AGROBACTERIUM TUMEFACIENS MEDIATED GENETIC TRANSFORMATION IN KUDANGAL (*CENTELLA ASIATICA* L. URBAN.)

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DECLARATION

I hereby declare that this thesis entitled "***Agrobacterium tumefaciens* mediated genetic transformation in kudangal (*Centella asiatica* L. Urban.)**" 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 entitled "***Agrobacterium tumefaciens* mediated genetic transformation in kudangal (*Centella asiatica* L. Urban.)**" is a record of research work done independently by Ms. Nanditha Krishnan, V. (2003-11-54) 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

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Live in the spirit of this creed,
Yet seek thy firm support, according to their need"

I bow before ***Lord, the Almighty*** *for his bountiful blessings.*

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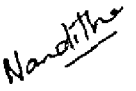
"After the countless songs, or long or short, all tongues, all lands,
Still something not yet told in poesy's voice or print -something lacking,
Who knows? the best yet unexpressed and lacking."



Nanditha Krishnan, V.

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LIST OF ABBREVIATIONS

ADS	Adenine sulphate
BA	Benzyl adenine
2, 4-D	2, 4-dichlorophenoxyacetic acid
DMF	Dimethyl formamide
GA	Gibberellic acid
gus	β-D-Glucuronidase
Kn	Kinetin
M	Molar
μM	Micromolar
MS medium	Murashige and Skoog medium
NAA	α-Naphthalene acetic acid
npt	Neomycin phosphotransferase
PCR	Polymerase Chain Reaction
pH	Per Hydrogen
vir	Virulence genes
X-gluc	5-Bromo-4-chloro-3-indolyl β-D glucuronide
YEP	Yeast Extract Peptone

Introduction

1. INTRODUCTION

Medicinal plants are the most important source of life saving drugs for majority of the world's population. Human beings have been utilizing plants for basic preventive and curative health care since time immemorial. The World Health Organization has estimated that more than 80% of the world's population in developing countries depends primarily on herbal medicine for basic healthcare needs. Recent estimates suggest that over 50,000 plants have known medicinal applications in various cultures and countries. Medicinal plants have played significant role in many ancient traditional systems of medicine in Asia such as the Ayurveda, Unani and Chinese medicine. Increasing demand for medicinal plants during the past 20 years has brought surging markets and production opportunities for these plant species.

Medicinal plants contain many secondary metabolites, which are responsible for their medicinal properties. Yield and quality of medicinal plants can be improved by biotechnological tools by gene alteration, or introduction of a new gene. Rapid progress in the area of crop biotechnology has enabled the development of efficient regeneration and suitable genetic transformation protocols for different crop species. Similar success can be achieved in medicinal plants by developing efficient regeneration and genetic transformation protocols, which in turn could be used for the enhancement of their secondary metabolite content. Genetic transformation has been reported for various medicinal plants. Efficient transgene delivery system based on *Agrobacterium tumefaciens* are well established for *Taxus* spp, *Echinacea purpurea, Scrophularia, Digitalis lanata, Thalictrum* and *Artemisia annua.*

Although the primary target for trait manipulation in medicinal plants is to improve the active compounds, the basic agronomic characters related to uniformity, stability, growth and development and resistance to biotic and abiotic stresses must also be improved. Metabolic engineering is emerging as one of the important approaches to

improve and modify secondary metabolite contents of medicinal plants. A major line of research has been to identify enzymes of a metabolic pathway and then manipulate these enzymes to provide better control of that pathway. Recently, some examples of successful genetic manipulation of secondary metabolite pathway through metabolic engineering for increased metabolite content and exploitation of the plants as bioreactors for the production of natural or recombinant secondary metabolites of commercial interest have been reported.

Centella asiatica, commonly known as Indian Pennywort belonging to the family Apiaceae, native to India and Southeast Asia is revered as one of the great multipurpose miracle herbs of oriental medicine. *Centella asiatica* has a long history of use to promote wound healing and treat skin diseases. It is a useful tonic and cleansing herb for skin problems and digestive disorders. In India, it is chiefly valued as a revitalizing herb that strengthens nervous function and memory. *Centella asiatica* contains a blend of compounds including at least three triterpenes (asiatic acid, madecassic acid and asiaticoside) that appear to have antioxidant properties and tissue regenerative property by stimulating collagen synthesis. The wound healing property of the species is ascribed to the triterpenoid saponin, asiaticoside. The anti tumour property is attributed to madecassic acid.

The triterpene asiaticoside present in the Indian cultivars is only one per cent. For the commercial extraction, the asiaticoside content should be at least three per cent. Hence, there is a need to improve the asiaticoside content in the plant. Conventional breeding methods have limitations in improving the asiaticoside content. Genetic transformation is a viable alternative to improve the content of asiaticoside in *Centella.* Till date, no report has been published regarding genetic transformation of the plant, though experiments have been done to develop reliable protocol for shoot regeneration and somatic embryogenesis.

Agrobacterium tumefaciens mediated transformation is simple and efficient in providing stable integration of transferred DNA into the plant genome. The present study was undertaken with the main objective of evolving a protocol for *Agrobacterium tumefaciens* mediated genetic transformation in *Centella asiatica*, which could further be utilized for the metabolic engineering of *Centella* to enhance the level of secondary metabolites.

Review of Literature

2. REVIEW OF LITERATURE

Plants play a dominant role in the introduction of new therapeutic agents. Drugs from plants continue to occupy an important niche in modern medicine. The study "*Agrobacterium tumefaciens* mediated genetic transformation in kudangal (*Centella asiatica* L. Urban.)" aims at standardization of protocol for *Agrobacterium tumefaciens* mediated transformation in *Centella* for transferring desirable genes.

Centella is a very valuable plant from medicinal point of view. It is an important ingredient of many ayurvedic formulations. *Centella asiatica* contains a blend of compounds including triterpenes (asiatic acid, madecassic acid and asiaticoside) that appear to have antioxidant, tissue regenerative and memory enhancing 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.

Till date, no report has been published regarding genetic transformation of *Centella*, though experiments have been done to develop reliable protocol for shoot regeneration and somatic embryogenesis. In this chapter, literature on *Agrobacterium tumefaciens* mediated genetic transformation of medicinal plants has been reviewed.

2.1. MEDICINAL PROPERTIES OF *CENTELLA ASIATICA*

Appa 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.

Giardina *et al.* (1987) reported that modern drugs comprising the pharmacologically active triterpenoid fractions and glycosides such as asiaticoside and madecassoside 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).

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 may 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. AF 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 asiaticoside, an isolated constituent of *Centella asiatica*, 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 highest during the initial stages of treatment (Shukla *et al.*, 1996).

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. According to Rao (2003) Ayurveda describes a group of plant drugs as "medhyarasayanas" (brain tonics). Among these brahmi and *Centella asiatica* occupy the place of pride.

2.2 *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 MS medium supplemented with 2 mg l^{-1} Kn and 4 mg l^{-1} NAA. The rate of shoot bud regeneration was the highest after 4 weeks of subculture on 4 mg l^{-1} BA, 2 mg l^{-1} Kn, 0.25 mg l^{-1} NAA and 20 mg l^{-1} adenine sulphate.

Rao *et al.* (1999) studied the influence of auxins and cytokinins on the production of callus in *Centella asiatica*. The stem explant stolon, proved to be the best for callus induction followed by leaf base. Kinetin supplementation at 0.25 and 0.5 mg l^{-1}along with auxin, 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 in the production of multiple shoots. BAP (2 mg l^{-1}) and IBA (0.1 mg l^{-1}) produced multiple shoots within 2 weeks. Maximum number of healthy shoots/ explant and rapid elongation of shoots was observed at 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 syngergistic 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 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 0.1mg 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.

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.

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 found 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 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 1mg l^{-1} NAA. *In vitro* rooting was induced when MS medium was supplemented with IAA 1mg 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.

2.3 PRODUCTION OF SECONDARY METABOLITES FROM MEDICINAL PLANTS

Plants are the traditional source of many chemicals used as pharmaceuticals. 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 (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.

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).

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. Hahn *et al.* (2003) reported the production of ginsenoside from adventitious root cultures of *Panax ginseng* through large-scale bioreactor system (1-10 ton).

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 Murashige and Skoog 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 to 1.53 and 1.41 fold respectively. Maximum asiaticoside production was achieved following the 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).

2.4 IMPROVEMENT OF MEDICINAL PLANTS BY GENETIC MANIPULATION

2.4.1 Gene Transfer Methods in Medicinal Plants

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.

The *Agrobacterium* mediated gene transfer system requires physically injured plant cells that the bacteria can enter. The process also requires the regeneration of the explant into a viable plant. 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 phosphotransferase 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.

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%). 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.

Although the success of *Agrobacterium* vectors was paramount, the technique continues to have problems and limitations. Since its discovery, microprojectile mediated transformation has gained considerable attention, primarily due to its reputation as a universal delivery system. Genetically transformed *Catharanthus roseus* plantlets were obtained after bombardment of nodal explants, which were then micropropagated, with DNA coated particles with green fluorescent protein (*gfp*) or β-glucuronidase (*gus*) reporter genes. Histological studies showed that the gene insertion method proved effective with many cells and different tissues displaying the reporter gene signals, showing that gene expressions were rather stable (Zarate *et al.*,1999).

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 per cent 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.

2.4.1.1 *Genes Transferred by Various Methods*

Genes of interest, including marker or reporter genes, are vectored on short units of DNA called 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).

Yun *et al.* (1992) reported that hydroxylase gene from *Hyoscyamus niger* under the control of the CaMV 35S promoter was introduced by a leaf disc transformation system into *Atropa belladona* which accumulates hyoscyamine as its main alkaloid.

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 was 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.

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 (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.

Panax ginseng, resistant to the herbicide Basta, has been generated by transformation with the enzyme phosphonithricin acetyl transferase (Choi *et al.*, 2003). Morphological variation introduced to *Taraxacum platycarpum* regenerated from hairyroot cultures was ascribed to the developmental effects of *rol* genes introduced from *A. rhizogenes* (Lee *et al.*, 2004).

2.4.2 Metabolic Pathway Engineering in Medicinal Plants

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. Charlwood and Pletsch (2002) reported that pathway engineering will lead to improvement of potential value in the breeding of medicinal plants.

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 transferring 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*. 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. 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.

2.4.3 Engineering Agronomic Traits in Medicinal Plants

The first targets for medicinal plant biotechnology were resistance to herbicides, pests and diseases. There have been relatively few reports of biotechnology applied to

non-biotic stresses and other aspects of agronomic performance, although the approach is considered to have great potential.

Somatic hybridization, the fusion of somatic cells from tissue culture and atrazine selection was used to regenerate herbicide-tolerant *Solanum nigrum* (black nightshade) (De Donato., 1990). Saito (1992) developed transgenic *Atropa* plants resistant to the herbicides bialaphos and glufosinate using Ri binary vector.

Geng (2001) observed 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. Chen and Punja (2002) obtained *Panax quinquefolium* (American ginseng) transformed with either a chitinase or a thaumatin-like antifungal gene which regenerated successfully.

Choi *et al.* (2003) reported the generation of *Panax ginseng*, resistant to the herbicide Basta, by transferring the enzyme phosphinothricin acetyl transferase. Morphological variation introduced to *Taraxacum platycarpum* regenerated from hairy root cultures was ascribed to the developmental effects of the *rol* genes introduced from *A. rhizogenes (*Lee *et al.*, 2004).

2.5 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION IN MEDICINAL PLANTS

2.5.1 Factors Affecting *Agrobacterium* Mediated Gene Transfer in Medicinal Plants

2.5.1.1 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

weeks 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 californica)* 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 californica* by co-cultivation of California poppy 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).

2.5.1.2 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 we get the genotype/strain combination. 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 pNOS 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).

2.5.1.3 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 mgl^{-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 at 600 nm) for 15 minutes.

2.5.1.4 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 1 day on regeneration medium was 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.

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 2-5 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.

2.5.1.5 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.

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.

2.5.1.6 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.

Vergauwe *et al* (1996) working on transformation of *Artemisia annua* L found that cefotaxime at 50 mg l^{-1} was effective as a decontaminating antibiotic; but it caused retardation in callus formation. Then they tried vanomycin at 750 mg l^{-1} which was not 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.

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.

2.5.1.7 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 analyse 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, 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. Neomycin phosphotransferase II (*nptII*) gene from transposon Tn5, detoxifies neomycin, kanamycin and G418 by phosphorylation. (Fraley *et al.*, 1983). It is a widely used selectable marker 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 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 seed (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).

2.5.1.8 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 not be so toxic to plant cells, 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 $\mu g\ ml^{-1}$ 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).

For selection and regeneration of plantlets in *Echinacea purpurea*, 640 leaf sections were co-cultivated with *Agrobacterium* for 48 hrs and transformed 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 mgl^{-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 7 days and selection by 100mg l^{-1} kanamycin.

2.5.1.9 Histochemical Analysis of GUS Expression

Reporter genes produce a product that has a distinct and easily detectable phenotype. With most reporter genes, successful transgene expression can be determined through a visual assay. The reporter gene assay is an invaluable step as it eliminates many of the transformants to a selected number of possible successful integrations. The most common reporter gene used in plant transformation is the *gus* gene isolated from *E coli* (Jefferson *et al.*, 1986). It encodes a stable enzyme that is not normally present in plants and that catalyzes the cleavage of a range of β-D- glucuronides. The GUS activity in transformed plant tissues can be localized by observing the blue colour that is formed

after hydrolysis of the uncoloured substrate 5-bromo 4-Chloro 3-indolyl β-D-glucuronic acid (X-gluc).

Vergauwe *et al.* (1996) in *Artemisia annua* performed GUS histochemical staining of transformed tissues and found that 94% of the transgenic plants showed a β-glucuronidase positive response. Dronne *et al.* (*1999*) in lavandin confirmed transgene expression using histoenzymatic β-glucuronidase assays, leaf callus assays and RT-PCR. Results showed that both β-glucuronidase and neomycin phosphotransferase II genes were expressed at a high level in at least 41 of the transgenic plants regenerated.

In *Echinacea*, Koroch *et al.* (2002) reported that leaf sections were assayed for GUS activity for 4 days after the co-cultivation period. Several morphologically normal appearing kanamycin resistant plantlets did not exhibit GUS staining in histochemical GUS assays, while most regenerated plantlets were GUS positive. Zaldivar *et al.* (2003) reported that histochemical GUS assay of infected hypocotyls from two *Bixa orellana* varieties showed transient *gus* gene expression between 3 and 12 days after inoculation.

Histochemical assay to detect the GUS activities in the four putative transformants were performed on the leaves of kanamycin resistant brahmi plants selected at random (Nisha *et al.*, 2003). The leaves showed pronounced GUS activity along the midrib region. Sugimura *et al.* (2005) performed histochemical assay of transformed tissues in patchouli. Results showed GUS expression in every organ of transformants.

2.5.1.10 PCR Analysis of Transformants

Confirmation of the putative transformants is usually done by Polymerase Chain Reaction (PCR) using the primer designed for the marker gene.

Vergauwe *et al.* (1996) in *Artemisia annua* performed PCR of transformed tissues and DNA sequencing of the amplification products. The results revealed that 75% 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.

Materials and Methods

3. MATERIALS AND METHODS

The experiments on *Agrobacterium tumefaciens* mediated genetic transformation in kudangal (*Centella asiatica L.* Urban.) were carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during January 2004 to February 2006. 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 and Laboline to remove soil particles. They were trimmed and subjected to surface sterilization using mercuric chloride (0.08%) for five minutes. They were 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, Plastic ware and Other Materials

Borosilicate glassware and disposable sterile petridishes were purchased from Tarsons, India. The membrane filters used were from Sartorius, Germany.

3.2.3 Composition of Media

Basal MS medium (Murashige and Skoog, 1962) supplemented with various plant growth substances, 2, 4-dichlorophenoxyacetic acid (2,4-D), benzyl adenine (BA),

α-naphthalene acetic acid (NAA), kinetin (Kn), adenine sulphate (ADS), gibberellic acid (GA), indole acetic acid (IAA) and organic supplements like coconut water were used for plant tissue culture experiments (Appendix I).

For maintenance of *Agrobacterium* strains, Yeast Extract Peptone (YEP) medium (An *et al.*, 1988) and AB minimal medium were used (Appendix II).

3.2.4 Preparation of Medium

Standard procedures (Murashige and Skoog, 1962) were followed for the preparation of the plant tissue culture media. The pH of the medium was adjusted to 5.7 using 0.1N NaOH / HCl. Agar was added at the rate of 6.0 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 1.05 kg cm^{-2} and a temperature of 120^0 C for 15 min. The medium was then stored in culture room at 25 ± 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 in culture room 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 -80^0 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 -80^0 C.

3.2.5.3 Rifampicin

A stock solution (10^4 mg l^{-1}) was prepared by dissolving rifampicin first in ethanol and then in sterile water. It was then filter sterilized and stored at -80^0 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 40^0C.

3.2.6 *Agrobacterium* Strains

Two strains of *Agrobacterium tumefaciens viz.,* LBA4404 and EHA105 were used for the study. EHA105 by virtue of harbouring the 'supervirulent', *'vir'* gene, exhibit broader host range and higher transformation efficiency. The bacterial kanamycin resistance gene in EHA101 was deleted to develop the '*vir*' helper strain EHA105.

Two *Agrobacterium* strains with the binary vector, pCAMBIA2301 were used for the study.

3.2.6.1 pCAMBIA2301 (Hajdukiewicz *et al.*, 1994)

The T-DNA of this binary vector contains the *gus A* gene and the *nptII* (Kanamycin resistance) gene under the control of CaMV 35S promoter (Appendix III).

3.2.6.2 Maintenance of Agrobacterium Strains

The *Agrobacterium tumefaciens* strains *viz.*, LBA4404 and EHA105 harbouring the binary vector, pCAMBIA2301 were grown on YEP medium supplemented with kanamycin 100 mg l^{-1} and rifampicin 10 mg l^{-1}.

3.3 INOCULATION AND OTHER ASEPTIC MANIPULATIONS

All the aseptic procedures 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 hr photoperiod (1000 lux) supplied by cool white fluorescent tubes. 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, NAA and BA. Cultures were incubated at 25 ± 2^0 C in culture room with 16 h photoperiod (1000 lux). Observations were recorded on the percentage establishment of explants and percentage of callusing.

3.6 REGENERATION FROM THE CALLUS

The callus obtained were transferred to MS medium with varying concentrations and combinations of Kn, NAA, 2, 4-D, BA, ADS, and GA 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, 300, 400 and 500 mg l^{-1}) or cefotaxime (0, 5, 25, 50, 75, 100, 125, 150, 200, 300, 400 and 500 mg l^{-1}). The petriplates were sealed with parafilm and kept at 25 ± 2^0 C. The culture response of the explants 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 YEP medium was transferred to YEP medium with different concentrations of kanamycin or cefotaxime (5, 25, 50, 75, 100, 125, 150, 200, 300, 400 and 500 mg l^{-1}). Petriplates were then sealed with parafilm and it was incubated at 28^0C 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* strains *viz.*, LBA 4404 and EHA 105 with the binary vector pCAMBIA2301 were grown on petriplates containing AB minimal medium with kanamycin 100 mg l^{-1} at 28^0 C overnight. *Agrobacteruium* suspension for co-cultivation was prepared by picking a single colony from the plate and inoculating into AB broth supplemented with suitable antibiotics (kanamycin 100 mg l^{-1}). AB broth with the bacterial strain was kept in a shaker overnight at 28^0C at 10 rpm. The next day the culture was spun in a centrifuge at 5000 rpm at 4^0C for 5 minutes. The pellets obtained were resuspended in 1ml 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 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.

3.9.3 Co-cultivation

The pre-cultured calli were used for co-cultivation. They were cut using sterile blade to facilitate wounding and infection process.

The callus pieces were placed in a sterile petriplates and wetted with liquid half MS medium to avoid drying of explants. The explants were then mixed thoroughly with the prepared *Agrobacterium* suspension (containing100μM acetosyringone) by gentle swirling for 15 and 20 min to standardize the optimum time required for the infection process. The explants were blot dried with sterile filter paper and transferred to a petriplate containing solidified half MS medium. Petriplates were sealed with parafilm and kept for co-cultivation in dark for three/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 75 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 75 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 mg l^{-1}) and cefotaxime (75 mg $l^{-1)}$. The tissues were maintained by subculturing once in seven days in the same medium. After two rounds of subculture, the transformed and non-transformed tissues were scored based on the colour formed. Then the transformed tissues were subjected to GUS histochemical assay.

3.10 GUS HISTOCHEMICAL ASSAY

A histochemical assay was used to detect the expression of *gus* gene, β-glucuronidase activity (Jefferson *et al.*, 1987). The putative transformants were incubated in GUS substrate, X-gluc (5-bromo-4-chloro-3-indolyl β-D glucuronide) for 24-30 hours in dark. X-gluc solution was prepared by mixing the following; l0 mg x-gluc in 100 µl dimethylformamide (DMF), 1 ml 1M sodium phosphate (pH 7.0), 1 ml triton (from a 20% stock solution) and 18 ml sterile water. The enzyme (β glucuronidase) converts X-gluc to an insoluble, intense, indigo-blue chromophore. GUS expressing cells were detected as blue spots on the explant under a microscope. Each spot was scored as one transformation event, regardless of its size (Puddephat *et al.*, 1999). The GUS expressing cells were then transferred to regeneration medium.

Results

4. RESULTS

The results of the experiments on *Agrobacterium tumefaciens* mediated genetic transformation in *Centella asiatica* carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during January 2004 to February 2006 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

The effect of different growth regulators on callus induction was studied. There was a considerable variation in the percentage of callus induction and time taken for callus induction in the different treatments. The minimum time taken for callus induction from leaf (20.50 days) was recorded in MS medium containing Kn 2 mg l^{-1} and NAA 3 mg l^{-1} (C5). The per cent induction of callus was 80. The maximum callus induction (85.70%) was observed in the medium containing Kn 2 mg l^{-1} and NAA 4 mg l^{-1} (C6). However, the time taken for callus induction was 25.50 days. Treatments C1, C2, C3, C15 and C18 induced callus from leaves in 24.50, 24.50, 24.33, 24.00 and 24.00 days, respectively. Treatments C7 and C8 took more time for callus induction from leaves (29.00 and 30.00 days, respectively). The per cent induction of callus was also less (< 70 per cent). The lowest callus induction percentage from leaves (60.00) was observed in the treatments C2 and C7 (Table 1).

Time taken for callus induction from node (21.00 days) was minimum in the medium containing 2, 4-D 1 mg l^{-1} and Kn 1 mg l^{-1}. The callus induction percentage was 78.50. The maximum callus induction percentage (86.67) was recorded in the medium containing Kn 2 mg l^{-1} and NAA 3 mg l^{-1}(C5) and that supplemented with Kn 1 mg l^{-1} and NAA 3 mg l^{-1} (C1). The time taken for callus induction in the above media was 23.67 and 22.00 days, respectively. Treatments C2, C5, C7, C13, C17 and C18 induced callus from nodes in 22.50, 22.00, 22.33, 21.50, 22.25 and 22.40 days,

respectively. The minimum callus induction (66.60%) was observed in the treatment C2. (Table 1)

4.1.1.2 Nature of Callus

The nature of the callus varied with the type of explants used. Brown friable callus was obtained from leaves and cream friable callus from node (Plate 1 and 2).

4.1.2 Regeneration of *Centella asiatica* from Callus

4.1.2.1 Effect of Growth Regulators on in vitro Regeneration from Callus

The effects of growth regulators on *in vitro* regeneration from callus were studied (Table 2). Of the different media composition tried, regeneration from callus was obtained on MS medium supplemented with Kn 2mg l^{-1}, BA 4 mg l^{-1}, NAA 0.25 mg l^{-1} and ADS 20 mg l^{-1} (R3) in 73.00 days (Plate 3). The percentage regeneration of callus in the above treatment was 16.67. The other treatments did not produce regeneration, however, the callus remained green in most of the regeneration media tried (R1, R2, R4, R5, R6, R7, R8, R9, R10, R14, R15, R16, R17, R18, R19, R20, R21, R22, R38) showing the regeneration potential of the callus. In the treatments R11, R12, R13, R23, R24, R25, R26, R27,R28, R33, R34, R35, R36 and R37, the callus produced was cream.

4.1.2.2 Effect of Coconut Water on in vitro Regeneration from Callus

Addition of coconut water 150 and 200 ml l^{-1} along with other growth regulators did not have any effect on regeneration from callus (Table 3). However, the callus turned green, indicating the regeneration ability.

4.1.2.3 Nature of Callus in Regeneration Medium

The callus remained green in most of the regeneration media tried, indicating the regeneration potential. However, regeneration was not observed even after three months of culture in the same regeneration media.

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

Medium: MS (full strength) + sucrose 30 g l^{-1} + agar 6g l^{-1}

Treatment No.	Growth regulators (mg l^{-1})	Time taken for callus induction(days)		Percent induction of callus	
		Leaf	Node	Leaf	Node
C1	Kn 1 + NAA 3	24.50	23.67	73.34	86.67
C2	Kn 1 +NAA 4	24.50	22.50	60.00	66.60
C3	Kn 1.5 +NAA 3	24.33	23.00	84.60	81.80
C4	Kn 1.5 +NAA 4	23.66	29.00	77.78	73.34
C5	Kn 2+ NAA 3	20.50	22.00	80.00	86.67
C6	Kn 2+ NAA 4	25.50	24.50	85.70	80.00
C7	2,4-D 2+BA 0.5	29.00	22.33	60.00	78.50
C8	2,4-D 2 +BA 1	30.00	27.33	66.60	73.30
C9	2,4-D 1+BA 0.5	25.67	25.25	84.60	80.00
C10	2,4-D 1+ BA 1	27.33	24.00	66.60	75.00
C11	2,4-D 0.5+ BA 2	32.50	32.50	84.60	86.60
C12	2,4-D 1+ BA 2	25.50	25.00	73.34	80.00
C13	2,4-D 2+Kn 1	25.00	21.50	80.00	85.70
C14	2,4-D 1+Kn 0.5	25.33	20.66	84.60	85.70
C15	2,4-D 1+Kn 1	24.00	21.00	73.34	78.50
C16	2,4-D 0.5+Kn 1	21.75	24.00	84.60	80.00
C17	2,4-D 0.5+Kn 2	23.00	22.25	78.50	73.30
C18	2,4-D 1+Kn 2	24.00	22.40	80.00	78.50

Table 2. Response of *Centella* callus to different compositions of regeneration medium

Medium: MS (full strength) + sucrose 30 g l^{-1} + agar 6g l^{-1}

Regeneration medium No.	Composition of regeneration medium (mg l^{-1})	Response of callus
R1	BA 2 + Kn 1	Callus turned green
R2	BA 3+ Kn 2 + NAA 0.25+ ADS 20	Callus turned green
R3	BA 4+ Kn 2 + NAA 0.25+ ADS 20	Regeneration of shoots was observed from callus
R4	BA 4+ Kn 3+ NAA 0.25+ ADS 20	Callus turned green
R5	BA 5+ Kn 2 + NAA 0.25+ ADS 20	Callus turned green
R6	BA 2+ Kn 1 + NAA 0.25+ ADS 20	Callus turned green
R7	BA 3+ Kn 1 + NAA 0.5+ ADS 20	Callus turned green
R8	BA 3+ Kn 1 + NAA 0.5	Callus turned green
R9	BA 3+ Kn 2 + NAA 0.25	Callus turned green
R10	NAA 0.5+ Kn 2 +ADS 20	Callus turned green
R11	BA 3+ NAA 1	Callus turned cream
R12	BA 4+ NAA 1	Callus turned cream
R13	BA 3+ NAA 0.5	Callus turned cream
R14	BA 3+ NAA 0.5+ADS 20	Callus turned green
R15	BA 1+Kn 2 + NAA 0.25+ ADS 20	Callus turned green
R16	BA 1+Kn 1+NAA 0.25 +ADS 20	Callus turned green
R17	BA 1+Kn 4 +NAA 0.25 +ADS 20	Callus turned green
R18	BA 2+Kn 2+NAA 0.25 +ADS 20	Callus turned green
R19	BA 2+Kn 4 +NAA 0.25 +ADS 20	Callus turned green
R20	BA 4+Kn 1+NAA 0.25 +ADS 20	Callus turned green
R21	BA 4+Kn 4+NAA 0.25 +ADS 20	Callus turned green
R22	BA 4+Kn 2+NAA 0.25	Callus turned green
R23	BA 4+Kn 2	Callus turned cream
R24	BA 3	Callus turned cream
R25	Kn 3	Callus turned cream
R26	BA 2	Callus turned cream
R27	BA 2+IAA 1	Callus turned cream
R28	ADS 20	Callus turned cream
R29	BA 2+ Kn 2+ GA 2	Callus turned brown
R30	BA 4+ GA 2	Callus turned brown
R31	BA 4+ Kn 2+ GA 5	Callus turned brown
R32	GA 5	Callus turned brown
R33	2,4-D 0.02+ BA 0.8	Callus turned cream
R34	2,4-D 0.05+ BA 2	Callus turned cream
R35	2,4-D 0.02+ BA 2	Callus turned cream
R36	2,4-D 0.02+ BA 1	Callus turned cream
R37	2,4-D 0.02+ BA 2+ NAA 1	Callus turned cream
R38	2,4-D 0.02+ BA 2 +ADS 20	Callus turned green

Table 3. Response of *Centella* callus to different compositions of regeneration medium

Medium: MS (full strength) + sucrose 30 g l^{-1} + agar 6g l^{-1}

Regeneration medium	Composition of regeneration medium (mg l^{-1})	Response of callus
R39	BA 4 + Kn 3+ NAA 0.25 + ADS 20 + coconut water 150ml l^{-1}	Callus turned green
R40	BA 4 + Kn 3+ NAA 0.25 + ADS 20 + coconut water 200ml l^{-1}	Callus turned green
R41	BA 4 + Kn 3+ NAA 0.25 + coconut water 150ml l^{-1}	Callus turned green
R42	BA 4 + Kn 3+ NAA 0.25 + coconut water 200mg l^{-1}	Callus turned green
R43	BA 2 + coconut water 150mg l^{-1}	Callus turned green
R44	coconut water 200ml l^{-1}	Callus turned green

Plate 1: Callus induced from node (Kn 1 mg l^{-1} + 2, 4-D 1 mg l^{-1})

Plate 2: Callus induced from leaf (Kn 2 mg l^{-1} + NAA 3 mg l^{-1})

Plate 3: Regeneration from callus

Plate 4: Multiple shoot induction from node

4.1.3 Multiple Shoot Induction from Nodal Explants

Multiple shoots were induced from nodes in the medium supplemented with BA 1.5 mg l^{-1} and IAA 1 mg l^{-1} in 20.50 days. An average of 3.5 shoots per node was obtained (Plate 4).

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 (5-500 mg l^{-1}) was recorded. In kanamycin 5 mg l^{-1}, the tissues remained green up to six weeks, later became partially discoloured and bleached after eight weeks. The percentage survival of callus in kanamycin 5 mg l^{-1} after eight weeks was 33.2.

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 22.2 and 16.7, respectively.

The *Centella* 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 of inoculation. The percentage survival of callus after eight weeks was 11.2.

The tissues remained green up to three weeks in medium containing 100 mg l^{-1} of kanamycin. They were partially discoloured after four weeks and bleached after five weeks of inoculation. The tissues turned brown and dead after six weeks of inoculation and the percentage survival of callus was found to be 11.2 after eight weeks.

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

Sl No.	Kanamycin mg l^{-1}	Sensitivity (weeks)								Survival per cent (after 8 weeks)
		I	II	III	IV	V	VI	VII	VIII	
1	Nil	++++	++++	++++	++++	++++	++++	++++	++++	100
2	5	++++	++++	++++	++++	++++	++++	+++	++	33.2
3	25	++++	++++	++++	++++	+++	+++	++	++	22.2
4	50	++++	++++	++++	++++	+++	++	+	+	16.7
5	75	++++	++++	++++	++++	+++	++	+	+	11.2
6	100	++++	++++	++++	+++	++	+	+	+	11.2
7	125	++++	++++	++++	+++	++	+	+	+	0
8	150	++++	++++	+++	++	++	+	+	+	0
9	200	++++	++++	+++	++	+	+	+	+	0
10	300	++++	++++	+++	++	+	+	+	+	0
11	400	++++	++++	+++	++	+	+	+	+	0
12	500	++++	+++	++	+	+	+	+	+	0

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

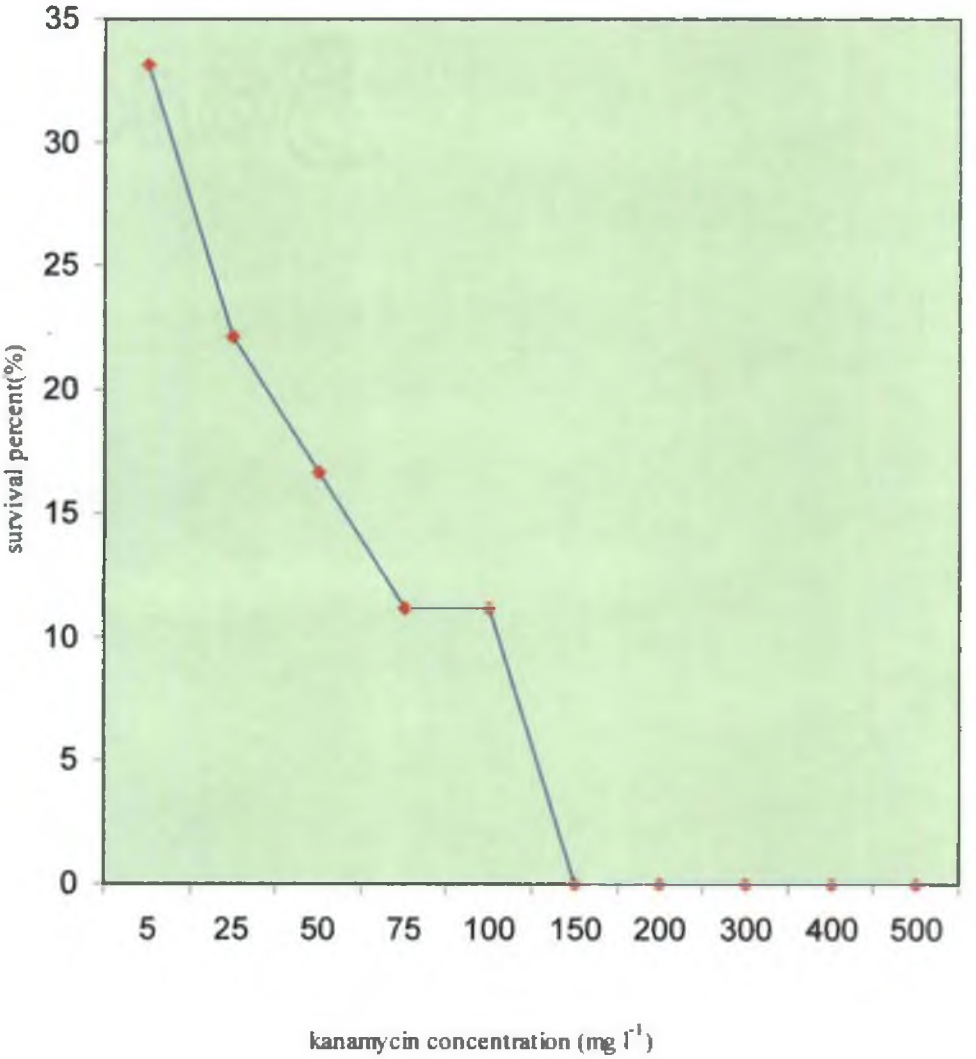
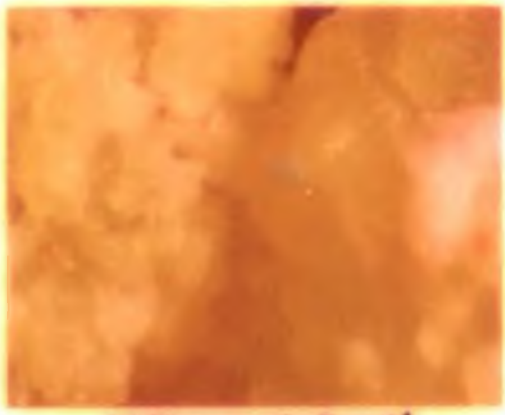


Fig.1. Survival of *Centella* tissues in kanamycin



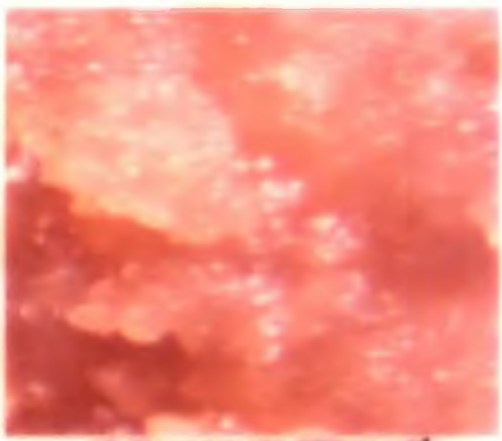
(a) Kanamycin 5 mgl^{-1}

(b) Kanamycin 75 mgl^{-1}

(c) Kanamycin 100 mgl^{-1}

(d) Kanamycin 150 mgl^{-1}

(e) Kanamycin 300 mg l^{-1}



(f) Kanamycin 500 mg l^{-1}

Plate 5: Sensitivity of *Centella* callus to different concentrations of kanamycin

In kanamycin 150-400 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 kanamycin 150 - 500 mg l^{-1}.

The tissues were green only up to one week after inoculation in kanamycin 500 mg l^{-1}. Later 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 (Table 4).

The maximum survival percentage (33.2) was observed in kanamycin 5 mg l^{-1} and minimum survival percentage was observed in kanamycin 75 and 100 mg l^{-1} (Figure1). For the selection of putative transformants, kanamycin was used at a concentration of 100 mg l^{-1} (Plate 5).

4.2.2 Cefotaxime

Sensitivity of *Centella* callus to varying concentrations of cefotaxime (5-500 mg l^{-1}) was studied. 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 71.5.

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 42.9 per cent of the callus survived after eight weeks in 25 mg l^{-1} 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 33.4 and 22.3, respectively.

Table 5. Sensitivity of *Centella* callus to different doses of cefotaxime

Sl No.	Cefotaxime mg l^{-1}	Sensitivity (weeks)								Survival per cent (after 8 weeks)
		I	II	III	IV	V	VI	VII	VIII	
1	Nil	++++	++++	++++	++++	++++	++++	++++	++++	100
2	5	++++	++++	++++	++++	++++	++++	+++	++	71.5
3	25	++++	++++	++++	++++	++++	+++	++	++	42.9
4	50	++++	++++	++++	++++	+++	++	++	+	33.4
5	75	++++	++++	++++	++++	+++	++	++	+	22.3
6	100	++++	++++	++++	+++	+++	++	++	+	22.3
7	125	++++	++++	++++	+++	+++	++	+	+	11.2
8	150	++++	++++	++++	+++	++	++	+	+	0
9	200	++++	++++	++++	+++	++	+	+	+	0
10	300	++++	++++	++++	+++	++	+	+	+	0
11	400	++++	++++	+++	+++	++	+	+	+	0
12	500	++++	+++	+++	+++	++	+	+	+	0

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

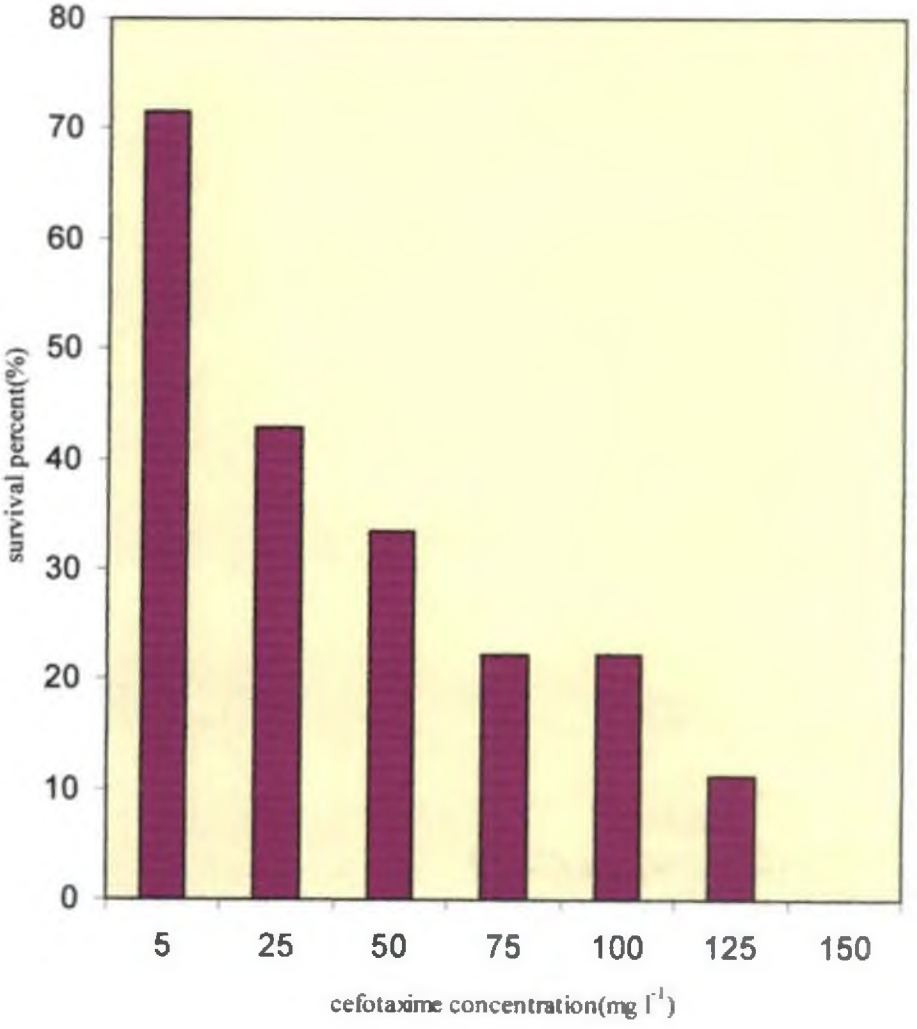
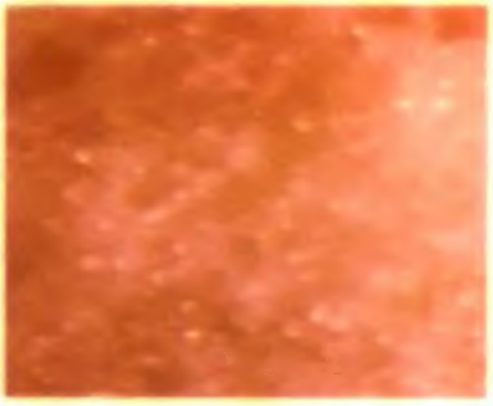
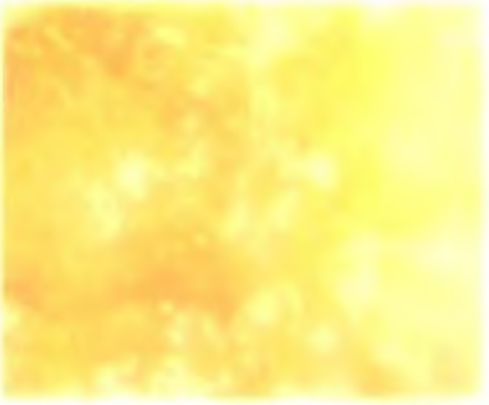


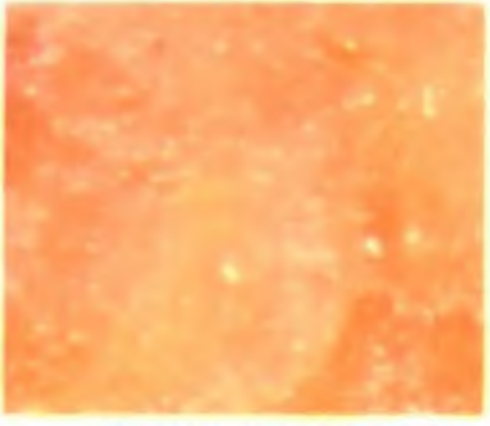
Fig.2. Survival of *Centella* tissues in cefotaxime



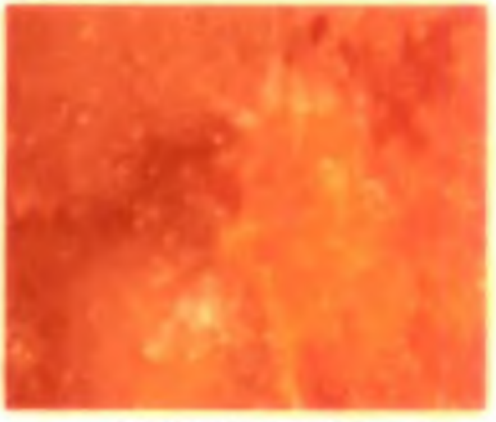
(b) Cefotaxime 5 mgl^{-1}



(b) Cefotaxime 75 mgl^{-1}

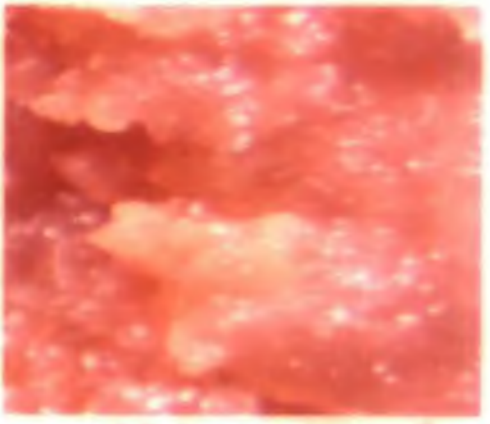


(c) Cefotaxime 100 mgl^{-1}



(d) Cefotaxime 150 mgl^{-1}

(e) Cefotaxime 300 mgl^{-1}



(f) Cefotaxime 500 mgl^{-1}

Plate 6: Sensitivity of *Centella* callus to different concentrations of cefotaxime

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 22.3. In cefotaxime 125 mg l^{-1}, the tissues remained green up to three weeks, were partially discoloured after four weeks and bleached after six weeks. After seven weeks of inoculation, the tissues turned brown and dead and the percentage survival of callus after eight weeks was 11.2.

When cefotaxime 150 mg l^{-1} was used, the tissues remained green up to three weeks, became partially discoloured after four weeks and bleached after five weeks. After seven weeks of inoculation, the callus turned brown and dead. The percentage survival of callus after eight weeks was zero.

In cefotaxime 200 mg l^{-1} and 300 mg l^{-1}, the tissues were green up to three weeks after inoculation, were partially discoloured after four weeks and bleached after five weeks. The tissues turned brown and dead after six weeks of culture and the survival percentage of callus after eight weeks was zero.

The tissues remained green up to two weeks in cefotaxime 400 and 500 mg l^{-1}. They were partially discoloured after three weeks and bleached after four weeks. The callus turned brown and dead after eight weeks and none of the callus survived after eight weeks of inoculation in the media containing cefotaxime 400 and 500 mg l^{-1} (Table 5).

The maximum survival percentage (71.5) was observed in cefotaxime 5 mg l^{-1} and minimum survival percentage was observed in cefotaxime 125 mg l^{-1} (Figure 2). For the elimination of *Agrobacterium* after co-cultivation, cefotaxime was used at a concentration of 75 mg l^{-1} (Plate 6).

4.3 SENSITIVITY OF *AGROBACTERIUM* STRAINS TO ANTIBIOTICS

The sensitivity of the bacterial strains LBA4404 and EHA105 harbouring pCAMBIA2301 to different doses of antibiotics is presented below.

4.3.1 Kanamycin

The growth of *Agrobacterium* strains LBA4404 and EHA105 containing the plasmid vector pCAMBIA2301 in YEP medium containing different concentrations of kanamycin (5-500 mg l^{-1}) after two days of culture was observed (Table 6). The bacterial cultures were resistant to kanamycin up to a concentration of 300 mg l^{-1}. No bacterial growth was observed in YEP medium containing kanamycin 350, 400 and 500 mg l^{-1}.

4.3.2 Cefotaxime

The growth of *Agrobacterium* strains LBA4404 and EHA105 containing the plasmid vector pCAMBIA2301 in YEP medium containing different concentrations of cefotaxime (5-500 mg l^{-1}) after two days of culture was observed (Table 7). Bacterial growth was observed in YEP medium containing cefotaxime 5, 25 and 50 mg l^{-1}. No bacterial growth was observed in YEP medium containing cefotaxime at concentrations above 50 mg l^{-1} for both the strains.

4.4 GENETIC TRANSFORMATION IN *CENTELLA ASIATICA*

4.5.1. Effect of Infection Time

The effect of infection time on transformation efficiency was recorded. Infection was carried out for 15 and 20 minutes to standardize the optimum time required for transformation. Transformation was effective both in 15 and 20 minutes infection period. With LBA4404 (pCAMBIA2301) in 15 minutes of infection period the transformation efficiency was 16.67 per cent. As the infection time was extended to 20 minutes an increased transformation efficiency of 35.71 per cent was observed. When transformation was done using node, the transformation efficiency obtained was 40 per cent with 20 minutes infection (Table 8).

Table 6. Sensitivity of *Agrobacterium tumefaciens* strains to kanamycin

Sl No.	Kanamycin mg l^{-1}	Bacterial growth *	
		LBA4404	EHA105
1	0	+	+
2	5	+	+
3	25	+	+
4	50	+	+
5	75	+	+
6	100	+	+
7	150	+	+
8	200	+	+
9	300	+	+
10	350	-	-
11	400	-	-
12	500	-	-

* Mean of three replications
\+ Bacterial growth
\- No bacterial growth

Table 7. Test for bacteriocidal activity of cefotaxime

Sl No.	Cefotaxime mg l^{-1}	Bacterial growth*	
		LBA4404	EHA105
1	0	+	+
2	5	+	+
3	50	+	+
4	75	-	-
5	100	-	-
6	125	-	-
7	150	-	-
8	200	-	-
9	300	-	-
10	400	-	-
11	500	-	-

* Mean of three replications
\+ Bacterial growth
\- No bacterial growth

Using the strain EHA105 (pCAMBIA2301) the transformation efficiency was 50 per cent, when callus was used for co-cultivation with infection carried out for 20 minutes.

4.5.2. Effect of Number of Days of Co-cultivation

Agrobacterium with the binary vectors and the plant tissues after infection were co-cultivated in dark for three and four days at 28^0C in a culture room. The effect of number of days of co-cultivation on the transformation efficiency was studied.

Transformation was effective with co-cultivation period for three and four days. As the co-cultivation period was increased from three to four days, the efficiency of transformation increased with LBA4404 containing pCAMBIA2301. When callus was used for co-cultivation, the efficiency of transformation was increased by 19.04 per cent by increasing the co-cultivation from three to four days (Table 9).

Transformation efficiency of 50 per cent was obtained when the callus was co-cultivated with EHA105 harbouring pCAMBIA2301, for four days.

4.5.3 Effect of Acetosyringone

Though transformation was effective without acetosyringone, transformation efficiency could be increased further by adding acetosyringone (100 μM) to infection and co-cultivation medium. Transformation efficiency of 33.33 per cent was obtained when acetosyringone 100 μM was used in the infection and co-cultivation medium. In the absence of acetosyringone the transformation efficiency was only 17.6 per cent.

4.5.4 Effect of Different Bacterial Strains

The effect of different bacterial strains on transformation efficiency was tested. Among the two strains with the vector pCAMBIA2301, the maximum efficiency of transformation (50%) was obtained with EHA105 (Table 10). With the strain LBA4404 out of the 44 tissues co-cultivated, 10 primary transformants were obtained resulting in 22.73 per cent transformation (Figure 3).

Table 8. Effect of infection time on transformation efficiency

Infection time (min)	Percent explants retained				Transformation efficiency (%)	
	After co-cultivation		After incubation			
	LBA4404	EHA105	LBA4404	EHA105	LBA4404	EHA105
15	93.33	-	83.33	-	16.67	-
20	92.85	83.33	78.57	66.67	35.71	50

Table 9. Effect of co-cultivation time on transformation efficiency

No. of days of co- cultivation	Percent explants retained				Transformation efficiency (%)	
	After co-cultivation		After incubation			
	LBA4404	EHA105	LBA4404	EHA105	LBA4404	EHA105
3	93.33	-	83.33	-	16.67	-
4	92.85	83.33	78.57	66.67	35.71	50

4.5.5 Survival of Tissues in Selection Medium

The survival of the tissues in selection medium was observed after two weeks. Using the strain LBA4404, 76.92 per cent tissues survived in the selection medium containing kanamycin 150 mg l^{-1}.With the same strain, the survival percentage was increased to 80.65 in selection medium containing kanamycin100 mg l^{-1} (Table 11). The survival per cent of callus was 66.67 in selection medium containing kanamycin 100 mg l^{-1} with the strain EHA105.

4.5.6. GUS Histochemical Assay

GUS histochemical assay was performed after selection. Among the 31 kanamycin resistant calli obtained with the strain LBA4404, eight were GUS positive (25.8 %). Of the four kanamycin resistant calli obtained with the strain EHA105, three were GUS positive (75 %). Among the kanamycin resistant nodes, 50 per cent showed GUS activity (Table 12).

The staining pattern varied among the callus from those partially stained and stained in a particular spot (Plate 7). The staining pattern also differed with the callus co-cultivated with different strains of *Agrobacterium* LBA4404 and EHA105 containing the vector pCAMBIA2301. In LBA4404, GUS activity was observed as blue spots, in contrast with EHA105, where the GUS activity was seen as blue patches.

Table 10. Transformation frequency and the number of putative transformants obtained with different bacterial strains

Bacterial strains and plasmid	No. of tissues co-cultivated	No. of putative transformants obtained	Percent transformation
LBA4404(pCAMBIA2301)	44	10	22.73
EHA105(pCAMBIA2301)	6	3	50

Table 11. Survival per cent of tissues in selection medium

Kanamycin concentration (mg l^{-1})	Bacterial strain	No. of tissues remained green in selection medium	No. of tissues turned brown in selection medium	Survival of tissues in selection medium (%)
150	LBA4404	10	3	76.92
100	LBA4404	25	6	80.65
100	EHA105	4	2	66.67

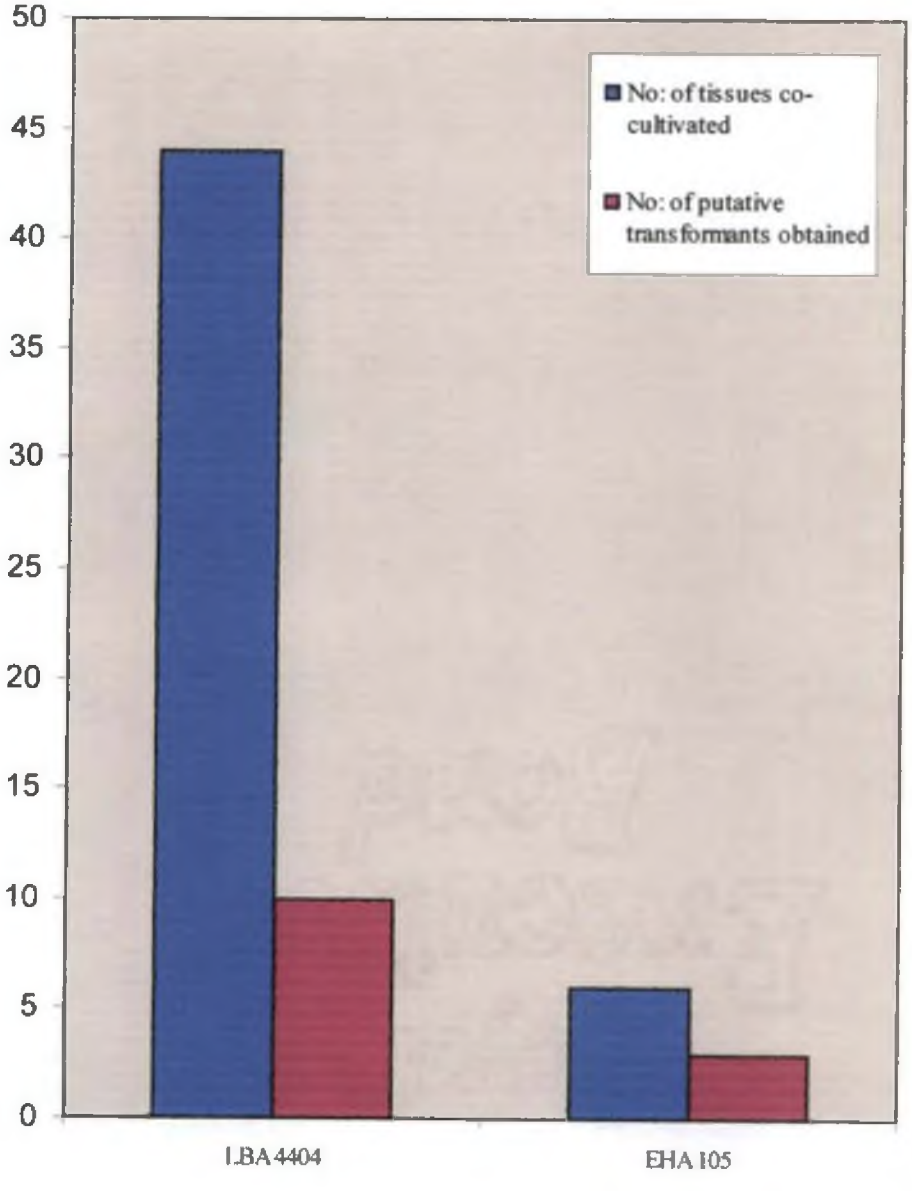


Fig.3. No. of tissues co-cultivated and no: of putative transformants obtained with different bacterial strains

Table 12. Transient GUS expression of *Centella* tissues following co-cultivation with *Agrobacterium* containing pCAMBIA2301

Explant	Plasmid and *Agrobacterium* strains	No. of explants retained in selection medium	No. of GUS positives
Callus	LBA4404(pCAMBIA 2301)	31	8 (25.80%)
Node	LBA4404(pCAMBIA 2301)	4	2 (50%)
Callus	EHA105(pCAMBIA2301)	4	3(75%)

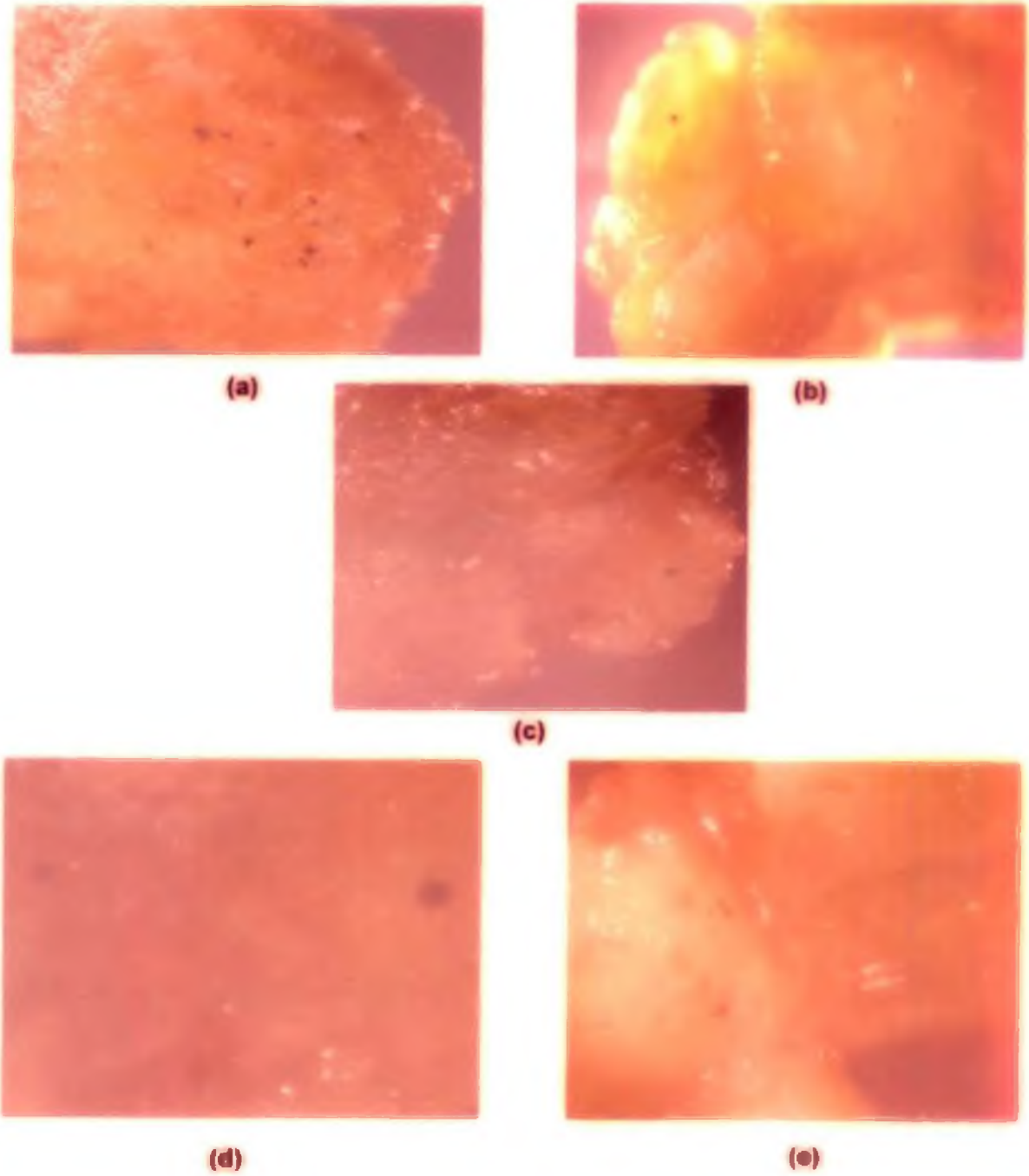


Plate 7 : GUS expression in putative transformants

a,b,c,d- GUS expression in transformed callus
e- GUS expression in transformed node

Discussion

5. DISCUSSION

Centella asiatica has a considerable reputation in the Indian system of medicine. It is used as a general and brain tonic. *Centella* has been found to improve memory and strengthen the central nervous system. It is also believed to be a rejuvenating medicament as well as an agent directly lowering blood pressure.

Globally there is an increasing demand for medicinal plants. The advent of transgenic technology allows for accelerated modification and improvement in medicinal plants. Genetic transformation is a powerful tool for enhancing the productivity of novel 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.

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 genetic transformation in *Centella asiatica.*

The present study was undertaken at the Department of Plant Biotechnology, College of Agriculture, Vellayani, to standardize the protocol for *Agrobacterium tumefaciens* mediated genetic transformation in *Centella asiatica.*

For molecular breeding the tissue to be genetically engineered must give rise to plants. In the present study callus derived from leaf and node explants and nodes of *Centella* established *in vitro* were used for genetic transformation. For inducing callus, leaves and nodes were collected from naturally grown plants and subjected to surface sterilization. Surface sterilization with mercuric chloride 0.08 per cent for five minutes was very effective. Mercuric chloride 0.08 per cent for more than five minutes resulted in browning and drying of explants. Mercuric chloride is the preferred surface sterilant for the crops grown in humid tropical conditions.

For callus induction, different combinations of growth regulators in MS medium were tried. Maximum callus induction percentage was recorded with a combination of Kn 2 mg l^{-1} and NAA 4 mg l^{-1} from leaf tissues. 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.* Kn supplementation at 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. 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 an important factor that affects the multiplication and establishment of transformed plants. Regeneration was attempted with different combinations of plant growth regulators in MS medium. Regeneration from the callus (16.67%) was obtained on MS medium supplemented with Kn 2 mg l^{-1}, BA 4mg l^{-1}, NAA 0.25 mg l^{-1} and ADS 20 mg l^{-1}. Patra *et al.* (1998) reported shoot bud regeneration in *Centella* after four weeks of subculture of callus on MS medium supplemented with 4 mg l^{-1} BA, 2 mg l^{-1}, Kn 0.25mg l^{-1} NAA and 20 mg l^{-1} ADS. The other treatments tried 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.

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 pCAMBIA2301 was used. The T-DNA of this binary vector contains *nptII* (kanamycin resistance) gene as the selectable marker (Hajadukiewicz *et al.*, 1994). This necessitates 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 percent of callus in kanamycin 100 mg l^{-1} after eight weeks was 11.2 per cent and this dose was 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 500 µg ml^{-1} kanamycin. These reports indicate that there is variation in the sensitivity to antibiotic depending on the genotype, physiological condition, size and type of explant and the tissue culture conditions (Yang *et al.*, 1999).

Elimination of bacteria from the plant tissues after co-cultivation is very important, as the survival of bacteria in plant tissue may affect the growth and regeneration of transformed tissues. Commonly used bacteriostatic agent, cefotaxime was used for the elimination of *Agrobacterium* in the present study. At a concentration

of 75 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. 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*, 500 mg l^{-1} cefotaxime was used (Negoianu *et al.*, 2002).

In the present study two strains of *Agrobacterium tumefaciens, viz.*, LBA4404 and EHA105 were used for standardization. There are reports on difference in the susceptibility between plant species and even between cultivars and genotypes of the species. (Desgagnes *et al.*, 1995). As *Centella* tissues were sensitive to kanamycin, the vector with kanamycin resistance was selected for transformation. The vector, pCAMBIA2301 contains the kanamycin resistance gene as selectable marker and *gus* as reporter gene.

The genetic transformation was done using callus and node explants of *Centella.* Feeney and Punja (2003) used suspension cells for *Agrobacterium* transformation in *Cannabis sativa.* 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 weeks 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 californica)* by co-cultivation of excised cotyledons with *A. tumefaciens.* In the present study, callus was pre-cultured 15 days before co-cultivation in MS media supplemented with Kn 2 mg $l^{-1}$and NAA 4 mg l^{-1}, for the cells to be 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.

Experiments were undertaken to standardize the optimum time required for the *Agrobacterium* to infect the plant cell. Among the different treatments, infection time of 20 minutes gave the maximum percentage of putative transformants (33.3) in callus and (40) in node compared to 15 minutes. The infection time varies according to the plant species and the bacterial strains. In *Echinacea purpurea* 30 minutes (Lichtenstein and Draper, 1986) and in brahmi 15 minutes (Nisha *et al.*, 2003) of infection time resulted in transformation.

In the present study, wounding of *Centella* tissues was beneficial 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. Addition of acetosyringone 100 µM in the infection and co-cultivation medium increased the transformation efficiency. Several workers have reported the use of acetosyringone in genetic transformation to increase the efficiency of the process. 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. Hence acetosyringone is added to the infection and co-cultivation medium to increase the transformation efficiency.

Standardization of the number of days required for co-cultivation suggested that transformants were could be obtained from the explants co-cultivated with *Agrobacterium* in dark for three and four days. 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 putatively transformed *Centella* tissues was done by the use of cefotaxime 75 mg l^{-1}. Cultures were transferred to selection medium containing kanamycin 100 mg l^{-1}. After two weeks, the non-transformed cells turned brown, while the putative transformants remained green. 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 500 µg ml^{-1} kanamycin. A concentration of 20 mg l^{-1} kanamycin 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 50 mg l^{-1} kanamycin (Koroch *et al.*, 2002).

There were differences in the transformation efficiency with respect to the different strains of *Agrobacterium* used. Among the two strains *viz.*, LBA4404 and EHA105 harbouring the vector pCAMBIA2301, the maximum percentage of transformants (50) was obtained with EHA105. The difference in the transformation efficiency may be due to the difference in interaction between bacteria and plant cell and other factors affecting infection process. It is reported that the *Agrobacterium*

infectivity was improved by the use of right strain of the bacteria, manipulating explant physiology, inoculation and co-cultivation conditions (Godwin *et al.*, 1992). *Agrobacterium* mediated transformation has been reported to be dependent on the bacterial strain, conditions of co-cultivation, selection method and mode of regeneration (Mathis and Hinchee, 1994).

Confirmation of transformation was done by histochemical GUS assay. The vector pCAMBIA2301 harbour the *gus* gene as reporter gene. The *gus* gene encodes a stable enzyme that is not normally present in plants and that catalyze the cleavage of a range of β-D glucuronides. Thus the GUS activity in transformed plant tissues can be localized by observing the blue colour that is formed after the hydrolysis of the substrate (X-gluc).

Out of the 31 kanamycin resistant calli, 25.8 per cent showed GUS activity for strain LBA4404. Using the strain EHA105, 75 per cent of the kanamycin resistant calli showed GUS activity. GUS negatives among the selected calli may be the escapes. Vergauwe *et al.* (1996) reported that out of the total transformed tissues, 94 per cent showed β-glucuronidase positive response. Dronne *et al.* (1999) obtained glucuronidase gene expression at a high level in at least 40 per cent of the transgenic plants regenerated in lavandin.

The X-gluc staining pattern varied among the callus from those partially stained to those stained at a particular spot. The staining pattern also differed with the callus co-cultivated with different strains of *Agrobacterium* LBA4404 and EHA105 containing the vector pCAMBIA2301, indicating the difference in the transformation efficiency. Portion of the callus showing GUS activity was transferred to regeneration medium.

In the present study, various parameters of genetic transformation in *Centella* were optimized like type of the explant, infection time, co-cultivation time, selection agent and suitable *Agrobacterium* strains. The *Agrobacterium* strain, EHA105 with pCAMBIA2301 was more efficient for transformation in *Centella asiatica*. The most effective infection time was 20 minutes, followed by a co-cultivation period of four days. Node and callus derived from node were more suitable for transformation. There were some problems associated with the regeneration of the transformed *Centella* callus and the protocol for regeneration needs to be refined further.

Till date no report has been published on genetic transformation in *Centella asiatica*. Hence this study provides a protocol for genetic transformation in *Centella* which can be used for transferring desirable genes in future. The use of tissue specific promoters and positive selection agent which render developmental advantage to the transformed cell and promote faster proliferation and regeneration could also be standardized.

Summary

SUMMARY

A study on "*Agrobacterium tumefaciens* mediated genetic transformation in kudangal (*Centella asiatica* L. Urban.)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2004-2006. The salient features of the above study are summarized in this chapter.

Centella asiatica is an important medicinal plant of India and is used in many ayurvedic formulations. *Centella asiatica* has a long history of use to promote wound healing and treat skin diseases. It is chiefly valued as a revitalizing herb that strengthens nervous function and memory. *Centella asiatica* contains a blend of compounds including at least three triterpenes (asiatic acid, madecassic acid and asiaticoside) that appear to have antioxidant properties and tissue regenerative property by stimulating collagen synthesis.

Agrobacterium tumefaciens mediated transformation is simple and efficient in providing stable integration of transferred DNA into the plant genome. The present study was undertaken with the main objective of evolving a protocol for *Agrobacterium tumefaciens* mediated genetic transformation in *Centella asiatica*, which could further be utilized for metabolic engineering to enhance the level of secondary metabolites.

Nodes established *in vitro* and callus derived from leaf and nodes were used for the experiment. Surface sterilization of leaf and node explants with mercuric chloride 0.08 per cent for five minutes was found to be very effective. Protocol for callus induction was standardized. Among the various treatments tried, MS medium supplemented with Kn 2 mg l^{-1} and NAA 4 mg l^{-1} was proved to be the best in terms of callus induction percentage (85.7) from leaf explant in 25.50 days. With node explants, the maximum callus induction (86.67%) was obtained in MS medium supplemented with Kn 2 mg l^{-1} and NAA 3 mg l^{-1} and also in that supplemented with Kn 1 mg l^{-1} and NAA 3 mg l^{-1} in 23.67 and 22.00 days, respectively.

Significant difference was observed among the different treatments for shoot regeneration from callus. Out of the various treatments, 16.67 regeneration per cent from callus was obtained on MS medium supplemented with Kn 2mg l^{-1}, BA 4mg l^{-1}, NAA 0.25 mg l^{-1} and adenine sulphate 20 mg l^{-1}.

Two strains of *Agrobacterium viz.*, LBA4404 and EHA105 harbouring the plasmid pCAMBIA2301 were used for genetic transformation. As the plasmid harbours *nptII* gene, kanamycin was used as the selection pressure to select the transformants. Hence, experiments were conducted to evaluate the sensitivity of *Agrobacterium* strains and *Centella* callus to different concentrations of kanamycin. It was observed that the lethal dose of kanamycin to *Agrobacterium* and *Centella* callus were 350 and 125 mg l^{-1} of the medium respectively. For the selection of transformed cells, kanamycin100 mg l^{-1} was used.

For elimination of *Agrobacterium* after co-cultivation cefotaxime was used. Hence experiments were conducted to evaluate the sensitivity of *Agrobacterium* and calli to different doses of cefotaxime. It was observed that the *Agrobacterium* was effectively killed by cefotaxime 75 mg l^{-1} and the lethal dose of cefotaxime to *Centella* callus was 150 mg l^{-1}.

Agrobacterium mediated genetic transformation was achieved by co-cultivating callus and node with bacterial suspension. In a study conducted to optimize the time required for infection, it was found that as the infection time increased from 15 to 20 minutes the percentage of transformants obtained also increased from 15.38 to 33.3 with the strain LBA4404 using callus explants. With nodal explants, the percentage of transformants obtained with LBA4404 was 40 when the infection time was 20 minutes. When callus was transformed using the strain EHA105 the transformation efficiency was 50 per cent with an infection time of 20 minutes.

In an experiment to standardize the co-cultivation time, it was observed that transformation efficiency of 33.33 per cent was obtained when callus was co-cultivated with LBA4404 (pCAMBIA2301) for four days. Maximum transformation efficiency of 50 per cent was obtained when callus was co-cultivated with EHA105 (pCAMBIA2301) for four days. After co-cultivation the explants were transferred to a medium containing cefotaxime 75 mg l^{-1} to kill the *Agrobacterium*.

The co-cultivated tissues were then transferred to selection medium containing kanamycin100 mg l^{-1} to select the putative transformants. Survival percentages of tissues in selection medium, using the strains LBA4404 and EHA105, were 80.65 and 66.67 respectively. The transformation efficiency was increased when acetosyringone 100 μM was added to infection and co-cultivation medium. Among the two strains, the maximum percentage of transformants (50) was obtained with EHA105.

Successful transformation was confirmed by histochemical assay for GUS activity. Out of the kanamycin resistant callus, 25.8 and 75.0 per cent showed GUS activity for strains LBA4404 and EHA105, respectively. Later the putative transformants were transferred to regeneration medium.

During this study various parameters of genetic transformation in *Centella* like type of the explant, infection time, co-cultivation time, selection agent and suitable *Agrobacterium* strains were optimized. The *Agrobacterium* strain EHA105 with pCAMBIA2301 was more efficient for transformation in *Centella*. The most effective infection time was 20 minutes, followed by a co-cultivation period of four days. Node and callus derived from node were more suitable for transformation. There were some problems associated with the regeneration of the transformed *Centella* callus and the protocol for regeneration needs to be refined further. This study provides a protocol for genetic transformation in *Centella* which can be used for transferring desirable genes.

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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*

Yeast Extract Peptone Medium (YEP Medium) (gl^{-1})

Yeast Extract	10
Peptone	10
Sodium chloride	5
Agar	15
p^H	7

AB Minimal Medium

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
p^H	7

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

K_2HPO_4	60
NaH_2PO_4	23

Glucose	0.5%
Agar	1.5%

Working solution 1X

APPENDIX III

1: AF234316. Binary vector pCA...[gi:7638149] Links

```
LOCUS       AF234316               11633 bp    DNA     circular SYN 24-APR-2000
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ACCESSION   AF234316
VERSION     AF234316.1  GI:7638149
KEYWORDS    .
SOURCE      Binary vector pCAMBIA-2301
  ORGANISM  Binary vector pCAMBIA-2301
            artificial sequences; vectors.
REFERENCE   1  (sites)
  AUTHORS   Hajdukiewicz,P., Svab,Z. and Maliga,P.
  TITLE     The small, versatile pPZP family of Agrobacterium binary vectors
            for plant transformation
  JOURNAL   Plant Mol. Biol. 25 (6), 989-994 (1994)
  MEDLINE   95002787
   PUBMED   7919218
REFERENCE   2  (bases 1 to 11633)
  AUTHORS   Roberts,C., Rajagopal,S., Smith,L.M., Nguyen,T.A., Yang,W.,
            Nugrohu,S., Ravi,K.S., Vijayachandra,K., Harcourt,R.L.,
            Dransfield,L., Desamero,N., Slamet,I., Hadjukiewicz,P., Svab,Z.,
            Maliga,P., Mayer,J.E., Keese,P.K., Kilian,A. and Jefferson,R.A.
  TITLE     A comprehensive set of modular vectors for advanced manipulations
            and efficient transformation of plants
  JOURNAL   Unpublished
  REMARK    Full description of constructs
REFERENCE   3  (bases 1 to 11633)
  AUTHORS   Roberts,C., Rajagopal,S., Smith,L.M., Nguyen,T.A., Yang,W.,
            Nugrohu,S., Ravi,K.S., Vijayachandra,K., Harcourt,R.L.,
            Dransfield,L., Desamero,N., Slamet,I., Hadjukiewicz,P., Svab,Z.,
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  TITLE     Direct Submission
  JOURNAL   Submitted (15-FEB-2000) CAMBIA, Clunies Ross St, Black Mountain /
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                     RTSHYPYAEEMLDWADEHGIVVIDETAAVGFQLSLGIGFEAGNKPKELYSEEAVNGET
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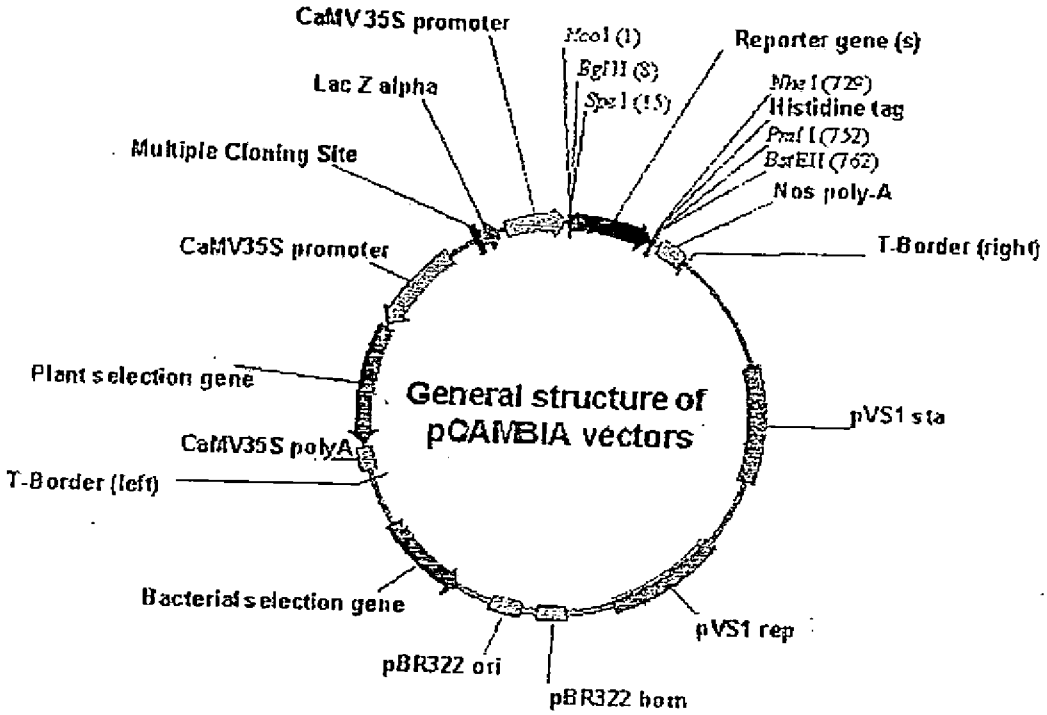

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AGROBACTERIUM TUMEFACIENS MEDIATED GENETIC TRANSFORMATION IN KUDANGAL (*CENTELLA ASIATICA* L. URBAN.)

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Abstract of the
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ABSTRACT

A study on "*Agrobacterium tumefaciens* mediated genetic transformation in kudangal (*Centella asiatica* L. Urban.)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2004-2006.

Centella asiatica is an important medicinal plant of India and is used in many ayurvedic formulations. *Centella asiatica* contains a blend of compounds including triterpenes (asiatic acid, madecassic acid and asiaticoside) that appear to have antioxidant, tissue regenerative and memory enhancing properties. The present study was undertaken with the main objective of evolving a protocol for *Agrobacterium tumefaciens* mediated genetic transformation in *Centella asiatica*, which could further be utilized for the metabolic engineering of *Centella* to enhance the level of secondary metabolites.

Callus was induced from leaf and node explants of *Centella* on MS medium supplemented with growth regulators. MS medium supplemented with Kn 2 mg l^{-1} and NAA 4 mg l^{-1} was proved to be the best in terms of callus induction percentage (85.7) from leaf explant in 25.50 days. With node explants, the maximum callus induction (86.67%) was obtained on MS medium supplemented either with Kn 2 mg l^{-1} and NAA 3 mg l^{-1} or with Kn 1 mg l^{-1} and NAA 3 mg l^{-1} in 23.67 and 22.00 days respectively. Of the various regeneration treatments, 16.67 per cent regeneration from callus was obtained on MS medium supplemented with Kn 2mg l^{-1}, BA 4mg l^{-1}, NAA 0.25 mg l^{-1} and ADS 20 mg l^{-1}.

Two strains of *Agrobacterium tumefaciens viz.*, LBA4404 and EHA105 harbouring the plasmid pCAMBIA2301 were used for genetic transformation. As the plasmid harbour *nptII* and *gus* reporter genes, the sensitivity of *Agrobacterium* strains and *Centella* callus to different concentrations of kanamycin was evaluated. The lethal dose of kanamycin to *Agrobacterium* and *Centella* callus were 350 and 125 mg l^{-1}

respectively. The effective dose of cefotaxime for the elimination of bacterial strains LBA4404 and EHA105 was 75 mg l^{-1} and the lethal dose of cefotaxime to *Centella* callus was 150 mg l^{-1}.

Genetic transformation was achieved by co-cultivating callus and node with bacterial suspension. Conditions like infection and co-cultivation time, type of the explant, selection agent and suitable *Agrobacterium* strains were optimized. The *Agrobacterium* strain, EHA105 with pCAMBIA2301 was more efficient for transformation in *Centella*. The most effective infection time was 20 minutes, followed by a co-cultivation period of four days. The survival of tissues transformed by the strains LBA4404 and EHA105 on the selection media were 80.65 per cent and 66.67 per cent respectively. Maximum transformation efficiency of 50 percent was obtained when callus was co-cultivated with EHA105 (pCAMBIA2301) for four days.

The transformation efficiency was increased when acetosyringone 100 μM was added to infection and co-cultivation media. Transformation was confirmed by histochemical GUS assay of putative transformants. This study provides a protocol for genetic transformation in *Centella* which can be used for transferring desirable genes.