GENETIC TRANSFORMATION AND HAIRY ROOT CULTURE IN ADA-KODIEN

(Holostemma ada kodien K. Schum.)

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled "**Genetic transformation and hairy root culture in ada-kodien (*Holostemma ada-kodien* K.Schum.)**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society

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We, the undersigned members of the Advisory Committee of **Mr.Karmarkar Shirish Hari**, a candidate for the degree of **Master of Science in Horticulture** with major in Plantation Crops and Spices agree that the thesis entitled "**Genetic transformation and hairy root culture in ada-kodien** (***Holostemma ada-kodien*** **K.Schum.**) may be submitted by **Mr. Karmarkar Shirish Hari**, in the partial fulfilment of the requirement for the degree

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Abbreviations

%	per cent
∝	alpha
β	beta
R_1 plasmid	root inducing plasmid
T-DNA	Transfer DNA
°C	Degree Celsius
g l^{-1}	grams per litre
mg l^{-1}	milligrams per litre
mg ml^{-1}	milligrams per millilitre
lux	lux
h	Hour
µM	micro molar
min	Minutes
µg	micrograms
$AgNO_3$	Silver nitrate
$KMnO_4$	Potassium per magnate
H_2SO_4	Sulphuric acid
ml	millilitre
nm	nanometers
$CHCl_3$	Chloroform
N	Normal
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
w/v	weight per unit volume
cm	centimetre
psi	pounds
NAA	Naphthalene acetic acid
$HgCl_2$	Mercuric chloride
µl	micro litre
rpm	Revolutions per minute
M	Molar
v	Volume
V/cm	Volt/centimetre
NH_4OH	Ammonium hydroxide
ϵ°	Epsilon zero
mm	millimetre
ppm	parts per million
UV	Ultra violet

Introduction

INTRODUCTION

Rejuvenation and restoration of vigour and potentiality at any age have been the fantasies of mankind 'Ayurvigyan' has shown that the self-confidence of an individual, the health and balance of one's personality depends on his preservation or recovery of vital power and potency Therefore, from the earliest dawn of civilisation, men have continually sought new and efficacious means for the recovery of both mental and physical potency 'Rasayanas' and 'Bajikaranas' (aphrodisiacs) have received special position in 'Ayurvedic' system of treatment for their capacity to maintain vigour, strength and vitality

Charak treated 'jivanti' as one of the important 'rasayana' drug capable of maintaining the youthful vigour and strength Probably, for this reanimating property, the jivanti was denoted as 'jivanti', 'jivani', 'jiva' and 'jivaniya' The drug is regarded as 'tridoshaghna' (curer of vata, pitta and kapha disorders)

'Jivanti' is one of the controversial drugs of the 'Ayurvedic' system of medicine, the identity of which is still unsettled (Gupta, 1997) *Holostemma ada-kodien* K Schum is a commonly used plant in South India as 'jivanti' The other plants used as 'jivanti' in different parts of India include *Leptadenia reticulata, Dregea volubilis, Sarcostemma brevistigma* (all Asclepiadaceae), and *Flickingnia nodosa* (Orchidaceae) (Gupta, 1997)

Holostemma ada-kodien K Schum is a laticiferous, climber belonging to the family Asclepiadaceae The tuberous roots are medicinally important and are utilised as a major ingredient of the drug 'jivanti' (Kolammal, 1979) There is a huge demand for the root tubers of *Holostemma* in the South Indian pharmacies and more than 150 metric tons of root tubers are needed every year (Nair *et al*, 1992) The destructive and ruthless collection of tubers, in recent times, has led to acute scarcity of the plant and consequently it is listed out as vulnerable and rare in the FRLHT red list (FRLHT, 1997) Commercial cultivation of

Holostemma is problematic due to increasing land pressure and its inherent difficulty to propagate Another alternative to meet the increasing demand is to produce the active chemicals in *Holostemma* through *in vitro* techniques

In vitro techniques have been extensively utilized to produce different secondary metabolites from various medicinal plants With the success of commercial production of shikonin from *in vitro* cultures of *Lithospermum erythrorhizon*, the horizons of this field have widened greatly The inherent problems of slow growth rates and unstable secondary metabolite production in *in vitro* cultures have now been overcome successfully by the induction of 'hairy root cultures' It would be a significant contribution to the mankind if the compounds in *Holostemma* could be successfully obtained through 'hairy root cultures'

Hence, this study was undertaken to genetically transform *Holostemma* to induce hairy roots The objectives of this study were

- To standardise the procedure to genetically transform *Holostemma ada-kodien* and to induce hairy root cultures
- To standardise the biochemical techniques for the estimation of secondary metabolites in the roots of *Holostemma*

Review of Literature

REVIEW OF LITERATURE

Over the last two decades, major efforts have been directed in enhancement of the ability of plant cell cultures to synthesise and accumulate secondary metabolites Successful attempts have been made in the case of *Lithospermum erythrorhizon* where the production levels of the quinone 'shikonin' achieved, have been high enough to be of commercial interest

Synthesis of secondary metabolites during distinct phases of plant development by specific tissues, poor understanding of regulatory mechanisms governing the expression and function of metabolic pathways (Rhodes *et al*, 1987), genetic instability and heterogeneity of disorganised cell cultures/cell suspension cultures are some of the limitations encountered during the *in vitro* production of secondary metabolites

Amongst the approaches to overcome these problems, the strong correlation between secondary metabolite production and morphological differentiation gives more impetus to the application of organised cell cultures, particularly the root cultures for the large-scale production of phytochemicals

However, slow growth rates of root cultures due to highly organised nature of normal roots is another limitation in the commercialisation of technology using root biomass as the source for secondary metabolite production

Transformed root cultures, the so called 'hairy root cultures' obtained after the insertion of T-DNA from the root-inducing (Ri) plasmid of *Agrobacterium rhizogenes* (David *et al*, 1984) into the plant genome, has emerged as an important tool for the production of secondary metabolites *in vitro* Hairy roots have advantages of fast growth, organ differentiation and stable secondary metabolite production

Holostemma ada-kodien K Schum is a laticiferous climber belonging to the family Asclepiadaceae and is widely distributed in South India The roots of *Holostemma* are sweet, refrigerant, ophthalmic, emollient, alterant, tonic, stimulant, aphrodisiac, expectorant and galactagogue They are useful in opthalmopathy, orchitis, cough, burning sensation, stomachalgia, fever and to cure 'tridosha' (Warrier *et al*, 1995) The sugars and amino acids present in the root tubers are responsible for the medicinal properties (Ramiah *et al*, 1981)

The literature pertaining to the topic is reviewed here

2.1 *Holostemma ada-kodien* K.Schum.

Use of *Holostemma ada-kodien* in the traditional systems of medicine is reported from all parts of India In each language it has a specific name, indicating the wide spread recognition of the medicinal properties of the plant It is called 'Jivanti' in Sanskrit, 'Chirvel' in Hindi and 'Atapatiyan', 'Atapotiyan' or 'Atakotiyan' in Malayalam (Warrier *et al*, 1995)

2 1 1 Botany of *Holostemma ada-kodien* K Schum

Holostemma ada-kodien K Schum exudes white latex from stem, leaves and immature pods and is classified under the family Asclepiadaceae The flowers are conspicuous, purple, borne in axillary umbellate cymes The fruits are thick follicles bearing hundreds of seeds per fruit (Warrier *et al*, 1995)

The genus *Holostemma* has several species distributed in the tropical Himalayas, Western ghats and Deccan and Tropical Peninsular India Several species have also been reported in SriLanka, Burma and Western China (Sivarajan and Balachandran, 1994)

FRLHT (1997) have reported the ecological status of *Holostemma ada-kodien* as 'vulnerable' and 'rare' in India

2 1 2 Biochemicals in *Holostemma ada-kodien* K Schum

CSIR (1959) published the economic importance of *Holostemma annulare* (*syn H ada-kodien* K Schum), with uses and chemical composition Analysis of root powder revealed that the roots contained moisture (10 8%), protein (4 07%), sugar (24 0%), starch (32 54%), fibre (12 2%) and ash (3 07%) Ash contained Ca and P (2 50%)

Ramiah *et al* (1981) isolated and identified different sugars such as ∝-amyrin, lupeol and β-sitosterol from the benzene extract of roots and six amino acids viz alanine, glycine, serine, aspartic acid, threonine and valine from the ethanol extracts of the roots

Meera (1994) isolated the free amino acids and total amino acids in the ethanol extract of the root tubers of *Holostemma* using ion exchange chromatography

Apart from this, a detailed, systematic biochemical analysis of the root tubers of *Holostemma* is lacking

2.2 Hairy root cultures

'Hairy root' is a neoplastic plant disease syndrome that occurs as a consequence of infection of plant with a gram-negative soil bacterium, the *Agrobacterium rhizogenes*

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

Hairy roots appear at the site of infection and are characterised by fast growth, highly branched adventitious nature and continuous growth *in vitro*, on a hormone free culture medium They have high genetic stability and are capable of producing the secondary metabolites of the mother plant (Doran,1989, Flores, 1986, 1992)

2 2 1 Hairy root cultures in medicinal plants

Hairy root culture in medicinal plants has specifically been utilised 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)

Among the important medicinal plant families in which hairy roots have been induced, include Apiaceae, Apocyanaceae, Asteraceae, Araliaceae, Boraginaceae, Brassicaceae, Campanulaceae, Chenopodiaceae, Compositae, Cucurbitaceae, Fabaceae, Gentianaceae, Geraniaceae, Labiatae, Leguminosae, Malvaceae, Myrtaceae, Polygonaceae, Rubiaceae, Scorphulariaceae, Umbelliferae, Valerianaceae and Verbenaceae (Banerjee *et al*, 1995)

Some important medicinal plants in which hairy roots have been successfully induced and the secondary metabolite detected in hairy roots are listed in Table 1

2 2 2 Induction of hairy roots

2 2 2 1 *Agrobacterium rhizogenes* strains

Agrobacterium rhizogenes is the causal organism for induction of hairy roots A large variety of *A rhizogenes* strains are present in nature The strains are classified on the basis of opine(s) they catabolise (Table 2)

Table 1 List of medicinal plants in which hairy roots have been induced and secondary metabolite detected

Sl No	Plant species	*A rhizogenes* strain used	Secondary metabolite obtained	Degree of hyper production of metabolite than normal root
1	*Atropa belladonna*	ATCC 15834	Scopolamine	5
		A_4	Atropine and Hyoscyamine	2
2	*Cinchona ledgeriana*	LBA 9402	Quinine, quinidine and cinchomdine	3
3	*Datura innoxia*	ATCC 15834	Hyoscyamine and scopolamine	6
4	*D queritolia*	LBA 9402	Scopolamine and Hyoscyamine	20
5	*D candida*	ATCC 15834	-do-	2
6	*Duboisia leichhardtii*	ATCC 15834 and A_4	Scopolamine	2
7	*Hyoscyamus niger*	ATCC 15834	Hyoscyamine and scopolamine	2
8	*Rubia tinctoria*	ATCC 15834	Anthraquinone	19
9	*Tagetes patula*	LBA 9365	Thiophene	25
10	*Valeriana officinalis*	R 1601	Valpotriates	7

(Banerjee *et al*, 1995)

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 strains have a wider host range when compared to mannopine and cucumopine strains (Rhodes *et al*, 1989) The wider host range of agropine strains has been attributed to the additional presence of T_R

fragment of T-DNA with its auxin biosynthesis genes which on transfer to the plant cells may, by causing elevated auxin production, enhance the induction of root-initial formation in adjacent cells (Filetici *et al*, 1987)

Table 2 Classification of *A rhizogenes* strains on the basis of opine catabolism

Sl No	Opine type	Characteristic opine markers	*A. rhizogenes* strains
1	Agropine	Agropine, Mannopine, Agropinic acid, Mannopinic acid and Agrocinopine	P_cA_4, 15834, A_4, LBA 9402, HRI
2	Mannopine	Mannopine, Mannopinic acid, Agropinic acid and Agrocinopine	8196
3	Cucumopine	Cucumopine and Cucumopine lactam	2659
4	Mikimopine	Mikimopine, Mikimopine lactam and Mannopine	MAFF 03-01724

(Dessaux *et al*, 1991)

2 2 2 1 1 Culture media for *A rhizogenes* strains

Several workers have suggested different media for growth of *A rhizogenes* strains

Petit *et al* (1983) suggested LB medium for the growth of *A rhizogenes* strains 15834 and 8196 YEB agar medium was found to be the best for culturing *A rhizogenes* strains 15834, A_4 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 31798 and A_4 (Jazin *et al*, 1988 and Mugnier, 1988)

Kittipongpatana *et al* (1998) found yeast mannitol broth as the best medium for growth of *A rhizogenes* strain 15834

2 1 2 Culture conditions for *A rhizogenes*

Temperature is the most critical factor for good growth of *A rhizogenes* strains Most of the strains prefer a growth temperature of 25 ± 2°C The optimum growth temperature for *A rhizogenes* strains HRI, 8196, 15834 and A_4 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 The optimum temperature for the growth of SV_2 and SV_4 strains of *A rhizogenes* was reported to be 37°C by Sarma *et al* (1997)

Yoshikawa and Furuya (1987) reported that the strain A_4 grows best at 25°C in dark conditions

2 2 2 2 Explants

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

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

Mugnier (1988) used discs of potato and sweet potato for induction of hairy roots Sarma *et al* (1997) used leaf segments of *Rauvolfia serpentina* for inducing hairy roots

The type of explant influences the transformation frequency The type and age of explant is critical for good response to *Agrobacterium* infections Yonemitsu *et al* (1990) used 20 days old seedling and shoot buds of *Lobelia inflata* to induce hairy roots Hairy roots were induced by seedlings only They concluded that juvenility of explant was necessary for hairy root induction

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

2 2 2 3 Wounding of explants

Wounding is a pre-requisite for transformation by *Agrobacterium* The method of wounding depends upon the type of explant used Kamada *et al* (1986) wounded the shoot tips of *Atropa belladonna* by pricking with sterile needle while Deno *et al* (1987) punctured the shoots of *Duboisia myoporides* with tooth picks Mugnier (1988) cut the tubers of potato and sweet potato with sterile blade Christen *et al* (1989) wounded seedlings of *Datura candida* by pricking with sterile needle Mano *et al* (1989) wounded leaf disks and stem segments of *Duboisia leichhardtii* with a sharp scalpel Yoshikawa and Furuya (1987) wounded the calli of *Panax ginseng* by initial treatment with 2 per cent cellulase and 0 5 per cent macerozyme followed by 0 3 per cent mannitol treatment Xu *et al* (1997) wounded alfalfa protoplasts by treatment with 0 45 M mannitol

2 2 2 4 Inoculation of explants with *Agrobacterium*

There are a number of approaches for inoculation of explants with *Agrobacterium* One approach involves inoculation of bacteria on the wound using sterile scalpel or needle In another approach the explants after wounding are co-cultured with a suspension of *Agrobacterium* in liquid growth media (Rhodes *et al*, 1989)

2 2 2 4 1 Direct inoculation of bacteria

This is the simplest method of inoculation This method is particularly used for inoculation of stem segments, seedlings and leaf explants with

Agrobacterium The bacterial inoculum used affects the transformation frequencies Patena *et al* (1988) reported that colonies were superior to suspension cultures for inducing hairy roots in carrot, kalanchoe and apple They attributed the superior activity of bacterial cell colonies to the greater concentration of bacteria in the colonies as compared to suspensions

Benjamin *et al* (1993) smeared bacterial from single cell colonies on wounds of shoot explants of *Rauvolfia serpentina* Mugnier (1988) applied bacterial suspension on potato and sweet potato discs to induce hairy roots Davioud *et al* (1989) wounded explants of *Catharanthus trichophyllus* with sterile scalpel dipped in fresh bacterial culture Yonemitsu *et al* (1990) dipped sterile scalpel in bacterial suspension and cut the base of seedlings of *Lobelia inflata* for inoculation with bacteria

Kittipongpatana *et al* (1998) stabbed four-week old plants of *Solanum avicularae* with sterile toothpicks previously loaded with bacteria

2 2 2 4 2 Co-culturing of explants with bacteria

Co-culturing of explants with bacteria is used when more direct methods fail to induce transformation Yoshikawa and Furuya (1987) induced hairy roots from calli of *Panax ginseng* by co-culture of cellulase, macerozyme and mannitol treated calli with *Agrobacterium rhizogenes* suspension for 3 days They could not get hairy roots by directly inoculating bacteria on wounded calli Hawes *et al* (1988) have reported that the motile strains of *Agrobacterium* exhibit virulence only in liquid medium but mutant strains (non-motile) exhibit virulence when inoculated directly on wounds

The co-culture time and the bacterial concentration affects the transformation frequencies (Mihaljevic *et al*, 1996)

Shimomura *et al* (1991) co-cultured leaf discs of Solanaceous plants with *Agrobacterium* for 24 h O'Keefe *et al* (1997) also co-cultured leaf discs of *Catharanthus roseus* with bacterial suspension for 24 h Ando *et al* (1997) co-cultured stem segments and leaf segments of *Wahlenbergia marginata* with 200 µl bacterial suspension in 30 ml ½ MS liquid medium for 2 days in dark Bakkali *et al* (1997) co-cultured leaf segments with 100 µl bacterial suspension in ½ MS liquid medium at 25°C in dark at 100 rpm for 24 h

2 2 2 5 Culturing of infected explants

2 2 2 5 1 Culture media

Infected explants were cultured on the best basal medium for the particular species and the explants were inoculated by direct method, by several workers

Kamada *et al* (1986) placed the inoculated shoot tips of *Atropa belladonna* on solid MS basal medium Deno *et al* (1987) inoculated the shoots of *Duboisia myoporoides* with bacteria and placed on LS basal medium for hairy root induction Aoki *et al* (1997) inoculated the stem bits of *Atropa belladonna* with bacteria and cultured on ½ MS (macro) solid medium for hairy root induction Sarma *et al* (1997) cultured the infected leaves of *Rauvolfia serpentina* on MS solid medium On occurrence of whitish bacterial growth on media, the leaves were transferred on MS basal medium supplemented with 400 mg l^{-1} ampicillin for hairy root induction In co-culture method the infected explants were placed directly on basal nutrient medium supplemented with antibiotics Ando *et al* (1997) transferred the co-cultured stem segments of *Wahlenbergia margmata* on ½ MS solid medium containing 0 5 g l^{-1} cefotaxime for hairy root induction Bakkali *et al* (1997) cultured the infected leaf discs of *Lawsonia inermis* on ½ MS solid medium supplemented with 0 5 g l^{-1} cefotaxime

Culture medium affects the hairy root induction Mano *et al* (1989) inoculated stem segments and leaf discs of *Duboisia leichhardtii* on LS solid medium without sucrose for 1 week and then transferred to 1 per cent agar 4F medium with 1 mg ml^{-1} carbenicillin and obtained hairy roots However, when the shoots were retained on 1 per cent LS medium with or without sucrose, no hairy roots were formed They concluded that culture medium affected the hairy root induction

Benjamin *et al* (1994) smeared the base of shoots of *Rauvolfia serpentina* with bacteria and incubated them on MS solid medium for 3 days Then the shoots were transferred on MS solid medium for 3 days Later the shoots were transferred on MS solid medium containing 1 g l^{-1} of carbenicillin for elimination of bacteria, for 30 days The shoots were finally sub-cultured on MS basal medium, LS medium, Knop's medium and White's medium for hairy root induction The hairy roots failed to be induced on Knop's medium and MS medium They concluded that culture medium affects hairy root induction

2 2 2 5 2 Photoperiod

The photoperiod requirement of explants to induce hairy roots varies from species to species Jazin *et al* (1988) incubated the infected seedlings of *Datura stramonium* and *Hyoscyamus niger* under continuous light (2000 lux) and obtained hairy roots Similarly, hairy roots were induced on 18-day old plantlets of *Datura candida* by culture in continuous light by Christen *et al* (1989)

Toivonen *et al* (1989) incubated the infected leaves of *Catharanthus roseus* in continuous dark and obtained hairy roots Similarly, Sarma *et al* (1997) were able to induce hairy roots on leaf segments of *Rauvolfia serpentina* by culture in continuous dark

Kamada *et al* (1986) incubated the infected stems of *Atropa belladonna* in 18 h light/6 h dark photoperiod (4000 lux) for hairy root induction A

photoperiod of 12 h per day (2500 lux) was found optimum for induction of hairy roots from infected stem segments and leaf discs of *Duboisia lechhardtii* by Mano *et al* (1989)

2 2 2 6 Effects of exogenous auxins on hairy root induction

Use of auxins to increase the transformation frequencies has been reported by several workers

Ryder *et al* (1985) studied the virulence properties of various *Agrobacterium rhizogenes* and *A tumefaciens* strains They found that most of the strains under study showed polar transformation They grouped those strains as polar strains They found that the polarity effect is due to the absence of T_R fragments of T-DNA in these strains, which contain auxin synthesis genes They further could solve the problem either by inserting T_R region in the strains or by exogenous application of auxins Similar results were obtained by Cardarelli *et al* (1987), while infecting carrot discs with mannopine type *Agrobacterium rhizogenes* strains

Xu *et al* (1997) cultured callus of alfalfa (*Medicago sativa*) for one hour in liquid SH medium containing 2 26 μM 2,4-D and then co-cultured with A_4 strain of *A rhizogenes* Control was not treated with 2,4-D They reported that 2,4-D treatment increased the transformation frequencies

2 2 2 7 Time period for hairy root induction

In majority of the plant species, hairy root induction is achieved in a time period of 2-4 weeks regardless of the explant and the *A rhizogenes* strain used (Mugnier, 1988, Yazaki *et al*, 1998, Kittipongpatana *et al*, 1998 and Zarate, 1999)

2 2 3 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 detecting the presence of opines Opine synthesis by plant cell is the essential feature of integration of T-DNA in the plant genome (Petit *et al*, 1983, De Paolis *et al*, 1985, Brevet *et al*, 1988, Bouchez and Tourneur, 1990) Opines are not synthesised by uninfected plant tissues Opine analysis is done by high voltage paper electrophoresis (Petit *et al*, 1983)

Hairy roots are more sensitive to auxins as compared to normal roots This phenomenon can be utilised as a test for confirmation of transformation (Shen *et al*, 1988 and Shen *et al*, 1990)

The presence of T-DNA in the plant genome can be confirmed by southern blotting and hybridisation It was first utilised by Ackermann (1977)

2.3 Biochemical estimation of amino acids

2 3 1 Paper chromatography for amino acids

Paper chromatography is the most simple and popular technique for the qualitative determination of amino acids Retnam and De Britto (1998) did a preliminary phytochemical screening of three medicinal plants namely *Mukia maderuspatana, Hybanthus enneaspermus* and *Enicostema axillare* They estimated the amino acids from benzene, chloroform, methanol and petroleum ether extracts of powdered plant material by paper chromatography (Whatman

No 1 chromatography paper) using different solvent systems and visualising the amino acids using 0 2 per cent ninhydrin They identified the amino acids by comparing the Rf values of standard amino acids

2 3 2 Extraction of amino acids

Ramiah *et al* (1981) extracted the amino acids from the roots of *Holostemma annulare* using absolute ethanol Pari *et al* (1998) extracted gigantiene, a non-protein amino acid from the root bark of *Calotropis gigantea* using absolute methanol Das and Dayanand (1983) analysed the amino acids in red sanders (*Pterocarpus santalinus* Linn F) from the ethanolic extracts

2 3 3 Separation of amino acids

Plummer (1971) suggested a two-dimensional paper chromatography for the separation of amino acids He suggested the use of two solvent systems viz n-butanol-acetic acid-water (12 3 5) and phenol-water (2 1) He suggested the use of Whatman No 1 chromatographic paper for the estimation of amino acids

Bhushan (1981) subjected the plant extract to two-dimensional paper chromatography with butanol-acetic acid-water (4 1 1) and phenol-water (1 4) as running solvent systems

Antures and Campos (1995) separated the amino acids from 8 species of *Phaseolus,* 3 species of *Vigna* and *Lablab purpureus* by two dimensional paper chromatography and ion-exchange chromatography using phenol-water (1 1) as running solvent system

2 3 4 Identification of amino acids

Bhushan (1981) developed the chromatograms for visualising the amino acids by spraying with 0 2 per cent ninhydrin

Parui and Mandal (1998) used 0 4 per cent ninhydrin in acetone as spray reagent for the identification of amino acids on paper chromatograms

2.4 Biochemical estimation of essential oil

2 4.1 TLC of essential oil

TLC is widely used for the large-scale separation of essential oil (Harborne, 1973) TLC is the only separation technique for essential oil in absence of GLC Even when GLC is available, TLC is useful at all stages for separation and analysis of essential oil (Hoerhammer *et al*, 1964)

Rossini *et al* (1995) developed a TLC method for evaluation of 1,8-cineole content of eucalyptus essential oil The samples were developed on silica gel G 60 with light petroleum-chloroform (70 30) as a mobile phase Detection was done with 4-dimethylaminobenzaldehyde-sulphuric acid reagent They confirmed the results using GLC They concluded that TLC method is advantageous because it allows several simultaneous determinations on the same plate with only one calibration curve and upto 10 samples could be analysed in 30 min They found the limit of detection of 1,8-cineole as 5 µg

2 4 2 Extraction of essential oil

Volatile components of essential oil are classically separated from fresh tissues by stem distillation (Guenther, 1948) Mono and sesquiterpene essential oil are separated by extraction with ether, petroleum or acetone (Harborne, 1973) Ramani and Mathur (1990) extracted the essential oil from aerial parts of an aromatic grass *Bothriochloa adorates* (Lisboa) in hexane followed by methanol

2 4 3 Separation of essential oil

Silica gel is the most commonly used absorbent with solvents such as benzene, chloroform, benzene-chloroform (1 1) and benzene-ethyl acetate (1 1)

To separate terpenes according to the number of double bonds, TLC plates coated with silica gel slurry with 2 5 per cent $AgNO_3$ are used The solvent system employed with $AgNO_3$ treated plates is methylene dichloride-chloroform-ethyl acetate-n-propanol (45 45 4 5 4 5) (Harborne, 1973)

2 4 4 Detection of essential oils

Essential oils are detected by spraying the eluted plated with 0 2 per cent aqueous $KMnO_4$, 5 per cent antimony chloride in chloroform, concentrated H_2SO_4 or Vanillin-H_2SO_4 Bromine vapour is used for detecting terpenes with double bonds while terpenes with ketonic groupings can be detected by using 2,4-dinitrophenyl hydrazine (2,4-DNP) (Harborne, 1973)

Ramani and Mathur (1990) visualised the essential oil under UV light after elution as pink fluorescent spots

2.5 Biochemical estimation of triterpenoids

2 5 1 TLC for triterpenoids

All types of triterpenoids are separated based on the technique of TLC TLC is particularly carried out on layers of silica gel

Lin and Cordell (1989) extracted the triterpenoids from the roots of *Salvia prionitis* by ethanol and partitioning into $CHCl_3$ and water The organic layer was further subjected to column chromatography and preparative TLC Yellow needles of salvonitin were obtained

2 5 2 Extraction of triterpenoids

In surveying plants for triterpenes and their derivatives, the dried tissue is first defatted with ether and then extracted with hot methanol The concentrated methanol extracts are used directly for the examination of triterpenoids (Harborne, 1973)

Jarvis *et al*, (1999) extracted the triterpenoids from neem seed using dichloromethane The extract was fractionated rapidly with economic use of solvent using 'Biotage T M flash chromatography system' The fractionated extract was then analysed for triterpenoids using TLC

2 5 3 Separation of triterpenoids

Argentative TLC (on plates of silica get treated with $AgNO_3$) is employed for separating triterpenoids according to the number of isolated double bonds present in the molecule (Harborne, 1973) They suggested that the triterpenoids are readily separated in solvents such as hexane-ethyl acetate (1 1) and chloroform-methanol (10 1) Attalah and Nicholas (1971) used n-butanol-2 M NH_4OH (1 1) for the separation of ∝ and β-amyrin that did not separate by any other solvent system Galgon *et al*, (1999) separated betulinic acid from *Betulus pendula* using petroleum-dichloromethane-acetic acid (50 50 0 7)

2 5 4 Detection of triterpenoids

Lisboa (1969) and Neher (1969) studied more that fifty detection reagents for the detection of triterpenoids They suggested the use of Carr-Price reagent, i e 20 per cent antimony tri chloride in chloroform for the detection of triterpenoids They also suggested the use of Libermann-Burchard reagent (1 ml concentrated H_2SO_4, 20 ml acetic anhydride and 50 ml chloroform) for sensitivity up to 2 5 µg

Tallevi and Kurz (1991) visualised the terpenoids from *Ginkgo biloba* leaves by heating the eluted TLC plates 110°C for 35-60 min followed by visualisation with UV light (365 nm)

2.6 Biochemical estimation of sterols

2 6 1 TLC for sterols

Sterols are readily separated from plant samples using TLC Separation of common sterols from their di-hydroderivatives is done by argentative TLC (Ikan and Cudzinovski, 1965)

Gupta and Sil (1983) separated sterols from *Madhuca butyracea* on silica gel G using n-hexane-ether-acetic acid (20 20 1) and also by argentative TLC on silica gel G using chloroform-acetone (95 5) for development They found that argentative TLC was more dependable

2 6 2 Extraction of sterols

Mahadevappa and Raina (1981) isolated free sterol, esterified sterol, and sterol glycoside from the chloroform/methanol extracts of *Vigna unguiculata* Miglani *et al* (1978) used petroleum ether for extraction of sterols from the corms of *Arisaema tortuosum* Meyre-Silva *et al* (1998) extracted the sterols from leaves of *Aleurites moluecana* using hexane as extraction solvent Anjaneyulu *et al* (1998) extracted sterols from whole plant of *Pergularia extensa* using hexane as extraction solvent

2 6 3 Separation of sterols

Sterols are separated from their dihydro derivatives using argentative TLC The plates are prepared by spraying fresh silica gel layers with

concentrated aqueous methanolic $AgNO_3$ and then activating them for 30 min at 120°C Separation is achieved in chloroform (Ikan and Cudzinovski, 1965)

Sitosterol, cholesterol and stigmasterol are separated by chromatography on Anasil B plates by continuous development for 2 hour with hexane-ether (97 3) as solvent system (Harborne, 1973)

Lockwood *et al* (1974) separated sterol and steroidal sapogenin in plant extracts by densitometric TLC on silica gel G developed in hexane acetone (4 1)

Purushothaman and Saradhavasanth (1986) pulverised *Vicoa indica* plant and extracted with light petroleum followed by $CHCl_3$ The sterols were eluted on silica gel plate using ethyl acetate as solvent

2 6 4 Detection of sterols

Lisboa (1969) and Neher (1969) recommended the Carr-Price reagent, i e 20 per cent antimony trichloride in chloroform for detection of sterols Ikan and Cudzinovski (1965) used H_2SO_4-Water (1 1) for the detection of sterols Lockwood *et al* (1974) used saturated solution of antimony trichloride in chloroform followed by heating for 8 min at 100°C for developing the chromatograms for the detection of sterols The absorbence was measured at 477 nm

Materials and Methods

MATERIALS AND METHODS

The study entitled 'Genetic transformation and hairy root cultures in Ada-kodien (*Holostemma ada-kodien* K Schum)' was carried out in the Plant Tissue Culture Laboratory of the Centre for Plant Biotechnology and Molecular Biology, and the Biochemistry Laboratory of the All India Co-ordinated Research Project on Medicinal and Aromatic Plants, College of Horticulture, Vellanikkara, from May 1999 to October 2000 The materials used and the methodologies adopted in this study are described below

3.1 Materials

3 1 1 Chemicals

The major and minor nutrients required for the preparation of media were of analytical grade and procured from M/S Sisco Research Laboratories (SRL), British Drug House (BDH) and M/S Merck India Ltd The amino acid standards and chemicals used for biochemical analysis were obtained from M/S Merck India Ltd, SRL and Sigma Chemicals, USA

3 1 2 Glasswares

Borosilicate glassware of Corning/Borosil brand was used for the study The glassware were cleaned initially by soaking in potassium dichromate solution for 12 h followed by thorough washing with jets of tap water in order to remove completely all traces of potassium dichromate solution They were further cleaned with 0 1 per cent Teepol detergent solution and were washed thoroughly with water and rinsed twice with double distilled water These were then dried in a hot air oven at 105°C for 24 h and later stored in cupboards free of dust till further use

3 1 3 Culture media

3 1 3 1 Composition of media

Murashige and Skoog (1962) medium (MS medium) was reported to be the best basal medium for the growth of explants of *H ada-kodien* by John (1996) and hence, was used as the basal medium in the present study The composition of the medium is given in Appendix I

For culturing of different *Agrobacterium rhizogenes* strains, LB medium and YEM medium were used Their composition is given in Appendix II

3 1 3 2 Preparation of medium

3 1 3 2 1 Preparation of MS medium

The MS medium was prepared according to the standard procedure adopted by Gamborg and Shyluk (1981) Stock solutions of major and minor elements were prepared and stored in pre-cleaned amber coloured bottles in refrigerated conditions

A clean steel vessel, rinsed with distilled water was used to prepare the medium Aliquots from all stock solutions were pipetted in proportionate volumes in the vessel Little amount of distilled water was added to it and later on, required quantities of sucrose (carbohydrate source) and inositol were added and dissolved in it The desired volume was made up by adding distilled water The pH of the medium was adjusted between 5 5 to 5 8 using 0 1 N NaOH or HCl

For solid medium, agar was added at 0 75 per cent (w/v) concentration, after adjusting the pH The medium was stirred and heated to melt the agar, and was poured when hot into culture vessels and were plugged with non-absorbent cotton For solid media, test tubes (15 cm × 2 5 cm) were used whereas for

liquid media conical flasks (100 and 250 ml volume) were used as culture vessels Fifteen ml medium was poured in each test tube, 30 ml medium in 100 ml conical flask and 75 ml medium in 250 ml conical flask Vessels containing media were sterilised in an autoclave at 121°C by applying 15 psi pressure for 20 min After this, the culture vessels were kept in culture room on racks till further use

3 1 3 2 2 Preparation of LB medium and YEM medium

Clean steel vessels, rinsed with distilled water were used to prepare the media The ingredients were weighed on electronic balance and were added into the vessels Little amount of distilled water was added to it and the ingredients were dissolved The desired volume was made up by adding distilled water The pH of the media were adjusted to 7 0 using a standard pH meter by adding either 0 1 N NaOH or HCl

For solid media, agar was added at 1 8 per cent (w/v) concentration for LB medium The media were stirred and heated to melt the agar and were poured when hot into jam bottles Hundred ml medium was poured in each jam bottle The jam bottles were covered with lid and sealed using clear adhesive tape Bottles containing media were sterilised in an autoclave at 121°C by applying 15 psi pressure for 20 min The sterilised bottles were kept in the culture room on racks till further use

3 1 4 Growth regulators

NAA (auxin) was incorporated in the MS basal medium at 1, 2 and 3 mg l^{1} concentrations for improving the transformation efficiency and hairy root growth

3 1 5 Antibiotics

Different antibiotics (ampicillin, kanamycin, cefotaxime and carbenicillin) were used in MS medium, LB medium and YEM medium for killing the bacteria and for testing the resistance of bacteria for antibiotics

The stock solutions of antibiotics were prepared fresh under sterile conditions Aliquots were taken from them and were added to the sterilised media The solid media were first melted, cooled to 40°C and then the desired quantities of aliquots of antibiotics were added to them

3 1 6 Transfer area and aseptic manipulations

All the aseptic manipulations such as surface sterilisation of explants, preparation and inoculation of explants, subsequent subculturing and preparation of antibiotic media were carried out under the hood of a laminar flow cabinet

3 1 7 Culture room

The cultures were incubated at 26±2°C in air conditioned culture room with 16 h photoperiod (1000 lux) from fluorescent tubes Humidity in the culture room varied from 60 to 80 per cent according to the climate prevailing Cultures were also incubated in the dark to study the effect of light and dark conditions on hairy root induction and growth

3 1 8 Source of explants

In vitro grown plantlets of *Holostemma* were used as the source of shoot buds, internodal segments, leaves and callus The seedling hypocotyls were obtained from seeds in the mature green pods of *Holostemma*, harvested from field grown plants in the medicinal plant garden attached to the Department of

Plantation Crops and Spices, College of Horticulture, Vellanikkara The *in vitro* seedlings were obtained by incubating the seeds on MS basal medium from which the hypocotyls were obtained

3 1 9 *Agrobacterium rhizogenes* strains

Agrobacterium rhizogenes strains of mannopine family (A_4, 15834, P_cA_4 and 8196) and cucumopine family (2659) were used for the study

3.2 Methods

3 2 1 Culturing of *A rhizogenes* strains

The bacterial strains were tested for a suitable growth medium They were cultured on LB medium and YEM medium

Each strain was streaked on plates containing either LB or YEM medium The growth of bacteria on each medium was observed The medium that induced faster growth was observed The colony characteristics of bacteria on each medium were also noted

To study the temperature requirements for the growth of bacteria, one set of culture was kept at room temperature (30-35°) while another set was kept in the culture room at 26±2°C The observations regarding the growth of bacteria were noted

3 2 2 Isolation of single cell colonies

To isolate single cell colonies, the bacteria were streaked on plates containing appropriate culture medium To streak the bacteria on to the plate, the transfer loop was flamed, cooled and plunged into a well grown bacterial colony The lid of petriplate containing sterile, solid medium was lifted from one side

and the loop loaded with bacteria was drawn gently on about 1/3rd of the plate surface, to make three lines close together, but separate from each other The loop was again flamed, cooled and drawn across one end of the second streaked area and the remaining 1/3rd area of the plate was streaked The plate was closed, sealed with parafilm and kept in the culture room on a rack The observations regarding growth of bacteria were documented

3 2 3 Screening of *A rhizogenes* strains for antibiotic resistance

The *A rhizogenes* strains used for the study were tested for resistance to antibiotics The antibiotics used for testing resistance were ampicillin, kanamycin and cefotaxime

The best culture medium for growth of each bacterial strains was supplemented with 100 mg l^{-1}, 250 mg l^{-1}, 500 mg l^{-1} and 1000 mg l^{-1} of each antibiotic, separately The bacteria from a single cell colony were streaked gently on the antibiotic medium in petriplates The observations regarding the growth of bacteria were documented

3 2 4 Maintenance of strains

Stabs of the best growth medium for each strain were prepared and the bacterial strains were maintained as stabs for further use

3 2 4 1 Preparation of stabs

The best growth medium containing marker antibiotic for each strain was sterilised, poured in sterilised culture tubes and allowed to solidify The transfer loop was flamed, cooled and plunged in an isolated single cell bacterial colony The loop loaded with bacteria was used to stab the solid medium in the culture tube Similarly, stabs for all the bacterial strains were prepared following the same procedure mentioned above, and allowed to stand in the culture room on

racks for growth of bacteria in the medium The stabs showing good growth of bacteria were further stored in refrigerator at 4-6°C till further use

3 2 5 Studying the growth of *A rhizogenes* strains

Bacteria show a characteristic growth when grown in liquid medium The initial lag phase is followed by log phase (phase of active rapid growth) The log phase is followed by a stationary phase and finally decline in growth is observed This growth pattern is obtained by measuring the optical density of liquid growth medium inoculated with bacteria and plotting it against time In the present study, the growth curve of the *A rhizogenes* strains was studied to find out the time required for each strain to attain a log phase

Culture tubes containing 5 ml of sterilised liquid MS medium were inoculated with 100 µl of bacterial suspension ($OD_{560\ nm}^{1}$) The tubes were then cultured on rotary shakers at 150 rpm The optical density (OD) of the medium at 560 nm was noted at 2 h interval and the observations were documented

A graph was plotted keeping time on X axis and the OD value on Y axis to obtain the growth curve The time required for each bacterial strain to attain the log phase was documented

3 2 6 Standardisation of optimum concentration of antibiotic to kill the bacterium

The optimum concentration of antibiotic to kill the bacterium was determined by two methods In the first method, the bacteria were plated on solid growth medium containing various concentrations of antibiotics (hereafter referred to as plating method) In the second method, the bacteria were cultured in liquid medium containing various concentrations of antibiotics (hereafter referred to as optical density or OD method)

3 2 6 1 Plating method

Sterilised petriplates with solid growth medium containing 0, 50, 100, 250, 500 and 1000 mg l^{-1} of antibiotics were streaked with bacteria The growth of bacteria was observed and the observations were documented

3 2 6 2 Optical density (OD) method

Culture tubes containing 5 ml of sterilised liquid medium were added with aliquots taken from freshly prepared stock solutions of antibiotics to obtain tubes containing 0, 1, 2, 5, 10, 50, 100, 250, 500, 1000, 2000 and 3000 mg l^{-1} of antibiotic medium 100 µl of bacterial suspension ($OD_{560\,nm}^{-1}$) was added in each tube The OD values of medium in each tube was measured using a Spectronic 20 spectrophotometer and the observations were documented

3 2 7 Effect of plant extract of *Holostemma* on growth of *A rhizogenes* strains

The success of genetic transformation via *Agrobacterium* depends on the compatibility of the plant and the bacterial strain In determining the compatibility reaction, we assume that the host plant and a particular strain of *A rhizogenes* may be compatible if the host plant extract does not inhibit if not favour the growth of the strain

To study the effect of plant extract of *Holostemma* on growth of *A rhizogenes* strain under study, 10 ml of water extract of *in vitro* plantlet of *Holostemma* was added to 100 ml of YEM medium The bacteria obtained from single cell colonies were grown on the medium The observations regarding the growth of the bacterial strains were documented For control, the bacteria were grown on YEM medium without plant extract

3 2 8 Explants

Various explants viz leaves, shoot buds, internodal segments, seedling hypocotyls and callus were screened for their transformation ability

3 2 8 1 Raising of *in vitro* seedlings

Mature green pods obtained from field grown plants were surface sterilised by wiping with absolute alcohol followed by dipping in 0 1 per cent $HgCl_2$ solution for 20 min The $HgCl_2$ treated pods were rinsed three times with sterile distilled water to remove all traces of $HgCl_2$ solution The pods were then blotted on sterile filter paper and cut open with the help of sterile blade and forceps The seeds were transferred to sterile tubes containing MS solid medium Three to four seeds were transferred to each culture tube All the operations were carried out in sterile conditions under the hood of a laminar air flow cabinet The tubes were kept in the culture room on racks

3 2 8 2 Preparation of explants

The *in vitro* plantlets were taken out of the culture tubes in a laminar flow cabinet on pre-sterilised steel plates The plantlets were cut with a sterile blade to separate the leaves, shoot buds, internodal segments and the callus formed at the base The *in vitro* seedlings having two cotyledonary leaves were taken out of the culture tubes and the base of each seedling was cut to obtain seedling hypocotyls with two cotyledonary leaves

3 2 8 3 Wounding of explants

The explants were wounded with a sterile blade and/or with a sterile needle The leaf margins were cut from all sides with a sterile blade to obtain leaf discs Alternatively, the leaves were wounded by pricking with a sterile needle

A fresh cut was made at the base of shoot buds The shoot buds were then pricked with sterile needle Both ends of internodal segments were freshly cut and the segments were pricked with a sterile needle The base of seedling hypocotyls was freshly cut and the hypocotyl portion was pricked with a sterile needle

The callus was cut into small pieces with a sterile blade Alternatively, a freshly cut piece of callus was pricked with sterile needle

The pricks were made carefully to make injuries Fifteen pricks each were made on seedling hypocotyl, shoot buds and internodal segments while 10 pricks were made on leaf midrib and lamina

3 2 9 Standardisation of inoculation method

The wounded explants were infected either by direct application of bacteria on wounds (hereafter referred to as direct inoculation method) or by culturing the wounded explants in sterilised liquid MS medium containing bacteria (hereafter referred to as co-culture method) To test the effect of wound-exudates on transformation, in one set of explants in each infection method, the exudates from wound were blotted on sterile filter paper and then the explants were infected whereas in the other set, the explants were infected without wiping the wound exudates

3 2 9 1 Direct inoculation method

3 2 9 1 1 Effect of bacterial inoculum on transformation

To test the effect of type of bacterial inoculum used for infection, on transformation, either the bacteria from isolated single cell colonies were used as inoculum or bacterial suspension (OD_{560nm} [1]) was used as inoculum For control,

sterilised liquid MS medium was applied on wounds The observations regarding the response of infected explants to transformation were documented

3 2 9 1 2 Effect of intensity of bacterial inoculum applied

To test the effect of lesser intensity of bacterial inoculum applied on the wounds, on transformation, the needle was either dipped in bacterial suspension (OD_{560nm}^{-1}) or in an isolated single cell colony and pricking was done with the needle loaded with bacteria, on the explants To test the effect of more bacterial inoculum applied on the wounds, a drop of bacterial suspension (OD_{560nm}^{-1}) or bacteria picked from a single cell colony was smeared on each wound on the explants For control, sterilised liquid MS medium was applied on each wound on the explants The observations regarding the response of infected explants to transformation were documented

3 2 9 1 3 Effect of NAA on transformation

To study the effect of NAA application on wounds prior to infection, a drop of NAA was apphed on the wounds The bacterial inoculum was then applied on the wounds Various concentrations of NAA (1, 2 and 3 mg l^{-1}) were tested for their effect on transformation For control, only a drop of NAA was applied on the wounds The explants were also soaked in different concentration of NAA solution (1,2 and 3 mg l^{-1}) for 5 min and then infected The observations regarding the transformation of infected explants were documented

3 2 9 1 4 Co-culture method

3 2 9 1 4 1 Effect of amount of bacteria present during co-culture and the co-culture time on transformation

To study the effect of less number of bacteria in the co-culture medium, on transformation, 100 µl of bacterial suspension (OD_{560nm}^{-1}) was added to 30 ml

sterilised liquid MS medium in 100 ml conical flasks Freshly wounded explants were transferred to the flasks and the flasks were kept on rotary shakers at 100 rpm The co-culture time was standardised by co-culturing different sets of flasks containing explants for variable time viz 12, 24, 36, 48 and 72 h

To study the effect of more intensity of bacteria in the co-culture medium, on transformation, 100 µl of bacterial suspension (OD_{560nm}^{1}) was added to 30 ml sterilised liquid medium in 100 ml conical flasks The flasks were kept on rotary shakers at 100 rpm for 36 h Freshly wounded explants were then inoculated and were kept on rotary shakers at 100 rpm The co-culture time was standardised by inoculating separate sets of cultures at different time periods viz 5, 10, 15, 30, 60 and 120 min One set of explants cultured in MS liquid medium without bacterial inoculum was treated as control The observations regarding the explant response to transformation were noted

3 2 9 2 2 Standardisation of shaker speed

To standardise the optimum shaker speed, co-culturing was done at 50, 100 and 150 rpm The explant response to transformation was noted

3 2 9 2 3 Effect of NAA on co-culture

The effect of NAA (1, 2 and 3 mg l^{1}) in co-culture medium, transformation was tested The wounded explants and bacteria were co-cultured for 24 h in 100 ml conical flasks containing 30 ml liquid MS medium with various concentrations of NAA viz 1, 2 and 3 mg l^{1}, separately The observations regarding the effect of NAA on transformation frequency were noted For control, the explants were cultured in liquid MS medium with various concentrations (1, 2 and 3 mg l^{1}) of NAA

3 2 10 Culturing of infected explants

The infected explants were cultured on solid media for 24 h for the multiplication of bacteria in the explant tissues One set of explants was cultured on 1 per cent agar in water while another set of explants was placed on MS solid medium The observations regarding the growth of bacterium on media were noted

3 2 11 Elimination of bacteria from explant tissues

For removing the bacteria from explant tissues, one set of explants was cultured on MS solid medium containing 250 mg l^{-1} of appropriate antibiotic (ampicillin or cefotaxime) Another set of explants was cultured in liquid MS medium containing 250 mg l^{-1} antibiotic for 24 h and then sub-cultured on MS solid medium containing 250 mg l^{-1} antibiotic If the bacterial growth was seen after a few days, on the media, the explants were again sub-cultured on MS solid media containing 250 mg l^{-1} of the antibiotic This was done until no bacterial growth was seen on the media

3 2 12 Effect of media on hairy root induction

To study the effect of media on hairy root induction, one set of infected explants was cultured on ½ MS solid medium and second set of infected explants was cultured on MS solid medium Third set of infected explants was placed in ½ MS liquid , medium while fourth set of explants was placed in MS liquid medium The observations regarding the hairy root induction were documented at regular intervals

3 2 13 Effect of photoperiod on hairy root induction

To study the effect of photoperiod on hairy root induction, one set of infected explant was given a photoperiod of 16 h light (1000 lux) while another

set of infected explants was placed in dark The observations regarding the hairy root induction were documented at regular intervals

3 2 14 Effect of various concentrations of antibiotics on explants

The effect of various concentrations of ampicillin and cefotaxime on explants was studied The wounded explants were cultured in liquid MS medium containing 250, 500, 1000, 2000 and 4000 mg l^{1} concentrations of either ampicillin or cefotaxime For control the explants were cultured in MS liquid medium without antibiotic The cultures were incubated on rotary shakers at 100 rpm. The observations were documented

3 2 15 Confirmation of transformation

The confirmation of transformation was done on the basis of the morphological features of the roots obtained from wounds after infection with *Agrobacterium*

The roots emerging from wounds of infected explants were confirmed to be transformed by opine analysis using the modified procedure mentioned by Dessaux *et al* (1991) The normal roots and bacterial strains were also tested for the presence of opines

The sensitivity of normal and hairy roots to NAA was also studied

3 2 15 1 Morphological features

The roots obtained from wounds of infected explants were tested for the morphological features such as colour, presence of root hairs, response to geotropism and the growth rate

3 2 15 2 Opine analysis

Opine analysis was done according to the modified procedure given be Dessaux *et al* 1991

3 2 15 2 1 Preparation of reagents

A buffer system of 1 1 M acetic acid/0 7 M formic acid, pH 1 9 was used for the separation of opines The buffer system was prepared by mixing acetic acid-formic acid-water (50 4 46 v/v/v)

Various reagents were used for the detection of opines

- Solution I 0 4 per cent silver nitrate in 99 1 acetone water mixture was prepared The reagent was stored in black coloured bottle in refrigerated conditions
- Solution II 2 per cent NaOH in 90 per cent ethanol in water was prepared
- Solution III Reducer solution was prepared by using the reducer concentrates A, B and C obtained from Sigma Company, USA One ml of reducer A concentrate was mixed with 2 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

3 2 15 1 2 Extraction of opines

300 mg fresh root tissue was placed in an eppendorf tube Distilled water (3 ml/g of tissue) was added, and the tube was heated for 10 min at 100°C

Softened tissues were crushed, briefly vortexed and separated from the liquid phase by centrifugation for 5 min at 13,000 gravity at room temperature

The supernatant was collected and rotary evaporated at 40°C under vacuum using Savant vacuum freeze drier to yield a dried plant extract This extract was resuspended in distilled water (0 5 µl/mg of dried sample) and used for the detection of opines Remaining extract was kept frozen for further studies

3 2 15 2 3 Separation of opines

Ten µl of plant extract was spotted on Whatman No 1 chromatography paper strip (26 × 8 cm) A mixture of standard agropine and mannopine was spotted at a distance of 1 5 cm from the plant sample The paper strip was moistened with butter excluding 0 5 cm area on both sides of the spots. The moistened paper strip was placed on the supporter of horizontal electrophoresis unit (Biotech Pvt. Ltd , Chennai), containing equal volumes of buffer in both wells, such that both ends of paper were dipped in the buffer

The spotted end of the strip 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 min Following electrophoresis, the paper was dried in a stream of hot air using a hair drier

3 2 15 2 4 Detection of opines

Opines were detected using alkaline silver nitrate reagent Dried paper was first dipped into the silver nitrate reagent (solution I), allowed to dry in a stream of cold air, and dipped in sodium hydroxide solution (solution II) The paper was dried in hot air using a hair dryer The background colour was reduced by dipping the developed electrophorograms in reducer solution (solution III), followed by drying in a flow of hot air The observations regarding the presence or absence of opines were documented

3 2 15 3 Testing the relative sensitivity of hairy roots and normal roots to NAA

The hairy roots and the normal roots were cultured in liquid MS medium containing 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M of NAA The flasks were kept on rotary shaker at 100 rpm in dark The observations regarding the growth of roots were regularly documented.

3 2 16 Observations on hairy root induction

a) Number of days taken for hairy root induction

The days taken for induction of roots from wounds of infected explants were noted

b) Percentage of cultures initiating hairy roots

Of all infected tubes, those which induced roots from wounds upon infection were counted and were expressed as percentage of total number of infected tubes

c) Number of hairy roots emerging per explant per wound

The number of hairy roots emerging from the wounds of each infected explant was noted

d) Period of treatment with *A rhizogenes* effective for hairy root induction

In co-culture method, the co-culture time effective for hairy root induction was noted

e) Effect of strain on hairy root induction

Of all the strains, those capable of inducing roots from wounds on infection were noted

3 2 17 Biochemical analysis of root tubers and *in vitro* induced callus of *Holostemma*

Biochemical analysis was carried out to isolate the different secondary metabolites namely essential oil, triterpenoids and sterols The free amino acids were also isolated and identified Both the tubers from field grown plants and the *in vitro* callus were analysed

3 2 17 1 Collection of sample

Fresh root tubers were collected from field grown plants and were washed free of adhering dust particles using clean water The callus formed at the base of *in vitro* grown shoot bud explants was separated and used for biochemical estimation The callus was washed free of the media constituents adhering to it using clean water The clean tubers and callus were blotted on clean filter papers to remove the adhering water droplets

3 2 17 2 Estimation of amino acids

Paper chromatography method was developed for the isolation and identification of free amino acids The experiments conducted to standardise the method are described here

3 2 17 2 1 Extraction of amino acids

The clean tubers and callus were weighed and 1 g each of tubers and callus were ground separately in a mortar and pestle with approximately 5 ml of 10 per cent iso-propanol The extracts were transferred to eppendorf tubes and centrifuged at 10,000 rpm for 3 min The supernatent were transferred to clean eppendorf tubes and used for the estimation of amino acids The remaining extracts were stored in a refrigerator for further use

3 2 17 2 2 Spotting on chromatographic paper

Whatman No 1 chromatographic paper manufactured by M/S Merck India Ltd was used The paper was cut to obtain strips of 30 cm length and 4 cm breadth These strips were used for spotting the sample

Spotting was done with the help of capillary tubes The capillary tubes were calibrated to 5, 10, 15 ad 20 µl volumes The respective volumes of distilled water were successively pipetted using micropipettes These volumes were sucked by capillary tubes and the respective levels of water in them were marked to get calibrated capillary tubes

The amount of sample to be applied and the spot diameter were standardised by spotting 5, 10 and 20 µl of sample on Whatman No 1 chromatographic paper in the form of circular spots of various diameter ranging from 2 mm, 5 mm and 10 mm

Spotting was done 2 cm above the lower edge of the chromatographic paper strips Spotting was done to obtain circular spots Sample was not allowed to spread and exceed the specified limits This was done by intermittent application by blowing hot air from a hair dryer After drying, a separate dose of sample was applied on the same spot This was done till the desired volume of sample was completely applied

3 2 17 2 3 Running solvent system

Running solvent system was prepared by mixing different solvents in a desired ratio For the estimation of amino acids, a solvent mixture of n-butanol-acetic acid-water (12 3 5) was used About 50 ml of this solvent system was sufficient to elute 15-20 strips successively in a CAMAG TLC developing glass tank The solvent system was poured and the lid was placed tightly Adjacent to

the walls of the tank, filter paper sheets were placed The tank was shaken once vigorously and then retained as such for 30 min to saturate with the volatile components of the solvent system The spotted strip was then placed in the tank such that, the edge of the strip below the spot was immersed in the solvent system, but the spot was above the surface of the solvent system The lid was closed tightly to avoid the loss of volatile solvents All chromatograms were eluted between 29 to 30°C and 72-75 per cent relative humidity, to maintain uniformity in the elution pattern After approximately 6 h, the solvent eluted the spot vertically upto 2/3rd the length of the strip Then the strip was removed for developing

3 2 17 2 4 Preparation of spray reagent

To detect the amino acids on the chromatographic paper, ninhydrin spray reagent was used It was prepared by mixing 0 2 per cent ninhydrin in acetone

3 2 17 2 5 Developing the chromatogram

The eluted chromatographic paper strip was air-dried Using a Vensil reagent sprayer, the paper strip was uniformly sprayed with minimum 5 ml of ninhydrin spray reagent The sprayed paper was kept at 110°C for 3-5 min in a chromatographic oven to develop spots and the observations were documented

3 2 17 2 6 Determination of standard Rf values

3 2 17 2 6 1 Preparation of standard samples of amino acids

Standard amino acids from M/S Sisco Research Laboratories Pvt Ltd were used Ten mg of each amino acid was dissolved in 200 µl of 10 per cent iso-propanol solution A drop of acid (1 N HCl) or alkali (1 N NaOH) was added if the amino acid did not dissolve in 10 per cent iso-propanol, in order to

enhance the dissolution Standard solutions of 24 amino acids were prepared as mentioned above

3 2 17 2 6 2 Spotting on chromatographic paper

Spotting was done on strips of Whatman No 1 chromatographic paper as mentioned in 3 2 17 2 2 One amino acid was spotted per strip

3 2 17 2 6 3 Running solvent system

n-Butanol-acetic acid-water (12 3 5) was used as the running solvent system as in the case of plant sample The elution procedure was same as mentioned in 3 2 17 2 3

3 2 17 2 6 4 Preparation of spray reagent

Ninhydrin in acetone (0 2 %) was prepared and used as spray reagent

3 2 17 2 6 5 Developing the chromatogram

The various steps in developing the chromatogram were same as that mentioned in 3 2 17 2 5 The Rf values were documented after developing each chromatogram All the standard amino acids were run three times and the observations were documented

3 2 17 2 7 Identification of amino acids

The Rf values of amino acids from root tubers and callus were compared with those of the standard amino acids

The amino acid standards having matching Rf values, or those having closer Rf values to the plant samples were run by spotting one over the other, as

a single spot The plant sample was run along with the standard amino acid on the same paper at a distance of 1 5 cm from the previous spot Third run was also done on the same paper by spotting the standard amino acid along with the plant sample as a single spot This spot was at a distance of 1 5 cm from the previous spot The spotting procedure and the method of elution and detection were same as that in the case of plant samples and standard amino acids

The Rf values of standard amino acids and those of the plant samples were again compared and the Rf values of amino acids in plant samples that did not match with the corresponding standard amino acids were recorded as unidentified amino acids

In case where the Rf values of more than one standard amino acid matched with the corresponding amino acid in the plant sample, the plant sample was spotted with one of the matching standard amino acid at a time and the spotting, elution and detection procedure were done similar to that in the case of plant samples and standards Following the above procedure, the Rf values of all the spots in the case of tubers and callus were compared and the amino acids were identified

3 2 17 3 Estimation of essential oil, triterpenoids and sterols

Thin layer chromatography methods were developed for the isolation and separation of essential oil, triterpenoids and sterols The experiments conducted to standardise each method are described separately here

3 2 17 3 1 Extraction of essential oil

The clean tubers and callus were weighed and 1 g each of tubers and callus were ground separately in a mortar and pestle with approximately 5 ml of absolute diethyl ether, each time The extracts were transferred to eppendorf

tubes These extracts were used for the estimation of essential oil The remaining extracts were stored in the refrigerator for further use

3 2 17 3 2 Extraction of triterpenoids

The clean tubers and callus were weighed and 1 g each of tubers and callus were ground separately in a mortar and pestle with approximately 5 ml of diethyl ether The ether extracted samples were ground with 5 ml of absolute methanol, each time The extract was centrifuged at 10,000 rpm for 3 min to separate the solid impurities The supernatant was collected in clean eppendorf tubes and used for the estimation of triterpenoids The remaining extract was stored in a refrigerator for further use

3 2 17 3 3 Extraction of sterols

The extraction procedure was same as that mentioned in 3 2 17 3 1 except that absolute methanol was used as extraction solvent.

3 2 17 3 4 Preparation of TLC plates

TLC plates were prepared by coating 300 μm thick layer of silica gel-G on glass plates of 20×20 cm Forty-nine g silica gel-G of SRL brand was weighed Approximately 50 ml of clean distilled water was added and the mixture was stirred to form a homogeneous slurry The slurry was then poured in the trough of the TLC plate gel applicator of CAMAG brand The plates were passed one by one beneath the trough and each was coated with a 300 μm thick coat of silica gel-G The plates were coated quickly, to avoid setting of the silica gel-G in the applicator itself The plates were allowed to dry for about 10 min , after which they were placed in aluminium racks and then kept in chromatographic oven for heating at 110°C for 30 min This desiccated the plates and activated them for further use

To separate the essential oil on the basis of number of double bonds present, $AgNO_3$ treated silica gel-G was used for preparation of TLC plates Here, 2 5 per cent $AgNO_3$ solution in water was added to silica gel-G, to make homogeneous slurry, instead of water The plates were coated as mentioned earlier

To separate the triterpenoids according to the number of isolated double bonds present in the molecule and to separate common sterols from their dihydro derivatives, argentative TLC was done For the preparation of argentative TLC plates, the silica gel-G was impregnated with $AgNO_3$ The method is described below

Forty-nine g of silica gel-G of SRL brand was weighed and added to 350 ml water The mixture was filtered through Whatman no 1 filter paper using Buchner's funnel The silica gel-G was washed thoroughly 2-3 times in distilled water during filtration A few drops of absolute alcohol were added at the end of filtration to ensure easy transfer of silica gel-G

After filtration, 9 ml of 1N $AgNO_3$ and 45-50 ml of water were added to the filtered silica gel-G and the mixture was stirred for 5 min in a stoppered flask After the gel formed a homogeneous slurry, it was used for coating the TLC plates The method of coating was same as that mentioned for normal TLC plate coating

3 2 17 3 5 Spotting on TLC plate

Spotting of sample on TLC plates was done with the help of capillary tubes The capillary tubes were calibrated to 5,10,15 and 20 µl as mentioned previously in 3 2 17 2 2

The amount of sample to be applied, and the spot diameter were standardised by spotting 5,10,15,20,30 and 40 µl of sample on TLC plates in the form of circular spots of various diameters ranging from 5 mm, 8 mm, 10 mm and 15 mm Spotting procedure was same as the one mentioned in 3 2 17 2 2 A distance of 1 5 cm was maintained between two successive spots

3 2 17 3 6 Running solvent systems

For the purpose of estimation of essential oil, methylene-di-chloride-chloroform-ethyl acetate-n-propanol (47 5 45 2 4 5) was used as the solvent system

For the estimation of triterpenoids, hexane-chloroform-methanol (1 3 1) was used as the solvent system

For the estimation of sterols, chloroform and chloroform-hexane (1 4) were used as the solvent system

The elution procedure was same as that mentioned in 3 2 17 2 3 The time required for elution of spots to 2/3rd the length of the plates was documented

3 2 17 3 7 Preparation of spray reagents

For the detection of essential oil, Vanillin-H_2SO_4 reagent was used The reagent was prepared in two solutions and sprayed separately, first to be sprayed was 5 per cent H_2SO_4 in ethanol followed by 3 per cent vanillin in ethanol

For the detection of triterpenoids and sterols, saturated solution of antimony chloride in chloroform was prepared

3 2 17 3 8 Developing the chromatograms

The eluted plates were placed under an exhaust flow of air to evaporate the solvents from the silica gel coat on the plate When the uncoated side of the plate became free of all moisture droplets, the plate was considered to be free of the running solvent The chromatogram was then uniformly sprayed with the spray reagent using a Vensil reagent sprayer in an exhaust chamber The sprayed plates were kept at 110°C for 5-10 min in a chromatographic oven to develop spots The plates were removed and observations were documented

3 2 17 3 9 Experiments conducted to standardise the running solvent system and spray reagents in the estimation of essential oil, triterpenoids and sterols

3 2 17 3 9 1 Standardisation of running solvent system

The composition of running solvent system is a crucial factor in the resolution of spots The standardisation of composition of running solvent system was done by using different components of the solvent system in various ratios The solvent systems tried for elution of each of, the essential oil, triterpenoids and sterols are listed in Table 3

3 2 17 3 9 2 Standardisation of spray reagent

The spray reagents evaluated for detecting essential oil, triterpenoids and sterols, and their method of preparation are given in Table 4

Table 3 Running solvent systems tried for their suitability to elute the essential oils, triterpenoids and sterols in the tubers and callus of *Holostemma ada-kodien* on thin layer chromatograms

Compound	Running solvent systems	Ratio of solvents
Essential oils	- Methylene di chloride-chloroform-ethyl acetate-n-propanol	45 45 4 5 4.5
	- Methylene di chloride-chloroform-ethyl acetate-n-propanol	47 5 45 2 4 5
Triterpenoids	- Hexane	
	- Chloroform	
	- Methanol	
	- Chloroform-methanol	10 1
	- Hexane-Chloroform-Methanol	1 1 1
	- Hexane-Chloroform-Methanol	1 1 1
	- Hexane-Chloroform-Methanol	1 1 1
	- Hexane-ethyl acetate	1 1
	- n-Butanol 2 M NH4OH	1 1
	- n-Butanol 2 M NH4OH	2 1
Sterols	- Hexane	
	- Chloroform	
	- Chloroform-methanol	1 1
	- Chloroform-methanol	3 4
	- Chloroform-methanol	4 1

3 2 17 3 10 Observations on essential oil, triterpenoids and sterols

The following observations were recorded for the extracts from tubers and callus

- RF values of spots

  The RF values of distinct spots were recorded for each compound

- Colour of spots

  The colour of spots in the case of essential oil, triterpenoids and sterols were recorded

Table 4 Spray reagents tried for their ability to detect essential oil, triterpenoids and sterols

Compound	Running solvent systems	Ratio of solvents
Essential oils	- Vanillin sulphuric acid	It was prepared in two solutions and sprayed separately one after other, each approximately 5 ml First was 5 per cent (v/v) sulphuric acid in ethanol and second was 4% (w/v) vanillin in ethanol
	- Antimony chloride	Saturated solution of antimony trichloride in chloroform
Triterpenoids	- Antimony chloride	Saturated solution of antimony trichloride in chloroform
	- P-anisaldehyde sulphuric acid	In 50 ml acetic acid 0 5 ml acetic acid was added 1 ml sulphuric acid was added to it at the time of spraying
	- Sulphuric acid ethanol	1 per cent sulphuric acid in ethanol
	- Phenol-Bromine	50 per cent phenol in carbon tetra chloride spray and then expose to bromine vapour
	- Ferric chloride (anhydrous)	Saturated solution of anhydrous ferric chloride in methanol
Sterols	- Water Bromine sulphuric acid	Expose to bromine vapour and then spray 50 per cent sulphuric acid in water
	- P-anisaldehyde sulphuric acid	In 50 ml acetic acid 0 5 ml acetic acid was added 1 ml sulphuric acid was added to it at the time of spraying
	- Water	Distilled water
	- Sulphuric acid	50 per cent sulphuric acid in water

Results

RESULTS

The results of the study on "Genetic transformation and hairy root cultures in Ada-kodien (*Holostemma ada-kodien* K Schum)" are presented in this chapter under four major heads viz

- Culturing of *Agrobacterium rhizogenes* strains
- Standardisation of transformation technique
- Confirmation of transformation
- Standardisation of biochemical techniques

4.1 Culturing of *A. rhizogenes* strains

4 1 1 Influence of culture media

The *A rhizogenes* strains differed in their growth on the two media tested The results are given in Table 5

Table 5 Influence of culture media on growth of *A rhizogenes* strains

A rhizogenes strains	Growth	
	LB medium	YEM medium
P_cA_4	+	+++
15834	++	+++
A_4	++	+++
8196	+++	+++
2659	+++	+++

+ Poor, ++ Good, +++ Very good

Both media favoured the growth of *A rhizogenes* strains In general, YEM medium was found to be the best for the growth of *A rhizogenes* strains under study The optimum growth temperature for all strains was 26 ± 2°C

4 1 2 Screening of *A rhizogenes* strains for antibiotic resistance

The response of *A rhizogenes* strains to ampicillin, kanamycin and cefotaxime is given in Table 6

Table 6 Effect of antibiotics on growth of *A rhizogenes* strains

A rhizogenes strains	Growth							
	Ampicillin				Kanamycin			
	100 mg l^{-1}	250 mg l^{-1}	500 mg l^{-1}	1000 mg l^{-1}	100 mg l^{-1}	250 mg l^{-1}	500 mg l^{-1}	1000 mg l^{-1}
P_cA_4	-	-	-	-	-	-	-	-
15834	-	-	-	-	+	+	+	+
A_4	-	-	-	-	+	+	+	+
8196	+	+	+	+	-	-	-	-
2659	-	-	-	-	+	+	+	+

-No growth, + Growth

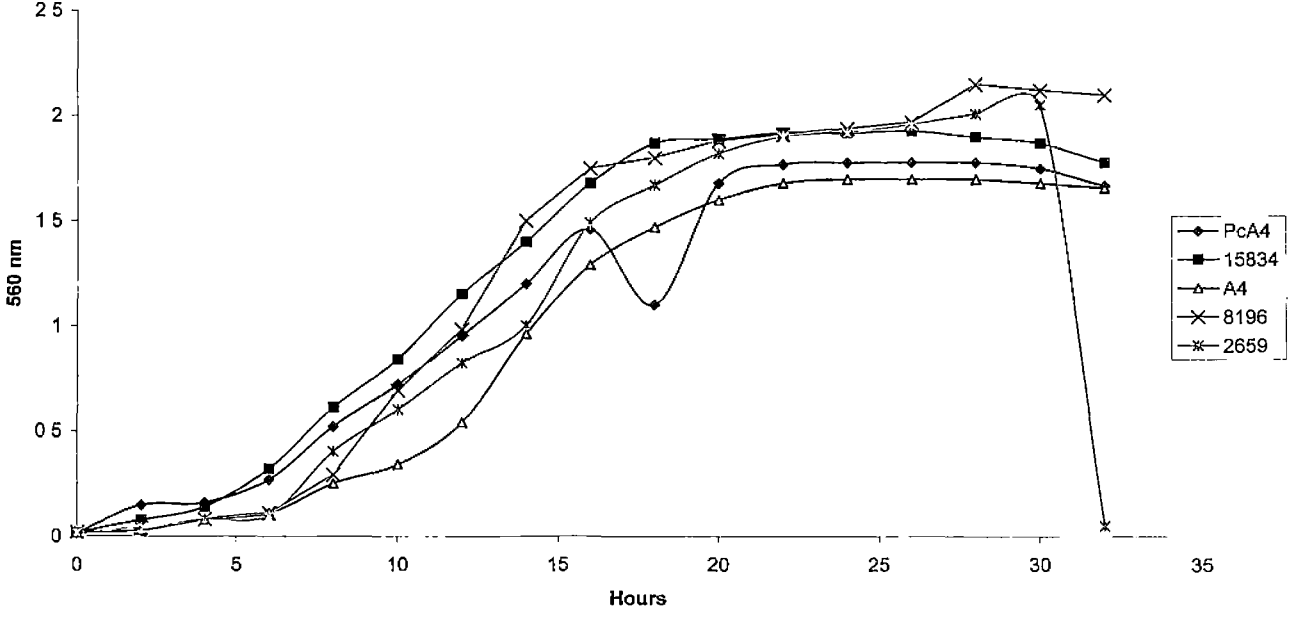
The strains 15834, A_4 and 2659 showed kanamycin resistance even at 1000 mg l^{-1} concentration The strain 8196 showed ampicillin resistance at all concentrations tested whereas the strain P_cA_4 did not show resistance to any of the antibiotics tested

4 1 3 Studying the growth curves of *A rhizogenes* strains

The growth curves of *A rhizogenes* strains are given in Fig 1

All the strains showed a typical sigmoid growth curve comprising of the lag phase, followed by the log phase, then the stationary or transition phase and finally decline in growth The time taken for each strain to attain the log phase differed slightly In general, all the strains showed a period of active growth between 8 and 20 h

Fig.1. Growth of *A. rhizogenes* strains in MS liquid medium (at 25±2°C)



4 1 4 Standardisation of optimum concentration of antibiotic

4 1 4 1 Plating method

The growth response of *A rhizogenes* strains on solid growth medium containing various concentrations of antibiotics is given in Table 7

Table 7 Effect of various concentrations of antibiotics on growth of *A rhizogenes* strains

A rhizogenes strains	Growth											
	Ampicillin						Cefotaxime					
	0 mg l^{-1}	50 mg l^{-1}	100 mg l^{-1}	250 mg l^{-1}	500 mg l^{-1}	1000 mg l^{-1}	0 mg l^{-1}	50 mg l^{-1}	100 mg l^{-1}	250 mg l^{-1}	500 mg l^{-1}	1000 mg l^{-1}
P_oA_4	++	+	-	-		-	++	+	-	-	-	-
15834	++	+	-	-	-	-	++	-	-	-	-	-
A_4	++	+	-	-	-	-	++	-	-	-	-	-
8196	++	++	++	++	++	++	++	+	-	-	-	-
2659	++	+	-	-	-	-	++	+	-	-	-	-

-No growth + Restricted growth ++ Good growth

All the strains were killed at 100 mg l^{-1} concentration of cefotaxime Strain 8196 showed ampicillin resistance whereas all other strains were killed at 100 mg l^{-1} concentration of ampicillin So optimum concentration of either ampicillin or cefotaxime to kill all the *A rhizogenes* strains under study is 100 mg l^{-1}

4 1 4 2 Optical density (OD) method

The response of various *A rhizogenes* strains to various concentrations of ampicillin and cefotaxime is given in Fig 2 and 3 The response of strain 8196 to ampicillin is given in Fig 4

Fig. 2. Effect of ampicillin on bacteria (*A. rhizogenes* strains 2659, A_4, PcA_4 and 15834

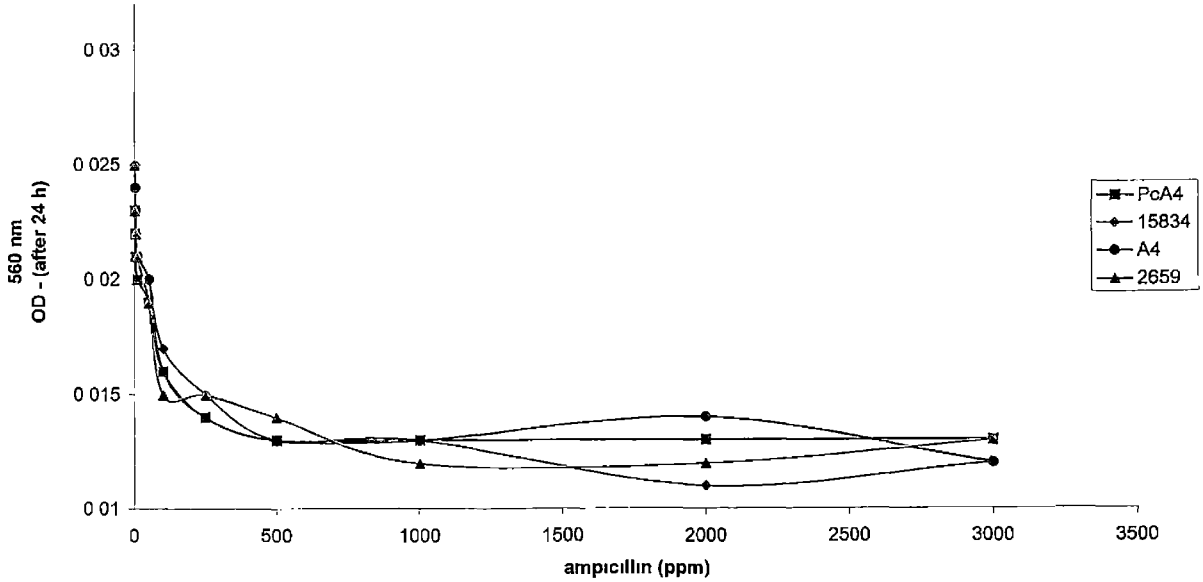


Fig.3. Effect of cefotaxime on bacteria (*A. rhizogenes* strains 8196, 2659, A_4, 15839 and PcA_4)

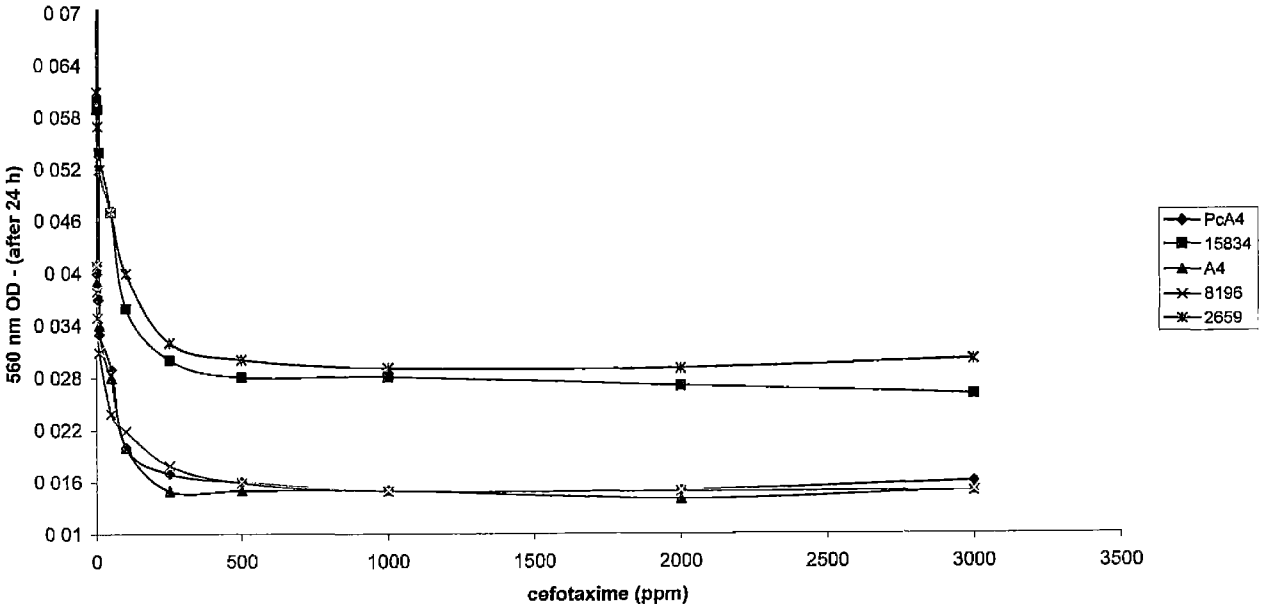
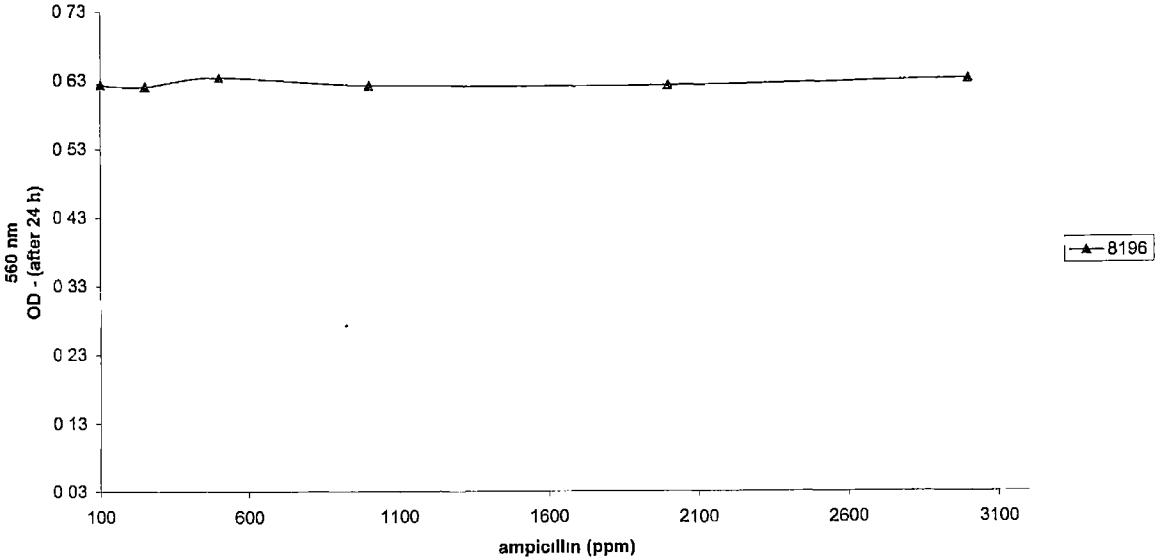


Fig. 4. Effect of ampicillin on bacteria (A. rhizogenes strain 8196)



The lowest OD values obtained for all the *A rhizogenes* strains for cefotaxime were at 250 mg l^{-1} concentration and any further increase in the concentration of antibiotic did not show significant decrease in the OD value The same was the trend with ampicillin except that strain 8196 showed ampicillin resistance even up to 3000 mg l^{-1} concentration

The optimum concentration of antibiotic to kill the bacteria was 250 mg l^{-1} according to the OD method

4 1 5 Effect of plant extract of *Holostemma* on growth of *A rhizogenes* strains

The response of *A rhizogenes* strains to plant extract of *Holostemma* is given in Plate IIIa In general the plant extract favoured the growth of *A rhizogenes* strains under study, when compared to control (Plate IIIb)

The strains grew very fast on the medium containing plant extract of *Holostemma* as compared to the control

4 1 6 Culturing of infected explants

The infected explants were cultured on one per cent agar in water and on solid MS medium The bacterial growth was seen within 24 h on both the media

4 1 7 Elimination of bacteria from explant tissues

The infected explants, cultured on one per cent agar or MS solid medium were sub-cultured on MS solid medium containing antibiotics (250 mg l^{-1} cefotaxime or ampicillin) In both the cases the bacteria could not be eliminated in the first subculture on antibiotic medium The bacteria were effectively eliminated on second and or third subculture on same antibiotics Among the various explants, the elimination of bacteria from callus needed 4 to 6 subcultures on MS solid media containing 250 mg l^{-1} cefotaxime or ampicillin To reduce the number of subcultures, the infected calli were cultured on 1 per

cent agar or MS solid medium for 24 h The calli were then placed in liquid MS medium containing 250 mg l^{-1} cefotaxime or ampicillin for 24 h Then the calli were sub-cultured on MS solid medium containing antibiotic The bacteria could be eliminated on the first or second subculture of callus on solid MS medium containing antibiotic

For the elimination of strain 8196, cefotaxime was used at 250 mg l^{-1} concentration as it showed ampicillin resistance All the other strains could be eliminated using either cefotaxime or ampicillin at 250 mg l^{-1} concentration

4 1 8 Effect of various concentrations of antibiotics on explants

The effect of various concentrations of cefotaxime and ampicillin on different explants under study is given in Table 8

Cefotaxime showed auxin like effects at concentrations 500 mg l^{-1} and above All the explants showed callusing above 500 mg l^{-1} concentration of cefotaxime (Plates Ia to Id) Ampicillin showed auxin like effects only at 200 mg l^{-1} and above Also the effects were not as pronounced as that in the case of cefotaxime

4.2 Standardisation of transformation technique

4 2 1 Standardisation of inoculation method

4 2 1 1 Direct inoculation method

4 2 1 1 1 Influence of bacterial inoculum

The bacterial inoculum in the form of bacterial cells in a single cell colony and the bacterial suspension (OD_{560nm}^{-1}) used for infection, varied in their response to transformation The results are given in Table 9

Plate I Effect of different concentrations of cefotaxime on explants

Ia Effect of 500 mg l^{1} concentration of cefotaxime on explants

Ib Effect of 1000 mg l^{-1} concentration of cefotaxime on explants

Ic Effect of 2000 mg l^{1} concentration of cefotaxime on explants

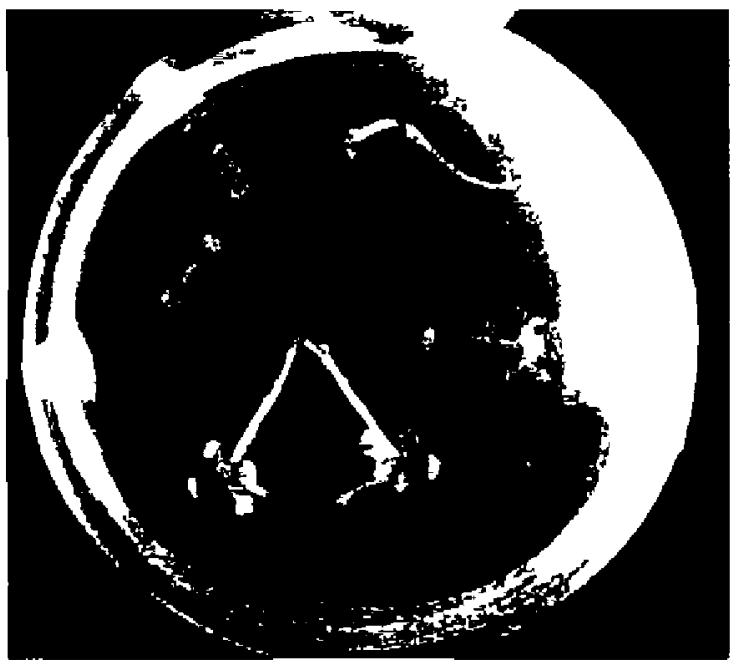
Id Effect of 4000 mg l^{1} concentration of cefotaxime on explants

Plate I

Ia

Ib

Ic



Id

Table 8 Response of different explants to various concentrations of cefotaxime and ampicillin

Explant	Explant response											
	Cefotaxime concentration (mg l^{-1})						Ampicillin concentration (mg l^{-1})					
	250	500	1000	2000	4000	Control	250	500	1000	2000	4000	Control
Leaf	No callusing	No callusing	Slight callusing	Slight callusing	Good callusing	No callusing	No callusing	No callusing	No callusing	No callusing	No callusing	No callusing
Internodal segment	Slight callusing	Slight callusing	Good callusing	Slight callusing	Slight callusing	No callusing	No callusing	No callusing	No callusing	Slight callusing	Slight callusing	No callusing
Callus	No callus growth	No callus growth	Slight callus growth	Good callus growth	Slight callus growth	No callus growth	No callus growth	No callus growth	No callus growth	Slight callus growth	Slight callus growth	No callus growth
Shoot bud	Slight callusing	Slight callusing	Good callusing	Good callusing	Slight callusing	Slight callusing	No callusing	No callusing	No callusing	Slight callusing	Slight callusing	Slight callusing
Seedling hypocotyls	No callusing	Slight callusing	Good callusing	Slight callusing	Slight callusing	No callusing	No callusing	No callusing	No callusing	Slight callusing	Slight callusing	No callusing

Table 9 Influence of bacterial inoculum on transformation of different explants

Bacterial inoculum	Percentage transformation				
	Leaf	Shoot bud	Internodal segment	Seedling hypocotyls	Callus
Cells from single cell colonies	-	-	-	-	-
Bacterial suspension (OD_{560nm}^{-1})	-	-	-	7 14	-

The bacterial cells from single cell colonies when used for infection did not show any transformation The bacterial suspension ($OD_{560\,nm}^{-1}$) when used for infection gave a transformation percentage of 7 14

Among the various explants used, only the seedling hypocotyls responded to transformation Similarly, the different *A rhizogenes* strains used for infection differed in their transformation ability The results are given in Fig 5 The strain P_cA_4 gave highest transformation (23 05%) followed by 15834 (14 28%) and A_4 (13 34%) In the case of leaf, shoot buds and internodal segments, wiping of wound exudates before infection or vice versa did not have any effect on transformation of these explants All the explants did not show any transformation

4 2 1 1 2 Influence of amount of bacterial inoculum applied per wound

The amount of bacterial inoculum applied per wound, influenced the transformation The results are given in Table 10

Less amount of bacterial inoculum gave a higher transformation percentage (18 42%) than the higher amount of bacterial inoculum (15 21%) applied per wound Among the different explants used, the seedling hypocotyls responded to transformation when infected with either less or more amount of bacterial inoculum

Fig 5 Ability of different *A. rhizogenes* strains to transform seedling hypocotyls

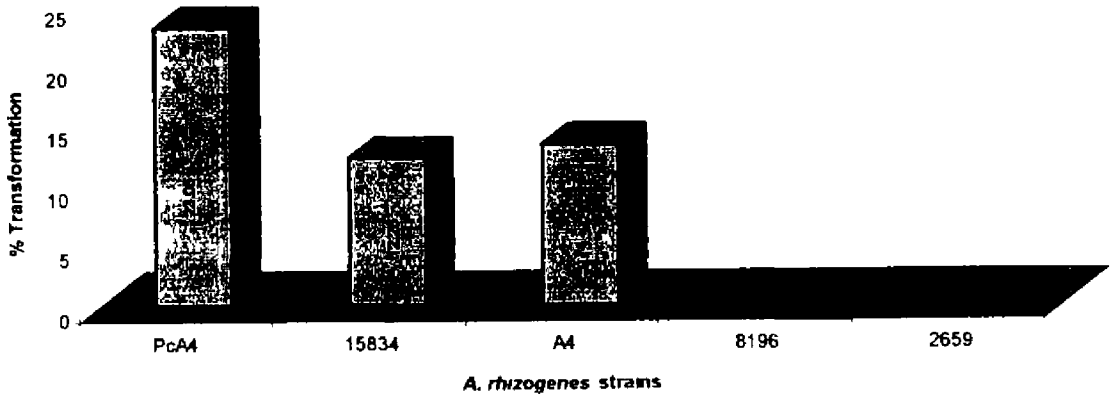


Fig 6 Transformation percentage of seedling hypocotyls using different amounts of bacterial inoculum

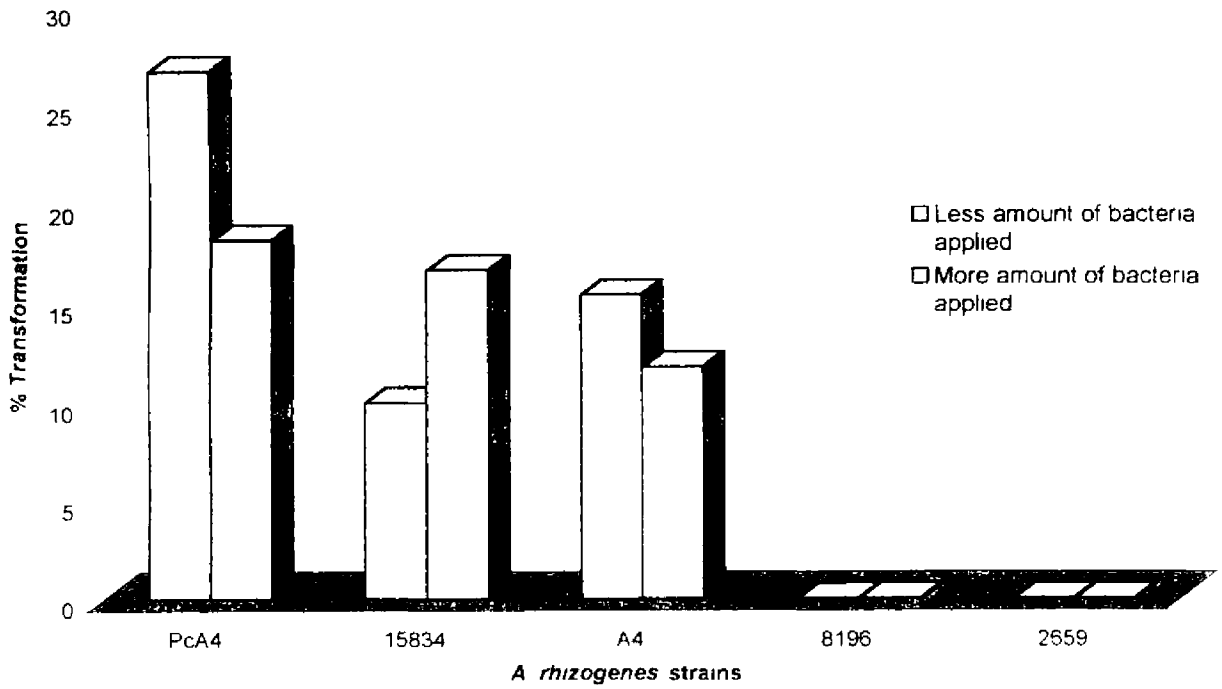


Table 10 Influence of amount of bacterial inoculum applied per wound

Bacterial inoculum	Percentage transformation				
	Explants				
	Leaf	Shoot bud	Internodal segment	Seedling hypocotyls	Callus
Less	-	-	-	18 42	-
More	-	-	-	15 21	-

Less- Needle dipped in bacterial suspension/single cell colony before making a wound

More- A drop of bacterial suspension ($OD_{560\ nm}^{1}$) applied on each individual wound/bacterial cells from single cell colony smeared on each wound

- No transformation

The different *A rhizogenes* strains under study, differed in their transformation ability The transformation percentage obtained by infecting seedling hypocotyls with a particular *A rhizogenes* strain differed when variable amount of bacterial inoculum was used The results are given in Fig 6

The strain P_cA_4 showed the highest transformation percentage (26 66 and 18 18) irrespective of the amount of bacterial inoculum applied, followed by the strain 15834 (10% and 16 66%) and A_4 (15 38% and 11 76%) In the case of strain 15834, higher transformation percentage (16 66) was obtained when more amount of bacterial inoculum was applied, when compared to less amount of bacterial inoculum (10 00%)

In the case of shoot buds, leaf and internodal segments, wiping of wound exudates prior to infection or vice versa did not show any effect on transformation All the explants did not show any transformation

4 2 1 1 3 Effect of NAA applied on wounds

The auxin (NAA 1, 2 and 3 ppm) when applied on wounds before and after infection with different *A rhizogenes* strains did not show any positive

effect on transformation of *Holostemma* explants All the explants *viz* leaf, internodal segment, shoot buds and callus did not show any transformation

Similarly, the explants when soaked with NAA solution (1,2 and 3 ppm) before and after infection, did not show any transformation of the explants viz leaf, shoot buds, internodal segments and callus

Callus was induced at the wound sites in the case of all explants under studies upon NAA treatment Callus induction was observed both in the case of infected explants and non-infected explants (control) However, no rooting was observed in any of the explants from the wounds

In the case of shoot buds, leaf and internodal segments, wiping of latex prior to infection or vice versa and NAA treatment did not show any effect on transformation All the explants did not show any transformation Similarly, irrespective of latex wiping all these explants induced callus from wounds on NAA treatment

4 2 1 2 Co-culture method

4 2 1 2 1 Influence of bacterial population and co-culture time on transformation

The amount of bacteria present during co-culture and the co-culture time influenced transformation of the different explants studies The different *A rhizogenes* strains also differed in their transformation ability The results are given in Table 11a and 11b

Among the different explants tried, the shoot buds and seedling hypocotyls responded to transformation Only seedling hypocotyls responded when less amount of bacterial cells were present at the time of co-culture The transformation ability varied among the different bacterial strains used and the co-culture time The results are given in Fig 7a and 7b

A greater response of seedling hypocotyls to induce hairy roots was seen when they were cultured for 24 h, when compared to 36 h of co-culture The seedlmg hypocotyls responded to strains P_cA_4 and 15834 when co-cultured for 24 h or 36 h while they responded to strain A_4 when co-cultured for 24 h The highest transformation percentage (22 22%) was obtained when the hypocotyls were co-cultured with strain P_cA_4 for 24 h followed by 15834 (14 28%) and A_4 (13 63%) (also cultured for 24 h) (Fig 7a and 7b)

Comparative evaluation of the varying intensity of bacterial population and the co-culture time showed that both, less intensity of bacterial population coupled with greater co-culture time and vice versa showed similar transformation frequencies (14 40% and 14 51% respectively)

On comparison of the relative transformation percentage of individual bacterial strains in both the methods, the strains P_cA_4 and 15834 showed almost same transformation frequency (Table 11a and 11b) In the case of A_4 strain, higher transformation percentage was obtained when seedling hypocotyls and shoot bud were co-cultured for 24 and 36 h with less bacterial population

The other explants *viz* leaf internodal segments and callus showed callusmg at wound sites (plates II) The callus grew further in sub-cultures but no root induction was observed The callus when cultured in liquid medium gave cell suspensions within a period of 15 days

The number of days taken for hairy root induction differed depending upon the method of inoculation used In general, the direct inoculation method induced hairy roots in 6 to 8 days while co-culture method induced hairy roots in 12 to 14 days (Table 12)

Fig 7a. Response of seedling hypocotyls to various strains of *A. rhizogenes* with 24 h co-culture

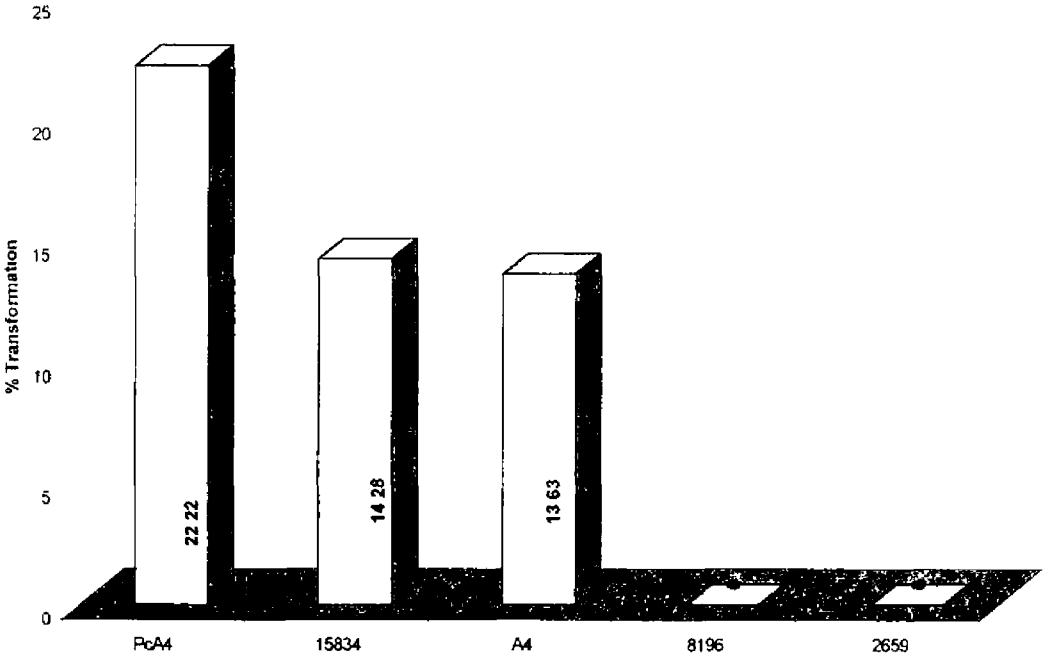


Fig 7b Response of seedling hypocotyls to various strains of *A rhizogenes* with 36 h co-culture

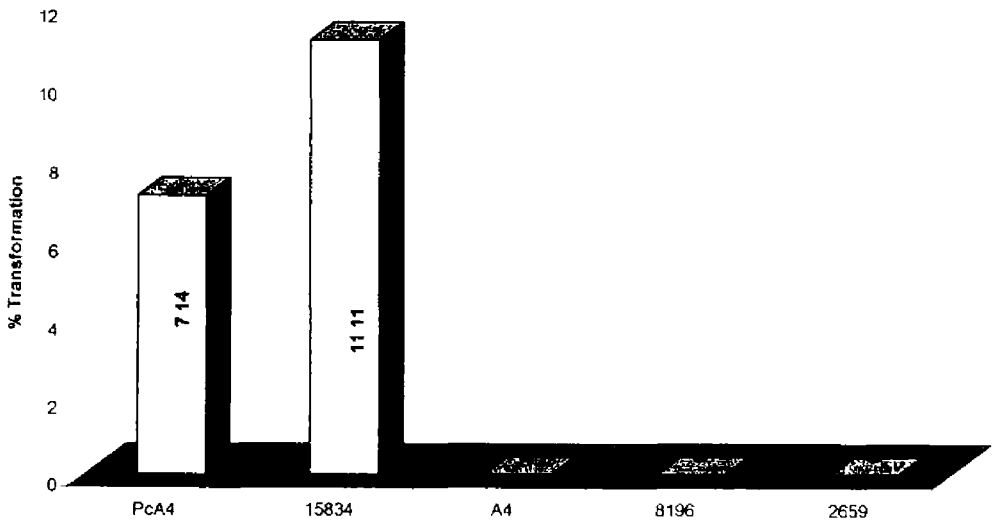


Fig 8a Response of seedling hypocotyls to various strains of *A rhizogenes* with co-culture at 50 rpm and 100 rpm

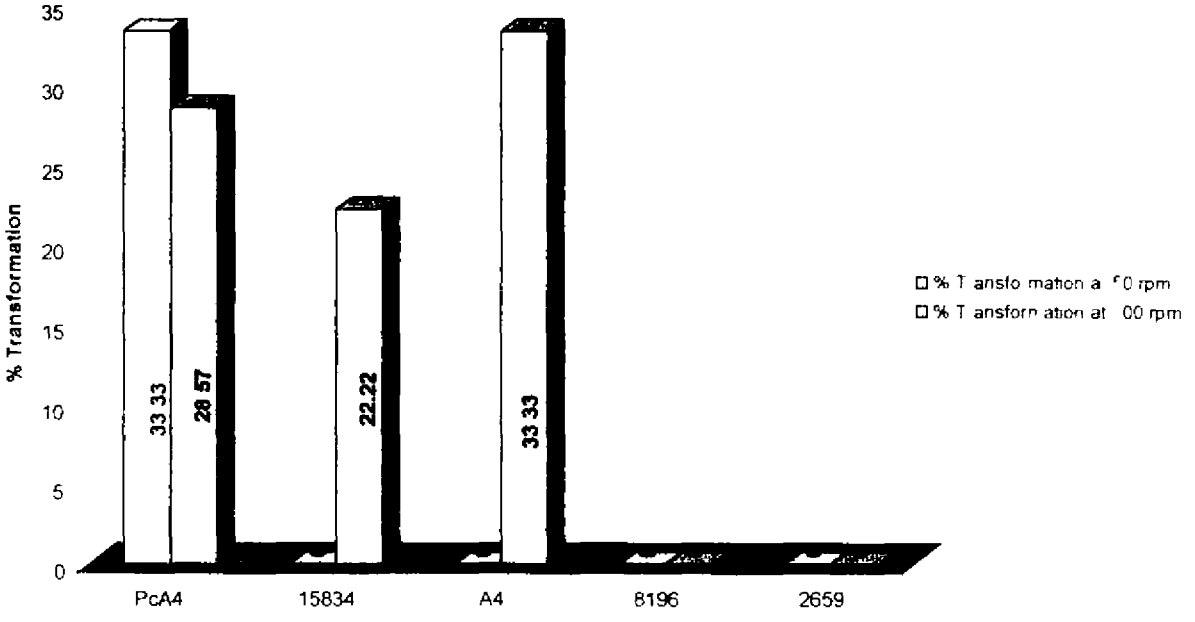


Fig 8b Response of shoot buds to various strains of *A rhizogenes* with co-culture at 50 rpm and 100 rpm

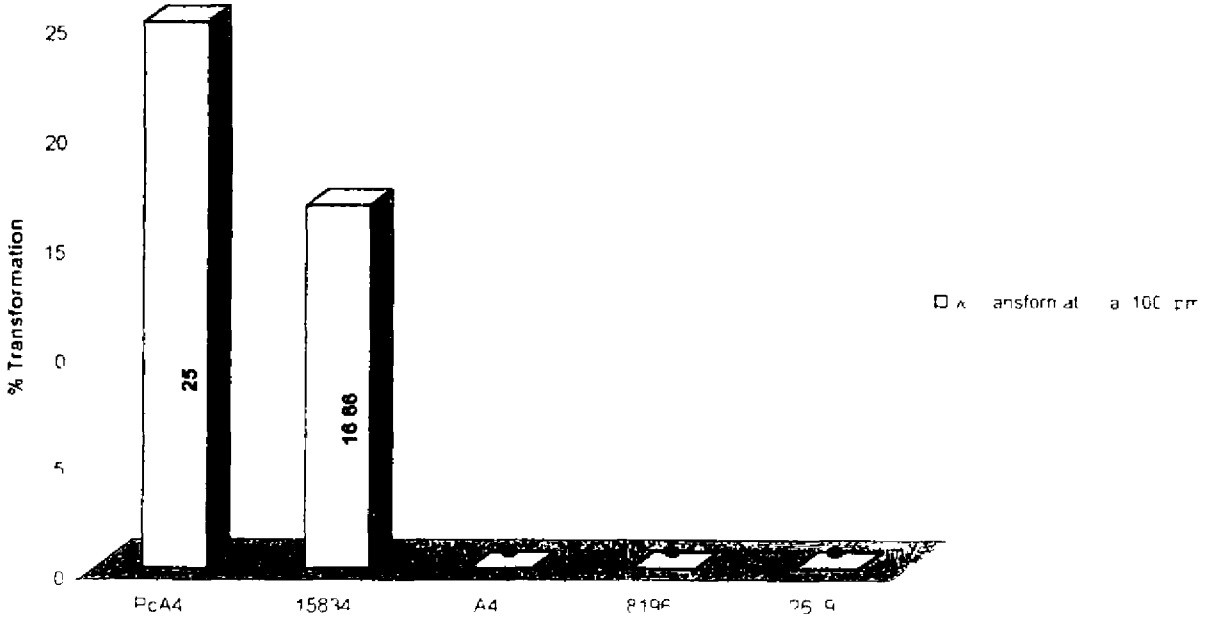


Table 11a Influence of less bacterial population and co-culture time on transformation

A rhizogenes strain	Explant	Photo-period	Co-culture time (h)									
			12		24		36		48		72	
			% transformation	Wound response	% transformation	Wound response	% transformation	Wound response	% transformation	Wound response	% transformation	Wound response
P_cA_4	Leaf	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Less callus	0 00	Less callus	0 00	Less callus	0 00	Less callus	0 00	Less callus
	SB	L	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots
		D	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots
	IS	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SH	L	0 00	Roots	25 00	Roots	7 14	Roots	0 00	Roots	0 00	Roots
		D	0 00	Roots	16 66	Roots	0 00	Roots	0 00	Roots	0 00	Roots
	C	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
15834	Leaf	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Less callus	0 00	Less callus	0 00	Less callus	0 00	Less callus	0 00	Less callus
	SB	L	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots
		D	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots
	IS	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus

Table 11a Contd

		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SH	L	0 00	Roots	15 38	Roots	11 11	Roots	0 00	Roots	0 00	Roots
		D	0 00	Roots	12 50	Roots	0 00	Roots	0 00	Roots	0 00	Roots
	C	L	0 00	More callus	0 00	More callus	0 00	More callus	0 00	More callus	0 00	More callus
		D	0 00	Less callus	0 00	Less callus	0 00	Less callus	0 00	Less callus	0 00	Less callus
A_4	Leaf	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SB	L	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots
		D	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots
	IS	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SH	L	0 00	Roots	20 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots
		D	0 00	Roots	8 33	Roots	0 00	Roots	0 00	Roots	0 00	Roots
	C	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
8196	Leaf	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SB	L	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots
		D	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots
	IS	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SH	L	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots
		D	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots

Table 11a Contd

	C	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
2659	Leaf	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SB	L	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots
		D	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots
	IS	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SH	L	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots
		D	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots
	C	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
Control	Leaf	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SB	L	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots
		D	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots
	IS	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SH	L	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots
		D	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots
	C	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus

Table 11b Influence of more bacterial population and co-culture time on transformation

A rhizogenes strain	Explant	Photo-period	Co-culture time (min)											
			5		10		15		30		60		120	
			% T	WR	% T	WR	% T	WR	% T	WR	% T	WR	% T	WR
P_cA_4	Leaf	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SB	L	0 00	Callus& Roots	12 50	Callus& Roots	0 00	Callus& Roots	18 18	Callus& Roots	0 00	Callus &Roots	14 28	Callus& Roots
		D	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus &Roots	0 00	Callus& Roots
	IS	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SH	L	0 00	Roots	17 64	Roots	0 00	Roots	18 75	Roots	8 33	Roots	0 00	Roots
		D	0 00	Roots	20 00	Roots	0 00	Roots	14 28	Roots	25 00	Roots	0 00	Roots
	C	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
15834	Leaf	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SB	L	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	18 18	Callus& Roots	0 00	Callus &Roots	0 00	Callus& Roots
		D	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus &Roots	0 00	Callus& Roots
	IS	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SH	L	0 00	Roots	0 00	Roots	0 00	Roots	11 76	Roots	0 00	Roots	0 00	Roots
		D	0 00	Roots	0 00	Roots	0 00	Roots	8 33	Roots	0 00	Roots	0 00	Roots
	C	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus

Table 11b Contd

		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
A_4	Leaf	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SB	L	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus &Roots	10 00	Callus& Roots
		D	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus &Roots	0 00	Callus& Roots
	IS	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SH	L	0 00	Roots	0 00	Roots	0 00	Roots	12 50	Roots	0 00	Roots	0 00	Roots
		D	0 00	Roots	0 00	Roots	0 00	Roots	10 00	Roots	0 00	Roots	0 00	Roots
	C	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
8196	Leaf	L	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus &Roots	0 00	Callus& Roots
		D	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus &Roots	0 00	Callus& Roots
	SB	L	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus &Roots	0 00	Callus& Roots
		D	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus &Roots	0 00	Callus& Roots
	IS	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SH	L	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots
		D	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots
	C	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus

Table 11b Contd

2659	Leaf	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SB	L	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus &Roots	0 00	Callus& Roots
		D	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus& Roots	0 00	Callus &Roots	0 00	Callus& Roots
	IS	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
	SH	L	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots
		D	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots	0 00	Roots
	C	L	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus
		D	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus	0 00	Callus

Table 12 Number of days taken for hairy root induction

Inoculation method	Explant	*A rhizogenes* strain	No of days taken for root induction	No of roots per wound
Direct Inoculation	Seedling hypocotyls	P_cA_4	8-25	1
		15834	5-21	1
		A_4	11-26	1
Co-culture method 24 h	Seedling hypocotyls	P_cA_4	14 19	1
		15834	12 16	1
		A_4	12 19	1
36 h	Seedling hypocotyls	P_cA_4	16 19	1
		15834	14 14	1
5 min	Seedling hypocotyls	P_cA_4	15 24	1
	Shoot bud	P_cA_4	16 30	1
30 min	Seedling hypocotyls	P_cA_4	18 26	1
	Shoot bud		21 23	1
	Seedling hypocotyls	15834	15 23	1
	Shoot bud		20 24	1
	Seedling hypocotyls	A_4	19 26	1
60 min	Seedling hypocotyls	P_cA_4	15 22	1
120 min	Shoot bud	P_cA_4	14 28	1
	Shoot bud	A_4	17 19	1

4 2 1 2 2 Standardisation of shaker speed (rpm) during co-culture

The explants differed in their ability to produce roots when co-cultured at different shaker speeds Among the explants, only the seedling hypocotyls and shoot buds gave hairy roots The different *A rhizogenes* strains used during co-culture exhibited different effect on the percentage of seedling hypocotyls and shoot buds generating transformed roots The comparison is given in Fig 8a and 8b

Plate II Callus induction in leaf explants

IIa Leaf explants infected with *A rhizogenes* strain P_cA_4

IIb Leaf explants infected with *A rhizogenes* strain 15834

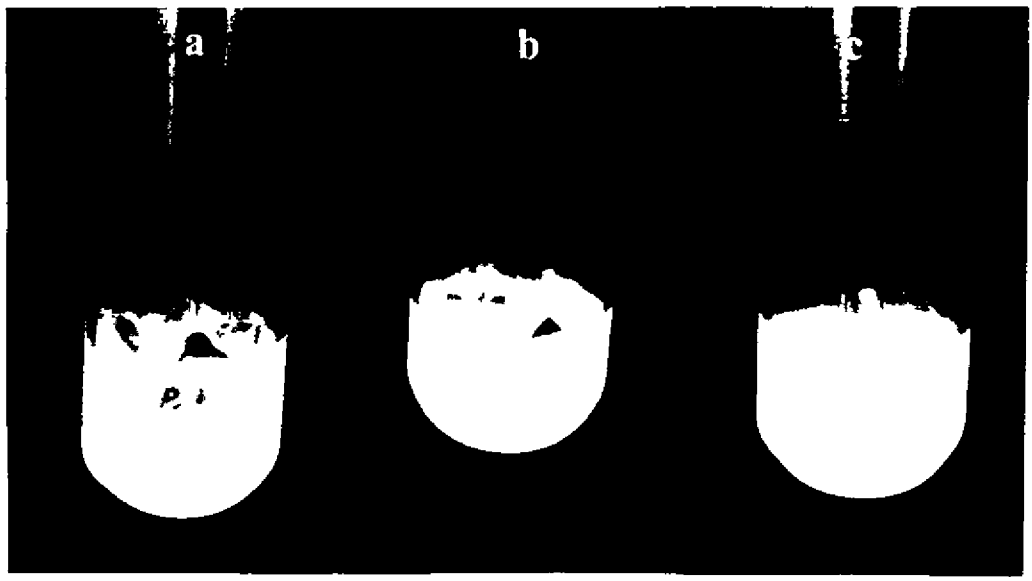
IIc Leaf explants infected with *A rhizogenes* strain 8196

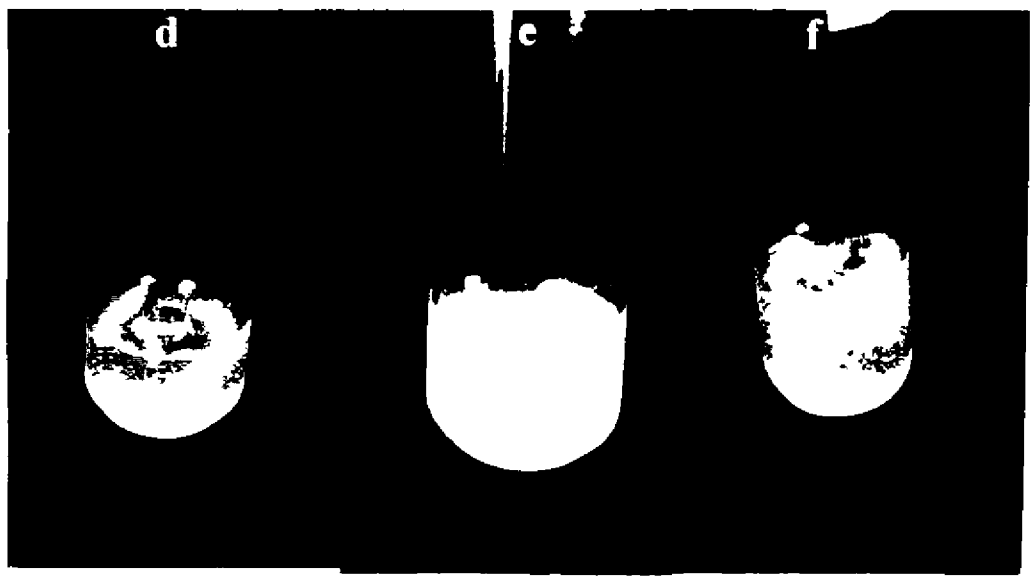
IId Leaf explants infected with *A rhizogenes* strain A_4

IIe Leaf explants infected with *A rhizogenes* strain 2659

IIf Non infected leaf explants

Plate II





At 50 rpm the seedling hypocotyls produced hairy roots when co-cultured with strain P_cA_4 At 100 rpm, the shoot buds and seedlings produced hairy roots when co-cultured with strains P_cA_4 and 15834 The strain A_4 when co-cultured with seedling hypocotyls at 100 rpm also gave hairy roots In general, at 100 rpm more number of explants gave hairy roots

Among the other explants, callus readily formed cell suspension cultures at 100 and 150 rpm The leaf and internodal segments gave callus at wound sites when cultured on solid MS medium The control explants also formed callus at the wound sites

4 2 1 2 3 Influence of NAA during co-culture on transformation

The explants differed in their ability to produce hairy roots when co-cultured with various strains of *A rhizogenes* in MS liquid medium containing various concentrations of NAA The results are given in Table 13

Among the different explants, the seedling hypocotyls responded to transformation The highest transformation percentage (75%) was obtained when seedling hypocotyls were co-cultured with strain P_cA_4 in MS liquid medium containing 2 mg l^{-1} NAA All the other explants gave friable callus when cultured on solid MS medium The callus grew very fast in subsequent subcultures on MS basal solid medium The callus was obtained from control explants also

The callus obtained from seedling hypocotyls was cultured in liquid MS medium in dark at 100 rpm It gave thin yellowish roots after four weeks of culture (Plate VIId) These roots were positive to the presence of opines The callus obtained from all other explants when cultured in liquid MS medium in dark gave very good cell suspension but did not produce roots The callus irrespective of the explant and bacterial strain, when cultured in liquid MS medium in day/night regime (16 h photoperiod) failed to produce roots

Table 13 Response of different explants when co-cultured with different *A rhizogenes* strains in liquid MS medium containing NAA

A rhizogenes	Explant	NAA content								
		1 mg l^{-1}			2 mg l^{-1}			3 mg l^{-1}		
		Wound response	% transformation		Wound response	% transformation		Wound response	% transformation	
			Light	Dark		Light	Dark		Light	Dark
P_cA_4	Leaf	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	IS	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	Callus	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	SB	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	SH	Callus	0 00	66 66	Callus	0 00	75 0	Callus	0 00	0 00
15834	Leaf	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	IS	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	Callus	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	SB	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	SH	Callus	0 00	0 00	Callus	0 00	20 0	Callus	0 00	0 00
A_4	Leaf	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	IS	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	Callus	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	SB	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	SH	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
8196	Leaf	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	IS	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	Callus	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	SB	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	SH	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
2659	Leaf	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	IS	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	Callus	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	SB	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00
	SH	Callus	0 00	0 00	Callus	0 00	0 00	Callus	0 00	0 00

4 2 4 Effect of media on hairy root induction

Among the different media tried for culturing the infected explants, the hairy roots were induced only on the solid MS medium (Table11a and 11b) The explants did not induce hairy roots on any other media used

4 2 5 Effect of photoperiod on hairy root induction

The effect of photoperiod on transformation of explants by different strains of *A rhizogenes* using direct or co-culture method of infection is given in Fig 9a, 9b and 9c, respectively

In the direct infection method the strain P_c A_4 gave the highest transformation percentage (25%) when cultured in 16 h photoperiod The strain 15834 gave greater percentage transformation (16 66%) when cultured in the dark The while the strain A_4 gave greater percentage transformation (18 18%) when cultured in dark

In the co-culture method the shoot buds produced hairy roots when cultured at photoperiod of 16 h whereas the hairy root production was inhibited in the dark The strain 15834 gave the highest transformation percentage (17 64%) followed by P_cA_4 (15 15%) and A_4 (10%)

The seedling hypocotyls produced hairy roots under both the dark and light The highest transformation percentage (31 03) was obtained when seedling hypocotyls were co-cultured with strain P_cA_4 in dark In the case of strains 15834 and A_4 greater transformation percentage was observed when seedling hypocotyls were cultured in light

In general, light favoured the hairy root formation by shoot buds and seedling hypocotyls irrespective of the strain and the method of infection followed

Fig 9a Effect of photoperiod on transformation of seedling hypocotyls on infection with different *A rhizogenes* strains by direct method

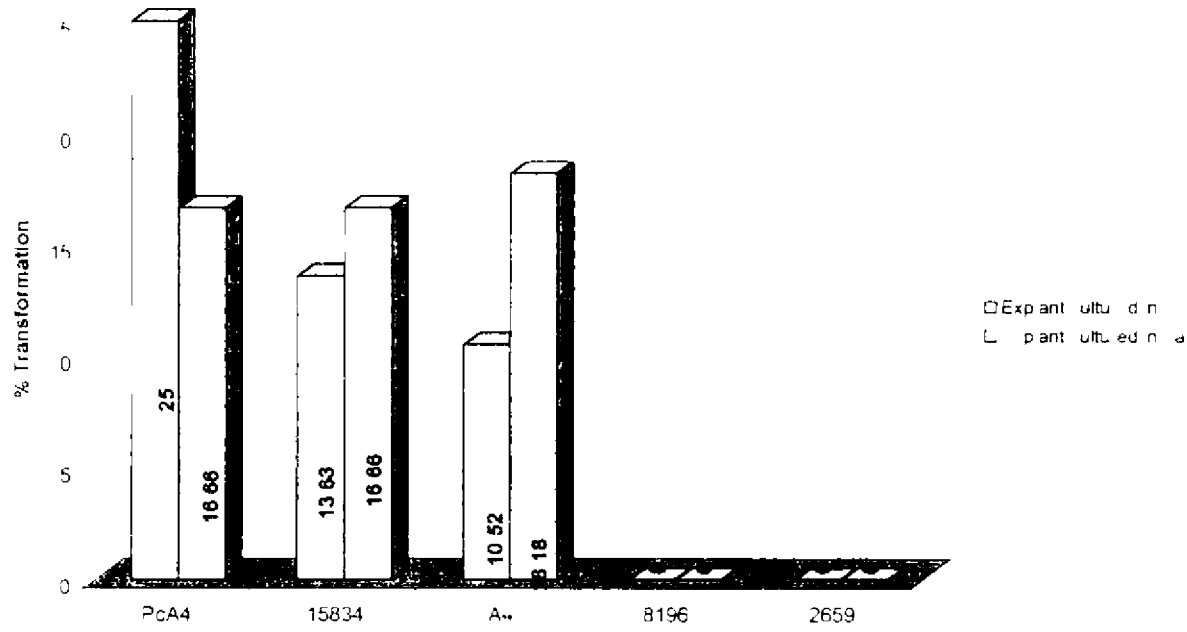


Fig 9b Effect of photoperiod on transformation of seedling hypocotyls on infection with different *A rhizogenes* strains by co-culture method

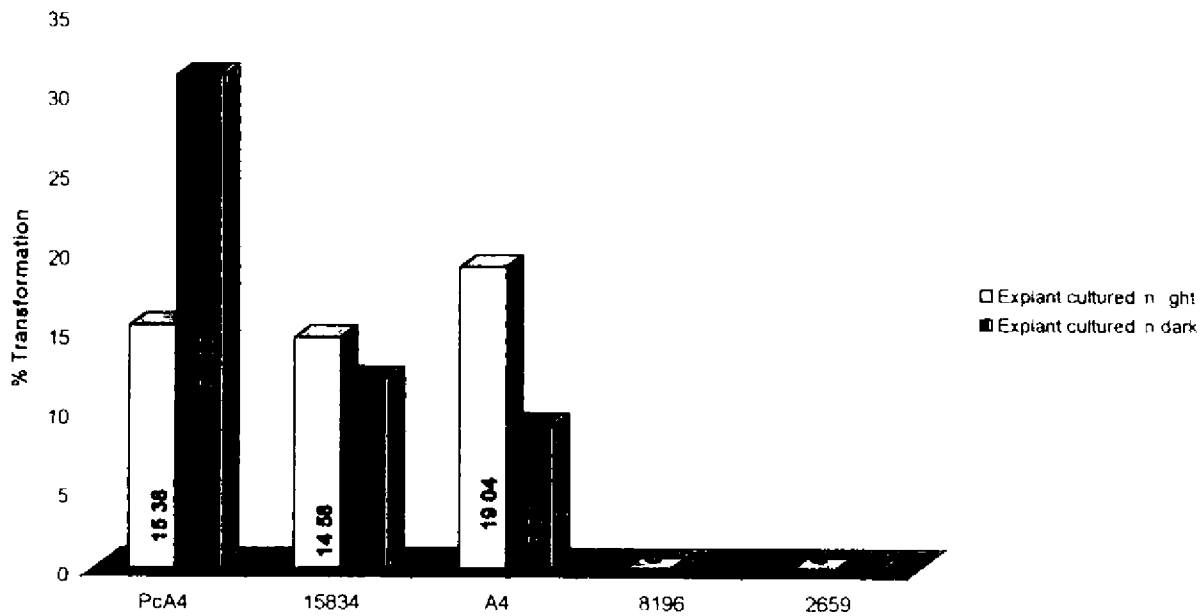
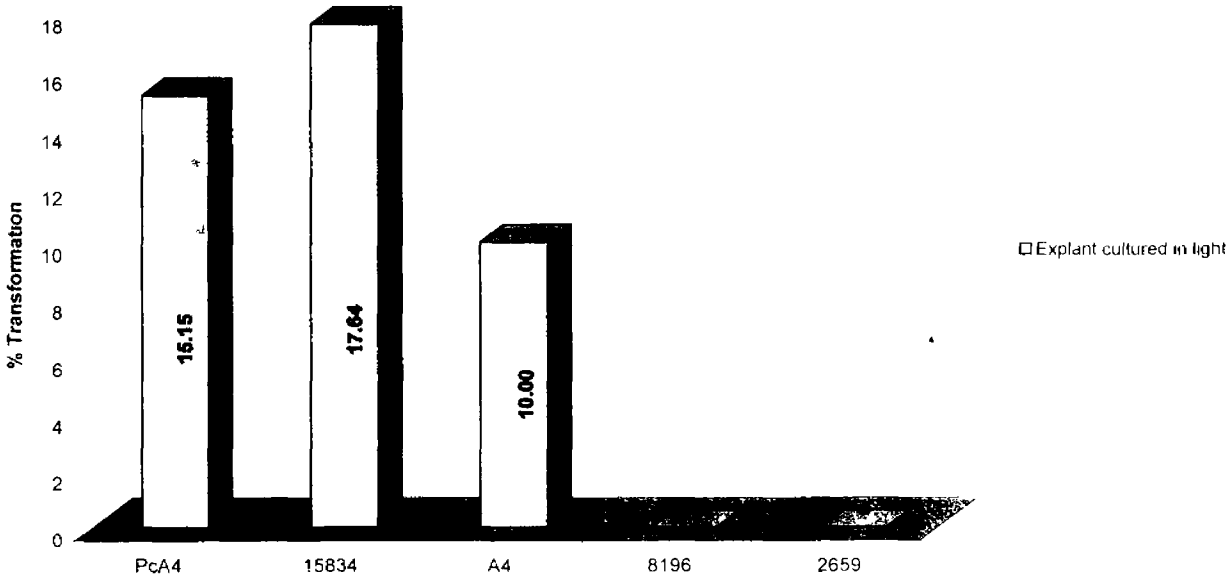


Fig. 9c. Effect of photoperiod on transformation of shoot buds on infection with different *A rhizogenes* strains by co-culture method



4.3 Confirmation of transformation

4 3 1 Opine detection

The response of normal roots and transformed roots to the presence of opines is given in Plate IV Opines were detected in the transformed roots The transformed roots obtained from P_cA_4, 15834 and A_4 strains of *A rhizogenes* showed the presence of agropine in the form of a blackish spot 6 cm from the origin None of the transformed roots showed the presence of mannopine

The normal roots or the bacterial strains did not show the presence of opines The callus formed at the wound sites after infection also did not show presence of any opine

The profile of opines detected in various tissues on infection with different strains of *A rhizogenes* is given in Table 14

Table 14 Response of transformed and non-transformed tissues to the presence of opines

Tissue/strain	Opine detected
Transformed root – 15834	Agropine
Transformed root – P_cA_4	Agropine
Transformed root – A_4	Agropine
Non-transformed root	–
Infected callus	–
Non-infected callus	–
Strain P_cA_4	–
Strain 15834	–
Strain A_4	–

4 3 1 1 Standardisation of quantity of sample and sample concentration on visibility to opines

The quantity of sample applied, and the sample concentration influenced the visibility of opines The results are given in Table 15

Plate III Effect of plant extracts on growth of *A rhizogenes* strains

IIIa On YEM medium

IIIb On YEM medium supplemented with plant extract

Plate IV Electrophorogram showing presence of opines

Lane 1 Standard agropine and mannopine

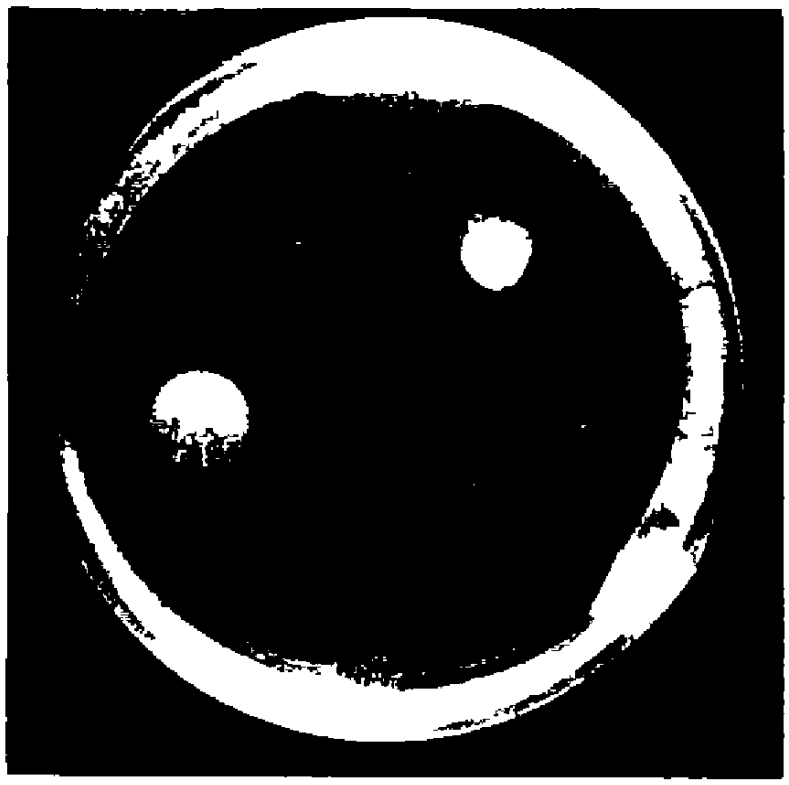
Lane 2 Hairy root induced by strain P_CA_4

Lane 3 Hairy root induced by strain 15834

Lane 4 Hairy root induced by strain A_4

Lane 5 Normal roots

IIIa



IIIb

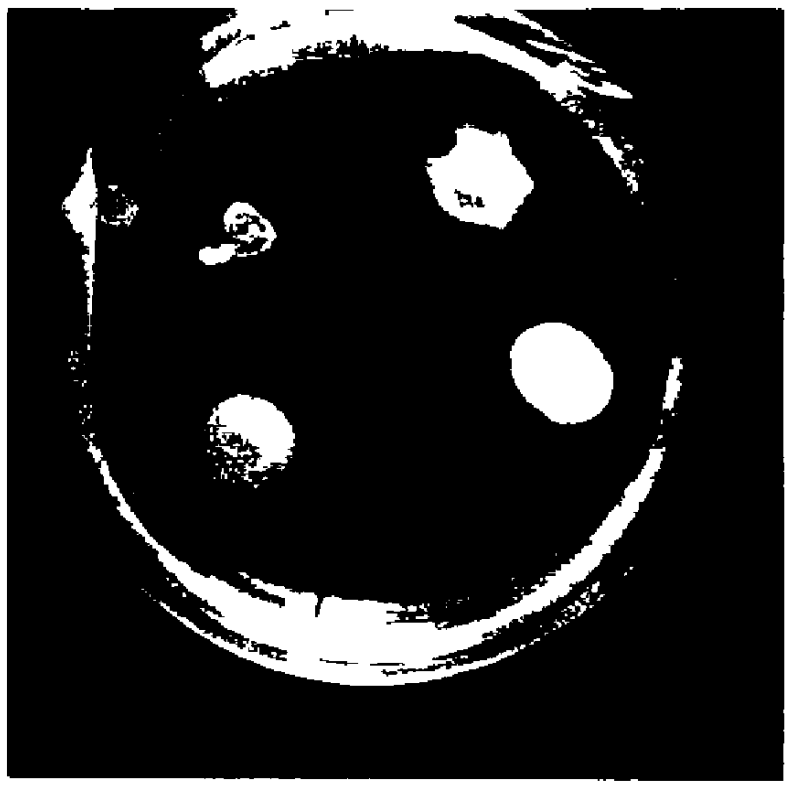


Plate IV

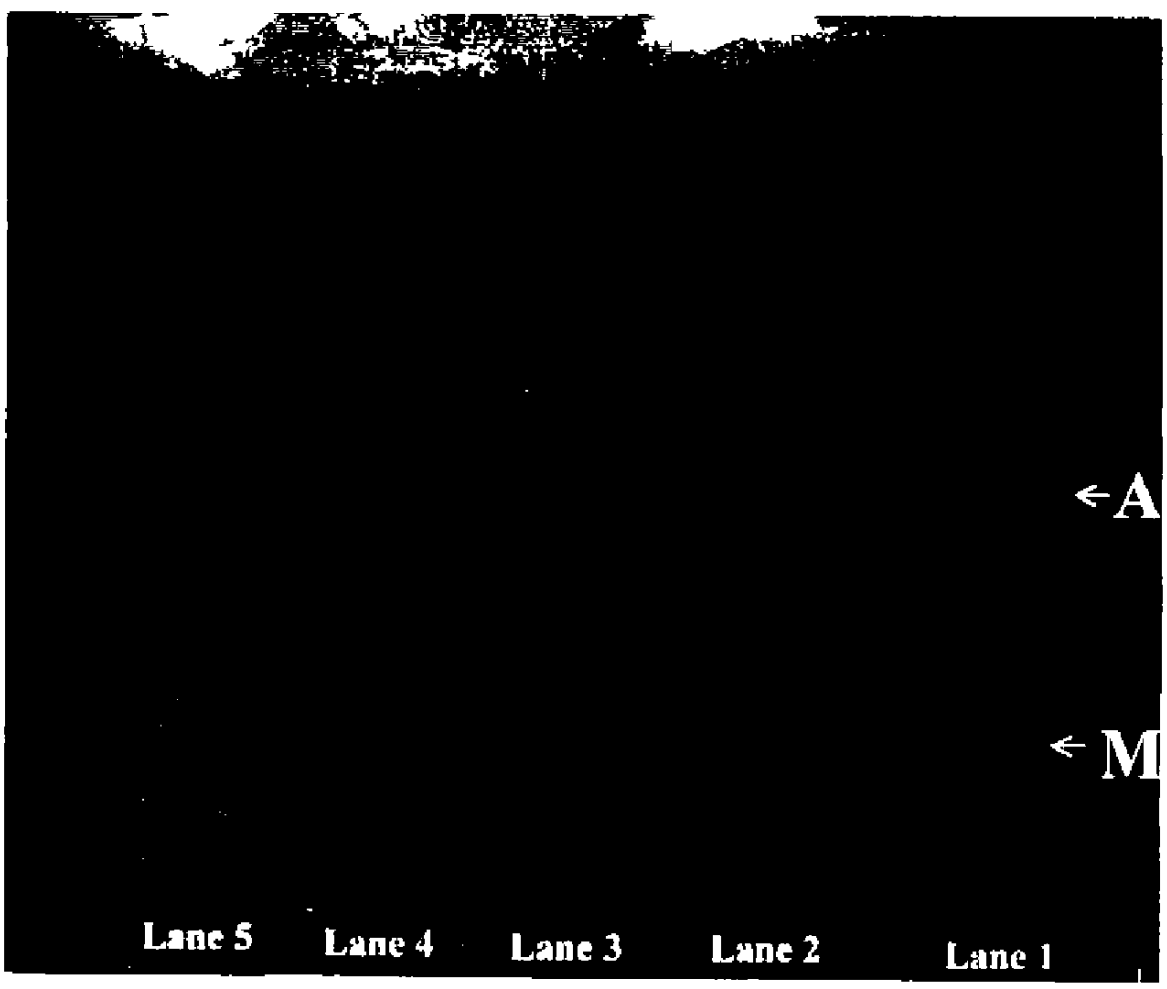


Table 15 Influence of quantity of sample and sample concentration on visibility

Quantity of sample (µl)	Visibility of opines	
	Before concentration	After concentration
2	Poor	Poor
3	Poor	Poor
4	Very faint spots	Faint spots
5	Faint spots	Good (faint)
6	Fair	Good (dark)
10	Good	Good (dark)

A minimum of 6 µl concentrated sample was required to get good dark spot of opine

4 3 2 Sensitivity of transformed roots and normal roots to NAA

The response of transformed roots and normal roots to low concentrations of NAA (10^{-11}M to 10^{-6}M) was studied Plates Va to Vc show the comparative response of transformed roots and normal roots to low concentration of NAA Hairy roots showed lateral branching The branches initiated within 72 h of culture at 10^{-9}M NAA concentration The branches extended in the same medium (Plate Vc and Vd)

In control (no NAA) no lateral branching was seen in either normal or transformed roots

4.4 Standardisation of biochemical techniques

4 4 1 Biochemical estimation of amino acids

4 4 1 1 Application of samples

Samples were applied as circular spots of various diameters A combination of different quantities of sample and the spot diameter influenced the visibility of spots The results are given in Table 16

Plate V Response of normal roots and hairy roots to lower concentrations of NAA

Va Response of normal roots and hairy roots to 10^{-11} M concentration of NAA after 48 h

Vb Response of normal roots and hairy roots to 10^{-9} M concentration of NAA after 72 h

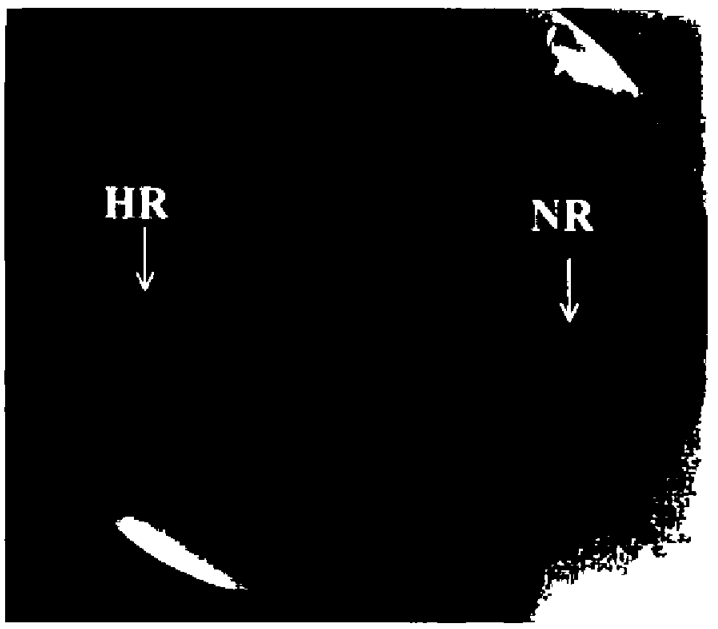
Vc Response of normal roots and hairy roots to 10^{-9} M concentration of NAA after one week

Vd Proliferation of lateral branches in liquid MS medium containing 10^{-9} M concentration of NAA

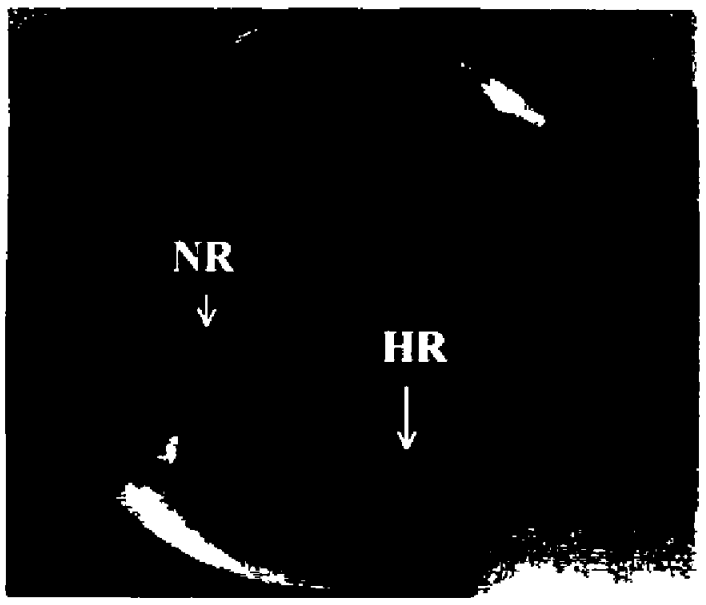
Plate V

Va

Vb



Vc



Vd

Table 16 Influence of spot diameter and quantity of sample on visibility

Spot diameter (mm)	Visibility of spots			
	Quantity of sample applied per spot (µl)			
	2	5	10	15
5	Poor	Good	Poor	Poor
8	Poor	Poor	Good	Poor
10	Poor	Poor	Good (faint)	Good (dark)

It can be seen from the Table that combinations of 5µl sample and 5 mm diameter, 10 µl sample and 8 mm diameter, and 15 µl sample and 10 mm diameter gave good visibility of spots

4 4 1 2 Standardization of optimum concentration of spray reagent

Various concentrations of ninhydrin in acetone were analysed for their efficiency in expressing the amino acids as specific coloured spots Table 17 gives an idea of the effect of concentration of ninhydrin on the visibility of amino acids

Table 17 Influence of ninhydrin concentration of visibility

Concentration of ninhydrin (%)	Visibility
0 1	Very faint, violet spots
0 2	Clear, dark violet spots
0 3	Clear, dark violet spots
0 4	Clear, dark violet spots

The minimum concentration of ninhydrin to obtain a clear dark violet spot was 0 2 per cent. Further increase in concentration did not increase the quality of the spot

4 4 1 3 Determination of Rf values of amino acids in root tubers and callus

In all, six amino acids were found in the root tubers as distinct spots of violet colour where as in the callus, apart from six distinct spots of violet colour

an additional spot (Rf 0 48) of yellow colour was found Thus, callus gave an additional seventh amino acid when compared to the root tubers The Rf values of amino acids in the root tubers and the callus are given in Table 18

Table 18 The Rf values of spots obtained for root tubers and callus

Spot No	Rf value		Colour of spot
	Root tubers	Callus	
1	0 22	0 18	Violet
2	0 31	0 31	Violet
3	0 39	0 40	Violet
4	-	0 48	Yellow
5	0 53	0 55	Violet
6	0 67	0 68	Violet
7	0 83	0 83	Violet

The Rf values of amino acids in root tubers and callus were almost same So also the colour obtained was same The additional amino acid showed yellow colour and was the characteristic of callus only

4 4 1 4 Determination of standard Rf values

The Rf values obtained for standard amino acids are given in Table 19

Table 19 The Rf values obtained for standard amino acids

Amino acid	Rf value	Colour
DL Alanine (DL Ala)	0 46	Violet
Dl 2 Amino Butyric acid (DL2 ABA)	0 75	Violet
L-Arginine mono hydro chloride (L Arg MHC)	0 18	Violet
DL Aspartic acid (DL Asp Acid)	0 30	Violet
L Cysteine mono hydro chloride (L cys MHC)	0 53	Yellow
L Cysteine (L cys)	0 09	Violet
DL DOPA	0 25	Yellow
L-Glutamic acid (L Glu acid)	0 63	Violet
L-Glycine (L Gly)	0 28	Violet
L Histidine monohydro chloride (L His MHC)	0 24	Violet
L Hydroxy proline (L Hydr Pro)	0 30	Yellow

L-ISO Leucine (L Iso-Leu)	0 07	Violet
DL-Nor-Leucine (DL-nor leu)	0 71	
L-Leucine (L leu)	0 52	Violet
L-Lysine mono hydro chloride (L Lys MHC)	0 15	Violet
DL Methionine (DL Met)	0 54	Violet
L-Ornithine (L Orn)	0 08	Violet
DL Phenyl alanine (DL Phe Ala)	0 71	Violet
L-Proline (L Pro)	0 43	Yellow
DL-Serine (DL Ser)	0 29	Violet
DL Threonine (DL Thr)	0 34	Violet
L Tyrosine (L Tyr)	0 49	Violet
DL Tryptophan (DL Try)	0 51	Violet
DL Valine (DL Val)	0 59	Violet

4 4 1 5 Identification of amino acids in root tuber and callus

The amino acids in root tubers and callus were identified on the basis of their Rf values by comparing them with standard amino acids The results are given in Table 20

Table 20 Comparative evaluation of amino acids present in root tubers and callus with the standard amino acids

Spot No	Rf value			Colour of spot	Identified amino acid
	Root tubers	Callus	Standard		
1	0 22	0 22	0 22 0 22	Violet Violet	L His MHC L Gly
2	0 21	0 31	0 31 0 31	Violet Violet	DL Asp acid DL ser
3	0 39	0 40	0 39 0 39	Violet Violet	DL Thr DL Ala
4	-	0 48	0 48 0 49	Yellow Yellow	L Pro L cys MHC
5	0 53	0 54	0 53 0 53	Violet Violet	L Leu DL Met
6	0 67	0 67	0 67	Violet Violet	DL Phe Ala
7	0 83	0 83	0 83	Violet Violet	DL 2 ABA

An additional amino acid (spot 4) was found in callus that was not observed in the root tubers

4 4 2 Biochemical estimation of essential oils

4 4 2 1 Application of samples

Samples were applied as circular spots of varying diameter, in various quantities The results obtained are given in Table 21

Table 21 Influence of spot diameter and quantity of sample on visibility

Spot diameter (mm)	Visibility of spots					
	Quantity of sample applied per spot (µl)					
	5	10	15	20	30	40
5	Poor	Poor	Good (dark)	Poor	Poor	Poor
8	Poor	Poor	Good (faint)	Good (dark)	Poor	Poor
10	Poor	Poor	Poor	Good (faint)	Good (dark)	Poor
15	Poor	Poor	Poor	Poor	Good (faint)	Good (dark)

Both, the spot diameter and the quantity of sample applied, influenced the visibility of spots The combinations, 15 µl sample with 5 mm diameter spot, 20 µl sample with 8 mm diameter spot, 30 µl sample with 10 mm diameter spot and 40 µl sample with 15 mm diameter spot gave good spots

4 4 2 2 Standardisation of elution technique

Methylene di chloride-chloroform-ethyl acetate-n propanol (45 45 4 5 4 5) was used for the elution of essential oil as suggested by Harborne (1973) However, this combination gave a cluster of spots which may be due to the large number of compounds of near affinity

So the ratio of the running solvent system was changed to 47 5 45 2 4 5 It gave good separation of spots This combination was found to be the best for elution of essential oils in *Holostemma*

The normal TLC plates and $AgNO_3$ treated plates were tested for their ability in separation of spots The results obtained are given in Fig 10

The normal plates and $AgNO_3$ treated plates varied in their ability to elute the spots Furthermore, the Rf values of spots obtained in both the cases were different The $AgNO_3$ treated plates gave the best separation of spots appearing on the plate

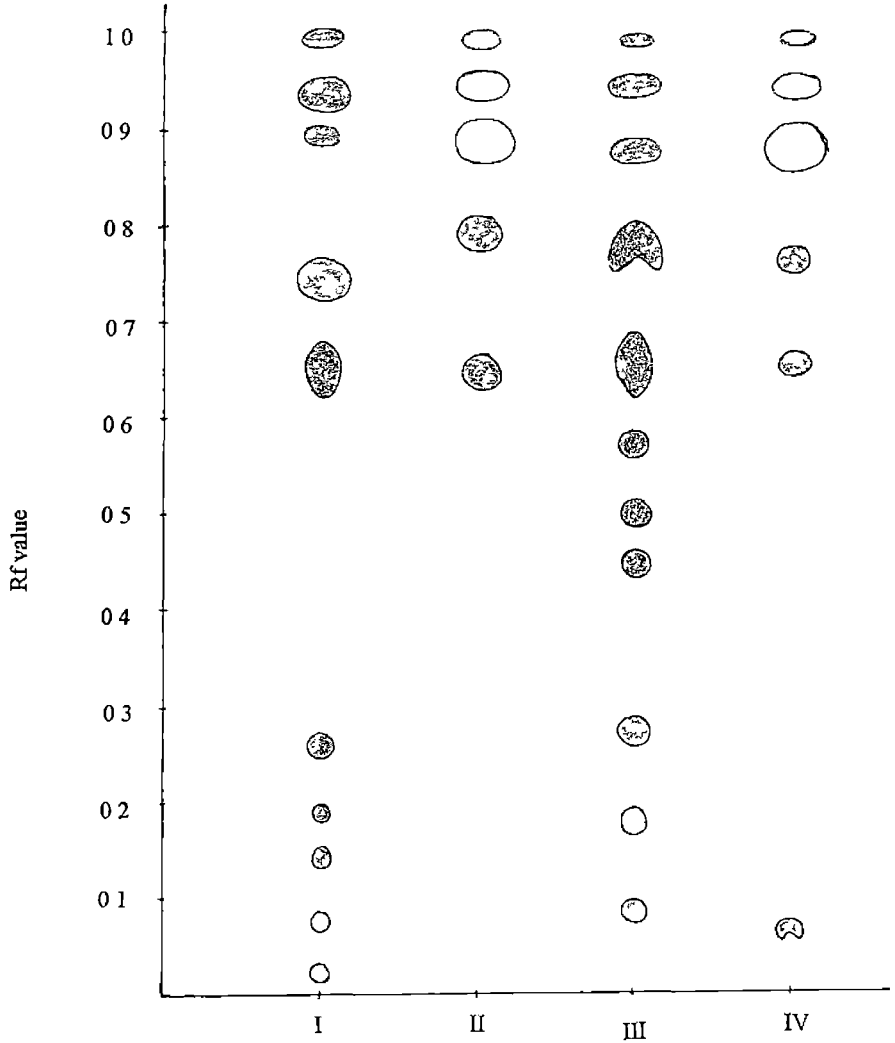
4 4 2 3 Standardisation of spray reagents

Spray reagents were evaluated for finding out the optimum reagent for visibility of the chromatogram Ability of the spray reagents to detect the terpene essential oils with double bonds was also tested

The colour of various spots obtained from root tuber extract with different spray reagents and their Rf values are given in Table 22a and those from callus are given in Table 22b

It can be seen from the tables that the spray reagents differed in their ability to detect the compounds on TLC plates Compounds having similar Rf values were detected by different spray reagents, however, the colour of the compound varied and was the characteristic of the particular spray reagent

On comparison of Table 22a and 22b it is clear that lesser compounds are present in callus as compared to the root tubers Their Rf values differed from those of the corresponding compounds in the root tubers Similarly, the colours developed on spraying a particular spray reagent also differed between the spots obtained from the root tubers and the callus



I Normal TLC plate – root tubers
II Normal TLC plate – callus
III $AgNO_3$ treated TLC plate – root tubers
IV $AgNO_3$ treated TLC plate – callus

Fig 10 Elution pattern of essential oils in root tubers and callus of *Holostemma* on normal and $AgNO_3$ treated TLC plates

Table 22a Development pattern of essential oils with various spray reagents in callus

Spot No	Vanillin-sulphuric acid		Antimony chloride		Bromine vapour		Bromine vapour followed by 50% H_2SO_4 spray	
	Rf Value	Colour of spot	Rf Value	Colour of spot	Rf Value	Colour of spot	Rf Value	Colour of spot
1	0 04	Brownish black	0 62	Yellow GF	0 62	Whitish	0 62	Bluish fading to black
2	0 58	Pink	0 85	Yellow SF	0 90	Whitish	0 90	Brown
3	0 62	Pink	0 95	Pinkish yellow GF	0 99	PF	0 95	Brownish
4	0 75	Purplish blue					0 66	Bluish black
5	0 90	Pinkish yellow						
6	0 95	Pinkish						
7	0 99	Pinkish blue turning purple on prolonged heating						

GF - Brilliant green fluorescence under UV (366 nm)
SGF - Slight green fluorescence under UV (366 nm)
PF - Pinkish fluorescence

Table 22b Development pattern of essential oils with various spray reagents in root tubers

Spot No	Vanillin-sulphuric acid		Antimony chloride		Bromine vapour		Bromine vapour followed by 50% H_2SO_4 spray	
	Rf Value	Colour of spot	Rf Value	Colour of spot	Rf Value	Colour of spot	Rf Value	Colour of spot
1	0 08	Greenish SGF	0 44	Brownish charring to black	0 65	Whitish	0 08	Brownish black
2	0 16	Greenish	0 49	Brownish charring to black	0 79	Whitish	0 44	Bluish black
3	0 26	Greenish SGF	0 56	Brownish charring to black	0 4	GF	0 49	Bluish black
4	0 44	Bluish brown SGF	0 65	Yellowish	0 98	GF	0 56	Bluish black
5	0 49	Brownish	0 79	Bluish				
6	0 56	Pinkish brown	0 89	Yellow				
7	0 65	Purplish blue						
8	0 79	Greenish blue						
9	0 89	Pinkish yellow turning brown						
10	0 94	Pinkish brown SGF						
11	0 98	Pink GF						

GF - Brilliant green fluorescence under UV (366 nm)
SGF - Slight green fluorescence under UV (366 nm)

4 4 3 Biochemical estimation of triterpenoids

4 4 3 1 Estimation of triterpenes

4 4 3 1 1 Application of samples

Samples were applied as circular spots of varying diameters in various quantities The results obtained are given in Table 23

Table 23 Influence of spot diameter and quantity of sample on visibility

Spot diameter (mm)	Visibility of spots					
	Quantity of sample applied per spot (µl)					
	5	10	15	20	30	40
5	Poor	Poor	Good (faint)	Good (dark)	Poor	Poor
8	Poor	Poor	Good (faint)	Good (dark)	Poor	Poor
10	Poor	Poor	Poor	Good (faint)	Good (dark)	Poor
15	Poor	Poor	Poor	Poor	Good (faint)	Good (dark)

Both, the spot diameter and the quantity of sample applied, influenced the visibility of spots The combinations, 20 µl sample with 5 mm and 8 mm diameter, 30 µl sample with 10 mm diameter and 40 µl sample with 15 cm diameter spot, gave good spots

4 4 3 1 2 Standardisation of elution technique

As suggested by Harborne (1973), hexane-ethyl acetate (1 1), chloroform-methanol (10 1) and n-butanol-2M NH_4OH (1 1 and 2 1) and also hexane, chloroform and hexane-chloroform-methanol (1 1 1, 1 3 1 and 1 4 1) were evaluated

Similarly, argentative TLC and normal TLC were tested for their ability to separate triterpenes The elution pattern of triterpenes in the case of

argentative TLC and normal TLC for the different running solvents is given in Fig 11

The combination of hexane-chloroform-methanol (1 3 1) gave good spots for argentative TLC while n-butanol-2M NH_4OH (1 1) was best in the case of normal TLC

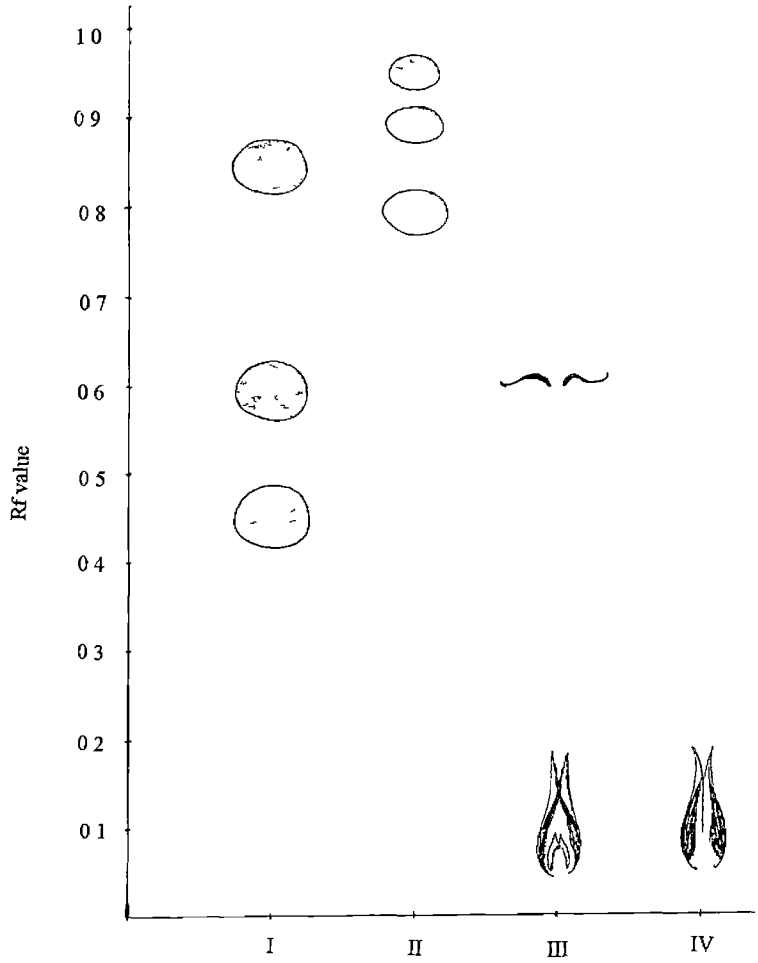
4 4 3 1 3 Standardisation of spray reagent

Spray reagents were evaluated for efficacy in expressing more number of spots of triterpenes The colour of various spots obtained from root tuber extract with different spray reagents and their Rf values are given in Table 24a whereas those from callus are given in Table 24b

It can be seen from Table 24a and 24b that spray reagents differed in their ability to detect triterpenes So also the variation in separation was observed in normal and argentative TLC plates

Among the different spray reagents tried, antimony chloride and vanillin-sulphuric acid gave maximum number of spots

On comparison of Table 24a and 24b it can be seen that lesser compounds are present in callus as compared to root tubers Their Rf values differed from each other The colours developed by spraying with a particular spray reagent also differed



I Normal TLC plate – root tubers
II Normal TLC plate – callus
III Argentative TLC plate – root tubers
IV Argentative TLC plate – callus

Fig 11 Elution pattern of triterpenoids in root tubers and callus of *Holostemma* on normal and argentative TLC plates

Table 24a Development pattern of triterpenes with various spray reagents in root tubers on normal and argentative TLC plates

Spot No	Normal plate							
	Antimony chloride		Vanillin-sulphuric acid		1% H_2SO_4 in ethanol		Phenol-Bromine	
	Rf value	Colour of spot	Rf value	Colour of spot	Rf value	Colour of spot	Rf value	Colour of spot
1	0 46	Purplish blue	0 22	Yellowish green		No sports obtained		No sports obtained
2	0 59	Purplish blue	0 30	Blue				
3	0 84	Purplish blue	0 46	Bluish black				
			0 59	Bluish black				
			0 97	Bluish purple				
Impregnated plate								
1	0 13	Red turning brown on heating	0 32	Bluish green		No spots obtained		No spots obtained
	0 63	Brown turning purplish black on heating	0 72	Bluish black				
			0 84	Bluish				
			0 95	Bluish black				

Table 24b Development of triterpenes with various spray reagents in callus on normal and argentative TLC plates

Spot No	Normal plate							
	Antimony chloride		Vanillin-sulphuric acid		1% H_2SO_4 in ethanol		Phenol-Bromine	
	Rf value	Colour of spot	Rf value	Colour of spot	Rf value	Colour of spot	Rf value	Colour of spot
1	0 80	BF	0 23	Greenish		No spots obtained	0 93	BF
2	0 88	PF	0 35	Greenish				
3	0 97	Pinkish purple	0 80	Bluish black				
			0 90	Bluish black				
			0 97	GF				
Impregnated plate								
1	0 13	Red turning brown on heating	0 32	Greenish	0 13	Red spot turning yellow-orange on heating		No spots obtained
			0 72	Faint bluish black spots				
			0 84	Faint bluish black spots				
			0 95	Faint bluish black spots				

PF - Pink fluorescence under UV (366 nm), GF - Brilliant green fluorescence under UV (366 nm)
BF - Bluish fluorescence under UG (366 nm)

4 4 3 2 Estimation of sterols

4 4 3 2 1 Application of samples

Samples were applied as circular spots of varying diameters in various quantities The results obtained are given in Table 25

Table 25 Influence of spot diameter and quantity of sample on visibility

Spot diameter (mm)	Visibility of spots					
	Quantity of sample applied per spot (µl)					
	5	10	15	20	30	40
5	Poor	Good (faint)	Good (dark)	Poor	Poor	Poor
8	Poor	Good (faint)	Good (dark)	Poor	Poor	Poor
10	Poor	Poor	Good (faint)	Good (dark)	Poor	Poor
15	Poor	Poor	Poor	Good (faint)	Good (dark)	Poor

Both, the spot diameter and the quantity of sample applied, influenced the visibility of spots The combinations 15 µl sample with 5 mm and 8 mm diameter spot, 20 µl sample with 10 mm diameter spot, 30 µl sample with 15 mm diameter spot gave clear distinct spots

4 4 3 2 2 Standardisation of elution technique

Chloroform and hexane were tried separately to test their ability to elute the sample as suggested by Ikan and Cudzinovski (1965) However, major part of sample remained uneluted with both chloroform and hexane So different combinations of hexane and chloroform were tried The combination 3 2 chloroform-hexane was the best to isolate the compounds in TLC

4 4 3 2 3 Standardisation of spray reagent

Various spray reagents were evaluated for their ability to detect sterols and related compounds The reagents used in this study, the Rf values of spots obtained in the case of each reagent and the colour of the spots in the case of root tubers and callus is given in Table 26

The compounds present both in callus and root tubers showed similar Rf values Similarly, the colour obtained for the corresponding spots were same

A bluish spot was obtained on P-anisaldehyde spray having Rf 0 15 It may be a sterol or 17 21 and 16 17 diol of P series

Concentrated sulphuric acid-ethanol-water (40 9 1) did not show any spot This is referred to as 'Allen's test' for detection of steroids. It detects 16-hydroxy steroids Since no spots were obtained it in presumed that this group of steroids were not present

Similarly water spray did not show any spot which confirmed that no steroids were present

Table 26 Detection of sterols with various spray reagents in root tubers and callus

Spot No	Root tubers							
	Bromine-sulphuric acid		P-anisaldehyde		Water		Conc Sulphuric acid ethanol-water (40 9 1)	
	Rf value	Colour of spot	Rf value	Colour of spot	Rf value	Colour of spot	Rf value	Colour of spot
1	0 15	Brownish black	0 15	Brownish black		No spots obtained		No spots obtained
2	0 62	Faint brown	0 62	Faint pink to mauve				
Callus								
1	0 11	Brownish black	0 11	BF		No spots obtained		No spots obtained
2	0 62	Faint brown	0 62	Faint pink to mauve				

BF - Bluish fluorescence under UG (366 nm)

Discussion

DISCUSSION

The results obtained in the study on "Genetic transformation and hairy root cultures in Ada-kodien (*Holostemma ada-kodien* K. Schum)" are discussed in this chapter

5.1 Standardisation of the transformation technique

5 1 1 Effect of explants on transformation

In the present study, the explants viz leaf, shoot buds, internodal segments, seedling hypocotyls and callus showed different responses to transformation The hairy roots were induced from shoot buds and seedling hypocotyls only

Yonemitsu *et al* (1990) induced hairy roots from 20 days old seedling of *Lobelia inflata* by infection with *A. rhizogenes* strain 15834 They observed that the shoot buds could not induce hairy roots and they concluded that the juvenility of explant is necessary for hairy root induction. Similar result was reported by Trypsteen *et al* (1991) in *Enhinacea purpurea,* wherein the seedling hypocotyls induced hairy roots whereas older plants did not induce hairy roots They concluded that the type and age of explant is crucial for good response to *Agrobacterium* infections

Citovsky *et al* (1996) have reported that specificity of *Agrobacterium transformation* is closely connected with the physiology and growth of the plant Nin *et al* (1997) have reported that the age and hormonal balance of host tissue affect the transformation frequencies

Stachel *et al* (1986) reported that the induction of vir genes was not merely a response of *Agrobacterium* to necrotic plant cells, but it required an active plant cellular metabolism Kahl (1982) and Citovsky *et al* (1996) stated

that cell division and DNA synthesis are involved in the incorporation of T-DNA into the plant genome Therefore, absence of cell division may prevent successful T-DNA transfer

Villemont *et al* (1997) studied the role of host cell cycle in the *Agrobacterium* – mediated genetic transformation of *Petunia hydrida* They found that no transformation occurred when the cell division was stopped at the G1 phase If the cell division was stopped at early G2 phase, the T-DNA transfer occurred but the transformation frequencies were less and the T-DNA transformation was not stable If the cell division was stopped after M phase, the T-DNA integration was stable They concluded that the S phase (DNA synthesis phase) and M phase (cell division phase) were very important for the stable integration of T-DNA in the plant cells

Potrykus (1990) did a critical assessment of the *Agrobacterium* –mediated gene transfer process He stated that the most important factor for successful transformation by *Agrobacterium* was the wound response and that the plant tissues differ in their wound response He further stated that the explants with a pronounced wound response develop larger populations of wound adjacent competent cells for regeneration and transformation He suggested that the explants or plant species recalcitrant to transformation with *Agrobacterium* probably do not express appropriate wound response

It is thus quite evident that different explants vary in their wound response i e to produce the number of competent cells for regeneration and transformation The explant cells differ in their DNA synthesis and cell division ability due to the difference in physiological maturity of the cells The seedling hypocotyls being juvenile in nature have the most competent cells for regeneration and transformation, and have the highest rate of cell division and DNA synthesis as a consequence of which they show the highest transformation frequencies. The shoot buds show a lower transformation frequency, which might be due to lower wound adjacent competent cells and a lower cell division

rates as compared to seedling hypocotyls The inability of leaf, internodal segments and callus top induce hairy roots may be due to lack of wound adjacent competent cells or difference in physiological maturity of this tissues

5.1 2 Influence of *A rhizogenes* strains on transformation

In the present study, among the five *A rhizogenes* strains used, the strains P_cA_4, 15834 and A_4 induced hairy roots The strains 8196 and 2659 did not induce hairy roots Among the successful strains, P_cA_4 showed the highest transformation frequencies followed by 15834 and A_4

Patena *et al* (1988) reported marked differences in the ability of four *A rhizogenes* strains to induce hairy roots on carrot discs, kalanchoe leaves and apple shoots The strains A_4 and 232 produced hairy roots The strains R 1000 and 178 × A_4 did not produce hairy roots Similarly, Constable and Towers (1988) observed that out of seven *A rhizogenes* strains used, only two strains viz TR7 and A_2/83 produced hairy roots in *Chaenactis douglasii*

Trypsten *et al* (1991) observed that two strains viz ATCC 15834 and R 1601 produced hairy roots in *Echinacea purpurea* whereas the strains LMG 63 and LMG 150 did not produce hairy roots Benjamin *et al* (1995) found that the *A rhizogenes* strains $A_4^{10\text{-}19}$ and $A_4^{u\,1\text{-}19}$ did not induce hairy roots in *Rauvolfia serpentina* while the strain 15834 produced hairy roots

Kittipongpatana *et al* (1998) found that the *A rhizognes* strains 15834, A_4, 43057 and 11325 were capable of inducing hairy roots in *Solanum aviculare* However, the strains differed in their transformation frequencies and the strain 15834 showed the highest transformation frequency (90%) followed by A_4 (83%)

Petit *et al* (1983) studied the pathogenicity of different strains of *A rhizognes* They found that the strains A_4, 15834 and HRI were the most virulent on all hosts tested The strains 8196, TR7 and TR 101 were found to be less virulent and transformed only a limited number of hosts They suggested that this difference in host range and pathogenicity of *A rhizogenes* strains is due to the difference in the plasmids they harbour Similarly Cordarelli *et al* (1987) attributed the difference in transformation ability of different strains of *Agrobacterium* to the plasmids harboured by them

Rhodes *et al* (1989) reported that the agropine strains (15834, A_4, TR-7 etc) have wider host range that is attributed to the presence of TR-DNA fragment of the T-DNA harbouring genes for auxin synthesis (tms 1 and tms 2) These genes trigger cellular division by auxin synthesis due to which these strains are able to transform a wide range of species On contrary, the mannopine strains and cucumopine strains are deficit in the TR-DNA fragment hence they cannot trigger auxin synthesis and so can infect only a limited number of hosts In the present study, it was found that only the agropine strains viz P_cA_4, 15834 and A_4 induced hairy roots while the mannopine strain 8196 and the cucumopine strain 2659 did not induce any hairy roots

Gelvin (1990) reviewed the physiological basis of hairy root disease He pointed out that the TR fragment of T-DNA need not be present, to exhibit hairy root phenotype He suggested the existence of two mechanisms of hairy root tumerogenesis, one depending on the auxin over production directed by T_R T-DNA of certain *A rhizogenes* strains and the other apparently independent of transfer and expression of genes directing the biosynthesis of auxins

Spencer and Towers (1989) reported that different *A rhizogenes* strains respond differently to the wound induced compounds The wide host range (WHR) strains like 15834 and A_4 are more sensitive to the wound induced compounds than the limited host range (LHR) strains like 8196 and 2659 So the lack or low levels of inducer molecules from wounded plant cells inhibit the

virulence of these LHR strains resulting in inhibition of transformation So also, in addition to the vir-inducing compounds certain compounds induced from wound are inhibitory to the vir induction Not all the *Agrobacterium* strains are capable of degrading the inhibitory wound induced compounds So they differ in the transformation ability In the present study, the WHR strains P_CA_4, 15834 and A_4 are showed transformation This may be due to their ability to respond to the wound inducer molecules as well as to degrade the inhibitory wound induced compounds On the contrary, the strains 8196 and 2659 were either unable to respond to the levels of inducer molecules from wound or to degrade the inhibitory compounds

Leroux *et al* (1987) reported that the host range of WHR and LHR strains may be affected by the differences in structure of Vir A protein Though the vir-A proteins in both types are structurally related, the proteins in their amino acid termini are quite divergent So they differ in signal recognition and hence the transformation ability The strain P_CA_4, 15834 and A_4 are from the WLR type agropine family so they possess similar type of vir A proteins The strains 8196 and 2659 may possess different type of vir A proteins due to which their vir gene system is not sufficiently activated resulting in their failure to induce transformation The vir gene system of agropine strains, however might be fully activated resulting in successful T-DNA transfer

5 1 3 Standardisation of infection technique

5 1 3 1 Direct inoculation method

5 1 3 1 1 Influence of nature of bacterial inoculum on transformation

Patena *et al* (1988) studied the effect of nature of bacterial inoculum on transformation of kalanchoe leaves, carrot discs and apple shoot buds They found that the colonies were more effective than bacterial suspensions when used as inoculum They attributed the better performance of colonies to the greater quantity of bacteria in the colonies when compared to bacterial suspensions

In the present study, bacterial suspensions when used as inoculum were able to induce transformation. No transformation was obtained when the colonies were used as the inoculum These observations are contradictory to the above report The superior performance of bacterial suspension over the single colonies, in the present study, may be due to the optimum concentration of bacteria in suspensions as compared to colonies Secondly, when suspensions are applied, the bacteria are motile and can take advantage of the liquid medium to facilitate better attachment to the wounded cells than they get when applied as colonies

5 1 3 1 2 Influence of amount of bacteria applied per wound

Kumar *et al* (1991) studied the effect of quantity of bacteria applied on wound, on production of transformed roots They found that the optimum concentration of bacteria was 1×10^{8} cells ml^{1} They showed that the concentration below optimum may result in fewer competent bacteria being available to transform the plant cells while the supra optimum levels were responsible for competitive inhibition of competent bacteria resulting in decrease in transformation frequencies In the present study, when the bacterial suspension ($OD_{560\ nm}^{1}$) was applied as a drop on each wound, the transformation frequency was less where as when the needle was dipped in bacterial suspension and used for inoculation, the transformation frequencies were high. In this study, the lesser amount of bacterial inoculum (needle dipped in bacterial suspension prior to inoculum) may have coincided with the optimum levels of bacteria at wounds resulting in greater transformation frequencies and vice versa.

5 1 3 2 Co-culture method

5 1 3 2 1 Influence of quantity of bacterial inoculum and the co-culture time

Mihaljevic *et al* (1996) induced hairy roots in *Pinus nigrum* explants using *A rhizogenes* strains 15834 and 8196. They observed that the co-culture time affected the transformation frequency

Xu *et al* (1997) reported that the co-culture time affected the transformation frequencies of alfalfa suspension cultures They found that the co-culture period of 72 h to be the best as it gave maximum number of transformants After 6 days, of co-culture, the sensitivity of plant cells to bacteria was very low and no transformation was achieved

In the present study, the amount of bacterial cells present at the time of co-culture influenced the co-culture time When less bacterial cells were present at the time of co-culture, the co-culture time of a minimum of 24 h and maximum of 36 h gave good transformation frequencies On the contrary, when more number of bacterial cells was present during co-culture a co-culture time ranging from 10 min to 120 min gave good transformation frequencies

These results are on par with the results obtained by Kumar *et al* (1991) that the optimum concentration of bacterial cells is essential for transformation with *Agrobacterium*

The bacterial cells multiplied in the co-culture medium and after 24h, the optimum quantity of bacteria were available and hence transformation was achieved After 36 h the level of bacterial cells reached supra optimum level and the competitive inhibition of competent bacterial cells resulted in inhibition of transformation

When more bacterial cells were present at the time of co-culture, the co-culture time of more than 120 min allowed the bacterial cell concentration to reach the supra – optimum level resulting in inhibition of transformation

5 1 3 2 2 Influence of shaker speed

Bakkali *et al* (1997) reported that the speed of rotation at the time of co-culture influenced the transformation frequencies of leaf segments of *Lawsonia*

inermis They found that the speed of 100 rpm was the best as it gave the maximum number of transformants

In the present study, the explants were co-cultured at 50, 100 and 150 rpm The maximum numbers of transformants were obtained at 100 rpm followed by 50 rpm The speed of 150 rpm did not give any transformant So the optimum speed of rotation was 100 rpm in the present study

Attachment of bacterial cells to plant cells is a pre-requisite for transformation by *Agrobacterium* (Pueppke and Hawes, 1985) When the explants are co-cultured with bacteria, the success of transformation depends on the attachment of bacterial cells to plant cells, which is greatly influenced by the speed of rotation When speed of rotation is 50 rpm, some bacterial cells are able to attach to the wounded cells and hence transformation is achieved. At 100 rpm, the rotation favours the bacterial movement towards the wounded cells and more transformation frequencies are observed. Finally at 150 rpm, the bacterial cell movement is hampered and the bacterial cells are unable to attach with the plant cell resulting in inhibition of transformation

5 1 3 2 3 Influence of NAA in co-culture medium on transformation

NAA has been reported as the most potent auxin for callus induction in *Holostemma* (John, 1996, John and Keshavachandran, 1996) NAA when applied in co-culture medium increased the transformation frequency remarkably The transformation frequency was increased from 25 per cent to 75 per cent on application of NAA in co-culture Among the different concentrations of NAA tested, concentrations of 1 ppm and 2 ppm were effective and 3 ppm NAA did not give any transformants Aronen *et al* (1996) have reported that 0 5 M IBA increased the transformation frequency of fascicular shoots of scots pine from 16 per cent to 40 per cent

Sevrenia *et al* (1996) reported that the transformation efficiency of *Agrobacterium* strain EHA 105 was increased remarkably on addition of 2-isopentenyl adenine, in the co-cultivation medium Similarly, Krens *et al* (1996) reported that addition of NAA and 2,4-D during co-culture gave high transformation frequencies in *Beta vulgaris* transformed with *Agrobacterium*

Xu *et al* (1997) reported that 2,4-D addition in the co-culture medium was indispensable to growth and transformation of alfalfa suspension cultures The optimum 2,4-D concentration was 2 26 μM, transformation was inhibited which also occurred when 2,4-D was added in combination with BA

Cradarelli *et al* (1987) showed that addition of auxin increased the virulence of cucumopine and mannopine strains of *Agrobacterium rhizogenes* by relieving the deficiency caused by the absence of T_RDNA

Auxins influence a variety of cellular processes including cell elongation, cell division, differentiation and morphogenesis (Brummell and Hall, 1987, Davis, 1987 and Dudits *et al*, 1993) Auxins induce cell division as a consequence of activation of a multicomponent cascade system that includes binding of hormone to receptor molecules, triggering of signaling pathway, reprogramming of gene expression and structural re-organization of the cyto-architecture The altered gene expression leads to stimulation of cell proliferation and morphogenesis

In the perview of above theory, and the fact that active cell division and cellular metabolism aids in transformation via *Agrobacterium rhizogenes* (Villemont *et al*, 1997), the addition of auxins during co-culture resulting in enhancement of the transformation frequencies is quite evident

Since optimum concentration of auxin is the key for such cellular responses, the concentration of auxin in co-culture should be optimum and slight

alterations in concentrations do not increase, but in fact decrease the transformation frequencies In the present study, the observations that only NAA at 1 and 2 mg l^{-1} concentrations induced hairy roots and at 3 mg l^{-1} concentration no transformation was achieved, were on par with the above theory

5 1 4 Effect of culture media on transformation

The shoot buds and seedling hypocotyls produced hairy roots on MS solid medium only In co-culture method, when NAA was used during co-culture, calli developed from wounds These calli produced hairy roots only in liquid MS medium No hairy root induction was observed in ½ MS solid or ½ MS liquid medium

Mano *et al* (1989) observed that the culture medium strongly influences the hairy root induction on stem segments and leaf disks of *Duboisia leichhardtii* upon infection with *A rhizogenes* The inoculated stem segments and leaf disks when cultured for 1 week on 1 per cent LS medium without sucrose in light and then incubated in dark for 3-6 weeks on 1 per cent HF medium containing 1 mg ml^{-1} carbemicillin induced hairy roots But when the explants from 1 per cent LS medium without sucrose were incubated in dark on 1 per cent agar LS medium with or without sucrose, no hairy roots were induced This observation hints that the sucrose concentration is most important for hairy root induction In ½ MS medium the sucrose concentration is reduced and hence no transformation was achieved

Benjamin *et al* (1994) observed that in *Rauvolfia serpentina* hard tumorous mass was induced on wound sites after *A rhizogenes* infection When the tumorous mass was excised and cultured on MS medium with 3 per cent sucrose and 1g l^{-1} carbencillin in dark, friable callus was formed After 3 passages of 30 days, roots developed from callus Root formation was sporadic on Knop's medium and MS ½ basal medium They concluded that culture media affected the hairy root formation

Xu *et al* (1997) cultured alfalfa suspension cultures treated with Agrobacterium, on different basal media without hormones More transformants were obtained on SH medium and B_5 medium with $Fe2^+$ salts of MS medium than on ½ MS or V-KM (Gamborg *et al*, 1968, Binding *et al* 1981) medium They reported that B_5 and SH media were more suitable for transformation Our observation that no transformation was achieved on ½ MS medium is on par with the above observations that culture medium affects the transformation

5 1 5 Effect of photoperiod on hairy root induction

In the present study, photoperiod markedly affected the hairy root induction The hairy root induction was observed when seedling hypocotyls were cultured in continuous dark and also when cultured in a photoperiod of 16 h In the case of shoot buds hairy root induction was observed only in 16 h photoperiod and never in dark

Different workers have reported the effect of photoperiod on hairy root induction Jazin *et al* (1988) cultured hypocotyls of *Datura stramonium* in dark and induced hairy roots Sarma *et al* (1997) incubated the inoculated leaf segments of *Rauvolfia serpentina* on MS medium in dark and induced hairy roots Light did not induce hairy roots This report is contradictory to the present observation that no transformation was achieved in dark

Ando *et al* (1997) reported that stem segments of *Wahlenbergia marginata* induced hairy roots only when incubated in dark Similar, observation was reported in the case of leaf segments of *Lawsonia inermis* (Bakkali *et al*, 1997)

Christen *et al* (1989) reported that in *Datura candida* the inoculated seedlings induced hairy roots in continuous light. Similar, observation was

reported in seedlings of *Lithospermum erythorhizon* upon *A rhizogenes* infection by Yazaki *et al* (1998)

Kamada *et al* (1986) reported that photoperiod of 18 h (4000 lux) favoured hairy root induction in shoot tips of *Atropa belladonna* upon *A rhizogenes* infection Mano *et al* (1986) reported that stem segments and leaf disks of *Duboisia leichhardtii* induced hairy roots on infection with *A rhizogenes* when incubated in a photoperiod of 12 h Seedlings of *Lobelia* inflata induced hairy roots on *A rhizogenes* infection when cultured in a photoperiod of 16 h

It is quite evident that the photoperiod requirement for hairy root induction differs from species to species and also on the types of explant used In *Holostemma* a 16 h photoperiod was found to be a pre-requisite for transformation of shoot buds whereas seedling hypocotyls showed greater transformation when cultured in dark

5.2 Induction of hairy roots

In the present study, different routes of hairy root induction were observed depending upon the explant used and the culture conditions The thin whitish roots having numerous hairs were induced from seedling hypocotyls by direct inoculation of bacteria or by co-culture In the case of shoot buds the hairy roots emerged from the callus formed after infection In the case of shoot buds, hairy roots were induced from the basal cut portion of the explant and not from the top portion They were whitish with numerous hairs Callus was developed when NAA (1 mg l^{-1}, 2 mg l^{-1} and 3 mg l^{-1}) was added in the co-culture medium Thus callus when cultured in MS liquid medium induced hairy roots No roots were induced from calli of uninfected tissues These roots were dull whitish in colour and turned brownish yellow on subsequent culture in liquid medium They lacked root hairs (Plate VI)

Plate VI Induction of normal roots and hairy roots

VIa Induction of normal roots in MS medium

VIb Induction of hairy roots in MS medium

VIc Hairy roots induced from an infected seedling hypocotyl

VId Hairy roots induced from an infected shoot bud

VIe Induction of hairy roots from shoot buds by co-culture method

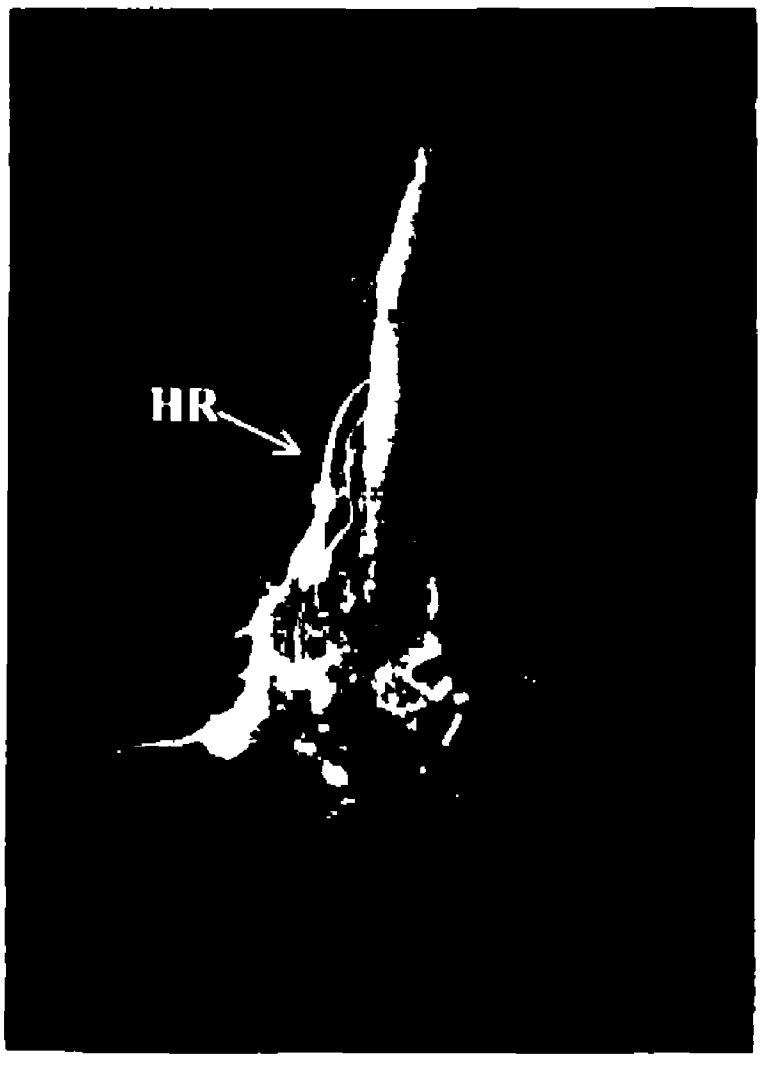
VIf Induction of hairy roots from seedling hypocotyls by direct inoculation method

Plate VI

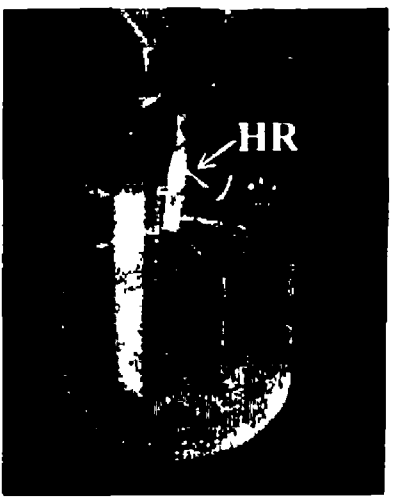
VIa

VIb

VIe



VIc



VId

VIf

Mano *et al* (1986) have reported slight callus formation on explants of *Scopolia japonica* on infection with *A rhizogenes* strains 15834, A_4, 1855 and 2659 After 4 weeks hairy roots were induced from the calli Inomata *et al* (1993) reported the induction of unorganized tissues upon infection of root segments of *Panax ginseng* with *A rhizogenes* Further these tissues many roots were generated Similarly, Benjamin *et al* (1993) reported the induction of tumors on multiple shoot cultures of *Rauvolfia serpentina* on infection with *A rhizogenes* strain 15834 The tumorous mass gave rise to hairy roots when cultured on MS medium or White's medium or LS medium In the present study, it was found that the callus mass obtained from infected seedling hypocotyls when placed in liquid MS medium induced hairy roots

Callus and disorganized morphology have often been observed on infection of explants with *A rhizogenes* (Williams and Ellis, 1993, Vozumi *et al*, 1994) In the present study, a high amount of callus formation in leaf explants, shoot buds and seedling hypocotyls after infection with *A rhizogenes* was observed

Toivonen and Rosengvist (1995) induced hairy roots from seedling of *Glycyrrhiza glabra* on infection with A_4 strain of *A rhizogenes*. They reported that the hairy roots did not form root hairs that are otherwise a typical feature of transformed root cultures of several species It was observed in the present study that the hairy roots obtained from seedling hypocotyls on treatment with NAA did not show presence of root hairs

The induction of hairy roots on addition of auxins during co-culture has been reported previously by Yoshikawa and Furuya (1987) and Aronen *et al* (1996) A steep rise in transformation frequency (25 % to 75 %) on addition of NAA in co-culture medium was observed in the present study

5.3 Confirmation of transformation

5 3 1 Morphology of hairy roots

Hairy roots are whitish in colour with numerous root hairs and show a high degree of lateral branching (Banerjee *et al*, 1995) Presence of many lateral root tips promotes rapid growth (Hamill *et al*, 1986, Hashimoto *et al*, 1986, Quattrocchio *et al*, 1986)

In the present study, the roots induced from seedling hypocotyls and shoot buds were whitish with numerous root hairs However, they did not show any lateral branching As a consequence, the growth rate of the roots was slow

The roots developed from unorganized calli induced when co-culture medium was supplemented with NAA, were dull whitish in colour turning brownish yellow on subsequent culture in liquid MS medium They lacked the presence of root hairs and lateral branching (Plate VIId)

Slight lateral branching was induced when NAA (10^{-1M}) was added to the culture medium The induction of lateral branching on hairy roots on IBA supplementation has been reported in hairy root culture of *Panax ginseng* (Yoshikawa and Furuya, 1987)

Aoki *et al* (1997) attributed the differences in morphological features of hairy roots to the differences in copy number and/or the position in the genome of plant where the T-DNA is integrated

5 3 2 Response to gravitropism

Banerjee *et al* (1996) have reported that the hairy roots generally showed negative gravitropism or reduced gravitropism, while normal roots show strong gravitropism

Plate VII Effect of NAA on hairy root induction

VIIa Response of seedling hypocotyls to 1 mg l^{-1} NAA

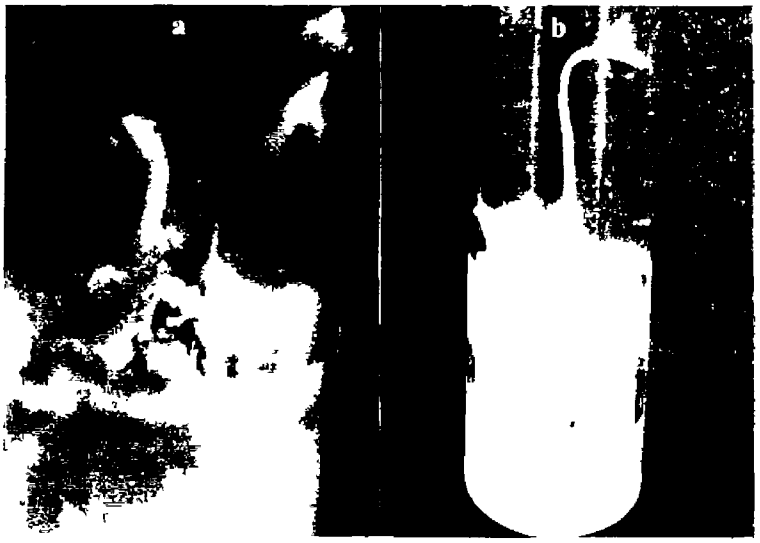
VIIb Response of seedling hypocotyls to 2 mg l^{-1} NAA

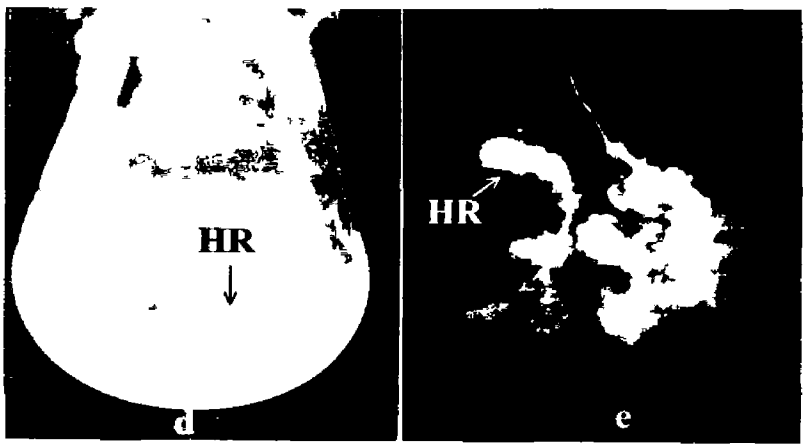
VIIc Response of seedling hypocotyls to 3 mg l^{-1} NAA

VIId Proliferation of hairy roots in liquid MS medium in dark

VIIe Hairy root induction on solid MS medium from disorganized callus formed due to NAA supplementation during co-culture

Plate VII





In the present study, the hairy roots induced from *Holostemma* explants showed reduced gravitropism while normal roots (obtained from the uninfected explants) showed strong gravitropism

5 3 3 Response to production of opines

Production of opines is a characteristic feature of hairy roots The presence of opines is a strong evidence of T-DNA transfer into the plant genome (Petit *et al*, 1983) The presence of opines in hairy roots have been reported by several workers who have utilized it as a strong tool for confirmation of transformation (Kamada *et al*, 1986, Patena *et al*, 1988, Kumar *et al*, 1991, Trypsteen *et al*, 1991, Dobigny *et al*, 1996) Opines are absent in normal roots (Petit *et al*, 1983)

In the present study, agropine was detected in the roots induced by infection of *A rhizogenes* strains 15834, P_cA_4 and A_4 (Plate IV) This is a strong evidence to support the confirmation of transformation

5 3 4 Sensitivity to auxins

Hairy roots are more sensitive to auxin than normal roots (Shen *et al*, 1988, Shen *et al*, 1990) Shen *et al* (1988) hypothesised that the modified response to auxin sensitivity of hairy roots can be attributed to the modification of the reception-transduction system of the auxin signal Guren (1987) suggested that the transformed cells have increased number of auxin receptor sites or an increased efficiency of the transduction system rendering them more sensitive to auxins They showed that the proton extrusion machinery of transformed cells was not affected whereas the effect of auxin on proton extrusion was drastically affected confirming that modification of response to auxin is due to early events of the hormone action such as reception or transduction

In this study, the transformed roots and normal roots showed different responses when treated with lower concentration of NAA The transformed roots showed a higher degree of callusing even at 10^{-11} M concentration of NAA and a minimal duration of callus induction phase where as the normal root did not induce any callus (Plate Va)

The transformed roots produced lateral branching at 10^{-9} M concentration of NAA where as the normal roots did not show any lateral branching (Plate Vb) The growth of hairy roots was induced on treatment with low concentrations of NAA that was not observed in the case of normal roots

These observations were on par with the earlier reports of lateral branch induction on treatment with auxin (Len *et al*, 1988 and Aronen *et al*, 1996) The reduced duration of callus induction phase in transformed roots may be due to higher cell division activity in transformed cells and the stimulation by NAA Similar observation was documented in the case of hairy roots of potato by Ottavini *et al* (1989)

5.4 Plant hormone like effect of antibiotics

The antibiotics viz cefotaxime and ampicillin were used for the elimination of bacteria from transformed roots and the infected explant tissues The toxicity of different concentrations of antibiotics or the effects of antibiotics on various explants of *Holostemma* was tested

The cefotaxime showed auxin like effect and induced callusing in all explants at 500 mg l^{-1} and above (Plate I) However, ampicillin did not show such effect below 2000 mg l^{-1} concentrations Neither antibiotic was toxic to explant tissues of *Holostemma* within the range of concentrations tested

The toxicity of antibiotics to plant cell cultures *Nicotiana plumbaginifolia* has been studied by Pollock *et al* (1982) They suggested that ampicillin has a

broader spectrum of activity and is relatively less toxic to plant cell cultures The lack of phytotoxicity of ampicillin has been noted by several other workers (Bancroft *et al*, 1975 and Phillips *et al*, 1981) Cefotaxime was reported to be toxic to protoplast cultures of *Nicotiana plumbaginifolia* only above 100 mg l^{1} (Pollock *et al*, 1982)

Lin *et al* (1995) studied the plant hormone effects of carbenicillin and cefotaxime They reported these antibiotics to have minimal toxicity to most plant tissues and that they are efficiently utilized to eliminate *Agrobacterium* cells They however found that both antibiotics showed hormone-like effect on the different plant tissues They attributed the auxin like effects of the two antibiotics to the structural similarity of the antibiotics to auxins They reported that carbenicillin was more toxic to plant cells above 1000 mg l^{1} due to high auxin activity levels The hormone-like effect of carbenicillin and cefotaxime has been reported by several other workers (Mathias and Boyd, 1986, Okkels and Pedersen, 1988 and Yazaki *et al*, 1988) This effect has been seen in the present study as well

5.5 Comparative evaluation of biochemical attributes

5 5 1 Amino acids in root tubers and callus

5 5 1.1 Method of application of samples

In the present study the plant sample and standard amino acids were applied as circular spots of various diameters The spot diameter and quantity of sample applied per spot affected the visibility of spots The combinations of 5 µl sample and 5 mm diameter, 10 µl sample and 8 mm diameter and 15 µl sample and 10 mm diameter were found to be the best for obtaining good spots, in both root tubers and callus

Hamilton and Hamilton (1987) have reported that, during the elution of chromatogram, in general, different compounds elute vertically upwards and

accumulate in the form of spots at their characteristic Rf values Compounds with similar elutropic abilities accumulated at adjacent Rf values Now, when the quantity of applied sample is high, the amount of each compound in the sample is also high This causes the spots or chromatograms to spread beyond their respective Rf values This causes mixing of adjacent spots, thus reducing the clarity of the chromatogram (Hamilton and Hamilton, 1987) When the quantity of sample increases even more, all the spots enlarge and merge with respective neighbouring spots to form big smear of compounds This results in poor visibility

The quantity of applied sample when coupled with the spot diameter, a clear chromatogram showing distinct spots can be obtained even at higher quantities of sample, with a larger spot diameter Touchstone and Dobbins (1978) have reported that the elution of sample is in a vertical direction When the running solvent system moves vertically upwards, it elutes all the compounds present in each vertical column Now, when a high quantity of sample is applied in the form of a bigger spot, the quantity of sample per unit vertical column remains the same So, even when the quantity of applied sample is increased with a proportionate increase in spot diameter we get good spots

5 5 1 2 Standardization of optimum concentration of spray reagent

The spray reagent 0 2 ninhydrin in acetone was found to be the best to detect amino acids in root tubers and calli of *Holostemma* When the concentration of ninhydrin increased, the colour intensity of the spots increased, thus improving the visibility Beyond 0 2 per cent concentration, the visibility did not show further improvement This suggested that the quantity of ninhydrin available to react with the amino acids might be insufficient at 0 1 per cent concentration When the concentration was increased, the quantity of ninhydrin reacting with amino acids increased and thus the visibility increased Since the content of amino acids in a specific sample was constant, once a threshold level of ninhydrin was available, all the amino acids reacted to show colour When the

concentration of ninhydrin was increased beyond the threshold level, the quantity of amino acids available for the reaction, being constant, no further increase in colour intensity was noticed The threshold level of ninhydrin was 0 2 per cent in the present study

5 5 1 3 Comparison of Rf values of amino acids in plant sample and standards

The comparison of Rf values of amino acids in plant samples and standards is given in Table 20 Table Rf values of amino acids in the callus and root tubers were same except that in callus an additional amino acid (Rf 0 48) was detected The probable amino acids were identified by comparison of Rf values of amino acids in plant sample and the amino acid standards (Table 20) In five spots, the Rf values of compounds matched with more than one amino acid standard In such a case, it was reported that either of the amino acid might be present

5 5 1 4 Comparison of amino acids in root tubers and callus

The root tubers and callus showed presence of six amino acids with similar Rf values and similar spot colour conforming the presence of same amino acids The callus showed the presence of an additional amino acid (Rf 0 48) with yellow coloured spot suggesting either the *de novo* synthesis of amino acids in *Holostemma* callus or that this amino acid has not been utilized in the metabolism of the callus tissues This may be the reason for the altered secondary metabolism observed in these tissues

5 5 2 Essential oils in root tubers and callus

5 5 2 1 Method of sample application

The plant samples and standards were applied as circular spots of varying diameter The quantity of sample applied per spot and the spot diameter affected

the visibility of spots The spot diameter of 5 mm and 15 µl sample, 8 mm diameter and 20 µl sample, 10 mm diameter and 30 µl sample and 15 mm diameter and 40µl sample gave good spots As stated earlier in 5 5 1, that the compounds present in a spot are eluted upwards by the running solvent system The quantity of sample unit vertical column, thus, needs to be constant, and at a particular threshold level Now on increasing the quantity of sample keeping the spot diameter constant or on decreasing the spot diameter keeping the quantity of applied sample constant, the quantity of sample per unit vertical column changes So a large variation is seen in the visibility of spots Doing this a combination of the spot diameter and the quantity of applied sample is obtained, such that, the quantity of sample per unit vertical column attains the threshold level and good spots are obtained

5 5 2 2 Standardisation of elution technique

Hamilton and Hamilton (1987) have stated that the success of a running solvent system having different solvents of various strengths to elute a particular sample depends upon the ratio of the solvents in the running solvent system and the nature of compounds present in the sample They have reported that the solvent strength parameter is ϵ° and it differs for each solvent The solvents are graded according to their ϵ°values (Touchstone and Dobbins, 1978 and Hamilton and Hamilton, 1987) More the ϵ° value more is the ability of the solvent to elute any compound to higher Rf Thus ϵ° determines the carrying capacity of the sample Solvents with lower polarity have higher ϵ° values and vice versa The solvents used in elution of essential oil are listed increasing elutropic abilities viz , methylene di chloride, chloroform, ethyl, acetate and finally n- propanol Methylene di chloride has the least ϵ° values while and n-propanol has the highest

Touchstone and Dobbins (1978) have stated that the Rf value of any spot depends on the polarity of sample itself, the eluting solvent and the adsorbent

(silica gel in this case) The theory states that, while moving upwards, the running solvents dissolve the compounds present in the sample and carry them upwards along with themselves A competition arises between the silica gel on the TLC plate and the running solvent for retention of the eluting sample Though both of them try to retain the sample, only the one having better ability to adhere will bond Adhesion of sample can also occur due to the attraction between the ions on the sample with those on the solvent or silica gel

Solvents with lower polarity have better adhesion to samples of lower polarity and vice versa When a competition occurs between the silica gel and the solvent, the low-polarity solvents retain the low-polarity samples while losing the highly polar solvents to the silica gel So the solvents of lower polarity carry samples of lower polarity along with them to greater Rf values Samples of higher polarity have more affinity to silica gel and hence adhere to the plate without much elution Thus a solvent of low polarity produces low Rf values for highly polar samples and high Rf values for low-polarity samples On contrary, polar solvents have high affinity for both non-polar and polar samples So they elute all the samples to higher Rf values Thus solvents with high E° move samples to produce spots of high Rf values

In the present study the initial ratio of running solvent system 45 45 4 5 4 5 could not elute the *Holostemma* sample satisfactorily However, when the quantity of highly polar methylene di chloride increased and the low polar ethyl acetate decreased, the separation of compounds and spot formation was satisfactory

5 5 2 3 Selection of suitable spray reagent

Harborne (1973) suggested the use of 0 2 per cent aqueous $KMnO_4$, 5 per cent antimony chloride in chloroform, concentrated H_2SO_4 or Vanillin – H_2SO_4 for the detection of essential oil They have also suggested selective agents like

bromine vapour (for detecting terpenes with double bonds) and 2,4-dinitrophenyl hydrazine (for detecting terpenes with ketonic groupings

From the above-mentioned reagents, vanillin- H_2SO_4 antimony chloride, bromine vapour and bromine- H_2SO_4 were used to detect essential oil Vanillin H_2SO_4 and antimony chloride gave more number of spots (Table 22a & 22b) as they detect terpenes, sesquiterpenoids, diterpenes, sugars and compounds with oxygen mediated bonds Bromine vapour gave less number of spots as it detects terpenes with double bonds only

5 5 2 4 Comparison of essential oils in root tubers and callus

From Table 22a and 22b it is quite evident that *Holostemma* callus produced lesser essential oils then that of the root tubers The callus and root tubers showed presence of similar compounds Vanillin – H_2SO_4 spray showed two spots (Rf 0 90 and 0 56) that were present both in root tubers ad callus The colour of the spots was same So these compounds may be the same Antimony chloride spray showed presence of two spots (Rf 0 85 and 0 65) in root tubers and callus The compounds in callus however, showed fluorescence, which was not seen in the case of tubers Thus, these compounds may be terpenes differing in their side chain structure Bromine vapour showed presence of similar compound (Rf 0 62) in callus and root tubers This characteristic result is a clear indication that the secondary metabolism in the callus tissue slightly altered from the normal metabolism in the root tubers at a particular stage(s)

5 5 3 Triterpenoids in root tubers and callus

5 5 3 1 Method of sample application

The spot diameter and the quantity of sample applied per spot, influenced the visibility The combinations, 20µl sample with 5 mm or 8mm diameter, 30

µl sample with 10 mm diameter and 40 µl sample with 15mm diameter gave good spots

As stated earlier in 5 5 1 a combination of the spot diameter and the quantity of applied sample per spot should be such that the quantity of sample per unit vertical column attains a threshold level At this threshold level we get good spots The combinations of spot diameter and quantity of sample applied, mentioned above, helped the amount of sample applied per unit vertical column to reach the threshold level and gave good spots

5 5 3 2 Standardisation of elution technique

The success of elution of compounds depends upon a proper combination of polar and non- polar components of the solvent system and also the adsorbent

In this study hexane- chloroform – methanol (1 3 1) gave good spots in the case of argentative, TLC, while the same did not work out for normal TLC In argentative TLC the silica gel (adsorbent) was washed free of chloride ions and impregnated with nitrate (NO_3) ions This helped in separation of triterpenoids on the basis of number of isolated double bonds present The running solvent system hexane – chloroform – methanol provided a perfect balance of more polar and less polar solvents so this solvent system in combination with argentative TLC showed good separation of spots

The running solvent system n butanol – 2M NH_4OH (1 1) was used specifically, to separate ∝- amyrin and β - amyrin by Attalah and Nicholas (1971) Ramiah *et al* (1981) have reported the presence of ∝ - amyrin in the root tubers of *Holostemma* In this study, the running solvent system n-butanol – 2 M NH_4OH was used in combination with normal TLC using silica gel as adsorbent It gave two good spots The two compounds may be ∝ - amyrin and β - amyrin When the above running solvent system is used, the amyrins that

differ in their basic structure at α and β positions show good separation due to lagging of one component during elution

5 5 3 3 Selection of suitable spray reagent

Lishoa (1969) and Neher (1969) suggested the use of Carr- Price reagent i e 20 per cent antimony trichloride in chloroform for the detection of triterpenoids This reagent was used in the present study to detect triterpenoids Vanillin – H_2SO_4 was also used

Either the reagents detect terpenes, diterpenes, sequiterpenes and triterpenes or sugars or oxygen mediated bonds So they gave more number of spots

5 5 3 4 Comparison of triterpenoids in root tubers and callus

From Table 26a and 26b it is quite evident that *Holostemma* callus produces lesser triterpenoids compounds when compared to tubers The normal TLC and argentative TLC showed the presence of similar compounds in root tubers and callus when Vanillin – H_2SO_4 was used as spray reagent. Argentative TLC showed a same spot (Rf 0 84), which had bluish colour in callus and root tubers It may be the same compound Similarly, antimony chloride spray showed similar triterpenoid showing whitish colour (Rf 0 13) in callus and root tubers, which may also be a similar compound

In normal TLC, Vanillin – H_2SO_4 when used as spray reagent, showed compounds with similar Rf values (0 97 and 0 22) in root tubers and callus Their colour however, differed which is an indication that the compounds differ only in their side chain structure The 1 per cent H_2SO_4 – ethanol spray and phenol – bromine spray showed presence of two compounds (Rf 0 13 and 0 93 respectively) in callus only, which suggests the *de novo* synthesis of triterpenoids

in *Holostemma* callus The difference in triterpenoid compounds observed in the root tubers and callus is a clear indication of the alteration in triterpenoid formation pathway in the callus

5 5 4 Sterols in root tubers and callus

5 5 4 1 Method of application of sample

The spot diameter and the quantity of applied sample per spot, influenced the visibility of compounds both in root tubers and callus The combinations 15 µl sample with 5 mm and 8 mm diameter spot, 20 µl sample with 10 mm diameter spot and 30 µl sample with 15 mm diameter spot gave good spots

The above combination helped in achieving the threshold quantity of sample per unit vertical column that is the basis for obtaining good spots by TLC (Hamilton and Hamilton, 1987, Touchstone and Dobbins, 1978)

5 5 4 2 Standardisation of elution technique

Among the various solvent systems used, chloroform or hexane alone could not elute the sample at all This may be due to the uneven polarity of these solvents and the polarity of the components in the sample However, a combination of chloroform – hexane (3 2) provided a perfect balance between the polarity of running solvent system and the polarity of components in the sample, giving good spots

5 5 4 3 Standardisation of spray reagent

Lisboa (1969) and Neher (1969) suggested the Carr-Price reagent i e 20 per cent antimony tri chloride in chloroform for the detection of sterols

Gritter and Albers (1962) mentioned the use of water for detecting steroids on TLC plate Ikan and Cudzinovski (1965) used H_2SO_4 water (1 1) for the detection of steroids

The later two reagents were tested for their ability to detect steroids Both of them did not show any spots So it can be concluded that steroids may be absent in the sample

P- anisaldehyde is a reagent that detects sterols and bile acids When the reagent was used, a bluish spot Rf (0 15) was detected This may be a sterol or bile acid

Concentrated sulphuric acid – ethanol – water (40 9 1) commonly referred as 'Allen's Test' for detecting steroids was used It detects 16 – dehydro steroids It did not show any spots So 16- dehydro steroids may be absent in the sample

5 5 4 4 Comparison of sterols in root tubers and callus

From Table 26 it is quite evident that *Holostemma* callus produced same sterols as that in the plant samples Bromine – H_2SO_4 spray and P-anisaldehyde spray showed the presence of spots with same Rf values (0 15 and 0 62 respectively) They had the same colour in the case of callus and root tubers So these compounds may be one and the same This characteristic result is a clear indication that the secondary metabolism of production of sterols was not hampered in the *in vitro* induced callus This reflects the potential of *Holostemma* callus to produce sterols of the mother plant *in vitro*

Summary

SUMMARY

The study entitled "Genetic transformation and hairy root cultures in Ada-kodien (*Holostemma ada-kodien* K Schum)" was carried out at the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara The salient findings of the study are stated below

1 Among the five *Agrobacterium rhizogenes* strains tested, the strains P_cA_4, 15834 and A_4 were able to induce hairy roots in *Holostemma ada- kodien*
2 Of the three successful strains, P_cA_4 showed a greater potential for transformation, followed by 15834 and A_4
3 Among the various explants used, the seedling hypocotyls and shoot buds were able to induce hairy roots
4 Seedling hypocotyls showed a greater percentage of hairy root formation than the shoot buds
5 The plant extract of *Holostemma ada-kodien* did not inhibit the growth of any of the *Agrobacterium rhizogenes* strains tried, but on contrary, enhanced the growth of all strains used in this study
6 Among the different infection methods tried, the co-culture method gave a greater transformation percentage
7 In the direct infection method, the form of bacterial inoculum used for inoculation and the amount of bacterial inoculum applied per wound, influenced the transformation frequencies
8 Bacterial suspension ($OD_{560\ nm}^{1}$) gave a transformation percentage of 7 14 whereas application of bacterial cells obtained form single cell colonies did not show any transformation of explants
9 In co-culture method of inoculation, the amount of bacteria present during co-culture and the co-culture time, affected the transformation frequencies
10 The highest transformation percentage (22 22%) was obtained by strain P_cA_4 when seedling hypocotyls were co-cultured with less amount of

bacteria for 24 h followed by co-culture with strain 15834 (14 28%) and A_4 (13 63%)

11 In the direct infection method, only seedling hypocotyls induced hairy roots whereas in co-culture method the seedling hypocotyls and shoot buds induced hairy roots

12 The shaker speed, in the co-culture method affected the transformation frequencies At 100 rpm, all the three strains viz P_cA_4 15834 and A_4 transformed the seedling hypocotyls and shoot buds and hairy roots

13 The transformation frequency of seedling hypocotyls was greatly enhanced by the incorporation of NAA in the co-culture medium A transformation percentage of 75 per cent was obtained when seedling hypocotyls were co-cultured with strain P_cA_4 in MS liquid medium containing 2 mg l^{-1} NAA

14 Among the different antibiotics tested, cefotaxime (250 mg l^{-1}) and ampicillin (250 mg l^{-1}) were effective for elimination of bacteria from the explant tissues

15 Among the different strengths and forms of MS medium tried, the explants induced hairy roots on MS solid medium (with full strength of all salts)

16 Photoperiod influenced the transformation frequencies

17 In direct infection method, hairy roots were induced only when given a photoperiod of 16 h light and no hairy roots were induced in dark

18 In co-culture method, the shoot buds produced hairy roots when cultured at a photoperiod of 16 h light whereas hairy root production was inhibited in dark The seedling hypocotyls produced hairy roots in both 16 h light and continuous dark conditions The transformation percentage in both 16 h light and continuous dark was almost the same

19 The antibiotic cefotaxime showed auxin like effects at concentration of 500 mg l^{-1} and above Ampicillin showed auxin like effects at 2000 mg l^{-1} concentration and above

20 One hairy root was produced per wound in both seedling hypocotyls and shoot buds irrespective of the bacterial strain and infection method used

21 Hairy roots were white in colour with numerous root hairs and showed neoplastic growth

22 Hairy roots produced upon incorporation of auxin (NAA) in co-culture medium were pale whitish and turned brownish yellow

23 In no case, did the hairy roots show lateral branching in MS basal medium

24 The hairy roots showed the presence of agropine while it was absent in uninfected (normal) roots

25 Hairy roots showed higher sensitivity to auxin than normal roots and induced lateral branching when supplemented with 10- 9M NAA

26 Roots of *Holostemma ada-kodien* showed the presence of six amino acids while the *in vitro* callus showed the presence of an additional amino acid (Rf 0 48)

27 Vanillin-sulphuric acid was found to be the best spray reagent for the detection of essential oils in *Holostemma* tubers and *in vitro* callus

28 The root tubers and *in vitro* callus of *Holostemma* showed different profile of essential oils although some similar compounds were noticed

29 Antimony tri chloride and vanillin-sulphuric acid were found to be the best spray reagents for the detection of triterpenoids in the root tubers and the *in vitro* callus of *Holostemma*

30 A different profile of triterpenoids was found in the root tubers of *in vitro* callus of *Holostemma*

31 Similar sterols were detected in the root tubers and callus of *Holostemma*

32 Bromine-sulphuric acid and P-anisaldehyde were found to be the best spray reagents for detection of sterols in *Holostemma*

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* Originals not seen

Appendix

APPENDIX-I

Composition of Murashige and Skoog plant tissue culture medium, used as basal medium in the study

Constituents	Concentrations (: mg l^{-1})
Inorganic	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2$ $2H_2O$	440
$MgSO_4$ $7H_2O$	370
KH_2PO_4	170
KI	0 83
HBO_3	6 2
$MnSO_4$ $4H_2O$	22 3
$ZnSO_4$, $7H_2O$	8 6
Na MoO_4 $2H_2O$	0 25
$CuSO_4$ $5H_2O$	0 025
CoCl $6H_2O$	0 025
$FeSO_4$ $7H_2O$	27 8
Na_2EDTA $2H_2O$	37 3
Organic	
Sucrose	30000
Inositol	100
Nicotinic acid	0 5
Pyridoxine HCI	0 5
Thiamine HCI	0 1
Glycine	2

APPENDIX-II

Composition of bacterial culture media used for the study

Constituents	Concentrations (mg l^{-1})	
	LB	YEM
Inorganic		
NaCl	-	100
$MgSO_4$ $7H_2O$	1000	200
KH_2PO_4	-	500
Organic		
Peptone	10000	-
Yeast extract	2000	400
Mannitol	-	10000

GENETIC TRANSFORMATION AND HAIRY ROOT CULTURE IN ADA-KODIEN

(*Holostemma ada kodien K. Schum*)

By

KARMARKAR SHIRISH HARI

ABSTRACT OF THESIS

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ABSTRACT

The present study was undertaken at the Department of Plantation Crops and Spices, College of Horticulture, Kerala Agricultural University Vellanikkara during May 1999 to October 2000 The study was undertaken to standardize the procedure to genetically transform *Holostemma ada-kodien* and induce hairy roots It was also envisaged to standardise the biochemical techniques for the estimation of secondary metabolites in the roots of *Holostemma*

Hairy roots were induced by infection of *Holostemma* explants with a gram negative soil bacterium, *Agrobacterium rhizogenes* Leaf segments, shoot buds, internodal segments, seedling hypocotyls and callus were used as explants for hairy root induction Among them, the seedling hypocotyls showed highest potential for hairy root induction followed by shoot buds Leaf segments, internodal segments and callus did not induce hairy roots Different strains of *Agrobacterium rhizogenes viz* P_cA_4, 15834, A_4, 8196 and 2659 were evaluated for their ability to induce hairy roots in *Holostemma* explants The strain P_cA_4 showed the highest potential for hairy root induction, followed by strains 15834 and A_4 The strains 8196 and 2659 did not induce hairy roots

Direct inoculation of bacteria on wounds induced hairy roots on seedling hypocotyls only Co-culture of wounded explants with bacteria induced hairy roots on seedling hypocotyls and shoot buds In the direct inoculation method, the nature of bacterial inoculum and the intensity of bacterial inoculum applied on wounds influenced the transformation Bacterial cell suspensions when applied on wounds induced transformation Less intensity of bacterial inoculum when applied on wounds gave greater transformation frequencies and vice versa In the co-culture method, the intensity of bacteria present during co-culture, the co-culture time and the shaker speed influenced the transformation Comparative evaluation of varying intensity of bacterial population during co-culture and the co-culture time showed that, both less intensity of bacterial inoculum with more co-culture time and vice versa showed almost similar transformation frequencies (14 40 % and 14 51 % respectively) The shaker speed of 100 rpm gave the

highest transformation percentage than 50 rpm speed Shaker speed of 150 rpm did not induce any transformation

Application of different concentration of NAA prior to direct inoculation of bacteria on wounded explants did not aid in transformation Addition of 2 mg l^{-1} NAA in the co-culture medium, however, increased the transformation frequency from 25 per cent to 75 per cent

Photoperiod influenced the transformation frequencies A photoperiod of 16 h light was found to be the best for hairy root induction in *Holostemma* Media influenced the hairy root induction Full strength MS medium favoured hairy root induction while ½ strength MS medium did not favour hairy root induction

Hairy roots were induced in a period of one to four weeks in all the treatments The induced hairy roots showed altered phenotypes The hairy root obtained directly from explants without NAA treatment were whitish, hairy and showed negative geotropism The hairy roots obtained after NAA treatment were brownish yellow and were induced from calli formed on wounds after infection Hairy roots obtained on infection with strains P_cA_4, 15834 and A_4 showed the presence of agropine confirming their transformed nature Normal roots did not show presence of opine(s) Hairy roots showed greater sensitivity to lower concentrations of NAA than the normal roots At 10^{-9} M concentration of NAA the hairy roots showed lateral branch formation

The tubers and *in vitro* induced callus of *Holostemma* were tested for the presence of amino acids, essential oils, triterpenoids and sterols Six amino acids were found in the root tubers while an additional amino acid (Rf 0 48) that may be L Proline or Cysteine MHC was found in the callus

Root tubers and callus showed the presence of essential oils, triterpenoids and sterols Root tubers showed the presence of more number of essential oils and triterpenoids than the callus The callus showed the presence of new terpene compounds The two sterols present in root tubers and calli were identified to be α-amyrin and β-amyrin