# *AGROBACTERIUM TUMEFACIENS* MEDIATED TRANSFER OF EXOGENOUS HYDROXY METHYL GLUTARYL CoA (HMG CoA) REDUCTASE GENE TO *CENTELLA ASIATICA* L.

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## DECLARATION

I hereby declare that this thesis entitled **"*Agrobacterium tumefaciens* mediated transfer of exogenous hydroxy methyl glutaryl CoA (HMG CoA) reductase gene to *Centella asiatica* L."** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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## CERTIFICATE

Certified that this thesis "***Agrobacterium tumefaciens* mediated transfer of exogenous hydroxy methyl glutaryl CoA (HMG CoA) reductase gene to *Centella asiatica* L.**" is a record of research work done independently by Mrs. Lekshmi, R. S (2005-11-134) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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*Dedicated to*
*My Beloved Parents*
*and Teachers*

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ADS | Adenine sulphate |
| BA | Benzyl adenine |
| bp | base pair |
| CTAB | Cetyl trimethyl ammonium bromide |
| 2, 4-D | 2, 4-dichlorophenoxyacetic acid |
| DNA | Deoxy ribonucleic acid |
| dNTPs | deoxy Nucleotide Tri Phosphates |
| HCl | Hydrochloric acid |
| *hmgr* | Hydroxy methyl glutaryl CoA rductase |
| IAA | Indole acetic acid |
| Kn | Kinetin |
| LB | Luria-Bertani medium |
| lux | Luminous intensity |
| M | Molar |
| µM | Micromolar |
| µl | Microlitre |
| MS medium | Murashige and Skoog medium |
| NAA | α-Naphthalene acetic acid |
| NaOH | Sodium hydroxide |
| *npt II* | Neomycin phosphotransferase II |

| | |
|---|---|
| OD | Optical Density |
| PCR | Polymerase Chain Reaction |
| pH | Per Hydrogen |
| TAE | Tris acetic acid EDTA |
| TE | Tris EDTA |
| TLC | Thin layer chromatography |
| *vir* | Virulence genes |

# *Introduction*

# 1. INTRODUCTION

*Centella asiatica*, described by Charaka as the anti–aging plant, is a herbaceous annual belonging to the family Apiaceae. It is believed to have originated in Asia. Common names include, Asiatic Pennywort, Asiatic coinwort, Brahmi, Gotu Kola, Hydrocotyle, Indian Pennywort, Marsh Penny, Pegaga, Marsh, Spadeleaf, Thick-leaved Pennywort, White Rot and Vallarai.

*Centella asiatica* is a widely used medicinal herb. It is chiefly valued as a revitalizing herb that strengthens nervous function, thereby improving memory. It is sometimes burnt in incense prior to meditation. It is regarded as useful in balancing the right and left hemispheres of the brain. The whole plant is alterative, cardio-depressant, hypotensive and weakly sedative. It is a rejuvenating diuretic herb that clears toxins and digestive disorders, reduces inflammations and fevers, and improves healing and immunity. There are reports that *Centella asiatica* reduces scarring and improves circulatory problems in the lower limbs. It has been used as a tonic and cleansing herb for skin problems. It is also used internally in the treatment of wounds, chronic skin conditions like leprosy, venereal diseases, malaria, varicose veins, ulcers and senility. Externally, the herb is applied to wounds, hemorrhoids and rheumatic joints (Rao et al., 2006). In the Ayurvedic tradition, it is recommended for treatment of mental disorders, immune system deficiencies, circulatory problems, chronic skin conditions, liver ailments, epilepsy, asthma, bronchitis, hair loss, tetanus, inflammation, rheumatism, in combating depression, urinary disorders and intestinal complaints. *Centella asiatica* is regarded as the primary herb for promoting longevity.

*Centella asiatica* contains a variety of constituents, which are responsible for its medicinal properties of which the triterpenoids have attracted the most attention

from researchers. These include asiaticoside A and B, madecassoside, brahminoside, brahmoside, thankuniside, isothankunoside, vallarine as well as triterpene acids such as asiatic acid, 6- hydroxyl asiatic acid, madecassic acid, madasiatic acid, brahmic acid, isobrahmic acid, betulinic acid and isothankunic acid (Chaudari, S. 1978).

Among the various secondary metabolites in *Centella asiatica*, asiaticoside possesses an anti-inflammatory, antitumour, neuroprotective, skin care and toning effects and is used clinically as a wound-healing agent (Pointel et al., 1987). Asiaticoside is used in the treatment of Alzheimer's disease, leprosy, lupus, eczema, psoriasis and ulcers of the duodenum, skin and cornea, tuberculosis and venous diseases (Giardiana et al., 1987).

For commercial extraction, the asiaticoside content should be at least three per cent. In Indian cultivars it is only about one per cent. Hence most of the pharmaceutical companies are importing this plant from African countries to meet their industrial needs. By improving the asiaticoside content in the plant, the utilization of our ecotypes can be enhanced and the cost of medicinal preparations can be reduced. Conventional breeding methods have limitations in improving the ecotypes by targeting on to a particular trait. Many researchers have attempted overproduction of asiaticoside through cell/tissue culture and elicitation using enhancers (Kim et al., 2005). This necessitates development of economic methods for large scale *in vitro* production of secondary metabolites.

Metabolic engineering is becoming a popular approach for the modification of medicinal plants for altering the metabolite contents. Medicinal plants with quantitatively and qualitatively improved pharmacological properties have been developed by metabolic pathway engineering (Kim et al., 2005).

Hydroxy methyl glutaryl coenzyme A reductase (HMGR) is an important enzyme which act at the upstream of the mevalonate pathway producing mevalonic acid, further down stream produce IPP, squalene, β-amyrin and finally asiaticoside. 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGR) catalyzes the irreversible conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate and is considered a key regulatory step controlling isoprenoid metabolism (Chappell et al., 1995). Through metabolic engineering the content of asiaticoside in *Centella asiatica* can be improved. Till date no report has been published regarding metabolic enigineering of *Centella asiatica*, though experiments have been done to develop reliable protocol for shoot regeneration and somatic embryogenesis (Paramageetham et al., 2004). *Agrobacterium* mediated genetic transformation in *Centella asiatica* has been standardized in the Department of Plant Biotechnology, College of Agriculture, Vellayani (Krishnan, 2006).

The present study was taken up with the objective to enhance the production of asiaticoside in *Centella asiatica* L. by introducing exogenous hydroxy methyl glutaryl CoA reductase gene (*hmgr*), using *Agrobacterium tumefaciens*.

# *Review of literature*

# 2. REVIEW OF LITERATURE

The practice of using natural plant substances (botanicals) to treat and prevent illness existed since prehistoric times and flourishes today as the primary form of medicine for perhaps as much as 80 per cent of the world's population. Medicinal plants are the most important source of life saving drugs for the majority of the world's population. Over 80,000 species of plants are in use throughout the world. *Centella asiatica,* belonging to the family Apiaceae, is an important medicinal herb that is widely used and is becoming increasingly popular. It is used as a medicinal herb in Ayurvedic and traditional Chinese medicine, as a tonic, cleansing herb for skin problems, remedy for digestive disorders, alterative, cardio-depressant, hypotensive and sedative. It is also used in the treatment of wounds, chronic skin conditions (including leprosy), venereal diseases, malaria, varicose veins, ulcers, nervous disorders and senility. As per Ayurveda it is chiefly valued as a revitalizing herb that strengthens nervous function, memory and hence known as a brain food in India.

Plants are the major sources of natural products used as pharmaceuticals. Many of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances. Pharmacological value of medicinal plants is mainly due to the presence of secondary metabolites in it. The evolving commercial importance of secondary metabolites has in recent years resulted in a great interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites by means of various technologies. Plant cell culture technologies were introduced at the end of the 1960's as a possible tool for both studying and producing plant secondary metabolites. Now different strategies as *in vitro* enhancement of metabolites using different elicitors, metabolic pathway

engineering through genetic transformation technique etc. have been extensively tried to improve the production of plant chemicals.

*Centella asiatica* contains a blend of compounds including triterpenes such as asiaticoside, asiatic acid and madecassic acid that are found to be responsible for its medicinal properties. Many studies have been conducted to improve the medicinal plants in terms of the secondary metabolite content. Yield and quality of medicinal plants can be improved by biotechnological tools by gene alteration or specific gene transfer. Since the first successful report on plant genetic transformation using *Agrobacterium tumefaciens* in tobacco, this gene delivery system has been used for the transfer of desirable genes in several crop plants. The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants. *In vitro* regeneration holds tremendous potential for the production of high-quality plant-based medicine. *In vitro* production of secondary metabolites has been reported from various medicinal plants. Metabolic pathway engineering mediated by *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* may be a powerful tool for enhancing the productivity of novel secondary metabolites.

The study "*Agrobacterium tumefaciens* mediated transfer of exogenous hydroxy methyl glutaryl CoA (HMG CoA) reductase gene to *Centella asiatica* L." aims at enhancement of asiaticoside production in *Centella asiatica*. The review of literature related to the above work such as enhancing secondary metabolite production through various *in vitro* techniques, metabolic pathway engineering through genetic transformation, various biotechniques used to confirm transformation etc are presented in this chapter.

## 2.1 MEDICINAL PROPERTIES OF *CENTELLA ASIATICA*

*Centella asiatica* has been used for centuries in traditional Indian systems of medicine. For the last 3,000 years it has been used in Ayurvedic medicine for wound healing, boosting memory, improving concentration and alertness. It is used as a mild diuretic, anti-anxiety and anti-stress drug. It has also been used for centuries in the treatment of liver and kidney problems. *Centella asiatica* was first accepted as a drug in France in the 1880's. *Centella asiatica* is a mild adaptogen that allows the body to counter adverse physical, chemical or biological stresses by raising nonspecific resistance towards such stress, thus allowing the organism to "adapt" to the stressful circumstances.

Rao (1973) studied the effects of *Centella asiatica* on mentally challenged children. Half of the children were given 500 mg tablets of dried *Centella asiatica* (whole plant), and half placebo. Intelligence quotient tests were conducted at the outset of the study, and again after three months. Results indicated that children who took the *Centella* tablet showed significant improvements in co-operation, memory, concentration, attention, vocabulary and social adjustment.

Moharana and Moharana (1994) reported the use of *Centella asiatica* plant extracts in memory enhancing tonics and for the treatment of mental and stress-related disorders. Herbert et al. (1994) studied the *in vitro* effect of an indigenously produced dry powder of *Centella asiatica* (CA) on the acid-fastness and viability of *Mycobacterium tuberculosis*. The results indicate that CA did not have any direct action on the acid-fastness or viability of *M. tuberculosis* H37Rv *in vitro*

Babu et al. (1995) tested both a crude extract of *Centella asiatica* (CE) and its partially purified fractions (AF) for their antitumor activity. The partially purified

fractions significantly inhibited the proliferation of the transformed cell lines in Ehrlich ascites tumor cells and Dalton's lymphoma ascites tumor cells with no toxic effects on normal human lymphocytes.

The plant is grown and consumed as a vegetable in many Asian countries and is finding acceptance in the western world (Peiris and Kays, 1996). Cheng et al. (2000) examined the effect of *Centella asiatica* on the prevention of ethanol induced gastric lesions in rats. The results suggested that *Centella* extract prevented ethanol induced gastric lesions by strengthening the mucosal barrier and reducing the damaging effects of free radicals.

Sharma and Sharma (2002) reported that *Centella asiatica* has a considerable reputation in the Indian system of medicine. It is a rasayana (general tonic), brain tonic, improves memory and strengthens the central nervous system. Ayurveda describes a group of plant drugs as "medhyarasayanas" (brain tonics). Among these brahmi and *Centella asiatica* occupy the place of pride (Rao, 2003).

Gupta et al. (2003) studied the effect of *Centella asiatica* on pentylenetetrazole-induced kindling, cognition and oxidative stress in rats. Their studies showed that the administration of aqueous extract of *Centella asiatica* (300 mg/kg orally) decreased the PTZ-kindled seizures and showed improvement in the learning deficit induced by PTZ kindling as evidenced by decreased seizure score and increased latencies in passive avoidance behavior. The findings suggested the potential of aqueous extract of CA as adjuvant to antiepileptic drugs with an added advantage of preventing cognitive impairment.

*Centella asiatica* possess central nervous system activity, such as improving intelligence. In addition, it has been demonstrated that *Centella asiatica* has cognitive-enhancing and anti-oxidant properties in normal rats. Oxidative stress or

an impaired endogenous anti-oxidant mechanism is an important factor that has been implicated in Alzheimer's disease (AD) and cognitive deficits seen in the elderly. In this study, the effect of an aqueous extract of *C. asiatica* (100, 200 and 300 mg/kg for 21 days) was evaluated in intracerebroventricular (i.c.v.) streptozotocin (STZ) induced cognitive impairment and oxidative stress in rats. Rats treated with aqueous extract of *C. asiatica* (200 and 300 mg/kg) showed a dose-dependent increase in cognitive behaviour in both paradigms (Kumar and Gupta., 2003).

Somchit et al. (2004) evaluated the antinociceptive and anti-inflammatory effects of *Centella asiatica* (CA) extracts in rodent models. The results suggested that water extract of CA revealed significant antinociceptive activity with the model. The activity was stastitically similar to aspirin but less potent than morphine. the CA extract also revealed significant anti-inflammatory activity. This effect was statistically similar to the non-steroidal anti-inflammatory drug, mefenamic acid.

Rao et al. (2006) investigated the effect of *Centella asiatica* fresh leaf extract during the rat growth spurt period on the dendritic morphology of hippocampal CA3 neurons, one of the regions of the brain concerned with learning and memory. The results showed a significant increase in the dendritic length and dendritic branching points along the length of both apical and basal dendrites treated with *Centella asiatica* fresh leaf extract. It has been concluded that the constituents/active principles present in *Centella asiatica* fresh leaf extract have a neuronal dendritic growth stimulating property; hence, the extract can be used for enhancing neuronal dendrites in stress and neurodegenerative and memory disorders.

Shetty et al. (2006) evaluated the effect of ethanolic extract of *Centella asiatica* on normal and dexamethasone-suppressed wound healing on Wistar albino rats. The results indicated that the leaf extract promotes wound healing significantly

and is able to overcome the wound healing suppressing action of dexamethasone in a rat model.

## 2.2 SECONDARY METABOLITES IN *CENTELLA ASIATICA*

*Centella asiatica* contains a variety of constituents which are responsible for its medicinal properties of which the triterpenoids have attracted the most attention from researchers. These include asiaticoside A and B, madecassoside, braminoside, brahmoside, brahminoside, thankuniside, isothankunoside, vallarine as well as triterpene acids such as asiatic acid, 6- hydroxyl asiatic acid, madecassic acid, madasiatic acid, brahmic acid, isobrahmic acid, betulinic acid and isothankunic acid. *Centella asiatica* also contains the flavones such as quercitin, kaempferol and astragalin, the alkaloid hydrocotylin, and phytosterols, stigmasterols and sitosterol. The fresh and recently dried plant contains an essential oil comprised primarily of sesquiterpenoids such as β-caryophyllene, α-humulene and germacrene. Additional constituents include tannins, amino acids such as asparate, glutamate, serine, threonine, alanine, lysine, histidine, vitamin B- complex and K, magnesium, calcium, sodium and a resin (Chaudari, S. 1978).

Mathur et al. (2000) described variation in the Indian accessions of the medicinal plant *Centella asiatica* (L.) Urban morphologically, physiologically and biochemically.

Major active principles in *Centella asiatica* are the triterpenes - asiatic acid and madecassic acid, and, their derived triterpene ester glycosides, asiaticoside and madecassoside (Kartnig, 1988). A new triterpenoid glycoside 3-O-[a-L-arabinopyranosyl] 2 a, 3 ß, 6 ß, 23-a tetrahydroxyurs-12-ene-28-oic acid (1) accompanied by 6ß-hydroxyasiatic acid and asiatic acid were also isolated from *Centella asiatica* by Shukla et al. (1996).

*Centella asiatica* from Madagascar is used for most standardized extracts, and its four main triterpene properties are: Asiatic acid (29-30%), madecassic acid (29-30%), asiaticoside (40%), madecassoside (1-2%). *Centella asiatica* also contains the following: volatile oil of a terpene acetate, camphor, cineole, glycerides of some fatty acids, plant sterols (campesterol, stigmasterol, sitosterol), polyacetylene compounds, flavanoids (Kampferol, quercetin), myo- inositol, sugars, vellarin, amino acids and resins (Sharon Crawford, 2007). Triterpenes of the plant are known to be highly useful for skin care and toning, and, prevent pre-mature wrinkles (Kumar et al., 2007).

*2.2.1 TRITERPENOIDS*

Giardina et al. (1987) reported that modern drugs comprising the pharmacologically active triterpenoid fractions and glycosides such as asiaticoside and madacassoside are being currently used in the treatment of leprosy, lupus, eczema, skin lesions, psoriasis, wound healing, burns, ulcers of the duodenum, skin and cornea, tuberculosis and venous diseases.

A three week treatment of a triterpene fraction of *Centella asiatica* in clients with postphlebitic syndrome significantly reduced the number of circulating endothelial cells, as compared to normal subjects (Montecchio et al., 1991). *Centella asiatica* has been found to have a GABAnergic activity (Chatterjee et al., 1992).

Asiatic acid, madecassic acid, and asiaticoside, were tested separately and in combination on skin human fibroblast collagen I synthesis *in vitro*. In the absence of ascorbic acid, the mixture as well as each individual component stimulated collagen I synthesis. In the presence of ascorbic acid, the level of collagen I secretion was

found higher for each individual component and for the mixture. A comparison of asiaticoside and asiatic acid showed that the sugar moiety of the molecule is not necessary for the biological activity (Bonte et al., 1994).

Lee et al. (2000) modified the chemical structure of asiatic acid and obtained 36 derivatives of asiatic acid to prepare neuroprotective compounds that were more efficient than asiatic acid itself. The neuroprotective activities of these derivatives were evaluated using primary cultures of rat cortical neurons insulted with the neurotoxin, glutamate, as an *in vitro* screening system and the results showed that among these 3 of these derivatives exerted significant neuroprotective effects on cellular oxidative defense mechanism. It was also patented as a treatment for dementia and an enhancer of cognition.

Incandela et al. (2001) suggested that total triterpenic fraction of *Centella asiatica* (TTFCA) is effective in improving venous wall alterations in chronic venous hypertension and in protecting the venous endothelium.

Coldren et al. (2003) studied the gene expression changes in the human fibroblast induced by *Centella asiatica* triterpenoids and the results showed that *Centella* triterpenes evoked a gene-expression response consistent with their prevailing medical uses in the treatment of connective tissue disorders such as wound healing and microangiopathy. The extent of gene expression change was found to be largely dose-dependent, and also there was an increase in expression over 48 hours of treatment. They also suggested that identification of genes modulated by these compounds will provide the basis for a molecular understanding

of *Centella's* bioactivity, and opportunities for the quantitative correlation of its activity with clinical effectiveness at a molecular level.

### *2.2.1.1 Asiaticoside*

Asiaticoside stimulate the reticuloendothelial system where new blood cells are formed and old ones destroyed, fatty materials are stored, iron is metabolized, and immune responses and inflammation occur or begin. Asiaticoside also stimulate the synthesis of lipids and proteins necessary for healthy skin. *Centella asiatica* extract has been administered as a cosmetic, dermatologic, or pharmaceutical composition to promote collagen synthesis. Administration of the composition results in the firming of the skin improves healing and is suitable for treating various pathologies accompanied with a deficiency in collagen. The extract can also be added to cell culture medium used in the culture of skin cells, particularly skin fibroblasts. The composition contains ingredients like madecassic acid, asiatic acid, madecassoside, asiaticoside, collagenase inhibitors etc.

Modern drugs comprising the pharmacologically active triterpenoid fractions and glycosides such as asiaticoside and madecassoside are used in the treatment of leprosy, lupus, eczema, skin lesions, psoriasis, wound healing, burns, ulcers of the duodenum, skin and cornea, tuberculosis and venous diseases (Boiteau et al. 1949; King, 1950a, b; Boiteau and Ratsimamanga 1956; Ratsimamanga *et al.* 1958; Boiteau and Ratsimamanga 1959; Tsurumi et al. 1973; Allegra et al. 1981; Giardina et al. 1987).

Asiaticoside has an anti-inflammatory and wound-healing effect (Pointel et al., 1987). It is also economically important as drugs, detergents, sweeteners and cosmetics (Hostettmann and Marston, 1995).

Grimaldi et al. (1990) studied the pharmacokinetics of the total triterpene fraction of *Centella asiatica* after single and multiple administrations to healthy volunteers and could also explain the phenomenon by a metabolic interaction between asiatic acid and asiaticoside, which transformed into asiatic acid *in vivo*.

Asiaticoside significantly increased the levels of super oxide dismutase, catalase, glutathione peroxidase, vitamin E and ascorbic acid in excision-type cutaneous wounds in rats. The level of antioxidant activity was the highest during the initial stages of treatment (Shukla et al., 1996).

Mook-Jung et al. (1999) tested asiaticoside derivatives for potential protective effects against Aβ-induced cell death. He concluded asiaticoside and asiatic acid as reasonable candidates for a therapeutic Alzheimer's disease drug that protects neurons from Aβ toxicity.

Cheng et al. (2004) studied the healing effects of *Centella asiatica* water extract and asiaticoside on acetic acid induced gastric ulcers in rats. Asiaticoside in *Centella asiatica* extract was quantitatively determined with the use of HPLC analysis. They were found to reduce the size of the ulcers in a dose-dependent manner, with a concomitant attenuation of myeloperoxidase activity at the ulcer tissues. Epithelial cell proliferation and angiogenesis were promoted. The expression of basic fibroblast growth factor, an important angiogenic factor, was also up regulated in the ulcer tissues in treated rats. These results further suggest the potential use of *Centella asiatica* and its active ingredient as anti-gastric ulcers drugs.

Guo et al. (2004) studied the effect of *Centella asiatica* water extract (CE) and asiaticoside (AC), on the expression and activity of inducible nitric oxide (NO)

synthase during gastric ulcer healing in rats. Their findings indicated that CE and AC had an anti-inflammatory property that is brought about by inhibition of NO synthesis and thus facilitates ulcer healing.

Huang et al. (2004) reported the anti- tumor effect of asiaticoside. They studied the inhibitory effect of asiaticoside on proliferation of several cancer cell lines and apoptosis. The apoptosis rates were much higher in asiaticoside plus vincristine groups than in vincristine or asiaticoside groups. They concluded asiaticoside, as a biochemical modulator, that can induce apoptosis, and enhance anti-tumor activity of vincristine in cancer cells, which might be useful in cancer chemotherapy.

## 2.3 *IN VITRO* PROPAGATION OF *CENTELLA ASIATICA*

Patra et al. (1998) reported a successful procedure for *in vitro* regeneration of callus derived from stem and leaf explants of *Centella asiatica* on semisolid modified Murashige and Skoog (MS) medium supplemented with 2 mg $l^{-1}$ kinetin (Kn) and 4 $mgl^{-1}$ naphthalen acetic acid (NAA). The rate of shoot bud regeneration was the highest after 4 weeks of subculture on 4 mg $l^{-1}$ benzyl adenine (BA), 2 mg $l^{-1}$ Kn, 0.25 mg $l^{-1}$ NAA and 20 mg $l^{-1}$ adenine sulphate.

The influence of auxins and cytokinins on the production of callus in *Centella asiatica* was studied by Rao et al. (1999). The stem explant, stolon proved to be the best for callus induction followed by leaf base. Kn supplementation at 0.25 and 0.5 mg $l^{-1}$ along with auxin, 2, 4-dichlorophenoxyacetic acid (2, 4-D) at 2 mg $l^{-1}$ proved to be beneficial for the growth of callus. The best combination of growth regulators for maximum callus induction was 2 mg $l^{-1}$ NAA + 0.5 mg $l^{-1}$ Kn.

Banerjee and Zehra (1999) developed a protocol for *in vitro* multiplication of *Centella asiatica* from leaf explants. Leaf segment devoid of petiole was more responsive multiple shoots within 2 weeks. Maximum number of healthy shoots/ explant and rapid elongation of shoots was observed at 6-benzylaminopurine (BAP) 3 mg $l^{-1}$ and NAA 0.05 mg $l^{-1}$. Root initiation was observed on half strength MS medium supplemented with 1 mg $l^{-1}$ IBA within 2 weeks.

Tiwari et al. (2000) described a protocol for rapid and large scale *in vitro* clonal propagation of *Centella asiatica* by enhanced axillary bud proliferation in nodal segments isolated from mature plants. The synergistic combination of 22.2 µM BA and 2.68 µM NAA induced optimum frequency (91%) of shoot formation as well as shoot number (4-5 shoots per node). Subculturing of nodal segments harvested from the *in vitro* derived axenic shoots on the multiplication medium enabled continuous production of healthy shoots with similar frequency. MS medium supplemented with 6.7 µM BA and 2.88 µM IAA was found most suitable for shoot elongation. Rooting was the highest (90 %) in full strength MS medium containing 2.46 µM IBA.

Stem node explant of naturally grown *Centella asiatica* was used for *in vitro* regeneration of multiple shoots. A combination of 1 mg $l^{-1}$ BAP and 0.5 mg $l^{-1}$ NAA was found superior in the optimum production of multiple shoots. 0.2 mg $l^{-1}$ IBA was found most effective in the production of roots (Hossain et al., 2000).

Sangeetha et al. (2003) developed a rapid clonal propagation system for *Centella asiatica* by shoot tip (2-3 cm long) culture. The shoot tips isolated from mature plants were inoculated on MS medium incorporated with BA alone or in combination with NAA and Kn. The optimum number of shoots (3.38) with optimum number of leaves per shoot (4.25) was attained on MS medium

supplemented with 4.0 mg $l^{-1}$ BA and 0.1 mg $l^{-1}$ NAA. On transferring the microshoots on full strength MS medium supplemented with various concentrations of IBA (1.0-3.0 mg $l^{-1}$) and NAA (0.5-2.0 mg $l^{-1}$), profuse rooting (46.8 per shoot) was obtained in MS medium with 2.0 mg $l^{-1}$ IBA with root length of 19.7 cm.

Tiwari et al. (2004) described a protocol for rapid and large-scale *in vitro* propagation of *Centella asiatica* (L.) by enhanced release of axillary bud proliferation in nodal segments with a combination of 22.2 µM BA and 2.68 µM NAA. Subculturing of nodal segments harvested from the *in vitro* derived axenic shoots on the multiplication medium enabled continuous production of healthy shoots. MS medium supplemented with 6.7 $\mu$M BA and 2.88 $\mu$M IAA was found most suitable for shoot elongation. Rooting was highest (90%) on full-strength MS medium containing 2.46 $\mu$M IBA.

Paramageetham et al. (2004) standardized somatic embryogenesis in *Centella asiatica* by culturing leaf explants on MS medium with 9.29 µM Kn and 2.26 µM 2,4-D. Martin (2004) reported high frequency somatic embryogenesis and plant regeneration on callus derived from leaf (petiole and lamina) and internode explants of *Centella asiatica*. Growth regulators significantly influenced the frequency of somatic embryogenesis and plant regeneration. Callus developed on MS medium fortified with 4.52 µM 2, 4-D or 5.37 µM NAA, both with 2.32 µM Kn were superior for somatic embryogenesis. Callus developed on NAA and kinetin supplemented medium favoured induction and maturation of embryos earlier compared to that on 2, 4-D and Kn.

The effects of sucrose, indole-3-acetic acid (IAA) and 6-benzylaminopurine (BAP) concentrations on cell growth of *Centella asiatica* cell suspension culture were studied by Omar et al. (2004). Only sucrose as a single factor was positively

significant on cell growth. Increasing sucrose concentration from 3.32 to 6.68 per cent (w/v) resulted in an increase in dry cell weight from 16 to 27 g $l^{-1}$. IAA and BAP as single factors and other possible interactions effect were insignificant. The optimum values predicted were 6.68 per cent (w/v) sucrose, 0.84 mg $l^{-1}$ IAA and 1.17 mg $l^{-1}$ BAP yielding 27.4 g $l^{-1}$ dry cell weight.

Panimalar et al. (2005) reported a viable tissue culture protocol for the commercial propagation of *Centella asiatica*. They reported that for direct shoot regeneration from nodal segments, a combination of BAP 2.0 mg $l^{-1}$ and Kn 1 mg $l^{-1}$ was optimum. Callusing was observed in nodal segments and leaf bits when 3 mg $l^{-1}$ NAA along with 0.3 mg $l^{-1}$ BAP was used. Half strength MS medium supplemented with IBA 0.5 mg $l^{-1}$ and NAA 0.2 mg $l^{-1}$ induced rhizogenesis of regenerated shoots.

Hossain et al. (2005) studied the effect of carbon sources on *in vitro* regeneration of *Centella asiatica*. Effect of glucose, sucrose, maltose, gur and growth regulators on *in vitro* plantlet formation in *Centella* from nodal segments were studied. Among carbon sources, sucrose was optimum for shoot formation in presence of BA or Kn. Sucrose + BA or sucrose + Kn caused 100 or 90 % shoot formation. Longest shoots (2.6±0.059 cm) were produced with sucrose + BA. The other carbon sources were less effective than sucrose both in BA and Kn containing media.

Nodal segments of *Centella asiatica* were used as explants for *in vitro* regeneration in MS media supplemented with 2 mg $l^{-1}$ BA and 1 mg $l^{-1}$ NAA. *In vitro* rooting was induced when MS medium was supplemented with IAA 1 mg $l^{-1}$ (Shashikala et al., 2005). Nath and Buragohain (2005) developed methods for induction of callus and cell suspension cultures of *Centella asiatica*. TLC and HPLC

analysis showed the presence of asiaticoside in the *in vitro* grown leaves, callus and cell suspension cultured cells.

Sivakumar et al. (2006) developed a protocol for high frequency *in vitro* multiplication of *Centella asiatica* (L.) Urban, using shoot tip culture. High frequency bud break (88 %) and multiple shoot formation (16.8 shoots/shoot tip) were observed when shoot tip segment, was cultured on MS medium supplemented with (BAP) (17.76 µM) and gibberillic acid ($GA_3$) (1.44 µM). Half-strength MS medium supplemented with (NAA) (10.74 µM) induced the maximum (27.66) number of roots.

Sharma and Shalini (2007) optimized the *in vitro* culture conditions of *Centella asiatica* for mass multiplication and callus culture study has also been initiated. They have found that MS Basal + 3 mg $l^{-1}$ BAP + 0.025 mg $l^{-1}$ of IAA + 30 g $l^{-1}$ Sugar + 8.0 g $l^{-1}$ agar gave the best result for culture initiation as well as in axillary shoot proliferation for no of leaves/shoot, no of shoots/node, no of nodes/explant and explant height. For rooting, MS + 0.5 mg $l^{-1}$ IBA + 30 g $l^{-1}$ of sugar + 8 g $l^{-1}$ agar gave higher primary roots/shoot with higher secondary roots. Green globular callus was best induced and proliferated in MS + 3 mg $l^{-1}$ NAA + 1 mg $l^{-1}$ Kn + 30 g $l^{-1}$ sugar + 8 g $l^{-1}$ of agar.

## 2.4 IMPROVEMENT OF SECONDARY METABOLITE PRODUCTION IN MEDICINAL PLANTS

Plants are the source of many of our important pharmaceuticals. The types and concentrations of secondary metabolites vary with plant species, tissue type, physiological development, and conditions to which the plant is exposed. Manipulating plants to optimize secondary metabolite concentration and dry matter

production contributes to the economics of medicinal plant cultivation. Some efforts have been made to discover high-yielding chemotypes of medicinal plants, but there have been few targeted breeding programmes to increase yields of secondary metabolites. Cultural practices to optimise pharmaceutical production in field (or greenhouse) grown plants have not been rigorously determined or have been of little benefit in increasing levels of the desired compounds. Considerable effort has been made to generate plant-derived pharmaceuticals economically in plant cell or tissue culture, with relatively few successes. Biotechnology offers new tools that might improve production of many phytopharmaceuticals. These approaches include cultural manipulations, elicitation, tissue culture and genetic engineering.

Cultural manipulation such as providing shade has shown to improve the pharmaceutical quality of the plant. Genetic resources of the medicinal and vegetable plant *Centella asiatica*, collected from different parts of India, were screened for their herb and asiaticoside yields under different levels of shading under sub-tropical field conditions of Indo-Gangetic plains at Lucknow in the winter season (Mathur et al., 2000). Considering all the accessions together, 50% shading of plants resulted in higher yields of herbage and asiaticoside. Thirteen out of the 16 accessions studied had shading requirements for high yields of fresh and dry herb and asiaticoside. The accession from Meghalaya, was identified as very high herbage and asiaticoside yielding but requiring a shading of about 50 per cent. On the other hand accessions from Orissa and West Bengal gave high herb and asiaticoside yields under full light. It is concluded that triterpenoid saponin rich *C. asiatica* for high yields can be cultivated in the field under shade or full light by selecting the genotype adapted to the respective growth conditions.

### *2.4.1 PRODUCTION OR ENHANCEMENT OF SECONDARY METABOLITES IN MEDICINAL PLANTS IN VITRO*

Most valuable phytochemicals are products of plant secondary metabolism. The improved *in vitro* plant cell culture systems have the potential for commercial exploitation of secondary metabolites.

The accumulation of secondary products in plant cell culture depends on the composition of the culture medium and on environmental conditions (Stafford et al., 1986). Scragg et al. (1992) isolated quinoline alkaloids in significant quantities from globular cell suspension cultures of *Cinchona ledgeriana*. Park and Yoon (1992) obtained significant amounts of sanguinarine in cell suspension cultures of *Papaver somniferum* using bioreactors. Pradel et al. (1997) observed that the biosynthesis of cardenolides was maximal in the hairy root cultures of *Digitalis lanata* compared to leaf.

Tripathi et al. (2003) discussed the applications of biotechnology for regeneration and genetic transformation for enhancement of secondary metabolite production *in vitro* from medicinal plants.

Hahn et al. (2003) reported the production of ginsenoside from adventitious root cultures of *Panax ginseng* through large-scale bioreactor system.

Roots cultivated in bioreactors have been found to release medicinally active compounds, including the anticancer drug isolated from various *Taxus* species, into the liquid media of the bioreactor which may then be continuously extracted for pharmaceutical preparations (Murch et al., 2000).

*2.4.1.1 Using Elicitors*

Parisi et al. (2002) obtained high yields of proteolytic enzymes from the callus tissue culture of garlic (*Allium sativum* L.) on MS medium supplemented with NAA and BAP.

Bais et al. (2002) developed a novel cell culture system for *in vitro* growth and production of *Hypericum perforatum* suggesting a possible technology for large scale production of hypericin. Leaf explants grown in MS salts supplemented with 2, 4-D (0.90 µM) and Kn (0.11 µM) gave maximum percentage callus formation.

Kim et al. (2004) studied the effects of a number of different elicitors on asiaticoside production in whole plant cultures of *Centella asiatica*, including yeast extract, $CdCl_2$, $CuCl_2$ and methyl jasmonate (MJ). Only MJ and yeast extract stimulated asiaticoside production, 1.53 and 1.41 fold respectively. Maximum asiaticoside production was achieved following treatment with 0.1 mM MJ (116.8 mg $l^{-1}$). The highest asiaticoside production (342.72 mg $l^{-1}$) was obtained after 36 days of elicitation in cultures treated with 0.1 mM MJ and 0.025 mg $l^{-1}$1-phenyl-3-(1,2,3-thidiazol-5-yl) urea (TDZ).

Kim et al. (2004) investigated the regulation of saponin biosynthesis in higher plants using *Centella asiatica* (L.) Urban as a model plant. Effects of a feeding precursor on asiaticoside production from leave and on the level of two-type 2, 3-oxidosqualene cyclaes (OSCs) mRNA were investigated. As a feeding precursor, squalene negatively affected the levels of cycloartenol synthase (CYS) and β-amyrin synthase (bAS) mRNA, but it also decreased the production of asiaticoside from whole plants. Plant hormones regulate secondary metabolism, and

in plant tissue cultures they could affect both culture growth and secondary metabolite production.

Susana et al. (2006) studied the effect of methyl jasmonate on triterpene and sterol metabolisms of *Centella asiatica* cultured plants and results showed that elicited plantlets of *C. asiatica* increased (upto 152 times) their content of triterpenoids directly synthesized from 2,3-oxidosqualene. In contrast, the free sterol content of *C. asiatica* decreased notably. The result also suggested that methyl jasmonate can be used as an inducer of enzymes involved in the triterpenoid synthesis downstream from 2, 3-oxidosqualene in *C. asiatica* plantlets.

### *2.4.1.2 Using Genetic Engineering Methods*

#### 2.4.1.2.1 Hairy root induction

A number of plant species including many medicinal plants have been successfully transformed with *Agrobacterium rhizogenes*. Transformed hairy roots mimic the biochemical machinery present and active in the normal roots (Chilton et al., 1982). Yun et al. (1992) increased the production of scopolamine in *Atropa belladona* from the naturally occuring chemical precursor hyocyamine by transformation with the enzyme hyoscyamine 6β- hydroxlase from *Hyoscyamus niger*. Plant transformation and genetic modification using *A. rhizogenes*, can be used to boost the production of those secondary metabolites, which are naturally synthezised in the roots. Cai et al. (1995) reported the establishment of hairy root culture system of *Artemisia annua* by infection with *Agrobacterium rhizogenes* and the optimum concentration of artimisin was 4. 8 mg $l^{-1}$.

Pradel et al. (1997) developed a system for producing transformed plants from root explants of *Digitalis lanata*. They reported higher amounts of anthraquinones and flavanoids in the transformed hairy roots than in untransformed roots. Argolo et al. (2000) reported the regulation of solasodine production by *Agrobacterium rhizogenes* transformed roots of *Solanum aviculare*.

Transgenic hairy root cultures have served as a useful model system to investigate the biosynthesis of alkaloids, and a variety of other secondary metabolites. Giri et al. (2001) used different strains of *Agrobacterium rhizogenes*, *viz.*, $A_4$, 15834, $K_{599}$, LBA9402, 9365 and 9340 for induction of hairy roots in *Artemisia annua* using shoot tip meristem as explant. Hairy root lines induced by strain 9365 were found to contain highest amount of artemisinin (0.23%).

Shi and Kintzios (2003) reported the genetic transformation of *Pueraria phaseoloides* with *Agrobacterium rhizogenes*. The content of puerarin in hairy roots reached a level of 1-2 mg $g^{-1}$ dry weight and was 1.067 times the content in the roots of untransformed plants.

Aziz et al. (2004) studied the production of asiaticoside and madecassoside in *Centella asiatica in vitro* and *in vivo*. The localization was determined of the triterpenoids, asiaticoside and madecassoside, in different organs of glasshouse-grown plants and cultured material, including transformed roots, of two phenotypes of *Centella asiatica*. Methanolic extracts of asiaticoside and madecassoside were prepared for gradient HPLC analysis. Terpenoid content was highest in leaves, with asiaticoside (0.79 ± 0.03 and 1.15 ± 0.10 % of dry mass) and madecassoside [0.97 ± 0.06 and 1.65 ± 0.01 % (d.m.)]. Transformed roots were induced using *Agrobacterium rhizogens* and their growth was maximal on MS basal medium

supplemented with 60 g $dm^{-3}$ sucrose. However, asiaticoside and madecassoside were undetectable in transformed roots and undifferentiated callus.

Lee et al. (2004) produced transformed hairy roots from the seedlings of *Taraxacum platycarpum* by infection with *Agrobacterium rhizogenes* 15834. Transgenic plantlets showed considerable differences in their morphology when compared to the corresponding non transgenic plants. The differences reflect the modification of morphological root characters by introduction of *rol* genes.

Hairy root cultures have been proven to be an efficient means of producing secondary metabolites that are normally biosynthesized in roots of differentiated plants. Furthermore, a transgenic root system offers tremendous potential for introducing additional genes along with the Ri plasmid, especially with modified genes, into medicinal plant cells with *A. rhizogenes* vector systems. The cultures have turned out to be a valuable tool with which to study the biochemical properties and the gene expression profile of metabolic pathways. Moreover, the cultures can be used to elucidate the intermediates and key enzymes involved in the biosynthesis of secondary metabolites Zhi-Bi Hu et al. (2006).

Kim et al. (2007) transformed *C. asiatica* using *Agrobacterium rhizogenes* strain R1000 that harbors pCAMBIA1302 encoding the hygromycin phosphotransferase (*hpt*) and green fluorescence protein (*mgfp5*) genes and the hairy culture was coupled with elicitation technique. 0.1 mM methyl jasmonate (MJ) was applied as an elicitor to the culture medium for 3 weeks, a large quantity of asiaticoside was generated (7.12 mg/g, dry wt). In the case of gene expression, 12 h after MJ treatment the expression of the *CabAS* (*C. asiatica* putative β-amyrin synthase) gene in the hairy roots was significantly different from that of the control and this level of transcripts was maintained for 14 days. The results showed that

production of *C. asiatica* hairy roots could be optimized and the resulting cultures could be elicited with MJ treatment for enhanced production of asiaticoside.

**2.4.1.2.2 *Agrobacterium* Mediated Genetic Transformation in Medicinal Plants**

Plant transformation mediated by the soil plant pathogen *Agrobacterium tumefaciens* has become the most used method for plant transformation. *A. tumefaciens* naturally infects the wound sites in dicotyledonous plant causing the formation of the crown gall tumours (Smith and Townsend, 1907).

*A. tumefaciens* is capable to transfer a particular DNA segment (T-DNA) of the tumour-inducing (Ti) plasmid into the nucleus of infected cells where it is subsequently stable integrated into the host genome and transcribed (Nester et al., 1984; Binns and Thomashaw, 1988).

The first plant transformed by Agrobacterium tumefaciens was tobacco (Herrera-Estrella, 1983). However *Agrobacterium*-mediated transformation has remarkable advantages over direct transformation methods in reducing the copy number of the transgene, potentially leading to fewer problems with transgenic cosuppresion and instability (Koncz et al., 1994, Hansen et al., 1997). In addition, it is a single-cell transformation system and avoids the obtainment of mosaic plants, which are more frequent when direct transformation is used (Enríquez-Obregón., et al 1997; 1998).

*Agrobacterium* Ti/Ri plasmids are natural gene vectors, by which a number of attempts have been made in genetic engineering of secondary metabolism in pharmaceutically important plants in the last few years. Opines are biosynthesized by transformed crown galls and hairy roots integrated with T-DNAs of Ti/Ri plasmids. The hairy roots induced by Ri plasmid of *Agrobacterium rhizogenes* have

been proved to be an efficient means of producing secondary metabolites that are normally biosynthesized in roots of differentiated plants. A second and more direct way to manipulate secondary pathways is performed by transferring and expressing specifically modified genes into medicinal plant cells with *Agrobacterium* vector systems. The genes encoding neomycin phosphotransferase and beta-glucuronidase have been used as model genes under the transcriptional control of appropriate promoters. Recently some specific genes that can eventually modify the fluxes of secondary metabolism have been integrated and expressed in medicinal plant cells (Saito et al., 1992).

The gene transfer method and the gene expression vector to be used must be compatible with the plant genotype and the tissue to be treated. The generally used gene transfer methods include *Agrobacterium* mediated transformation, particle bombardment, electroporation etc. Genes of interest, including marker or reporter genes, are vectored on plasmids. The plasmids have various gene promoters and other sequences which affect the ability of the plant cell to transcribe the gene and to translate it into a protein product. Use of plasmid with inappropriate promoters and markers may mask the effectiveness of a specific gene transfer method (Kuehnle, 1997).

Leo et al. (2000) reported an efficient whole plant transformation system for *Hyoscyamus muticus,* an important medicinal plant of the solanaceous family. They developed a system using a plasmid carrying the *nptII* and *gus A* genes, which was delivered into leaf explants by particle bombardment. Ten percent of bombarded leaf explants formed kanamycin resistant callus, from which putative transgenic plants were recovered. The *nptII* gene conferring kanamycin resistance was found to be incorporated into the genome of all transgenic plants screened. Hosokawa et al.

(2000) produced transgenic gentian plants by particle bombardment of suspension-culture cells.

Chen (2000) reported a three fold enhancement in production of the putative antimalarial, anticancer agent artemisinin in transgenic *Artemisia annua* plants over expressing farnesyl diphosphate synthase, the enzyme immediately preceding the first committed biosynthetic step. Geng et al. (2001) reported that when the bacterial gene *ipt*, which promotes the endogenous production of cytokinin growth hormones, is expressed in *Artemisia* there is a coordinated increase in hormone, chlorophyll and artemisinin levels.

Souret et al. (2002) demonstrated that the transformed roots of *Artemisia annua* are superior to whole plants in terms of yield of the sesquisterpene artemisinin.

Lievre et al. (2005) reported *Agrobacteium tumefaciens* mediated genetic transformation in *Ruta graveolens*. Transformation was obtained by co-cultivation of hypocotyls of 2-3 weeks old plants and *Agrobacterium tumefaciens* strain C58C1Rif $^{R}$ containing a plasmid harbouring neomycin phosphotransferace and β-glucuronidase encoding genes.

Bae et al. (2005) reported an efficient transformation protocol for stable introduction of *hmgr* into *Taraxacum platycarpum* plants. The *Agrobacteium tumefaciens* strain EHA105 containing the binary vector, pCAMBIA1301, with *gus* and *hmgr* genes, showed high transformation efficiency after 3-5 weeks of hygromycin selection.

A high-frequency and simple procedure for *Agrobacterium tumefaciens*-mediated genetic transformation of the medicinal plant *Salvia miltiorrhiza* was developed by Yan et al. (2007) with *A. tumefaciens* strain EHA105 harboring the plasmid pCAMBIA 2301.

### *2.4.2 METABOLIC PATHWAY ENGINEERING IN MEDICINAL PLANTS TO ENHANCE SECONDARY METABOLITE PRODUCTION*

Genetic transformation is a powerful tool for enhancing the productivity of novel secondary metabolites of limited yield. Direct manipulation of DNA sequences to alter gene expression in medicinal plants is an area that is ripe for expansion. One approach is to identify enzymes of a metabolic pathway and then manipulate these enzymes to provide better control of that pathway. Another approach is to introduce new genes for the increased yield of secondary metabolites. Metabolically engineered medicinal plants will complement conventional breeding efforts for the production of quantitatively and qualitatively improved pharmacological properties.

3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the irreversible conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate and is considered a key regulatory step controlling isoprenoid metabolism in mammals and fungi. A constitutively expressing hamster HMGR gene was introduced into tabacco (*Nicotiana tabaccum* L.) plants to obtain unregulated HMGR activity to investigate whether HMGR activity could be limiting in plants. The impact of the resulting enzyme activity on the biosynthesis and accumulation of particular isoprenoids was evaluated. Expression of the hamster HMGR gene led to a 3- to 6-fold increase in the total HMGR enzyme activity (Chappell et al., 1995).

Transgenic expression of an intron containing GUS and a gene encoding for the terpenoid indole alkaloid biosynthetic enzyme strictosidine synthase was demonstrated by Cardoso et al. (1997) through *Agrobacterium* mediated transformation of pharmacologically important plant species *Tabernaemontana pandacaqui*.

Nabha et al. (1999) reported transformation of calli of *Papaver somniferum* with the *sam-1* gene from *Arabidiopsis thaliana* which encodes on S-adenosyl methionine synthetase (SAM Synthetase) and was subcultured over a four year period. The stability of the expression and the level of SAM synthetase activity were evaluated in transgenic cell lines and in the control. All transgenic cell lines exhibited a level of SAM synthetase activity higher than that of control.

Sato et al. (2000) did metabolic engineering in plants such as *Atropa belladonna, Nicotiana sylvestris, Coptis japonica* and *Eschscholzia californica* to enhance alkaloid biosynthesis. Charlwood and Pletsch (2002) reported that pathway engineering lead to improvements of potential value in the breeding of medicinal plants. Kim et al. (2004) isolated a cDNA encoding farnesyl diphosphate synthase from *Centella asiacita* (L.) Urban. They have reported the cloning and expression of an FPS cDNA as a first step in investigating the saponin pathway.

A homology-based PCR method was used to clone a cDNA encoding oxidosqualene cyclase from *Centella asiatica*, which produces a large quantity of triterpene saponins such as asiaticoside and madecassoside by Kim et al. (2005). Sequence analysis of one clone found sequences related to β-amyrin synthase.

The genetic manipulation of both the mevalonic acid (MVA) and methylerythritol-4-phosphate (MEP) pathways, leading to the formation of

isopentenyl diphosphate (IPP), has been achieved in tomato using 3-hydroxymethylglutaryl CoA (*hmgr-1*) and 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*) genes, respectively. Transgenic plants containing an additional *hmgr-1* from *Arabidopsis thaliana*, under the control of the cauliflower mosaic virus (CaMV) 35S constitutive promoter, contained elevated phytosterols (up to 2.4-fold). Phytoene and β-carotene exhibited the greatest increases (2.4- and 2.2-fold, respectively) (Enfissi et al., 2005).

*Arabidopsis thaliana HMG1* cDNA, coding the catalytic domain of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR1S), the key enzyme of the Mevalonate (MVA) pathway, was expressed in the cardenolide-producing plant *Digitalis minor* (the main industrial source of cardiac glycosides, steroidal metabolites derived from mevalonic acid via the triterpenoid pathway) by pathway engineering (Sales et al., 2007). Constitutive expression of *HMG1* resulted in an increased sterol and cardenolide production in both *in vitro*- and greenhouse-grown plants. Thus the genetic improvement of *Digitalis* species was achieved through metabolic engineering.

## 2.5 FACTORS INFLUENCING *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION IN MEDICINAL PLANTS

### *2.5.1 EXPLANT FOR GENETIC TRANSFORMATION*

For molecular breeding to be feasible, the tissue to be genetically engineered must give rise to plants. The gene transfer method and the gene expression vectors used must be compatible with the plant genotype and the tissue to be treated.

According to Vergauwe et al. (1998) transformation of *Artemisia annua* was accomplished by co-cultivation of sterile leaf, stem and root explants from 12 to 18

week old plants, cotyledons and hypocotyls from eight day old seedlings with *A. tumefaciens*. The first transgenic peppermint plants have been obtained by *Agrobacterium* mediated transformation by co-cultivation with morphogenically responsive leaf explants (Niu et al., 1998).

Park and Facchini (2000) obtained transformants in California poppy (*Eschscholzia californa)* by co-cultivation of excised cotyledons with *A. tumefaciens* strain GV1301 carrying the pBI121 binary vector. Leaf and hypocotyls explants from 35-40 old lavender seedlings were inoculated with the EHA105 strain of *A. tumefaciens* to obtain transgenic plants of the aromatic shrub *Lavandula latifolia* (Nebauer et al., 2000).

Lee and Pedersen (2001) obtained transformants in *Eschscholzia californa* by co-cultivation of leaves with *A. tumefaciens*. Hypocotyls were used as explants for genetic transformation in *Bixa orellana*. Hypocotyls from annatto seedlings were inoculated with *Agrobacterium tumefaciens* harbouring a binary vector pBI121 or pCAMBIA2301 (Zaldivar et al., 2003). Feeney and Punja (2003) reported that suspension cells of hemp *(Cannabis sativa)* were transformed with *A. tumefaciens* strain EHA101 carrying the binary vector pNOV3635.

Complete plants regenerated *in vitro* which possessed no or trace amount of patchouli oil in leaf tissues were used as explant source in *Pogostemon cablin* to generate the transformants (Sugimura et al., 2005).

### *2.5.2 STRAIN SPECIFICITY OF AGROBACTERIUM*

There are differences in the susceptibility of *Agrobacterium* infection between species and even between cultivars and genotypes of the species. Hence the

best method is to transform with different strains harbouring a good selectable marker, till a genotype/strain combination is obtained. Several *Agrobacterium tumefaciens* strains varying in chromosomal background, *vir* helper plasmid and binary vector plasmid should be tested for their competence to transform.

Yun et al. (1992) reported the transfer of binary vectors pHY8 and pGA482 to *Agrobacterium tumefaciens* LBA4404 by direct transfer method and the ex-conjugants were used to transform *Atropa belladona* leaf explants, basically as described for tobacco leaf disc transformation. Lee and Pederson (2001) obtained stable genetic transformation of *Eschscholzia californica* (California poppy) using the disarmed *A. tumefaciens* LBA4404 encoding a synthetic green fluorescent protein reporter gene that is further controlled by an enhanced cauliflower mosaic virus 35S promoter.

*Agrobacterium tumefaciens* strain GV3010 containing the plasmid vector pMP90 containing the marker gene for kanamycin resistance and a reporter gene for *gus,* both controlled by the NOS promoter derived from nopaline synthase gene was used to transform *Atropa belladonna* plants (Negoianu et al., 2002).

Fenny and Punja (2003) reported that suspension cells of *Cannabis sativa* were transformed with *Agrobacterium tumefaciens* strain EHA101 carrying the binary vector pNOV3635 with a gene encoding phosphomannose isomerase (PMI). Shanjun et al. (2005) in *Hyoscyamus* niger developed a method for transformation *in vitro* with *Agrobacterium* strain harboring binary vector (pGSGluc1).

Conditions for transformation were examined using two *Agrobacterium tumefaciens* strains containing different chimeric plasmid in Patchouli. Leaf explants were infected with strain EHA 101/pIG121-Hm carrying β-glucuronidase and

hygromycin phosphotransferase (*hpt*) gene and another strain LBA4404/pBI 121-PaCP1 encoding the coat protein precursor gene of patchouli mild mosaic virus (CP-P) and neomycin phosphotransferase (*nptII*) gene to obtain transformants (Sugimura et al., 2005).

*2.5.3 BACTERIAL DENSITY*

Concentration of bacterial cells in the induction medium is an important factor to be considered for efficient transformation. Very low density of bacterial population could lead to ineffective transformation, whereas very high density may lead to necrosis and death of the explant. Some species are very sensitive to bacterial infection and hence very low density of bacterial population is used.

Lichtenstein and Draper (1986) attempted transformation from the cultured bacterial cells obtained from the bacterial suspension by centrifugation and resuspended in liquid MS medium and the final OD value was adjusted to 0.6-0.8. Leaf sections of *Echinacea purpurea* were immersed into this bacterial suspension for 30 minutes, and blotted on sterile filter paper and then co-cultivated to generate the transformants.

Negoianu et al. (2002) used the *Agrobacterium* grown on liquid LB medium containing 50 mg $L^{-1}$ rifampicin in the dark at 28°C for two days until the optical density at 600 nm reached the value of 0.5 to obtain transformants in *Atropa belladonna*. Nisha et al. (2003) reported that in *Bacopa monniera* transformants were obtained when leaf discs were immersed in over night culture of *A. tumefaciens* diluted to 5 x $10^8$ cells / ml (as measured by $OD_{600}$) for 15 minutes.

*Agrobacterium*-mediated transformation of hypocotyl explants of cauliflower variety Pusa Snowball K-1 was standardized by Chakrabarty et al. (2002). Many parameters (and treatments) were tested. Among them density of bacterial culture, 0.5 OD600 and its dilutions 1: 10, 1: 20 were found effective.

The establishment of an efficient *Agrobacterium*–mediated transformation protocol for sunflower genotypes, cv. Capella and SWSR2 inbred line were studied. The protocol requires the identification and optimization of parameters affecting T-DNA delivery and plant regeneration. *Agrobacterium tumefaciens*–mediated transformation was performed using strains LBA4404 and GV3101 respectively harboring the pBI121 plasmid with the *gus* gene under the control of 35S promoter. The flourometric GUS activity was increased in cv. Capella and SWSR2 inbred line 1.9 and 1.6 fold, respectively, relative to transformation without inducers, by optimizing the bacterial density to OD600=1.0 (Mohamed et al., 2004).

*2.5.4 CO-CULTIVATION*

The explants used for co-cultivation with *Agrobacterium* carrying the desired vector include cells, callus, tissue slices, protoplasts etc. For transformation to be efficient, the induced *Agrobacterium* should have access to cells that are competent for transformation.

Nebauer et al. (2000) obtained optimal transformation rates in *Lavandula latifolia* when leaf explants pre-cultured for one day on regeneration medium were subcultured on selection medium after a 24 h co-cultivation with *Agrobacterium*.

Lee and Pedersen (2001) reported that stably transformed *Eschscholzia californica* cells appear 3 weeks after initial co-cultivation of *A. tumefaciens* with

poppy leaves, stems or roots. Koroch et al. (2002) reported *Agrobacterium tumefaciens* mediated genetic transformation of *Echinacea purpurea* by co-cultivation of leaf sections using the strain EHA105, containing the binary vector pBISNI for 48 hours.

*Agrobacterium*-mediated transformation of hypocotyl explants of cauliflower variety Pusa Snowball K-1 was standardized by Chakrabarty et al. (2002). Many parameters (and treatments) were tested. Among them co-cultivation temperature and cocultivation duration were found to be effective at 22°C and 26°C and 2, 4 days respectively.

Negoianu et al. (2002) performed gene transfer in *Atropa belladonna* via bacterial infection and further co-culture of plant leaf explants in *Agrobacterium* suspension, for 2 to 5 minutes. After gentle shaking, the leaves were dried on sterile Whatman paper to remove excess bacterial suspension and plated on solid LS medium. After two days of co-culture, explants were transferred to bacterial selection medium.

In *Bacopa monniera,* Nisha et al. (2003) reported that the incubation of leaf segments in bacterial suspension EHA105 (pBE2113) for 15 minutes and then co-cultivation for 48 hours, resulted in efficient transformation.

### *2.5.5 USE OF ACETOSYRINGONE DURING TRANSFORMATION*

The Ti plasmid virulence (*vir*) loci encode functions essential for the transfer of the T-DNA element from *Agrobacterium tumefaciens* to plant cells. These *vir* genes and thus the virulence of *Agrobacterium* are stimulated by compounds secreted from the wounded plant tissue. These compounds such as acetosyringone

greatly enhance transformation and are now routinely added to transformation experiments.

Mathews et al. (1990) reported that leaf segments from young seedlings of *Atropa belladona* were infected with a disarmed strain of *Agrobacterium tumefaciens* C58C1, harboring the plasmid pGV3850:1103 containing the coding sequences of neomycin phosphotransferase. Explants were infected with *A. tumefaciens*, and cultured overnight with and without acetosyringone (AS). The mean frequencies of explants producing calli on the two kanamycin-containing media were 21% and 71% when the explants were infected with bacteria cultured in the presence of AS compared with 2% in those infected with bacteria grown without AS.

Use of surfactants such as Silwet L77 and pluronic acid F68 in inoculation medium greatly enhanced T-DNA delivery by aiding *A. tumefaciens* attachment and or by elimination of certain substances that inhibit *A. tumefaciens* attachment.in immature embryos of wheat (Cheng et al., 1997).

The use of surfactant Silwet L77 prooved to be successfull with the floral dip method of *Arabidopsis thaliana* transformation. Surfactant added to the inoculation medium may play a role similar to vacuum infiltration, facilitating the delivery of *A. tumefaciens* cells to closed ovules, the primary target for *A. tumefaciens* during in planta transformation of *A. thaliana* (Ye et al., 1999; Bechold et al., 2000; Desfeux et al., 2000).

*Agrobacterium*-mediated transformation of hypocotyl explants of cauliflower variety Pusa Snowball K-1 was standardized by Chakrabarty et al. (2002). Many

parameters were tested. Among them transformation enhancers acetosyringone 50 and 100 µM; 5-azacytidine 100 µM were found to be effective

In *Agrobacterium* transformation of white clover, Voisey et al. (1994) found that acetosyringone (100 µM) when added to co-cultivation medium helped in stabilizing the rate of transformation, which varied considerably in its absence. Sales et al. (2003) reported that among the factors influencing T-DNA transfer to *Digitalis* plants, the EHA105 strain and the addition of acetosyringone to the co-culture medium increased transformation.

The establishment of an efficient *Agrobacterium*–mediated transformation protocol for sunflower genotypes, cv. Capella and SWSR2 inbred line were studied. The protocol requires the identification and optimization of parameters affecting T-DNA delivery and plant regeneration. *Agrobacterium tumefaciens*–mediated transformation was performed using strains LBA4404 and GV3101 respectively harboring the pBI121 plasmid with the *gus* gene under the control of 35S promoter. The addition of 200 µM acetosyringone during the co-cultivation increased the fluorometric GUS activity 1.9 fold in cv. Capella and 1.6 fold in SWSR2 inbred line, while the addition of 100 µM coniferyl alcohol increased the fluorometric GUS activity 1.1 fold in both genotypes compared with the transformed without inducer (Mohamed et al., 2004).

Enhancement of acetosyringone-mediated virulence gene induction in *Agrobacterium tumefaciens* by polyamines was reported by Kumar et al. (2005). The results suggested the involvement of diamine putrescine and triamine spermidine in *vir* gene induction and T-DNA transfer when *agrobacterial* cells were treated with polyamine prior to acetosyringone addition. This has been found useful

in achieving a high transformation frequency in those plant species, which show minimal *vir* gene induction.

### *2.5.6 ELIMINATION OF BACTERIA AFTER CO-CULTIVATION*

Complete elimination of bacteria from the explant after co-cultivation is very essential; otherwise it will interfere with the growth and organogenesis of the explant. Over growth of bacteria causes death of the explant and disrupts the experiment. Elimination of bacteria from the explant is done by the use of antibiotics. The antibiotic should be such that it kills the bacteria without affecting the growth and organogenesis of the explants. The most commonly used antibiotic for this purpose is cefotaxime. However, the effect of antibiotic on the explant need to be studied before choosing it as bacteriostatic agent as they are also reported to have detrimental effect on some species.

Antibiotics such as cefotaxime, carbenecillin and timentin have been used regularly in *Agrobacterium*-mediated transformation of crops following co-culture to suppress or eliminate *Agrobacterium* (Cheng et al., 1996; Bottinger et al., 2001; Sunikumar and Rathore, 2001).

Vergauwe et al. (1996) working on transformation of *Artemisia annua* found that cefotaxime at 50 mg $l^{-1}$ was effective as a decontaminating antibiotic; but it caused retardation in callus formation. When a concentration of vanomycin at 750 mg $l^{-1}$ was tried, it was found to be non-toxic to the tissue. However, it could not control the bacteria effectively. Curtis et al. (1999) reported that in *Datura* after two days of co-cultivation period, the leaf explants were transferred to the shoot regeneration medium supplemented with 200 mg $l^{-1}$ kanamycin sulphate and 200 mg $l^{-1}$ cefotaxime. Cefotaxime was used as the bacteriostatic agent.

Carbenicillin has been the antibiotic of choice in *Agrobacterium*-mediated transformation of wheat and maize (Cheng et al., 1997, 2003; Zhang et al., 2003).

Park and Facchini (2000) reported that after co-cultivation, *Eschscholzia californica* cotyledons were transferred to a medium containing 200 mg $l^{-1}$ timentin to eliminate the *Agrobacterium.* Koroch et al. (2002) co-cultivated *Echinacea purpurea* leaf sections with *Agrobacterium* for different periods of time ranging from 0 to 72 hrs before transfer to shoot induction media containing 300 mg $l^{-1}$ timentin, which was used for the control of *Agrobacterium.* Negoianu et al. (2002) performed gene transfer in *Atropa belladona* via bacterial infection and further co-culture of plant leaf explants in *Agrobacterium* suspension for 2 to 5 minutes. After two days of co-culture, explants were first transferred to a fresh Linsmaier and Skoog (LS) medium supplemented with 500 mg $l^{-1}$ cefotaxime for bacterial selection.

Tang et al. (2005) evaluated the effect of three antibiotics carbenicillin, claforan, timentin on the elimination of *Agrobacterium tumefaciens* from loblolly pine (*Pinus taeda*) zygotic embryo explants and on transgenic plant regeneration. The results showed that all the three antibiotics at 500 mg $l^{-1}$ were effective in elimination of bacteria after transformation and also increased callus growth and shoot regeneration.

Ogawa et al. (2006) evaluated the usefulness of β-lactam antibiotics, meropenem (MEPM) and moxalactam (LMOX) for *Agrobacterium*-mediated transformation of tobacco, tomato and rice in comparison with commonly used β-lactams, carbenicillin (CBPC) and cefotaxime (CTX). It was concluded that introduction of MEPM or LMOX at 25 mg $l^{-1}$ for non-β-lactamase-producing strains and MEPM at 25 mg $l^{-1}$ or LMOX at 50 mg $l^{-1}$ for β-lactamase-producing strains,

respectively, to *Agrobacterium*-mediated transformation is an efficient strategy to improve transformation efficiency.

Wiebke et al. (2006) carried out an experiment to determine the efficacy of different antibiotics in suppressing *Agrobacterium tumefaciens* strain LBA4404 in soybean genetic transformation. On tissue culture conditions, carbenicillin at 500 and 1000 mg $L^{-1}$ was not active against *Agrobacterium*. Treatments with cefotaxime at 350 and 500 mg $L^{-1}$, and cefotaxime + vancomycin efficiently suppressed *Agrobacterium* during 49 days.

*2.5.7 MARKER AND REPORTER GENES*

Selection of transformed cells is a key factor in developing a successful genetic transformation system (Chia et al., 1994). Single dominant gene encoding suitable resistance to a selective agent is used as a marker. The reporter genes are used to analyze the function of promoters and other gene regulatory sequences. These genes do not disrupt the plant regeneration, but allow the selection of transformed cells. Transformation vectors are constructed with a reporter gene or a selectable marker, which indicates successful incorporation and expression of the introduced genes. The reporter gene and selectable marker gene are fused to the same plant promoter that is fused to the foreign gene of interest. Commonly, resistance to antibiotics and herbicides are used as selectable markers (Hinchee et al., 1994).

The most widely used antibiotic marker is kanancycin and was used in the first transformation experiments. (Fraley et al., 1983) Neomycin phosphotransferase II (*nptII*) gene from transposon Tn5, detoxify neomycin, kanamycin and G418 by phosphorylation. It is widely used in dicotyledon system including tobacco, potato

and tomato (An et al., 1986). Hygromycin phosphotransferase (*hptIV*) governs resistance to hygromycin. This gene isolated from *E. coli* has been placed under various promoters and has been successfully used in strawberry (Nehra et al., 1990).

Herbicide resistance markers function in the same way as antibiotic markers. The *bar* gene isolated from *Streptomyces hygroscopicus*, confers resistance to the herbicide phosphinothricin, the active ingredient of Bilaphos and Basta. The *'bar'* gene codes for phosphinothricin acetyl transferase (PAT) which inactivates phosphinothricin, an irreversible inhibitor of glutamine synthase. This gene has been inserted and expressed in rape (De Block et al., 1989) and alfalfa (Krieg et al., 1990).

An alternative to antibiotic selection is the use of the firefly luciferase gene (Chia et al., 1994) as a marker. The product of this gene produces light upon reaction with luciferin, which can be detected with a camera photomultiplier. Another reporter gene commonly used in the transformation is anthocyanin. Upon activation, this reporter system produces a reddish purple pigment in transformed tissue (Ludwig et al., 1990).

### *2.5.8 SELECTION OF TRANSFORMED CELLS*

Selection of transformed cells is an important aspect of transformation work (Chia et al., 1994). Several factors affect the choice of chemicals used for selection. The selection agent must be toxic to plant cells, though not so toxic that the products from the dying non-transformant cells kill adjacent transformed cells. Thus the most effective toxins are those which either inhibit growth of untransformed cells or slowly kill the untransformed cells. Optimal selection pressure will use the lowest level of toxin needed to kill the untransformed tissues.

Yun et al. (1992) obtained transformants in *Atropa belladona* by selection in media containing 500 μg $ml^{-1}$ kanamycin. A transformation system was developed for *Artemisia annua* plants from leaf explants using *Agrobacterium tumefaciens* strain (58 C1 $Rif^{R}$ (pGV2260) (pTJK136). A concentration of 20 $mgl^{-1}$ kanamycin was used to select transformed tissue (Vergauwe et al., 1996).

Dronee et al. (1999) obtained transformed callus lines in lavandin and transgenic shoots were regenerated from the kanamycin resistant calli and rooted on selective media with 150 mg $l^{-1}$ kanamycin. Niu et al. (2000) observed enhanced transformation efficiency in peppermint by increased selection pressure mediated by higher concentrations of kanamycin in the medium during shoot induction, regeneration and rooting (from 20 to 50 mg $l^{-1}$ in shoot induction / regeneration medium and from 15 to 30 mg $l^{-1}$ in rooting medium).

*Echinacea purpurea* leaf sections were co-cultivated with *Agrobacterium* for 48 hrs and transferred to shoot induction media containing timentin for control of *Agrobacterium* and kanamycin (50 mg $l^{-1}$) for selection of transformed tissues (Koroch et al., 2002). Negoianu et al. (2002) reported that a selective medium containing 500 mg $l^{-1}$ kanamycin was used for the early transformed tissue selection in *Atropa*. They were then transferred to a fresh stepwise diluted (400, 300 and 200 mg $l^{-1}$) selective medium.

Alsheikh et al. (2002) observed that *Fragaria vesca* showed a high sensitivity to kanamycin and therefore, a selection regime from 10mg $l^{-1}$ to 25 mg $l^{-1}$ kanamycin over a period of 8 weeks was used. Nisha et al. (2003) observed that the selection medium comprised of shoot regeneration medium supplemented with 15 mg $l^{-1}$ kanamycin and 300 mg $l^{-1}$ cefotaxime in brahmi. Four weeks after bacterial infection, co-cultivated explants showed callusing on the selection medium, while

the control uninfected explant inoculated into the selection medium showed browning and senescence.

Sugimura et al. (2005) selected greenish calli with adventitious shoots, following co-cultivation for three days and selection by 50 mg $l^{-1}$ hygromycin B from which putative transformants with roots were regenerated in Pogostemon *cablin* using *A. tumefaciens* strain EHA101 carrying the vector p1G 121-Hm. Using another strain LBA 4404/pBI121-PaCP1 Patchouli mild mosaic virus (CP-P) and neomycin phospotransferase (*nptII* gene) putative transformants were also obtained after co-cultivation for seven days and selection by 100mg $l^{-1}$ kanamycin.

*2.5.9 CONFIRMATION OF TRANSFORMATION*

***2.5.9.1 PCR Analysis***

Confirmation of the transformants is usually done by Polymerase Chain Reaction (PCR) using the primer designed for the marker gene. Integration of our gene of interest can be confirmed by a PCR reaction with gene specific primers.

Vergauwe et al. (1996) in *Artemisia annua* performed PCR of transformed tissues and DNA sequencing of the amplification products. The results revealed that 75 per cent of the regenerants contained the foreign genes. Dronne et al. (1999) in lavandin confirmed the presence of the introduced β-glucuronidase and neomycin phosphotransferase II genes by PCR and Southern blot analysis.

Cucu et al. (2002) confirmed the presence of the marker *nptII* gene in the entire plant using PCR. The obtained genotype and phenotype profiles confirm both the integration and the expression of the *nptII* gene into the *A.belladonna* genome.

In *Echinacea*, Koroch et al. (2002) detected *nptII* sequences in kanamycin-resistant plantlets by PCR analysis using the primer sequences to amplify a 320 bp region of the *nptII* gene. PCR analysis for the presence of the *nptII* transgene correlated positively with GUS assay results. Sales et al. (2003) in *Digitalis minor* obtained evidence of stable transgene integration by PCR, growth on media selective for *nptII* or *bar* genes, and expression of the *gus A* gene.

Nisha et al. (2003) studied the presence of *gus* and *nptII* genes in the genomes of the transformants in brahmi by PCR amplification using specific primers for *nptII* and *gus* genes. The DNA isolated from five randomly selected kanamycin resistant plants showed amplification of 1 kb fragment with GUS primers and a 600 bp fragment with the *nptII* primers, while there was no amplification with the DNA of the control plant.

Shanjun et al. (2005) confirmed transformation in *Hyoscyamus niger* calli by β -glucuronidase histochemical and flurometric assays, PCR and Southern blot analysis. Sugimura et al. (2005) reported that using total DNAs from the transformants in patchouli the full length of CP-P encoding the coat protein precursor gene of patchouli mild mosaic virus was detected by PCR reaction.

### *2.5.9.2 Southern Hybridization*

A Southern blot is a method used to check for the presence of a DNA sequence in a DNA sample. Southern blotting combines agarose gel electrophoresis for size separation of DNA with methods to transfer the size-separated DNA to a filter membrane for probe hybridization. Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contains DNA

sequence that is complementary to the probe. By this technique copy number of gene integration can also be found out.

Bae et al. (2005) successfully transformed the medicinal plant *Taraxacum platycarpum* using *Agrobacterium tumefaciens* with GUS and HMGR genes. Southern blotting demonstrated stable integration of one copy of HMGR gene into the plant cell genome.

Kim et al. (2005) cloned a cDNA encoding oxidosqualene cyclase from *Centella asiatica*, which produces triterpene saponins as asiaticoside and madecassoside. Sequence analysis of one clone found sequences related to β-amyrin synthase (*CabAS*). Southern analysis showed that *Centella asiatica* genome contains one copy of the *CabAS* gene.

Kim et al. (2007) confirmed transformation of *Centella asiatica* with *Agrobacterium rhizogenes* strain R1000 that harbors pCAMBIA1302 encoding the hygromycin phosphotransferase (*hpt*) and green fluorescence protein (*mgfp5*) genes by Southern blot analyses.

### *2.5.9.3 Gene Expression Analysis by RT-PCR*

Reverse transcription polymerase chain reaction (RT-PCR) is a laboratory technique for amplifying a defined piece of a ribonucleic acid (RNA) molecule. The RNA strand is first reverse transcribed into its DNA complement or complementary DNA, followed by amplification of the resulting DNA using polymerase chain reaction. The exponential amplification via RT-PCR provides for a highly sensitive technique, where a very low copy number of RNA molecules can be detected. This

technique is used in the determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression.

Diemer et al. (1999) achieved the stable integration of *gus* and *npt II* genes in *Mentha arvensis* and *M. spicata* by *Agrobacterium tumefaciens*-mediated gene transfer. Either GV2260/GI or EHA105/MOG *Agrobacterium* strains. Transgene presence and structure was studied by the use of PCR analysis and Southern blot hybridization. Transgene expression was evaluated by RT-PCR.

Rezmer et al. (1999) identified and localized transformed cells in *Agrobacterium tumefaciens*-induced plant tumors by polymerase chain reaction (PCR) and reverse transcriptase-PCR analyses by using β-glucuronidase (gus)-gene-containing wild-type bacteria (A281 p35S gus-int).

Sturtevant (2000) performed differential-display reverse transcription PCR (DDRT-PCR) that allows extensive analysis of gene expression among several cell populations. DDRT-PCR has been used to address biological questions in mammalian systems, including cell differentiation, cell activation, cell stress, and identification of drug targets. In microbial pathogenesis and plant pathogenesis, it allowed the identification of virulence factors, genes involved in cell death, and signaling genes. In Candida albicans, DDRT-PCR studies identified TIF-2, which may play a role in the up regulation of phospholipases, and the stress-related genes, CIP1 and CIP2.

Jennifer et al. (2001) successfully developed a method to detect the presence of virus in infected plants, transgenes such as neomycin phosphotransferase II in transformed plants, and various plant genes including RubiscoL and 1,3-b-D-glucanase from small tissue samples (≤1 mg) of fresh leaves from wheat, clover,

tobacco, broad bean, grape, tomato, lettuce, or asparagus taken using glass micro capillaries, combined with RNase inhibitor and subjected to RT-PCR either directly or after a DNase treatment.

Zhou et al. (2005) performed Single Microspore Reverse Transcriptase PCR to study the dynamic change of gene expression during microsporogenesis in Rice.

Bae et al. (2005) successfully transformed the medicinal plant *Taraxacum platycarpum using Agrobacterium tumefaciens* with *gus* and *hmgr* genes and demonstrated stable integration of the gene by RT-PCR analysis.

## 2.6 QUANTIFICATION OF ASIATICOSIDE

### *2.6.1 SOLVENT EXTRACTION OF ASIATICOSIDE*

Asiaticoside was resolved by Verma et al. (1999) from other constituents by using an octadecyl silane-packed column eluted with water (containing 1 per cent trifluoroacetic acid): methanol (30:70, v/v) for their experiment to determine asiaticoside using simple reverse-phase high performance liquid chromatography (HPLC). This optimized analytical conditions allowed the determination of asiaticoside with a recovery of 97 per cent.

Singh et al. (2004) isolated asiaticoside from plant constituents by methanol extraction and separated from other terpenes by silica gel column chromatography. This protocol was a modification of that followed by Matsuda et al. (2001).

Jumpatong et al. (2006) used electrocoagulation for dechlorophyllation of alcoholic extracts of secondary metabolites as the results showed that for every plant

extract studied; electrocoagulation was more efficient than the classical solvent extraction method in removing plant pigments, while not affecting the important secondary metabolites in those extracts. For *C. asiatica*, the method consisted of extracting the dry leaves with absolute ethanol, diluting the ethanolic solution with water before dechlorophyllating by electrolysis for 2 hours using aluminium electrodes, filtering the resulting electrolysed mixture, evaporating the filtered solution to dryness, and extracting the residue with a little ethanol to afford a crude extract containing 4 per cent (by HPLC) of asiaticoside after evaporation of the ethanol.

Rai et al. (2006) developed an effective spectroscopic method for quantitative determination of asiaticoside in a polyherbal formulation. They have also standardized that asiaticoside has an absorption maxima at 278nm and the molecule obeys Beer's law in the concentration range 10-100µg/mL.

### *2.6.2 THIN LAYER CHROMATOGRAPHY TO QUANTIFY ASIATICOSIDE CONTENT*

Thin layer chromatography (TLC) is a widely used chromatographic technique used to separate chemical compounds. It involves a stationary phase containing of a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose immobilized on to a flat, inert carrier sheet. A liquid phase consisting of the solution to be separated dissolved in an appropriate solvent is drawn through the plate via capillary action, separating the experimental solution. It is used to determine the pigments a plant contains, in forensics to analyze the dye composition of fibers, or to identify compounds present in a given substance. It is a quick generic method for organic reaction monitoring.

Nath et al. (2004) showed the presence of asiaticoside in the *in vitro* grown leaves, callus and cell suspension cultured cells of medicinally important herb *Centella asiatica* by TLC and high performance liquid chromatography (HPLC) analysis.

Bonfill et al. (2006) identified four principal triterpenoid components of *Centella asiatica* by TLC on silica gel plates and mass spectrometry by using ethyl acetate and methanol as the mobile phase. The spots were detected with anisaldehyde solution. The separated compounds were confirmed by MALDI – TOF mass spectrometry.

*2.6.3 CONFIRMATION BY MALDI-TOF MASS SPECTROMETRY*

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) on cellulosic surfaces is shown to be a suitable method for examining highly oxidized terpenoids, which are otherwise too difficult to determine by other techniques. By crystallization of a 2, 5-dihydroxybenzoic acid (DHB) matrix and the sample solution on cellulose-coated thin layer chromatography (TLC) plates, spectra with good signal/noise ratios are obtained.

Crecelius et al. (2002) used particle suspension matrices for Thin-layer chromatography-matrix-assisted laser desorption ionization-time-of-flight mass spectrometry for analyzing tetracycline antibiotics.

The validity of the method was tested by Scalarone et al. (2005) on natural di- and triterpenoid resins used as paint varnishes. The samples were analyzed before and after artificial light ageing. Di- and triterpenoid compounds, being very

sensitive towards photo-oxidation, were found as oxidized molecules even in the raw resins and in the unexposed varnish layers.

Bonfill et al. (2006) identified four principal triterpenoid components of *Centella asiatica* by TLC on silica gel plates and mass spectrometry by using ethyl acetate and methanol as the mobile phase. The spots were detected with anisaldehyde solution. The separated compounds were confirmed by MALDI – TOF mass spectrometry.

### *2.6.4 HIGH PRESSURE LIQUID CHROMATOGRAPHY*

High-Pressure liquid chromatography (HPLC) is a form of liquid chromatography to separate compounds that are dissolved in solution. The different components in the mixture pass through the column at different rates due to 33differences in their partitioning behavior between the mobile liquid phase and the stationary phase.

A column–switching high-pressure liquid chromatography (HPLC) method was described by Baek et al. (1999) for the determination of asiaticoside in rat plasma and bile using column switching and ultraviolet (UV) absorbance detection. Detection of asiaticoside was accurate and repeatable. This method has been successfully applied to determine the level of asiaticoside in rat plasma and bile samples from pharmacokinetics and biliary excretion studies.

Verma et al. (1998) developed a rapid and simple reverse-phase HPLC method for the quantitative determination of asiaticoside, a pharmacologically active constituent from *Centella asiatica*. Using an octadecyl silane-packed column eluted with water (containing 1% trifluoroacetic acid): methanol (30:70, v/v), asiaticoside



was well resolved from other constituents. These optimized analytical conditions allowed the determination of asiaticoside with a recovery of 97 per cent.

Schaneberg et al. (2003) developed an improved HPLC for qualitative and quantitative determination of six triterpenes (asiaticoside, madecassoside, asiatic acid, madecassic acid, terminolic acid, and asiaticoside-B) in *Centella asiatica.* They used a Phenomenex Aqua 5µ C18 (200 Å) column as the stationary phase, a gradient mobile phase of water (0.1% TFA), acetonitrile (0.1% TFA), and methyl tert-butyl ether (0.1% TFA), and UV detection at 206 nm. The correlation coefficients for the calibration curves and the recovery rates ranged from 0.995 to 0.999 and from 98.39 per cent to 100.02 per cent, respectively.

Chauhan et al. (2003) described a simple and reproducible reverse phase HPLC method for the determination of asiaticoside in *Centella asiatica.* The method involves the separation of asiaticoside using an isocratic mobile phase consisting of acetonitrile, water and orthophophoric acid in the ratio of 32:67:01 and detection of chromatogram at 210 nm using photodiode array detector.

Cheng et al. (2004) quantitatively determined the concentration of asiaticoside in *centella asiatica* extract with the use of HPLC while they were studying the healing effects of *Centella asiatica* water extract and asiaticoside on acetic acid induced gastric ulcers in rats.

Guo et al. (2004) used HPLC method to quantify asiaticoside concentration in *centella asiatica* extract during their study on the effect of *centella asiatica* water extract and its active constituent asiaticoside on the expression and activity of inducible nitric oxide synthase during gastric ulcer healing in rats.

Schieffer (2005) used solid-phase extraction (SPE) for solving interference problems observed in simple HPLC assays. Anion-exchange SPE is used to remove an interfering compound co eluting with the triterpene glycoside madecassoside in *Centella asiatica* for improving HPLC assays of triterpene glycosides in *Centella asiatica*.

# *Materials and methods*

# 3. MATERIALS AND METHODS

The study on *Agrobacterium tumefaciens* mediated transfer of exogenous hydroxy methyl glutaryl CoA (HMG CoA) reductase gene to *Centella asiatica L.* was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during March 2006 to February 2008. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

## 3.1 SOURCE OF EXPLANT

Leaves and nodes collected from naturally grown healthy *Centella asiatica* plants were used as explants. Explants were washed in tap water containing a few drops of laboline. They were trimmed and surface sterilized using mercuric chloride (0.08%) for five minutes, washed using sterile water 3-4 times and used for inoculation.

## 3.2 CULTURE MEDIUM

### 3.2.1 Chemicals

All the chemicals used for the preparation of the culture media were of analytical grade and procured from Sisco Research Laboratories (SRL), India. The antibiotics and plant growth substances were purchased from Himedia Laboratories, India.

### 3.2.2 Glassware, Plasticware and Other Materials

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

### 3.2.3 Composition of Media

Basal MS medium (Murashige and Skoog, 1962) supplemented with various plant growth substances, *viz.*, 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA), kinetin (Kn), adenine sulphate (ADS), benzyl Adenine (BA), indole acetic acid (IAA) and proline were used for plant tissue culture experiments (Appendix I).

For the maintenance of *Agrobacterium* strains, AB minimal medium and Luria-Bertani (LB) medium were used (Appendix II).

### 3.2.4 Preparation of Medium

Standard media (Murashige and Skoog, 1962) for plant tissue culture was used for the study. The pH of the medium was adjusted to 5.7 using 0.1N NaOH / HCl. Agar was added at the rate of 4 - 4.5 g $l^{-1}$ and the medium was heated to dissolve agar. The medium was then dispensed into culture vessels at the rate of 15ml/ culture tube and 50 ml /conical flask. The test tubes and conical flasks were plugged with non-absorbent cotton and sterilized by autoclaving under steam at a pressure of 1.06 kg $cm^{-2}$ and a temperature of $121^0$ C for 20 min (Dodds and Roberts, 1982). The medium was then stored at $25 \pm 2^0$ C.

For bacterial culture, the pH of the medium was adjusted to 7.0 and agar was added at the rate of 1.5 per cent. The medium was sterilized by autoclaving and stored at $28^0$ C.

### 3.2.5 Preparation of Stock Solution of Antibiotics

#### *3.2.5.1 Kanamycin*

A stock solution ($10^4$ mg $l^{-1}$) was prepared by dissolving kanamycin monosulphate in sterile water. It was then filter sterilized and stored at -$20^0$ C.

#### *3.2.5.2 Cefotaxime*

A stock solution ($10^4$ mg $l^{-1}$) was prepared by dissolving cefotaxime in sterile water. It was then filter sterilized and stored at -$20^0$ C.

#### *3.2.5.3 Rifampicin*

A stock solution ($10^4$ mg $l^{-1}$) was prepared by dissolving rifampicin in small volume of ethanol and then the required volume was made up with sterile water. It was then filter sterilized and stored at -$20^0$ C.

For the antibiotic sensitivity studies, the required concentrations of kanamycin and cefotaxime were diluted from the stock and added to the sterilized molten medium at $40^0$C.

### 3.2.6 *Agrobacterium* Strains

*Agrobacterium tumefaciens* strain EHA105 harbouring the plasmid pBE 2113 was used for the study.

#### *3.2.6.1. Vector*

The vector pBE 2113 contains the *hmgr* (Hydroxy methyl glutaryl CoA reductase gene) and the *npt II* (*Neomycin* phosphotransferase II, kanamycin resistance) genes under the control of CaMV 35S promoter (Appendix III).

### *3.2.6.2 Maintenance of Agrobacterium Strains*

The *Agrobacterium tumefaciens* strain EHA105 harbouring the binary vector, pBE 2113 were grown on YEP medium supplemented with kanamycin 50 mg $l^{-1}$ and rifampicin 20 mg $l^{-1}$.

## 3.3 INOCULATION AND OTHER ASEPTIC MANIPULATIONS

All the aseptic manipulations were carried out in a laminar air flow cabinet (Thermadyne).

## 3.4 CULTURE CONDITIONS

The cultures were incubated at 25 ± $2^0$ C in air-conditioned culture room with 16 hour photoperiod (1000 lux) supplied by cool white fluorescent tubes. Relative Humidity in the culture room varied between 50 and 60 per cent.

## 3.5 PRODUCTION OF CALLUS

Callus was induced from leaf and node explants. Surface sterilized leaves were cut into square pieces and inoculated on MS media containing different concentrations and combinations of 2,4-D, Kn, and NAA. Cultures were incubated at 25 ± $2^0$ C in culture room with 16h photoperiod (1000 lux). Observations were recorded on the percentage establishment of explants and percentage of callusing.

## 3.6 REGENERATION FROM THE CALLUS

The calli obtained were transferred to MS medium with varying concentrations and combinations of Kn, NAA, BA, and ADS for regeneration. Observations were recorded on the percentage of regeneration.

## 3.7 EVALUATION OF *CENTELLA ASIATICA* CULTURES FOR SENSITIVITY TO ANTIBIOTICS

Sensitivity of *Centella* cultures to antibiotics was evaluated to utilize it as a marker system for selection process after transformation. The callus produced on callus induction medium (MS medium supplemented with Kn 2mg $l^{-1}$ and NAA 4mg $l^{-1}$) were transferred to Petriplates containing medium of same composition with different concentrations of kanamycin (0, 5, 25, 50, 75, 100, 125, 150, 200, 250, 300 and 350 mg $l^{-1}$) or cefotaxime (0, 5, 25, 50, 75 and 100 mg $l^{-1}$). The Petriplates were sealed with parafilm and kept at 25 ± $2^0$ C. The response of the callus to varying concentrations of kanamycin and cefotaxime was evaluated for a period of eight weeks.

The following scoring method was used for evaluation.

| **Score** | **Culture response** |
|---|---|
| '++++' | Fully green |
| '+++' | Partially discoloured |
| '++' | Bleached tissues |
| '+' | Tissues turning brown and dead |

## 3.8 SCREENING OF *AGROBACTERIUM* STRAINS FOR SENSITIVITY TO ANTIBIOTICS

*Agrobacterium* maintained in LB medium was transferred to LB medium with different concentrations of kanamycin (5, 25, 50, 75, 100, 125, 150, 200, 300, 400 and 500 mg $l^{-1}$) and cefotaxime (0, 5, 10, 15, 20, 25, 50, 75, 100 mg $l^{-1}$). Petri plates were then sealed with para film and incubated at $28^0$C and bacterial growth was observed for two days.

## 3.9 GENETIC TRANSFORMATION OF *CENTELLA ASIATICA*

Sterile cultures of nodes/callus were used for transformation.

### 3.9.1. Preparation of *Agrobacterium* Suspension for Co-cultivation

The *Agrobacterium tumefaciens* EHA 105 with the binary vector pBE2113 was grown on Petriplates containing AB minimal medium with kanamycin 50 mg $l^{-1}$ at $28^0$ C overnight. *Agrobacterium* suspension for co-cultivation was prepared by picking a single colony from the plate and inoculating into AB broth supplemented with kanamycin 50 mg $l^{-1}$. It was kept in a shaker overnight at $28^0$C at 10 rpm. The next day the culture was spun in a centrifuge at 5000 rpm at $4^0$C for 5 min. The pellet obtained was resuspended in 1 ml of half strength MS broth (with 100 µM acetosyringone) for co-cultivation.

### 3.9.2 Preparation of Plant Material

The calli of *Centella* were pre-cultured on MS medium containing 4 mg $l^{-1}$ Kn and 2 mg $l^{-1}$ NAA for 15 days before co-cultivation to maintain cells in active cell division stage.

### 3.9.3 Co-cultivation

The pre-cultured calli were used for co-cultivation. They were wounded using sterile disposable syringe to facilitate the infection process.

The pieces of callus were placed in a sterile Petriplate and wetted with liquid half MS medium to avoid drying of explants. The calli was then mixed thoroughly with the prepared *Agrobacterium* suspension (containing100 µM acetosyringone) by gentle swirling for 20 min for the infection process. The explants were blot dried with sterile filter paper and transferred to a Petriplate containing solidified half MS

medium. Petri plates were sealed with para film and kept for co-cultivation in dark for four days at $28^0$C.

### 3.9.4. Incubation on Bacteriostatic Medium

After co-cultivation, callus pieces were washed in half strength liquid MS medium containing 50 mg $l^{-1}$ cefotaxime to kill the bacteria. The tissues were blot dried with sterile filter paper and transferred to sterile petriplates containing half MS medium supplemented with 50 mg $l^{-1}$ cefotaxime for eradication of the remaining *Agrobacterium.*

### 3.9.5 Selection of Transformed Tissues

The transformed tissues were selected on MS medium containing kanamycin (100 - 350 mg $l^{-1}$) and cefotaxime (50 mg $l^{-1}$). The tissues were maintained by subculturing once in seven days in the same medium. After four rounds of subculture, the transformed and non-transformed tissues were scored based on their response in the medium.

## 3.10. CONFIRMATION OF TRANSFORMATION

### 3.10.1 Isolation of Genomic DNA

Total genomic DNA was isolated using modified protocol of Murray and Thompson (1980). Callus (0.2g) was taken and washed in distilled water and blot dried. It was ground into fine powder using liquid nitrogen with mortar and pestle. The powder was then transferred quickly to a 2 ml eppendorf tube containing 1 ml of pre warmed Cetyl trimethyl ammonium bromide (CTAB) extraction buffer (Appendix IV). The tube was incubated at 65° C for 30 min with occasional mixing. The mixture was cooled to room temperature and centrifuged at 5000 rpm for 5 min. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant and mixed well to get an emulsion by inverting the tube several times for

15 min. It was centrifuged at 10,000 rpm for 10 min and the aqueous phase was taken. To this equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed gently and centrifuged at 10,000 rpm for 10 min. To the aqueous phase, two volumes of absolute alcohol was added and mixed carefully. It was kept at –20° C for 30 min. The precipitated DNA was pelleted at 10,000 rpm for 10 min in a refrigerated centrifuge. The pellet was washed twice using 70 per cent ethanol; air dried, and was dissolved in 50 µl *Tris EDTA* (*Ethylene diamene tetra acetic acid*) buffer (pH 8.0) (Appendix IV).

### 3.10.2 Quantification of DNA

Quantification of DNA was carried out with UV – Visible spectrophotometer (Spectronic Genesys 5). The optical density of the DNA samples was recorded at both 260 and 280 nm wave lengths. The concentration of DNA was calculated using the following formula:

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

Where $A_{260}$ = absorbance at 260 nm

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

### 3.10.3 Polymerase Chain Reaction (PCR)

The PCR analysis was performed with DNA isolated from the callus. A standard PCR mix was prepared for 20 µl total volume containing 20 ng of template

DNA, 1mM of each dNTPs, 10pM of each primer, 1 unit of *Taq* DNA polymerase and 10X PCR buffer.

The genomic DNA of the transformed and the non-transformed plants obtained were amplified with the gene specific primers for *nptII* gene.

The following conditions were provided for the amplification of the specific region in the genomic DNA in a Thermal cycler (PTC-150 mini cycler).

| Step | Stage | *npt II* | |
|---|---|---|---|
| | | Temperature (°C) | Duration |
| 1. | Initial denaturation | 94 | 3 min |
| 2. | Denaturation | 94 | 30 sec |
| 3. | Annealing | 52 | 30 sec |
| 4. | Extension | 72 | 1 min |
| 5. | Final extension | 72 | 7 min |

The steps 2-4 were allowed to repeat 29 times.

### 3.10.4 Preparation of positive control

#### *3.10.4.1 Isolation of plasmid DNA*

A single colony of bacteria was inoculated in a 2 ml of LB broth containing appropriate antibiotics and incubated for 12-16 hrs at 28°C. The broth was transferred into microfuge tubes and centrifuged for 5 min at 10,000 rpm. The supernatant was discarded and the tube was inverted on a paper towel and blotted. 100 µl of ice cold solution I (Appendix V) was added and the cells were resuspended

in it by vigorous vortexing. This was followed by the addition of 200 µl of freshly prepared solution II (Appendix V) and the contents were mixed by inverting it four times. The tube was then incubated for 5 min in ice. To this mixture 150 µl of ice-cold solution III (Appendix V) was added and incubated at room temperature for 5 min. The bacterial lysate was centrifuged at 12,000 rpm for 5 min. The cleared lysate was transferred to an eppendorf tube. Equal volume of saturated phenol: chloroform: isoamyl alcohol (25:24:1) was added and the contents were mixed well. The contents were centrifuged at 12,000 rpm for 5 min to separate the aqueous layer. To the aqueous layer equal volume of chloroform: isoamyl alcohol (24:1) was added and the contents were centrifuged at 12,000 rpm for 5 min. The aqueous layer was separated and two volumes of 100 per cent ethanol at room temperature were added to precipitate the DNA. The contents were mixed well and were allowed to stand for 2 min at room temperature. Then it was spun at 5,000 rpm for 5 min at 4°C to collect the pellet. The pellet was washed with 70 per cent ethanol, air dried and dissolved in 50 µl 1X TE (pH 8.0) and stored at -20°C.

### *3.10.4.2 PCR of the Plasmid DNA*

The plasmid DNA was subjected to polymerase chain reaction, with *npt II* primer. A standard PCR mix was prepared for 20 µl final volumes. The reactions were set up with 20 ng of template DNA, 1mM of each dNTPs (2 µl), 10pM primer (0.5 µl each of forward and reverse primer), 1 unit of *Taq* DNA polymerase (0.25 µl) and 10X PCR buffer (2 µl). The final volume was made up with sterile water.

The following conditions were provided for the amplification of the specific regions in the plasmid DNA in a Thermal Cycler (PTC-150 mini cycler).

| Step | Stage | Temperature(°C) | Duration |
|---|---|---|---|
| 1. | Initial denaturation | 94 | 5 min |
| 2. | Denaturation | 94 | 30 sec |
| 3. | Annealing | 53 | 20 sec |
| 4. | Extension | 72 | 1 min |
| 5. | Final extension | 72 | 7 min |

### 3.10.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit. Required amount of agarose (1.0%) was weighed out and melted in 1X TAE buffer (0.04 M Tris acetate, 0.001 M EDTA, pH 8.0) by boiling. After cooling to about 50°C, ethidium bromide ($10^{-3}$ mg $ml^{-1}$) was added. The mixture was then poured to a pre set template with appropriate comb. After the gel was set, the comb and the sealing tapes were removed and the gel was mounted in an electrophoresis tank and was flooded with 1X TAE buffer, to about 1mm above the gel. Required volume of DNA sample and gel-loading buffer (6 x loading dye *i.e.*, 40 per cent sucrose and 0.25 per cent bromophenol blue) were mixed. One of the wells was loaded with 5 µl of the 100 bp molecular weight marker with required volume of gel loading buffer. Electrophoresis was carried out at 50 volts until the loading dye reached three fourth length of the gel. The gel was documented using a gel documenting system (BIO-RAD).

### 3.10.6 Quantification of Asiaticoside

#### *3.10.6.1 Extraction of Asiaticoside*

The protocol for the extraction was a modification of that followed by Matsuda et al. (2001). Air dried callus (5 g) was powdered with the help of pestle

and mortar. The contents were extracted with equal volume of methanol. This step was repeated three more times with the left-over residue. The three methanol extracts were combined in a porcelain container. The solvent was evaporated in an oven under reduced pressure. The dried extract was subjected to partition in equal volume of 1:1 mixture of ethyl acetate and water. Water dissolved fraction was dried in an oven. The dried water fraction was extracted with 0.5 ml methyl alcohol.

### *3.10.6.2 Thin Layer chromatography (TLC)*

Ready to use TLC plates (silica gel 60) of size 20x20 cm (MERCK India Ltd.) was used for TLC. The developing solvent was used for equilibrating the TLC tank for one hour. The samples along with the standard (2 mg/ml) were spotted on the plate 1.5 cm apart. The spots were dried at 50°C for 15 minutes. The TLC plates were developed with the mobile phase (methanol: water 60: 40) at 19 ± 2°C. After development the TLC plate was dried at room temperature. Anisaldehyde was sprayed and the plate was heated at 100-105°C. The dried plate was visualized at daylight.

# Results

# 4. RESULTS

The results of the present study on "*Agrobacterium tumefaciens* mediated transfer of exogenous hydroxy methyl glutaryl CoA (HMG CoA) reductase gene to *Centella asiatica* L." carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during March 2006 to February 2008 are presented below.

## 4.1 ESTABLISHMENT OF *IN VITRO* CULTURE

### 4.1.1 Establishment of *Centella asiatica* Callus Cultures

#### *4.1.1.1 Effect of Growth Regulators on Callus Induction*

There was considerable variation in the percentage of callus induction and time taken for callus induction in the different treatments tried (Table 1). Among these treatments the highest percentage of callus induction (100% from nodal explant and 92.85% from leaf explant) was obtained in (C1), where MS medium was supplemented with NAA 2 mg $l^{-1}$ and Kn 4 mg $l^{-1}$ (Plate 1). Minimum callus induction percentage was from C3 with 26.08 from leaf explant and 76.24 from nodal explant where NAA 1 mg $l^{-1}$ and 2,4-D 0.8 mg $l^{-1}$ were supplemented to MS medium (Plate 2). Earlier callus induction (21 days) was observed in C2 (MS media supplemented with 2, 4-D 2 mg $l^{-1}$) when leaf was used as explant. Nodal explants produced more callus compared to leaf explants in all the treatments.

In the treatment C2, along with callus, multiple shoots (an average of 4 shoots per node) were also induced (Plate 3). Profuse rooting could be induced from leaf and nodal explant as well as from callus in C3 when NAA 1 mg $l^{-1}$ and 2, 4-D 0.8 mg $l^{-1}$ were supplemented (Plate 4 and 5).

**Table 1.** **Effect of growth regulators on *in vitro* callus induction in *Centella asiatica***

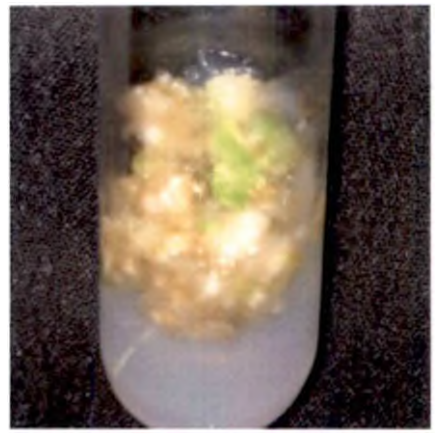
Medium: MS (full strength) + sucrose 30 g $l^{-1}$ + agar 5 g $l^{-1}$

| Treatment No. | Growth regulators (mg $l^{-1}$) | Time taken for callus induction (days) | | Callus induction (%) | |
|---|---|---|---|---|---|
| | | Leaf | Node | Leaf | Node |
| C1 | Kn 4.0 + NAA 2.0 | 25.00 | 23.00 | 92.85 | 100.00 |
| C2 | 2,4-D 2.0 | 21.00 | 24.00 | 66.07 | 80.35 |
| C3 | NAA 1.0 + 2,4-D 0.8 | 25.00 | 22.00 | 26.08 | 76.24 |

**Response of different explants of *Centella asiatica* in callus induction media**

**Plate 1: NAA 2 mg $l^{-1}$ and Kn 4 mg $l^{-1}$**

**Callus from node**



**Callus from leaf**

**Plate 2: NAA 1 mg $l^{-1}$ and 2, 4-D 0.8 mg $l^{-1}$**

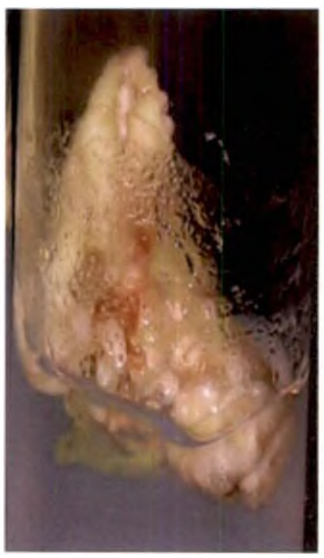
**Callus from node**

**Callus from leaf**

Plate 3: 2, 4-D 2 mg $l^{-1}$

**Multiple shoots and callus induced from node**

**Multiple shoots and callus from leaf**



**Callus induced from leaf**

**Plate 4: NAA 1 mg $l^{-1}$ and 2, 4-D 0.8 mg $l^{-1}$**

**Root and callus induction from leaf**

**Root and callus induction from node**

**Plate 5: NAA 1 mg $l^{-1}$ and 2, 4-D 0.8 mg $l^{-1}$**

**Root induction and callus multiplication on sub culturing**

*4.1.1.2 Nature of Callus*

The nature of the callus varied with the type of explants used. Light green friable callus was obtained from leaves and cream coloured friable callus from node. The callus obtained from C1 (NAA 2 mg $l^{-1}$ and Kn 4 mg $l^{-1}$) was cream coloured and friable whereas green hard callus was formed in C2 (2, 4-D 2 mg $l^{-1}$).

**4.1.2 Regeneration of *Centella asiatica* from Callus**

***4.1.2.1 Effect of Growth Regulators on in vitro Regeneration from Callus***

Of the different media compositions tried (Table 2), the highest regeneration was obtained on MS medium supplemented with NAA 2 mg $l^{-1}$ and Kn 4 mg $l^{-1}$ (Plate 6). Percentage regeneration was 0.052 and period taken for regeneration was 24 days. Regeneration (0.018 per cent) was obtained on MS medium supplemented with Kn 2 mg $l^{-1}$, BA 4 mg $l^{-1}$, NAA 0.25 mg $l^{-1}$ and ADS 20 mg $l^{-1}$ (R8) in 32 days (Plate 7). There was proliferation of callus in the other treatments but the callus remained green without any regeneration in most of the media tried showing the regeneration potential of the callus. In the treatments R16, R17, R22, R23, R24 and R25 the proliferation of callus was observed but it remained cream coloured without any regeneration. The treatments R36, R37 and R38 formed dark green hard callus and remained without any regeneration. In treatments R31, R32, R33, R34 and R35 callus multiplication could be observed but the callus remained pale green coloured and friable in nature.

***4.1.2.2 Effect of Proline on in vitro Regeneration from Callus***

Addition of proline 10 and 20 mg $l^{-1}$ along with other growth regulators did not have any effect on regeneration from callus (Table 2). The callus turned green in this medium.

**Table 2. Response of callus in regeneration medium**

Medium: MS (full strength) + sucrose 30 g $l^{-1}$ + agar 4.5 g $l^{-1}$

| Regeneration medium No. | Composition (mg $l^{-1}$) | Response of callus |
|---|---|---|
| R1 | BA 3.0 + Kn 2.0 | Callus turned green |
| R2 | BA 3.0 + Kn 1.0 | Callus turned green |
| R3 | BA 3.0 + Kn 0.5 | Callus turned green |
| R4 | BA 3.0 + Kn 0.5 + IAA 1.0 | Callus turned green |
| R5 | BA 2.0 + Kn 1.0 | Callus turned green |
| R6 | BA 3.0 + Kn 2.0 + NAA 0.25 + ADS 20.0 | Callus turned green |
| R7 | Half MS+ BA 4+ Kn 2 + NAA 0.25+ ADS 20.0 | Callus turned green |
| R8 | BA 4.0 + Kn 2.0 + NAA 0.25 + ADS 20.0 (Agar 5 g $l^{-1}$) | Shoot induction |
| R9 | BA 4.0 + Kn 1.0 +NAA 0.25 +ADS 20.0 (Agar 4 g $l^{-1}$) | Callus turned green |
| R10 | BA 4.0 + NAA 1.0 (Agar 5 g $l^{-1}$) | Callus turned green |
| R11 | BA 4.0 + NAA 1.0 (Agar 4 g $l^{-1}$) | Callus turned green |
| R12 | Half MS+ BA 2.0 + 2,4- D 0.5 | Callus turned green |
| R13 | BA 4.0 + Kn 3.0 | Callus turned green |
| R14 | R6 + Proline 40.0 | Callus turned green |
| R15 | R6 + Proline 20.0 | Callus turned green |
| R16 | R6 + CH 20.0 | Callus turned cream |
| R17 | (R6 + CH 20.0) - ADS | Callus turned cream |
| R18 | BA 8.0 + NAA 1.0 | Callus turned green |
| R19 | BA 6.0 + NAA 1.0 | Callus turned green |
| R20 | BA 4.0 + NAA 1.0 | Callus turned green |
| R21 | BA 2.0 + NAA 1.0 | Callus turned green |
| R22 | BA 8.0 + IAA 1.0 | Callus turned cream |
| R23 | BA 6.0 + IAA 1.0 | Callus turned cream |
| R24 | BA 4.0 + IAA 1.0 | Callus turned cream |
| R25 | BA 2.0 + IAA 1.0 | Callus turned cream |
| R26 | NAA 2.0 + Kn 4.0 | Shoot induction |
| R27 | NAA 1.0 + Kn 4.0 | Callus turned green |
| R28 | NAA 0.5 + Kn 4.0 | Callus turned green |
| R29 | NAA 0.25 + Kn 4.0 | Callus turned green |
| R30 | Kn 4.0 | Callus turned green |
| R31 | R26 + Proline 20.0 | Callus turned pale green |

| | | |
|---|---|---|
| R32 | R27 + Proline 20.0 | Callus turned pale green |
| R33 | R28 + Proline 20.0 | Callus turned pale green |
| R34 | R29 + Proline 20.0 | Callus turned pale green |
| R35 | R30 + Proline 20.0 | Callus turned pale green |
| R36 | BAP 3.0 + NAA 1.0 | Callus turned dark green |
| R37 | BAP 3.0 + NAA 0.5 | Callus turned dark green |
| R38 | BAP 3.0 + NAA 0.25 | Callus turned dark green |
| R39 | BA 1.0 + Kn 3.0 + NAA 0.25+ ADS 20.0 | Callus turned green |
| R40 | BA 2.0 + Kn 2.0 + NAA 0.25+ ADS 20.0 | Callus turned green |
| R41 | BA 5.0 + Kn 2.0 + NAA 0.25+ ADS 20.0 | Callus turned green |
| R42 | BA 6.0 + Kn 1.0 + NAA 0.25+ ADS 20.0 | Callus turned green |

**Regeneration of *Centella* callus on MS medium supplemented with various hormones**

**Plate 6: NAA 2 mg $l^{-1}$ and Kn 4 mg $l^{-1}$**

**Plate 7: Kn 2 mg $l^{-1}$, BA 4 mg $l^{-1}$, NAA 0.25 mg $l^{-1}$and ADS 20 mg $l^{-1}$.**

*4.1.2.3 Effect of Casein Hydrolysate on in vitro Regeneration from Callus*

Casein hydrolysate (20 mg $l^{-1}$) added along with other growth regulators did not have any effect on regeneration from callus (Table 2). Callus remained cream coloured, with no indication of regeneration.

4.2 SENSITIVITY OF *CENTELLA* CALLUS TO ANTIBIOTICS

The sensitivity of callus to different doses of antibiotics in MS medium was tested.

**4.2.1 Kanamycin**

The sensitivity of callus to different doses of kanamycin (5-350 mg $l^{-1}$) was recorded (Table 3). In kanamycin 5 mg $l^{-1}$, the tissues remained green up to six weeks, later they became partially discoloured and bleached after eight weeks. The percentage survival of callus in kanamycin 5 mg $l^{-1}$ after eight weeks was 33.33.

In kanamycin 25 and 50 mg $l^{-1}$, the tissues remained green up to four weeks. The percentage survival of callus in kanamycin 25 and 50 mg $l^{-1}$ after eight weeks was 22.22 and 19.44, respectively.

The callus remained green up to four weeks in kanamycin 75 mg $l^{-1}$ after inoculation. The tissues were partially discoloured and bleached after six weeks of inoculation and turned brown and dead after seven weeks. The percentage survival of callus after eight weeks was 13.88.

The tissues remained green up to three weeks in medium containing kanamycin 100 mg $l^{-1}$ and 125 mg $l^{-1}$. They were partially discoloured after four weeks and bleached after five weeks of inoculation. The tissues turned brown and

**Table 3. Sensitivity of *Centella* callus to different doses of kanamycin**

| Sl No. | Kanamycin (mg $l^{-1}$) | Sensitivity | | | | | | | | Survival (%) after 8 weeks |
|---|---|---|---|---|---|---|---|---|---|---|
| | | I week | II week | III week | IV week | V week | VI week | VII week | VIII week | |
| 1 | 0 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | 100 |
| 2 | 5 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | 33.33 |
| 3 | 25 | ++++ | ++++ | ++++ | ++++ | +++ | +++ | ++ | ++ | 22.22 |
| 4 | 50 | ++++ | ++++ | ++++ | ++++ | +++ | ++ | + | + | 19.44 |
| 5 | 75 | ++++ | ++++ | ++++ | ++++ | +++ | ++ | + | + | 13.88 |
| 6 | 100 | ++++ | ++++ | ++++ | +++ | ++ | + | + | + | 11.11 |
| 7 | 125 | ++++ | ++++ | ++++ | +++ | ++ | + | + | + | 2.70 |
| 8 | 150 | ++++ | ++++ | +++ | ++ | ++ | + | + | + | 0 |
| 9 | 200 | ++++ | ++++ | +++ | ++ | + | + | + | + | 0 |
| 10 | 250 | ++++ | ++++ | +++ | ++ | + | + | + | + | 0 |
| 11 | 300 | ++++ | ++++ | +++ | ++ | + | + | + | + | 0 |
| 12 | 350 | ++++ | +++ | ++ | + | + | + | + | + | 0 |

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

**Plate 8: Sensitivity of *Centella* callus to different doses of kanamycin**

**(A) Kanamycin 5 mg $l^{-1}$**

**(B) Kanamycin 50 mg $l^{-1}$**

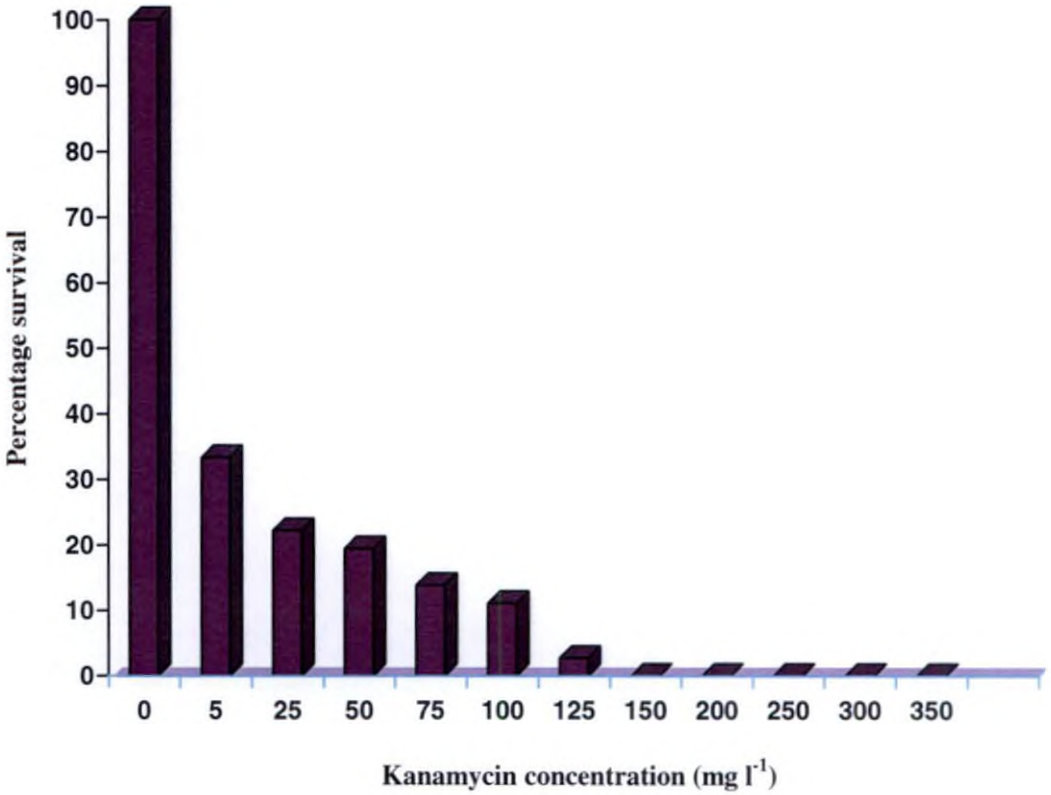
**(C) Kanamycin 100 mg $l^{-1}$**

**(D) Kanamycin 150 mg $l^{-1}$**

**(E) Kanamycin 250 mg $l^{-1}$**

**(F) Kanamycin 350 mg $l^{-1}$**

**Fig.1. Sensitivity of *Centella* callus to different doses of kanamycin**



dead after six weeks of inoculation and the percentage survival of callus was found to be 11.11 and 2.70, respectively after eight weeks.

In kanamycin 150, 200, 250 and 300 mg $l^{-1}$, the callus remained green only up to two weeks after inoculation, became partially discoloured after three weeks and turned brown and dead after five weeks. None of the callus survived after eight weeks in these concentrations. The tissues were green only up to one week after inoculation in kanamycin 350 mg $l^{-1}$. They became discoloured after two weeks and bleached after three weeks. After four weeks the tissues turned brown and dead. The percentage survival after eight weeks was zero.

The kanamycin became toxic to the tissues from concentrations 75, 100 and 125 mg $l^{-1}$ (Figure 1). For the selection of the transformants, kanamycin was used at concentrations of 200, 250 and 300 mg $l^{-1}$ (Plate 8).

### 4.2.2 Cefotaxime

Sensitivity of *Centella* callus to varying concentrations of cefotaxime (5 - 100 mg $l^{-1}$) was recorded (Table 4). In the medium containing cefotaxime 5 mg $l^{-1}$, the tissues remained green up to six weeks, were partially discoloured after seven weeks and became bleached after eight weeks. The percentage survival of callus after eight weeks was observed to be 73.33.

In cefotaxime 25 mg $l^{-1}$, the tissues remained green up to five weeks after inoculation. They were partially discoloured after six weeks and bleached after seven weeks. Only 44.44 per cent of the callus survived after eight weeks in this concentration.

**Table 4. Sensitivity of *Centella* callus to different doses of cefotaxime**

| Sl No. | Cefotaxime (mg $l^{-1}$) | Sensitivity | | | | | | | | Survival (%) after 8 weeks |
|---|---|---|---|---|---|---|---|---|---|---|
| | | I week | II week | III week | IV week | V week | VI week | VII week | VIII week | |
| 1 | Nil | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | 100 |
| 2 | 5 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | 73.33 |
| 3 | 25 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | ++ | 44.44 |
| 4 | 50 | ++++ | ++++ | ++++ | ++++ | +++ | ++ | ++ | + | 27.77 |
| 5 | 75 | ++++ | ++++ | ++++ | ++++ | +++ | ++ | ++ | + | 26.66 |
| 6 | 100 | ++++ | ++++ | ++++ | +++ | +++ | ++ | ++ | + | 20.00 |

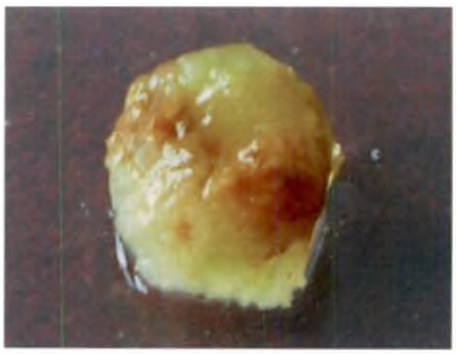
++++ Fully green
+++ Partially discoloured
++ Bleached tissues
+ Tissues turning brown and dead

**Plate 9: Sensitivity of *Centella* callus to different doses of cefotaxime.**

**(A) Cefotaxime 0 mg $l^{-1}$**

**(B) Cefotaxime 5 mg $l^{-1}$**

**(C) Cefotaxime 25 mg $l^{-1}$**

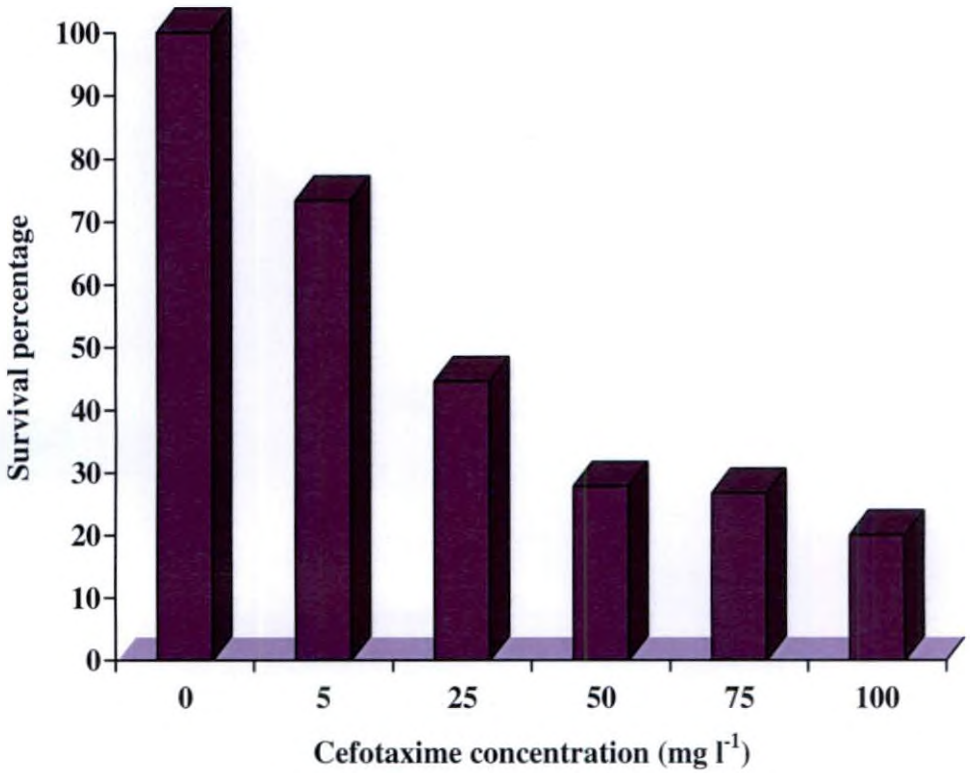


**(D) Cefotaxime 50 mg $l^{-1}$**

**(E) Cefotaxime 75 mg $l^{-1}$**

**(F) Cefotaxime 100 mg $l^{-1}$**

**Fig.2. Sensitivity of *Centella* callus to different concentrations of cefotaxime**



The tissues were green up to four weeks, became partially discoloured after five weeks and bleached after six weeks in cefotaxime 50 and 75 mg $l^{-1}$. The tissues turned brown and dead after eight weeks and the percentage survival of callus after eight weeks was 27.77 and 26.66, respectively.

The tissues were green up to three weeks, became partially discoloured after four weeks and bleached after six weeks in medium supplemented with cefotaxime 100 mg $l^{-1}$. The percentage survival of callus after eight weeks was 20.

Cefotaxime was found to be toxic tissues at 100 mg $l^{-1}$ (Figure 2). Hence, for the elimination of *Agrobacterium* after co-cultivation, cefotaxime was used at a concentration of 50 mg $l^{-1}$ (Plate 9).

## 4.3 SENSITIVITY OF *AGROBACTERIUM* STRAINS TO ANTIBIOTICS

The sensitivity of the bacterial strain EHA105 harbouring pBE2113 to different doses of antibiotics is presented below.

### 4.3.1 Kanamycin

The growth of *Agrobacterium* strain EHA105 containing the plasmid vector pBE2113 in LB medium containing different concentrations of kanamycin (5 - 500 mg $l^{-1}$) after two days of culture was recorded (Table 5). The bacterial cultures survived in the presence of kanamycin up to a concentration of 300 mg $l^{-1}$. No bacterial growth was observed in LB medium containing kanamycin 350, 400 and 500 mg $l^{-1}$.

**Table 5.** **Sensitivity of *Agrobacterium tumefaciens* strain EHA 105 (pBE2113) to kanamycin**

| Sl No. | Kanamycin (mg $l^{-1}$) | Bacterial growth |
|---|---|---|
| 1 | 0 | + |
| 2 | 5 | + |
| 3 | 25 | + |
| 4 | 50 | + |
| 5 | 75 | + |
| 6 | 100 | + |
| 7 | 150 | + |
| 8 | 200 | + |
| 9 | 300 | + |
| 10 | 350 | - |
| 11 | 400 | - |
| | 500 | - |

\+ Bacterial growth
\- No bacterial growth

**Table 6. Bacteriocidal activity of cefotaxime**

| Sl No. | Cefotaxime ($mg\ l^{-1}$) | Bacterial growth |
|---|---|---|
| 1 | 0 | + |
| 2 | 5 | + |
| 3 | 10 | + |
| 4 | 15 | - |
| 5 | 20 | - |
| 6 | 25 | - |
| 7 | 50 | - |
| 8 | 75 | - |
| 9 | 100 | - |

\+ Bacterial growth
\- No bacterial growth

### 4.3.2 Cefotaxime

The growth of *Agrobacterium* strain EHA105 containing the plasmid vector pBE2113 in LB medium containing different concentrations of cefotaxime (5 -100 mg $l^{-1}$) after two days of culture was recorded (Table 6). Bacterial growth was observed in LB medium containing cefotaxime 5 and 10 mg $l^{-1}$. No bacterial growth was observed in LB medium containing cefotaxime at concentrations 15 mg $l^{-1}$ and above.

## 4.4 GENETIC TRANSFORMATION IN *CENTELLA ASIATICA*

### 4.4.1 Identification of Transformants

#### *4.4.1.1 Survival of Tissues in Selection Medium*

The tissues after transformation were placed in selection medium and survival percentage was recorded after three weeks (Table 7). A survival percentage of 18.75 were obtained in selection medium containing kanamycin 200 mg $l^{-1}$. The survival per cent of callus was 19.35 in selection medium containing kanamycin 250 mg $l^{-1}$. 13.63 per cent of callus survived in selection medium containing 300 mg $l^{-1}$ (Plate 10).

#### *4.4.1.2 Polymerase Chain Reaction (PCR)*

The transgenes were confirmed by PCR using *nptII* gene specific primers. All samples (tissues selected on kanamycin 200, 250 and 300 mg $l^{-1}$) gave appreciable quantity of the product of size 700 bp. The PCR product and the positive control were of the same size (Plate 11).

**Table 7. Survival of tissues in selection medium after transformation with *Agrobacterium* EHA 105 (pBE2113)**

| Kanamycin concentration (mg $l^{-1}$) | No. of tissues remained green in selection medium | No. of tissues turned brown in selection medium | Survival of tissues in selection medium (%) |
|---|---|---|---|
| 200 | 6 | 26 | 18.75 |
| 250 | 6 | 25 | 19.35 |
| 300 | 3 | 19 | 13.63 |

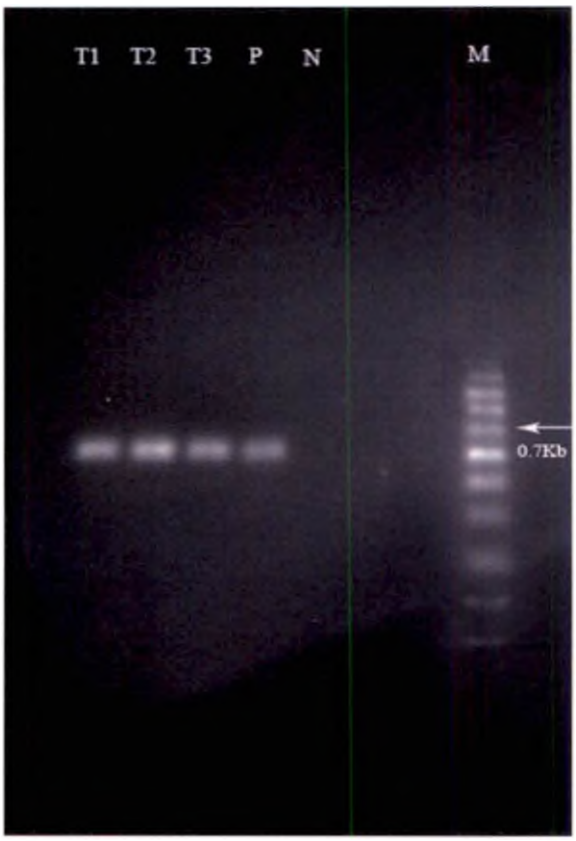
**Plate 10: Survival of tissues in selection media containing kanamycin**

a) 200 mg $l^{-1}$ (18.75%)

b) 250 mg $l^{-1}$ (19.35%)

c) 300 mg $l^{-1}$ (13.63%)

**Plate 11: Confirmation of transgenes by PCR using *nptII* gene specific primers**




Lane 1- T1- npt II gene amplified from transformed plant DNA

Lane 2- T2- npt II gene amplified from transformed plant DNA

Lane 3- T3- npt II gene amplified from transformed plant DNA

Lane 4- P- Positive control

Lane 5- N- Non-transformed control

Lane 6- M- 100bp DNA marker

## 4.5 ANALYSIS OF ASIATICOSIDE USING TLC

Asiaticoside content in the transformants were analysed using TLC. Asiaticoside was extracted from the callus and TLC was carried out using silica gel plate along with the standard. Two different developers were tried for developing the TLC plates, using methanol: water (60:40) v/v and by using ethyl acetate and methanol (60:40) v/v as the mobile phase. After development the plates were sprayed with anisaldehyde and heating upto 105°C. In the present study no spots corresponding to asiaticoside could be detected.

# *Discussion*

# 5. DISCUSSION

*Centella asiatica* has considerable reputation in the Indian system of medicine. It is used as a brain tonic, cardio-depressant and sedative. It is used internally in the treatment of wounds, chronic skin conditions including leprosy, venereal diseases, malaria, varicose veins, ulcers, nervous disorders and senility. *Centella* has been found to improve memory and strengthen the central nervous system. It is also used in rejuvenating nervous tissues and lowering blood pressure.

Currently there is an increasing demand for medicinal plants and its pharmacologically important constituents. Secondary metabolites in the plants are primarily responsible for its medicinal properties. In *Centella asiatica* there are metabolites having great pharmacological value. Among them asiaticoside is of wide demand, especially in drug preparation. Asiaticoside has anti-inflammatory, antitumour, neuroprotective, skin care and toning effects and is used clinically as a wound-healing agent (Pointel et al., 1987). Asiaticoside is used in the treatment of Alzheimer's disease, leprosy, lupus, eczema, psoriasis and ulcers of the duodenum, skin, cornea, tuberculosis and venous diseases (Giardiana et al., 1987).

For commercial extraction asiaticoside content should be at least three per cent, whereas Indian ecotypes contain only one per cent of this compound. The advent of pathway engineering allows accelerated modification and improvement in medicinal plants by altering or integrating genes responsible for key enzymes in the metabolic pathway. This technology has now turned up as a powerful tool for enhancing the productivity of secondary metabolites of limited yield. Metabolically engineered medicinal plants will complement conventional breeding efforts for the production of plants with quantitatively and qualitatively improved pharmacological properties. In the present study attempts have been made to transform *Centella*

*asiatica* with exogenous hydroxy methyl glutaryl CoA reductase (*hmgr*) gene. It is an important gene coding for the enzyme hydroxy methyl glutaryl CoA reductase (HMGR) which acts at the upstream of the mevalonate pathway producing mevalonic acid, further downstream produce IPP, squalene, β-amyrin and finally asiaticoside. 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGR) catalyzes the irreversible conversion of 3-hydroxy-3-methyl glutaryl coenzyme A to mevalonate and is considered a key regulatory step controlling isoprenoid metabolism (Chappell et al., 1995). *Arabidopsis thaliana HMG1* cDNA, coding the catalytic domain of 3-hydroxy-3-methyl glutaryl CoA reductase (HMGR1S), was expressed in the cardenolide-producing plant *Digitalis minor* (the main industrial source of cardiac glycosides, steroidal metabolites derived from mevalonic acid via the triterpenoid pathway) by pathway engineering. Constitutive expression of *HMG1* resulted in an increased sterol and cardenolide production in both *in vitro* and greenhouse-grown plants (Sales et al., 2007).

One of the important requirements for a successful genetic transformation is the selection of an efficient gene delivery system. Among the different gene delivery systems available, *Agrobacterium tumefaciens* mediated method is widely used to introduce foreign genes into dicots (Weising et al., 1988). High frequency of transformation, broad host range, high rate of expression and stable integration of inserted genes have made *Agrobacterium* based gene transfer system the most popular one. Genetic transformation has been reported in various medicinal plants like *Echinacea purpurea, Digitalis lanata, Artemisia annua,* and *Bacopa monniera.* Till date no reports have been published on *Agrobacterium tumefaciens* mediated metabolic pathway engineering in *Centella asiatica.*

A preliminary study was conducted for developing protocol for *Agrobacterium* mediated genetic transformation in *Centella asiatica* in the

Department of Plant Biotechnology, College of Agriculture, Vellayani (Krishnan, 2006) and *gus* A gene was successfully transferred to callus tissues. The present study was conducted in the view of the above facts with an objective to enhance the production of asiaticoside in *Centella asiatica* L. by introducing exogenous *hmgr* gene, using *Agrobacterium tumefaciens.*

Callus tissues were used for the transformation experiments. Node and leaf explants were used for initiating callus on MS medium containing different combinations of growth regulators. Maximum callus induction from leaf (92.85%) and nodal explants (100%) was recorded in medium containing Kn 4 mg $l^{-1}$ and NAA 2 mg $l^{-1}$. Patra et al. (1998) reported the *in vitro* induction of callus from leaf explants of *Centella* in MS medium supplemented with 2 mg $l^{-1}$ Kn and 4 mg $l^{-1}$ NAA. Rao et al. (1999) observed the influence of auxin and cytokinins on the production of callus in *Centella asiatica.* According to them kinetin supplementation (0.25 and 0.5 mg $l^{-1}$) along with auxin (2, 4-D 2 mg $l^{-1}$ ) proved to be beneficial for the growth of callus and the best combination of growth regulators for maximum callus induction reported was 2 mg $l^{-1}$ NAA + 0.5 mg $l^{-1}$ Kn.

Regeneration of the transformed tissues is a basic requirement for the multiplication and establishment of the genetically modified plants. Regeneration was attempted with different combinations of plant growth regulators in MS medium. The regeneration event was sporadic. Of the different media compositions tried, maximum regeneration was obtained on MS medium supplemented with NAA 2 mg $l^{-1}$ and Kn 4 mg $l^{-1}$. Percentage regeneration was 0.052 and period taken for regeneration was 24 days. Regeneration (0.018 per cent) was obtained on MS medium supplemented with Kn 2 mg $l^{-1}$, BA 4 mg $l^{-1}$, NAA 0.25 mg $l^{-1}$ and ADS 20 mg $l^{-1}$ (R8) in 32 days. Patra et al. (1998) have also reported successful regeneration of callus derived from stem and leaf explants of *Centella asiatica* on the same

medium; reproducibility of the result was very less. The other treatments did not result in regeneration from the callus, however, calli turned green showing the regeneration potential. The *in vitro* callus induction and regeneration are regulated by the interaction and balance between the growth substances supplied in the medium and produced endogenously by cultured cells (Sharma and Shalini. 2007).

Plant cells are sensitive to certain antibiotics and they affect regenerative capacity of the explant. Since the genes encoding resistance to antibiotics are used as selectable marker, the sensitivity of tissues to different doses of antibiotics needs to be evaluated. The most effective selection agents are those, which either inhibit growth or slowly kill the untransformed tissues. In the present study, the plasmid vector pBE2113 was used. The T-DNA of this binary vector contains *nptII* (kanamycin resistance) gene as the selectable marker (Hajadukiewicz et al., 1994). This necessitated the evaluation of sensitivity of plant tissue to kanamycin and to determine the optimum concentration of kanamycin that is required to select transformed tissues without affecting their regenerative ability.

Bleaching, discolouration and death of callus were observed beyond 100 mg $l^{-1}$ of kanamycin from the fourth week of treatment. The survival percentage of callus in kanamycin 100 mg $l^{-1}$ after eight weeks was 11.11 and hence higher doses above this (200, 250 and 300 mg $l^{-1}$) were selected for screening the transformants. Kanamycin is a widely used marker for plant transformation and has been used by several workers. Dronne et al. (1999) reported the use of kanamycin at strength of 150 mg $l^{-1}$ for selection of lavandin transformants. Yun et al. (1992) obtained transformants in *Atropa belladona* by selection in media containing kanamycin 500 $\mu g\ ml^{-1}$. These reports indicate that there is variation in the sensitivity to antibiotics depending on the genotype, physiological condition, size and type of explant and the tissue culture conditions (Yang et al., 1999).

After co-cultivation, elimination of *Agrobacterium* from the plant tissues is very important, as the survival of bacteria in plant tissue may affect the growth and regeneration of transformed tissues. Elimination of bacteria from the explant is done by the use of antibiotics. The antibiotic should be such that it kills the bacteria without affecting the growth and organogenesis of the explants. Commonly used bacteriostatic agent, cefotaxime was used for the elimination of *Agrobacterium* in the present study. At a concentration of 50 mg $l^{-1}$ cefotaxime, complete elimination of the bacteria was effected without affecting the growth of the callus. Cefotaxime has been used successfully for the elimination of *Agrobacterium* in transformation in a number of crops. Vergauwe et al. (1996) working on transformation of *Artemisia annua* found that cefotaxime at 50 mg $l^{-1}$ was effective as a decontaminating antibiotic. It was successfully used at a strength 200 mg $l^{-1}$ in eliminating *Agrobacterium* from the inoculated leaf explant during transformation in *Datura* (Curtis et al., 1999). In *Atropa belladona*, cefotaxime 500 mg $l^{-1}$ was used (Negoianu et al., 2002).

In the present study *Agrobacterium tumefaciens* strain EHA105 was used. The vector, pBE2113 contains *hmgr* (encoding HMGR enzyme) and kanamycin resistance gene (*npt II*) under 35S CaMV promoter.

The genetic transformation was done using callus of *Centella.* The calli were pre-cultured on MS medium containing 2 mg $l^{-1}$ Kn and 4 mg $l^{-1}$ NAA for 15 days before co-cultivation to maintain cells in active cell division stage. The greater the number of actively dividing cells in the explant to be infected with *Agrobacterium*, the higher was the probability of obtaining stable expression of the transformed gene. Park and Facchini (2000) obtained transformants in California poppy (*Eschscholzia california)* by co-cultivation of excised cotyledons with *A. tumefaciens*.

For transforming callus of *Centella asiatica*, an infection time of 20 minutes was given in half strength MS broth in which the bacterial pellet was suspended. In *Echinacea purpurea,* 30 minutes (Lichtenstein and Draper, 1986) and in brahmi 15 minutes (Nisha et al., 2003) of infection time resulted in transformation. Callus tissues were wounded with sterile disposable syringe to facilitate efficient infection of explants with *Agrobacterium tumefaciens*. Wounded cells release polyphenolic compounds like acetosyringone, which activate the *Agrobacterium vir* genes (Zambryski, 1992). When the *vir* genes were activated they facilitate the transfer of T-DNA to the plant cell. There are reports on the positive influence of acetosyringone on transformation efficiency. Voisey et al. (1994) found that acetosyringone 100 µM when added to co-cultivation medium helped in stabilizing the rate of transformation, which varied considerably in its absence. Confalonieri et al. (1995) reported the use of 200 µM acetosyringone to enhance the frequency of leaf disc transformation. The quantity of acetosyringone released by the wounded cultured cells may not be sufficient to activate the *Agrobacterium vir* genes. Krishnan (2006) observed that the transformation efficiency in *Centella* could be increased by adding acetosyringone (100 µM) to infection and co-cultivation medium. Hence acetosyringone (100 µM) was added to the infection and co-cultivation medium to increase the transformation efficiency in the present study.

Co-cultivation was carried out for four days in dark. When co-cultivated for more than four days, overgrowth of bacteria was observed. Nebauer et al. (2000) obtained optimal transformation rates in *Lavandula latifolia* when leaf explants pre-cultured for one day on regeneration medium were sub cultured on selection medium after a 24 h co-cultivation with *Agrobacterium.* Koroch et al. (2002) reported *Agrobacterium tumefaciens* mediated genetic transformation of *Echinacea purpurea* by co-cultivation of leaf sections using the strain EHA105, containing the binary vector pBISNI for 48 hours. In *Bacopa monniera,* Nisha et al. (2003) reported that

the incubation of leaf segments in bacterial suspension EHA105 (pBE2113) for 15 minutes and then co-cultivation for 48 hours, resulted in efficient transformation.

After co-cultivation, elimination of bacteria from the transformed *Centella* tissues was done by the use of cefotaxime 50 mg $l^{-1}$. Vergauwe et al. (1996) working on transformation of *Artemisia annua* found that cefotaxime at 50 mg $l^{-1}$ was effective as a decontaminating antibiotic. Negoianu et al. (2002) performed gene transfer in *Atropa belladonna*. After two days of co-culture, explants were first transferred to a fresh medium supplemented with cefotaxime 500 mg $l^{-1}$ for bacterial selection.

The vector, pBE2113 contains the kanamycin resistance gene as selectable marker. Hence for selection of transformants the cultures were transferred to selection medium containing kanamycin 200, 250 and 300 mg $l^{-1}$. After two weeks, the non transformed cells turned brown, while the transformants remained green. Selection of transformed tissues was carried out with three different concentrations of kanamycin for ensuring the elimination of untransformed cells. Screening of transformed cells was done by several workers by using various antibiotics according to the antibiotic resistant genes in the vector. Yun et al. (1992) obtained transformants in *Atropa belladonna* by selection in medium containing kanamycin 500 mg $l^{-1}$. Kanamycin 20 mg $l^{-1}$ was used to select transformed tissue of *Artemisia annua* (Vergauwe et al., 1996). Nisha et al. (2003) observed that the selection medium comprised of shoot regeneration medium supplemented with 15 mg $l^{-1}$ kanamycin in brahmi. Sugimura et al. (2005) obtained putative transformants in patchouli by selection on medium containing 100 mg $l^{-1}$ kanamycin. For selection of transformed tissues in *Echinacea purpurea*, the leaf sections after co-cultivation were transferred to shoot induction medium containing kanamycin 50 mg $l^{-1}$ (Koroch et al., 2002).

Confirmation of transformation was done by PCR analysis using *nptII* gene specific primers. All the three samples gave appreciable quantity of the product at 700 bp. The PCR product and the positive control were of the same size. Dronne et al. (1999) in lavandin confirmed the presence of the introduced β-glucuronidase and neomycin phosphotransferase II genes by PCR. Cucu et al. (2002) confirmed the presence of the marker *nptII* gene in the entire plant using PCR. In *Echinacea*, Koroch et al. (2002) detected *nptII* sequences in kanamycin-resistant plantlets by PCR analysis using the primer sequences to amplify a 320 bp region of the *nptII* gene. Sales et al. (2003) in *Digitalis minor* obtained evidence of stable transgene integration by PCR, growth on media selective for *nptII* or *bar* genes, and expression of the *gus A* gene. Nisha et al. (2003) studied the presence of *gus* and *nptII* genes in the genomes of the transformants in brahmi by PCR amplification using specific primers for *nptII* and *gus* genes.

Asiaticoside content in the transformants was analysed using TLC. Nath et al. (2004) showed the presence of asiaticoside in the *in vitro* grown leaves, callus and cell cultures of *Centella asiatica* by TLC. The content of the triterpenoid, asiaticoside in different organs of glasshouse-grown plants and cultured material, including transformed roots, of two phenotypes of *Centella asiatica* was determined by Aziz et al. (2004). Terpenoid content was the highest in leaves, with asiaticoside content 0.79 ± 0.03 and 1.15 ± 0.10 per cent of dry mass. Asiaticoside was also undetectable in undifferentiated callus and roots induced by *Agrobacterium rhizogens*. In the present study, eventhough regeneration was obtained from non transformed tissues (treatments R8 and R26 in 32 and 24, days respectively), the transformed tissues failed to regenerate even after 34 days of inoculation. Hence an attempt was made to analyse asiaticoside content in the callus using TLC. But ther was no detectable quantity of asiaticoside inthe callus. Bonfill et al. (2006)

identified four principal triterpenoid components of *Centella asiatica* by TLC on silica gel plates and mass spectrometry by using ethyl acetate and methanol as the mobile phase. The spots were detected with anisaldehyde solution. Absence of detectable spot in the present study may be due to the absence of asiaticoside in the undifferentiated callus. Asiaticoside is accumulated preferably in leaf tissues. Hence a better protocol for regeneration should be standardized so that further studies on triterpenoid analysis could be carried out.

Indian ecotypes of *Centella asiatica* can be made a reliable source of asiaticoside for the pharmaceutical industries if the metabolite content could be increased. Metabolic pathway engineering has a wide scope in the improvement of medicinal plants because this will be having no resistance from the people as it is a non-food crop, which is neither directly nor indirectly forming part of the diet. In the present study *hmgr* gene was successfully integrated to *Centella* calli. Development of protocols for better regeneration is necessary for further studies in improving *Centella* with respect to its metabolite content.

# *Summary*

## SUMMARY

A study on "*Agrobacterium tumefaciens* mediated transfer of exogenous hydroxy methyl glutaryl CoA (HMG CoA) reductase gene to *Centella asiatica* L." was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2006-2008.

Metabolic engineering is becoming a popular approach for the modification of medicinal plants for altering the metabolite contents. Many crops including medicinal plants with quantitatively and qualitatively improved secondary metabolite contents have been produced by metabolic pathway engineering. *Centella asiatica* contains a blend of compounds including triterpenes such as asiaticoside, asiatic acid and madecassic acid which are found to be responsible for its medicinal properties. Among them, asiaticoside is of high pharmacological value, as it possesses anti-inflammatory, antitumour, neuroprotective, skin care and toning effects. It is used as an ingredient in drugs, detergents, sweeteners and cosmetics. The asiaticoside content in the Indian ecotypes is comparatively very less and hence it is being imported by the industries for various purposes. The present study was undertaken with an objective to enhance the asiaticoside content by introducing *hmgr* gene to *Centella asiatica* using *Agrobacterium tumefaciens*. This gene is responsible for coding hydroxy methyl glutaryl CoA reductase (HMGR) enzyme that acts at the upstream of the metabolic pathway and produce mevalonic acid.

Since regeneration of the transformed tissues is a basic requirement in genetic transformation studies, an attempt was made to develop a reliable protocol for the callus regeneration in *Centella asiatica*. Among the various treatments tried, MS medium supplemented with Kn 4 mg $l^{-1}$ and NAA 2 mg $l^{-1}$ was proved to be the best for callus induction. This medium yielded 100 per cent callus induction from node

explants in 23 days and 92.85 % from leaf explant in 25 days. Regeneration was less in the various media tried, with the highest regeneration (0.052%) on MS medium supplemented with Kn 4mg $l^{-1}$ and NAA 2 mg $l^{-1}$. MS medium supplemented with Kn 2 mg $l^{-1}$, BA 4 mg $l^{-1}$, NAA 0.25 mg $l^{-1}$ and ADS 20 mg $l^{-1}$ produced 0.018 per cent regeneration.

*Agrobacterium tumefaciens* strain EHA105 containing plasmid pBE2113 harbouring *hmgr* and *nptII* gene was used for the study. Experiments were conducted to evaluate the sensitivity of *Agrobacterium* strains and *Centella* callus to different concentrations of kanamycin. The lethal dose of kanamycin to *Agrobacterium* and *Centella* callus was found to be 350 and 125 mg $l^{-1}$, respectively. For the selection of transformed cells, kanamycin 200, 250 and 300 mg $l^{-1}$ were used.

Cefotaxime was used for the elimination of *Agrobacterium* after co-cultivation. The experiments conducted to evaluate the sensitivity of *Agrobacterium* and *Centella* callus to different doses of cefotaxime indicated that the *Agrobacterium* was effectively killed by cefotaxime 50 mg $l^{-1}$ and up to a concentration of 100 mg $l^{-1}$ it was not toxic to *Centella* callus.

Genetic transformation was achieved by co-cultivating the callus with bacterial suspension ($OD_{600}$ of 0.1) containing 100 µM acetosyringone. An infection time of 20 min and co-cultivation period of four days were given. The transformed tissues were selected on MS medium containing 200, 250 and 300 mg $l^{-1}$ of kanamycin. The survival of the tissues on these media after three weeks was 18.75, 19.35 and 13.63 percent, respectively.

The survived tissues were transferred to regeneration medium with selection pressure. Transformation was confirmed by PCR analysis with *npt II* gene specific

primer. All the samples (tissues selected on 200, 250 and 300 mg $l^{-1}$ of kanamycin) yielded appreciable quantity of the product of size 700 bp that was comparable to the positive control.

Asiaticoside content in the transformants were analysed using thin layer chromatography (TLC). In the present study regeneration of the transformed tissues was not obtained in the media standardized. Therefore only callus tissues were analysed for asiaticoside content using TLC. However no spots corresponding to asiaticoside could be detected.

The attempt made to transfer an exogenous *hmgr* gene to *Centella asiatica* was successful. Since asiaticoside accumulates in the leaf tissues, for further analysis a better protocol needs to be developed for the regeneration of transformed tissues.

# Reference

## REFERENCES


Alsheikh, M., Suso, H. P., Robson, M., Battey, N. and Wetten, A. 2002. Appropriate choice of antibiotic and *Agrobacterium* strain improves transformation of antibiotic sensitive *Fragaria vesca* and *Fragaria vesca* Semperflorens. Pl. Cell Rep. 20 (12): 1173-1180

An, G., Watson, B. D. and Chiang, C. C. 1986. Transformation of tobacco, tomato, potato and *Arabidopsis thaliana* using Ti vector system. Pl. Physiol. 81: 301-305

Appa Rao, M. V. R. 1973. The effect of Mandookaparni (*Centella asiatica*) on the general mental ability (medhya) of mentally retarded children. J. Indian Med. 7: 9-12

Argolo, A. C., Charlwood, B. V. and Pletsch, M. 2000. The regulation to solasodine production by *Agrobacterium rhizogenes* transformed roots of *Solanum aviculare*. Pl. Medica. 66: 448-451

Aziz, Z. A., Davey, M. R., Power, J. B., Anthony, P., Smith, R. M. and Lowe, K. C. 2007. Production of asiaticoside and madecassoside in *Centella asiatica in vitro* and *in vivo*. Biologia Plantarum. 51(1): 34-42

Babu, T. D., Kuttan, G. and Padikkala, J. 1995. Cytotoxic and anti-tumour properties of certain taxa of Umbelliferae with special reference to *Centella asiatica*. (L.) Urban. J. Ethnopharmacol. 48(1): 53-57

Bae, T. W., Park, H. R., Kwak, Y. S., Lee, H. Y. and Ryu, S. B. 2005. *Agrobacterium tumefaciens*-mediated transformation of a medicinal plant *Taraxacum platycarpum*. Pl. Cell Tiss. Org. Cult. 80 (1): 51-57

Baek, M., Rho, Y. S. and Kim, D. H. 1999. Column-switching high performance liquid chromatographic assay for determination of asiaticoside in rat plasma and bile

with ultraviolet absorbance detection. J. Chromatogr. B. Biomed. Sci. Appl. 732(2): 357-363

Bais, H. P., Walker, T. S., McGrew, J. J. and Vivanco, J. M. 2002. Factors affecting growth of cell suspension cultures of *Hypericum perforatum* L. (St. John's wart) and production of hypericin. *In vitro* Cell Dev. Biol. Pl. 28(1): 58-65

Banerjee, S. and Zehra, M. 1999. *In vitro* multiplication of *Centella asiatica*, a medicinal herb from leaf explants. Curr. Sci. 76(2): 147–148

Bechold, N., Jaudeau, B., Jolivet, S., Maba, B., Vezon, D., Voisin, R. and Pelletier, G. 2000. The maternal chromosome set is the target of T-DNA in planta transformation of *Arabidopsis thaliana*. Genetics. 155:1875- 1887.

Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. Nucl. Acids Res. 12(22): 8711-8720

Binns, A. N. and Thomashow, M. F. 1988. Cell biology of *Agrobacterium* infection and transformation of plants. Ann. Rev. Microbiol. 42:575-606

Boiteau, P. and Ratsimamanga, A. R. 1956. Asiaticoside extracted from *Centella asiatica*, its therapeutic uses in the healing of experimental or refractory wounds, leprosy, skin tuberculosis and lupus. Therapie. 11:125–149

Boiteau, P. and Ratsimamanga, A. R. 1959. Important cicatrizants of vegetable origin and biostimulins of Filatov. Bulletin Soc. Sci. Bretagne. 34:307-315

Bonfill, M., Mangas, S., Cusido, R. M., Osuna, L., Pinol, M. T. and Palazon, J. 2006. Identification of triterpenoid compounds of *Centella asiatica* by thin-layer chromatography and mass spectrometry. Biomed Chromatogr. 20(2): 151-153

Bonte, F., Dumas, M., Chaudagne, C. and Meybeck, A. 1994. Influence of asiatic acid, madecassic acid, and asiaticoside on human collagen I synthesis. Planta Med. 60(2): 133-135

Cai, G., Li, G., Ye, H. and Li, G. 1995. Hairy root culture of *Artemisia annua* L. by Ri plasmid transformation and biosynthesis of artemisinin. Chin. J. Biotechnol. 11: 227-235

Cardoso, L. M. I., Meijer, A. H. and Hoge, J. H. C. 1997. *Agrobacterium*-mediated transformation of the terpenoid indole alkaloid-producing plant species *Tabernaemontana pandacaqui*. Pl. Cell rep. 17(2): 150-154

Chakrabarty, R., Viswakarma, N., Bhat, S. R., Kirti, P. B., Singh, B. D. and Chopra, V. L. 2002. *Agrobacterium*-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. J. Biosci. 27(5): 495-502

Chappell, J., Wolf, F., Proulx, J., Cuellar, R. and Saunders, C. 1995. Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase a rate-limiting step for isoprenoid biosynthesis in plants. Pl. Physiol. 109(4): 1337-1343

Charlwood, B.V. and Pletsch, M. 2002. Manipulation of natural product accumulation in plants through genetic engineering. J. Herbs Spices Med. Pl. 9:139-151

Chatterjee, T. K., Chakraborty, A., Pathak, M. and Sengupta, G. C. 1992. Effects of plant extract *Centella asiatica* on cold restraint ulcer in rats. Indian J. Exp. Biol. 30 (10): 889-891

Chaudhari, S. 1978. Use of common Indian Herb Mandukparni in the treatment of Leprosy (preliminary report). J. Indian Med. Ass. 70: 177-180

Chauhan, S. K., Singh, B. and Agarwal, S. 2003. A HPLC Determination of asiaticoside in *Centella asiatica*. Indian. J. Natural Products. 19(4): 21-23

Chen, D. H. 2000. Expression of a chimeric farnesyl diphosphate synthase gene in *Artemisia annua* L. transgenic plants via *Agrobacterium tumefaciens* mediated transformation. Pl. Sci. 155: 179-185

Chen, W. P. and Punja, Z. K. 2002. *Agrobacterium*-mediated transformation of American ginseng with a rice chitinase gene. Pl. Cell Rep. 20: 1039–1045

Cheng, M., Fry, J. E., Pang, S., Zhou, I., Hironaka, C., Duncan, D. R. I., Conner, T. W. L. and Wang, Y. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*, Pl. Physiol. 115: 971-980

Cheng, C. L. and Koo, M. W. 2000. Effects of *Centella asiatica* on ethanol induced gastric mucosal lesions in rats. Life Sci. 67(21): 2647-2653

Cheng, C. L., Guo, J. S., Luk, J. and Koo, M. W. 2004. The healing effects of *Centella* extract and asiaticoside on acetic acid induced gastric ulcers in rats. Life Sci. 74(18): 2237-2249

Chia, T. F., Chan, Y. S. and Chua, N. H. 1994. The firefly luciferase gene as a noninvasive reporter for *Dendrobium* transformation. Pl. J. 6: 441– 446

Chilton, M. D., Tepfer, D., Petit, A., David, C. and Casse- Delbart, F. T. 1982. *Agrobacterium rhizogenes* inserts T-DNA into the genome of the host plant root cells. Nature. 295: 432-434

Choi, Y. E., Jeong, J., In, J. and Yang, D. 2003. Production of herbicide resistant trans genic *Panax ginseng* through the introduction of the phosphinothricin acetyl transferase gene and successful soil transfer. Pl. Cell Rep. 2: 563-568

Coldren, C. D., Hashim, P., Ali, J. M., Oh, S. K., Sinskey, A. J. and Rha, C. 2003. Gene expression changes in the human fibroblast induced by *Centella asiatica* triterpenoids. Planta Med. 69(8): 725-732

Confalonieri, M., Balestrazzi, A., Bisoffi, S. and Cella, R. 1995. Factors affecting *Agrobacterium tumefaciens* mediated transformation in several black poplar clones. Pl . Cell Tiss. Org. Cult. 43(3): 215-222

Crecelius, A., Clench, M. R., Richards, D. S. and Parr, V. 2002. Thin-layer chromatography-matrix-assisted laser desorption ionization-time-of-flight mass spectrometry using particle suspension matrices. J. Chromatogr. A. 958(1-2): 249-260

Cucu, N., Gabriela, and Gavrila, L. 2002. Genetically modified medicinal plants II. Transfer and expression of a marker kanamycin resistance gene in *Atropa belladona* plants. Rom. Biotechnol. Lett. 7: 869–874

Curtis, I. S., Power, J. B., Hedden, P., Ward, D. A., Phillips, A., Lowe, K. C. and Davey, M. R. 1999. A stable transformation system for the ornamental plant *Datura metaloides.* Pl. Cell Rep. 18: 554-560

DeBlock, M., De Brouwer, D. and Tenning, P. 1989. Transformation of *Brassica napus* and *Brassica Oleracea using Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Pl. Physiol. 91: 694–701

Desfeux, C., Clough, S. J. and Bent, A. F. 2000. Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the *Arabidopsis* floral-dip method. Pl. Physiol. 123: 8599-9041.

Dronne, S., Moja, S., Jullien, F., Berger, F. and Caissard, J. C. 1999. *Agrobacterium* mediated transformation of lavandin (Lavandula x intermedia Emeric ex Loiseleur). Transgenic Res. 8(5): 335–347

Enfissi, E. M. A., Fraser, P. D., Lois, L. M., Boronat, A., Schuch, W. and Bramley, P. M. 2005. Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. J. Pl. Biotechnol. 3(1): 17-27

Enríquez-Obregón G. A., Vázquez-Padrón R. I., Prieto-Samsónov D. L., Pérez M. and Selman-Housein G. 1997. Genetic transformation of sugarcane by *Agrobacterium tumefaciens* using antioxidants compounds. Biotecnología Aplicada. 14:169-174.

Feeney, M. and Punja, Z. K. 2003. Tissue culture and *Agrobacterium* mediated transformation of Hemp (*Cannabis sativa*.L.). *In vitro* Cell Dev. Biol. 39(6): 578–585

Fraley, R. T., Rogers, S. C., Horsch, R. B., Sanders, P. R., Flick, J. S., Fink, C., Hoffman, N. and Sanders, P. 1983. Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. 80: 4803 – 4807

Geng, S. 2001. Effects of *ipt* gene expression on the physiological and chemical characteristics of *Artemisia aunna* L. Pl. Sci. 160: 691-698

Giardina, A., Castelli, G. P. and Ali, A. 1987. Use of *Centella asiatica* in the topical treatment of tropic ulcers of the lower limbs. Chronol. Dermatol. 18: 109–112.

Grimaldi, R., De Ponti, F., D'Angelo, L., Caravaggi, M., Guidi, G., Lecchini, S., Frigo, G. M. and Crema, A. 1990. Pharmacokineticsof the total triterpenic fraction of *Centella asiatica* after single and multiple administrations to healthy volunteers. A new assay for Asiatic acid. J. Ethnopharmacol. 28(2): 235-241

Guo, J. S., Cheng, C. L. and Koo, M. W. 2004. Inhibitory effects of *Centella asiatica* water extract and asiaticoside on inducible nitric oxide synthase during gastric ulcer healing in rats. Planta Med. 70(12): 1150-1154

Gupta, Y. K., Veerendra Kumar, M. H. and Srivastava, A. K. 2003. Effect of *Centella asiatica* on pentylenetetrazole-induced kindling, cognition and oxidative stress in rats. Pharmacol. Biochem. Behav. 74(3): 579-585.

Hahn, E. J., Kim, Y. S., Yu, K. W., Jeong, C. S. and Paek, K. Y. 2003. Adventitious root cultures of *Panax ginseng* cv Meyer and ginsedoside production through large-scale bioreactor system. J. Pl. Biotechnol. 5: 1-6.

Herbert, D., Paramasivam, C. N., Prabhakar, G. and Swaminathan, G. 1994. *In vitro* experiments with *Centella asiatica*: investigation to elucidate the effect of an indigenously prepared powder of this plant on the acid-fastness and viability of *Mycobacterium tuberculosis*. Indian J. Lepr. 66(1): 65-68

Herrera-Estrella, L. 1983. Transfer and expression of foreign genes in plants. PhD thesis. Laboratory of Genetics, Gent University, Belgium

Hosokawa, K., Matsuki, R., Oikawa, Y. and Yamamura, S. 2000. Production of transgenic gentian plants by particle bombardment of suspension culture cells. Pl. Cell Rep. 19(5): 454–458

Hossain, M. A., Hossain, M. Y., Ali, M. R. and Rahman, S. 2005. Effect of different carbon sources on *in vitro* regeneration of Pennywort. ( *Centella asiatica*). Pakist. J. Biol. Sci. 8(7): 963-968

Hossain, S. N., Rahman, S., Joydhar, A., Islam, S. and Hossain, M. 2000. *In vitro* propagation of Thankuni (*Centella asiatica* L.). Pl. Tiss. Cult. 10(1): 17-23

Hu, Z. and Du, M. 2006. Hairy roots and its application in plant genetic engineering. J. Integrative Pl. Biol. 48(2): 121-127

Huang, Y. H., Zhang, S. H., Zhen, R. X., Xu, X. D. and Zhen, Y. S. 2004. Asiaticoside inducing apoptosis of tumor cells and enhancing anti-tumor activity of vincristine. Ai Zheng. 23(12): 1599-1604

Incandela, L., Cesarone, M. R., Cacchio, M., De Sanctis, M. T., Santavenere, C., D'Auro, M. G., Bucci, M. and Belcaro, G. 2001. Total triterpenic fraction of *Centella asiatica* in chronic venous insufficiency and in high-perfusion microangiopathy. Angiology. 52(2): 09-13

Jumpatong, K., Phutdhawong, W. and Buddhasukh, D. 2006. Dechlorophyllation by electrocoagulation. Molecules. 11: 156-162

Kartnig, T. 1988. Clinical applications of *Centella asiatica* (L.) Urb. J. Herbs Spices Med. pl. 3: 146-173.

Kim, O. T., Lim, M. Y., Hong, M. H., Alm, J. C. and Hwang, B. 2004. Stimulation of asiaticoside accumulation in the whole plant cultures of *Centella asiatica* (L.) Urban. by elicitors. Pl. Cell Rep. 23(5): 339-344

Kim, O. T., Ahn, J. C., Hwang, S. J. and Hwang, B. 2005. Cloning and expression of a farnesyl diphosphate synthase in *Centella asiatica* (L.) Urban. Mol. Cells. 19(2): 294-299

Kim, O. T., Kim, M. Y., Huh, S. M., Bai, D. G., Ahn, J. C. and Hwang, B. 2005. Cloning of a cDNA probably encoding oxidosqualene cyclase associated with asiaticoside biosynthesis from *Centella asiatica* (L.) Urban. Pl. Cell Rep. 24(5): 304-311

Kim, O. T., Kim, M. Y., Ahn, J. C., Li, M. Y. and Hwang, B. 2005. Effects of precursor and thidiazuron on triterpene saponin biosynthesis in *Centella asiatica* (L.) Urban. Korean. J. Med. Crop Sci. 13(2): 91-94

Koncz, C., Németh, K., Redei, G. P. and Scell, J. 1994. J. Homologous recombination and gene silencing in plants. 167-189

Koroch, A., Kapteyu, J., Juliani, H. R. and Simon, J. E. 2002. *In vitro* regeneration and *Agrobacterium transformation* of *Echinacea purpurea* leaf explants. (eds. Janick,J. and Whipkey,A.). Trends in New Crops and New Uses. ASHHS Press, Alexandria. 522-526

Krishnan, N. 2006. *Agrobacterium tumefaciens* mediated genetic transformation in kudangal (*Centella asiatica* L. Urban). Dept of Plant Biotechnology. Kerala Agricultural University

Kuehnle, A. R. 1997. Molecular biology of orchids. *Orchid Biology- Review and perspectives.* (eds Arditti, J. and Pridgeon, A.M.I.) VII. Kluwer Academic, London. 75-115

Kumar, M. H. V. and Gupta, Y. K. 2002. Effect of different extracts of *Centella asiatica* on cognition and markers of oxidative stress in rats. J. Ethanopharmacol. 79(2): 253-260

Kumar, M. H. V. and Gupta, Y. K. 2003. Effect of *Centella asiatica* on cognition and oxidative stress in an intracerebroventricular streptozotocin model of Alzheimer's disease in rats. J. Clinical Exptl. Pharmacol. Physiol. 30(5-6): 336-342

Kumar, V. S and Rajam, M. V. 2005. Polyamines enhance *Agrobacterium tumefaciens vir* gene induction and T-DNA transfer. Pl. Sci. 168(2): 475-480

Lee, J. and Pedersen, H. 2001. Stable genetic transformation of *Eschscholzia Californica* expressing synthetic green fluorescent proteins. Biotechnol. Prog. 17(2): 247-251

Lee, M. H., Yoon, E. S., Jeong, J. H. and Choi, Y. E. 2004. *Agrobacterium rhizogenes* mediated transformation of *Taraxacum platycarpum* and changes of morphological characters. Pl. Cell Rep. 22: 822-827

Lee, M. K., Kim, S. R., Sung, S. H., Lim, D., Kim, H., Choi, H., Park, H. K., Je, S. and Ki, Y. C. 2000. Asiatic acid derivatives protect cultured cortical neurons from glutamate-induced excitotoxicity. Res. Commun. Mol. Pathol. Pharmacol. 108(1-2): 75-86

Leo, A. H. Z., Christon, P. and Leek, M. J. 2000. Transformation of the tropane alkaloid producing plant *Hyoscyamus muticus* by particle bombardment. Transgenic Res. 9(3): 163-168

Lichtenstein, C. and Draper, J. 1986. DNA cloning: A practical approach. Genetic Engineering of Plants. (ed. Glover,D.M.). IRL Press, Oxford. pp. 67-119.

Lievre, K., Hehn, A., Gravot, A., Thomasset, B., Bourgand., Gontier, E. 2005. Genetic transformation of the medicinal plant *Ruta graveolans* L. by an *Agrobacterium tumefaciens* mediated method. Pl. Sci.168(4): 883-888

*Mamtha, B., Kavitha, K., Srinivasan, K. K. and Shivananda, P. G. 2004. An in vitro study* of the effect of *Centella asiatica* (Indian pennywort) on enteric pathogens. Indian J. Pharmacol. 36(1): 41-44

Mangas, S., Bonfill, M., Osuna, L., Moyano, E., Tortoriello, J., Cusido, R. M., Pinol, M. T. and Palazon, J. 2006. The effect of methyl jasmonate on triterpene and sterol metabolisms of *Centella asiatica, Ruscus aculeatus, Galphimia glauca* cultured plants. Phytochemistry. 67(18): 2041-2049

Mathews, H., Bharathan, N., Litz, R. E., Narayana, K. R., Rao, P. S. and Bhatia, C. R. 1990. The promotion of *Agrobacterium* mediated transformation in *Atropa belladonna* L. by acetosyringone. J. Pl. Physiol. 136(4): 404-409

Mathis, N. L. and Hinchee, M. A. W. 1994. *Agrobacterium* inoculation techniques for plant tissues. *Plant Molecular Biology Manual* (eds. Gelving, S.B. and Schilperoort, R.A). Kluwer Academic Publishers, Dordrecht. 1-9.

Mathur, S., Varma, R. K., Gupta, M. M., Ram, M., Sharma, S. and Kumar, S. 2000. Screening of genetic resources of medicinal–vegetable plant *Centella asiatica* for herb and asiaticoside yields under shaded and full sunlight conditions. J. Hortl. Sci. Biotechnol. 75: 551–554.

Matsuda, H., Morikawa, T., Ueda, H. and Yoshikawa, M. 2001. Medicinal foodstuff XXVII. Saponin constituents of Gotu Kola (2): Structures of new Ursane- and Cleanane- type triterpene glycosides, centellasaponins B, C and D from *Centella asiatica* cultivated in Sri Lanka. Chem. Pharm. Bull. 49(10): 1368-1371.

Mohamed, Sh., Boehm, R., Binsfeld, P. C. and Schnabl, H. 2004. *Agrobacterium*-mediated transformation of two high oleic Sunflower (*Helianthus annus* L.) genotypes: Assessment and optimization of important parameters. HELIA. 27(40): 25-40

Moharana, D and Moharana, S. 1994. A clinical trial of mental health in children with various types of epilepsy. Probe. 33: 160–162.

Montecchio, G. P., Samaden, S., Carbone, M., Vigotti, S., Siragusa. and Piovella, F. 1991. *Centella asiatica* triterpenic fraction reduces the number of circulating endothelial cells in subjects with post phlebitic syndrome. Haematologica. 76(3): 256-259

Murch, S. J., Krishnaraj, S. and Saxena, P. K. 2000. Tryptophan is a precursor for melatonin and serotonin biosynthesis in *in vitro* regenerated St. John's wort (*Hypericum perforatum* L. cv. Anthos) plants. Pl. Cell Rep. 19: 698-704

Nabha, S., Lamblin, F., Gillet, F., Lauraen, D., Fliniax, M., Daud, A. and Jacques, A. 1999. Polyamine content and somatic embryogenesis in *Papaver somniferum* cells transformed with *sam-1* gene. J. Pl. Physiol. 154: 729-734.

Nath, S. and Buragohain, A. K. 2003. *In vitro* method for propagation of *Centella asiatica* (L) Urban by shoot tip culture. J. Pl. Biochem. Biotechnol. 12: 167-169

Nath, S. and Buragohain, A. K. 2005. Establishment of callus and cell suspension cultures of *Centella asiatica*. Biologia. Pl. 49(3): 411-413

Nebauer, S. G., Arrillaga, I., Agudo, L. D. C. and Segura, J. 2000. *Agrobacterium tumefaciens* mediated transformation of the aromatic shrub *Lavandula latifolia*. Mol. Breed. 6(6): 539-552

Negoianutenea, G., Calin, A. and Cucu, N. 2002. Genetic Modified Medical Plants. *Agrobacterium tumefaciens* mediated reporter *gus* gene transfer and expression in *Atropa belladonna* plants. Roum. Biotechnol. Lett. 7(3): 773-778

Nehra, N. S., Chibbar, R. N., Kartha, K. K., Datta, R. S. S., Crosby, W. L. and Stushnoff, C. 1990. Genetic transformation of strawberry by *Agrobacterium tumefaciens* using a leaf disc regeneration system. Pl. Cell Rep. 9: 293-298

Nester, E. W., Gordon, M. P., Amasino, R. M. and Yanofsky, M. F. 1984. Crown gall: a molecular and physiological analysis. Ann. Rev. Pl. Physiol. 35: 387-413.

Nisha, K. K., Seetha, K., Rajmohan, K. and Purushothama, M. G. 2003. *Agrobacterium tumefaciens* mediated transformation of Brahmi [*Bacopa monniera* (L.) Wettst], a popular medicinal herb of India. Curr. Sci. 85 (1): 85-89

Niu, X., Li, K., Hasegawa, P. M., Bressan, R. A. and Wellar, S. C. 1998. Trangenic Peppermint (*Mentha piperita* L.) plants obtained by co-cultivation with *Agrobacterium tumefaciens*. Pl. Cell Rep. 17(3): 165-171

Niu, X., Li, X., Veronese, R., Bressan, A., Weller, S. C. and Hasegawa, P. M. 2000. Factors affecting *Agrobacterium tumefaciens* mediated transformation of Peppermint. Pl. Cell Rep. 19(3): 304-310

Ogawa, Y., Mii, M. 2007. Meropenem and moxalactam: Novel β-lactam antibiotics for efficient *Agrobacterium*-mediated transformation. Pl. Sci. 172(3): 564-572

Omar, R., Abdullah, M. A., Hasan, M. A. and Marziah, M. 2004. Development of growth medium for *Centella asiatica* cell culture via response surface methodology. Am. J. Appl. Sci. 1(3): 215-219

Opabode, J. T. 2006. *Agrobacterium* mediated transformation of plants: emerging factors that influence efficiency. Biotech. Mol. Biol. Rev. 1(1): 12-20

Panimalar, V., Vadivel, E. and Kavitha, P. S. 2005. *In vitro* studies in *Centella asiatica* (Linn.). Urban. *Proc of ICAR National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and strategies. January 10-12, 2005* (eds.Rao,G.S.L.H.V. and Nazeem,P.A.) Kerala Agricultural University, Thrissur. 128-131.

Paramageetham, C., Babu, G. P. and Rao, T. V. S. 2004. Somatic embryogenesis in *Centella asiatica* L. an important medicinal & neutraceutical plant in India. Pl. Cell Tiss. Org. Cult. 79(1): 19-24

Parisi, M., Moreno, S. and Fernandez, G. 2002. Characterization of a novel cysteine peptidase from tissue culture of garlic (*Allium sativum* L.). *In-vitro* Cell Dev. Biol. Pl. 38: 608-12.

Park, J. M. and Yoon, S. Y. 1992. Production of sanguinarine by suspension culture of *Papaver somniferum* in bioreactors. J. Ferm. Bioeng. 74: 292-296.

Park, S. U. and Facchini, P. J. 2000. *Agrobacterium* mediated genetic transformation of California poppy, *Eschscholzia californica* chem., via somatic embryogenesis. Pl. Cell Rep. 19(10): 1006-1012

Patra, A., Rai, B., Rout, G.R., Das, P. 1998. Successful plant regeneration from callus cultures of *Centella asiatica* (Linn.) Urban. Pl. Growth Regult. 24(1): 13-16

Peris, K. H. S. and Kays, S. J. 1996. Asiatic pennywort (*Centella asiatica* (L.) Urb. : little-known vegetable crop. Am. Soc. Hort. Sci. 6(1): 13-18.

Pradel, H., Dumkelehmann, U., Diettrich, B. and Luckner, M. 1997. Hairy root cultures of *Digitalis lanata* secondary metabolism and plant regeneration. J. Pl. Physiol. 151: 209-215

Rai, P. and Mishra, S. H. 2006. Development of a simple and sensitive spectrophotometric method for the simultaneous determination of asiaticoside and wedelolactone in a ployherbal formulation. Phcog. Mag. 47-51

Rao, K. G. M., Rao, S. M. and Rao, S. G. 2006. *Centella asiatica* (L.) leaf extract treatment during the growth spurt period enhances hippocampal CA3 neuronal dendritic arborization in rats. Evid. Based Complement. Alternat. Med. 3(3): 349-357

Rao, K. P., Rao, S. S. and Sadanandam, M. 1999. Tissue culture studies of *Centella asiatica*. Indian J. Pharmaceutical Sci. 61(6): 392-394

Rao, V. E. 2003. Herbal drugs for memory enhancement : An appraisal. Recent Progress in Medicinal Plants: Phytochemistry and Pharmacology. (eds. Govil, J.N. and Singh, V.K. ) Stadium Press, Texas, USA. pp. 191-199

Rezmer, C., Schlichting, R., Wachter, R., Ullrich, C. I. 1999. Identification and localization of transformed cells in *Agrobacterium tumefaciens*-induced plant tumors. Planta. 209(4): 399-405

Sales, E., Segura, J. and Arrillaga, I. 2003. *Agrobacterium tumefeciens* mediated genetic transformation of the cardenolide producing plant *Digitalis minor* L. Planta Med. 69(2): 143-147

Sales, E., Munoz-Bertomeu, J., Arrillaga, I. and Segura, J. 2007. Enhancement of cardenolide and Phytosterol Levels by Expression of an N-Terminally Truncated 3-Hydroxy-3-methylglutaryl CoA Reductase in Transgenic *Digitalis minor*. Planta Med. 73: 605-610

Sampson, J. H., Raman, A., Karlsen, G., Navsaria, H. and Leigh, I. M. 2001. *In vitro* keratinocyte antiproliferant effect of *Centella asiatica* extract and triterpenoid saponins. Phytomed. 8(3): 230-235

Sangeetha, N. and Alak, K. B. 2003. *In vitro* method for propagation of *Centella asiatica* (L) Urban by shoot tip culture. J. Pl. Biochem. Biotechnol. 12: 167-169

Sato, F., Hashimoto, T., Hachiya, A., Tamura, K., Choi, K., Morishige, T., Fujimoto, H. and Yamada, Y. 2001. Metabolic engineering of plant alkaloid biosynthesis. Proc. Natl. Acad. Sci. 98(1): 367-372

Scalarone, D., Duursma, M. C., Boon, J. J. and Chiantore, O. 2005. MALDI-TOF mass spectrometry on cellulosic surfaces of fresh and photo-aged di- and triterpenoid varnish resins. J. Mass. Spectrom. 40(12): 1527-1535

Schaneberg, B. T., Mikell, J. R., Bedir, E. and Khan, I. A. 2003. An improved HPLC method for quantitative determination of six triterpenes in *Centella asiatica* extracts and commercial products. Pharmazie. 58(6): 381-384

Schieffer, G. W. 2005. Use of solid-phase extraction for improving HPLC assays of triterpene glycosides in gotu kola (*Centella asiatica*), parthenolide in feverfew (*Tanecetum parthenium*), and valerenic acids in valerian (*Valeriana officinalis*). J. Liquid Chromatogr. Related Tech. 28(4): 581-592

Shanjun, T., Sangwan, R. S., Raghavan, V., Verma, D. P. S. and Sangwan Norreel, B. S. 2005. Transformation of pollen embryo derived explants by *Agrobacterium tumefaciens* in *Hyoscyamus niger*. Pl. Cell Tiss. Org. Cult. 81(2): 139-148

Sharma, J. and Sharma, R. 2002. Radioprotection of Swiss albino mouse by *Centella asiatica* extract. Phytotheraphy Res. 16(8): 785-786

Shashikala, C. M., Shashidhar, S. and Rajashekaran, P. F. 2005. *In vitro* regeneration of *Centella asiatica* L. Pl. Cell Biotechnol. Mol. Biol. 6: 53-56

Shetty, B. S., Udupa, S. L., Udupa, A. L., Somayaji, S. N. 2006. Effect of *Centella asiatica* L (Umbelliferae) on normal and dexamethasone-suppressed wound healing in wistar albino rats. The International J. Lower Extremity Wounds. 5(3): 137-143

Shi, H. P. and Kintzios, S. 2003. Genetic transformation of *Puerania phaseoloides* with *Agrobacterium rhizogenes* and puerarin production in hairy roots. Pl. Cell Rep. 21: 1103–1107

Shukla, A., Rasik, A. M. and Dhawan, B. N. 1996. Asiaticoside induced elevation of antioxidant levels in healing wounds. Phytother. Res. 13(1): 50-54

Shukla, Y. N., Srivastava, R., Tripathi, A. K., Prajapathi, V. 2000. Characterization of an ursane triterpenoid from *Centella asiatica* with growth inhibitory activity against *Spilarctia oblique*. Pharm. Biol. 38(4): 262-267

Sivakumar, G., Alagumanian, S. and Rao, M. V. 2006. High frequency *in vitro* multiplication of *Centella asiatica*: An important industrial medicinal herb. Engg. Life Sci. 6(6): 597-601

Smith E. F. and Towsend C. O. 1907. A plant tumour of bacterial origin. Science. 25: 671-673.

Somchit, M. N., Sulaiman, M. R., Zuraini, A., Samsuddin, L., Somchit, N., Israf, D. A and Moin, S. 2004. Antinociceptive and anti-inflammatory effects of *Centella asiatica*. Indian. J. Pharmacol. 36(6): 377-380

Souret, F. F., Weathers, P. J. and Wobbe, K. K. 2002. The mevalonate independent pathway is expressed in transformed roots of *Artemisia annua* and regulated by light and culture age. *In vitro* Cell Dev. Biol. Pl. 38: 581-588

Stafford, A., Morris, P., Fowler, M. W. 1986. Pl. Cell Biotechnology: A perspective. Enzyme Microbial. Tech. 8: 578-597

Sturtevant, J. 2000. Applications of differential-display reverse transcription-PCR to molecular pathogenesis and medical mycology. Clinical Microbiol. Rev. 13(3): 408-427

Sugimura, Y., Kadotani, N., Ueda, Y., Shima, K. and Sakihitokitajima. 2005. Transgenic Patchuoli plants produced by *Agrobacterium* mediated transformation. Pl. Cell Tiss. Org. Cult. 82(3): 251-257

Tang, W., Luo, H., Newton, R. J. 2004. Effects of antibiotics on the elimination of *Agrobacterium tumefaciens* from loblollo pine (Pinus Taeda) zygotic embryo explants and on transgenic plant regeneration. Pl. Cell. Tiss. Org. Cult. 79: 71-81

Tiwari, K. N., Sharma, N. C., Tiwari, B. and Singh, B. D. 2000. Micropropagation of *Centella asiatica* (L), a valuable medicinal herb. Pl. Cell Tiss. Org. Cult. 63(3): 179–185

Tripathi, L., Tripathi, J. N. 2003. Role of biotechnology in medicinal plants. Trop. J. Pharma. Res. 2(2): 243-253

Upadhyay, S. C., Khosa, R. L., Sharma, D. N., Kumar, M. and Chansauria, J. P. N. 1991. Total glycoside content and antistress activity of Indian and Mauritius *Centella asiatica* – A comparison. Indian Drugs. 28(8): 388-389

Kumar. V. M. H. and Gupta, Y. K. 2003. Effect of *Centella asiatica* on cognition and oxidative stress in an intracerebroventricular streptozotocin model of Alzheimer's disease in rats. Clin. Expt. Pharmacol. Physiol. 30(5-6): 336-342.

Vergauwe, A., Cammaert, R., Vandenberghe, D., Genetello, C., Inze, D., Van Montagu, M. and Van Den Eeckhout, E. 1996. *Agrobacterium tumefaciens* mediated transformation of *Artemisia annua* L. plants and the regeneration of transgenic plants. Pl. Cell Rep. 15: 929-933.

Vergauwe, A., Van Geldre, E., Inze, D., Van Montagu, M. and Van den Eeckhovt, E. 1998. Factors influencing *Agrobacterium tumefaciens* mediated transformation of *Artemisia annua* L. Pl. Cell. Rep. 18: 105 – 110

Verma, R. K., Bhartariya, K. G., Gupta, M. M. and Kumar, S. 1999. Reverse phase High Performance Liquid Chromatography of Asiaticoside in *Centella asiatica.* Phytochemical Analysis. 10(4): 191-193.

Voisey, C. R., White, D. W. R., Dudas, B., Appleby, R. D., Eaung, P. M. and Scolt, A. G. 1994. *Agrobacterium* mediated transformation of white clover using direct shoot organogenesis. Pl. Cell Rep. 13: 309 – 314

Wiebke, B., Ferreira, F., Pasquali, G., Bodanese-Zanettini, M. H. and Droste, A. 2006. Influence of antibiotics on embryogenic tissue and *Agrobacterium tumefaciens* suppression in soyabean gentic transformation. Bragantia. 65(4): 543-551

Yang, J., Lee, H. J., Shin, D. H., Oh, S. K., Seon, J. H., Paek, K. Y. and Han, K. H. 1999. Genetic transformation of *Cymbidium* orchid by particle bombardment. Pl. Cell Rep. 18: 978-984

Ye, G. N., Stone, D., Pang, S. Z., Creely, W., Gonzalez, K. and Hinchee, M. 1999. *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. Pl. J. 19: 249-257

Yun, D. J., Hashimoto, T., Yamad, T. 1992. Metabolic engineering of medicinal plants: transgenic *Atropa belladonna* with an improved alkaloid composition. Proc. Natl. Acad. Sci. 89: 11799–11803

Zaldivar, J. M., Ballina, H., Rodriguez, E., Berzuza, A. and Hernandez, G. C. 2003. *Agrobacterium* mediated transient transformation of annatto (*Bixa orellana*). hypocotyls with the *gus* reporter gene. Pl. Cell Tiss. Org.Cult. 73(3): 281-284

Zarate, R., Memelink, J., Heijden, R. V. and Verpoorte, R. 1999. Genetic transformation via particle bombardment of *Catharanthus roseus* plants through adventitious organogenesis of buds. Biotechnol. Lett. 21(11): 997-1002

Zhou, B., Sauter, M. and Sun, M. 2005. Single microspore reverse transcription-polymerase chain reaction: dynamic change of gene expression during microsporogenesis. Biosci. Biotechnol. Biochem. 69(11): 2240-2243

# *Appendices*

## APPENDIX I

Chemical composition of media employed for the *in vitro* culture of *Centella asiatica*

**MS medium**

**Macro-nutrients ($mgl^{-1}$)**

| | |
|---|---|
| $Mg\ SO_4.7H_2O$ | 370 |
| $CaCl_2.2H_2O$ | 440 |
| $KNO_3$ | 1900 |
| $NH_4NO_3$ | 1650 |
| $KH_2PO_4$ | 170 |

**Micro-nutrients ($mgl^{-1}$)**

| | |
|---|---|
| $MnSO_4.4H_2O$ | 22.3 |
| $ZnSO_4.7H_2O$ | 8.6 |
| $CuSO_4.5H_2O$ | 0.025 |
| $AlCl_3$ | 0.025 |
| KI | 0.83 |
| $H_3BO_3$ | 6.2 |
| $Na_2MoO_4.2H_2O$ | 0.25 |

**Iron Source ($mgl^{-1}$)**

| | |
|---|---|
| $FeSO_4.7H_2O$ | 27.85 |
| $Na_2EDTA$ | 37.25 |

**Vitamins ($mgl^{-1}$)**

| | |
|---|---|
| Nicotinic acid | 0.5 |
| Pyridoxine HCl | 0.5 |
| Thiamine HCl | 0.1 |

**Amino acid source ($mgl^{-1}$)**

| | |
|---|---|
| Glycine | 2.0 |
| Inositol ($mgl^{-1}$) | 100 |
| Sucrose ($gl^{-1}$) | 30 |
| Agar ($gl^{-1}$) | 6 |

## APPENDIX II

Chemical composition of media employed for the culture of *Agrobacterium tumefaciens*

### AB Minimal Medium (Working solution 1X)

***AB salts ($gl^{-1}$) 20 X stock***

| | |
|---|---|
| $NH_4Cl$ | 20 |
| $MgSO_4.7H_2O$ | 25 |
| KCl | 3 |
| $CaCl_2$ | 0.2 |
| $FeSO_4.7H_2O$ | 0.05 |
| | |
| pH | 7 |

***AB buffer ($gl^{-1}$) 20 X stock***

| | |
|---|---|
| $K_2HPO_4$ | 60 |
| $NaH_2PO_4$ | 23 |
| | |
| Glucose | 0.5% |
| Agar | 1.5% |

### Luria-Bertani (LB) medium ($gl^{-1}$)

| | |
|---|---|
| Yeast Extract | 5 |
| Tryptone | 10 |
| Sodium chloride | 10 |
| Agar | 15 |
| | |
| pH | 7 |

## APPENDIX III

Nucleotide sequence and structure of the vector, pBE 2113 in *Agrobacterium* strain EHA 105

*hmgr1* of *Arabidopsis thaliana.*

SOURCE: - Dr. S. G. Purushothama, Plant Molecular Biology Lab, Rajiv Gandhi Centre for Biotechnology, Trivandrum.

| | |
|---|---|
| LOCUS | X15032 2385 bp mRNA linear PLN 18-APR-2005 |
| DEFINITION | *Arabidopsis thaliana* mRNA for 3-hydroxy-3-methylglutaryl CoA reductase (EC 1.1.1.34). |
| ACCESSION | X15032 |
| VERSION | X15032.1 GI: 16335 |
| KEYWORDS | hydroxymethylglutaryl-CoA reductase; transmembrane protein. |
| SOURCE | *Arabidopsis thaliana* (thale cress) |
| ORGANISM | *Arabidopsis thaliana*<br>Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; eurosids II; Brassicales; Brassicaceae; Arabidopsis. |
| REFERENCE | 1 (bases 1 to 2385) |
| AUTHORS | Caelles,C., Ferrer,A., Balcells,L., Hegardt,F.G. and Boronat,A. |
| TITLE | Isolation and structural characterization of a cDNA Encoding *Arabidopsis thaliana* 3-hydroxy-3-methylglutaryl coenzyme A reductase |
| JOURNAL | Plant Mol. Biol. 13 (6), 627-638 (1989) |
| PUBMED | 2491679 |
| REFERENCE | 2 (bases 1 to 2385) |
| AUTHORS | Caelles,C. |
| TITLE | Direct Submission |
| JOURNAL | Submitted (18-APR-1989) Caelles C., University of Barcelona, Unit of Biochemistry, School of Pharmacy, Plaza Pio XII s/n, 08028 Barcelona, Spain |
| FEATURES | Location/Qualifiers source 1..2385<br>/organism="*Arabidopsis thaliana*"<br>/mol_type="mRNA"<br>/strain="columbia"<br>/db_xref="taxon: 3702"<br>/clone="lambda cAT1, lambda cAT12" |

/ecotype="Columbia"

Coding sequence: - 71-1849

```
   1  atcacgccacctcaccacctctctcctctctcctctctcccccctggagagattattc
  61  attccctccaatggatctccgtcggaggcctcctaaaccaccggttaccaacaacaacaa
 121  ctccaacggatctttccgttcttatcagcctcgcacttccgatgacgatcatcgtcgccg
 181  ggctacaacaattgctcctccaccgaaagcatccgacgcgcttcctcttccgttatatct
 241  cacaaacgccgttttcttcacgctcttcttctccgtcgcgtattacctcctccaccggtg
 301  gcgtgacaagatccgttacaatacgcctcttcacgtcgtcactatcacagaactcggcgc
 361  cattattgctctcatcgcttcgtttatctatctcctagggtttttttggtattgactttgt
 421  tcagtcatttatctcacgtgcctctggtgatgcttgggatctcgccgatacgatcgatga
 481  tgatgaccaccgccttgtcacgtgctctccaccgactccgatcgtttccgttgctaaatt
 541  acctaatccggaacctattgttaccgaatcgcttcctgaggaagacgaggagattgtgaa
 601  atcggttatcgacggagttattccatcgtactcgcttgaatctcgtctcggtgattgcaa
 661  aagagcggcgtcgattcgtcgtgaggcgttgcagagagtcaccgggagatcgattgaagg
 721  gttaccgttggatggatttgattatgaatcgattttggggcaatgctgtgagatgcctgt
 781  tggatacattcagattcctgttgggattgctggtccattgttgcttgatggttatgagta
 841  ctctgttcctatggctacaaccgaaggttgtttggttgctagcactaacagaggctgcaa
 901  ggctatgtttatctctggtggcgccaccagtaccgttcttaaggacggtatgacccgagc
 961  acctgttgttcggttcgcttcggcgagacgagcttcggagcttaagtttttcttggagaa
1021  tccagagaactttgatactttggcagtagtcttcaacaggtcgagtagatttgcaagact
1081  gcaaagtgttaaatgcacaatcgcggggaagaatgcttatgtaaggttctgttgtagtac
1141  tggtgatgctatggggatgaatatggtttctaaaggtgtgcagaatgttcttgagtatct
1201  taccgatgatttccctgacatggatgtgattggaatctctggtaacttctgttcggacaa
1261  gaaacctgctgctgtgaactggattgagggacgtggtaaatcagttgtttgcgaggctgt
1321  aatcagaggagagatcgtgaacaaggtcttgaaaacgagcgtggctgctttagtcgagct
1381  caacatgctcaagaacctagctggctctgctgttgcaggctctctaggtggattcaacgc
```

1441 tcatgccagtaacatagtgtctgctgtattcatagctactggccaagatccagctcaaaa

1501 cgtggagagttctcaatgcatcaccatgatggaagctattaatgacggcaaagatatcca

1561 tatctcagtcactatgccatctatcgaggtggggacagtgggaggaggaacacagcttgc

1621 atctcaatcagcgtgtttaaacctgctcggagttaaaggagcaagcacagagtcgccggg

1681 aatgaacgcaaggaggctagcgacgatcgtagccggagcagttttagctggagagttatc

1741 tttaatgtcagcaattgcagctggacagcttgtgagaagtcacatgaaatacaatagatc

1801 cagccgagacatctctggagcaacgacaacgacaacaacaacaacatgatctgaatctga

1861 atcatcatcctctcaaagaaggacaacaatccaaaacaagggcaggctttttacaacgca

1921 ttcactcaaaactcgctggtggacagattttagccatgtgcgtatgcgtttgccctttg

1981 ttaaataaaaaaactatttgttttgtttgtttgacttgatatctttttttgggattgagg

2041 attgagagagatagagagattttacaaactttctctctttctctctttctctctttctca

2101 tggataattcgtgtctctttgatttgtctaaggtttgtctttgtttgttaggaagtggtc

2161 tatatgaacgaaaaatttgtgtatggtgcagttgcgtttggggacatttttgagattttt

2221 tctctgttttgtttcctctcttcgtttttattgtttgttacatataaaatatttctctgt

2281 atgttggaacatctctctctctttagttgttgttggtaaaagatacggatcttctttcct

2341 ccagaagaatccatctatataatattaccatctatgtgttctact

/translation="MDLRRRPPKPPVTNNNNSNGSFRSYQPRTSDDDHRRRATTIAPP

PKASDALPLPLYLTNAVFFTLFFSVAYYLLHRWRDKIRYNTPLHVVTITELGAIIALI

ASFIYLLGFFGIDFVQSFISRASGDAWDLADTIDDDDHRLVTCSPPTPIVSVAKLPNP

EPIVTESLPEEDEEIVKSVIDGVIPSYSLESRLGDCKRAASIRREALQRVTGRSIEGL

PLDGFDYESILGQCCEMPVGYIQIPVGIAGPLLLDGYEYSVPMATTEGCLVASTNRGC

KAMFISGGATSTVLKDGMTRAPVVRFASARRASELKFFLENPENFDTLAVVFNRSSRF

ARLQSVKCTIAGKNAYVRFCCSTGDAMGMNMVSKGVQNVLEYLTDDFPDMDVIGISGN

FCSDKKPAAVNWIEGRGKSVVCEAVIRGEIVNKVLKTSVAALVELNMLKNLAGSAVAG

SLGGFNAHASNIVSAVFIATGQDPAQNVESSQCITMMEAINDGKDIHISVTMPSIEVG

TVGGGTQLASQSACLNLLGVKGASTESPGMNARRLATIVAGAVLAGELSLMSAIAAGQ

LVRSHMKYNRSSRDISGATTTTTTTT"

**Vector(s) description (the plasmid map)**

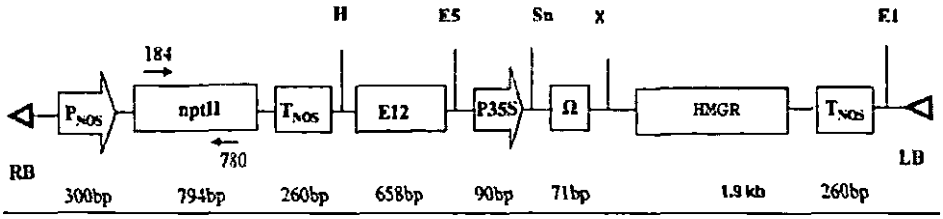



Figure represents schematic map of the T-DNA region of pBE2113 (RB, Right border; LB, Left border; $P_{NOS}$, Nopaline synthase promoter; *npt*II, Gene for neomycin phosphotransferase; $T_{NOS}$, Nopaline synthase terminator; E12, 5'-upstream sequence of CaMV 35S promoter; P35S, 5'- upstream sequence of CaMV 35S promoter; Ω, 5'- untranslated sequence of TMV; HMGR, Gene for Hydroxy methyl glutaryl CoA reductase; H, *Hind*lll; E5, *Eco*RV; Sn, *Sna*BI; X, *Xba*I; E1, *Eco*RI. Bold arrows above the boxes indicate the position of primers used for PCR analysis.

## APPENDIX IV

Chemicals for isolation of DNA from *Centella asiatica*

**CTAB BUFFER**

| | |
|---|---|
| CTAB | 2 % |
| Tris- HCl (pH-8.0) | 100 mM |
| NaCl | 1.4 M |
| EDTA | 20 mM |
| 2-mercapto ethanol | 0.1 % w/v |

**TE BUFFER (pH 8.0)**

| | |
|---|---|
| Tris buffer | 10mM |
| EDTA | 1mM |

**50 X TAE BUFFER (pH 8.0)**

| | |
|---|---|
| Tris Buffer | 24.20 g |
| Glacial acetic acid | 5.71 ml |
| EDTA | 0.05 M |

## APPENDIX V

Chemicals for isolation of plasmid DNA from *Agrobacterium* strain EHA 105

**(i) Solution I**

| | |
|---|---|
| 20 % glucose | 2.25 ml |
| 0.5 M EDTA (pH – 8.0) | 1 ml |
| 1 M Tris (pH – 8.0) | 1.25 ml |
| Sterile distilled water | 45.50 ml |

**(ii) Solution II**

| | |
|---|---|
| 10 N NaOH | 0.4 ml |
| 20 % SDS | 1 ml |
| Sterile Distilled Water | 18.6 ml |

**(iii) Solution III**

| | |
|---|---|
| 5 M Sodium acetate | 60 ml |
| Glacial acetic acid | 11.5 ml |
| Sterile distilled water | 28.5 ml |

# *AGROBACTERIUM TUMEFACIENS* MEDIATED TRANSFER OF EXOGENOUS HYDROXY METHYL GLUTARYL CoA (HMG CoA) REDUCTASE GENE TO *CENTELLA ASIATICA* L.

LEKSHMI, R. S

Abstract of the
thesis submitted in partial fulfillment of the requirement
for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

2008

Department of Plant Biotechnology
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM 695 522

## ABSTRACT

A study on "*Agrobacterium tumefaciens* mediated transfer of exogenous hydroxyl methyl glutaryl CoA (HMG CoA) reductase gene to *Centella asiatica* L." was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2006-2008.

*Centella asiatica* is an important medicinal plant, which is used in many ayurvedic formulations. It contains a blend of compounds including triterpenes (asiatic acid, madecassic acid and asiaticoside), which are responsible for its medicinal properties. Among the various secondary metabolites in *Centella asiatica*, asiaticoside possesses remarkable pharmaceutical value due to its anti-inflammatory, antitumour, neuroprotective, skin care and toning effects. Since the asiaticoside content in the Indian ecotypes is less, the industrial demands are met by importing this plant from African countries. By improving asiaticoside content, utilization of Indian ecotypes could be improved and thereby the cost of medical preparations could be reduced.

Metabolic engineering is becoming a popular approach for the modification of medicinal plants for altering the metabolite content. Medicinal plants with quantitatively and qualitatively improved pharmacological properties have been produced by metabolic pathway engineering. The present study was undertaken with an objective to enhance the asiaticoside content by introducing exogenous *hmgr* gene to *Centella asiatica*. This gene is responsible for coding hydroxy methyl glutaryl CoA reductase enzyme which acts at the upstream of the triterpene biosynthetic pathway and produce mevalonic acid.

Callus was induced from leaf and node explants of *Centella* and MS medium supplemented with Kn 4 mg $l^{-1}$ and NAA 2 mg $l^{-1}$ was found to be the best for callus induction. Callus initiation was faster (23 days) from node compared to leaf (25 days), with 100 and 92.85 per cent induction respectively. Of the various media tried for regeneration, the highest regeneration (0.052%) was obtained on MS medium supplemented with Kn 4mg $l^{-1}$ and NAA 2 mg $l^{-1}$.

*Agrobacterium tumefaciens* strain EHA105 harbouring the plasmid pBE2113 containing *nptII* and *hmgr* gene was used for transformation. The sensitivity of *Agrobacterium* strains and *Centella* callus to different concentrations of kanamycin was evaluated. The lethal doses of kanamycin to *Agrobacterium* and *Centella* callus were 350 and 125 mg $l^{-1}$, respectively. The effective dose of cefotaxime for the elimination of bacteria was 50 mg $l^{-1}$ and the lethal dose of cefotaxime to callus was 100 mg $l^{-1}$.

Genetic transformation was achieved by co-cultivating callus with bacterial suspension ($OD_{600}$ of 0.1) containing 100 µM acetosyringone. An infection time of 20 min and co-cultivation period of four days were given. The transformed tissues were selected on MS medium containing 200, 250 and 300 mg $l^{-1}$ of kanamycin. The survival of the tissues on these media after three weeks was 18.75, 19.35 and 13.63 per cent, respectively.

Transformation was confirmed by PCR analysis with *npt II* gene specific primer. All the three samples gave appreciable quantity of the product of size 700 bp which was comparable to positive control.

In the present study regeneration of the transformed tissues was not obtained in the media standardized. Even though an attempt was made to analyse asiaticoside

content in the transformed callus using thin layer chromatography (TLC), there was no detectable quantity of asiaticoside. This could be due to the undifferentiated nature of the callus. As the accumulation of asiaticoside is mainly in the leaves, a better protocol for regeneration needs to be developed so that further studies on triterpenoid analysis could be carried out.