AGROBACTERIUM RHIZOGENES MEDIATED GENETIC TRANSFORMATION IN KODUVELI (*Plumbago* spp. Linn.)

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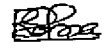
DECLARATION

I hereby declare that this thesis entitled "***Agrobacterium rhizogenes*** **mediated genetic transformation in *Koduveli (Plumbago* spp. L.)** " is a bonafide record of research work done by me during the course of my research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other university or society.



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CERTIFICATE

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Dedicated to

my beloved parents and husband

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LIST OF ABBREVATIONS

2,4-D	2,4 – dichlorophenoxyacetic acid
BA	N^6 –benzyl adenine
CI	callus index
Cm	centimeter
DNA	deoxyribonucleic acid
G	growth score
GA	gibberellic acid
H	hour
HCl	hydrochloric acid
IAA	indole-3-acetic acid
IBA	indole-3- butyric acid
Mg	milligram
MS	Murashige and Skoog
Na Cl	sodium chloride
Na OH	sodium hydroxide
NAA	α – naphthaleneacetic acid
°C	degree Celsius
µ l	microlitre
µ M	micromolar

Introduction

1. INTRODUCTION

Plants have served as the primary source of pharmaceutical compounds from the time immemorial. India is blessed with a wide variety of medicinal plants. Our ancient literatures like *Atharva veda*, *Ashtangahridaya* and *Charakasamhitha* contain references on various medicinal plants and their healing effect on human diseases. Medicinal plants are of special interest to researchers in the field of biotechnology as most of the pharmaceutical companies depend in part on plants for the production of pharmaceutical compounds (Rout *et al.*, 2000).

Earlier the sources of these plant derived phytomedicines were strictly limited to the natural stand, which consequently has reduced the natural plant population considerably. Non judicious harvest of medicinal plants has even resulted in the extinction of many plant species and many more have become endangered. But with the advent of biotechnological tools, many of the plant derived pharmaceuticals could be cultured *in vitro*. With the commercial production of shikonin *in vitro* from *Lithospermum erythrorhizon* the prospects of this technique has widened.

Plumbago spp. (family *Plumbaginaceae)* is an important medicinal plant containing Plumbagin (2-methoxy-5-hydroxy-1, 4-naphthoquinone) that possess various pharmacological activities *i.e.* antimalarial, antimicrobial, anticancer, cardiotonic, brain tonic and antifertility action. It is used in the treatment of diarrhoea and skin diseases like psoriasis and leucoderma. It has also been specially recommended in the treatment of rheumatism (Rout *et al.*, 2000). The most exploited plant source of plumbagin is the roots of *P. rosea* and *P. zeylanica*. Plumbagin is present in the roots of *Plumbago* to an extent of 0.99 per cent. However, these plants grow quite slowly and it takes long time until the roots are suitable for use. To overcome this drawback it is suggested to look

into means and methods for rapid proliferation of *roots in vitro* and also to search for alternative sources of plumbagin.

Because of the broad application of *Plumbago* roots in the indigenous system of medicine, *Plumbago* is in great demand and thereby in great shortage. Plant tissue culture is the most efficient way to solve plant shortage problems. But plant tissue culture has a tendency to be genetically and biochemically unstable, and they tend to synthesise very low levels of usefull secondary metabolites. Hairy root culture is the best alternative in this context.

Inoculation of plants with soil-borne bacterium *Agrobacterium rhizogenes* has been reported to induce the formation of hairy roots, which readily give rise to rapidly growing, permanent root cultures. Hairy roots transformed with Agrobacterium *rhizogenes*, have been found to be suitable for the production of secondary metabolites because of their stable growth and high productivity on hormone free culture conditions. The attractiveness of hairy root cultures in bioprocessing is attributed to their genetic and biochemical stability, rapid growth on hormone free medium and morphological differentiation. Hence the present study is undertaken to evolve a successful protocol for *in vitro* propagation and genetic transformation of *Plumbago* spp. Linn. to induce hairy root culture for secondary metabolite production.

Review of Literature

2. REVIEW OF LITERATURE

Plants are the important source of many chemicals having therapeutic value. Drugs from higher plants continue to occupy an important niche in modern medicine (Dev, 1997). Plumbagin (2-methoxy-5-hydroxy-1,4-naphthoquinone) which is a high valued compound is a natural naphthoquinone possessing pharmacological activities such as antimalarial, antimicrobial, anticancer, cardiotonic and antifertility action. At present, the most exploited source of plumbagin is the roots of *Plumbago* spp. (*Plumbago rosea* and *Plumbago. zeylanica*). However, these plants grow quite slowly and it takes long time for the roots to become suitable for use. Therefore, it is advisable to probe into methods for enhancing growth and production of plumbagin from *Plumbago* spp and also search for alternative sources of plumbagin.

Genetic transformation of medicinal plants is an important tool for induction of hairy roots. The genetically transformed roots of red beet have been shown, for the first time, to produce very high levels of peroxidase accounting for 1.21×10^6 Units l^{-1} (Rudrappa *et al.*, 2005).

In this chapter literature on *in vitro* propagation and genetic transformation of Plumbago has been reviewed. Wherever sufficient literature on *Plumbago* is lacking, literature on other similar plants are reviewed.

2.1 *IN VITRO* PROPAGATION

In vitro propagation method is used widely for the commercial propagation of a large number of plant species, including many medicinal plants (Rout *et al.*, 2000).

Murashige (1974) reported that propagule multiplication is achieved through enhanced release of axillary buds or production of adventitious shoots through organogenesis and somatic embryogenesis.

2.1.1 Enhanced Release of Axillary Buds

Axillary shoot proliferation has been the most simple and reliable route for the production of elite clonal plants in many medicinal plants (Dias *et al.*, 2002). Axillary shoot growth is stimulated by overcoming apical meristem dominance (Boxus, 1999). The rate of shoot bud regeneration was positively correlated with the concentration of cytokinins in the nutrient media.

2.1.1.1 Culture Medium

Composition of the culture medium is the most important factor that governs growth and morphogenesis of plant tissues *in vitro*. The purpose and plant species or the variety to be cultured determines the choice of a particular medium (Wang and Charles, 1991). The main components of most plant tissue culture media are mineral salts, sugar as carbon source and water. Organic supplements, growth regulators and a gelling agent are the other components (Gamborg *et al.*, 1968; Gamborg and Phillips, 1995).

A wide variety of media have been formulated for *in vitro* culture of plant tissues and organs. White's medium is one of the earliest plant tissue culture media formulated. Since 1962 most researchers have been using MS (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968) or SH (Schenk and Hidebrant, 1972) media. MS medium is characterized by high salt concentration.

Murashige and Skoog medium is the most widely used culture medium for *in vitro* propagation of medicinal plants. In medicinal plants *in vitro* plantlet regeneration

from nodal explants in MS medium was reported in several species *viz.*, *Aristolochia indica* (Manjula *et al.*,1997), *Atropa acuminata* (Ahuja *et al.*, 2002), *Hemidesmus indicus* (Saha *et al.*, 2003), *Leptadenia reticulata* (Arya *et al.*, 2003), *Baliospermum montanum* (Johnson and Manickam, 2003) and *Wedelia chinensis* (Martin *et al.*, 2003).

2.1.1.2 Plant Growth Regulators

The interaction and balance between the growth regulators supplied to the medium and the growth substance produced endogenously by the cultured cell regulates the growth and morphogenesis *in vitro*. The crucial factor determining the success of plant tissue culture is the selection and addition of growth regulators at the optimum level (Gamborg *et al.*, 1968; Gamborg and Phillips, 1995).

Cytokinin is used to overcome apical dominance of shoots and to enhance the emergence of lateral buds from each leaf axil (Wang and Charles, 1991). BA has been the most favourable cytokinin for initiation and multiplication of axillary buds in plant tissue culture. The potentiality of BA over other cytokinin has been well established in many plants including medicinal plants (Martin *et al.*, 2003).

The highest number of shoots per explant was observed on MS medium supplemented with 2.22 µM BA in *Baliospermum montanum* (Johnson and Manickam, 2003). BA and kinetin had a synergistic effect on multiplication and growth of shoots in medicinal plants (Sreekumar *et al.*, 2000 and Martin, 2002).

2.1.1.3 Culture Conditions

The pH of the culture medium affects the growth and differentiation of tissues in cultures. Plant cells in culture require an acidic pH of 5.5 to 5.8 (Gamborg and Shyluk, 1981).

Murashige (1977) reported that light intensity, light quality and duration affect growth of *in vitro* grown cultures. He found that the optimum light intensity for shoot formation in large number of herbaceous species to be around 1000 lux. The optimum day length was considered to be 16 hours for a wide range of plants.

Yoeman (1986) opined that, for better performance of plants under *in vitro* conditions the usual environment temperature of the species should be taken into account.

2.1.2 Somatic organogenesis

Somatic organogenesis may be direct or indirect. In direct organogenesis shoots arise directly from the tissues of the explant. In indirect organogenesis, shoots arise from previously formed callus.

Skoog and Miller (1957) proposed that organ differentiation in plants is regulated by interplay of auxins and cytokinins. A high concentration of auxin and a low concentration of cytokinins promote abundant proliferation of cells with formation of callus. On the other hand low auxin and high cytokinin levels in the culture medium results in the shoot morphogenesis.

2.1.2.1 Explant

Rapid plant regeneration from leaf and stem explant callus was achieved in *Plumbago zeylanica* (Rout *et al*,. 1999). Media containing higher concentrations of IAA or NAA failed to initiate adventitious buds. Green nodular structures developed into dark green shoots over the entire surface when calli were cultured in the medium supplemented with BA (4.44-6.66 μM) and IAA1.42 μM) after four weeks of subculture. Leaf explants were more responsive than stem explants.

2.1.2.2 Plant Growth Regulators

A high concentration of auxin to cytokinin favours callus formation. Whereas a high concentration of cytokinin to auxin is necessary for shoot initiation from callus. Satheesh Kumar *et al.* (2003) reported that callus was induced from leaf explants of *Plumbago zeylanica* in the media containing 1.5 mg l^{-1} NAA and 2.5 mg l^{-1} BA. Shoot regeneration was induced in half strength MS supplemented with 3 mg l^{-1} BA.

Semi friable callus was induced on young stem and leaf segments of *Plumbago zeylanica* in MS solid medium containing 1.5 to 2. 2 mg l^{-1} 2,4-D and 0.5 to 1.5 mg l^{-1} kinetin (Satheesh kumar and Seeni, 2002). Rapid plant regeneration was achieved in the callus cultures derived from the leaf and stem explants of *Plumbago zeylanica* on MS basal medium supplemented with 4.44 µM 6-BA, 1.42 µM IAA and three percent (w/v) sucrose. The leaf explants were more responsive than the stem explants (Rout *et al.*, 1999).

Rapid plant regeneration was achieved in callus cultures derived from leaf and stem explants of *Plumbago zeylanica* on MS basal medium supplemented with 4.4 µM 6-BA, 1.42 µM IAA and three per cent (w/v) sucrose (Rout *et al.*, 1999). Kavikishore *et al.* (2004) reported that somatic organogenesis was induced in suspension cultures of *Plumbago rosea* by acetylsalicylicacid (ASA) 8.32 µM and IAA 5.06 µM.

Wei *et al.* (2003) reported highest frequency of shoot regeneration in hypocotyl segments of *Plumbago zeylanica* cultured on basal medium supplemented with BA 2.0 mg l^{-1}, NAA 0.75 mg l^{-1}, adenine sulphate 50 mg l^{-1} and ten per cent (v/v) coconut water.

2.1.3 Rooting

2.1.3.1 In vitro rooting

The *in vitro produced* plantlets should have a strong and functional root system. Roots are mostly induced in the presence of suitable auxins (Razdan, 2003). IAA, IBA and NAA were found to induce rooting.

2.1.3.2 Plant Growth Regulators

Efficacy of IBA at lower concentrations in *in vitro* rooting has been reported in *Plumbago* spp. (Das and Rout, 2002). Roots were induced *in vitro* in *Plumbago spp.* cultured on basal medium supplemented with 0.10 mg l^{-1} (Satheesh Kumar *et al.*, 2003). Wei *et al.* (2003) reported that rooting was induced in *P. zeylanica* half MS without adding any plant growth regulator.

2.2 GENETIC TRANSFORMATION

Introduction of DNA into the cells of an organism by a method other than conventional sexual crossing is called genetic transformation. Transformation of plants with soil born bacterium *Agrobacterium rhizogenes* induces the formation of "hairy roots". *Agrobacterium rhizogenes* effects transformation by virtue of the *rol* genes present in the plasmid. These "hairy roots" can be used for regeneration of transformed plantlets. Hairy roots have been recognized as a potential source of secondary metabolites, since transformation does not impair the natural root synthetic activities.

2.2.1 *Agrobacterium rhizogenes*

The induction of hairy roots is the consequence of integration of a portion of bacterial plasmid (Ri plasmid), the T-DNA, into the plant genome (Ackermann, 1977).

The hairy roots are characterised by fast growth, highly balanced adventitious nature and continuous growth *in vitro*, on a hormone free culture medium. They have high stability and are capable of producing the secondary metabolites of the mother plant (Doran, 1989; Flores, 1986, 1992).

The function of Ri *rol* genes in T_L-DNA of the Ri plasmid of *Agrobacterium rhizogenes* has been previously studied in *Nicotiana tabacum* and *Daucus carota*, but it was reported that these plants have a T_L-DNA-similar sequence in their genome. The single gene Ri *rol*B induced adventitious roots in *Nicotiana. debneyi.* Introduction of a DNA fragment that contained Ri *rol*B, Ri *rol*C, RiORF13 and RiORF14 resulted in more intense and earlier root formation than that of RirolB. Ngrol genes (NgrolB, NgrolC, NgORF13, and NgORF14) in the genome of *Nicotiana glauca* that are similar in sequence to Rirol genes were also examined (Aoki and Syono, 2000).

In Agrobacterium *rhizogenes*, various Ri plasmids are reported which show some differences in terms of genetic organization as compared to the reference plasmid pRiA4. On this plasmid as well as on the pRi 2659 and pRi 8196, the T-DNA genes rolA, *rol*B and *rol*C (ORFS 10, 11&12) appear to suffice to induce root formation in most assayed plant species. However, the most conserved sequence of the *Agrobacterium rhizogenes* T-DNAs are not of the *rol* genes. But those defining ORF 8, 13 and14. Sequence comparisons have suggested that the gene product of ORF8 might be involved in mediating auxin-related effects. Hormone responses were analysed using transgenic tobacco plants expressing the ORF8 of pRi2659 under the control of the cauliflower mosaic virus (CaMV) 35S promoter (35S-ORF8). The fusion of the ORF8

promoter with uidA, encoding B- Glucuronidase (GUS), revealed that ORF8 transcription is auxin-induced (Akila Ouartsi et al., 2003).

The *rol*ABC gene cluster harboring the *rol*A, *rol*B and *rol*C genes were cut out of the pUC9-rolABC plasmid as a 4.3-kb fragment and ligated into pGreen to construct pRYG, the basic transformation vector for the induction of hairy root proliferation response. The pRYG vector harboring the three rol genes is a fast and effective method for the generation of transgenic hairy roots. The pRYG based transformation system can also be used for the rhizosecretion of heterologous proteins (Komarnytsky et al., 2004).

Rol genes belong to the T-DNA which is transferred by *Agrobacterium rhizogenes* into plant cells. Each of these genes affects plant development and the promoters of these genes are controlled by plant regulatory proteins. The aux genes of the T_R –DNA do not play a main role in the hairy root disease, whereas the *rol* genes of the T_L-DNA are necessary and sufficient per se to induce hairy roots (Altamura, 2004).

2.2.1.1 *Agrobacterium rhizogenes* strains

Agrobacterium rhizogenes strains present in nature are classified on the basis of opine(s) they catabolise.

Table1 Classification of *Agrobacterium rhizogenes* on the basis of opine catabolism

SI No.	Opine type	Characteristic opine markers	*A. rhizogenes* markers
1	Agropine	Agropine,Mannopine,Agropinic acid, Mannopinic acid and Agrocinopine.	PcA4, 15834, A4, LBA 9402, HRI
2	Mannopine	Mannopine, Mannopinic acid, agropinic acid and Agrocinopine	8196
3	Cucumopine	Cucumopine and Cucumopine lactum	2659
4	Mikimopine	Mikimopine, Mikimopine lactum and Mannopine	MAFF 03-01724

The genes for opine synthesis are located on the Ri plasmid of *A. rhizogenes* . The *A. rhizogenes* strains differ in their host range of infection (Dessaux *et al.*, 1991). Generally agropine strians have a wider host range than mannopine and cucumopine strains (Rhodes *et al.*, 1989).

The wider host range of agropine strains is attributed to the additional presence of T_R frgment of T-DNA wuth its auxin biosynthesis genera which on transfer to the plant cells causes an elevated auxin production, thereby enhancing the induction of root initial formation in adjacent cells (Filetici *et al.*, 1987).

2.2.1.2 Culture media for A.rhizogenes strains

Several scientists have suggested different media for the growth of *A. rhizogenes* strains.

Petit *et al.* (1983) suggested LB medium for the growth of *A. rhizogenes* strains 15834 and 8196. Yeast extract peptone (YEP) agar was found to be the best medium for culturing *A. rhizogenes* strains A4, 15834 and MAFF 03-01724 (Kamada *et al.*, 1986; Yoshikawa and Furuya, 1987; Yonemitsu *et al.*, 1990 and Sasaki *et al.*, 1998).

Yeast extract mannitol (YEM) medium was found to be the best for the growth of *A. rhizogenes* strains 15834, ATCC 319798 and A4 (Jaziri *et al.*, 1988 and Mugnier, 1988).

2.2.1.3 Hairy root cultures in medicinal plants

Hairy root culture in medicinal plants is specifically used for the production of secondary metabolites, *in vitro.* Several authors have reviewed the different medicinal plants used for induction of hairy roots (Mugnier, 1988; Tepfer, 1990; Saito *et al.* 1992 and Banerjee *et al.* (1995).

The different medicinal plant families which are exploited for secondary metabolite production include Apiaceae, Apocynaceae, Asteraceae, Araliaceae, Boraginaceae, Brassicaceae, Campanulaceae, Chenopodiaceae, Compositae, Cucurbitaceae, Fabaceae, Gentianaceae, Geraniaceae, Labiatae, Leguminosae, Malvaceae, Myrtaceae, Polygonaceae, Rubiaceae, Scophulariaceae, Umbelliferae, Velarianceae and Verbenaceae (Banerjee *et al.* 1995).

2.2.2 Explant

Different explants such as shoot buds leaf segments, seedlings, stem segments, root segments and callus are used for hairy root induction.

Shoot tips, shoot buds, stem segments and seedlings are the commonly used explants for hairy root induction (Kamada *et al.*, 1986; Deno *et al.*, 1987; Yonemitsu *et al.*, 1990 and Zarate, 1999).

Trypsteen *et al.* (1991) reported that successful responses to *Agrobacterium* infections were obtained with young etiolated seedlings and not from older plantlets.

Hairy root cultures were induced from *Plumbago rosea* after infecting the *in vitro* grown shoots with *Agrobacterium rhizogenes* and accumulation of plumbagin was detected (Komaraiah *et al.*, 2002).

Hairy roots were successfully established in leaf explants of *Clitoria ternatea* (Linn.) using *Agrobacterium rhizogenes* strain A13 (Malabadi and Nataraja, 2003).

Transformed hairy root cultures were efficiently induced from seedlings of *Taraxacum platycarpum* by infection with *Agrobacterium rhizogenes* 15834 (Lee *et al.*, 2004).

Xu *et al.* (2004) reported that hairy roots were induced from both cotyledon and hypocotyl explants of *Isatis indigotica* Fort. Hairy roots were successfully established from cotyledons, hypocotyls and true leaves of *Camptotheca acuminata* by *Agrobacterium rhizogenes* strains ATCC15834 and R-100 (Lorence *et al.*, 2004).

Leaf sections of tobacco were inoculated with *Agrobacterium rhizogenes* strain A4 and resulted in hairy root induction (Komarnytsky *et al.*, 2004).

Shoots of *Gentiana purpurea* and *Gentiana lutea* were inoculated with ATCC 15834 strain and resulted in hairy root formation (Momcilovik *et al.*,1997). Transformation was confirmed by GUS staining. Ray *et al.* (1996) reported that

Table 2 List of medicinal plants in which hairy roots have been induced and secondary metabolite detected

SI No	Plant species	*A. rhozogenes* strain used	Secondary Metabolite Obtained	Degree of hyper Production than normal root
1	*Atropa belladona*	ATCC 15834	Scopalamine	5
		A4	Atropine and Hyoscyamine	2
2	*Cinchona ledgeriana*	LBA 9402	Quinine, Quinidine and Cinchonidine	3
3	*Datura innoxia*	ATCC 15834	Hyoscyamine And scopalamine	6
4	*D. queritolia*	LBA 9402	Scopalamine and hyoscyamine	20
5	*D. candida*	ATCC 15834	-do-	2
6	*Duboisia* Leichardtii	ATCC 15834 and A4	Scopalamine	2
7	*Hyoscyamus niger*	ATCC 15834	Hyoscyamine and Scopalamine	2
8	*Rubia tinctoria*	ATCC 15834	Anthraquinone	19
9	*Tagetes patula*	LBA 9365	Thiophene	25
10	*Valeriana officinalis*	R 1601	Vaipotriates	7

(Banerjee *et al.* 1995)

transformed root cultures of *Withania somnifera* Dunal were obtained by infecting shoots cultured *in vitro* with *Agrobacterium rhizogenes* LBA 9402.

2.2.3 Strain Specificity

Different strains of *Agrobacterium rhizogenes* vary in their efficiency to bring about successful transformation. A study was conducted on hairy root induction in *H. ada-kodien*. Five strains of *Agrobacterium rhizogenes* were also tested for their transforming ability. Among the different *A. rhizogenes* strains tested, the strains PcA4, 15834 and A4 induced hairy roots. The strains 8196 and 2659 did not induce hairy roots (Karmarkar *et al.*, 2001)

Agrobacterium rhizogenes strain ATCC 15834 successfully transformed *Plumbago rosea* shoots (Komaraiah *et al.*, 2002). The hairy root cultures showed an enhancement in biomass and plumbagin production with an increase in the concentration of sucrose upto three per cent (w/v) level.

Malabadi and Nataraja (2003) reported that *Agrobacterium rhizogenes* strain A13 successfully transformed *Clitoria ternatea* (Linn).

Use of *Agrobacterium rhizogenes* strains (A4T and LB9402) with or without application of IBA can trigger root formation in explants of *Pinus radiata*. Strain LB9402 was more effective than A4T in increasing rooting percentage and root number. Rooting of adventitious shoots from three year old radiata pine was improved following inoculation with LBA9402, suggesting that this rooting treatment has potential to aid clonal propagation of radiate pine (Li and Leung 2003)

Hairy roots could be obtained directly from the cut edges of petioles of leaf explants or from callus of *Pueraria phaseoloids* by transforming with *Agrobacterium*

rhizogenes strain ATCC 15834. The highest frequency of explant transformation by *Agrobacterium rhizogenes* strain ATCC 15834 was about 70 per cent. Paper electrophoresis revealed that bacteria- free hairy roots of *Pueraria phaseoloids* could synthesise agropine and mannopine. Transformation was confirmed by PCR analysis (Shi and Kintzios, 2003).

Two strains of *Agrobacterium rhizogenes* (15834, LBA9402) and one *Agrobacterium tumifaciens* strain [GV 3101 (PMP 90RK, p35SGUS-2)] and four culture media were tested and compared for their ability to induce hairy root formation on wounded *Papaver somniferum* L. hypocotyls. The strain LBA 9402 was found to be more effective (Flem-Bonhomme *et al.*, 2004).

2.2.4 Co-culture

Hairy roots were successfully established in *Clitoria ternatea* (Linn.) using *Agrobacterium rhizogenes* strain A13 for biosynthesis of alkaloid. Co-cultivation was done for five days (Malabadi and Nataraja, 2003).

Transformed hairy roots were efficiently induced from seedlings of *Taxacum platycarpum* by infection with *Agrobacterium rhizogenes* 15834. Co-cultivation was done for three days. Plantlets formed from transformed roots had numerous fibrous roots with abundant lateral branches instead of thickened taproots in non-transformed plants (Lee *et al.*, 2004).

Withania somnifera (L) Dunal. was transformed by treating the stem, leaf and hypocotyl explants placed on the co-cultivation medium with a 10 µl aliquot of *Agrobacterium rhizogenes* suspension (Pawar and Maheshwari, 2004).co-cultivation was done for two days.

Komarnytsky *et al.* (2004) reported successful transformation of tobacco plants. The *rol*ABC gene cluster harboring the *rol*A, *rol*B and *rol*C genes were cut out of the pUC9-*rol*ABC plasmid as a 4.3-kb fragment and ligated into pGreen to construct pRYG, the basic transformation vector for the induction of hairy root proliferation response. The pRYG vector harboring the three *rol* genes is a fast and effective method for the generation of transgenic hairy roots. Co-culture was done for 2 days.

2.2.5. Time Peroid for Hairy Root Induction

Hairy root induction was achieved in a time period of 2-4 weeks regardless of the *Agrobacterium rhizogenes* strains and the type of explant used (Muringer 1988; Yazaki *et al.*, 1998; Kittipongpatana *et al.*, 1998 and Zarate, 1999).

2.2.6 Culture Conditions for *A. rhizogenes*

Temperature is the most critical factor for good growth of *A. rhizogenes.* The optimum growth temperature for the growth of *A. rhizogenes* strains HRI, 8196, 15834 and A4 was reported to be 25°C (Deno *et al.*, 1987 and Christen *et al.*, 1989). Davioud *et al.* (1989) reported the optimum temperature for growth of 15834 strain of *A.rhizogenes* as 28°C. Yoshikawa and Furuya (1987) reported that strain A4 grows best at 25°C in dark conditions.

2.2.7 Methods of Bacterial Inoculation.

The hypocotyl segments of radiata pine were inoculated with a loop of agrobacterial strain, whereas the adventitious shoots were inoculated *in vivo* with bacterial suspension using a syringe fitted with a 24G needle at the site of 10-15 cm from the top of the adventitious shoots (Li and Leung, 2003).

Withania somnifera (L) Dunal. was transformed by treating the stem, leaf and hypocotyl explants placed on the co-cultivation medium with a 10 µl aliquot of *Agrobacterium rhizogenes* suspension (Pawar and Maheshwari, 2004). Co-cultivation was done for two days.

Komarnytsky *et al.* (2004) reported successful transformation of tobacco plants by excising a vascular tissue of the major vein of the leaf explants by a sterile surgical blade that had been immersed into a 2-day-old culture of the bacteria. Co-cultivation was done for two days.

2.2.8 Bacterial Density

A study on induction of hairy roots in *H. ala-kodien* by infection with agropine type *Agrobacterium rhizogenes* strains was conducted. MS medium containing bacteria (108 cells ml^{-1}) was found to be the best for successsfull transformation (Karmarkar *et al.*, 2001).

Koike *et al.* (2003) reported successful transformation of *Angelonia salicarifolia* by immersing the leaf segments in the A*grobacterium rhizogenes* suspensionof optical density (OD_{600}) 1.0 for 20 seconds. Co-culture was done for three days and hairy roots appeared after 2-4 weeks.

Hairy roots were induced from both cotyledon and hypocotyls explants of *Isatis indigotica* Fort. (Indigo woad) through transformation with *Agrobacterium rhizogenes* strain A4, R1601 and ATCC15834. Bacteria at exponential growth phase (attenuance (D_{600}) 0.7), was used for inoculation. High voltage paper chromatography (HVPE) analysis confirmed successful transformation (Tiefeng Xu , 2004).

2.2.9 Effect of Acetosyringone

Celma *et al.* (2000) reported successful transformation of *Duboisia* plantlets by *Agrobacterium rhizogenes* strain A4. leaf discs of approximately 1cm in diameter were added to 10 ml of liquid MS medium in petridishes containing 100µl of a culture of *Agrobacterium rhizogenes* grown overnight at 28°C in YEB medium containing 100µM acetosyringone.

Significant differences were observed between transformation efficiency of different strains of *Agrobacterium rhizogenes.* The age and differentiation status of plant tissue also affect the chances of successful transformation. Successful infection of some species can be achieved by addition of acetosyringone (Sevo *et al.*,2002).

Korac *et al.* (2004) reported the positive effect of acetosyringone on transformation with *Agrobacterium rhizogenes.* After inoculating the androgenic embryos of horse chestnut with the bacterial culture, they were transferred to solid MS medium with and without acetosyringone(50 µM). The presence of 50 µM acetosyringone during co-culturing significantly increased the number of putative transformants.

2.2.10 Elimination of Bacteria

The *plumbago rosea* explants co-cultivated with *Agrobacterium rhizogenes* (ATCC 15834) for transformation, were transferred to MS medium containing 3 per cent sucrose and 0.8 per cent agar along with 500 mg l^{-1} carbenicillin to eliminate the *Agrobacterium* growth (Komarnytsky *et al.*, 2004).

Hairy roots were successfully established in *Clitoria ternatea* (Linn.) using *Agrobacterium rhizogenes* following transformation for alkaloid biosynthesis.The

culture was made bacteria free by transfering to a medium containing cefotaxime 300 mg l^{-1} (Malabadi and Nataraja, 2003).

Leaf discs of *Duboisia* were blotted dry with sterile filter paper and placed on one per cent (w/v) bacto-agar containing 500 mg l^{-1} cefotaxime for eleminating the bacteria. Adventitious roots appearing after 4-8 weeks were excised and grown separatelyon solidified B5 medium containing 30 g l^{-1} and cefotaxime 500 mg l^{-1}. After 1 to 2 subcultures on this medium for four weeks each, the roots were transferred to half strength B5 without antibiotics (Celma *et al.*, 2000).

Transformed root cultures of *Withania somnifera* Dunal were obtained by infecting root cultures in vitro with *Agrobacterium rhizogenes* LBA 9402. The roots which developed after 3-4 weeks were excised and transferred to MSO medium containing three per cent sucrose and 500 mg l^{-1}. They grew axenically in the absence of exogenous plant growth regulators in MS medium containing three per cent (w/v) sucrose. The root cultures synthesized several withanaloides of which withanaloide D was isolated and identified. The productivity of withanaloide D in transformed root was higher than in non-transformed root cultures (Ray *et al.*, 1996).

2.2.11 Confirmation of Transformation

The simplest criteria for confirming the transformation is the growth characteristics and morphology of hairy roots.

Hairy roots are characterised by fast growth and high degree of lateral branching on hormone free nutrient medium. They are negatively geotropic and possess numerous root hairs (Rhodes *et al.*, 1989 and Banerjee *et al.*, 1995).

Confirmation of transformation can be done by opine detection. Opine synthesis is an essential feature of integration of T-DNA into the plant genome (Petit *et al.*, 1983; De Paolis *et al.*, 1985; Brevet *et al.*, 1988; Bouchez and Tourner, 1990). Opines are not synthesised by uninfected plant tissues. Opine analysis is done by high voltage paper electrophoresis (Petit *et al.*, 1983).

Two series of *Atropa belladonna* hairy root lines were obtained, the first one transformed *viz.*, *Agrobacterium tumefaciens* harbouring rol C and npt II genes, and the other transformed with *rol* ABC and *npt II* genes. Thirteen hairy root lines were obtained and selected on hormone-free medium. The transformation was confirmed by PCR (Bonhomme *et al.*, 2000).

A study on induction of hairy roots in *H. ala-kodien* by infection with agropine type *Agrobacterium rhizogenes* strains was conducted. *Five A. rhizogenes* strains, i.e. PcA4, 15834, A4 (all agropine type), 8196 (mannopine type) and 2659 (cucumopine type), and different types of explant, *ie.* leaf, internodal segments, shoot buds, seedling hypocotyls and callus, were tested. The hairy roots were thin, whitish in colour and showed negatively geotropic growth. The transformed nature of hairy roots was confirmed by opine analysis (Karmarkar *et al.*, 2001).

Hairy roots were induced from *Phytolacca acinosa* leaves after co-culturing with *Agrobacterium rhizogenes* strain Ar1334 containing the agropine type pRi1855 and the binary vector pIG121HM. A 560-bp DNA fragment was amplified from the genomic DNA of hairy root by PCR using primers of *rol* C gene, one of the four genes related with hairy root induction on the T_L-DNA of the Ri plasmid. Blue dots were observed on the hairy root tips after dipping in X-glue solution at 37°C for one night (Cui- Hong *et al.*, 2004). Presence of T-DNA in the plant genome can be confirmed by Southern blotting and hybridisation. It was first utilised by Ackermann (1977).

Materials and Methods

3. MATERIALS AND METHODS

The present study, '*Agrobacterium* mediated genetic transformation of *Koduveli* (*Plumbago* spp. L)' was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2003- 2006. Attempts were made to evolve a successful protocol for *in vitro* propagation and genetic transformation in *Plumbago*. Propagation studies were carried out by enhanced release of axilary buds, and indirect organogenesis. Genetic tansformation was attempted by co-culture method using *Agrobacterium rhizogenes.*

The details of materials and methods adopted for the study are presented in this chapter.

3.1 *IN VITRO* PROPAGATION

3.1.1 Collection and Preparation

Explants of *Plumbago zeylanica and Plumbago rosea* from the medicinal plant garden of the Department of Plantation Crops and Spices, College of Agriculture, Vellayani, were used for this study.

3.1.2 Surface Sterilization

Nodes and leaves of *Plumbago rosea* and *Plumbago zeylanica* were collected and washed in running tap water. The explants were then immersed in water with a few

drops of wetting agent, for half an hour, followed by washing several times in distilled water. They were then transferred to a sterile beaker and treated with mercuric chloride (0.08 per cent) for 10 minutes. This was followed by washing five times in sterile distilled water.

3.1.2 Inoculation and Incubation

The glassware and tools required for inoculation were washed thoroughly in tap water, rinsed with distilled water, covered with aluminium foil and autoclaved at 121^0 C and 1.06 kg cm^{-2} pressure for 45 minutes. Horizontal type autoclave (NAT Steel) was used for autoclaving.

Inoculation was carried out in a laminar flow chamber (Klenzaids, Model 1104). Surface sterilized explants were cut using a sterile surgical blade. Single node cuttings (0.5-0.8 cm) and leaf pieces (0.5 cm^2) were inoculated in the culture medium.

The cultures were incubated in a culture room maintained at a light intensity of 3000 lux using white fluorescent light for 16 h per day. The temperature of the room was regulated using an air conditioner at 20 ± 2^0 C at a relative humidity (RH) of 60 per cent.

3.1.3 Culture Media

The culture media used for the study were MS (Murashige and Skoog, 1962), and White's. The chemicals used in the culture media were of analytical grade from

Sisco Research Laboratories (Mumbai), Merck (Mumbai) and British Drug House (Mumbai).

Standard procedures were followed for the preparation of basal medium (Thorpe, 1980). Stock solutions of major and minor nutrients, organic supplements and plant growth substances were prepared by dissolving the required quantity of chemicals in specific volume of distilled water / ethyl alcohol / 0.1N HCl or NaOH, depending on the chemical and were stored under refrigerated conditions (4 ^{0} C).

The glassware used in the experiment were washed with dilute liquid detergent (Labolene) and rinsed with distilled water. Specific quantities of stock solutions were pipetted out into 1000ml beaker. Sucrose, adenine sulphate and inositol were added fresh in required quantity, weighed using an electronic balance (Sartorius analytic A120S) and dissolved by constant stirring. The pH of the medium was adjusted between 5.6 and 5.8 using an electronic pH meter. The final volume was made up to 1000 ml, using a volumetric flask. Agar was added and melted using a heating mantle, stirring continuously while heating for uniform mixing. The melted medium was dispensed into pre-sterilized culture vessels such as test tubes (25x150 mm) and Erlenmeyer flasks (100ml). The culture vessels containing the medium were plugged firmly with non absorbent cotton. They were then autoclaved at 121 ^{0} C and 1.06 kg cm^{-2} pressure for 20 minutes.

3.1.4 Explant

Nodal segments excised from *ex vitro* raised plants were tried for enhanced release of axillary buds and leaf segments for indirect organogenesis in *Plumbago rosea* and *Plumbago zeylanica.*

3.1.5 Enhanced Release of Axillary Buds

3.1.6.1 *Plant Growth Substances*

Cytokinins and Auxins

Explants were subjected to treatments with different combinations of cytokinin (BA) auxin (IAA) with MS as the basal media. Treatments involved different levels of BA (0.25 mg l^{-1} to 1.5 mg l^{-1}) and IAA (0.05 to 0.5 mg l^{-1}) (Table 1). The treatments were replicated six times. In the case of nodal segments, observations were recorded on number of days for bud initiation, number of shoots, length of longest shoot and number of leaves.

3.1.6.2 Basal Media

The best treatment was later tried on MS half strength and White's media to assess its effect on shoot proliferation (Table 2). The treatments were replicated six times. Observations were recorded on number of days for bud initiation, number of shoots, length of longest shoot and number of leaves.

3.1.6 Indirect Organogenesis

3.1.7.1 Callus Initiation

Explants of *P.rosea* and *P. zeylanica* were subjected to different treatments for callus initiation. The treatments tried included different levels of 2,4-D (0.05 to 0.50 mg

Table 3 Plant growth regulators tested for shoot proliferation *via* enhanced release of axillary buds from nodal explants of *P. rosea* and *P. zeylanica.*

Treatments	Plant growth regulators (mg l^{-1})
P1	BA 0.25 + IAA 0.05
P2	BA 0.50 + IAA 0.05
P3	BA 1.00 + IAA 0.05
P4	BA 1.50 + IAA 0.05
P5	BA 0.25 + IAA 0.10
P6	BA 0.50 + IAA 0.10
P7	BA 1.00 + IAA 0.10
P8	BA 1.50 + IAA 0.10
P9	BA 0.25 + IAA 0.20
P10	BA 0.50 + IAA 0.20
P11	BA 1.00 + IAA 0.20
P12	BA 1.50 + IAA 0.20
P13	BA 0.25 + IAA 0.50
P14	BA 0.50 + IAA 0.50
P15	BA 1.00 + IAA 0.50
P16	BA 1.50 + IAA 0.50

Culture medium:Basal medium + Inositol (100 mg l^{-1}) + Sucrose (30g l^{-1}) + Agar (6.3 g l^{-1})

Table 4 Effect of basal media on shoot proliferation *via* enhanced release of axillary buds from nodal explants of *P. rosea* and *P. zeylanica.*

Treatment	Basal medium
M1	½ MS
M2	MS
M3	White's

Culture medium: Basal medium + Inositol (100 mg l^{-1}) + Sucrose (30g l^{-1}) +Agar (6.3 g l^{-1}) + Plant growth regulators

Table 5 Plant growth regulators tested for callus initiation from lesf explants of *P. rosea* and *P. zeylanica*

Treatment	Plant growth regulator(s) mg l^{-1}
C1	2,4-D 0.10
C2	2,4-D 0.20
C3	2,4-D 0.25
C4	2,4-D 0.50
C5	BA 1.00
C6	BA 1.50
C7	BA 2.00
C8	BA 2.50
C9	BA 1.00 + 2,4-D 0.10
C10	BA 1.00 + 2,4-D 0.20
C11	BA 1.00 + 2,4-D 0.25
C12	BA 1.00 + 2,4-D 0.50
C13	BA 1.50 + 2,4-D 0.10
C14	BA 1.50 + 2,4-D 0.20
C15	BA 1.50 + 2,4-D 0.25
C16	BA 1.50 + 2,4-D 0.50
C17	BA 2.00 + 2,4-D 0.10
C18	BA 2.00 + 2,4-D 0.20
C19	BA 2.00 + 2,4-D 0.25
C20	BA 2.00 + 2,4-D 0.50
C21	BA 2.50 + 2,4-D 0.10
C22	BA 2.50 + 2,4-D 0.20
C23	BA 2.50 + 2,4-D 0.25
C24	BA 2.50 + 2,4-D 0.50

Culture medium: MS + Inositol (100 mg l^{-1}) + Sucrose (30g l^{-1}) +Agar (6.3 g l^{-1})

l^{-1}) and BA (0.5 to 2 mg l^{-1}) (Table 5). The treatments were replicated six times. Observations were recorded on number of cultures initiating callus from the explant. Callus Index (CI) was computed by multiplying the per cent cultures initiating callus with growth score (G). Growth of callus was assessed based on visual rating (with score 1.0 to the smallest and score 4.0 to the largest). The meam score was expressed as growth score, 'G' (poor 1, Medium 2, Good 3, Profuse 4).

3.1.7.2 Shoot Regeneration

The callus was inoculated into medium with varying levels of auxins and cytokinins. Different concentrations of BA (0.5 to 2 mg l^{-1} and IAA (0.05 to 1.0 mg l^{-1}) and their combinations were tried to assess the effect of these plant growth substances on regeneration of shoot from callus (Table 6). The treatments were replicated six times. Observations were taken on number of cultures developing shoots, number of shoots and length of the longest shoot.

3.1.7 Rooting

3.1.8.1 In vitro Rooting

Well developed shoots having 3-4 cm length were subjected to different rooting treatments. The shoots were inoculated into medium with varying levels of IAA (0.05 to 1.0 mg l^{-1}) and NAA (0.05 to 1.0 mg l^{-1}) (Table 7). The treatments were replicated six times. Observations were taken on per cent cultures initiating roots, number of days for root initiation, number of roots and length of roots.

Table 6 Plant growth regulators tested for shoot regeneration from the calli of *P. zeylanica* and *P. rosea*

Treatment	Plant growth regulators (mg l^{-1})
SR1	BA 1.00 + IAA 0.05
SR2	BA 1.00 + IAA 0.10
SR3	BA 1.00 + IAA 0.15
SR4	BA 1.00 + IAA 0.20
SR5	BA 1.25 + IAA 0.05
SR6	BA 1.25 + IAA 0.10
SR7	BA 1.25 + IAA 0.15
SR8	BA 1.25 + IAA 0.20
SR9	BA 1.50 + IAA 0.05
SR10	BA 1.50 + IAA 0.10
SR11	BA I.50 + IAA 0.15
SR12	BA 1.50 + IAA 0.20
SR13	BA 1.75 + IAA 0.05
SR14	BA 1.75 + IAA 0.10
SR15	BA 1.75 + IAA 0.15
SR16	BA 1.75 + IAA 0.20
SR17	BA 2.00 + IAA 0.05
SR18	BA 2.00 + IAA 0.10
SR19	BA 2.00 + IAA 0.15
SR20	BA 2.00 + IAA 0.20

Culture medium: MS + Inositol (100 mg l^{-1}) + Sucrose (30 g l^{-1}) +Agar (6.3 g l^{-1})

Table 7 Plant growth regulators tested for rooting the regenerated shoots of *P. zeylanica* and *P. rosea*

Treatment	Plant growth regulators (mg l^{-1})
R1	IAA 0.05
R2	IAA 0.10
R3	IAA 0.25
R4	IAA 0.50
R5	IAA 1.00
R6	NAA 0.05
R7	NAA 0.10
R8	NAA 0.25
R9	NAA 0.50
R10	NAA 1.00

Culture medium: MS + Inositol (100 mg l^{-1}) + Sucrose (30 g l^{-1}) +Agar (6.3 g l^{-1})

Table 8 Plant growth regulators tested for multiplication of shoots in *P. zeylanica* and *P.rosea*

Treatment	Plant growth regulators (mg l^{-1})
MR1	IAA 0.25
MR2	IAA 0.50
MR3	IAA 1.00
MR4	IAA 1.25
MR5	IAA 1.50
MR6	NAA 0.25
MR7	NAA 0.50
MR8	NAA 1.00
MR9	NAA 1.25
MR10	NAA 1.50

Culture medium: MS + Inositol (100 mg l^{-1}) + Sucrose (30 g l^{-1}) +Agar (3.15 g l^{-1})

3.1.8 Root culture

Root segments from the *in vitro* cultured plants were innoculated into semisolid full strength MS medium with different concentrations and combinations of auxins to multiply the roots (Table 8). Observations were taken on number of days for root multiplication and per cent cultures showing root multiplication.

3.2 GENETIC TRANSFORMATION

3.2.1 Bacterial strains and binary vectors

Three strains of *Agrobacterium rhizogenes viz.,* A4, MTCC 532 and MTCC 2364 were used for the study. These strains by virtue of harboring the ***rol*** genes exhibit high transformation efficiency.

A4 is a virulent agropine strain possessing three plasmids. Plasmid a (pArA4a, 180 kb) is not necessary for plant transformation. Plasmid b (pRiA4, 250 kb) is the root inducing plasmid. Plasmid c (pArA4C) is a cointegrate of pArA4a and pRiA4.

3.2.2 Culturing of Bacteria

The *Agrobacterium rhizogenes* strains A4, MTCC 532 and MTCC 2364 harboring *rol* genes were cultured on plates with YEP medium.

3.2.3. Screening of *Agrobacterium* strains for sensitivity to antibiotics

The YEP medium was prepared in the required volume in conical flasks, sterilized and kept in the culture room until use. On the day of experiment the medium was melted and then cooled to 40°C.

3.2.3.1 Ampicillin

Ampicillin stock was prepared by dissolving ampicillin in water. The YEP medium was melted, cooled; ampicillin stock was added in the required quantity and swirled. The medium was then poured into sterile disposable petri plates. When the medium was cooled and solidified the bacterial strains were spread on the medium with an L shaped rod and then the Petri plates were sealed with parafilm and kept in dark. The growths of the bacterial strains were recorded for three days.

3.2.3.2 Cefotaxime

The stock solution of cefotaxime was prepared by dissolving it in sterile water. The YEP medium was melted, cooled, the cefotaxime stock solution added in the required quantity and swirled. The medium was then poured into sterile disposable petri plates and allowed to solidify. The bacterial strains were spread on the medium with an L shaped rod and then petri plates were sealed with parafilm and kept in the culture room. The growths of different strains were recorded.

3.2.6 Genetic Transformation of *Plumbago*

3.2.6.1. Preparation of Agrobacterium suspension for Co-cultivation

The three strains of bacteria were grown on petri plates with YEP medium. A single colony was inoculated in 20 ml liquid YEP medium. The liquid medium was kept in a shaker overnight. The bacterial suspension was spun in a centrifuge at 5000 rpm for five minutes at 4°C. The bacterial pellet obtained was resuspended in liquid MS medium with or without acetosyringone 200μM and kept in a shaker for one hour.

3.2.6.2. Preparation of Plant Material

Leaves, nodes, calli and roots were used as the explants for co-cultivation experiments. These explants were precultured for fourteen days before co-cultivation to maintain the cultures in active cell division and to get rid of the accumulated plant growth regulators from the previous sub cultures.

3.2.6.3 Co-culture

The precultured explants were used for co-cultivation experiments. They were cut by sterile knives and wounded to facilitate the infection process. *A. rhizogenes* responds to phenolic compounds like acetosyringone and hydroxyl acetosyringone, which are exuded by wounded plants. These small molecules act to induce the activity

of *vir* genes that are encoded on the plasmid. This helps in initiating the first step in the infection process *viz.*, attachment of *A. rhizogenes* to an open wound.

The precultured explants were transferred to the bacterial suspension in a laminar flow chamber. The explants were mixed thoroughly with the bacterial suspension by keeping it in a shaker at 90 rpm for one hour.

3.2.6.4. Addition of Acetosyringone

After mixing thoroughly with the bacterial suspension, one set of explants was blotted with sterile filter paper and placed a petri plate containing MS medium and acetosyringone at a concentration of 200 μM. The other set of explants was blotted and placed on MS medium without acetosyringone. The petri plates were sealed and kept in dark for two days at 26°C in a culture room.

3.2.6.5. Killing the Bacteria

After co-cultivation the explants were washed in sterile distilled water and then in a solution containing 500 mg l^{-1} cefotaxime. They were blotted dry with filter paper and transferred to petri plates containing MS medium with 500 mg l^{-1} cefotaxime.

3.2.6.6 confirmation of Transformation

3.2.6.6.1 Morphological Characters

The hairy roots produced by transformation were negatively geotropic and possessed numerous root hairs. Hairy roots were characterised by high degree of lateral branching. The root hairs were detected under a stereo microscope (plate No. 13).

3.2.6.6.2 Opine Analysis

Opine analysis was done according to the modified procedure given by Dessaux *et al.* (1991).

a) Preparation of reagents

A buffer system of 1.1M acetic acid and 0.7 M formic acid at pH 3.2 was used for the separation of opines. The buffer system was prepared by mixing acetic acid, formic acid and water 50:4:46. (v/v/v)

Various reagents were used for detection of opines.

1. Solution 1 – 0.4 per cent silver nitrate in 99:1 acetone: water mixture was prepared. The reagent was stored in amber coloured bottle under refrigerated conditions.

2. Solution 2- 2 per cent NaOH in 90 per cent ethanol in water was prepared.

3. Solution 3 – a) Reducer A concentrate: saturated potassium ferricyanide

b) Reducer B concentrate: saturated sodium thiosulphate

c) Reducer C concentrate: 25 per cent sodium carbonate

One ml of reducer A concentrate was mixed with two ml of reducer B concentrate and 0.35 ml of reducer C concentrate was added to the mixture. The mixture was diluted to 150 ml using distilled water.

b)Extraction of opines

500 mg of fresh root tissue was ground homogenously in 500 µl of 0.1 M HCL, and left at 4°C for two hours. It was then centrifuged at 9000g for five minutes (Xu *et al.*, 2004). The supernatant was collected and used for detection of opines.

c) Separation of opines

Ten µl of the root extract was spotted on Whatman No.1 chromatography paper strip. The standard agropinic acid, mannopine and mannopinic acid were dissolved in autoclaved double distilled water and used for spotting. Small volumes of samples were applied successively using micropipette and in between a current of warm air from drier was used to concentrate the spots. The moistened paper was placed on the support of horizontal electrophoresis unit (BIO RAD, SUB CELL GT) containing equal volumes of buffer in both the wells, such that both ends of the paper touches the buffer. Spotted end was kept at the anode end of the electrophoresis unit and the extract was subjected to high voltage paper electrophoresis at 400 v/cm for 45 minutes. Following electrophoresis, the paper was dried in a stream of hot air using a hair drier.

d) Detection of opines

Opines were detected using alkaline silver nitrate regent. The dried paper was first dipped into silver nitrate reagent, allowed to dry in a stream of cold air, and dipped in sodium hydroxide solution. The paper strip was dried in hot air using a hair drier. The background was reduced by dipping the developed electrophorograms in reducer solution, followed by drying in a flow of hot air. The observation regarding the presence or absence of opines were documented.

Results

4. RESULTS

The present study titled 'Genetic transformation in *Koduveli* (*Plumbago s*pp Linn.)' was carried out to standardise the protocols for *in vitro* propagation and genetic transformation in *Plumbago zeylanica* and *Plumbago rosea,* in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2004 –2006. The results of the study are presented in this chapter.

4.1 *IN VITRO* PROPAGATION

4.1.1 Enhanced Release of Axillary Buds

4.1.1.1 Explant(s)

Nodal segments and leaves excised from stem cuttings of green house grown *Plumbago zeylanica* and *Plumbago rosea* were used as explants for this experiment. Nodal segments gave rise to shoots. However, leaf explants did not give rise to shoots.

4.1.1.2 Plant Growth Substances

Cytokinins and Auxins

Sixteen treatments involving various concentrations and combinations of plant growth substances (BA and IAA) were tried to study their effect on shoot proliferation from nodal explants. Results (Table 9) of the study are presented below. Bud initiation is indicated by swelling of axillary buds, which later develops into shoots (Plate No 1) .

Table 9 Effect of plant growth regulators on enhanced release of axillary buds from nodal explants of *P. zeylanica* and *P.rosea*

Treatment	Plant growth regulators ($mg\ l^{-1}$)		*P. zeylanica: P. rosea*		
	BA	IAA	DBI	SpC	LLS
P1	0.25	0.05	9.00 : 9.33	2.50 : 2.3	3.50 : 3.50
P2	0.50	0.05	8.83 : 8.67	2.50 : 2.5	3.67 : 3.33
P3	1.00	0.05	5.17 : 6.33	5.50 : 5.00	4.67 : 5.83
P4	1.50	0.05	6.83 : 5.17	4.00 : 6.33	3.72 : 6.00
P5	0.25	0.10	11.17 : 10.33	2.30 : 2.80	3.50 : 3.33
P6	0.50	0.10	9.83 : 9.33	3.00 : 4.00	4.67 : 4.83
P7	1.00	0.10	10.17 : 10.50	4.67 : 4.40	5.00 : 4.67
P8	1.50	0.10	7.67 : 7.50	4.50 : 4.60	5.33 : 5.00
P9	0.25	0.20	11.00 : 11.33	3.17 : 3.80	4.50 : 5.33
P10	0.50	0.20	10.33 : 10.83	4.00 : 4.42	5.83 : 4.50
P11	1.00	0.20	8.00 : 8.33	4.83 : 4.98	5.80 : 5.83
P12	1.50	0.20	8.17 : 8.50	4.67 : 4.83	5.30 : 4.00
P13	0.25	0.50	11.33 : 8.62	3.00 : 3.33	5.17 : 5.80
P14	0.50	0.50	11.33 : 10.83	3.33 : 4.50	5.33 : 5.67
P15	1.00	0.50	7.83 : 7.50	4.83 : 5.00	4.17 : 4.67
P16	1.50	0.50	8.33 : 8.67	4.83 : 5.00	4.00 : 4.60

The data represent mean of six replications; Culture medium:MS
DBI: days for bud initiation; SpC: Shoots per culture
LpS: Leaves per shoot; LLS: Length of longest shoots

In *P. zeylanica* bud initiation was the earliest (5 days) when cultured in P3 (BA 1.0 mg l^{-1} + IAA 0.05 mg l^{-1}) and in *P. rosea* bud initiation was the earliest (6.33 days) when cultured in P4 (BA 1.5 mg l^{-1} + IAA 0.05 mg l^{-1}). In *P. zeylanica* bud initiation was late (11.7 days) when cultured in P6 (BA 0.50 mg l^{-1} + IAA 0.10 mg l^{-1}). In *P. rosea* bud initiation was late (11.33 days) in P9 (BA 0.25 mg l^{-1} + IAA 0.20 mg l^{-1}).

P. zeylanica produced maximum number of shoots (5.5) per culture in P3 (BA 1.00 mg l^{-1} + IAA 0.05 mg l^{-1}). Whereas *P. rosea* produced maximum number of shoots (6.33) per culture in P4 (BA 1.5 mg l^{-1} + IAA 0.05 mg l^{-1}). The least number of shoots (3.0) per culture was recorded by P5 (BA 0.25 mg l^{-1} + IAA 0.10 mg l^{-1}) in *P. zeylanica*. In *P. rosea* the least number of shoots (2.3) per culture was recorded by P1 (BA 0.25 mg l^{-1} + IAA 0.05 mg l^{-1}).

The longest shoot (5.17 cm) was obtained in the treatment P4 (BA 1.5 mg l^{-1} + IAA 0.05 mg l^{-1}), followed by P3 (BA 1.0 mg l^{-1} + IAA 0.05 mg l^{-1}) in *P. zeylanica*. In *P. rosea* the longest shoot (6.00 cm) was obtained in the treatment P4 (BA 1.5 mg l^{-1} + IAA 0.05 mg l^{-1}), followed by P3 (BA 1.0 mg l^{-1} + IAA 0.05 mg l^{-1}). In *P. zeylanica* the shortest shoot (3.33 cm) was obtained in the treatment P9 (BA 0.25 mg l^{-1} + IAA 0.20 mg l^{-1}). Whereas in *P. rosea* the shortest shoot (3.5 cm) was obtained in P1 (BA 0.25 mg l^{-1} + IAA 0.05 mg l^{-1}).

In *P. zeylanica* number of leaves per shoot (4.12) was the highest in treatment P3 (BA 1.0 mg l^{-1} + IAA 0.05 mg l^{-1}). In *P. rosea* maximum number of leaves per shoot (4.02) was recorded in P3 (BA 1.0 mg l^{-1} + IAA 0.05 mg l^{-1}).

In *P. zeylanica* the lowest number of leaves per shoot (2.68) in P1 (BA 0.25 mg l^{-1} + IAA 0.05 mg l^{-1}). However in *P. rosea* the lowest number of leaves per shoots (2.42) was recorded in P1 (BA 0.25 mg l^{-1} + IAA 0.05 mg l^{-1}).

4.1.1.3 Basal Medium

Basal media such as half strength MS, full strength MS and White's were compared to assess their effect on shoot proliferation in *P. zeylanica* and *P. rosea.* Results (Table 10) of the study are presented below.

Full strength MS was found to be superior to others with respect to number of shoots, length of longest shoot and number of leaves per shoot for*P. Zeylanica* and *P. rosea.* Maximum number of shoots in *P. zeylanica* (3.83) and in *P. rosea* (3.00) were produced by cultures in full strength MS. The length of the longest shoot was the highest (4.83 cm) for cultures in full strength MS in *P. Zeylanica.* In *P. rosea* the longest shoot (3.57 cm) was obtained in cultures on full strength MS. In *P. zeylanica* the longest shoot (2.67 cm) was recorded for cultures in White's medium. In *P. rosea* also, the longest shoot (2.42 cm) was obtained for cultures in White's medium.

Maximum number of leaves per shoot for *P. zeylanica* (3.33) was recorded by the cultures on full strength MS. In *P. rosea* the maximum number of leaves per shoot (3.07) was achieved in cultures on full strength MS medium. The lowest values in this regard (1.67 and 2.21 respectively for *P. zeylanica* and *P. rosea*) were obtained for cultures on White's medium.

4.1.2 Indirect Organogenesis

4.1.2.1 Callus Initiation

Twenty four treatments with different combinations of plant growth substances (2,4-D and BA) were tried to assess their effect on callus initiation, using leaf explants of *P. zeylanica* and *P. rosea* (Plate No 2 and 3). The results showed (Table 11) that cent per cent cultures initiated callus with leaf explants of *P. zeylanica.*

Table 10 Effect of basal media on shoot proliferation in *P. zeylanica* and *P. rosea*

Treatmen	Basal media	*P. zeylanica: P. rosea*		
		SpC	LLS	LpC
M1	½ MS	2.17 : 2.42	3.50 : 2.83	2.00 : 2.81
M2	MS	3.83 : 3.00	4.83 : 3.57	3.33 : 3.07
M3	White's	2.00 : 2.17	2.67 : 2.42	1.67 : 2.21

The data represent mean of six replications;Culture medium:MS
SpC: Shoots per culture: LLS: Length of longest shoot
LpS: Leaves per shoot

Plate No. 1 Enhanced release of axillary buds from nodal explants of *P. zeylanica*

Plate No. 2 Callus induction from the leaf explants of *P. zeylanica*

Plate No. 3 Callus induction from leaf explants of *P. rosea*

Plate No. 4 Regeneration of shoots from the leaf derived callus

Plate No. 5 Regeneration of shoots from the leaf derived callus of *P. zeylnica*

Plate No. 6 Regeneration of shoots from the leaf derived callus of *P. rosea*

Plate 1

Plate 2

Plate 3

Plate 4

Plate 5

Plate 6

Table 11 Effect of plant growth regulators on callus initiation from leaf explants of *P. zeylanica* and *P.rosea*

Treatment	Plant growth regulators (mg l^{-1})		*P. zeylanica: P. rosea*			
	BA	2,4-D	CIC(%)	G	CI	NC
C1	1.00		25 :	1.00 : -	25 : -	BF : -
C2	1.50		50 :	1.00 : -	50 : -	BF : -
C3	2.00		67 : 25	1.00 : 1.00	67 : 25	BF : BF
C4	2.50		67 : 67	1.00 : 1.00	67 : 67	BF : BF
C5		0.10	50 : 83	1.00 : 1.00	50 : 83	BC : BF
C6		0.20	33.33: 83	1.00 : 1.00	33.33 : 83	BC : BF
C7		0.25	50 : 83	1.50 : 1.50	75 : 124.5	BC : BC
C8		0.50	50 : 67	1.50 : 1.50	75 : 100.5	BF : BC
C9	1.00	0.10	83 : 17	2.00 : 1.00	166 : 17	BF : BC
C10	1.00	0.20	25 : 17	1.00 : 1.00	25 : 17	BF : BC
C11	1.00	0.25	25 : 50	1.00 : 1.50	25 : 75	BF : BF
C12	1.00	0.50	67 : 50	1.00 : 1.50	67 : 75	BF : BF
C13	1.50	0.10	67 : 50	1.00 : 1.00	67 : 50	BF : BF
C14	1.50	0.20	83 : 83	1.50 : 1.50	124.5 : 124.5	BF : BF
C15	1.50	0.25	83 : 83	2.00 : 1.50	166 : 124.5	BF : BF
C16	1.50	0.50	83 : 83	2.00 : 1.50	166 : 124.5	BF : BF
C17	2.00	0.10	100 : 100	1.50 : 2.00	150 : 200	BF : BF
C18	2.00	0.20	100 : 100	1.50 : 2.50	150 : 250	BF : BF
C19	2.00	0.25	100 : 100	2.00 : 2.50	200 : 250	BF : BF
C20	2.00	0.50	100 : 100	3.00 : 3.00	300 : 300	BF : BF
C21	2.50	0.10	100 : 100	1.50 : 1.50	150 : 150	BF : BF
C22	2.50	0.20	100 : 100	2.00 : 2.00	200 : 200	BF : BF
C23	2.50	0.25	100 : 100	2.00 : 2.00	200 : 200	BF : BF
C24	2.50	0.50	100 : 100	2.50 : 2.00	250 : 200	BF : BF

The data represent mean of six replications; Culture medium:MS
CIC: Cultures initiating callus; G: Growthscore; CI: Callus index
NC: Nature of callus; BF: Brown friable; BC: Brown compact

In *p. zeylanica* the highest callus index (300) was recorded by C20 (BA 2 mg l^{-1} + 2,4-D 0.50 mg l^{-1}). In *P. rosea* also the highest callus index (300) was recorded by C20 (BA 2 mg l^{-1} + 2,4-D 0.50 mg l^{-1}), followed by a callus index of 250 in C24 (BA 2.5 mg l^{-1} + 2,4-D 0.5 mg l^{-1}).

The lowest callus index (25) was registered by C1 (BA 1.00 mg l^{-1}), C10 (BA 1.00 mg l^{-1} + 2,4-D 0.20 mg l^{-1}) and C11 (BA 1.00 mg l^{-1} + 2,4-D0.25 mg l^{-1}) in *P. zeylanica*. In *p. rosea* the lowest callus index (25) was recorded by C3 (BA 2.00 mg l^{-1}), C4 (BA 2.50 mg l^{-1}), C5 (2,4-D 0.10 mg l^{-1}), C6 (2,4-D 0.20 mg l^{-1}) and C14 (BA 1.50 mg l^{-1} + 2,4-D 0.20 mg l^{-1}).

4.1.2.2 Shoot Regeneration

The calli obtained in the initiation media of the two plant species were sub-cultured into twenty different treatments with various growth regulators such as cytokinin (BA) and auxin (IAA) (Plate No 5 and 6). The rate of shoot regeneration (Table 12) from calli cultures varied widely in the two species studied. Green nodular structures developed into dark green shoots over the entire surface of the culture medium (Plate No 4).

In *P. zeylanica* the longest shoot (3.10 cm) was reported by SR13 (BA1.75 mg l^{-1} + IAA 0.05 mg l^{-1}). The longest shoot was recorded by SR17 (BA 1.75 mg l^{-1} + IAA 0.05 mg l^{-1}) in *P. rosea*. The shortest shoot (1.2 cm) was recorded by SR4 (BA 1.0 mg l^{-1} + IAA 0.2 mg l^{-1}) in *P. zeylanica*. Whereas in *P. rosea* SR3 (BA 1.0 mg l^{-1} + IAA 0.15 mg l^{-1}) recorded the shortest shoot.

Table 12 Effect of plant growth regulators on shoot regeneration from callus of *P. zeylanica* and *P.rosea*

Treatment	Plant growth regulators (mg l^{-1})		*P. zeylanica: P. rosea*		
	BA	IAA	SRP	SpC	LLS (cm)
SR1	1.00	0.05	- : -	- : -	- : -
SR2	1.00	0.10	- : -	- : -	- : -
SR3	1.00	0.15	50 : -	1.00 : -	1.35 : -
SR4	1.00	0.20	50 : 25	1.50 : 1.33	1.20 :1.20
SR5	1.25	0.05	25 : 25	1.50 : 1.83	1.50 : 1.30
SR6	1.25	0.10	50 : 50	1.83 : 2.00	2.00 : 1.50
SR7	1.25	0.15	67 : 67	2.25 : 2.17	2.00 : 1.80
SR8	1.25	0.20	83 : 67	2.25 : 2.25	2.15 : 2.00
SR9	1.50	0.05	83 : 83	2.55 : 2.50	2.10 : 1.80
SR10	1.50	0.10	83 : 83	2.50 : 3.17	2.50 : 2.50
SR11	1.50	0.15	83 : 83	3.33 : 3.27	2.15 : 2.25
SR12	1.50	0.20	83 : 100	3.83 : 4.00	2.30 : 2.25
SR13	1.75	0.05	100 : 100	4.83 : 4.33	3.10 : 2.80
SR14	1.75	0.10	100 : 100	4.33 : 4.17	2.80 : 2.50
SR15	1.75	0.15	100 : 100	3.50 : 3.83	2.75 : 2.50
SR16	1.75	0.20	100 : 100	3.50 : 3.83	2.90 : 2.80
SR17	2.00	0.05	100 : 100	3.83 : 3.50	2.80 : 2.90
SR18	2.00	0.10	100 : 100	3.17 : 3.67	2.90 : 2.60
SR19	2.00	0.15	100 : 100	3.17 : 3.50	2.70 : 2.50
SR20	2.00	0.20	100 : 100	3.33 : 3.50	2.80 : 2.50

The data represent mean of six replications;Culture medium:MS
DBI: Days for bud initiation; SRP: Shoots regeneration per cent
LLS: Length of longest shoot

In *P. zeylanica* number of shoots per culture was the highest for SR13 (BA1.75 mg l^{-1} + IAA 0.05 mg l^{-1}). In *P. rosea* the SR13 (BA 1.75 mg l^{-1} + IAA 0.05 mg l^{-1}) recorded the highest value (4.33) for the number of shoots per culture.

4.1.2.3 Rooting

4.1.2.3.1 in vitro Rooting

Ten treatments with various levels of auxins (IAA and NAA) were tried for *in vitro* rooting in the two plant species (Plate No 8 and 9). Results (Table 13) of the study are discussed below.

In *P. zeylanica* the least number of days for root initiation (10.5 days) was obtained for the treatment R4 (IAA 0.5 mg l^{-1}) in *P. zeylanica* and *P. rosea*. The number of days taken for root initiation was the highest (18 days) for R6 (NAA 0.05 mg l^{-1}) in both *P. zeylanica* and *P. rosea*. The treatments R4 (IAA 0.5 mg l^{-1}) and R5 (IAA I mg l^{-1}) recorded the maximum number of roots in *P. zeylanica* and *P. rosea*. The least number of roots (1.48) was recorded in the treatment R7 (NAA 0.1 mg l^{-1}) in *P. zeylanica*. In *P. rosea* the least number of roots (2.00) was recorded in the treatment R10 (NAA 2.00 mg l^{-1}) . The longest root (2.85 cm and 3.05 cm respectively for *P. zeylanica* and *P. rosea*) was obtained in R4 (IAA 0.5 mg l^{-1}) and the shortest (1.80 cm and 2.00 cm respectively for *P. zeylanica* and *P. rosea*) in R1 (IAA 0.05 mg l^{-1}).

4.1.2.3.2 Root Culture

Eight treatments with different levels of IAA and NAA were tested for root culture. The results (Table 14) showed that NAA at a concentration of 1.5 mg l^{-1} was the best for root culture (Plate No 10). Days for root initiation was the least (15.0) for MR8 (NAA 2.00 mg l^{-1}) and the highest (19.8 days) for MR1 (IAA 0.50 mg l^{-1}).

Table 13 Effect of plant growth regulators on root initiation regenerated shoots of *P. zeylanica* and *P.rosea*

Treatment	Plant growth regulators ($mg\ l^{-1}$)		*P. zeylanica: P. rosea*		
	IAA	NAA	DRI	NoR	MLR (cm)
R1	0.05		16.00 : 16.33	2.00 : 2.50	1.80 : 2.00
R2	0.10		14.37 : 15.00	2.50 : 2.50	2.00 : 2.50
R3	0.25		13.00 : 15.17	2.50 : 3.50	2.50 : 3.00
R4	0.50		10.50 : 12.17	3.50 : 4.00	2.85 : 3.05
R5	1.00		12.67 : 13.50	3.50 : 3.50	2.75 : 2.50
R6		0.05	17.33 : 18.33	3.00 : 2.50	1.90 : 1.20
R7		0.10	18.00 : 17.80	1.48 : 3.00	2.50 : 1.00
R8		0.25	16.33 : 16.00	1.50 : 2.50	2.75 : 1.35
R9		0.50	14.00 : 16.33	2.50 : 3.50	2.50 : 1.50
R10		1.00	14.00 : 15.50	1.50 : 2.00	2.50 : 1.35

The data represent mean of six replications; Culture medium:MS
DRI: days for root initiation; NoR: Number of roots
MLR: Mean length of roots

Table 14 Effect of plant growth regulators on root culture of *P. zeylanica* and *P.rosea*

Treatment	Plant growth regulators ($mg\ l^{-1}$)		*P. zeylanica*
	IAA	NAA	DRI
MR1	0.50		19.80
MR2	1.00		18.00
MR3	1.50		18.20
MR4	2.00		16.60
MR5		0.50	19.20
MR6		1.00	18.00
MR7		1.50	15.80
MR8		2.00	15.00

The data represent mean of six replications: Culture medium:MS
DRI: Days for root initiation

Plate No.7 Regenerated shoot of *P. zeylanica*

Plate No.8 Rooting of the regenerated shoots of *P. zeylnica*

Plate No. 9 Rooting of the regenerated shoots of *P. rosea*

Plate No. 10 Root culture of the *in vitro* induced roots in *P. zeylanica*

Plate No. 11 Growth stages of rooted plantlets of *P. zeylanica*

Plate 7

Plate 8

Plate 9

Plate 10

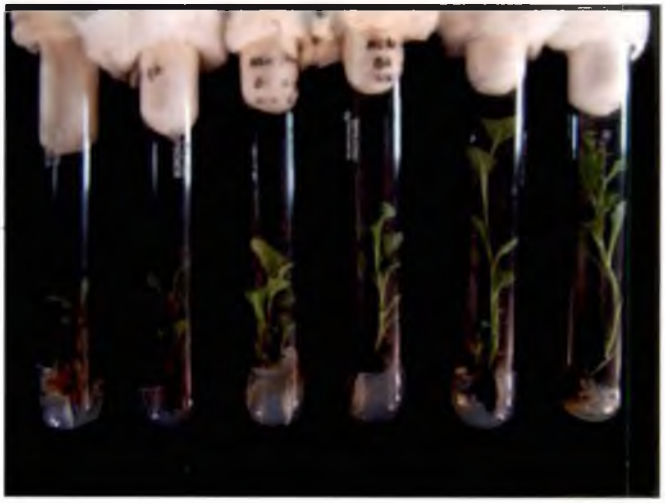


Plate 11

4.2 GENETIC TRANSFORMATION

Genetic transformation was carried out with an objective of inducing hairy roots in *P. zeylanica* and *P. rosea* . The *Agrobacterium rhizogenes* strains A4, MTCC 532 and MTCC 2364 were used for transformation. The results obtained are discussed below.

4.2.1 Growth of *Agrobacterium*

The establishment of bacterial culture is a prerequisite for transformation. The bacterial culture should be in active growth phase to bring about successful transformation. It is also necessary for storage of the bacterial cultures. The *Agrobacterium rhizogenes* produced white slimy and smooth colonies. The strains of *Agrobacterium rhizogenes* A4, MTCC 532 and MTCC 2364 took 36 h for growth with colonies.

4.2.2 Screening of *Agrobacterium* Strains for Antibiotic Sensitivity

The sensitivity of the bacterial strains to different doses of antibiotics *viz.*, ampicillin and cefotaxime was studied. The results of the tests were used in the elimination of bacteria after co- culture.

4.2.2.1 Test for Bacteriocidal Activity of Cefotaxime and Ampicillin

In the present study the bacteriocidal effects of cefotaxime and ampicillin were tested by culturing *Agrobacterium rhizogenes* strains in YEP medium containing different doses of ampicillin and cefotaxime ranging from 50 to 500 mg l^{-1}. At concentrations of 500 mg l^{-1} and above there was absence of bacterial growth. The result of this study was used for the elimination of bacteria after co- culture.

Plate No. 12 Hairy root induction from nodal explants of *P. zeylanica*

Plate No. 13 Morphology of hairy root

Plate No. 14 Hairy root induction from callus explants of *P. zeylanica*

Plate No. 15 Hairy root induction from leaf explants of *P. zeylanica*

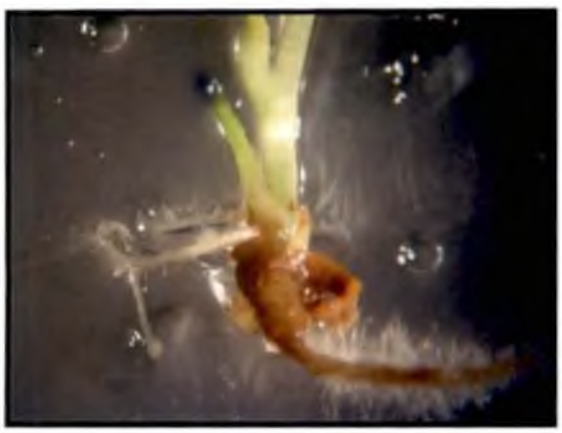


Plate 12

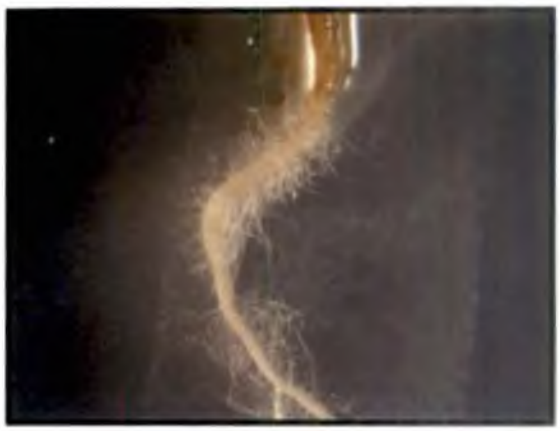


Plate 13

Plate 14

Plate 15

Table 15 Effect of type of explant on transformation per cent

Treatment	Type of Explant	Transformation per cent		
		A4	MTCC 532	MTCC 2364
1	Root segments	0	0	0
2	Calli	1	0	0
3	Leaf segments	20	10	10
4	Nodal segments	80	60	80

Co-culture time : 2 days; induction time: 1h

Table 16 Effect of infection time on transformation per cent

Treatment	Infection time	Transformation per cent		
		A4	MTCC 532	MTCC 2364
1	10 min	1	0	0
2	20 min	5	0	1
3	30 min	20	1 0	15
4	60 min	80	60	70

Co-culturing time: 2 days

Table 17 Effect of bacterial strains on transformation per cent

Treatment	Bacterial Strains	Transformation per cent
1	A4	80
2	MTCC 532	50
3	MTCC 2364	70

Co-culture time: 2 days; infection time: 1h

Table 18 Effect of co-culture time on transformation per cent

Treatment	Co-culture Time (days)	Transformation per cent of *A. rhizogenes*		
		A4	MTCC 532	MTCC 2364
1	1	20	5	10
2	2	80	50	70
3	3	30	10	20
4	4	10	2	10

4.2.3 Genetic Transformation

4.2.3.1 Effect of Type of Explant

Different types of explants like nodes, leaves, roots and calli were co-cultured to standardize best explant for transformation (Table 15).

Transformation efficiency was the highest for nodal explants (73.33 per cent) (Plate No 12). Leaf explants produced transformants, but only 13.33 per cent (plate No 15). The induction of hairy roots from callus (Plate No 14) was very low (0.33 per cent). The use of roots as explants did not yield any transformants. All the roots turned dark brown on inoculation with the bacterium.

4.2.3.2 Effect of Infection Time

Infection was carried out for 20, 30 and 60 minutes so as to standardize the optimum time required for infection (Table 16). An infection time of 60 minutes resulted in 70 per cent transformation. 11.67 per cent transformation was observed after an infection time of 30 minutes. Infection times of 10 minutes and 20 minutes recorded transformation percent of 0.33 and 2.00. The infection by diluting the bacterial suspension more than five times did not produce any transformants.

4.2.3.3 Effect of Different Bacterial Strains

Among the different strains of *Agrobacterium rhizogenes* strains, A4 recorded the highest transformation percent of 80 (Table 17). The result was consistent even in the absence of acetosyringone. The strain MTCC 2364 recorded an average of 70 per

cent transformation efficiency, whereas the strain MTCC 532 recorded an even lesser transformation of 50 per cent even in the presence of acetosyringone.

4.2.3.4 Effect of Co- culture time

Agrobacterium rhizogenes and the plant tissues were co-cultured in dark for two, three, four and five days at 26°C in a culture room. The effect of number of days of co-cultivation on the transformation efficiency was standardised (Table 18).

Among the different treatments, transformants were obtained from the explants co-cultivated with *Agrobacterium rhizogenes* in the dark for two days (66.67 per cent) . Per cent transformation was low when the explants were co-cultivated for more than two days. A co-cultivation time of three days resulted in 20 per cent transformation. However, a co-culture time of four days yielded only 7.30 per cent transformation. Heavy over growth of bacteria was seen surrounding the explants co-cultivated for more than two days. The tissues were turgid and dark brown in colour and produced an off smell when opened. 11.65 per cent transformation was obtained when the explants were co-cultured with the bacteria for one day.

Maximum transformation per cent was obtained when the bacterial strains and tissues were co-cultivated for two days and the infection was carried out with bacterial suspension of the pellets obtained by centrifugation of the overnight grown bacterial culture.

4.2.3.5 Effect of Acetosyringone

Among the different treatments the maximum transformation (80) per cent was recorded in the treatment without acetosyringone. Effect of acetosyringone was tested by adding 50, 100 and 200 µM of acetosyringone during infection time and co-

Table19 Effect of acetosyringone on transformation per cent

Treatment		Addition of Acetosyringone (μM)	Transformation per cent of *A.rhizogenes*		
			A4	MTCC 532	MTCC 2364
1	C 1	50	75	50	70
	C2		80	60	70
2	C1	100	60	60	50
	C2		75	60	60
3	C1	200	80	50	40
	C2		80	60	40

C1: co-cultivation with acetosyringone; C2: co-cultivation without acetosyringone

Table 20 Effect of different concentrations of antibiotics on bacterial growth

Treatment	Concentration of cefotaxime/ ampicillin (mg l^{-1})	Presence or absence of bacterial growth		
		A4	MTCC 532	MTCC 2364
1	100	+	+	+
2	200	+	+	+
3	300	+	+	+
4	400	+	+	+
5	500	-	-	-

+: Growth of bacteria

-: No bacterial growth

cultivation. There was no effect on transformation per cent by addition of acetosyringone during co-cultivation (Table 19). However, the number of hairy roots induced per explant was increased by the addition of acetosyringone.

4.2.3.4 Elimination of Overgrown Bacteria

The overgrown bacteria after co-cultivation were eliminated by the addition of cefotaxime. The co-cultured explants were washed in a solution of 500 mg l^{-1} cefotaxime, blotted dry on a sterile filter paper and transferred to solid MS medium containing cefotaxime at a concentration of 500 mg l^{-1}. If bacterial growth was detected even after the first subculture, the process was repeated at two days interval till no bacterial growth was detected.

4.2.4 Confirmation of transformation

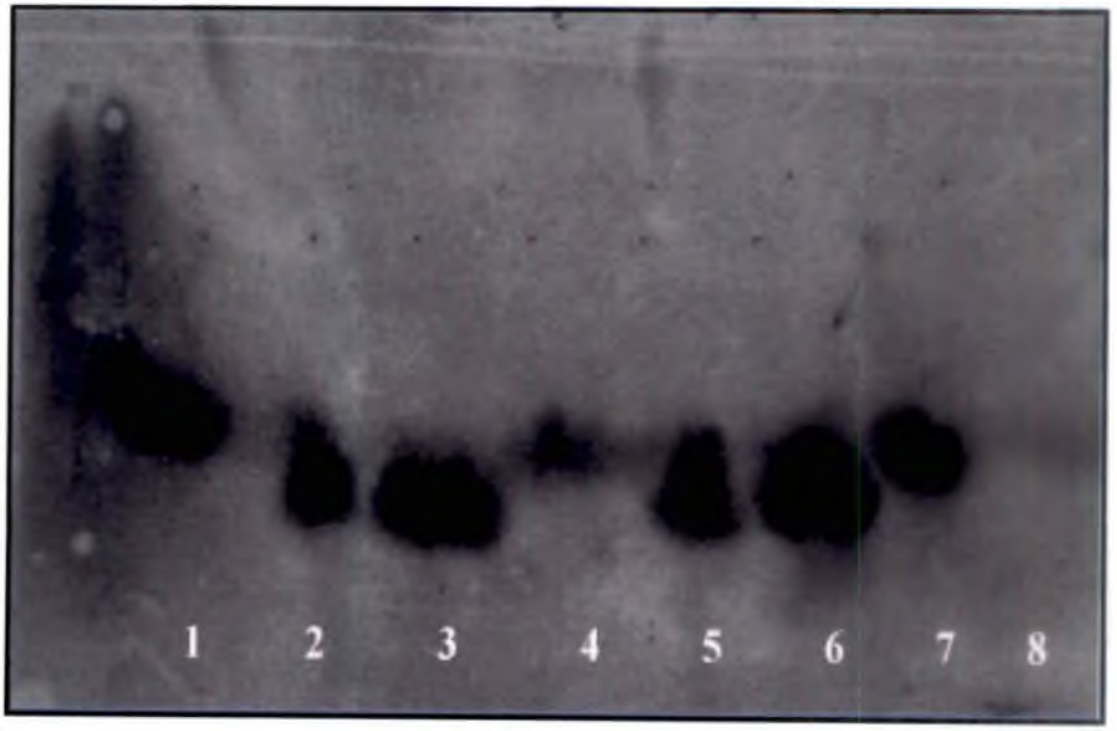
4.2.4.1 Confirmation by morphological appearance

The hairy roots produced by transformation were negatively geotropic and possessed numerous root hairs. Hairy roots were characterized by high degree of lateral branching. The root hairs were detected under a stereo microscope (Plate No. 13).

4.2.4.2 Opine detection

Opine detection was carried out according to the protocol given by Dessaux *et al.* (1991). Mannopine, mannopinic acid, agropinic acid and agropine were used as the standards. Opine were detected only in the transformed root tissues and not in the normal roots. This experiment confirmed transformation (Plate No 16).

Plate No. 16 Opine analysis



1. AGROPINE

2. MANNOPINIC ACID

3. MANNOPINE

4. AGROPINIC ACID

5. MTCC 2364

6. MTCC 532

7. A 4

8. CONTROL

PLATE 16

Discussion

5. DISCUSSION

Attempts were made to evolve a successful protocol for *in vitro* propagation as well as genetic transformation in *Plumbago* zeylanica and *Plumbago rosea* at the Department of Plant Biotechnology, College of Agriculture, Vellayani during the period from 2004-2006. The results of the project are discussed in this chapter.

Plumbago zeylanica and *Plumbago rosea* are propagated using stem cuttings. The seeds are not viable in *Plumbago rosea*. No alternative mode of multiplication is available to propagate this valuable medicinal plant. *In vitro* propagation can provide large number of propagules and disease free planting material. It is a requirement for genetic transformation also. In the present study, enhanced release of axillary buds and indirect organogenesis were tried for standardization of *in vitro* propagation techniques. The results obtained are discussed below.

Enhanced release of axillary buds was attempted using nodal segments. Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied to the medium and growth substances endogenously produced by the cultured cell. BA and IAA at various concentrations and combinations were tested to standardize their optimum levels for shoot proliferation in *P. zeylanica* and *P. rosea*. The nodal explants exhibited organogenesis. In *P. zeylanica* the best combination for shoot proliferation was BA 1.0 mg l^{-1} and IAA 0.05 mg l^{-1} with regard to number of shoots and shoots length. While in *P. rosea* the numbers of shoots and shoot length was the highest for the combination of BA 1.50 mg l^{-1} and IAA 0.05 mg l^{-1}. In *P. rosea* bud initiation was earliest in the treatments with BA, IAA and coconut water (100 ml l^{-1}). Whereas in *P. zeylanica* bud initiation was achieved without coconut water. The addition of BA to a concentration of 1.50 mg l^{-1} increased both the number

of shoots and shoot length. The addition of still higher concentrations of BA did not increase the bud initiation. This may be due to the fact that higher concentrations of BA above the optimum level interfered with the bud initiation. In the present study, half strength MS medium, full strength MS medium and White's medium were tested to standardize the best culture media suited for the enhanced release of axillary buds in *P. zeylanica* and *P. rosea*. The results showed that full strength MS medium was significantly superior to half strength MS and White's medium in the two plant species.

Dias *et al.* (2002) has reported that one of the most promising method for clonal propagation in many medicinal plants is *in vitro* propagation by axillary shoot proliferation. The beneficial effect of GA along with BA was reported in *Murraya koenigii* (Bhuyan *et al.*, 1997) and in *Vitex negundo* (Sahoo and Chand, 1998). Elongation of the shoots occurred at the combination BA 1.0 mg l^{-1} and IAA 0.50 mg l^{-1} in *P. zeylanica*. In *P. rosea* shoot elongation occurred at the combination of BA 1.50 mg l^{-1} and IAA 0.50 mg l^{-1}. Since sufficient amount of shoot elongation was obtained from the above treatments, GA was not tested for its effect on shoot elongation. The basal medium requirement by the cultures depends upon the plant species and the purpose of the cell tissue or organ culture. In the present study; full stregth MS was found to be significantly superior to half strength MS and White's medium in the two plant species. Similar reports have been made by Komalavalli and Rao (2000) that MS is the best medium for shoot sprouting, number of shoots and shoot length in *Gymnema sylvestre*.

Indirect organogenesis is characterized by redetermination of differentiated cells leading to callus formation. Among the different treatments tried for callus initiation C20 (BA 2.0 mg l^{-1} + 2,4-D 0.50 mg l^{-1}) gave maximum callus induction in leaf explants. Similar result has been obtained by Mercier *et al.* (1992). He reported callus induction from stem explants of *Gomphrena officinalis* in BA + NAA combination. Whereas BA in combination with NAA did not give any significant callus growth in

stem and leaf explants of *Centella asiatica* (Patra *et al.,* 1998). Rout *et al.* (1999) observed callus induction in leaf and stem explants of *P. zeylanica* in BA supplemented MS medium, though with a low callus induction per cent. Similar results were obtained in this study also *i.e.* callus index was very low in MS medium supplemented with BA alone, when compared to BA + 2,4-D combination. Both the plant species failed to produce callus in the media lacking plant growth regulators.

Leaf segments were used as explants for callus induction in *Plumbago zeylanica* and *Plumbago rosea.* Different combinations of plant growth regulators (2,4-D and BA) were tested to assess their effect on callus initiation, in the two plant species. Best treatment combination for callus initiation recorded, was the same with respect to the two plant species. The manipulation of appropriate levels of auxins and cytokinins is crucial to define the balance of growth regulators so that there is induction of callus in the explants (Franklin and Dixon, 1994).

The callus subsequently undergoes dedifferentiation to form separate shoot and root initials. Bhalsing (1999) reported that the standardization of *in vitro* plant regeneration protocols with intervening callus phase provides for genetic variation developing in many of its component cells. The *in vitro* regeneration protocol can be utilized for generating new genetic variability by somatic hybridization through protoplast fusion (Arcioni *et al.,* 2001).

The medium containing 2,4-D and BA induced callus in the leaf explants of the two plant species tested. But within four weeks of culture it turned black and did not give any regeneration. Shoot regeneration was observed with callus induced in the media with BA in combination with IAA. Calli from the induction media were sub cultured on to various regeneration media containing different concentrations and combinations of BA and IAA.

In *Plumbago zeylanica*, shoot regeneration was best in the treatment SR13 (BA 1.75 mg l^{-1} + IAA 0.05 mg l^{-1}). Green nodular structures developed into dark green shoots over the entire surface when calluses were cultured in SR13. Number of shoots (4.83) and the longest shoot (3.10) was obtained in the treatment SR13.

In *Plumbago rosea* shoot regeneration obtained was the best on MS media supplemented with 2.0 mg l^{-1} BA and 0.05 mg l^{-1} (treatment SR17) of IAA. Maximum number of shoots per culture (3.5) and the longest shoot (2.9) was also obtained in SR17. Light brown callus with green nodular structures developed into shoots when transferred to SR17 medium after four weeks of subculture. In the present study, low shooting response was observed in all treatments with lower concentrations of BA. Addition of adenine sulphate to the medium enhanced shoot regeneration and also reduced the time taken for regeneration in *P. rosea*. Skoog and Tsui (1948) reported that addition of adenine sulphate to the medium enhances growth and shoot formation.

In *P. zeylanica* and *P. rosea* MS medium supplemented with 0.50 mg l^{-1} IAA proved to be the best for *in vitro* rooting. This result is on par with the report of Rout *et al.* (1999) that in *P. zeylanica* best rooting was obtained in media supplemented with 0.57 μM IAA. It was observed that higher concentrations of auxins inhibited root elongation. On treatments with different concentrations of NAA, days for root initiation was higher. Number of roots and mean length of roots were lower in treatments with NAA than with IAA. In contrast to this, Howell *et al.* (1987) observed that rooting in *Indigofera potaninii*, was the best at higher levels of NAA (5 mg l^{-1}). Similar results have been obtained by George and Sherrington, (1984). He observed that efficient rooting of *in vitro* regenerated plants is a crucial step of rapid clonal propagation. Auxins frequently used for rooting are IAA, IBA and NAA (George and Sherrington, 1984).

Different *in vitro* propagation methods have been standardised for the two species of *Plumbago*. This has made possible the availability of pathogen free planting material. High ratio of cytokinins to auxins promoted shoot formation and proliferation in both enhanced release of axillary buds and callus mediated organogenesis. Auxins promoted *in vitro* rooting in the two plant species. Balance between plant growth regulators and additives like coconut water is a prerequisite for organogenesis.

In the present study root cultures were successfully established from *in vitro* induced roots. Root cultures were established in semisolid MS medium supplemented with 2.00 mg l^{-1} of NAA. Panichayupakaranant and Tewtrakul (2002) reported that *Plumbago rosea* root cultures using the initiated cultures on solid B5 medium supplemented with a combination of NAA and kinetin in the concentration ranges of 0.5-2.0 mgl^{-1} and 0.1-0.5 mgl^{-1}, respectively. Young leaves explants were used to obtain *Plumbago rosea* root cultures using the initiated cultures on solid B5 medium supplemented with a combination of NAA and kinetin in the concentration ranges of 0.5-2.0 mg l^{-1} and 0.1-0.5 mg l^{-1}, respectively.

Genetic transformation does not impair the natural root's synthetic capacities. Hence induction of hairy roots, which can grow vigorously and produce high levels of secondary metabolite, has been recognized as a potential way of producing important pharmaceuticals as secondary compounds from medicinal plants (Hamill *et al.*, 1987). Hairy root culture has been utilized for *in vitro* production of many secondary metabolites *viz.*, atropine, scopalamine, ajmalicin and hyoscyamine. However not much studies have been taken up for genetic transformation in *Plumbago* spp to produce plumbagin *in vitro*. Genetic transformation of *Plumbago* spp was attempted for inducing hairy roots *in vitro*.

Wound response is a function of explants. Different explants *viz.*, leaf segments, roots, calli and nodal segments showed different responses to transformation. There was no difference with regard to the two plant species, *Plumbago zeylanica* and *Plumbago rosea* in their response to genetic transformation. The hairy roots were induced from leaf segments and nodal explants. Per cent induction of hairy roots in nodal explants (66.67 per cent) was the highest, followed by leaf segments (13.33 per cent). The induction of roots in callus was very low and roots did not respond to hairy root induction.

Potrykus (1990) stated that the most important factor for successful transformation by *A. rhizogenes* was the wound response. Plant tissues differ in their wound response. He further observed that the explants with a pronounced wound response developed larger populations of wound adjacent competent cells for regeneration and transformation and that the explants or plant species recalcitrant to transformation with *A. rhizogenes* probably do not produce appropriate wound response.

Yonemitsu *et al.* (1990) induced hairy roots from 20 days old seedling of *Lobelia inflata* by infection with *Agrobacterium rhizogenes* strain 15834. They observed that juvenility of explant is necessary for hairy root induction. Similar results were obtained by Trypsteen *et al.* (1991) in *Enhinacea purpurea,* in which seedling hypocotyls induced hairy roots whereas older plants did not induce hairy roots. They concluded that the type and age of explant are critical factors influencing *A. rhizogenes* infections.

Citovsky *et al.* (1996) reported that specificity of *A. rhizogenes* is closely connected with the physiology and growth of the plant. Villemont *et al.* (1997) reported that host cell cycle play a crucial role in genetic transformation. They found that no transformation occurred when cell division was stopped at the G1 phase. If the cell

division stopped at early G2 phase, T-DNA transfer occurred but transformation frequencies were less. If cell division was stopped at M phase, the T-DNA integration was stable. They concluded that S phase and M phase were important for stable integration of T-DNA in the plant cells.

It is thus quite evident that different explants differ in their wound response. The explants differ in their DNA synthesis and cell division ability due to the difference in physiological maturity of the cells. The inability of roots and calli to induce hairy roots may be due to lack of wound response of adjacent competent cells or difference in physiological maturity of these tissues.

Different strains differ in their ability to induce transformation in different explants. This might be due to the interaction between the bacterial strains and the plant species. *A. rhizogenes* strain A4 (transformation per cent of 80) was found to be the best for induction of hairy roots in *Plumbago* spp. in the present study. Transformation per cent was lower for the strains MTCC 532 (50 per cent) and MTCC 2364 (70 per cent). Similar reports have been made by several scientists. Patena *et al.* (1998) reported marked differences in the ability of four *A. rhizogenes* strains to induce hairy roots on carrot discs. The strains A4 and 232 produced hairy roots. The strains R 1000 and 178 x A4 did not produce hairy roots. Gelvin (1990) reviewed physiological basis of hairy root disease. In contrast to this result, Li and Leung, (2003) reported that strain LB 9402 was more effective than A4 in increasing rooting percentage and root number.

Generally an addition of acetosyringone increases the efficiency of transformation. Certain plant phenolic substances like acetosyringone and hydroxyacetosyringone play a key role in gene transfer process by *Agrobacterium*. Acetosyringone molecules act to induce activity of virulence (*vir*) genes that are encoded on the plasmid. The *Vir genes* are induced when *Agrobacterium get* attached

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to the plant cell and influences the efficiency of transformation. So the addition of acetosyringone in the co-cultivation medium and in bacterial suspension might act synergistically to increase the infection and transformation efficiency. However in the present study, addition of acetosyringone did not increase the per cent transformation. Whereas the addition of acetosyringone increased the number of hairy roots induced per explant.

Celma *et al.* (2000) reported successful transformation of *Duboisia* plantlets by *Agrobacterium rhizogenes* strain A4. Leaf discs of approximately 1.0 cm in diameter were added to 10 ml of liquid Ms medium in Petri dishes containing 100 µl of a culture of *Agrobacterium rhizogenes* grown overnight at 28°C in YEB medium containing 100 µM acetosyringone. The ability of acetosyringone to induce higher number of roots per explant compared to the control might be due to the induction of *vir* genes by the phenolic produced by the explants as well as the added acetosyringone. This indirectly increases the quantity of secondary metabolite and hence the addition of acetosyringone is beneficial.

A*grobacterium rhizogenes* suspension of optical density (OD_{600}) 1.0 was found to the most effective one for successful transformation in the present study. When the bacterial suspension of optical density (OD_{600}) less than 1.0 was used, the efficiency of transformation was considerably reduced. Kumar *et al.* (1999) found that optimum concentration of bacteria was 1×10^8 cells ml^{-1}. They found that the optimum concentration of the bacterial concentration below optimum may result in fewer competent bacteria being available to transform the plant cells while the concentrations of bacterial culture above the optimum level resulted in competitive inhibition of competent bacteria resulting in decreased transformation frequencies. In this study the bacterial suspension of optical density (OD_{600}) 1.0 might have resulted in optimum levels of bacteria at the wounds resulting in greater transformation frequencies and vice versa.

The infection time required by the bacterial cells was found to influence the transformation percentage significantly. An optimum infection time is essential for the bacteria to get attached to the plant cells. Different plant species require different infection time for induction of hairy roots. In the present study, infection times of 10 min, 20 min, 30 min and 60 min were tried to standardize the optimum infection time for transforming the explants of *Plumbago* spp and it was found that an infection time of 60 minutes (70 per cent transformation) was the best for inducing hairy roots in the species studied. Infection times of 10, 20 and 30 min resulted in lower transformation percentage of 0.33, 2.00 and 21.66 respectively.

In contrast to this result, Lee *et al.* (2004) reported that an infetcion time of five minutes was optimum for the transformation of *Taraxacum platycarpum.* Babaoglu *et al.* (2004) obtained successful transformation in *Lupinus mutabilis* by immersing the leaf explant in the bacterial suspension for 30 minutes. Transformation in *Angelonia salicarifolia*, successful transformation was obtained by immersing the leaf explants in the bacterial susupension for 20 minutes. Xu *et al.* (2004) observed that an infection time of 10 minutes was the optimum for hairy root induction in *Isatis indigotica.*

In the present study, the amount of bacterial cells present at the time of co-culture time affected the transformation percentage. When optimum numbers of bacterial cells were present during the co-culture time, a co-culture time of two days gave good transformation percentage (6.66 per cent). On the contrary, when more number of bacterial cells were present or when the co-culture time was more than two days the result was over growth of bacteria over explants and reduced frequencies of transformation. The bacterial cells multiplied in the co-culture medium and after 48 h, the optimum quantity of bacteria were available and hence transformation was achieved. After 48 h the level of bacterial cells reached supra optimum level and resulted in competitive inhibition of competent bacterial cells and thereby inhibition of

transformation. co-culture time of 24 h resulted in lower transformation per cent of 11.66. Co-culture time of 72 h and 96 h resulted in lower transformation per cent of 70 and 7.33, respectively. Influence of co-culture time on successful transformation was reported by other scientists also. Mihaljevic *et al.* (1996) reported that hairy roots were induced in *Pinus nigrum* explants using *Agrobacterium rhizogenes* strains 15834 and 8196. They observed that co-culture time affected the transformation frequency. Xu *et al.* (1997) reported that transformation frequencies of alfalfa cultures were dependent on co-culture time. They suggested that a co-culture time of 72 h to be the best as it gave the maximum number of transformants.

In the present study, the roots induced from the nodal and leaf segments of the two plant species were whitish with numerous root hairs (Plate No: 4). But they did not show any lateral branching. As a result the rate of growth of roots was slow. Similar observations were made by Banerjee *et al.* (1995) that the hairy roots are whitish in colour. Presence of many lateral root tips promotes rapid growth. (Hamill *et al.*, 1986, Hashomoto *et al.*, 1986 and Quattrocchio *et al.*, 1986). Aoki *et al.* (1997) reported that the differences in the morphological features of hairy roots were due to the differences in copy number and or the position in the genome of plant where the T-DNA is integrated.

In the present study, the hairy roots induced from *Plumbago* spp. explants showed reduced gravitropism while normal roots showed strong gravitropism. Similar result has been reported by Banerjee et *al.* (1996). They observed that hairy roots generally showed negative gravitropism or reduced gravitropism, while normal roots show strong gravitropism.

Production of opines is a characteristic feature of hairy roots. Opines are produced by the transformed cells only. In the present study, agropine, mannopine and

mannopinic acid were detected in the roots induced by *A. rhizogenes* strains A4, MTCC 532 and MTCC 2364 (Plate No. 4).

The presence of opines is a strong evidence of T-DNA transfer into the plant genome (Petit *et al.*, 1983). The presence of opines has been utilised as a strong tool for confirmation of transformation (Patena *et al.*, 1988; Kumar *et al.*, 1991; Trypsteen *et al.*, 1991; Dobigny *et al.*, 1996; Shi *et al.*, 2004). Petit *et al.* (1983) reported that opines are absent in normal roots. Opine synthesis is a firm indication that the roots are transformed (Hamill *et al.*, 1987).

Summary

6. SUMMARY

The present study titled "*Agrobacterium rhizogenes* mediated genetic transformation in *Koduveli (Plumbago* spp. L.)" was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2004-2006. Two plant species *viz., Plumbago zeylanica* and *Plumbago rosea* were selected for the study. Investigations were carried out in two phases *viz., in vitro* propagation and genetic transformation in *Plumbago* spp *In vitro* propagation studies were carried out by enhanced release of axillary buds and somatic organogenesis and geentic transformation was carried out by co-culture method. The salient findings of the study are summerised below.

Among the various concentrations and combinations of cytokinins and auxins tested, full strength MS medium supplemented with BA 1.0 mg l^{-1} and IAA 0.05 mg l^{-1} was found to be the best for shoot proliferation from nodal explants in *P. zeylanica.* The hormonal combination BA 1.50 mg l^{-1} and IAA 0.05 mg l^{-1} recorded the highest shoot proliferation rate in *P. rosea.*

Full strength MS medium was superior to half strength MS and White's medium with regard to shoot proliferation in the two plant species.

With regard to rate of callusing, there was no significant difference between the two plant species. MS medium supplemented with BA 2.0 mg l^{-1} and 2,4-D 0.5 mg l^{-1} recorded the highest callusing rate from the leaf explants of *P. zeylanica* and *P. rosea.*

Shoot regeneration from callus was achieved in *P. zeylanica* on MS medium containing BA 1.75 mg l^{-1} , and IAA 0.05 mg l^{-1}. In In *P. rosea* MS medium supplemented with BA 2.00 mg l^{-1} and IAA 0.05 mg l^{-1} and adenine sulphate 20 mg l^{-1}

was identified as the best medium for shoot regeneration from leaf derived callus. Coconut water(100 ml l^{-1}) was found to increase the rate of shoot regeneration from callus in *P. rosea.*

Good rooting was obtained when *in vitro* raised shoots were cultured in full strength MS medium with IAA 0.50 mg l^{-1} in *P. zeylanica* and *P. rosea.* Root culture was successfully established in MS medium containaining NAA 2.00 mg l^{-1}.

The *Agrobacterium rhizogenes* strains A4, MTCC 532 and MTCC 2364 were used for the study. Among the three strains of bacteria utilised for transformation, A4 was the most efficient in transforming the explants with a transformation per cent of 80.

Among the various explants tried for transformation, nodal explants were the best (transformation per cent of 66.67) followed by, leaf segments. Hairy root induction was low in callus explants and roots did not give rise to any transformants.

An infection time of one hour proved to be the best for transformation in *Plumbago* spp. An infection time of 10 minutes, 20 minutes and 30 minutes reduced the rate of transformation .

Co-cultivation time of two days was the best for successful transformation. An increase in co-cultivation time reduced the per cent of transformation and resulted in bacterial overgrowth over the explants. The present study proved that acetosyringone did not increase the per cent of transformants, but increased the number of hairy roots induced per explant.

Agrobacterium rhizogenes strains were eliminated after co-cultivation by transfering the explants to MS medium containing cefotaxime or ampicillin, 500 mg l^{-1}.

Transformation was confirmed by root morphology. The roots were whitish and possessed numerous root hairs. Further confirmation was done by opine detection. This was done by high voltage paper electrophoresis using opine standards. Opines were detected only in transformed samples.

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* Originals not seen

Abstract

AGROBACTERIUM RHIZOGENES MEDIATED GENETIC TRANSFORMATION IN KODUVELI (*Plumbago* spp. Linn.)

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Abstract of the Thesis
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ABSTRACT

Investigations on "*Agrobacterium rhizogenes* mediated genetic transformation in *Koduveli (Plumbago* spp. L.)" were carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2003-2006. Two plant species *viz., Plumbago zeylanica* and *Plumbago rosea* having great medicinal value by virtue of plumbagin, a naphthoquinone, present in them were selected for the study. Standardisation of rapid *in vitro* propagation of these medicinal plants were attempted in the present study.

Enhanced release of axillary buds from nodal explants, with the highest shoot proliferation of 5.5 was obtained in MS medium supplemented with BA 1.0 mg l-1 + IAA 0.05 mg l-1 for *P. zeylanica* . In *P. rosea* the highest shoot proliferation of 5.0 was recorded in MS medium supplemented with BA 1.50 mg l-1 + IAA 0.05 mg l-1. Among the different basal media tested full strength MS medium was found to be the best for shoot proliferation.

In indirect organogenesis, the highest callus index (300) was recorded in MS medium with BA 2.00 mgl^{-1} and 2,4-D 0.50 mg l^{-1} for *P. zeylanica.* In *P. rosea* also maximum callus index (300) was obtained in the same medium.

In *P. zeylanica* BA 1.75 mg l^{-1}, IAA 0.05 mgl^{-1} and adenine sulphate 20 mg l^{-1} in MS medium was identified as the best medium for shoot regeneration from leaf derived callus. Whereas in *P. rosea* BA 2.00 mg l^{-1}, IAA 0.05 mg l^{-1} and adenine sulphate 20 mg l-1 in MS medium obtained the highest rate of shoot regeneration from callus. Coconut water (100 ml l^{-1}) was found to increase the rate of shoot regeneration in *P. rosea.*

Rooting of *in vitro* raised shoots was achieved by subculturing them on MS medium containing IAA 0.50 mg l^{-1}. *In vitro* root culture was carried out successfully in semi solid MS medium containing 1.50 mg l^{-1} NAA.

Genetic transformation was carried out by co-culture method. *Agrobacterium rhizogenes* strains A4, MTCC 532 and MTCC 2364 were utilised for transformation. YEP medium was used for culturing the bacteria.

The strain A4 was the best for transforming *Plumbago* spp. There was no significant difference between the two species *P. zeylanica* and *P. rosea* in their response to transformation.

Nodal explants recorded the highest transformation percentage followed by, leaf segments. Calli and root explants did not respond to transformation. Bacterial density of one ($O.D_{600}$) during transformation resulted in high transformation percentage. Co-culturing for two days resulted in high transformation percentage, whereas co culturing for more than two days resulted in bacterial over growth on the tissues and low transformation per cent.

After two days of co-culture, the bacteria were killed by transfering the tissues to MS medium containing cefotaxime or ampicillin 500 mg l^{-1}. The transformed tissues induced hairy roots in a period of seven to ten days. The hairy roots produced by transformation were negatively geotropic and possessed numerous root hairs.Transformation was confirmed by opine analysis, which was carried out by high voltage paper electrophoresis. Opines were detected only in transformed samples and not in normal roots. The hairy roots induced in *P. zeylanica* and *P. rosea* is a potential source for production of plumbagin *in vitro*.

Appendices

APPENDIX I

MS Medium

Constituents	Concentration (mg l^{-1})
$MgSO_4.7H_2O$	370.00
$CaCl_2.2H_2O$	440.00
KNO_3	1900.00
$NH_4.NO_3$	1650.00
KH_2PO_4	170.00
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
$CuSO_4.5H_2O$	0.025
$AlCl_3$	0.025
KI	0.830
H_3BO_3	6.20
$Na_2MoO_4.2H_2O$	0.25
$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25
Sucrose	30 g l^{-1}
Myo-inositol	100
Nicotinic acid	0.50
Pyridoxine HCl	0.50
Thiamine HCl	0.10
Glycine	2.00
Agar	6 g l^{-1}

APPENDIX II

White's Medium

Constituents	Concentration (mg l^{-1})
$MgSO_4.7H_2O$	720.00
$Na2SO_4$	200.00
KCl	65.00
KNO_3	80.00
$Ca(N_3)_2.4H_2O$	300.00
$NaH_2PO_4.H_2O$	16.50
$MnSO_4.4H_2O$	7.00
$ZnSO_4.7H_2O$	3.00
$Fe_2(SO_4)_3$	2.50
KI	0.75
H_3BO_3	1.50
Sucrose	20 g l^{-1}
Nicotinic acid	0.50
Pyridoxine HCl	0.10
Thiamine HCl	0.10
Calcium	1.00
Glycine	3.00
Cystenic HCl	1.00
Agar	6 g l^{-1}

APPENDIX III

YEP Medium

Constituent	Concentration ($g\ l^{-1}$)
Yeast extract	10
Peptone	10
NaCl	5
PH	7