGs. The maximum content of acteoside (36.951%) and isoacteoside (22.220%) was obtained in calli treated with yeast extract ($1.5\ gL^{-1}$).

The effect of elicitors on expression profile of acteoside biosynthesis genes such as *PAL*, *HCT* and *UGT* was analysed 24h and 48h after elicitation by real time PCR using SYBR® Green dye. Primers were designed using "Primer Express" software. RNA isolated from the callus after 24 and 48h of elicitation was converted to cDNA and the quality was confirmed by PCR using *ACTIN* gene specific primers. Cq values obtained in RT-qPCR for each gene was analysed using "qbase plus" software with *ACTIN* as the reference gene.

Expression of *PAL* gene which acts in the upstream of acteoside biosynthetic pathway was found upregulated by all the elicitors. The highest elicitation (26 fold) was shown by SA (40μM), followed by yeast extract (18 fold with $1.5\ gL^{-1}$), MJ (16 fold with 25μM) and ABA (11 fold with 50μM). Treatment with SA produced upregulation of *PAL* at 24h, while all other elicitors enhanced its expression at 48h. All the elicitors enhanced the expression of *UGT* at 48h of elicitation. The highest expression (42 fold) was with SA (100μM), followed by yeast extract (20 fold with $1\ gL^{-1}$) and MJ (10 fold with 25μM).

In this study, the two key genes, *PAL* and *UGT*, involved in the acteoside biosynthetic pathway were upregulated by all the elicitors. But only yeast extract significantly enhanced the *in vitro* production of six major phenyl propanoid glycosides (artanemoside, isoacteoside, leucosceptoside, martynoside and plantainoside) in *A. sesamoides*. The study shows a possible use of yeast extract in the *in vitro* production of phenyl propanoid glycosides.

Appendices

APPENDIX I

MS medium						
Stock	**Chemical**	**Quantity for 1L of MS (mg)**	**Conc. of stock**	**Volume of stock (mL)**	**Quantity for preparing stock (mg)**	**Volume for 1L MS media (mL)**
Macro nutrients						
A	NH_4NO_3	1650	5X	250 mL	8250	50 mL
	KH_2PO_4	1900			9500	
	KNO_3	370			1850	
	$MgSO_4.7H_2O$	170			850	
B	$CaCl_2.2H_2O$	440	5X	100 mL	2200	20 mL
Micro nutrients						
C	KI	0.83	100X	100 mL	83	1 mL
	H_3BO_3	6.2			620	
	$MnSO_4.H_2O$	16.9			1690	
	$ZnSO_4.7H_2O$	8.6			860	
	$Na_2MoO_4.2H_2O$	0.25			25	
D	$FeSO_4.7H_2O$	27.8	5X	100 mL	139	20 mL
	Na_2 EDTA. $2H_2O$	37.3			186.5	
E	$CuSO_4.5H_2O$	0.025	1000X	100 mL	25	0.1 mL
	$CoCl_2.6H2O$	0.025			25	
Organic supplements						
F	Nicotinic acid	0.5	250X	100 mL	125	0.4 mL
	Pyridoxine – HCl	0.5			125	
	Thiamine – HCl	0.1			25	
	Glycine	2			500	
Components directly added						
	MS supplement	3.3g				
	Myoinositol	100 mg				
	Sucrose	30g				
	Agar	6.5g				

APPENDIX II

TBE Buffer (5X) for 1 litre solution

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