ENHANCEMENT OF PHENYL PROPANOID GLYCOSIDES IN *Artanema sesamoides* Benth (VATHOMVARETTI) BY HAIRY ROOT INDUCTION

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Enhancement of phenyl propanoid glycosides in *Artanema sesamoides* Benth (vathomvaretti) by hairy root induction"** 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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CERTIFICATE

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Abbreviations	Full form
2, 4-D	2, 4-Dichlorophenoxyacetic acid
ATCC	American Type Culture Collections
BA	6- benzyl adenine
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxy ribonucleic acid
et al.	and others
Fig.	Figure
g	Gram
HPLC	High Pressure Liquid Chromatography
h	Hour
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
M	Molar
MTCC	Microbial Type Culture Collections
mg	Milligram
ml	Milliliter
min	Minute
mm	Millimolar
MS	Murashige and Skoog, 1962
NaOH	Sodium hydroxide
µM	Micromolar
pH	Potential of hydrogen
PGR	Plant growth regulator
PPGs	Phenyl propanoid glycocides
rpm	revolutions per minute
S	Second
T-DNA	Transfer DNA
TDZ	Thidiazuron
V	Volt
viz.	Namely
NAA	Naphthalene acetic acid
SH	Schenk & Hildebrandt Medium
°C	Degree Celsius
%	per cent
±	plus or minus

i.e.	That is
SDS	Sodium Dodecyl Sulphate
Sl. No	Serial number
Sp. or spp.	Species (Singular and plural)
PCR	Polymerase Chain Reaction
ppm	Parts per million
YEM	Yeast Extract Mannitol

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Introduction

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1. INTRODUCTION

Plants are valuable sources of secondary metabolites which are used as pharmaceuticals, agrochemicals, flavors, fragrances, colours, biopesticides and food additives (Gueven, 2012). Even though India is a rich repository of medicinal plants, only a lesser percentage of it is explored for industrial purpose.

Almost every civilization has a history of medicinal plant use and approximately 80 per cent of the people in the world rely on traditional medicine for their health care needs. As an average, about 85 per cent of the traditional medicines are derived from the plant extracts and clinically useful drugs are derived from plants (Payne *et al.*, 1991).

Artanema sesamoides Benth (family: Scrophulariaceae), an uncultivated, lesser known medicinal plant seen in Kerala is found to harbor a number of structurally close related biomolecules of pharmaceutical importance. This plant is commonly known as 'vathomvaretti' and seen along rice fields, forest, in humid and swampy localities and water sources up to 400 m altitude (Aiyer and Kolammal, 1963). Leaves and roots of *Artanema sesamoides* contain phenyl propanoid glycosides and an active ingredient acteoside/ verbascoside/ kusaginin having high antioxidant and anti-inflammatory properties (Joseph *et al.*, 2010).

Phenyl propanoid glycosides (PPGs) possess diverse biological activities. They are reported to have therapeutic properties against hypertension, tumors, fungal and viral infections and also immunomodulatory effect (Pan *et al.*, 2003). Most of these properties have been attributed to the antioxidant and free radical scavenging capacities of their structural components. Acteoside is a phenyl propanoid glycoside, which also has shown antioxidant, anti-inflammatory, hepato protective and cell apoptosis regulation activities (Jiang *et al.*, 2011).

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Despite the potential pharmacological properties, the low content of PPGs in plants (less than 5% w/w) has limited their isolation and commercial application. The leaves and root dry powder of *Artanema sesamoides* contain acteoside at 0.86 % and 0.80% respectively, but root in addition to acteoside, contain many more structurally related phenyl propanoids which constitute 46.55% of total sequential methanolic extractives on dry weight basis which in turn attribute to high antioxidant and anti-inflammatory power of root extractives (Joseph *et al.*, 2015). Chemical synthesis of PPGs is a time consuming process with low yield.

Hairy root cultures developed using *Agrobacterium rhizogenes* have been emerged as an important eco-friendly technique for the production of secondary metabolites. Hairy roots are reported to have growth rate and genetic stability.They grow in hormone free medium. Hairy root cultures had been established for numerous medicinal crops whereby a significant increase in the production of secondary metabolite has been observed. These cultures ensure continuous production of secondary metabolites independent of environment and seasonal factors.

The present study aims at developing hairy root culture of *A. sesamoides* using *A. rhizogenes* for enhancing the production of phenyl propanoid glycosides.

Review of Literature

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2. REVIEW OF LITERATURE

Plants are traditional source of many pharmaceuticals and these medicines are derived from secondary metabolism in plants. Common drugs like digitoxin, morphine, cocaine, theobromine etc. were extracted from plants only, since their artificial synthesis is difficult. Studies on these aspects have been increasing over the last fifty years. Literature pertaining to these aspects of research is discussed in this chapter.

2.1 *Artanema sesamoides* Benth

Artanema sesamoides Benth belong to 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 stout erect herb of 60 - 90 cm height. It is sparingly branched, glabrous and often tinged with purple on stem and leaf petioles and bears opposite leaves. Leaves are sessile or shortly petiolate with 18 - 25 mm width and 10 cm length. Flowers are large, bisexual, zygomorphic with dull violet – purple corolla which occurs as erect terminal racemes with 6 – 16 mm pedicel length and 8 – 11 mm long calyx. Sepals are ovate, acute, nearly glabrous and imbricate. Globose, glabrous capsules are 6-10 mm long with the valves when ripe separating from the broadly winged placentiferous axis. Seeds of *Artanema* are small, truncate, papillose or rugose (Aiyer and Kolammal, 1963).

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2.1.2 Occurrence and Distribution

A. sesamoides Benth is extensively seen in tropical Africa and Asia. The plant is found in Liberia to Cameroon, Congo, Uganda and Tanzania areas in tropical Africa. It is also seen in India and South East Asia. In Kerala, *Artanema* is commonly seen in Kollam, Idukki, Pathanamthitta, Kottayam, Palakkad, Kannur, Thiruvanthapuram, Thrissur and Malappuram districts. It favours marshy localities like margins of lakes or ponds and along the low lying banks of rivers for 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* (MEAS) 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 activity of the plant was reported by Gupta *et al.* (2008).

Joseph *et al.* (2010) evaluated the anti-inflammatory and anti-oxidant properties of the root extract. Ethanol, hexane, choloroform and water solvent extractives were tested in mouse erythema model in which ethanol extract showed

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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

In vitro propagation of *A. sesamoides* was not reported so far, but it is reported in the family Scrophulariaceae. Scrophulariaceae family, commonly known as figwort family of flowering plants consists of economically important crops like Foxglove (*Digitalis* L.), Snapdragon (*Antirrhinum majus* L.) and Mullein (*Verbascum* L.) (Encyclopedia Britannica, 2009).

Tissue culture studies on *Digitalis* were mainly done for the production of cardenolides. Effect of light and plant growth substances on digitoxin production was studied in undifferentiated cells and shoot forming cultures of *Digitalis purpurea* L. in liquid media (Hagimori *et al.*, 1982). An updated review of *in vitro* culture of various *Digitalis* species and production of cardenolides was given by Verma *et al.* (2016). According to them, seeds, root, seedlings, anther, pollen, leaves, shoot, axillary bud and hypocotyl were used as the explants for the *in vitro* culturing of different species of *Digitalis*. MS medium, Gamborg's B5 medium and SH (Schent and Hilderbrandt) medium with different combinations of growth regulators were used for the *in vitro* studies of *Digitalis*. Direct organogenesis, indirect organogenesis, direct embryogenesis and indirect embryogenesis were studied in *Digitalis* for the production of cardiac glycosides and its derivatives.

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In vitro cultures of snapdragon (*Antirrhinum majus* L.) was successfully established by using leaf, stem segments, seedling root, stem internode, shoots, nodal segments as explants. Multiple shoot formation, callus formation, indirect organogenesis and indirect embryogenesis of snapdragon were studied. A general protocol for the *in vitro* propagation of snapdragon was standardized with nodal segments as explants. MS medium supplemented with BAP 1 mg L^{-1} was identified as the best medium for multiple shoot formation (Iriondo and Torres, 1997).

Mullein (*Verbascum thapsus* L.), a famous species in Scrophulariaceae family was cultured *in vitro* on MS medium for shoot proliferation using leaves and petioles as explants. Explants were cultured in MS medium supplemented with BA and NAA as growth regulators for callus induction. Improvement of phenols in mullein using aminoacids was also studied (Al-Jibouri *et al.*, 2016). *In vitro* production of *A. sesamoides* is not reported so far.

2.2 SECONDARY METABOLITES IN *Artanema sesamoides* Benth

A. sesamoides Benth showed high antioxidant, anti-inflammatory activites and phenol content. Active compounds were chemically characterized from *A. sesamoides* and found to be iridoid, flavonoids and phenyl propanoid glycosides. Pectin which is generally reported to contribute to anti-inflammatory activity was detected to a level of 3.19 % in roots and 5.69 % in leaves of the plant. The root extract possessed very high antioxidant activity and was found to act through inhibition of 15- Lipoxygenase and Cyclooxygenase 2 enzymes in inflammatory pathways. All the compounds reported from *A. sesamoides* of molecular mass 624.2-954.3 belong to a compound family and have similar structures. All the members contain the moiety Acteoside / Verbascoside (2-(3, 4-Dihydroxyphenyl) ethyl 3-O- (6-deoxy-α-L-mannopyranosyl)- 4- O- [(2E)-3-(3, 4-dihydroxyphenyl)-2-propenoyl]-β-D-glucopyranoside). A few compounds have structural isomers. Two of the compounds have an additional sugar moiety ($C_6H_{10}O_4$) (Joseph *et al.*, 2010).

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2.2.1 Phenyl Propanoid Glycosides

Phenylethanoid glycosides are water soluble naturally occurring chemical compounds with remarkable 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. pseudoauriculata.* contains iridoid glycosides, saponins, phenyl ethanoid glycosides etc. Phenyl propanoid glycosides were also reported in *Scrophularia ningpoensis* by Li *et al.* (2000). Mainly phenyl propanoid glycosides named ningposides A, sibirioside A, cistanoside D, angoroside C, acteoside, decaffeoylacteoside and cistanoside F were obtained from the roots of *Scrophularia ningpoensis*. Monsef-Esfahani *et al.* (2010) isolated flavonoids, cinnamic acids and phenyl propanoids from aerial parts of *Scrophularia striata.*

2.2.1.1 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. Acteoside was biosynthesized from tyrosine through dopamine, where as caffeoyl meoity of acteoside was synthesized from phenylalanine *via* cinnamate pathway. Acteoside showed bioactivities like antioxidant, anti-inflammatory, antinephritis, hepatoprotective, immunoregulative, neuroprotective effects and also cell regulation (He *et al.*, 2011).

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

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pharmacologically beneficial properties like antioxidant, anti-inflammatory and antineoplastic properties. It was also produced *in vitro* using genetically transformed roots / hairy roots from *Paulownia fortunei* (Alipieva *et al.*, 2014).

2.3 ENHANCEMENT OF SECONDARY METABOLITES IN MEDICINAL PLANTS

Plant secondary metabolites are having immense role in human health and pharmaceutical world. Plant cell and organ cultures are used for the production of these metabolites and also by using various elicitors (Naik and Al-Khayri, 2016). Secondary metabolites have been enhanced by using *Agrobacterium rhizogenes* (hairy root cultures) (Sevon and Oksman-Caldentey, 2002).

2.3.1 Elicitors

Elicitors are chemical substances derived from the biotic and abiotic sources which can stimulate stress responses in plants leading to the synthesis and accumulation of secondary metabolites. Elicitors are mainly classified into abiotic and biotic elicitors. Abiotic elicitors used in the production of secondary metabolites includes copper (Purohit *et al.*, 1995), gibberellic acid (Yuan *et al.*, 2008), pH (Naik *et al.*, 2010), sucrose (Omer and Bengi, 2013), cadmium (Cai *et al.*, 2013), cobalt (Cai *et al.*, 2013), ultraviolent C (Xu *et al.*, 2015), ozone (Tonelli *et al.*, 2015), proline (Pratibha *et al.*, 2015), polyethylene glycol (Pratibha *et al.*, 2015), Jasmonic acid (Munish *et al.*, 2015), methyl jasmonate (Xiaolong *et al.*, 2015), salicyclic acid (Xiaolong *et al.*, 2015), sodium chloride (Fatima *et al.*, 2015), sorbitol (Arehzoo *et al.*, 2015) and silver (Arehzoo *et al.*, 2015). In the enhancement of secondary metabolites, biotic elicitors also have an important role. *Escherichia coli* (Chodisetti *et al.*, 2013), *Aspergillus niger* (Chodisetti *et al.*, 2013), *Agrobacterium rhizogenes* (Chodisetti *et al.*, 2013), chitin (Sonja *et al.*, 2014), pectin (Sonja *et al.*, 2014), yeast extract (Silja *et al.*, 2014), were some of the biotic elicitors used. Elicitor

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concentration, duration of elicitor exposure, age of culture and nutrient composition were some of the parameters highlighted on elicitation of some secondary metabolites (Naik and Al-Khayri, 2016).

2.4 HAIRY ROOT CULTURE FOR SECONDARY METABOLITES PRODUCTION

Agrobacterium rhizogenes, a soil bacterium causes a hairy root syndrome in plants and is characterized by the neoplastic growth of roots (Riker *et al.*, 1930). Chilton *et al.* (1977) and White *et al.* (1982) described the molecular basis of this phenomenon. The transfer and integration of "Transfer DNA" (T- DNA) of the root inducing (Ri) plasmid of *Agrobacterium rhizogenes* into the genome of plant causes neoplastic growth of roots. Ackermann (1977) did first transformation experiment using *A. rhizogenes* in *Nicotiana tabacum*.

High stability and productivity features of hairy root allow its exploitation as a valuable biotechnological tool for the production of secondary metabolites (Pistelli *et al.*, 2010). Some of the plants and secondary metabolites produced by hairy root culture are shown in the table 1.

Table 1. Secondary metabolites produced by hairy root cultures

Sl. No.	Plant	Secondary Metabolites	References
1	*Catharanthus roseus* L.	Terpenoid indole alkloids	Choi *et al.*, 2004
2	*Centella asiatica* L.	Asiaticoside	Kim *et al.*, 2007
3	*Coleus forskohlii* Briq	Forskohlin	Maheswari *et al.*, 2011
4	*Azadirachta indica* A. Juss	Azadirachtin	Srivastava and Srivastava, 2011
5	*Withania somnifera* L. Dual	Withanolide	Varghese *et al.*, 2014
6	*Berberis aristata* DC.	Berberin	Brijwal and Tamta, 2015
7	*Isatis tinctoria* L.	Flavonoids	Gai *et al.*, 2015
8	*Oldenlandia umbellata* L.	Anthraquinones	Krishnan and Siril, 2016
9	*Fagopyrum tataricum* Gaertn	Phenylpropanoid	Thwe *et al.*, 2016
10	*Achyaranthes aspera* Linn	2'0-hydroxyecdysone	John *et al.*, 2017
11	*Raphanus sativus* L.	Phenolics and flavonoids	Balasubramanian *et al.*, 2018

2.4.1 *Agrobacterium rhizogenes* in Hairy root induction

Agrobacterium rhizogenes, a gram negative soil bacterium is responsible for the hairy root disease in dicotyledous plants and various gymnosperms (Toshiya and Saito, 2010). A number of plant species were successfully transformed with *A. rhizogenes* and transformed root cultures were used as the potential source of pharmaceuticals (Sevon and Oksman-Caldentey, 2002).

2.4.1.1 Genes responsible for hairy root formation

A. rhizogenes contain a T-DNA region in the Ri plasmid which carries genes involved in root initiation and development (*rol* genes) (Hansen *et al.*, 1994). Sevon and Oksman-Caldentey (2002) studied the genes responsible for hairy root formation. The Ri plasmid of cucumopine and mannopine type *A. rhizogenes* strains consists of one T-DNA region but agropine strains have a split T-DNA, consisting of two T-DNA, TL and TR each ranging in size from 15 to 20 kb. These fragments are transferred independently during the transformation process and transfer of TL-DNA is important for the induction of hairy roots.

2.4.1.1.1 *rol* genes

Bulgakov (2008) described the functions of *rol* genes in plant secondary metabolism. *rolA, rolB, rolC* genes were found to be the potential activators of secondary metabolism in transformed cells from Araliaceae, Solanaceae, Rubiaceae, Vitaceae and Rosaceae.

The combined effect of *rol* genes were studied in several experiments and it was found that the accumulation of secondary metabolites in *rol ABC* transformed roots of plants were similar to that found in wild type transformed hairy roots (Palazon *et al.*, 1997; Hong *et al.*, 2006).

2.4.2 Culture of *Agrobacterium rhizogenes*

Yeast extract peptone (YEP) medium was found to be the best medium for culture *A. rhizogenes* strains ATCC 15834 and A4 (Kang *et al.,* 2006). But *A. rhizogenes* strain MTCC 2364 was cultured in both Nutrient Agar Medium (NAM) and yeast extract broth (YEB) medium (Pandey *et al.,* 2014).

A. rhizogenes strains A4, ATCC 15834, MTCC 2364 was cultured in Yeast Extract Broth (YBM) medium (Varghese *et al.*, 2014). Krishnan and Siril (2016), used yeast extract, beef extract and mannitol (YBM) medium for culturing wild type *A. rhizogenes* strain MTCC 532. pH of the medium was adjusted to 6.9.

The *A. rhizogenes* strains (wild type) such as MTCC 2364 and MTCC 532 were cultured in Luria broth (LB) medium (Balasubramanian *et al.*, 2018).

2.4.3 Culture Conditions for *Agrobacterium rhizogenes*

Growth of *Agrobacterium* is influenced by temperature given for culture. According to Sanita di Toppi *et al.* (1997), *Agrobacterium* should be cultured at temperature less than 28-30°C as loss of plasmid occurred at high temperatures.

According to Krishnan and Siril (2016), the *Agrobacterium* cultures at exponential growth phase were preferred for the transformation studies and bacteria inoculated liquid broth was incubated at 25°C for 16 h on an incubator shaker. Balasubramanian *et al.* (2018) incubated *Agrobacterium* in orbital shaker at 28°C for 14 h at 180 rpm.

2.4.4 Strain Specificity of *Agrobacterium rhizogenes*

Transformation efficiency of *A. rhizogenes* depends highly on strains of the bacteria taken for transformation. Karmarker *et al.* (2001) studied the hairy root induction in Adapathiyan (*Holostemma ada-kodien* K. Schum). Strains PcA4, ATCC

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15834 and A4 gave hairy root induction in the plant but the strains like ATCC 8196 and ATCC 2659 failed to induce the hairy root.

Suja *et al.* (2010) tried genetic transformation in sarpagandha *(Rauvolfia serpentine* (L.) Benth with *A. rhizogenes* strains ATCC 15834, ATCC 11325, TR7, A4, TR 107 and MTCC 532. But none of the strains induced hairy roots in the plant. But transformation with LBA 9402 strain induced hairy root (Ray *et al.*, 2014). Bansal *et al.* (2014) induced hairy root in *Bacopa monnieri* (L.) Wettst using A4, R1000, SA79, MTCC 532 and MTCC 2364 strains.

Hairy root cultures were established in *Raphanus sativus* also using MTCC 2364 and MTCC 532 strains with 77.6 % and 67.6 % transformation efficiency (Balasubramanian *et al.*, 2018).

2.4.5 Explants for Hairy Root Induction

Different explants were tried for hairy root induction using *A. rhizogenes*. Karmarker *et al.* (2001) tried leaf, shoot bud, internodal segment, seedling hypocotyl explants and callus of *Holostemma ada-kodien* for hairy root induction. Hairy root was induced from shoot bud and seedling hypocotyl part and no hairy root was observed from leaf, internodal segment and callus of the plant.

Leaves and nodal part of the stem were used as explants by Pandey *et al.* (2014) in coleus for establishing hairy root cultures. Cotyledonary leaf explants were used for transformation in *Lactuca serriola* L. (El – Ghamery *et al.*, 2017). Leaf explants were used in *Raphnus sativus* for hairy root induction and maximum 67.6 % transformation was reported (Balasubramanian *et al.*, 2018).

2.4.6 Wounding of Plants for Infection

Wounding of explants is a prerequisite for infection of *Agrobacterium,* because it triggers the secretion of *vir* gene inducing compounds (Stachel *et al.*, 1985). It can be done either with a sterile scalpel or syringe dipped in bacterial inoculum.

A. rhizogenes has to enter a wound to reach the phloem to induce hairy root induction (Hildebrand, 1934). According to Moore *et al.* (1974) most of the hairy roots are emerged from the pericycle tissue of vascular cylinder.

Mahesh and Jeyachandran (2011) studied transformation in *Taraxacum officinale* with proper wounding of explant with sterile scalpel. Explants were pinched and pricked with needle before transformation of coleus (Pandey *et al.*, 2014).

2.4.7 Role of Acetosyringone in Genetic Transformation

Acetosyringone and α-hydroxy acetosyringone are some of the phenolic compounds, which enhance T-DNA integration by inducing transcription of *vir* genes (Stachel *et al.*, 1985). Various concentrations of acetosyringone were tried in cotton to check the transformation efficiency. Maximum percentage of transformation was observed with 100 µM of acetosyringone (Afolabi- Balogun *et al.*, 2014). Different concentrations of acetosyringone were used to study the transformation efficiency in hairy root induction of *Oldenlandia umbellata* L. Acetosyringone at 200 µM concentration gave maximum percentage of response and further increase in the concentration of acetosyringone reduced the transformation efficiency (Krishnan and Siril, 2016). Acetosyringone 100 µM was used by Balasubramanian *et al.* (2018) in hairy root induction of radish and got the maximum hairy roots.

2.4.8 Co-cultivation

Rate of transformation in plants was highly influenced by the time of co-cultivation (Mihaljevic *et al.*, 1996). Hairy roots of *Catharanthus roseus* was induced with 48 h co-cultivation time (Choi *et al.*, 2004). Varghese *et al.* (2014) kept *Withania somnifera* cultures for 48 h co cultivation and got hairy roots.

Pandey *et al.* (2014) studied the hairy root induction in coleus and kept for co-cultivation for 24 h, 48 h, 72 h and 96 h. Maximum positive response was seen in 24 h and 48 h co-cultivation but all the explants were dried with 96 h co-cultivation.

According to Balasubramanian *et al.* (2018), 48 h co-cultivation showed the best transformation percentage. He tried transformation with two strains MTCC 532 and MTCC 2364. Hairy root induction was increased in both of these strains as co cultivation time increased, reaching maximum at 48 h followed by decrease thereafter.

2.4.9 Elimination of Bacteria

For hairy root induction in *Withania somnifera*, elimination of bacteria after transformation was done by using cefotaxime. The explants after transformation were washed with cefotaxime and transferred to solid MS containing cefotaxime 500 mg L^{-1} for removing bacteria (Varghese *et al.*, 2014).

Cefotaxime was also used as antibiotic in hairy root induction of *Holostemma ada-kodien K. schum.* (Karmarkar *et al.*, 2001), *Coleus forskohlii* (Pandey *et al.*, 2014), *Isatis tinctoria* L. (Gai *et al.*, 2015), *Catharanthus roseus* (Benyammi *et al.*, 2016), *Raphanus sativus* (Balasubramanian *et al.*, 2018).

Shanneja (2007) removed bacteria from infected explants of *Artemesia annua* by washing with MS medium containing 500 mg L^{-1} cefotaxime. After washing,

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explants were blot dried and transferred to solid MS medium containing 500 mg L^{-1} cefotaxime and cultures were incubated at 26 ± 2°C under diffused light.

Bansal *et al.* (2014) and Krishnan and Siril (2016) used ampicillin as an antibiotic for removing bacteria in their hairy root induction experiments.

2.4.10 Transformation Efficiency

LBA 9402 strain reported 85 per cent rooting in leaf explants and A4 strain had 10 percent transformation (Hu and Alfermann, 1993).

Shanneja (2007) compared the transformation efficiency of three *A. rhizogenes* strains in hairy root induction of *Rauvolfia serpentina.* In that ATCC 15834 showed 70%, A4 showed 50% and MTCC 2364 20% transformation efficiency.

According to Varghese *et al.* (2014) transformation efficiency of ATCC 15834, A4 and MTCC 2364 was 70%, 20% and 15% respectively in hairy root induction of *Withania somnifera.*

2.5 CONFIRMATION OF TRANSFORMATION

The integration of T-DNA in the genome of hairy roots is confirmed by using polymerase chain reaction (PCR) by amplifying specific genes like *rol A, rol B and rol C*. Confirmation of hairy root in *Bacopa monnieri* L. was done by amplifying *rol B* gene (Bansal *et al.,* 2014).

Suja (2010) confirmed the transformation with the help of morphological features, metabolite analysis and PCR analysis of *rol B* and *rol C* genes.

Transformation in *O. umbellata L.* was done by PCR amplification of *rol A, rol B* and *rol C* genes (Krishnan and Siril, 2016).

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Recombinant strains of *A. rhizogenes* were also used in hairy root studies and transformation was confirmed by GUS staining. Thus post transformational analysis of *Agrobacterium* mediated transformation in cotton was done by analysing GUS positive leaves (Afolabi-Balogun *et al.*, 2014).

2.6 QUANTIFICATION OF SECONDARY METABOLITES

High Performance Liquid Chromatography (HPLC) was used by several scientists for quantitative and qualitative analysis of secondary metabolites in plants. Schafer *et al.* (2016) quantified more than hundred primary and secondary metabolites by a single solid- phase extraction. Samples were analysed by Ultra High Performance Liquid Chromatography (UHPLC) and also by Heated Electro Spray Ionisation (HESI).

Anthraquinone from *O. umbellata* was extracted by sequential extraction method using ethanol and chloroform. Solvent was used at 10 ml for 1 g of dried hairy roots. HPLC analysis was done using final ethanol extract (Krishnan and Siril, 2016).

Quercetin content of radish was determined by Balasubramanian *et al.* (2018). Dried hairy roots of radish were ground into fine powder and extracted by using methanol in the rate of 1 g / 10 ml. HPLC was done in Waters 2998 liquid chromatography equipped with the photodiode array detector (PDA) and C18 column was used for the detection of quercetin.

Detection of metabolites from microbes was also done by HPLC technique. Secondary metabolites were detected from *Sclerotium rolfsii*, a microbe from chickpea using HPLC (Amber *et al.*, 2012).

Phenyl propanoids content in *Pedicularis densispica* was determined by using HPLC technique. Phenyl propanoids like salidroside, verbascoside, iso- verbascoside,

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leucoseptoside A, jionoside D and martynoside were determined. Separation was perfomed with C18 column with step-wise gradient elution with water (A) - methanol (B) as mobile phase (Chu *et al.*, 2017).

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled "Enhancement of phenyl propanoid glycosides in *Artanema sesamoides* Benth (vathomvaretti) by hairy root induction" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani and Aromatic and Medicinal Plants Research Station, Odakkali during 2016-2018. The study was conducted to develop a protocol to scale up the production of secondary metabolite content by inducing hairy root using *Agrobacterium rhizogenes*. The details regarding the experimental materials and methodology adopted for various experiments are described in this chapter.

3.1 COLLECTION OF PLANT MATERIALS

The seeds of *A. sesamoides* Benth used for the *in vitro* culturing were collected from Aromatic and Medicinal Plants Research Station, Odakkali, Ernakulam.

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

3.2.1 Chemicals

The chemicals for the preparation of media including major and minor nutrients were of analytical grade and procured from HIMEDIA, Sisco Research Laboratories (SRL), and Merck India Ltd. The plant growth regulators, vitamins, aminoacids, antibiotics and acetosyringone were obtained from HIMEDIA, SRL, Merck India Ltd and Sigma chemicals, USA. Primers for the PCR reaction were procured from Sigma Aldrich.

3.2.2 Glasswares and Plasticwares

Borosilicate glassware, membrane filters (Sartorius, Germany), disposable sterile petridishes (Tarsons, India) were used for the study.

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3.2.2.1 Cleaning and Sterilization of Glasswares and Other Equipments

Glasswares were soaked in detergent solution for 16 h and then washed with tap water followed by distilled water. After washing glassware were kept in hot air oven for 2 h for drying in a controlled temperature. Later glassware, forceps, scalpels, filtration units etc. were sterilized by autoclaving at 121^0 C, 100 Kpa for 45 minutes. Filterpapers, micropipette tip boxes and distilled water for the aseptic works were also autoclaved prior to the 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 by 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.

3.2.3 Composition of Medium

Murashige and Skoog (1962) medium (MS) was used for the *in vitro* propagation of *A. sesamoides*. The seeds of *A. sesamoides* were germinated in MS and half MS media. The composition of the MS medium is given in Appendix I.

The *A. rhizogenes* strains were cultured in Yeast Extract Mannitol (YEM) medium and Nutrient Agar (NA) media. The basal compositions of these media are given in Appendix II.

3.2.4 Preparation of Tissue Culture Medium

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 was stored in amber coloured bottle to avoid the photochemical reaction of iron.

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Medium was prepared in a clean beaker rinsed with distilled water. Working volumes of stock solutions were pipetted and added to the distilled water taken in the beaker. The required quantity of sucrose (30g L^{-1}), myoinositol (100 mg L^{-1}) and MS supplement (3.55g L^{-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 (6.3g L^{-1})/ Gelrite (4.5g L^{-1}) was added and heated in microwave oven. After melting the solidifying agent, medium was poured into clean, autoclaved culture bottles. Culture bottles containing the media were sterilized by autoclaving at 121°C in 100 KPa for 15 min and allowed to cool to room temperature. Inoculation was done only after checking media contamination.

3.2.5 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, IAA, 2,4-D) and cytokinins (BA) were prepared by dissolving in suitable solvents i.e., 2,4-D and IAA were dissolved in 90 per cent ethyl alcohol and NaOH was used to dissolve BA and NAA. Stock solutions were stored in refrigerator.

3.2.5 Antibiotics

Cefotaxime was used as the antibiotic to kill the *A. rhizogenes*. Stock solution of cefotaxime (10^4mgL^{-1}) was prepared. Cefotaxime was dissolved in sterile distilled water and filter sterilized in aseptic conditions. Aliquots were prepared and stored in refrigerator. Cefotaxime was added into MS medium for studying the sensitivity of the callus and transformation. Addition of cefotaxime to MS medium was done after autoclaving and cooling the medium to 40^0C.

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3.2.6 Culture Conditions

The culture bottles after inoculation were incubated at 25±2° C with 16 h photoperiod from fluorescent tubes. Humidity varied from 60-80 per cent according to the prevailing climate. Bacterial cultures were incubated at 25±°C in an incubator.

3.2.7 Seed Germination

Half ripen greenish yellow coloured pods were collected from *A. sesamoides*. Calyx of the pods was removed carefully without any breakage in the pods. Pods were washed in running tap water for 20 min followed by washing in laboratory detergent (Teepol) for 15 min. After washing in detergent, pods were washed in double distilled water for three to four times. Soaking in 100 per cent ethanol was also done for 1 min with washing in double distilled water. Surface sterilization of the pods was done in the laminar air flow chamber under sterile conditions with 0.1 per cent mercuric chloride ($HgCl_2$) for 2 min. After mercuric chloride treatment pods were washed three to four times with autoclaved double distilled water.

Surface sterilized pods were cut into two halves with sterile blade and taped the seeds inside the pods to the medium. Media used for seed germination were MS and half strength MS medium.

Cultures were incubated at 25±2°C in the dark for seed germination. Observations were taken after seed inoculation.

3.2.8 *In vitro* Multiplication of Shoots

Germinated seedlings were multiplied in MS medium with different concentrations of cytokinin (6-Benzyladenine) with basal MS medium as control. Node was used as the explant for multiplication.

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Table 2. Different concentrations of 6-Benzyladenine used for the multiplication of *A. sesamoides*

Treatment	6-Benzyladenine (mg L^{-1})
T1	0
T2	0.1
T3	0.2
T4	1
T5	2

Cultures were kept at 25±2°C temperature with 16 h light and 8 h dark photoperiod. Observations of each treatment were recorded after 45 days of inoculation.

3.2.9 Callus Induction

MS medium supplemented with different concentrations of plant growth regulators were used for callus induction. Explants for callus induction were node, leaf and root from *in vitro* raised *A. sesamoides*. Cultures were kept in dark at 25±2°C. Callus induction percentage was recorded after 60 days of inoculation.

Table 3. Plant growth regulators used in MS medium for callus induction.

Treatment	Plant growth regulators(mg L^{-1})			
	2,4 -D	BA	NAA	IAA
T1	0.5			
T2	1			
T3		0.1		
T4		0.2		
T5		0.5		
T6		0.5	0.5	
T7		0.5	0.2	
T8		2	0.1	
T9		4	0.1	
T10		1	1	
T11		0.5		0.2

3.3 HAIRY ROOT INDUCTION USING *Agrobacterium rhizogenes*

3.3.1 Sensitivity of *Artanema sesamoides* Callus to Cefotaxime

Sensitivity of *A. sesamoides* callus to bacteriostatic agent cefotaxime was evaluated. Calli produced in MS medium supplemented with BA 0.5 mg $L^{-1}$and NAA 0.5 mg L^{-1} were transferred to MS medium with different concentrations of cefotaxime. Cultures were kept in dark at 25±°C.

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Table 4. Different concentrations of cefotaximein basal MS medium used for sensitivity test

Treatment	Cefotaxime (mg L^{-1})
1	0
2	100
3	200
4	300
5	400
6	500

The response of the callus to varying concentrations of cefotaxime was studied for a period of four weeks.

The following scoring method was used for the evaluation of sensitivity test, depending on the appearance of the callus.

Score	Culture response
++++	Fully green
+++	Partially discoloured
++	Bleached tissues
+	Tissues turning brown and dead

3.3.2 Preparation of *Agrobacterium rhizogenes* Suspension

Three strains of *A. rhizogenes* MTCC 532(Microbial type culture collections and gene bank, Chandigarh), MTCC 2364 and ATCC 15834 (American type culture collections) were used for the present study.

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Three strains (MTCC 532, MTCC 2364, ATCC 15834) were cultured in Nutrient agar (NA) and Yeast extract mannitol (YEM). The solid medium was prepared by pouring the medium in sterile petriplates. The strains were streaked on plates containing the media and incubated at 26±2°C for 48 h. Bacterial culture was transferred to liquid medium by inoculating the single colony and incubated at 26±2°C in incubator shaker at 250 rpm for 16 h.

The next day, the culture at OD value 0.8-1 @ 600 nm was spun in a centrifuge at 5000 rpm for 5 min and re-suspended the bacterial pellet obtained in liquid MS medium containing acetosyringone, 100µM.

3.3.3 Preparation of Plant Material

Callus, leaf, internodal segment were used as explants for transformation experiments. Plants and calli were precultured before one week of infection by subculturing it in basal MS medium and low concentration hormone medium respectively. This is to maintain the cultures in active cell division stage and to get rid of the accumulated plant growth regulators from the previous sub cultures.

3.3.4 Infection

Precultured tissues were placed in sterile petriplates and wetted with liquid MS medium to avoid drying of the explants. Wounds were made in tissues by pricking with sterile needles. These wounded tissues were infected by immersing in bacterial suspension containing acetosyringone. A gentle swirling was given upto the time allotted for the infection process. Immediately after the infection time, tissues were taken from the suspension and blotted on sterile tissue paper and transferred the tissues on basal MS medium for co-cultivation.

3.3.5 Co - cultivation

Infected tissues were placed on basal MS medium for co-cultivation for 24 h, 48 h and 72 h. Cultures were kept in dark at 26±2°C.

3.3.6 Elimination of *Agrobacterium rhizogenes*

After co-cultivation, tissues were washed in 500 mg L^{-1} cefotaxime and blot dried on sterile tissue paper. Later the tissues were placed on basal MS medium containing 200 mg L^{-1} cefotaxime for hairy root induction. Cultures were kept in 25±2°C. Observations were taken at two weeks interval.

3.4 CONFIRMATION OF TRANSFORMATION

3.4.1 Isolation of Genomic DNA from roots

For PCR analysis, DNA was isolated from the roots obtained after transformation with *A. rhizogenes* and also from non-infected (control) roots.

3.4.1.1 Procedure

Roots induced after infection was taken, weighed (400 mg) and ground into fine powder using liquid nitrogen with mortar and pestle. To this powder 4 ml of pre warmed CTAB (Cetyl trimethyl ammonium bromide) buffer was added and homogenized. Later 1 ml of the extract was transferred into 2 ml eppendorf tubes. After incubation for about 30 min in a water bath (60°C), the extract was spinned at 10000 rpm and supernatant obtained was transferred to fresh sterile microfuge tubes. 500µl of chloroform: Iso Amyl Alcohol (24:1) was added to each tube and mixed the solution by inversion. Later tubes were spinned at 1000 rpm for 10 min. The upper aqueous phase obtained (contains the DNA) was transferred to a clean microfuge tube. To this, two volumes of ice cold absolute ethanol was added, mixed well and incubated the tubes at -20°C for 30 min to precipitate the DNA. Later DNA was pelleted by spinning the tube at 10000 rpm for 7 min. Supernatant was removed and

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DNA pellet was washed by adding ice cold 70% ethanol, air dried and dissolved in 50 µl sterile DNase free water / TE buffer.

3.4.2 Isolation of Plasmid DNA

Plasmid DNA was isolated from the *A. rhizogenes* and used as positive control in PCR analysis.

3.4.2.1 Procedure

A single bacterial colony was taken from the freshly streaked plate and inoculated into 5 ml YEM medium in test tubes and incubated overnight at 26°C in shaker (180 rpm). The culture was poured into 1.5 ml tube and bacterial cells were pelleted out by centrifuging at 4000 rpm for 4 min. The supernatant was removed and bacterial pellets were re-suspended in 200 µl GET (Glucose EDTA Tris) buffer. To this 300 µl freshly prepared 1% SDS was added, mixed the contents in the tubes by inversion and incubated in ice for 5 min. Solution was neutralized by adding 300 µl 3 M potassium acetate (pH- 4.8), mixed by inversion and incubated in ice for 5 min. Cellular debris was removed by spinning at 15000 rpm for 10 min at room temperature and supernatant was transferred to clean tubes. The supernatant was extracted twice with chloroform 400 µl. It was done by mixing for 30 seconds and separated the phases by centrifugation at 13000 rpm for 1 min and transferred the upper aqueous phase to a fresh tube. To this, 100% isopropanol was added in equal volume and mixed by inversion. The mixture was centrifuged (14000 rpm) for 10 min at room temperature. Pellet formed was washed with 70% ethanol; air dried the pellet for 30 min in laminar hood and re-suspended the pellet in 60 µl double distilled water.

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3.4.3 Quantification of DNA

Quantification of DNA was done by recording the optical density at 260 nm and 280 nm wavelength with the help of UV – visible spectrophotometer. The concentration of DNA was calculated using the formula given below.

Amount of DNA (µg/ µl) = A_{260} ×50× dilution factor/1000

Where A_{260} =absorbance at 260 nm

The DNA quality could be judged from the ratio of the OD values recorded at 260 nm and 280 nm. The A_{260}/A_{280} values between 1.6 and 1.8 indicate the best quality DNA.

3.4.4 Polymerase Chain Reaction (PCR)

The PCR analysis was carried out using DNA isolated from root after transformation. The DNA isolated from roots produced by non-transformed (control) plants of *A. sesamoides* was used as negative control. The plasmid of *A. rhizogenes* isolated was used as positive control.

The PCR primer sets used for amplifying *rol C* gene aregiven in table 5.

Table 5. Details of primer

Sl No.	Primer	Sequence (5′ - 3′)
1	Rol C F1	TGTACCTCTACGTCGACTGC
2	Rol C R1	AAGTACCCGGGGACAGAAAG

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Table 6. Composition of PCR reaction mixture

SL. No.	Components	Final concentration
1	10 X PCR buffer	1X
2	dNTPs	0.2 µM
3	Taq DNA polymerase	1.5 U
4	MgCl2	2.5 mM
5	Forward Primer	70 pmol
6	Reverse Primer	70 pmol
7	Genomic DNA	50 ng

Table7. Temperature profile used for PCR

Step No	Temperature (°C)	Duration	Cycles	Function
1	94	3 min		Initial denaturation
2	94	45 s	30	Denaturation
3	57	45 s		Annealing
4	72	1 min		Extension
5	72	5 min		Final extension
6	4	Hold		Final hold

Reaction mixture for PCR was prepared and a momentary spin was given for thorough mixing of the components. After mixing, PCR tubes were placed in a thermal cycler (Eppendorf master cycler gradient) for polymerase chain reaction.

3.4.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was done to check the quality of DNA isolated and for the separation of amplicons of PCR. It was done in horizontal gel electrophoresis unit. Required amount of agarose (0.8 per cent (w/v) for genomic DNA and 1.0 per cent (w/v) for PCR product) was weighed out and melted in 1X TAE buffer (0.04 M Tris acetate, 0.001M EDTA, pH 8.0) by boiling. After cooling to an ear bearing temperature, ethidium bromide (10^{-3} mg ml^{-1}) was added. The melted agarose with ethidium bromide was then poured to a preset template with appropriate comb. After the gel solidification, the comb was removed carefully and the gel was mounted in an electrophoresis tank which is flooded with 1X TAE buffer, to about 1 mm above the gel. Required volume of 6 X sample loading dye was mixed with DNA and added to each wells. One of the well was loaded with 3 µl of the 100 bp molecular weight DNA marker with required amount of gel loading dye. Electrophoresis was carried out at 75 volts until the loading dye reached three fourth length of the gel. The gel was documented using BIO-RAD gel documentation system.

3.5 EXTRACTION AND ESTIMATION OF PHENYL PROPANOID GLYCOSIDES

3.5.1 Preparation of Reference

Phenyl propanoid glycosides with 99 % purity were used as standards for quantification.

3.5.2 Sequential Extraction of Phenyl Propanoid Glycosides from Samples

Phenyl propanoid glycosides were extracted from *in vivo* (roots of field grown plant), callus and *in vitro* roots. Callus and roots were collected from the *in vitro*

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cultures. After taking the fresh weight of the samples were dried in hot air oven at 60°C and finely powdered.

Sequential extraction method was used to extract the phenyl propanoid glycosides. Extraction was done with hexane, chloroform and methanol. Extraction was done three times using each of these solvents. Solvents were added and kept in orbital shaker at 110 rpm for 16 h for extraction. Details of solvents added to each sample is given in table 8

Table 8. Details of sequential extraction

Sl. No.	Sample	Weight of dried sample (g)	Solvents		
			Hexane (ml)	Chloroform (ml)	Methanol (ml)
1	*In vivo* root	3	30	30	30
2	Callus	3	30	30	30
3	*Invitro* root	0.3	3	3	3

Methanol extract was injected into the HPLC system. Methanol extract obtained after 3 times extraction, was centrifuged at 4500 rpm for 5 min and residue was removed. The extracts were dried by using rotary vacuum flash evaporator (Heidolph, Germany). After drying, residue was weighed and HPLC grade methanol was used to re-dissolve the contents. 1 ml of methanol was added to *in vitro* root extract residue and callus extract residue was re-dissolved in 10 ml 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.

3.5.3 Details of HPLC Assay

Column : Merck LiCrospher 100 RP 18e (250 mm x 4.6 mm I.D, 5 : m

Mobile phase : Two solvents (A & B) ; A: H_2O with 0.1 % orthophosphoric acid v/v & B: (90% ACN and 10% A w/v)

Wavelength : 330 nm

Programme : Multistep gradient

20 % B (0-20 min)

20-30% B (20-40 min)

30-50 % B (40-55 min)

50-60 % (55-65 min)

60-70 % (65-75 min)

Flow rate : 1 ml/ min

Injection volume : 20µl

Detector : Dionex Ultimate 3000 with ultimate 3000 PDA detector

Data obtained are analysed and compared with the reference.

3.6 ECONOMIC ANALYSIS

Economic analysis was done to compare the cost of production of phenyl propanoid glycosides per gram dry weight of the callus to that of root collected from field grown plants (*in vivo* root).

Results

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4. RESULTS

The results of the study on "Enhancement of phenyl propanoid glycosides in *Artanema sesamoides* Benth (vathomvaretti) by hairy root induction" are presented in this chapter.

4.1 SEED GERMINATION

Seeds of *A. sesamoides* were surface sterilized and inoculated on different media. Seed germination percentage and days taken for seed germination is shown in the Table 9. Maximum percentage (94%) of seed germination was seen on basal MS medium within 10 days of inoculation. Half strength MS medium gave 87 % seed germination within 18 days of inoculation. Seedlings produced in MS and half strength MS medium are shown in plate 1.

Table 9. Germination of *A. sesamoides* Benth seeds

Sl. No.	Medium	Seed germination percentage	Days taken for seed germination
1	MS	94	10
2	½ MS	87	18

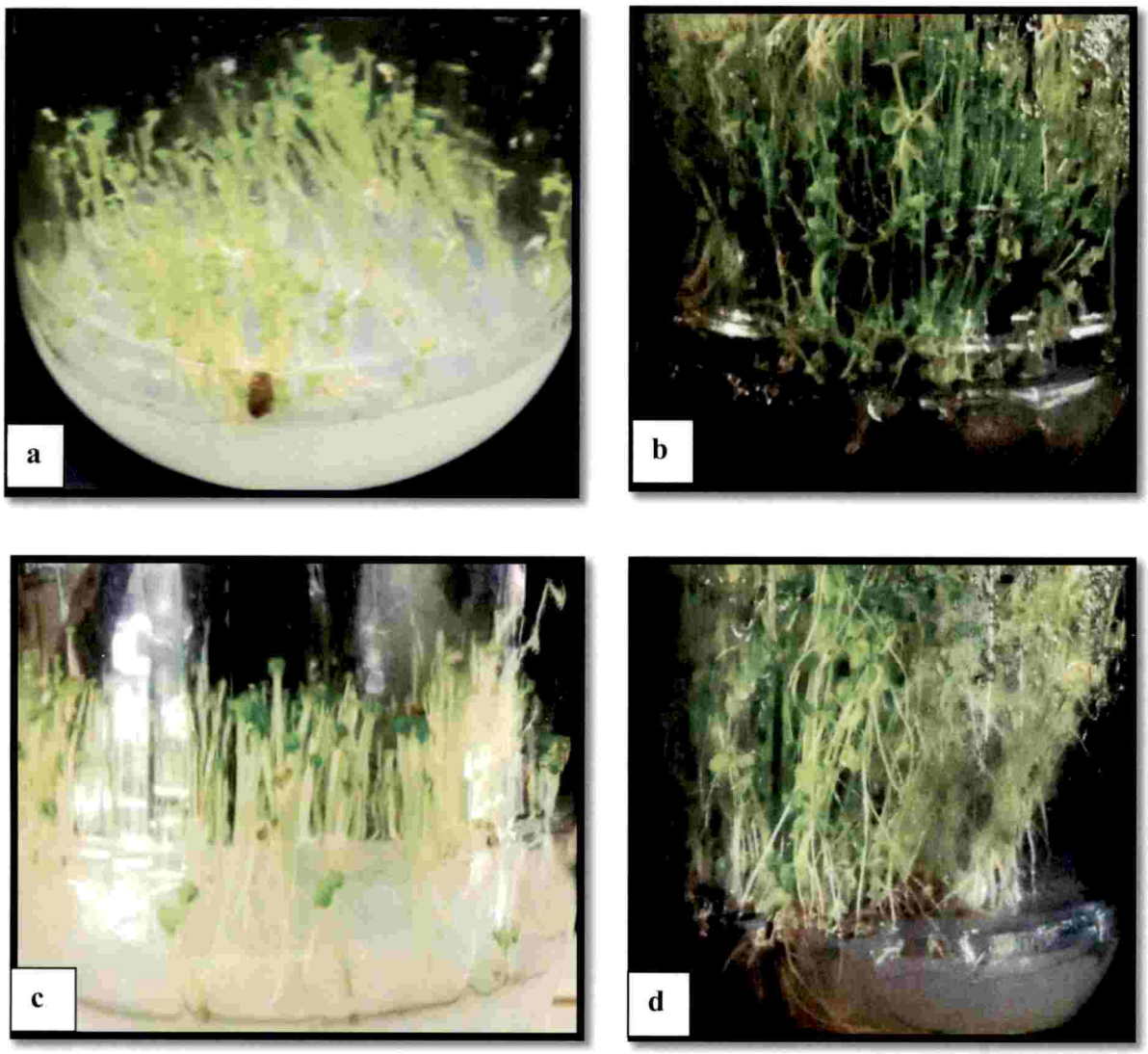

Plate 1. Germinated seedlings

a, b. In basal MS medium after 21 and 60 days

c, d. In half MS medium after 21 and 60 days

4.2 *IN VITRO* MULTIPLICATION OF SHOOTS

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In vitro shoots were raised as a source of explants for hairy root induction studies. Multiplication of shoots was done by using nodal segment from germinated seedlings as explant. Percentage of shoot induction, number of shoots produced per explant and morphology are shown in table 10. Basal MS medium supplemented with different concentrations of 6 -benzyladenine was used for multiple shoot induction. Observations were taken after 45 days of inoculation.

Table 10. Multiple shoot formation from nodal explant of *A. sesamoides*.

Sl. No.	6-Benzyladenine (mg L^{-1})	Percentage of shoot induction	Average no. of shoots produced per explant	Morphology
1	0	100.00	2	Stout purple shoots with large leaves
2	0.1	100.00	21	Numerous thin, slender and green shoots with small leaves
3	0.2	75.00	14	Numerous thin, slender and green shoots with small leaves
4	0.5	0.00	0	Green undifferentiated mass of cells
5	1	0.00	0	Green undifferentiated mass of cells
6	2	0.00	0	Green undifferentiated mass of cells

Maximum of shoot induction (100 %) was seen in basal MS medium and also in MS medium supplemented with BA 0.1 mg L^{-1}. But the average number of shoots produced per explant was maximum (21 nos) in MS medium supplemented with BA

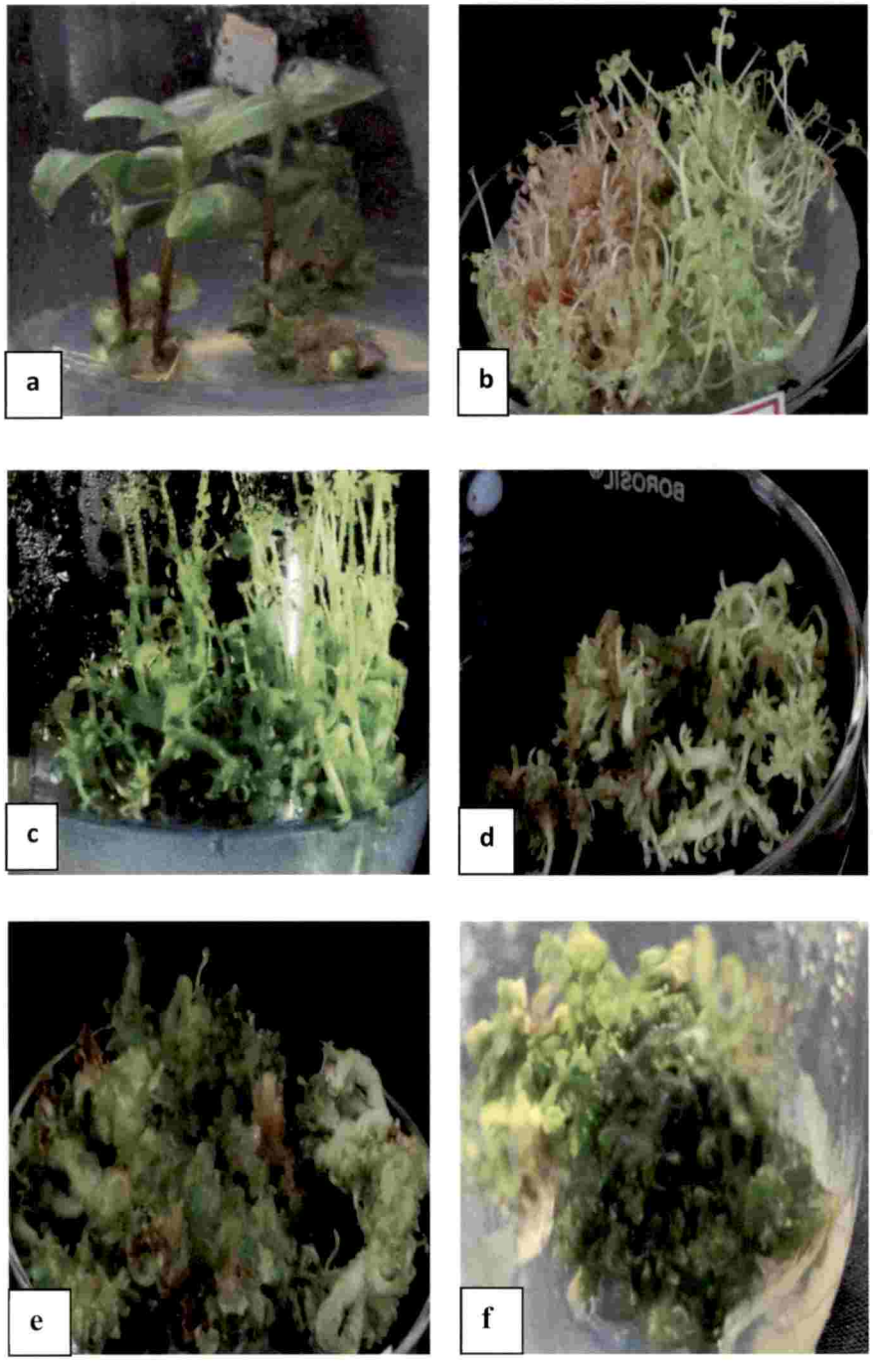

Plate 2. Multiple shoot formation in different concentrations of benzyl adenine in MS medium

a. 0 mg L^{-1} b. 0.1 mg L^{-1} c. 0.2 mg L^{-1} d. 0.5 mg L^{-1} e. 1 mg L^{-1} f. 2 mg L^{-1}

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0.1 mg L^{-1} and it was less (2 nos) in basal MS medium. As the concentration of BA increased to 0.2 mg L^{-1}, there was a decrease in the number of shoots produced, and further increase in BA concentration (0.5 mg L^{-1} to 2 mg L^{-1}) didn't produce any shoot, instead a green undifferentiated mass of cells was produced.

Morphology of shoots varied with each treatment (plate 2). Shoots formed in basal MS medium was same as that of field grown plants. Stout purple shoots with large leaves were formed in this medium. Thin, slender green shoots with small leaves were formed in MS medium supplemented with BA 0.1 mg L^{-1} and 0.2 mg L^{-1}. Further increase in BA concentration up to 2 mg L^{-1} gave a green undifferentiated mass of cells.

4.3 CALLUS INDUCTION

Callus induction in *A. sesamoides* was studied by using leaf, node, and root as explants. Effect of plant growth regulators on callus induction in MS medium for different explants is given in table 11.

Cent per cent callus induction was observed in MS medium supplemented with combinations of BA and NAA/IAA. All the explants formed callus in these combinations (plate 3, 4, 5).

Among the different explants tried, node and root showed better callus induction response (100%) than the leaf in almost all the media tried. All the explants gave friable callus in all the media except in MS medium supplemented with BA 1 mg L^{-1} and NAA 1 mg L^{-1}. Instead of friable callus, a compact mass of callus was obtained in this medium (plate 6c). Morphogenesis was observed in MS medium supplemented with BA 0.5 mg L^{-1} and IAA 0.2 mg L^{-1} after 60 days of inoculation.

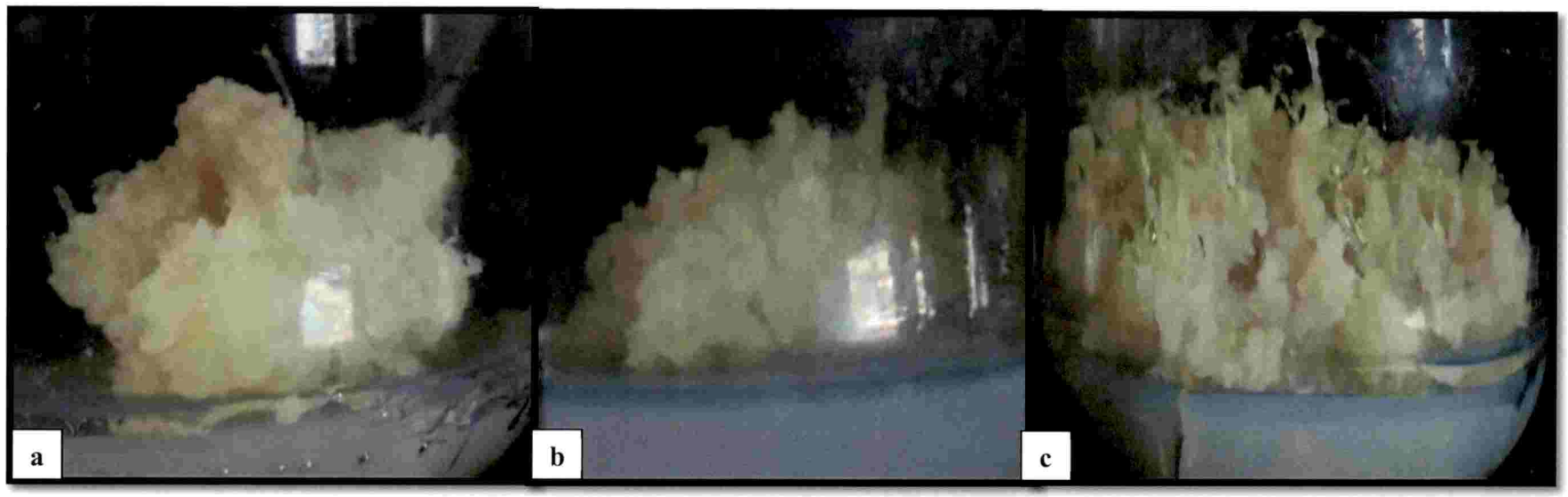

Plate 3. Callus induction using different explants in MS + BA 0.5 mg L^{-1} + NAA 0.5 mg L^{-1} medium

a. Node b. Leaf c. Root

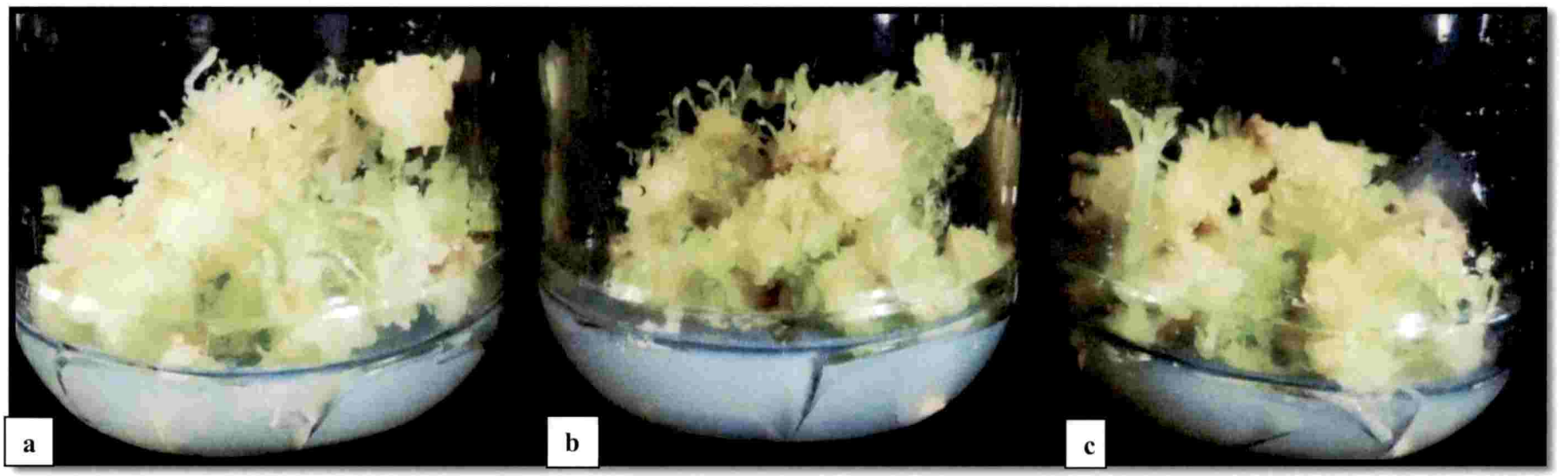

Plate 4. Callus induction using different explants in MS +BA 0.5 mg L^{-1}+IAA 0.2 mg L^{-1} medium

a. Node b. Leaf c. Root

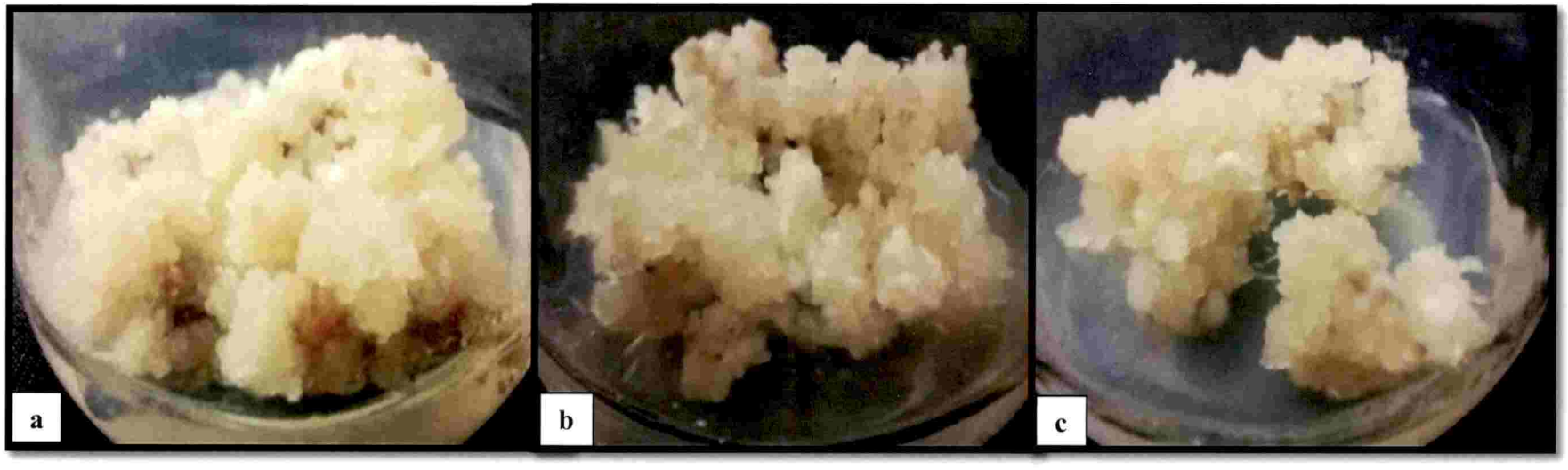

Plate 5. Callus induction using different explants in MS + BA 4 mg L^{-1} + NAA 0.1 mg L^{-1} medium

b. Node b. Leaf c. Root

Plate 6. Effect of different media in callus induction

a. node (MS + 2,4-D 0.5 mg L^{-1})

b. root (MS + 2,4-D 0.5 mg L^{-1})

c. node (MS + BA 1 mg L^{-1}+ NAA 1 mg L^{-1})

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Table 11. Effect of plant growth regulators on callus induction from different *in vitro* raised explants in MS medium after 60 days.

Sl. No.	Plant growth regulators ($mg\ L^{-1}$)				Callus induction percentage		
	2,4 - D	BA	NAA	IAA	Node	Leaf	Root
1	0.5				0.00	0.00	0.00
2	1				0.00	0.00	0.00
3		0.1			0.00	0.00	0.00
4		0.2			0.00	0.00	0.00
5		0.5			0.00	0.00	0.00
6		0.5	0.5		100.00	100.00	100.00
7		0.5	0.2		100.00	75.00	100.00
8		2	0.1		100.00	50.00	100.00
9		4	0.1		100.00	100.00	100.00
10		1	1		75.00	50.00	75.00
11		0.5		0.2	100.00	100.00	100.00

Addition of 2, 4-D or BA alone did not have any effect on callus induction. Addition of 2, 4 -D in the MS medium produced elongated shoots from the node (plate 6a and 6b). Roots produced good friable callus within 60 days of inoculation. Stages of callus induction after 14, 21, 30, 60 days of callus induction is shown in plate 7.

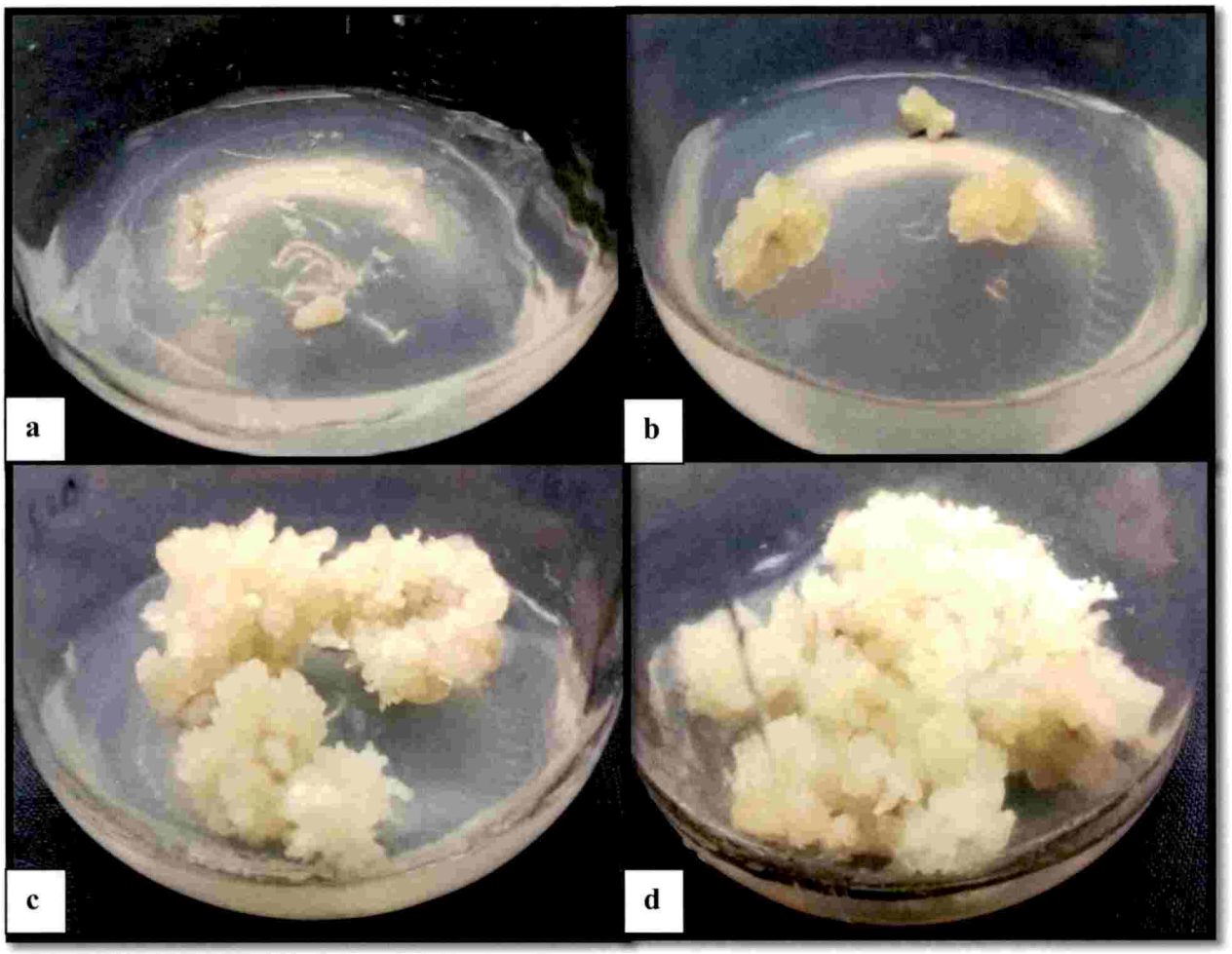

Plate7. Stages of callus induction on root explant in MS + BA 0.5 mg L^{-1} + NAA 0.5 mg L^{-1} medium

a. 14th day b. 21st day c. 30th day d. 60th day

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4.4 HAIRY ROOT INDUCTION USING *Agrobacterium rhizogenes*

4.4.1 Sensitivity of Callus to Cefotaxime

Sensitivity of *A. sesamoides* callus to different concentrations of cefotaxime was studied and the results are displayed in the table 12.

Callus remained healthy and fully green in all the concentrations of cefotaxime, except 500 mg L^{-1}. Callus kept in 500 mg L^{-1} of cefotaxime got partially discoloured after four weeks of inoculation. Hundred per cent survival of the callus was observed even after four weeks of inoculation up to cefotaxime concentration 500 mg L^{-1}. The entire calli got regenerated into shoots in MS medium supplemented with different concentrations of cefotaxime. Callus kept in MS medium supplemented with different concentration of cefotaxime after 2 weeks and 4 weeks is shown in plate 8 and 9. Shoot induction from callus was initiated after 2 weeks of inoculation. An increase in number of shoots was seen with increasing cefotaxime concentration upto 300 mg L^{-1}. But further increase in the concentration of cefotaxime showed a decrease in the rate of shoot induction.

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Table 12. Sensitivity of *Artanema sesamoides* callus to different doses of cefotaxime

Sl. No.	Cefotaxime ($mg\ l^{-1}$)	Sensitivity				Survival (%) after 4 weeks
		I Week	II Week	III Week	IV Week	100
1	0	++++	++++	++++	++++	100
2	100	++++	++++	++++	++++	100
3	200	++++	++++	++++	++++	100
4	300	++++	++++	++++	++++	100
5	400	++++	++++	++++	++++	100
6	500	++++	++++	++++	+++	100

++++ Fully green

+++ Partially discoloured

++ Bleached tissues

\+ Tissues turning brown and dead

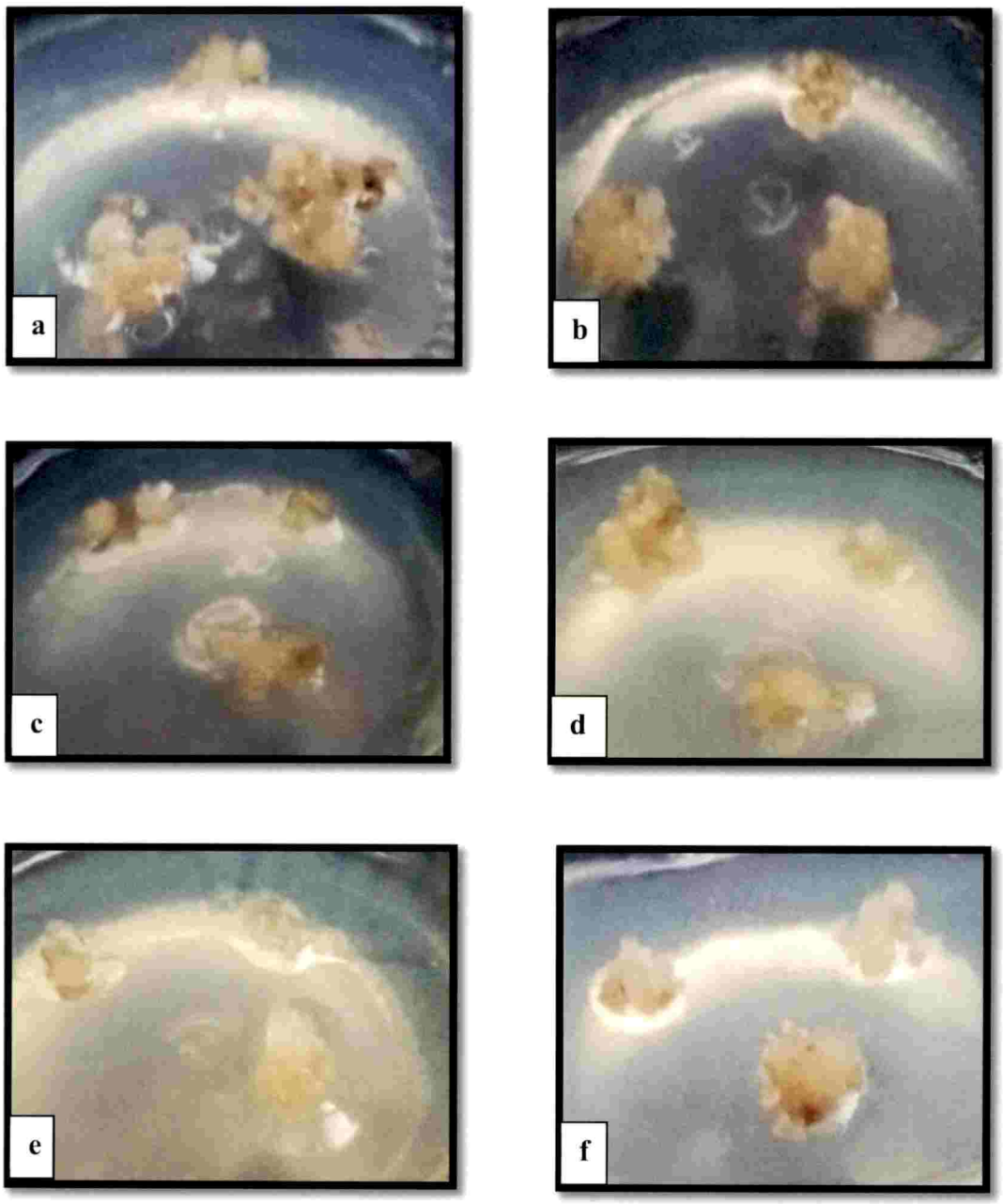

Plate 8. Sensitivity of callus to different concentrations of cefotaxime after 7 days of inoculation

a. 0 mg L^{-1} b. 100 mg L^{-1} c. 200 mg L^{-1} d. 300 mg L^{-1} e. 400 mg L^{-1} f. 500 mg L^{-1}

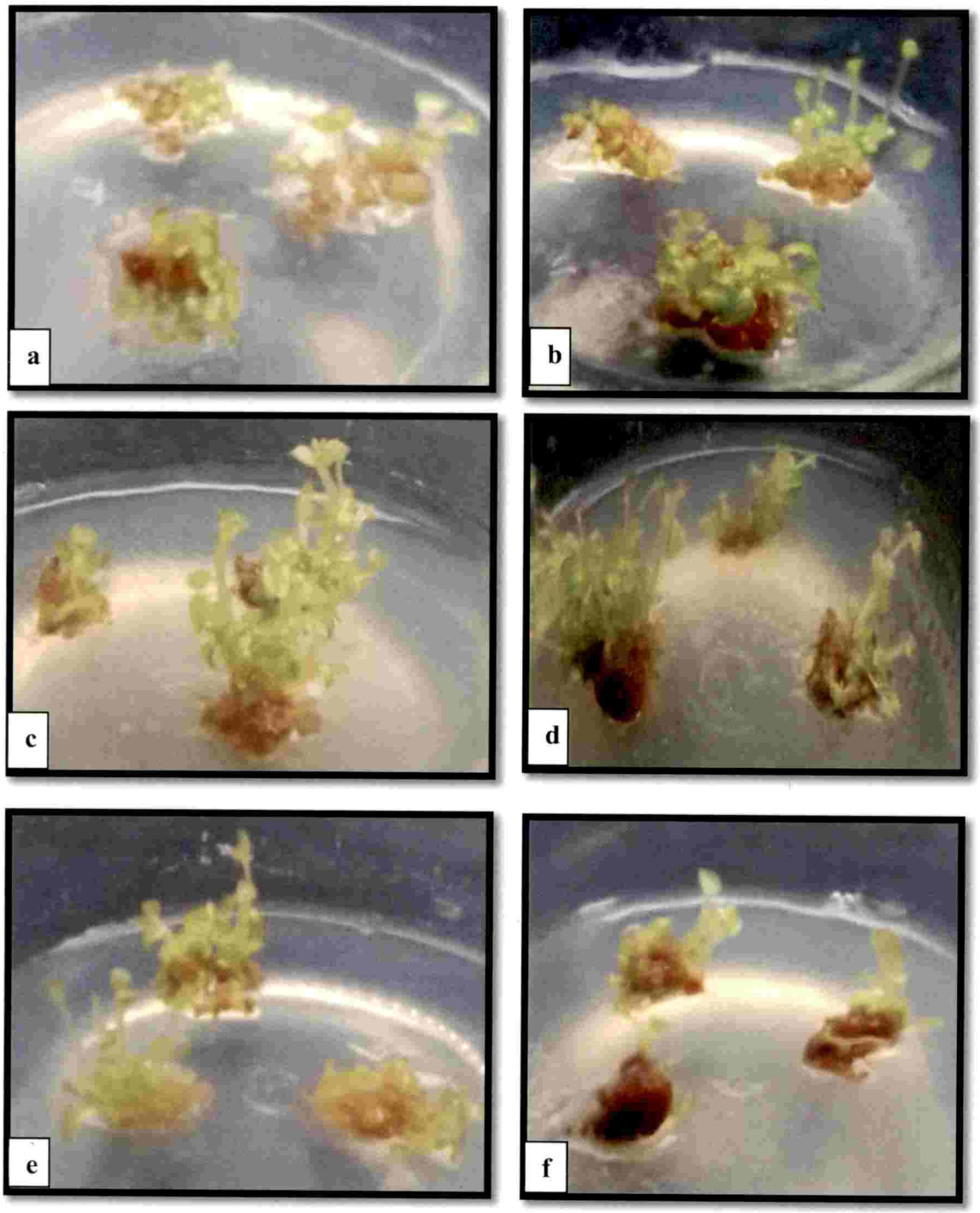

Plate 9. Sensitivity of callus to different concentrations of cefotaxime after 28 days of inoculation

a. 0 mg L^{-1} b. 100 mg L^{-1} c. 200 mg L^{-1} d. 300 mg L^{-1} e. 400 mg L^{-1} f. 500 mg L^{-1}

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4.4.2 Response of Explants after Transformation using *Agrobacterium rhizogenes*

Callus, leaf and stem segments of *A. sesamoides* were used as explants for transformation. Percentage of survival of explants after transformation in MS medium supplemented with cefotaxime 200 mg L^{-1} is given in table 13.

Calli infected with the strains MTCC 532, MTCC 2364 and ATCC 15834 survived in the MS medium supplemented with cefotaxime 200 mg L^{-1}. Leaf and stem segments dried off in the MS medium supplemented with cefotaxime after 28 days. Hundred per cent survival was noticed in the calli infected (MTCC 2364, MTCC 532) for 15 and 20 min with 48 h co-cultivation after 28 days of inoculation. Calli infected with MTCC 532 strain for 30 min and co-cultivated for 24 h also showed hundred per cent survival after 28 days. Almost all the calli survived in the cefotaxime containing medium regenerated into shoots and roots without growth regulators (Plate 10). Regeneration from calli started after two weeks of transformation. Discolouration of the callus was observed when callus infected for 60 min and co-cultivated for 72 h.

Untransformed calli (control) also showed regeneration in MS medium supplemented with cefotaxime 200 mg L^{-1}. There was no hairy root induction in any of the treatment tried. Roots developed from the calli after transformation, but their morphology was different from hairy roots. Infected calli were kept in both dark and light conditions for hairy root induction. Vigorous and fast growth of shoots and roots was seen from the callus kept in light. Chlorophyll content and size of the leaves was larger in light condition compared to that kept in dark conditions (Plate 11).

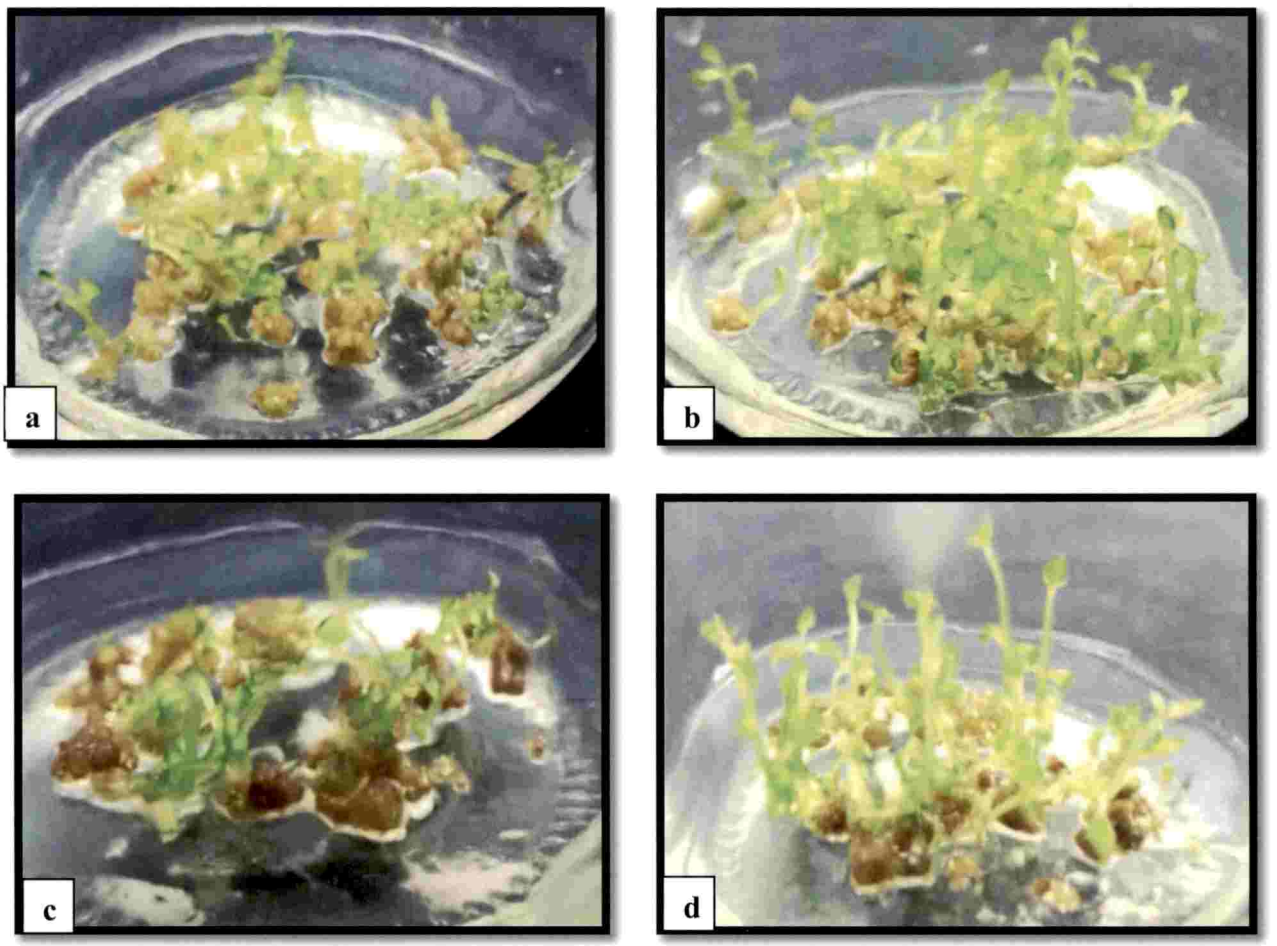

Plate 10. ***A. rhizogenes*** **infected callus survived in MS+ cefotaxime 200 mg L^{-1} medium**

a, b. MTCC 532 after 14 and 28 days

c, d. MTCC 2364 after 14 and 28 days

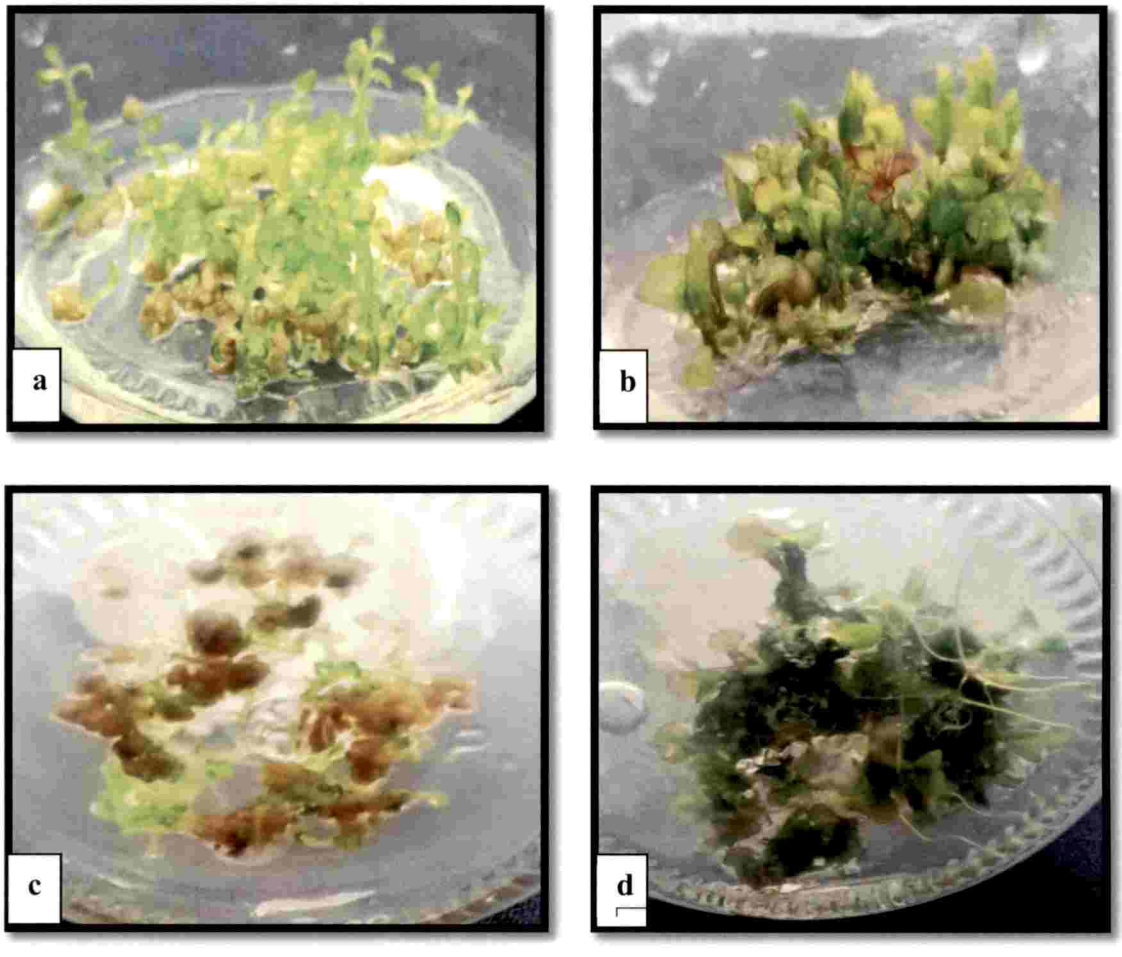

Plate 11. ***A. rhizogenes*** **infected callus incubated in dark and light conditions**

a, b. Shoots induced in dark and light conditions

c, d. Roots induced in dark and light conditions

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Table 13. Survival of *A. rhizogenes* strains infected tissues in MS + cefotaxime 200 mg L^{-1} after 28 days

Agrobacterium rhizogenes strains	Infection Period (min)	Co-cultivation period (h)	Percentage of tissues survived in cefotaxime		
			Callus	leaf	Internodal segment
MTCC 2364	0	24	33.30	0.00	0.00
	10		33.30	0.00	0 .00
	15		66.60	0.00	0.00
	20		66.60	0.00	0.00
	30		66.60	0.00	0.00
	60		0.00	0.00	0.00
	0	48	66.60	0.00	0.00
	10		66.60	0.00	0.00
	15		100.00	0.00	0.00
	20		100.00	0.00	0.00
	30		66.60	0.00	0.00
	60		0.00	0.00	0.00
	0	72	66.60	0.00	0.00
	10		33.30	0.00	0.00
	15		33.30	0.00	0.00
	20		33.30	0.00	0.00
	30		0.00	0.00	0.00
	60		0 .00	0.00	0.00

70A

Table 13. Survival of *A. rhizogenes* strains infected tissues in MS + cefotaxime 200 mg L^{-1} after 28 days (Contd.)

Agrobacterium rhizogenes strains	Infection Period (min)	Co-cultivation period (h)	Percentage of tissues survived in cefotaxime		
			Callus	Leaf	Internodal segment
MTCC 532	10	24	0.00	0.00	0.00
	15		33.30	0.00	0.00
	20		66.60	0.00	0.00
	30		100.00	0.00	0.00
	60		0.00	0.00	0.00
	0	48	66.60	0.00	0.00
	10		33.30	0.00	0.00
	15		100.00	0.00	0.00
	20		100.00	0.00	0.00
	30		100.00	0.00	0.00
	60		66.60	0.00	0.00
	0	72	66.60	0.00	0.00
	10		0.00	0.00	0.00
	15		33.30	0.00	0.00
	20		33.30	0.00	33.00
	30		0.00	0.00	0.00
	60		0.00	0.00	0.00

Table 13. Survival of *A. rhizogenes* strains infected tissues in MS + cefotaxime 200 mg L^{-1} after 28 days (Contd.)

Agrobacterium rhizogenes strains	Infection Period (min)	Co-cultivation period (h)	Percentage of tissues survived in cefotaxime		
			Callus	Leaf	Internodal segment
ATCC 15834	0	48	33.30	0.00	0.00
	10		33.30	0.00	0.00
	15		33.30	0.00	0.00
	20		66.60	0.00	0.00

4.4.3 Confirmation of Transformation by PCR Analysis

Polymerase chain reaction was used to confirm the presence of *rol C* genes in the roots obtained after transformation. Plasmid DNA of *A. rhizogenes* was kept as positive control and DNA from uninfected roots as negative control for PCR analysis. DNA samples isolated from plasmid and roots were analysed on 0.8 per cent agarose gel. Size of plasmid DNA was approximately 10 kb.

PCR analysis was carried out by using Rol Cf1r1 primer (Krishnan and Siril, 2016). The amplified fragments were separated on 1 per cent agarose gel (plate 12). Amplification of *rol C* genes was seen only in positive control (Ri plasmid DNA). No amplification was seen in roots obtained from infected and uninfected callus.

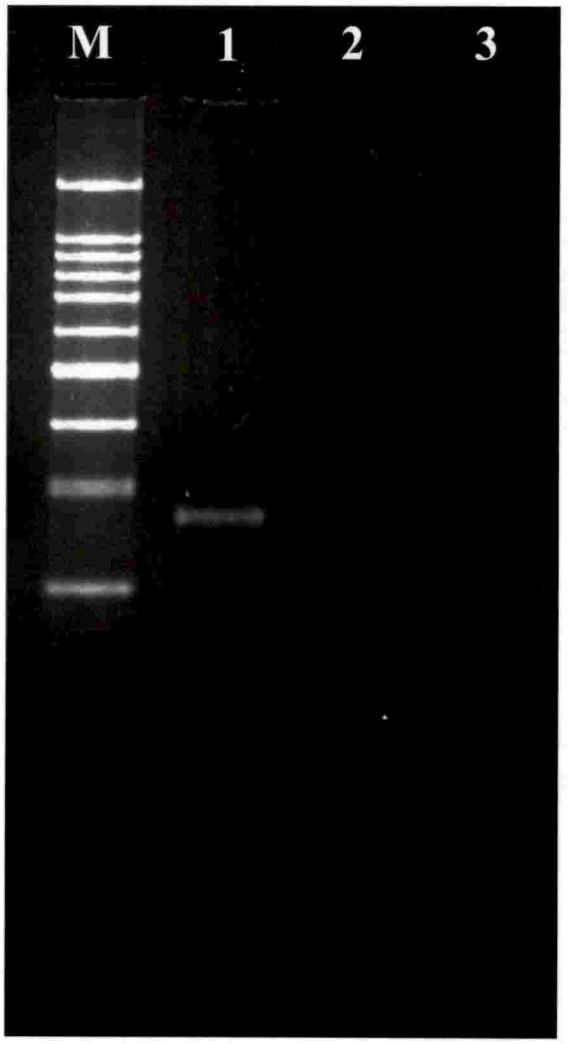

Plate 12. PCR profile of *rol C* genes

M -100 bp DNA marker

Lane 1- Positive Control (Ri plasmid)

Lane 2- Negative control

Lane 3- Roots induced from infected callus

4.5 QUANTIFICATION OF PHENYL PROPANOID GLYCOSIDES

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

Chromatograms obtained for roots of field grown plants (*in vivo* roots), callus and *in vitro* root are shown in Figures 1, 2 and 3 respectively. Details of the chromatogram peaks are shown in the tables 14, 15 and 16. After analysing with the standard graph of PPGs five active compounds (acteoside, artanemoside A, isoacteoside, leucoseptoside A and martynoside) were identified. Percentage of PPGs in dry powder in each sample was calculated based on the regression equations and is shown in the table 17. Percentage of all PPGs identified in the callus was more than that in *in vivo* root and *in vitro* root (Fig. 4).

Table17. PPGs content (%) in *A. sesamoides*

SL. No.	Phenyl propanoid glycosides	PPGs (%) in dry powder		
		In vivo root	Callus	*In vitro* root
1	Acteoside	0.25	0.98	0.13
2	Artanemoside A	0.30	2.14	0.29
3	Isoacteoside	0.05	0.11	0.02
4	Leucoseptoside A	0.01	0.08	0.006
5	Martynoside	0.10	0.21	0.003

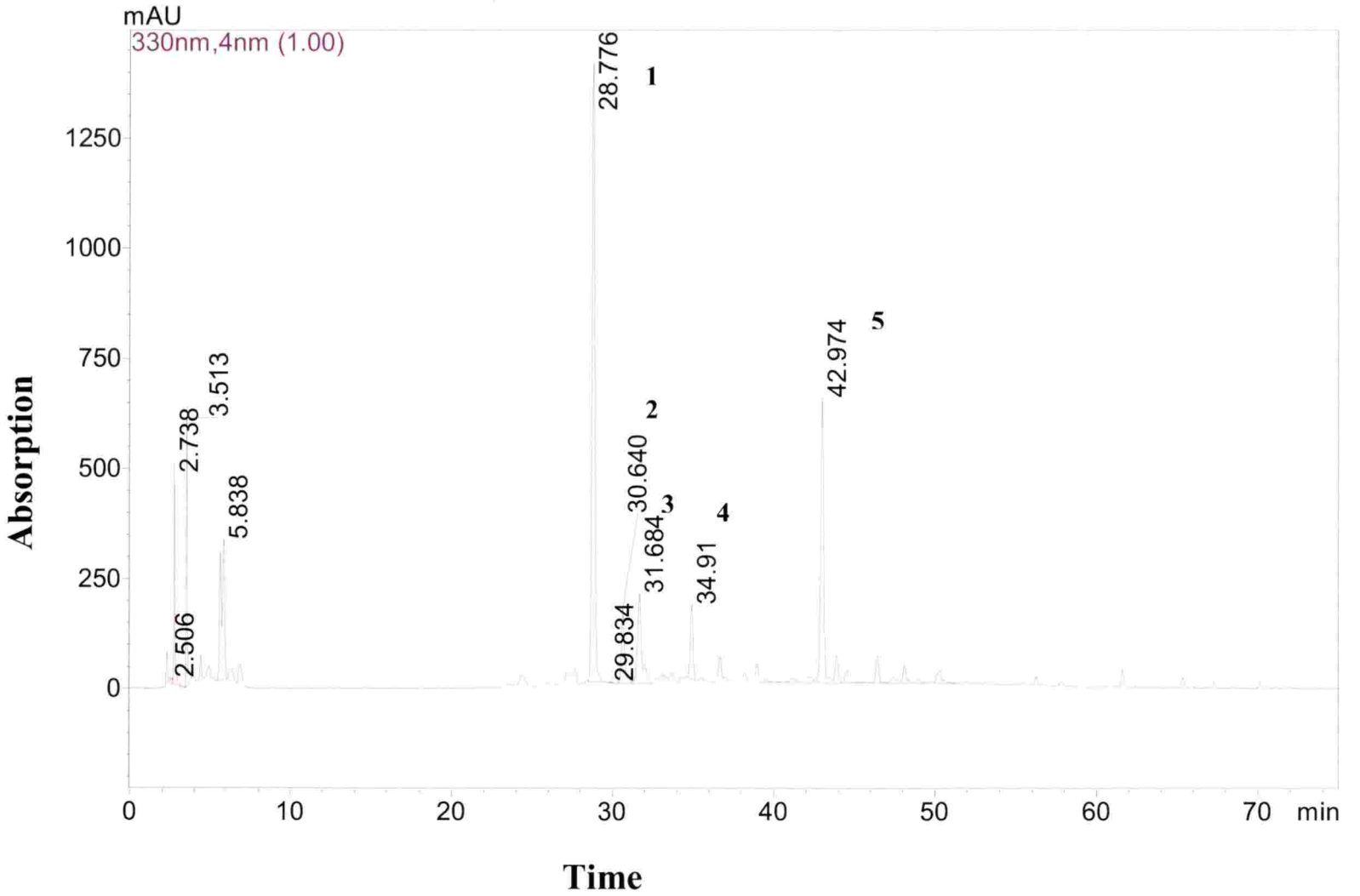

Fig. 1 Chromatogram of PPGs in field grown root of *A. sesamoides*

1-Acteoside, 2-Artanemoside A, 3-Isoacteoside, 4-Leucoseptoside A, 5-Plantainoside C, 6-martynoside

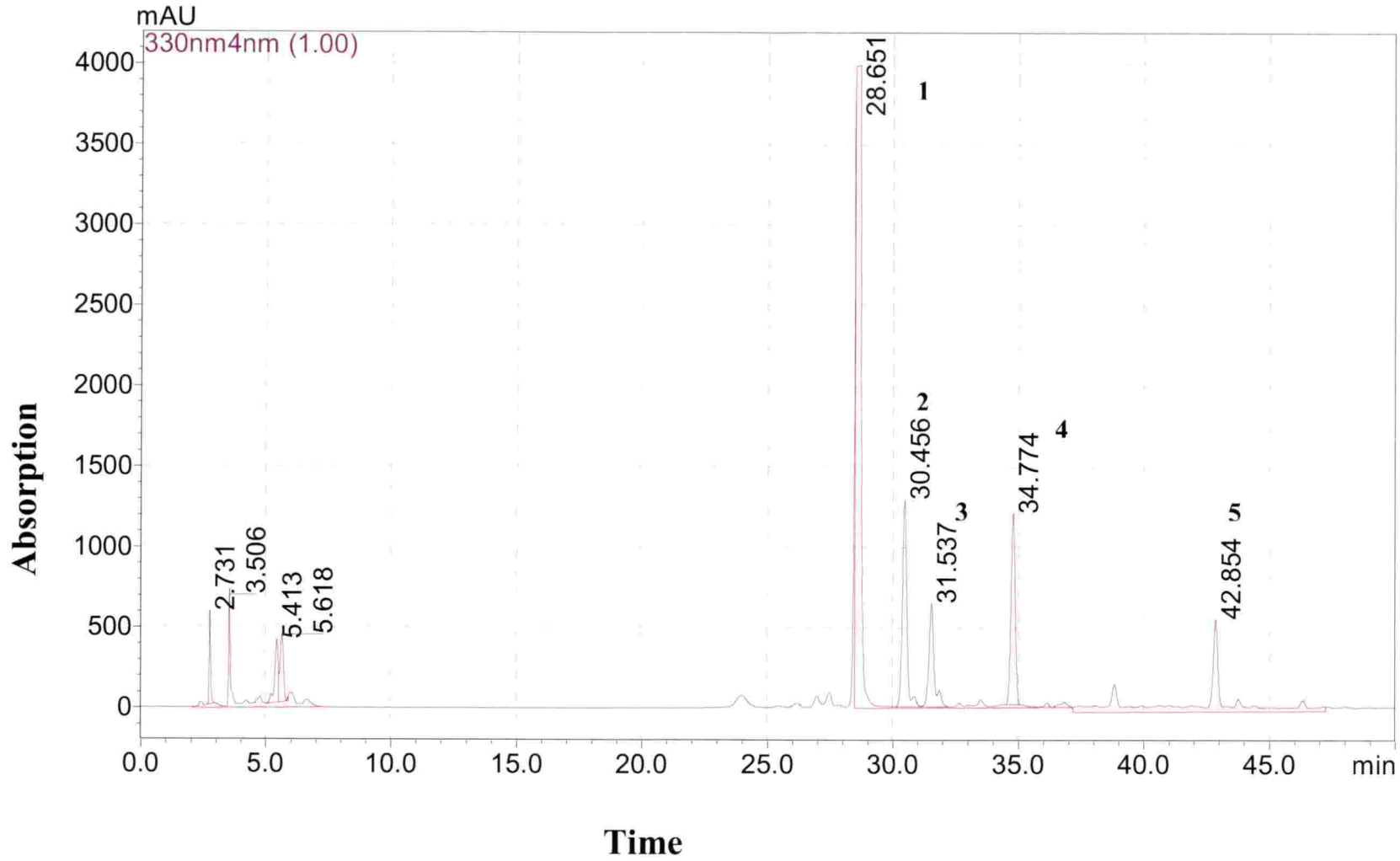

Fig. 2 Chromatogram of PPGs in callus of *A. sesamoides*

1-Acteoside, 2-Artanemoside A, 3-Isoacteoside, 4-Leucoseptoside A, 5-Plantainoside C, 6-martynoside

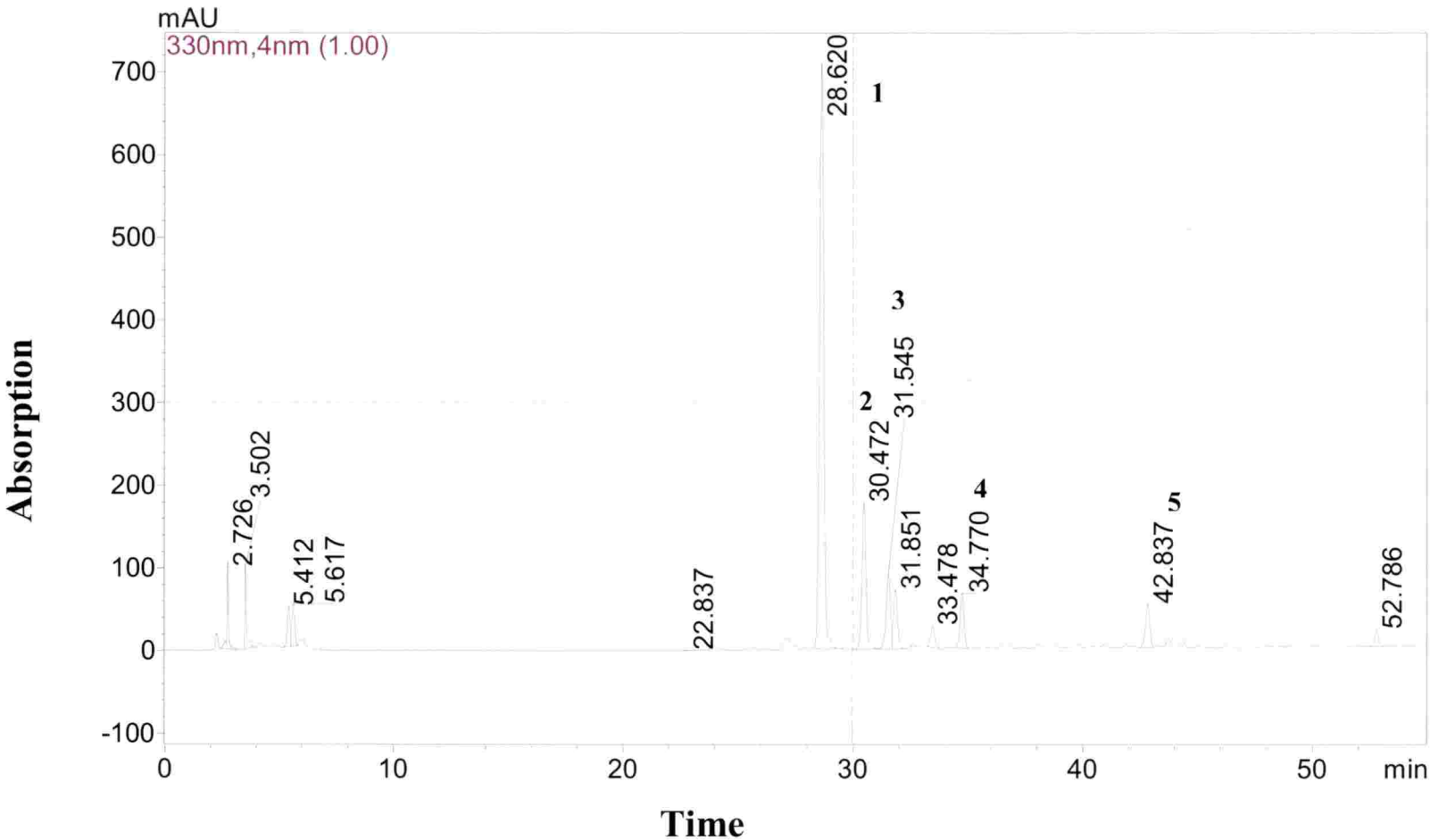

Fig. 3 Chromatogram of PPGs in *in vitro* of *A. sesamoides*

1-Acteoside, 2-Artanemoside A, 3-Isoacteoside, 4-Leucoseptoside A, 5-Plantainoside C, 6-martynoside

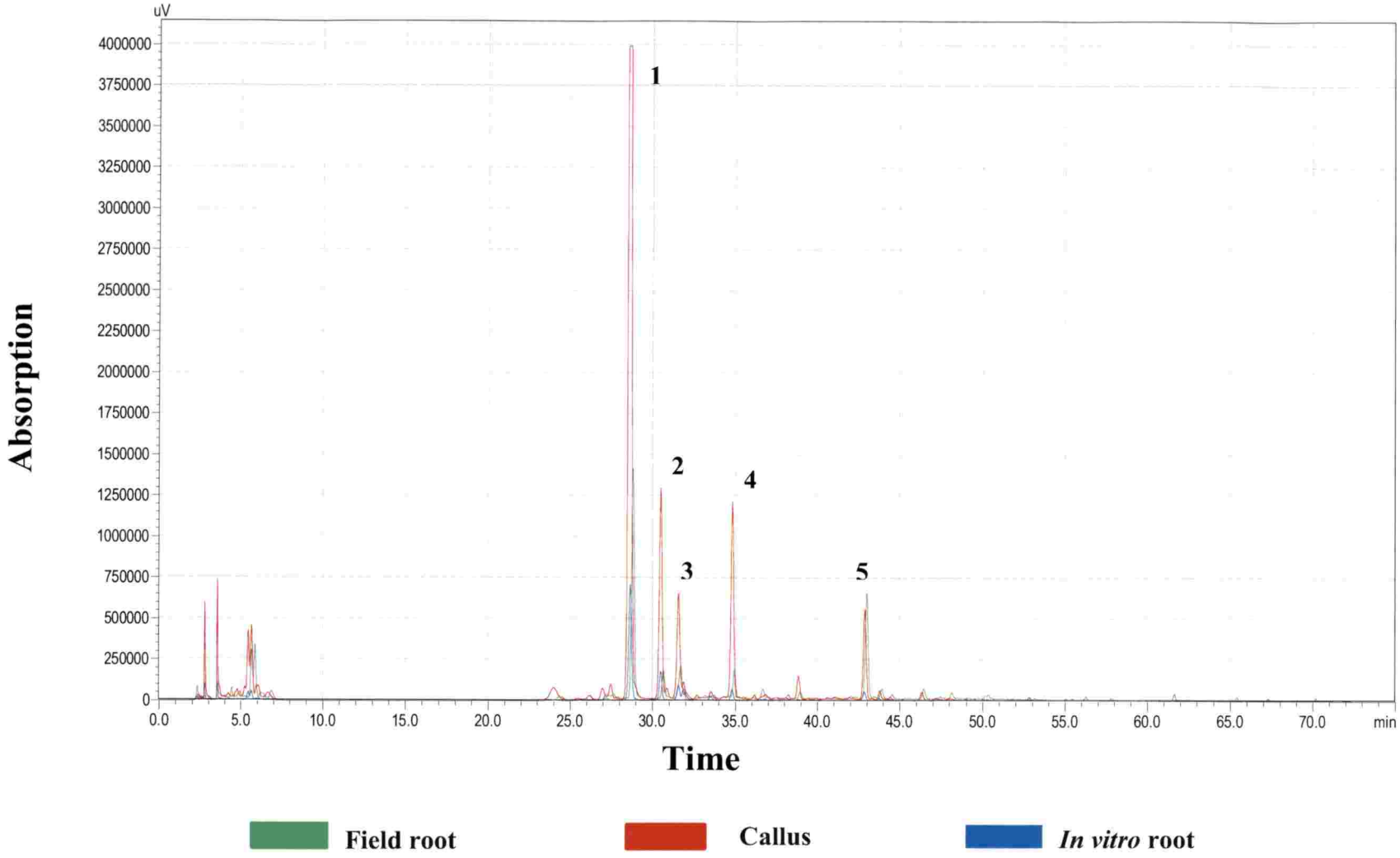

Fig. 4 Comparison between PPGs in field grown root, callus and *in vitro* root

1-Acteoside, 2-Artanemoside A, 3-Isoacteoside, 4-Leucoseptoside A, 5-Plantainoside C, 6-martynoside

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Table 14. Details of chromatogram peaks of *in vivo* root sample (PDA Multi 1/ 330nm 4nm)

Peak	Retention time (min)	Area (330 nm)	Height (330 nm)	Area%	Height%
1	2.506	130222	12209	0.268	0.299
2	2.738	2199199	505130	4.526	12.384
3	3.513	2578902	636674	5.308	15.609
4	5.838	5252200	320735	10.810	7.863
5	28.776	17255364	1406131	35.513	34.474
6	29.834	29048	1911	0.060	0.047
7	30.640	2288746	172131	4.710	4.220
8	31.684	3686867	205281	7.588	5.033
9	34.917	1922709	170135	3.957	4.171
10	42.974	13245085	648450	27.260	15.898
Total		48588342	4078787	100.000	100.000

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Table 15. Details of chromatogram peaks of callus sample (PDA Multi 1/330 nm 4nm)

Peak	Retention time (min)	Area (330 nm)	Height (330 nm)	Area%	Height%
1	2.731	2340199	582347	1.491	5.917
2	3.506	12316139	741793	7.849	7.537
3	5.413	3736377	397127	2.381	4.035
4	5.618	3938802	423965	2.510	4.308
5	28.651	66462818	3992835	42.357	40.571
6	30.456	16241223	1291143	10.351	13.119
7	31.537	9630864	645854	6.138	6.562
8	34.774	13706130	1189141	8.735	12.083
9	42.854	28538890	577488	18.188	5.868
Total		156911444	9841691	100.000	100.000

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Table16. Details of chromatogram peaks of *in vitro* root sample (PDA Multi 1/330 nm 4nm)

Peak	Retention time (min)	Area (330 nm)	Height (330 nm)	Area%	Height%
1	2.726	477233	105092	2.799	6.890
2	3.502	475430	103712	2.789	6.800
3	5.412	416267	49901	2.442	3.272
4	5.617	447721	52203	2.626	3.423
5	22.837	483	39	0.003	0.003
6	28.620	9005878	707648	52.822	46.396
7	30.472	2168351	177631	12.718	11.646
8	31.545	1181233	91241	6.928	5.982
9	31.851	847809	71617	4.973	4.695
10	33.478	328455	26590	1.926	1.743
11	34.770	789346	66461	4.630	4.357
12	42.837	680284	52742	3.990	3.458
13	52.786	231025	20357	1.355	1.335
Total		17049515	1525234	100.000	100.000

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4.6 ECONOMIC ANALYSIS

Economic analysis showed that the cost of production of PPGs per gram dry weight of the callus was less compared to that of *in vivo* roots (Appendix III). Since PPGs content in the callus is more than the *in vivo* root, large scale production of the compound is economically feasible in *in vitro* conditions.

Discussion

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5. DISCUSSION

Artanema sesamoides Benth, commonly known as vathomvaretti/ neermulli, is an important medicinal herb of Scrophulariaceae family. This lesser known, medicinal plant of Kerala is used by various traditional systems of medicine. The root of *A. sesamoides* is reported to have secondary metabolites known as phenyl propanoid glycosides (PPGs).

Extracts of *A. sesamoides* have shown to possess high antioxidant and antiinflammatory properties with several therapeutic activities against tumors, hypertension, viral and fungal infection (Selvan *et al.*, 2008; Gupta *et al.*, 2008). PPGs including acteoside, a well known pharmaceutical, were found to be effective in curing acute inflammation and rheumatoid arthritis and 47 % of the *A. sesamoides* root extract is PPGs in easily extractable form (Joseph *et al.*, 2010). But the roots of *Artanema* are spongy and dry weight is very less and it is difficult to obtain the phenyl propanoid glycosides in large scale from field grown plants. The present study aimed for establishing hairy root cultures using *Agrobacterium rhizogenes* to increase the PPGs content. Hairy root cultures are known valuable tool for the production of plant secondary metabolites due to the high stability and productivity features.

The present study was undertaken at the Department of Plant Biotechnology, College of Agriculture, Vellayani and Aromatic and Medicinal Plants Research Station, Odakkali under the title "Enhancement of phenyl propanoid glycosides in *Artanema sesamoides* Benth (vathomvaretti) by hairy root induction". The results obtained are discussed in this chapter.

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5.1 SEED GERMINATION

Seeds of *A. sesamoides* were germinated *in vitro* to raise seedlings as a source of sterile explants for hairy root induction. For seed germination basal MS medium and half strength MS medium were used. Maximum germination (94%) was recorded in basal MS medium compared to that in half strength MS medium (87%). Seeds kept in basal MS medium germinated within 10 days of inoculation.

In vitro propagation of *A. sesamoides*is reported for the first time in this study. *In vitro* propagation of a few plants of the same family (Scrophulariaceae) has been reported. Seeds of snapdragon, which is included in the family Scrophulariaceae when germinated *in vitro* showed 63% survival in the full strength MS medium (Hamza *et al.*, 2013). Seed germination of *Digitalis* (Verma *et al.*, 2016) and common mullein (Turker *et al.*, 2001) belonging to the same family also showed good results in basal MS medium. Same trend was shown by *A. sesamoides* in this study.

5.2 MULTIPLICATION OF SHOOTS

For the *in vitro* shoot multiplication, cytokinins like BA, Kinetin, TDZ etc. are commonly used (Pathak *et al.*, 2009; Mokhtari *et al.*, 2013; Siddique *et al.*, 2015).

In the present study, different concentrations of BA were tried for multiple shoot induction and maximum number of shoots (21 nos) per explant was obtained in MS medium supplemented with 0.1 mg L^{-1} BA. As the concentration of BA increased, number of shoots produced was reduced and when the concentration was increased to 2 mg L^{-1}, a green undifferentiated mass of cells was produced. Hamza *et al.* (2013) reported the use of BA and TDZ for *in vitro* multiplication of snapdragon (Family: Scrophulariaceae). In that study, MS medium supplemented with 0.5 and 1 mg L^{-1} of BA produced an average of 13 shoots per explant. They used TDZ concentrations upto 2 mg L^{-1} in MS medium and an increase in the concentration of TDZ to 1 and 2 mg L^{-1} produced an undifferentiated mass of cells instead of shoots.

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These results were in accordance with the results of the present study on *A. sesamoides*.

Baskaran *et al.* (2014) reported that the morphology of the plant varies in accordance with the concentration of plant growth regulators used. They did *in vitro* culturing of *Coleonema pulchellum* which is a rich source of phenyl propanoids, terpenoids and phenyl propenes and found that morphology varies with the concentration of TDZ. Green compact basal callus and hydrophilic roots were obtained in the shoot multiplication experiments of *Coleonema*. Same trend was also shown by *A. sesamoides* shoots, which is also a source of phenyl propanoids.

5.3 CALLUS INDUCTION

In the present study, callus induction was obtained in the MS medium supplemented with auxin and cytokinin in combination. BA in combination with NAA or IAA showed 100% callus induction. Hamza *et al.* (2013) studied the callus induction in snapdragon included in the same family Scrophulariaceae. In that study also 100 % callus induction was observed in MS medium supplemented BAP 0.5 mg L^{-1} and NAA 1 mg L^{-1}. In the similar way combination effect of BA and NAA in callus induction was also reported by Sarker *et al* (1996).

2, 4-D is successfully used as a plant growth regulator in callus induction experiments (Zheng and Konzak, 1999; Jala, 2014 and Zang *et al.*, 2016). But in the present study 2, 4-D didn't show any response to callus induction from any of the explants used and showed shoot initiation instead. Callus of *Artanema* also showed shoot initiation even without any plant growth regulators, an indicative of the presence of high endogenous cyokinin content in the plant. This may be the reason for the inhibition of the callus inducing effect of 2, 4-D in the experiment. Though shoot induction occurred in the presence of 2,4-D, the number of shoots were very less compared to other growth regulators.

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In the present investigation, while comparing the percentage of callus induction with different explants, callus induction was found to be high from nodal and root explants compared to leaf.

4.4 HAIRY ROOT INDUCTION USING *Agrobacterium rhizogenes*

4.4.1 Sensitivity of Callus to Cefotaxime

Since cefotaxime was used as the bacteriostatic agent in the transformation studies, sensitivity of *A. sesamoides* callus to cefotaxime was studied. Sensitivity of callus to different antibiotics was studied earlier in various medicinal plants like *Centella asiatica* (Lekshmi, 2008), *Plumbago* (Bhasker, 2004) etc. Studies showed that, sensitivity varied with the plant species and sensitivity test of callus to antibiotic is needed before every transformation experiment.

In the present study, *A. sesamoides* callus remained healthy in MS medium supplemented with different concentrations of cefotaxime up to 500 mg L^{-1}, but a small discolouration was seen in the callus in 500 mg L^{-1} after 4 weeks of inoculation. Another important observation was that the entire calli regenerated into shoots.

In majority of the sensitivity studies, plant calli were found sensitive to higher concentrations of cefotaxime. For example, *Centella asiatica* callus was sensitive to cefotaxime from 300 mg L^{-1} concentration onwards. Browning of callus occurred in higher concentrations of cefotaxime like 500 mg L^{-1} (Lekshmi, 2008). Similar effects were also noticed by Bhasker (2004) in *Plumbago*. But there are plant species which were found insensitive to cefotaxime upto 500 mg L^{-1}. For example Suja (2010) reported that the explants of *Rauvolfia serpentine* remained healthy up to 500 mg L^{-1} of cefotaxime concentration.

Cefotaxime has been considered as an antibiotic which induces negative effects on the plant tissues and regeneration. But in *Artanema*, the rate of shoot

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generation was positively influenced by the cefotaxime up to 300 mg L^{-1} concentration. Similar effects were reported by Naderi (2016) in melon. He studied the regeneration of melon by using different concentrations of cefotaxime and high frequency of shoot generation was observed in the MS medium supplemented with BA 1.5 mg L^{-1} and cefotaxime 250 mg L^{-1}.

4.4.2 Response of Explants Infected with *Agrobacterium rhizogenes*

In the present study, three wild strains of *A. rhizogenes* were used for the hairy root induction. But none of these induced hairy roots from the infected tissues. *Agrobacterium* infected calli were survived and differentiated into shoots and roots within two weeks of infection in the basal MS medium supplemented with cefotaxime 200 mg L^{-1}. Leaf and internodal segments after infection were dried off after 28 days of infection.

Susceptibility of plant species to *Agrobacterium* varies according to the plant species. Anderson and Moore (1979) studied the host specificity of *Agrobacterium*. Out of 89 *Agrobacterium* strains tested, none of the strains infected 11 host plant species. Host specificity of *A. rhizogenes* was also studied by Porter and Flores (1991) and reported the strain specificity of plants for infection. The role of host in *Agrobacterium* mediated transformation was also described by Karami *et al.* (2009).

Virulence of the *Agrobacterium* strain is also a factor which influences the infection. Suja (2010) attempted to induce hairy root using *A. rhizogenes* in *Rauvolfia serpentine* using ATCC 15834, ATCC 15834, TR7, A4, TR107, MTCC 532 strains. But none of strains induced hairy roots in the plant. Bansal (2014) used four different strains (A4, R1000, SA 79, MTCC 532, MTCC 2364) for hairy root culture of *Bacopa monnieri*. Out of these four strains, higher transformation efficiency was reported in SA 79 and A4, followed by R1000. MTCC 532 and MTCC 2364 strains were having less transformation efficiency compared with the other *Agrobacterium*

strains. Lack of virulence of the bacteria may be a reason for the lack of hairy root induction in the present study. Presence of bactericidal phyto-constituents such as acteoside and related molecules in the roots also may be responsible for lack of genetic transformation.

Genetic transformation of Ashwagandha was done by A4, ATCC 15834 and MTCC 2364 for inducing hairy roots (Varghese *et al.*, 2014). Transformation was successful and hairy roots were induced by A4, ATCC 15834 strains. But *A. rhizogenes* strain MTCC 2364 didn't produce transformation. This also clearly clarifies the strain specificity of *A. rhizogenes* to transformation.

Phenolic compounds like acetosyringone enhance the T-DNA integration with the host genome (Stachel *et al.*, 1985) and have been used in various plants for hairy root induction (Afolabi-Balogun *et al.,* 2014; Krishnan and Siril, 2016 and Balasubramanian *et al.*, 2018). But in the present study, addition of acetosyringone during infection process did not show effect.

Infected calli showed regeneration in the basal MS medium supplemented with cefotaxime 200 mg L^{-1}. Previous cefotaxime sensitivity experiments also produced same regeneration pattern with the callus in basal MS medium supplemented with cefotaxime 200 mg L^{-1}. This may be because of the effect of cefotaxime used in the medium.

Further detailed study is needed with more strains of *A. rhizogenes* for hairy root induction.

4.5 CONFIRMATION OF TRANSFORMATION

Confirmation of transformation in hairy root induction is usually studied by checking the presence of *rol* genes (*rol A, rol B, rol C*) using PCR (Bansal *et al.*, 2014; Suja 2010; Krishnan and Siril, 2016).

In the present study, roots induced from the callus didn't show any morphological similarities with hairy root and appeared as normal roots. To confirm the integration of T-DNA from the *Agrobacterium* to the plant genomic DNA, roots formed from the infected calli were subjected to PCR. Amplification of rol C genes was done using primers which had already shown amplification in other transformation experiments (Krishnan and Siril, 2016). But roots induced from infected callus showed no amplification of *rol C* genes, indicating the absence of T-DNA integration. The root induction from the infected calli may be due to the endogenous auxin in the plant cell.

4.6 QUANTIFICATION OF PHENYL PROPANOID GLYCOSIDES

Since there was no hairy root induction in *A. sesamoides*, the content of PPGs was studied in callus.

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

In the present study, PPGs content in the callus and *in vitro* raised root of *A. sesamoides* was quantified and compared with the PPGs in the roots produced in *in vivo* conditions (field grown roots) using HPLC. Five PPGs (acteoside, artanemoside A, isoacteoside, leucoseptoside A, martynoside), same as in the field conditions were identified in the *in vitro* conditions also. An increase in the PPGs percentage was seen in callus compared to *in vivo* roots, But *in vitro* roots showed a less PPGs per cent than *in vivo* roots.

Studies conducted by Hagimori *et al.* (1982) on *Digitalis* (Family: Scorphularaceae) showed that the undifferentiated cells showed more digitoxin than

the shoot forming cultures. According to them, plant growth regulators have a role in secondary metabolites production in the cells. Callus produced in MS medium supplemented with BA 2 mg L^{-1} and NAA 0.1 mg $L^{-1}$was found to have more digitoxin formation in long term culture of *Digitalis lanata*.

According to Coste *et al.* (2011) BA enhances the production of hypericins in *Hypericum hirsutum*. Effects of plant growth regulators in secondary metabolite production were also reported by Bienaime *et al.* (2015) in *Lycopodiella inundata* and they found that BAP was having maximum role in alkaloid production. These studies are supporting the results obtained in *A. sesamoides* in which callus showed more PPGs content. Stress produced during the callus induction in the plant may be the reason for the high production of secondary metabolites.

In future, detailed study is needed with more strains of *A. rhizogenes* for hairy root induction and reasons of non induction of hairy root need to be studied. Since callus is having high phenyl propanoid glycosides content, different elicitors can be used for enhancing the content. For cost effective production, the protocol needs to be standardized.

Summary

6. SUMMARY

The study entitled "Enhancement of phenyl propanoid glycosides in *Artanema sesamoides* Benth (vathomvaretti) by hairy root induction" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani and Aromatic and Medicinal Plants Research Station, Odakkali during 2016-2018. The salient findings of the study are summarized below.

The explants for the *Agrobacterium rhizogenes* mediated transformation were obtained from the *in vitro* raised seedlings of *A. sesamoides*. Seeds collected from AMPRS, Odakkali were germinated in basal Murashige and Skoog (MS) and half MS media. Maximum seed germination (94%) was observed in basal MS medium compared to half strength MS medium (87%).

Multiple shoot induction was done in MS medium supplemented with different concentrations of BA using node as explant. Hundred per cent multiple shoot induction was observed in basal MS medium and in MS medium supplemented with Benzyladenine (BA) 0.1 mg L^{-1}. Maximum number of shoots (21 nos) per explant was seen with BA 0.1 mg L^{-1}. Morphology of shoots varied with treatments. Shoots formed in basal MS medium were stout purple shoots with large leaves. Thin, slender green shoots with small leaves were formed in MS medium supplemented with BA 0.1 mg L^{-1} and 0.2 mg L^{-1}. Further increase in BA concentration up to 2 mg L^{-1} gave a green undifferentiated mass of cells.

Callus induction was tried in explants like node, leaf and root in basal MS medium supplemented with combinations of growth regulators like BA, NAA, IAA and 2,4-D. Node and root produced calli in combinations of BA with NAA or IAA and their response was better compared to leaf. MS medium supplemented with 2,4-D or BA did not produce any callus. All the explants produced friable callus in all the media except in MS medium supplemented with BA 1 mg L^{-1} and NAA 1 mg L^{-1}.

Sensitivity of *A. sesamoides* callus to different concentrations of cefotaxime (bacteriostatic agent) was studied and found insensitive to all concentrations studied up to 500 mg L^{-1}. Callus kept in 500 mg L^{-1} of cefotaxime only showed a partial discolouration after four weeks of inoculation. In all the treatment with cefotaxime calli got regenerated into shoots.

Hairy root induction in *A. sesamoides* was attempted with *A. rhizogenes* strains, MTCC 2364, MTCC 532 and ATCC 15834. Among the different explants tried for hairy root induction, calli infected with MTCC 2364 (Infection for 15 and 20 minutes and co-cultivation period of 48 h) and MTCC 532 (Infection for 15, 20 and 30 minutes and co-cultivation period of 48 h) showed 100% survival on MS medium supplemented with cefotaxime (200 mg L^{-1}). They also showed regeneration to produce shoots and roots in two weeks. No hairy root formation was observed in any of the treatments. The *in vitro* roots obtained were analysed for the presence of *rol* gene using PCR and no amplification was found.

Phenyl propanoid glycosides were quantified using HPLC with the help of class LC 10 software (Shimadzu, Japan) with PDA detector. Peaks related to acteoside, artanemoside A, isoacteoside, leucoseptoside A and martynoside were identified and quantified from roots of field grown (i*n vivo*) plants, *in vitro* roots and callus. Callus showed the highest quantity of all the five phenyl propanoid glycosides, followed by *in vivo* roots and *in vitro* roots.

Economics analysis showed that the cost of production of PPGs per gram dry weight of the callus is less compared to that of *in vivo* roots. Since phenyl propanoid glycosides content in the callus is more than the *in vivo* root, large scale production of the compound is economically feasible in *in vitro* conditions.

In the present study, *in vitro* multiplication of *A. sesamoides* is reported for the first time. Hairy roots could not be produced in this study, which may be due to the non virulence of the *A. rhizogenes* strains used or the presence of inhibitory

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substances in the explant, which needs further investigation. Increased phenyl propanoid glycosides content in callus indicates the scope of using an *in vitro* system for the continuous production of these compounds.

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ENHANCEMENT OF PHENYL PROPANOID GLYCOSIDES IN *Artanema sesamoides* Benth (VATHOMVARETTI) BY HAIRY ROOT INDUCTION

by

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Abstract of the Thesis

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ABSTRACT

The study entitled "Enhancement of phenyl propanoid glycosides in *Artanema sesamoides* Benth (vathomvaretti) by hairy root induction" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani and Aromatic and Medicinal Plants Research Station, Odakkali during 2016-2018. The objective of the study was to scale up the production of phenyl propanoid glycosides in *Artanema sesamoides* Benth by inducing hairy roots using *Agrobacterium rhizogenes*.

The explants for the *Agrobacterium rhizogenes* mediated transformation were obtained from the *in vitro* raised seedlings of *A. sesamoides*. Seeds collected from AMPRS, Odakkali were germinated in basal Murashige and Skoog (MS) and half MS media. Maximum seed germination was observed in basal MS medium with 94 per cent seed germination. Hundred per cent multiple shoot induction was observed in basal MS medium and in MS medium supplemented with Benzyladenine (BA) 0.1 mg L^{-1} using node as explant. Maximum number of shoots (21 nos) per explant was seen with BA 0.1 mg L^{-1}. Addition of BA at >0.2 mg L^{-1} resulted in green undifferentiated mass of cells.

Callus induction was tried in explants like node, leaf and root in basal MS medium supplemented with combinations of growth regulators like BA, NAA, IAA and 2,4-D. Node and root produced calli in combinations of BA with NAA or IAA and their response was better compared to leaf. MS medium supplemented with 2, 4-D or BA did not produce any callus.

Hairy root induction in *A. sesamoides* was attempted with *A. rhizogenes* strains, MTCC 2364, MTCC 532 and ATCC 15834. *A. sesamoides* callus was found insensitive to cefotaxime (bacteriostatic agent) upto 500 mg L^{-1}. Among the different explants tried for hairy root induction, calli infected with MTCC 2364 (Infection for 15 and 20 minutes and co-cultivation period of 48 h) and MTCC 532 (Infection for

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15, 20 and 30 minutes and co-cultivation period of 48 h) showed 100% survival on MS medium supplemented with cefotaxime (200 mg L^{-1}). They also showed regeneration to produce shoots and roots in two weeks. No hairy root formation was observed in any of the treatments. The *in vitro* roots obtained were analysed for the presence of *rol* gene using PCR and no amplification was found.

Phenyl propanoid glycosides were quantified using HPLC with the help of class LC 10 software (Shimadzu, Japan) with PDA detector. Peaks related to acteoside, artanemoside A, isoacteoside, leucoseptoside A and martynoside were identified and quantified from roots of field grown (i*n vivo*) plants, *in vitro* roots and callus. Callus showed the highest quantity of all the five phenyl propanoid glycosides, followed by *in vivo* roots and *in vitro* roots.

Economics analysis showed that the cost of production per gram dry weight of the callus was found to be less compared to that of *in vivo* roots. Since phenyl propanoid glycosides content in the callus is more than the *in vivo* root, large scale production of the compound is economically feasible in *in vitro* conditions.

In the present study, *in vitro* multiplication of *A. sesamoides* is reported for the first time. Hairy roots could not be produced in this study, which may be due to the non virulence of the *A. rhizogenes* strains used or the presence of inhibitory substances in the explant, which needs further investigation. Increased phenyl propanoid glycosides content in callus indicates the scope of using an *in vitro* system for the continuous production of these compounds.

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Appendices

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APPENDIX I

Stock solutions of Murashige and Skoog's medium

Constituent	Quantity (mg L^{-1})	Quantity required for preparing the stock (g)	Volume of stock (ml)	Concentration of stock	Volume required for 1L of medium (ml)
Stock A					
NH_4NO_3	1650	16.5	250 ml	40 x	25 ml
KNO_3	1900	19.0			
$MgSo_4.7\ H_2O$	370	3.7			
KH_2PO_4	170	1.7			
Stock B					
$CaCl_2.2H_2O$	440	8.8	100 ml	200 x	5 ml
Stock C					
H_3BO_3	6.2	0.62	100 ml	1000 x	1 ml
$MnSo_4.4\ H_2O$	22.3	1.69			
$Na_2MoO_4.2\ H_2O$	0.25	0.25			
KI	0.83	0.083			
$ZnSo_4.7\ H_2O$	8.6	0.860			
Stock D					
$Na_2EDTA2HO$	37.3	0.745	100 ml	200 x	5 ml
$FeSo_4.7H_2O$	27.8	0.556			
Stock E					
$CuSo_4.5H_2O$	0.025	0.125	250 ml	2000 x	0.5 ml
$COCl_2.6H_2O$	0.025	0.125			
Stock F					
Glycine	2	0.2			
Nicotinic acid	0.5	0.05	100 ml	1000 x	1 ml
Pryridoxine-HCl	0.5	0.05			
Thiamine-HCl	1	0.01			

APPENDIX II

Composition of bacterial culture media

Constituent	Nutrient Agar (g L^{-1})	Yeast Extract Mannitol (g L^{-1})
Beef extract	1	5
K_2HPO_4	-	-
Yeast extract	2	1
$MgSO_4.7H_2O$	-	0.2
Peptone / Trypton	5	-
Mannitol	-	10
NaCl	5	0.1
Agar	15	20
pH	7	7

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APPENDIX III

ECONOMIC ANALYSIS

FIELD CONDITIONS

Area - 1 cent

Plant spacing - 100 cm x 75 cm

Number of plants - 53

Duration of the crop is 4 months

Cost of production in the field

Sl. No.	Particulars	No. of units	Cost per unit (Rs)	Total cost (Rs)
1	Planting material	53	20	1060
2	LABOURS			
	Land preparation	2	750	1500
	Planting	1	750	750
	First weeding and fertilizer application	1	750	750
	Second weeding and fertilizer application	1	750	750
	Harvesting and processing	2	750	1500
3	Miscellaneous (fertilizer, pesticides etc.)			3000
	Total			**9310**

Yield of root powder

Dry weight of root powder per plant = 25g

Thus, dry weight of root powder for 1 cent = 1325 g

Cost for producing 1325g dried powder = Rs. 9310

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IN VITRO CONDITIONS

Duration of culturing – 4 months (same as the duration of one crop in the field)

Explants – roots

Average dry weight of powdered callus from 1 bottle = 0.6 g

So, to produce 1325 g powdered callus (same as in the field), we need 2120 bottles.

2120 bottles need 80 litres of media (1 litre media can be poured approximately into 26 bottles)

Cost of production for producing 1333g callus powder in 4 months

Sl. No.	Particulars	No. of units	Cost per unit (Rs.)	Total cost (Rs)
1	MS medium	80 Litres	75	6000
2	Equipment depreciation (4months)			300
3	Electricity charges (4 months)			1000
4	Labours	2	750	1500
	Total			8800

Conclusion

For producing 1325 g dry weight powder (root/callus), cost of production in the field is Rs. 9310 and Rs. 8800 in the *in vitro* conditions. Since the per cent dry weight of phenyl propanoid glycosides in the callus is more than that in the field conditions, it is economical to produce phenyl propanoid glycosides in the *in vitro* conditions.

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