GENETIC TRANSFORMATION IN *ARTEMESIA ANNUA* L. FOR HAIRY ROOT INDUCTION AND ENHANCEMENT OF SECONDARY METABOLITES

By
SHANEEJA. V. M.

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(Plant Biotechnology)

Faculty of Agriculture
Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2007

DECLARATION

I hereby declare that this thesis entitled **"Genetic transformation in *Artemesia annua* L. for hairy root induction and enhancement of secondary metabolites"** 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.

Vellanikkara

SHANEEJA V.M.

CERTIFICATE

Certified that this thesis, entitled **"Genetic transformation in *Artemesia annua* L. for hairy root induction and enhancement of secondary metabolites"** is a record of research work done independently by Ms. Shaneeja V.M. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Vellanikkara

R. Keshavachandran

Dr. R. Keshavachandran
Chairman, Advisory Committee,
Associate Professor,
Centre for Plant Biotechnology and Molecular Biology,
College of Horticulture,
Kerala Agricultural University,
Thrissur

ACKNOWLEDGEMENT

I humbly bow my head before the ALMIGHTY who filled me with his Holy Spirit and blessed me with health, will power, courage, happiness, peace and everything needed for me to complete this endeavour successfully.

It is with great pleasure I express my heartfelt gratitude to Dr. R. Keshavachandran, Associate Professor, CPBMB, COH, Vellanikkara and the chairman of my advisory committee, for the constant support, encouragement, valuable guidance and care rendered to me during the course of my research work. I am really grateful for the keen interest taken by him in the prompt correction of the manuscript.

I am highly thankful to Dr. P. A. Nazeem, Associate Professor and Head, CPBMB, COH, member of my advisory committee, for the encouragement, valuable guidance and timely suggestions during the conduct of experiments.

I am extremely thankful to Dr. Samuel Mathew, Associate Professor, Aromatic and Medicinal Plants Research Station, Odakkali, member of my advisory committee for his timely help and valuable guidance. His vast experience and expertise in the field of biochemistry has contributed a lot to the successful conduct of the experiments.

I am grateful to Dr. Asha Sankar, Assistant Professor, Department of Plantation Crops and Spices, COH, member of my advisory committee for the constant encouragement and valuable suggestions during the course of my study.

The frequent enquiries and suggestions of Dr. D. Girija, Dr. A. Augustine, Dr. P. A. Valsala, Dr. P.C. Rajendran and Smt. Lissamma Joseph during the progress of work have helped a lot to add quality to the investigation.

I am grateful to Dr. P. Ahamed and Dr. George Thomas for their inspiring words and guidance during the study.

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Ms. Shaneeja V.M.,** a candidate for the degree of **Master of Science in Agriculture,** agree that the thesis entitled **"Genetic transformation in *Artemesia annua* L. for hairy root induction and enhancement of secondary metabolites"** may be submitted by Ms.Shaneeja V.M., in partial fulfilment of the requirement for the degree.

R. Keshavachandran

Dr. R. Keshavachandran
(Chairman, Advisory Committee)
Associate Professor
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture
Kerala Agricultural University, Thrissur

Dr. P.A. Nazeem
(Member, Advisory Committee)
Associate Professor & Head
Centre for Plant Biotechnology and
Molecular Biology
College of Horticulture
Kerala Agricultural University
Vellanikkara, Thrissur

Asha Sankar

Dr. M. Asha Sankar
(Member, Advisory Committee)
Assistant Professor
Department of Plantation Crops & Spices
College of Horticulture
Kerala Agricultural University
Vellanikkara, Thrissur

Dr. Samuel Mathew
(Member, Advisory Committee)
Associate Professor
Aromatic and Medicinal Plant Research Station
Odakkali, Ernakulam

EXTERNAL EXAMINER

ACKNOWLEDGEMENT

I humbly bow my head before the ALMIGHTY who filled me with his Holy Spirit and blessed me with health, will power, courage, happiness, peace and everything needed for me to complete this endeavour successfully.

It is with great pleasure I express my heartfelt gratitude to Dr. R. Keshavachandran, Associate Professor, CPBMB, COH, Vellanikkara and the chairman of my advisory committee, for the constant support, encouragement, valuable guidance and care rendered to me during the course of my research work. I am really grateful for the keen interest taken by him in the prompt correction of the manuscript.

I am highly thankful to Dr. P. A. Nazeem, Associate Professor and Head, CPBMB, COH, member of my advisory committee, for the encouragement, valuable guidance and timely suggestions during the conduct of experiments.

I am extremely thankful to Dr. Samuel Mathew, Associate Professor, Aromatic and Medicinal Plants Research Station, Odakkali, member of my advisory committee for his timely help and valuable guidance. His vast experience and expertise in the field of biochemistry has contributed a lot to the successful conduct of the experiments.

I am grateful to Dr. Asha Sankar, Assistant Professor, Department of Plantation Crops and Spices, COH, member of my advisory committee for the constant encouragement and valuable suggestions during the course of my study.

The frequent enquiries and suggestions of Dr. D. Girija, Dr. A. Augustine, Dr. P. A. Valsala, Dr. P.C. Rajendran and Smt. Lissamma Joseph during the progress of work have helped a lot to add quality to the investigation.

I am grateful to Dr. P. Ahamed and Dr. George Thomas for their inspiring words and guidance during the study.

I am extremely thankful to ILRA, France for providing Agrobacterium strains earlier. I am thankful to Sri. Sreekumar, Farm Assistant for the prompt help rendered in the photographic work.

I express my sincere thanks to Rejith, Beena chechi, Shylaja chechi, Shaju chetan, Pradeep chetan, Sujith, Jithesh, Aneesh, Firoz, Shyam, Premjith, Fathima chechi, Preetha, Seena, Simi, Sandhya, Mega, Bindu, Neena, Hridya, Salmath, Ambili and all the RAs of CPBMB for their wholehearted help, encouragement and support during the course of this study.

I specially thank my friends Mable, Resmy, Smitha Jose, Blessy Paul, Smita Nair, Rethi, Smisha, Sree, Seena, Jaliya, Lincy, Nisha, Sini and Cinu who were always with me and provided all the encouragement and help during the entire course of study. I am thankful to all my juniors for their wholehearted co-operation.

I owe special thanks to my friend Smini Varghese for her moral support and encouragement.

I am thankful to Savithri chechi, Sara chechi, Shanta chechi, Manju chechi, Bhavani chechi and Draupathi chechi, labourers of CPBMB for their prompt help during the course of study.

The award of KAU Merit Scholarship is gratefully acknowledged.

I am grateful to Santhosh chetan of the computer club for his help.

I lovingly recall the earnest interest and unfailing inspiration of my beloved parents, sister, husband and Sanju I am forever indebted for their love, care, prayers and moral support.

Shaneeja V.M.

CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS

%	Per cent
°C	Degree Celsius
µg	Micro gram
µl	Micro litre
µ*M*	Micro molar
A	Ampere
$AgNO_3$	Silver nitrate
A. rhizogenes	*Agrobacterium rhizogenes*
AH	*Aspergillus* homogenate
ATCC	American Type Culture Collection
BAP	Benzyl amino purine
bp	base pair
$CaCl_2$	Calcium chloride
CCC	Cycocel
CH_3CN	Methyl cyanide
C_6H_6	Benzene
CH_3COOH	Acetic acid
CH_3OH	Methanol
$CHCl_3$	Chloroform
cm	Centi metre
CTAB	Cetyl Trimethyl Ammonium Bromide
$CuSO_4$	Copper sulfate
CW	Coconut Water
DICA	Direct Inoculation and Co-cultivation with acetosyringone
DIM	Direct Inoculation Method
DMSO	Dimethyl Sulphoxide
DNA	Deoxy ribo Nucleic Acid
dNTP	Deoxy ribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EtOAc	Ethyl Acetate
$FeCl_3$	Ferric Chloride
FW	Fresh weight
$g\ l^{-1}$	Grams per litre
GA_3	Gibberellic acid
GFP	Green Flouresecnt protein
GUS	β- glucuronidase
hrs	Hour (s)
H_2SO_4	Sulphuric acid
H_3PO_4	Phosphoric acid
ha	Hectare

HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
HPLC	High Pressure Liquid Chromatography
HVPE	High Voltage Paper Electrophoresis
IAA	Indole acetic acid
IBA	Indole butyric acid
IMTECH	Institute of Microbial Technology
kD	Kilo Dalton
kg	Kilo gram
KN	Kinetin
LBA	Luria Bretani Agar
LS	Leaf segment
lux	lux
M	Molar
MeOH	Methanol
mg l^{-1}	Milligrams per litre
min	Minute (s)
ml	Milli litre
mm	Milli metre
m*M*	Milli Molar
MS	Murashige and Skoog's medium
MTCC	Microbial Type Culture Collection
N	Normal
N	Nitrogen
NA	Nutrient Agar
NAA	Naphthalene acetic acid
Na_2SO_4	Sodium sulfate
NaOH	Sodium hydroxide
NaH_2PO_4	Monosodium phosphate
$Na_2H\ PO_4$	Disodium phosphate
NH_4^+	Ammonium
NH_4OAc	Ammonium acetate
Nm	Nano metre
NO_3	Nitrate
OD	Optical Density
PCR	Polymerase Chain Reaction
PEG	Poly ethylene glycol
pH	Negative log of hydrogen ion concentration
ppm	Parts per million
psi	pounds per square inch
rpm	revolutions per minute
$SbCl_3$	Antimony chloride
SCA	Suspension culture and Co-cultivation with acetosyringone

SDS	Sodium dodecyl sulphate
sec	second (s)
SM	Suspension culture method
SSC	Sodium chloride and sodium citrate
ST	Shoot tip
TAE	Tris acetate EDTA buffer
T-DNA	Transfer DNA
TDZ	Thidiazuron
TE	Tris EDTA buffer
TLC	Thin Layer Chromatography
TL-DNA	Transfer (Left) DNA
TR-DNA	Transfer (Right) DNA
UV	Ultra violet
v	Volume
V/ cm	Volt/ cm
WPM	Woody Plant Medium
W/V	Weight/ Volume
XM	Xanthomonas Media
YE	Yeast extract
YEB	Yeast Extract Broth
YEM	Yeast Extract Mannitol
YEP	Yeast Extract Peptone
YMB	Yeast Mannitol Broth
α	Alpha
2,4-D	2, 4-dichlorophenoxyacetic acid
2,ip	2, isopentenyl adenine

Dedicated to My Family

Introduction

1. INTRODUCTION

Plants are the traditional source for many chemicals used as pharmaceuticals, biochemicals, fragrances, food colours and flavors. Most valuable phytochemicals are products of plant secondary metabolism and possess sufficient chemical or structural complexity to make artificial synthesis difficult.

Artemisinin is the anti malarial principle isolated from annual wormwood *Artemesia annua* L. belonging to family Asteraceae. Chinese, in their traditional system of medicine have used the plant since ancient times for the treatment of malaria and fever. Artemisinin was isolated and structurally characterized by Chinese scientists in 1972 and shown to be a sesquiterpene lactone (Klayman, 1985). *Artemesia annua* is now among the top ten pharmaceutical crops, which are the sources of compounds that are being developed as life saving anti malarial drugs (Gupta *et al.*, 1997).

Every year more than 50 per cent of world population is exposed to malaria and there are three million deaths due to it. The present level of annual global incidents of malaria continues to be about 500 million. Malaria is endemic in as many as 91 countries. Around 800,000 children under the age of five die from malaria every year making this disease one of the major causes of infant and juvenile mortality (Walwalkar, 2004).

Malaria thus has social consequences and is a heavy burden on economic development. Currently large populations in India and parts of South East Asia are infested with the malarial parasite. Death due to malaria are occurring in increasing number because of frequent failure of conventional treatment using drugs such as chloroquinone and sulphadoxine against *Plasmodium falciparum*. However artemisinin resistant malaria is not at all recorded, thus increasing its significance as an anti malarial drug.

World Health Organization has recognized the use of Artemisinin Combination Therapies (ACTs) in the treatment of malaria as a long term measure to

control spread of disease under its Roll Back Malaria (RBM) programme. The gross insufficiency in supply of ACTs at present time is related to short supply of artemisinin and its derivatives and the high cost. Production of artemisinin is only upto one third of the need (Kumar and Sreevastava, 2005).

Artemisinin is mainly isolated from leaves and flowery tops of *Artemesia annua.* Artemisinin production in shoot is enhanced by the presence of root.

Artemisinin and its derivatives artemether, arteether dihydro artemisinin and artesunate are reported as potent drugs against chloroquinone and mefloquine resistant malarial parasite *Plasmodium falciparum* and cerebral malaria. Artemisinin has also been named in China as "Huanghauahaosu", "arteannuin" and quinghaosu. The name artemisinin has been adopted by *chemical abstracts* and has been proposed as International Non Proprietary Name (Trigg, 1989).

The herb is native to China and now grows in many countries such as Australia, Argentina, Bulgaria, France, Hungary, Italy, Spain and the United States (Chen and Zhang, 1987). In India it is introduced and being cultivated in the Himalayan region. It is cultivated on an experimental scale in temperate as well as subtropical conditions (Ram *et al.*, 1997).

To meet the new artemisinin supply challenge, *Artemesia annua* cultivation requires to be extended to geographical areas where high yields of artemisinin can be obtained. The relatively low concentration of artemisinin (0.01-0.5%) in *Artemesia annua* is however a serious limitation to the commercialization of the drug. Being unstable at high temperature due to the presence of endoperoxide bridge, the chemical synthesis of this compound is uneconomical and difficult (Abdin *et al.*, 2003).

In order to get 40 Kg artemisinin, *A.annua* should be cultivated in one hectare. To meet the global requirement, area of cultivation should be 20,000 hectares (Kumar and Sreevastava, 2005). Thus increasing area of cultivation and chemical synthesis is difficult. Therefore enhanced production of artemisinin in cell or tissue culture is desirable.

The soil born plant pathogen *Agrobacterium rhizogenes* is the causative agent of hairy root disease of plants. Hairy root cultures have several properties that have promoted their use for plant biotechnological applications. Their growth is fast and they ensure greater genetic stability. So they can be used as an alternative to plant cultures for the production of secondary metabolites of interest.

Only a few studies have been carried out for the induction of hairy roots in *Artemesia annua* L. Hence the present study on genetic transformation in *Artemesia annua* for hairy root induction and enhancement of secondary metabolites was carried out with the following objectives.

1. To standardize *in vitro* regeneration protocol in *Artemesia annua* from different explants
2. To standardize the genetic transformation in *Artemesia annua* using *Agrobacterium rhizogenes*
3. To standardize the biochemical techniques for estimation of secondary metabolites in *Artemesia annua*
4. To enhance the secondary metabolite production in hairy root cultures of *Artemesia annua* employing different techniques.

Review of Literature

2. REVIEW OF LITERATURE

Plant secondary metabolites serve as basic ingredients in production of important substances ranging from pharmaceuticals to flavorings. Many of these natural compounds cannot be easily synthesized artificially and must be extracted from the plants. Secondary metabolites are biosynthesized in multistep enzymatic reactions in specifically differentiated cells and production is often low.

Various strategies have been employed to improve the production of secondary plant compounds in *in vitro* systems. However the undifferentiated plant cells tend to be genetically unstable in culture and the metabolites they produce are sometimes chemically different from those produced in whole plants. They are often complexed with other substances making their extraction and use difficult. The strong correlation between secondary metabolite production and morphological differentiation laid more emphasis on plant organ culture as one of the alternate approaches. Plant roots seem to be most suitable for large-scale cultivation since roots are sites of synthesis or storage of certain chemicals of pharmaceutical importance (Kukreja *et al.*, 1997).

Slow growth rate of root is another limitation in commercialization of technology using root biomass as a source for secondary metabolite production. Hairy roots are fast growing and genetically stable and can grow in a hormone free media. The role of hairy roots for the production of plant chemicals of interest has been well documented (Doron, 1989). Hairy roots produce secondary metabolites at a level equal or higher to those of field grown plants. A number of plant species have been successfully transformed with *Agrobacterium rhizogenes*. *Artemesia annua* is a medicinal plant used for centuries in Chinese medicine against fever and malaria. The active principle artemisinin, a sesquiterpene lactone endoperoxide is a very powerful anti malarial compound also active against multi drug resistant *Plasmodium falciparum* strains. The high demand for artemisinin however cannot be met due to unavailability of resources. Since the natural production in plant is rather low, different approaches have already been made to increase the production of this drug.

From all reported attempts, overproduction of artemisinin in transgenic plants seems to be the most appropriate method (Van-Geldre *et al.*, 1997; Dhingra *et al.*, 2000). Literature available on these aspects are presented in this chapter.

2.1 Genus *Artemesia*

The genus *Artemesia* is one of the largest of Asteraceae family consisting of more than 800 species all over the world. In this genus, many species have been used since ancient times as folk remedies are and credited with a long last of medicinal uses, including anti malarial, antiviral, antitumour, spasmolytic and others (Tan *et al.*, 1998).

Artemesias generally known as sagebrush or wormwood belong to a genus of small herbs and shrubs and are found in temperate regions. The genus Artemesia is a rich source of sesquiterpenoids, which are receiving renewed phytochemical attention due to biological and chemical diversity. About 37 species are reported from India (Ali *et al.*, 2000).

2.1.1 Important medicinal plants under the genus *Artemesia* other than *Artemesia annua*

Artemesia californica. Less (California sage brush) produces camphor in addition to 1, 8 cineole and six other terpenoids (Halligan, 1975). *A. annua* appears to be the only species under the Genus that contain appreciable amounts of artemisinin. Scientists have reported that extracts from other species of *Artemesia* did not show anti malarial activity (Klayman, 1985). Balachandran *et al.* (1987) did not find artemisinin in various species of Indian origin.

Plant species of genus *Artemesia* that are better known than *A. annua* include *A. absinthium* used until 1920's to prepare the narcotic and now the illegal drink absinthe; *A. dracunculus* also known as tarragon, mugwort or dragon used as a spice in cooking and to flavour vinegar, sauce and salad dressing; and *A. tridentata* called sagebrush in Western United States (Klayman, 1985).

A. absinthium L. is commonly known as absinth wormwood produces three sesquiterpene phytotoxins β carophyllene, bisabolene and chamzuline (Grummer, 1961). The essential oil of *A. absinthium* contains mainly epoxyocimene, β thujone and many other compounds. It is having antifungal and anti bacterial property (Juteau *et al.*, 2002).

A. brevifolia contains santonin and it is having antihelminthic property.

A. gmelinii: Leaves of this plant is used for curing abdominal pain. The active principles are umbelliferone and genkwanin.

A. macrocephala jacq ex Bess: This herb is applied as a paste on joints to cure rheum and other pains.

A. maritime Linn *var fragrans*: Its santonin free essential oil is mainly used in perfumery.

A. maritime Linn *var thomsoniana*: These are santonin yielding. Flowers and tops are antihelminthic, useful in ascites and in high cough, applied against inflammation.

A. nilgirica synonym *A. vulgaris* Linn *var nilgirica* commonly known as Indian wormwood is used as febrifuge.

A. pallens: It is common in South India, known as Davana. Essential oil called oil of Davana is used in perfumery.

A. parviflora Roxb: This plant is found in greater parts of India. It contains coumarins. It is diuretic and anti viral.

A. persica Boiss: Leaves and stem yield scopoletin.

A. roxburghiana synonym is *A. grata*: Aerial parts of this plant contain hentriacontane, hentriacontanol, α-amyrin and β- amyrin.

A. vesitia: The plant is antihelmenthic, antiseptic. Essential oil is anti bacterial and anti fungal (Asolkar *et al.*, 1992).

A. sieversiana gives guanolide and two nonflavones (Chrysoplenetin and 5-hydroxy-3, 4,6,7-tetramethoxy flavones) (Tang *et al.*, 2000).

A. scoparia Waldst: Flower head contains scoparone. Volatile oil contains 73 components. The plant extract is used as purgative, to treat ear ache and fever and its smoke is used for burns. Terpinene and euginol are the main components of oil (Ali *et al.*, 2000).

2.2 *Artemesia annua*

The Chinese have used *Artemesia annua* L for the treatment of fever. Its active principle artemisinin, a sesquiterpene lactone is an active anti malarial drug (Klayman, 1985). It is used for reducing temperature and bringing down fever. In China, it is commonly known as Ginghao. The plant is now among the top ten pharmaceutical crops, which are the sources of compounds that are being developed as life saving anti malarial drugs (Gupta *et al.*, 1996). *A. annua* L. is an annual plant found as both wild and naturalized predominantly in temperate regions of world. The plant is also known commonly as Sweet Annie or Annual wormwood (Ferreira and Janick, 1996).

2.2.1 Botany of *A.annua* L.

A.annua plants are erect, glabrous and strong scented annuals. Stems are deeply grooved, leaves broad, three pinnatisect or decompound. The branches at the top bear the inflorescence with the capitulum as a basic unit. The capitula are inconspicuous, hemispherical, pendulous and about 1.5-3 mm in diameter (Bagchi *et al.*, 1997).

2.2.2 Climate and Soil

A.annua is adapted to a wide variety of soils from sandy loam to loam having proper drainage system. But light textured soils are ideal for its cultivation. It is a temperate plant, which requires cold winter and moderate summer. However it can also be cultivated as winter-summer crop, under subtropical conditions. It cannot be grown in low-lying areas prone to water logging (Ram *et al.*, 1997).

2.2.3 Cultivated areas

The herb *A.annua* L. is native to China and now grows in many countries such as Australia, Argentina, Bulgaria, France, Hungary, Italy, Spain and the United States (Chen and Zhang, 1987).

In India, the plant cultivated in Lucknow showed lower artemisinin content than those grown in Kashmir valley (0.1%) (Jha *et al.*, 1988). The plants grown in Kashmir valley contain good amount of artemisinin and viable achenes, the seed bearing fruits (Sharma *et al.*, 1991).

2.2.4 Chemical constituents of *A. annua*

You *et al.* (1982) isolated quinghaosu IV and V, quinghao acid, chrysophenol and paraffinic alcohol from *A. annua.* Artemisinin concentration varies from 0.05 to 0.2 per cent. The other main constituents are arteannuin A and arteannuin B, artemisic acid (artemisinic acid), artemesia - ketone, benzyl isovalerate, borneol acetate, cadinene, camphene, coumarin, scopoletin, scopalin and stigmasterol (Trigg, 1989).

Analysis of *A.annua* by chromatography resulted in isolation of 59 constituents. Volatile oil contains high amount of monoterpenes of which 1,8 cineole is the most predominant one. It contains α thujene, camphor and many other compounds (Ali and Siddiqui, 2000).

The phytochemicals isolated from different parts of the plant are different in different places. Chemical constituents are sesquiterpenes, triterpenes, coumarins, flavanoids and many others. *A.annua* leaves contain 3 per cent terpenoid. Compounds like absicic acid, annuic acid, artemisinic acid (0.005% to 0.6%), arteannuin A, arteannuin C (0.002%), artemisinin (0.05% - 0.2%), scopolin (0.004% to 0.02%), zeatin and stigmasterol are isolated from aerial parts of plants cultivated in India (Bhakuni *et al.*, 2001).

2.2.4.1 Sesquiterpenoid lactone artemisinin

Artemisinin (qinghaosu, QHS) is a sesquiterpenoid lactone peroxide naturally formed in *A. annua* plant. No other organism is known to synthesize artemisinin. Artemisinin production by *A. annua* is usually in the range of 0.01 to 0.4 per cent but some clones produced over 1 per cent. Artemisinin can also be obtained from artemisinic acid, which occurs at concentrations as much as ten fold than artemisinin (Acton *et al.*, 1985).

Artemisinin has been detected from aerial parts of the plant mostly in leaves and inflorescence with low levels in stems and none in pollen or roots. The occurrence of artemisinin in achene is due to the presence of floral remnants (Ferreira *et al.*, 1995). Artemisinin has been reported in green stems, buds, leaves, flowers and seeds (Ferreira *et al.*, 1995; Leirsch *et al.*, 1986). Although some authors reported artemisinin being highest during preflowering stages (Acton *et al.*, 1985; Leirsch *et al.*, 1986; El-Sohly, 1990; Woerdenbag *et al.*, 1990; Woerdenbag *et al.*, 1994), others reported artemisinin reaching its peak during full flowering (Singh *et al.*, 1988; Pras *et al.*, 1991; Morales *et al.*, 1993; Ferreira *et al.*, 1995; Laughlin, 1995).

Reports on distribution of artemisinin throughout the plant have been inconsistent. Artemisinin has been reported to be higher at the top of the plant in some clones (Charles *et al.*, 1990; Laughlin, 1995) and equally distributed in others (Laughlin, 1995). Artemisinin content in full bloom flowers were 4-5 times higher than in leaves (Ferreira *et al.*, 1995).

The content of artemisinin in *A.annua* has been reported to vary widely from 0.03 to 0.22 per cent (dry weight basis) in European wild plants (Delabays *et al.*, 1993), from 0.05 to 0.21 per cent (dry weight basis) in USA (Charles *et al.*, 1990), from 0.01 to 0.5 per cent (dry weight basis) in China, from 0.02 to 0.16 per cent (dry weight basis) in Indian cultivars. The highest level of artemisinin 0.86 per cent (dry weight basis) was reported in Vietnamese cultivars (Woerdenbag *et al.*, 1994). In temperate climate of Kashmir valley the average artemisinin content in leaves was 0.1 per cent while the leaves collected from the subtropical climate of Lucknow contains 0.06 per cent (Singh *et al.*, 1986). Unlike quinine and chloroquine artemisinin is non-toxic, rapid in effect and safe for pregnant woman (Usha and Swami, 1998). Oil of Artemisia is of interest to the perfumery, cosmetics industries and for dermatology. The oil is quite useful pharmaceutically because of its dermatological and specific fungicidal properties (Usha and Swami, 1998). Artemisinin content was shown to be 4 to 11 times higher in the inflorescence as compared to leaves and presence and development of glandular trichomes in the inflorescence was associated with artemisinin production (Ferreira *et al.*, 1995). After anthesis, artemisinin decreases and so does the number of intact glands. The association of artemisinin with glandular trichomes sequestration explains why artemisinin was not detected in parts of plant that do not bear glands such as pollen or roots.

2.2.4.2 Biological activity of artemisinin

Biological activities reported for the compounds isolated from *A.annua* are as follows.

Artemisinin is active against systemic lupus erythematosus and virusatic against influenza virus in chick embryos (Klayman, 1985). Artemether markedly reduced worm loads of *Schistosoma mansonni* in mice and dogs (Le *et al.*, 1982). Artemisinin and two of its derivatives artemether and artesunate have been shown to be effective in both chloroquine sensitive and chloroquine resistant *Plasmodiun falciparum* infection (Luo and Shen, 1987).

Artemisinin is a selective phytotoxin with herbicidal activity approximately equal to that of cinmethylin in some species. It inhibited germination of lettuce and growth of purslone, lettuce and *Ipomoea lacunose*. Artemisinin is apparently toxic to annual wormwood (Duke *et al.*, 1987). The growth inhibition caused by artemisinin appears to be due to a mechanism other than disruption of mitosis or amino acid synthesis or inhibition of respiration.

Artemisinic acid, a well-known precursor for semi synthesis of artemisinin has shown antibacterial activity (Roth and Acton, 1989). The plant exhibits anti oxidative activity and causes cytotoxicity on P 388 and L 1210 leukemia cell lines (Wei-chen *et al.*, 1992). Artemisinin related trioxane have anti HIV activity (Jung and Schinazi, 1994).

Scopoletin, a coumarin isolated from *A. annua* has been reported to possess anti-inflammatory activity. Artemisinin was also found to be effective against avian coccidiosis. It has been reported to cause significant reduction in the lesions caused by *Eimeria tenella* (Oh *et al.*, 1997).

Precursor of artemisinin, arteannuin B inhibited the growth of human pathogenic fungus *Candida albicans* and phyto pathogens *Gaumannomyces graminis* var *tritici, Rhizoctonia cerealis, Gerlachia nivalis* and *Verticillium dahliae* (Tang *et al.*, 2000).

Artemisinin and its derivatives have been reported to cause inhibition of plaque formation in *Toxoplasma gondii* and are active against *Leishmania major*. Artemisinin, arteannuin B and artemiscinic acid showed strong anti microbial activity against most of the organisms. Strong anti fungal activity was observed against *Aspergillus niger, Fusarium sp, Aspergillus flavus, Trichoderma viridae.* The sesquiterpene also showed positive response against yeast *Saccharomyces cerevisiae* (Dhingra *et al.*, 2000).

Selective phytotoxicity has encouraged development of artemisinin and its derivatives as novel and effective drugs for human cancers (Li *et al.*, 2001, Singh and Lai, 2001).

2.3 APPLICATION OF *IN VITRO* PLANT REGENERATION IN *Artemesia annua* L.

Plant tissue has long been recognized as an efficient tool for rapid clonal propagation. *In vitro* culture of plants has gained considerable importance during recent years in view of their possible application to the production of known and new aromatics. The plant regeneration systems with high efficiency are desirable for application of current practical studies and genetic transformation (Muthuvel *et al.*, 2005).

The organic synthesis of artemisinin although has been achieved, involves complicated steps and yields are very low. This renders the process economically unattractive. Artemisinin is unstable at high temperature due to the presence of endoperoxide. Commercial isolation of drug from *A.annua* has been limited because of the relative low yield. In addition, the artemisinin content extractable from leaves and flowering tops of the plant is influenced by several factors that include genotype, physiological conditions and geographic locations of plants (Acton *et al.*, 1985; Liersch *et al.*, 1986).

Plant is normally propagated through seeds. Local climatic conditions affect the viability of seeds. Plants, which were introduced to Calcutta, produced luxuriant growth with non-viable seeds (Singh *et al.*, 1988; Jha *et al.*, 1988).

The current global production of artemisinin is not adequate. A biotechnological approach has been considered to be an alternative for the production of artemisinin (Singh *et al.*, 1988; Pras *et al.*, 1991). *In vitro* methods therefore are now being exploited for the production of large number of propagules under controlled conditions and to assess the yield of artemisinin. As rapid large scale multiplication and flowering are essential prerequisites to enhance artemisinin

synthesis, *in vitro* propagation, flowering and artemisinin estimation at specific stages is extremely important (Gulati *et al.*, 1996).

2.3.1 *In vitro* distribution of artemisinin

Many investigators have successfully micropropagated *A. annua* although there are inconsistencies in literature regarding the presence of artemisinin in different organs and tissues of *in vitro* grown plants. Artemisnin is produced by differentiated shoot cultures (Martinez and Staba, 1988; Fulzele *et al.*, 1991; Whipkey *et al.*, 1992; Ferreira and Janick, 1996) but occur only in trace levels, if at all, in shoots without roots (Martinez and Staba, 1988; Jha *et al.*, 1988; Woerdenbag *et al.*, 1993; Paniego and Giulietti, 1994). Most workers (Martinez and Staba, 1988; Tawfiq *et al.*, 1989; Kim *et al.*, 1992) did not detect artemisinin in roots, although Nair *et al.* (1986) and Jha *et al.* (1988) reported trace amounts. It has been reported that low yield of artemisinin is produced from roots and rooted plantlets derived from leaf explants or callus cultures (Nair *et al.*, 1986).

Martinez and Staba (1988) reported an increase in artemisinin content *in vitro* when plants developed a root system. Ferreira and Janick (1996) reported that there is direct relationship between artemisinin production and rooting. Although there was no relationship between the number of shoots and artemisinin content, there was a significant correlation between root number and artemisinin content. The presence of extensive rooting in liquid medium was associated with both increased artemisinin production and concentration in shoots as compared to plants without roots. The role of roots in increasing shoot artemisinin is unclear. The possibility that roots may provide a precursor or promoter of artemisinin production that is translated to shoots warrants study (Ferreira and Janick, 1996).

Woerdenbag *et al.* (1991) reported presence of arteannuin B and artemisinic acid in roots of field grown plants. But they were not detected in *in vitro* roots grown in liquid or semisolid medium (Ferreira and Janick, 1996). Artemisinin is not present in roots, both *in vivo* and *in vitro* ((Ferreira *et al.*, 1995). Recent evidence indicates that artemisinin is compartmentalized and sequestered in glandular trichomes

present in leaves (Duke and Paul, 1993; Duke *et al.*, 1987) and flowers of *A. annua* (Ferreira and Janick, 1995).

Ferreira and Janick (1996) reported that specialized glands require roots to reach their full potential for artemisinin biosynthesis. Artemisinin is phytotoxic even to *A. annua* itself. This may be the reason for trace amounts or no artemisinin in non-glandular tissues (Duke *et al.*, 1987). Kudakasseril *et al.* (1987) reported that cell free system from shoot cultures and plants of *A. annua* contain enzyme related to artemisinin biosynthesis.

2.3.1.1 Artemisinin in cell cultures

Ferreira and Janick (1996) reported that there is no artemisinin in cell cultures, which is in agreement with results obtained by Liu *et al.* (1992) and Paniego and Giulietti (1994) who reported either trace or no artemisinin in cell cultures. However, Nair *et al.* (1986) reported 0.8 per cent (W/V) artemisinin in liquid culture medium.

2.3.2 Status of *in vitro* propagation in *A.annua*

A number of tissue culture studies have been conducted with the aim of inducing various target organs from a number of explant sources.

2.3.2.1 Source of explant

2.3.2.1.1 In vitro source

A. annua plant tissue cultures can be established from aseptically germinated seeds or seedlings (Martinez and Staba, 1988; Jha *et al.*, 1988; Tawfiq *et al.*, 1989; Fulzele *et al.*, 1995; Gulati *et al.*, 1996; Paniego and Giulietti, 1994; Usha and Swamy, 1998) and from aseptic explants such as stems, leaves or flowers (He *et al.*, 1983; Martinez and Staba, 1988; Nair *et al.*, 1986) hypocotyl and roots (Da Silva, 2003; Paniego and Giulietti, 1994).

2.3.2.1.2 Ex vitro source

Leaves and petiole segments from two-month-old field grown plants can be used as explants (Jha *et al.*, 1988). Any part of the plant can be induced to produce unorganized cells and tissues and organized shoot or root cultures in *A. annua* (Martinez and Staba, 1988).

Gulati *et al.* (1996) reported that different types of explants from field grown plants like stem, leaves, petioles, mature and immature inflorescence can also be used to induce regeneration. Using nodal explants from inflorescent segments, an efficient protocol has been developed for cyclic supply of *in vitro* micropropagated plants of *A.annua* (Mathur, 1998).

The parameters such as uninterrupted availability of explants, its dormancy status, polyphenol exudation level, uniformity and ease of decontamination should be taken in account while selecting explants for regeneration (Tiwari *et al.*, 2003).

2.3.2.2 Surface sterilization of explants

2.3.2.2.1 Seeds

Seeds of *Artemesia annua* previously soaked for 24 hrs. were sterilized in undiluted sodium hypochlorite solution (5%) for 2 min (Martinez and Staba, 1988), 0.1 per cent $HgCl_2$ treatment for 10 min (Jha *et al.*, 1988), 2 per cent sodium hypochlorite containing 1 per cent Triton X 100 for 15 min (Tawfiq *et al.*, 1989), immersed in 70 per cent ethanol followed by stirring for 20 min in 3 per cent W/V sodium hypochlorite solution (Woerdenbag *et al.*, 1993), 0.05 per cent $HgCl_2$ for10 min (Paniego and Giulietti, 1994) and washed with sterile water.

A. annua seeds were surface sterilized first with Dettol (3 min) and 70 per cent ethanol (2 min) followed by 0.1 per cent $HgCl_2$ (2 min) and subsequently washed with sterile water (Fulzele *et al.*, 1995). Ten per cent sodium hypochlorite followed 0.1 per cent $HgCl_2$ for 7 min and 70 per cent alcohol for 30 min (Gulati *et al.*, 1996) or washing with a detergent for 3 min and 70 per cent alcohol for 2 min followed by

$HgCl_2$ for 5 min (Usha and Swami, 1998) were also used to surface sterilize seeds of *A. annua.*

2.3.2.2.2 Other explants

Nair *et al.* (1986) reported that leaves, flower buds and stems were severed and washed under tap water for 15 min with sodium hypochlorite solution containing a detergent (10ml Chlorox and few drops of 1 per cent solution of Triton X 100 diluted to 100ml with double distilled water) and finally washed three times with sterile double distilled water.

Lamina and petiole from field grown plants of *A. annua* were sterilized in 0.1 per cent $HgCl_2$ for 5 min and rinsed 5 times with sterilized distilled water and cultured on semi solid medium (Jha *et al.*, 1988).

Leaves, stems, buds or flower explants from *A. annua* plants were sterilized with 70 per cent ethanol for 1-10 min and then sodium hypochlorite 0.5 per cent for 10-15 min. About 4.8 mm sized segments of explants like leaves, flower buds and stem were used for inoculation (Nair *et al.*, 1986; Martinez and Staba, 1988).

Gulati *et al.* (1996) surface sterilized leaves, inflorescence bits, petiole segments and stem segments with 10 per cent sodium hypochlorite for 30 min followed by 0.1 per cent $HgCl_2$ for 7 min and 70 per cent ethanol for 3 min.

2.3.2.3 In vitro germination of seeds

The surface sterilised seeds were germinated on moistened filter paper on petri plates sealed with parafilm (Martinez and Staba, 1988; Tawfiq *et al.*, 1989; Fulzele *et al.*, 1995) or on full strength MS medium with 5 per cent sucrose and 0.8 per cent agar (Paniego and Giulietti, 1994), ¼ strength of MS medium with out hormones with 2 per cent sucrose and 0.8 per cent agar (Usha and Swami, 1998; Woerdenbag *et al.*, 1993), MS medium with 0.6 per cent agar (Jha *et al.*, 1988). Eighty per cent seeds were germinated when they were exposed to continuous light (1400

lux) and a temperature of 23°C. Gulati *et al.* (1996) reported 75 per cent germination of seeds in MS media with 3 per cent sucrose and 800 μ*M* myo-inositol.

2.3.2.4 Basal media

Murashige and Skoog medium (1962) or B5 medium (Gamborg *et al.*, 1968) can be used for micropropagation of *A. annua* (Nair *et al.*, 1986; Da Silva, 2003). Many of the reports indicate use of MS nutrient medium for *in vitro* micropropagation of *A.annua* (Nair *et al.*, 1986; Martinez and Staba, 1988; Tawfiq *et al.*, 1989; Fulzele *et al.*, 1995; Ferreira and Janick, 1996; Gulati *et al.*, 1996; Usha and Swami, 1998). Aseptic young leaves, old leaves or stem explants produced callus on different media (B_5, N_6 and MS) that included soft friable callus within 5-10 days on N_6 media. But artemisinin was not present in the callus grown in N_6 medium (He *et al.*, 1983). Benjamin *et al.* (1990) reported that unorganized and organized cultures are induced from *in vitro* seedlings of *A. pallens* in MS media with growth regulators.

In other Asteraceae plants like Roman Chamomile (*Anthemis nobilis*), (Echeverrigaray *et al.*, 2000), *Glossocardia bosvallia* (Reddy *et al.*, 2001), MS media was used as basal media. MS media was also used as the nutrient medium for *in vitro* propagation of medicinal plant *Withania somnifera* (Vadawale *et al.*, 2004).

2.3.2.5 Carbon source

Many of the workers used sucrose as the carbon source (Nair *et al.*, 1986; Ferreira and Janick, 1996; Usha and Swami, 1998). Nair *et al.* (1986) reported that 2 per cent sucrose is optimum for organogenesis. But Usha and Swami (1998) reported that 3 per cent sucrose for callusing and multiple shoot production and 2 per cent sucrose to get *in vitro* roots from shoots. Woerdenbag *et al.* (1993) reported high artemisinin in shoot cultures grown in media containing 2 per cent sucrose.

2.3.2.6 Addition of growth regulators

The most important factor in successful tissue culture is the addition of growth regulators. Skoog and Miller (1957) hypothesized that the ratio of exogenously supplied auxin cytokinin triggers organogenesis. The concentrations of auxins and cytokinins are important as their ratio (Torres, 1989). Auxin at a moderate to high concentration is used to produce callus. In some species, a high concentration of auxin and low concentration of cytokinin in the medium promotes abundant cell proliferation with formation of callus.

Lowering the auxin and increasing the cytokinin is traditionally performed to induce shoot organogenesis from callus. IAA or IBA alone or in combination with low concentration of cytokinin is important in the induction of root primordium (Chawla, 2003).

NAA induces chlorophyllous callus with more compact texture while callus obtained with 2,4-D were white yellowish or brown. Higher zeatin concentration induced photosynthetic callus and poor organ regeneration in *Artemesia annua* (Paniego and Giulietti, 1994).

Ferreira and Janick (1996) reported that BA had greatest effect in increasing shoot proliferation but reduced rooting in *A. annua.* Shoot proliferation was found to be maximum at 5μ*M* BA. Prolonged use of BA results in the vitrification and death of clones. Shoot vitrification was eliminated in BA free medium.

Usha and Swami (1998) reported that the addition of adjutants like biotin (1 mg l^{-1}) and myo-inositol (100 mg l^{-1}) was found to enhance the proliferation of multiple shoots in *A. annua* along with BA (12 mg l^{-1}).

2.3.2.6.1 Effect of addition of growth regulators on artemisinin content

Martinez and Staba (1988) reported that GA_3 has no effect on artemisinin content of *Artemesia annua* shoot culture. Later Woerdenbag *et al.* (1991) observed

that addition of GA_3 increased artemisinin production but at a higher concentration, growth and production were negatively influenced.

IAA increases production of secondary metabolites while NAA suppresses production in *A. annua* (Tawfiq *et al.*, 1989). The artemisinin content varies depending on both NAA and sucrose concentration. The shoot cultures showed a better growth and produced more artemisinin in 2 per cent sucrose. A concentration of 0.05 mg l^{-1} NAA and 0.2 mg l^{-1} BA was found to be optimal for high artemisinin (Woerdenbag *et al.*, 1991)

Since artemisinin and its precursor (arteannuic acid, arteannuin B) were at maximum concentration in leaves, buds and small green stem (Woerdenbag *et al.*, 1991), the use of plant growth substances, which can improve leaf and herbage development, was suggested by Shukla *et al.* (1992) in order to increase the artemisinin production.

Various workers have reported increase in artemisinin concentration with growth regulators such as GA_3 (Woerdenbag *et al.*, 1991), casein hydrolysate or naftime (Woerdenbag *et al.*, 1993), CCC or miconazole (Kudakaseril *et al.*, 1987).

Whipkey *et al.* (1992) found that BA (1 mg l^{-1}) or kinetin 6.1 mg l^{-1} but not CCC or diaminozide, increased shoot yield of artemisinin due to an increase of dry matter, which overcome a decrease in artemisinin content. The artemisinin content was highest with 6.33μM CCC but at higher concentration of CCC or diaminozide there was 43 per cent or 36 per cent decrease in artemisinin content respectively. Increase in BA decreased artemisinin content. The highest concentration of artemisinin was obtained from BA free medium.

Kudakaseril *et al.* (1987) found enhanced production of artemisinin with 100 mg l^{-1} miconazole but later Woerdenbag *et al.* (1991) found that miconazole negatively influenced artemisinin production as well as growth of callus. In shoot culture, addition of 5 azacytidine did not enhance artemisinin (Woerdenbag *et al.*, 1991).

2.3.2.7 Regeneration from in vitro seedling

Tawfiq *et al.* (1989) obtained callus culture by transferring aseptic seedling on to solid (1.5% agar) MS medium containing 2, 4-D (1 mg l^{-1}), kinetin (0.1 mg l^{-1}) and sucrose 5 per cent.

Germinated seedlings of *A. pallens* showed profuse callusing when cultured on MS basal media supplemented with 2, 4-D 0.1 ppm and BA 1 ppm (Benjamin *et al.*, 1990). Callus cultures interspersed with shoot buds were initiated from seedling explants on MS medium supplemented with 1 mg l^{-1} BA and 1 mg l^{-1} NAA (Fulzele *et al.*, 1995).

All aseptic seedling explants responded positively within 10 days when compared to leaves, nodal and internodal explants. MS full strength medium supplemented with NAA (1 mg l^{-1}) and kinetin (0.1 mg l^{-1}) along with 3 per cent sucrose induced rapid proliferation of callus. A combination of BA (1 mg l^{-1}), biotin (1 mg l^{-1}) and myo-inositol (100 mg l^{-1}) along with 3 per ccent sucrose induced more number of multiple shoots directly from the aseptic seedling (Usha and Swami, 1998).

2.3.2.8 Regeneration from hypocotyls

Best results for callus induction in hypocotyls of *A. annua* seedling were obtained with NAA (5.4 μM) or with NAA/BA (5.4-0.22, 5.4-0.66 μM) or 2, 4-D (4.5 μM) in both light dark cycle and darkness (Paniego and Giulietti, 1994) or in media containing 0.5 μM NAA, 9 μM BA and 0.3 M GA_3 (Gulati *et al.*, 1996). Callus was formed also in 2, 4-D (1 mg l^{-1}), NAA (0.5 mg l^{-1}) and BA (0.5-2.5 mg l^{-1}) or NAA (2.5 mg l^{-1}) and also in media containing Zeatin (2 mg l^{-1}), NAA (1 mg l^{-1}) and BA (2 mg l^{-1}). Shoot regeneration was observed in media with BA (3 mg l^{-1}) and NAA (0.2 mg l^{-1}) (Da Silva, 2003).

2.3.2.9 In vitro regeneration from leaves

Callus formation

Callus was formed from leaf explants cultured on media containing 0.05 mg l^{-1} NAA and 0.1 to 0.2 mg l^{-1} BA with in 21 to 35 days. These calli turned to shoots in about 60 days (Nair *et al.*, 1986).

Unorganized or callus cultures were established in 14-21 days from aseptic leaf explants cultured on modified MS or B_5 media containing 0.02 to 0.05 mg l^{-1} NAA and 0.1 to 0.5 mg l^{-1}BA (Martinez and Staba, 1988), on a combination of NAA (2 mg l^{-1}) and Kinetin (1 mg l^{-1}) (Jha *et al.*, 1988), NAA (5.4 μM) or with NAA-BA (5.4-0.22, 5.4-0.66 μM) and 2,4-D 4.5 μM (Paniego and Giulietti, 1994), with 4.44 μM BA combined with 4.52 μM 2,4-D (Ferreira and Janick, 1996) or with 2.5 μM NAA and 9μM Kinetin (Gulati *et al.*, 1996) on MS or B_5 media with 2,4-D (0.05-2 mg l^{-1}) and BA (0.025-0.5 mg l^{-1}) and in MS with 0.67 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BA and 0.35 mg l^{-1} GA_3 (Da Silva , 2003).

The cotyledonary leaves produced friable light green callus on MS media supplemented with 0.5 μM NAA, 9 μM BA and 0.3 μM GA_3. Bright green regenerating callus was obtained from leaf explants cultured on MS medium supplemented with L- aspargine (300 μM), glutamine (700 μM), arginine (300 μM) cysteine hydrochloride (30 μM) in addition to NAA (0.5 μM), BA (13 μM) and GA_3 (0.3 μM) (Gulati *et al.*, 1996).

Rhizogenesis

The young leaf segments produced roots on modified MS or B_5 media (inorganic solvents supplemented with thiamine HCl (1 mg l^{-1}) inositol 100 mg l^{-1}, nicotinic acid 0.5 mg l^{-1}, pyridoxine HCl 0.5 mg l^{-1}, sucrose 20 gl^{-1} supplemented with IBA or NAA (0.05-2 mg l^{-1}) with in 6-14 days mostly from proximal end of mid rib (Nair *et al.*, 1986). The auxins IAA, IBA and NAA (1-2 mg l^{-1}) when used alone induced roots from cut ends of leaves and did not induced callus formation (Jha *et al.*, 1988).

Schizogenesis

Multiple shoots from leaf are reported on combination of Kinetin (0.1 mg l^{-1}) and 2, 4-D (1 mg l^{-1}) by Kudakaseril *et al.* (1987) on BAP (0.1 ppm) and IAA (1 ppm) (Fulzele *et al.*, 1995) and on NAA (0-0.5 mg l^{-1}) and BA (0-2.0 mg l^{-1}) (Woerdenbag *et al.*, 1993).

Mukherjee *et al.* (1995) reported that shoot organogenesis can be obtained in leaf callus by culturing NAA 1 mg l^{-1} and BA 1 mg l^{-1} and then transferring to 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA. MS medium supplemented with 1-4.5 μ*M* of BA and 1 μ*M* of NAA induced adventitious bud formation and shoot development in leaf explants of Roman chamomile (Echeverrigaray *et al.*, 2000).

Reddy *et al.* (2001) reported that leaf explants in *Glossocardia bosvallia* Roxb (ephemeral plants belonging to Asteraceae family) produced the highest frequency of shoot bud regeneration as well as number of shoots per explant. Regeneration was observed on low concentration of 2, 4-D and BA (0.2-0.5 mg l^{-1}). Leaf produced shoot in medias 0.05 mg l^{-1} NAA and 0.2 or 2 mg l^{-1} BAP, 1 mg l^{-1} NAA and 0.5 mg l^{-1} BA, 1 mg l^{-1} NAA, 3 mg l^{-1} BA and 0.1 mg l^{-1} GA_3 (Da Silva, 2003).

2.3.2.10 In vitro regeneration from shoot

Shoot cultures were established from the regenerating shoot and from shoot apices derived from sterile seedlings in a MS media with 2 mg l^{-1} BA (Jha *et al.*, 1988).

Organized shoot cultures could be established from either aseptic seedlings or apical meristems when they were transferred to MS medium containing BA (2.5 mg l^{-1}) and 0.8 per cent agar (Martinez and Staba, 1988). Mature stem segments produced callusing on MS with NAA (2.5 μ*M*) and Kinetin (9 μ*M*) (Gulati *et al.*, 1996). Shoot explants on *Glossocardia bosvallia* regenerated in lower concentration of 2, 4-D and BA (0.2-0.5 mg l^{-1}) (Reddy *et al.*, 2001).

Da Silva (2003) reported that stems of *A. annua* produced shoots when grown in a media containing 0.5 mg l^{-1} Kinetin, 0.2 mg l^{-1} IAA along with cotton fiber and produced callus in media with 0.05 mg l^{-1} NAA and 0.5 mg l^{-1} BA.

2.3.2.11 In vitro regeneration from inflorescence

Callus cultures were estsblished from aseptic flowers (5 mm) on MS medium supplemented with 2,4-D (0.5 mg l^{-1}) and Kinetin (1 mg l^{-1}). These calli produced shoots on media containing 0.1 mg l^{-1} IAA and 1 mg l^{-1} BA (Martinez and Staba, 1988). Gulati *et al.* (1996) reported that only mature inflorescence showed callusing on MS media with 0.5 μM NAA, 9 μM BA and 0.3 μM GA_3.

In immature inflorescence, shoots were developed on MS with 0.5 μM NAA, 0.3 μM GA_3 and 13 μM BAP or in MS with 1 μM NAA, 13 μM BAP and 2 per cent coconut milk (Gulati *et al.*, 1996).

Shoot cultures were established from inflorescence segments kept on MS medium supplemented with 1 gl^{-1} casein hydrolysate and 1mg l^{-1} BAP under light condition (Mathur, 1998).

2.3.2.12 Response of roots of A. annua

Paniego and Giulietti (1994) reported callusing of root in media with 5.4 μM NAA or with NAA-BA (5.4-0.22, 5.4-0.66 μM) and 2, 4-D (4.5 μM). Mukherjee *et al.* (1995) reported that green compact callus could be easily obtained in roots of *A. annua* with BA or kinetin (1 mg l^{-1}) and NAA (1 mg l^{-1}).

Gulati *et al.* (1996) reported that *in vitro* roots of *A. annua* plants produced friable light green callus on MS medium supplemented with 0.5 μM NAA, 9 μM BAP and 0.3 μM GA_3. Roots of *A. annua* produced callus in media containing 2 mg l^{-1} Zeatin, 1 mg l^{-1} NAA and 2 mg l^{-1} BA and shoot regeneration was observed in media with 3 mg l^{-1} BA and 0.2 mg l^{-1} NAA (Da Silva, 2003).

2.3.2.13 Regeneration from other explants

Nodal segments and petiole

Callusing of the petiole of *A. annua* plants were reported in 1 mg l^{-1} BA and 1.2 mg l^{-1} NAA (Jha *et al.*, 1988) and on MS media with 2.5 μ*M* NAA and 9 μ*M* Kinetin. (Gulati *et al.*, 1996). Calli were less compact. The nodal segments of *A. asplenifolia* produced shoots on media containing BAP (1 mg l^{-1}), IAA (0.1 mg l^{-1}) and GA_3 (0.025 mg l^{-1}) (Da Silva, 2003).

2.3.2.14 Regeneration from callus

Regenerated plants with a survival rate of 98 per cent were obtained from callus growing MS or B_5 media containing NAA (0.05 mg l^{-1}) and BA 0.1-0.2 mg l^{-1}. After 60 days, green shoots appeared and roots developed in medium with no growth regulators (Nair *et al.*, 1986).

Callus induced root formation in medium with BA (0.5 mg l^{-1}) and high NAA (2 mg l^{-1}). Callus showed profuse shoot regeneration in 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA (Jha *et al.*, 1988).

The friable green callus from various explants of *A.annua* showed slow growth in a media with high concentration of GA_3 and callus produced multiple shoots on 1.08 μ*M* NAA and 13.3 μ*M* BA or on media with 300 μ*M* aspargine, 700 μ*M* glutamine, 30 μ*M* cysteine hydrochloride, 300 μ*M* arginine supplemented with 0.05 μ*M* NAA, 9 μ*M* BAP and 0.02 μ*M* GA_3 (Gulati *et al.*, 1996; Paniego and Giulietti, 1994). Callus cultures interspersed with shoot buds when transferred to MS medium with 0.1 mg l^{-1} BA and 1 mg l^{-1} IAA produced multiple shoots (Fulzele *et al.*, 1995).

Ferreira and Janick (1996) reported that friable callus obtained from leaf produced roots on hormone free medium and shoots were induced on media with BA at concentration of 4.4 and 44.4μ*M*.

2.3.2.15 Influence of GA_3 on elongation

Gulati *et al.* (1996) reported elongation of *A. annua* shoots on MS medium with 0.3 μ*M* GA_3. Elongation of *in vitro* regenerated shoots was reported in *Glossocardia bosvallia* Roxb. where GA_3 (0.02-0.5 mg l^{-1}) was used in media along with BAP (0.1-0.5 mg l^{-1}) (Reddy *et al.*, 2001).

2.3.2.16 In vitro rooting

Nair *et al.* (1986) reported that the roots were developed from shoots kept on basal MS medium without any growth regulators for more than 3 months. Sterile plants obtained from shoot cultures rooted either in MS medium with NAA (3 mg l^{-1}) or MS medium without growth regulators (Martinez and Staba, 1988). The shoots excised from proliferating shoot cultures or callus cultures were rooted on basal medium with 2 mg l^{-1} IBA (Jha *et al.*, 1988; Usha and Swami, 1998). Shoot cultures placed in liquid MS medium containing 2, 4-D (1 mg l^{-1}) developed small random roots in 7 to 10 days (Martinez and Staba, 1988).

Benjamin *et al.* (1990) reported that *in vitro* shoots of *A. pallens* produced roots in medium devoid of phytohormones. When shoots were grown on MS medium with 1 mg l^{-1} NAA and 0.1 mg l^{-1} Kinetin, root formation occurred and plantlets developed further (Fulzele *et al.*, 1995). The *in vitro* shoots produced an external root system when transferred to medium containing 0.3 μ*M* GA_3 (Gulati *et al.*, 1996). *In vitro* shoots of Roman chamomile were rooted on MS medium containing 0.5 μ*M* IBA (Echeverrigaray *et al.*, 2000).

Leaf, stem and embryo explants of *Glossocardia bosvallia* Roxb exhibited rhizogenesis on MS medium supplemented with 2,4-D (2 mg l^{-1}) and IBA 0.2 mg l^{-1}). Fifty per cent of shoots were successfully rooted within 10-12 days when half strength MS medium without phytohormones was used (Reddy *et al.*, 2001).

2.3.2.17 Hardening

Cultures of *A. annua* were kept at room temperature for 6 weeks. Plants were then thoroughly washed in tap water and transferred to pots containing soil (Jha *et al.*, 1988).

2.3.2.18 In vitro flowering

Shoot cultures of *A. annua* growing on MS medium containing 0.8 per cent agar and BA (1 mg l^{-1}) occasionally developed flowers when grown at 29°C during light cycle of 12hrs and 22°C during dark cycle (Martinez and Staba, 1988). Gulati *et al.* (1996) reported flowering of *in vitro* shoots of *A. annua* in MS with 0.3 mg l^{-1} GA_3 after 12 weeks.

2.3.2.19 Effect of subculturing

MS medium with BA 2 mg l^{-1} (Martinez and Staba, 1988), 13.32 μ*M* BA (Paniego and Giulietti, 1994) or 4.44 μ*M* BA (Ferreira and Janick, 1996) were used for subculturing *A. annua* shoots.

2.3.2.20 Suspension cultures in A. annua

Leaf explants when cultured on modified MS or B_5 media under 16-8hr light-dark cycle with low concentration of NAA (0.02-0.05 mg l^{-1}) and BAP (0.1-0.5 mg l^{-1}) produced callus in 14-21 days. Two month old callus clumps removed from leaf explants were used to initiate suspension cultures. The media for suspension culture was modified MS or B_5 containing 1 mg l^{-1} NAA. The cultures were maintained in a rotary apparatus at 4 rpm in 25x100mm vials and were harvested after 2 to 3 weeks after inoculation (Nair *et al.*, 1986).

Cell suspension cultures were established by transferring third generation friable callus cells to liquid MS with 1mg l^{-1} 2, 4-D, 0.1mg l^{-1} Kinetin and sucrose 5 per cent. Cultures were maintained on orbital shaker (120 rpm) at 25°C under continuous illumination and subcultured into fresh medium every 4 weeks (Tawfiq *et al.*, 1989).

2.3.2.21 Bioreactor cultivation of plantlets and terpenoid synthesis

A. annua shoots were cultivated in 1 litre capacity bioreactor under submerged conditions using MS medium supplemented with 1 mg l^{-1} NAA and 0.1 mg l^{-1} kinetin. Fifteen day old shoot cultures were used as inoculum for biomass production. Gibberillic acid and ethephon are known to influence concentration of terpenoid and other secondary metabolites in bioreactor (Fulzele *et al.*, 1995).

2.3.2.22 Culture conditions for A. annua in vitro cultures

Most of the workers reported that *A.annua* cultures were grown at 25-26 ± 2°C, photo period of 16 hr light and relative humidity of 55 ± 5 per cent (Tawfiq *et al.*, 1989; Woerdenbag *et al.*, 1993; Fulzele *et al.*, 1995; Gulati *et al.*, 1996).

2.4 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

For the past few decades, considerable interest has been shown in the production of secondary metabolites. Many of these natural compounds cannot be artificially synthesized and must be extracted from plants. An alternative to field cultivation would be production of secondary products by cell cultures. There have been numerous examples for tissue culture based phytochemical production from medicinal plants (Ramachandra and Ravisankar, 2002). The success however is moderate mainly because the biosynthetic pathway of secondary metabolites including the enzymes and regulatory mechanism governing the expression and function of pathways are poorly understood (Rutenan, 1995). Metabolism of secondary products seems to correlate with the degreee of organization of cell cultures. Roots are capable of accumulating a large range of secondary metabolites reflecting biosynthetic capacity (Doron, 1989). The major problem associated with *in vitro* culture of conventional roots is usually slow growth and the plant tissue cultures have a great tendency to be genetically and biochemically unstable. They tend to synthesise very low levels of secondary metabolites. One recent advance in transgenic technology is the use of transgenic organ cultures such as shooty teratomas and hairy roots so as to attain overproduction and biotransformation of secondary metabolites (Saito *et al.*, 1992). Since the genetic transformation does not impair natural root synthetic

capacities, hairy roots induced by naturally occurring bacteria *Agrobacterium rhizogenes* have been explored and intensively used as a stable and high production system for useful secondary metabolites (Rhodes *et al.*, 1990). The hairy roots are fast growing and genetically stable which can also be successfully cultured in large scale bioreactors (Giri and Narasu, 2000).

2.4.1 Hairy root disease

A large number of fine hairy roots covered with root hairs originate directly from the explant in response to *Agrobacterium* infection. (Ricker, 1930) described and named the hairy root causing as *Phytomaonas rhizogenes* which was later renamed as *Agrobacterium rhizogenes*. The first directed transformation of higher plants using *A. rhizogenes* was made by Ackermann (1977).

2.4.2 Mechanism of *Agrobacterium* plant interaction

The soil borne gram negative bacteria *Agrobacterium* infects the crown region. The process of infection starts with attachment of bacterium to the plant. Wounding induces the infection. The wounded plant cell release phenolic substances such as acetosyringone to induce the expression of ***vir*** genes of plasmid (Binns and Thomasshow, 1988). Integration and expression of T- DNA genes in host plant leads to the development of hairy roots (Vanhala *et al.*, 1995; White and Nester, 1980; Tepfer, 1984). This bacterium transfers its transfer DNA (T-DNA) that is a portion of large plasmid called root inducing plasmid (Ri- plasmid). Plasmid contains *vir*-genes that are responsible for virulence of bacteria together with chromosomal genes and cause transfer of T-DNA. The border sequences (25bp) determine the mobility of T-DNA (Sevon and Oksman- Caldentey, 2002).

2.4.2.1 Steps involved in transfer of genetic material from Agrobacterium to plant cell (Bapat and Ganapathi, 2005)

- Wounding of plant cell and secretion of the phenolic compounds.
- Detection of the wound signal by the bacterium
- Activation of the bacterial gene machinery

- Induction of *vir* gene expression
- Generation of T-DNA
- Formation of T-DNA strand protein complex
- Movement of T-DNA complex through bacterial membranes
- Targeting of the T-DNA complex into and within the plant cell
- Targeting of the T- complex into cell nucleus and its stabilization
- Integration of the T- DNA strand into plant cell DNA at random
- Expression of the bacterial DNA in the plant cell

The interaction between the bacteria and plant is a multistep process involving recombination, replication and repair activities most likely mediated by host cell enzymes (Zambryski, 1988).

2.4.3 Characterization of *Agrobacterium* plasmids based on opine catabolism

The transformed hairy roots are able to synthesize specific secondary products, the opines (eg: Agropine, Mannopine, Cucumopine) which are catabolised by free living *Agrobacteria* (Rhodes *et al.*, 1990). Opines are classified according to their structure and biogenesis (Saito *et al.*, 1992).

Opines have no function in plant cells. The genes for opine biosynthesis is located inside T-DNA and genes for opine catabolism is located outside T-DNA. The Ri plasmids are grouped into three families according to different types of opines produced namely, agropine, mannopine and cucumopine (Petit *et al.*, 1983; Christey, 2001). Agropine type strains induce roots to synthesize agropine, mannopine and related acids, and mannopine type strains induce roots to produce mannopine and corresponding acids (Rhodes *et al.*, 1990). Agropine type strains are more virulent and more often used in establishment of hairy root cultures (Rhodes *et al.*, 1987).

Table 1 shows characterization of *Agrobacterium* plasmids on the basis of opines catabolism. The presence of TL-DNA in transformed tissues cannot be detected by opine markers only in agropine strain whereas sole presence of opine in tissue

demonstrates transformation status in mannopine and cucumopine strain (Huffman *et al.*, 1984).

Table 1. Characterization of *Agrobacterium* plasmids on the basis of opine catabolism

Sl No.	Opine type	Characterestic opine markers	*A.rhizogenes* strains
1.	Agropine	Agropine, Mannopine, Agropinic acid, Mannopinic acid, Agrocinopine	P_cA_4, 15834,A_4, LBA 9402, HR1, LBA 4404
2.	Mannopine	Mannopine, Mannopinic acid, Agropinic acid, Agrocinopine	8196
3.	Cucumopine	Cucumopine and Cucumopine lactam	2659, LBA8490
4.	Mikimopine	Mikimopine, Mikimopine lactam, and Mannopine	MAFF 03-01724

(White and Nester, 1980; Petit *et al.*, 1986; Dessaux *et al.*, 1991).

2.4.4 Genes responsible for hairy root formation

The genes encoded by Ri-T DNA apparently regulate the balance of endogenous hormones produced by transformed cells which results in the proliferation of fast growing roots at the wound site (Flores *et al.*, 1987). The physiological basis of hairy root disease is not totally understood. Alteration of auxin metabolism in transformed cells has been supposed to play an important role in expression of hairy root phenotype (Zambryski *et al.*, 1989; Gelvin, 1990).

Ri plasmids are large (200 to 800 kb) and contain the T-DNA and the virulence region (Gelvin, 1990). The T-DNA of the agropine Ri plasmid consists of two separate T-DNA regions TL-DNA and TR-DNA (White *et al.*, 1985). Each of the T-DNA fragments spans 15-20 Kb region and they are separated from each other by at least 15 kb of non integrated plasmid DNA (Vialine and Casse-Delbert, 1987). The expression of four root locus (rol) genes, *rol A*, *rol B*, *rol C* and *rol D* located on TL-DNA is having a role in the establishment of hairy roots (Taylor *et al.*, 1985). The products of these loci have been reported to show synergistic activities and are involved in rhizogenesis (Cardarelli *et al.*, 1987; Spena *et al.*, 1987). The product of these genes probably alters the sensitivity of plant cells to auxins (Gelvin, 1990).

TL-DNA is considered to be essential for the induction of hairy roots; since cucumopine and mannopine type Ri plasmids have a single T-DNA that is homologous to the TL-DNA of the agropine type Ri plasmid (Combard and Baucher, 1988).

The sequence analysis of TL-DNA revealed 18 open reading frames (ORFs) (Slightom *et al.*, 1986). ORFs 10,11,12 and 15 (*rol*A, B, C and D respectively) have been found to be essential for the hairy root syndrome (White *et al.*, 1985; Spena *et al.*, 1987; Jouanin *et al.*, 1989). The *rol* B gene plays a central role in hairy root induction while *rol* A, *rol* C and some other ORFs act in synergy to promote root induction (Aoki and Syono, 1999).

The *rol* genes from *A. rhizogenes* have been used to improve ornamental species (Mercury *et al.*, 2001). The exact role of these genes is still unclear and may vary among the plant species. The *rol* A in tobacco is mainly responsible for the development of hairy roots while *rol* B appears to be a factor in hairy root initiation (Cardarelli *et al.*, 1987). The transgenic potato and tobacco plants expressing *rol* A or derivatives of *rol* A and *rol* C had reduced gibberillic acid level (Dehio *et al.*, 1993).

ORF 8, ORF 13 and ORF 14 are conserved among different Ri plasmids (Brevet and Tempe, 1988). The right end of the T-DNA integrates more precisely than the left end (Krizkova and Hrouda, 1998). The gene encoding auxin synthesis (*tms* 1 and *tms* 2) and agropine synthesis (*ags*) have been localized on the TR-DNA of the agropine type Ri plasmid (White *et al.*, 1985). Auxin is needed for hairy root induction and does not play a role in T-DNA expression in transformed plant cells (Cardarelli *et al.*, 1987). Auxin synthesizing genes play a significant role in the morphology and alkaloid production of transformed roots of *Datura metel*, as they provide an additional source of auxin (Moyano *et al.*, 1999).

Christey (2001) reported that integration of Ri -T_L DNA and T_R DNA leads to the alterations in hormone metabolism, transcript properties and production of opines in transformed roots. This altered behaviour of roots has led to the hypothesis

that regulation of secondary metabolite pathway may be different in normal and transformed roots. The *rol* genes and genes from right border interact to change the metabolism of transformed cells in a number of ways facilitating excess auxin source to plant cells (Bapat and Ganapathi, 2005).

2.4.5 *Agrobacterium rhizogenes* mediated genetic transformation

A large number of plant species from a number of families have been successfully transformed by *A. rhizogenes.* Ooms *et al.* (1985) reported that Ri plasmids could be used as vectors to introduce any gene via Ri TDNA into potato. The use of *Agrobacterium* as a vector is based on its unique capacity to transfer a piece of its own DNA (T-DNA) into nuclear genome of plant cells. *A. rhizogenes* strains are more virulent than *A. tumefaciaens* strains. It has also been shown in several species that shoots can easily be regenerated from transformed hairy roots (Noda *et al.*, 1987; Ottaviani *et al.*, 1990).

A. rhizogenes can be used to insert non T-DNA genes into plants (Tepfer, 1990). A set of morphological markers is encoded by Ri T_L- DNA in regenerated plants (Ackermann, 1977; Durand-Tardif *et al.*, 1985; Sinkar *et al.*, 1988). These include wrinkled leaves, short internodes, altered flower morphology etc. Some of these are observed in particular species, some in certain clones within species and some only in certain individuals (Tepfer, 1984; Durand–Tardif *et al.*, 1985). These markers make up transformed phenotype and segregate with Ri T-DNA after meiosis (Tepfer, 1984). The transformed phenotype provides a simple and accurate way of identifying transformed materials.

2.4.6 Characteristics of hairy roots

2.4.6.1 Rapid growth

Hairy roots have more number of apical zones with high degree of cell division (Bapat and Ganapathi, 2005). High level of lateral branching in hairy roots due to presence of many lateral root tips result in high growth rate (Sevon and Oksman-Caldentey, 2002; Xu *et al.*, 2004) in growth regulator free media (Payne

et al., 1992). There is difference in growth of hairy roots among different species and between different root clones of the same species (Sevon and Oksman-Caldentey, 2002).

2.4.6.2 Secondary metabolite production

Hairy roots have been advocated for the production of plant secondary metabolites (Flores and Filner, 1985; Kamada *et al.*, 1986; Rhodes *et al.*, 1986; Mano *et al.*, 1986; Jung and Tepfer, 1987; Oksman- Caldenty and Hiltunen, 1996; Li *et al.*, 2000). The hairy roots excrete secondary metabolites into growth medium and the extent of release varies between species (Hamill *et al.*, 1986).

Alkaloid production decreased clearly when roots were induced to form callus and reappeared to form callus when allowed to redifferentiate (Flores *et al.*, 1987). Hairy roots can be a good alternate source of phytochemicals because transformed roots grow rapidly and produce secondary metabolites at levels comparable to those of parent plants (Bhadra *et al.*, 1993).

A most remarkable characteristic of hairy roots is the potential for biomass production. In addition they have also been shown to produce compounds not normally associated with the parental plant and could also be used as a source of new biologically active compounds (Wysokinska and Chmiel, 1997).

Till date many of the successful transformations have great commercial application particularly in secondary metabolites of medicinal importance (Giri and Narasu, 2000).

Secondary metabolite production of hairy roots is highly linked to cell differentiation. Hairy roots score over normal roots in their ability to grow without hormones since presence of hormones reduce secondary metabolite production in some cases (Bapat and Ganapathi, 2005).

The syringin and hispidulin content in transformed hairy root cultures of *Saussurea involucrata* were 40 and 3 times respectively higher than that in aerial parts of wild plant (Fu *et al.*, 2006).

2.4.6.3 High stability

The attractiveness of hairy root cultures in bioprocessing can be attributed to genetic and biochemical stability (Wysokinska and Chmiel, 1997; Li *et al.*, 2000).

2.4.7 Hairy roots as the source of pharmaceuticals

Ri T-DNA increased alkaloid production essentially by stimulating the growth (Jung and Tepfer, 1987). Transformed root cultures have proved amenable to growth and secondary metabolite production in fermenters. A vast amount of literature is available on production of high volume of phytochemicals such as alkaloids (Toivonen *et al.*, 1991) and terpenoids (Weathers *et al.*, 1997; Bapat and Ganapathi, 2005) in *Agrobacterium* transformed hairy root cultures.

2.4.8 Culture medium for *A. rhizogenes* strains

Benjamin *et al.* (1993) reported the use of AB minimal media for culturing 15834 strains. Mano *et al.* (1986) suggested nutrient broth as a good culturing medium for ATCC 15834, A_4, NCPB 1855 and NCPB 2659. YEB medium was found to be the best for the growth of MAFF 03-01724 (Sauerwein *et al.*, 1992; Jaziri *et al.*, 1994; Momocilovic *et al.*, 1997; Chen *et al.*, 1999; Shi and Kintzios, 2003; Yoshimatsu *et al.*, 2003; Xu *et al.*, 2004; Zdravkovic-Korac *et al.*, 2004). LB medium was used for culturing *A. rhizogenes* strains 15834 (Dobigny *et al.*, 1995; Lee *et al.*, 2004) AR-12 (Arellano *et al.*, 1996) 2659 (Dobigny *et al.*, 1995).

YMB medium (1% Mannitol, 0.05% K_2HPO_4, 0.01% NaCl, 0.04% Yeast extract, 0.02% $MgSO_4.7H_2O$) was used for culturing *A. rhizogenes* strains. ATCC 15834 (Hu and Alfermann, 1993; Vanhala *et al.*, 1995) LBA 9402 (Hu and Alfermann, 1993; Allan *et al.*, 2002; Chaudhuri *et al.*, 2005; Van-Wordragen *et al.*, 1992, Ooms *et al.*, 1985) A_4 (Hu and Alfermann, 1993; Chaudhuri *et al.*, 2005).

A rhizogenes strains A_4, R 1022, K599 and SA79 were propogated in yeast extract peptone medium (Batra *et al.*, 2004).

2.4.9 Culture conditions for *A. rhizogenes* strains

Temperature has influence on the growth of *A. rhizogenes* strains. Preferred temperature range is 25 ± 3°C. Jaziri *et al.* (1994) suggested 25°C for culturing strains like ATCC15834 and MAFF 03-01724. Optimum temperature for growth of *A.rhizogenes* strains like ATCC 15834, A_4 and LBA 9402 was found to be 28°C. (Shi and Kintzios, 2003; Momocilovic *et al.*, 1997; Celma *et al.*, 2001; Jha *et al.*, 2000; Lee *et al.*, 2004).

Xu *et al.* (2004) reported that A_4, R1601 and ATCC 15834 were cultured at 27°C and the culture media was shaken at 150 rpm.

2.4.10 Explants used for hairy root induction

The susceptibility of plant species to *Agrobacterium* strain varies. Xu *et al.* (2004) reported that root induction efficiency was influenced by the type of explants and *A. rhiozogenes* strains. The age and differentiation status of plant tissue and plant type can also affect the chances of successful transformation. The level of tissue differentiation also determines the ability to give rise to transformed roots after *A. rhizogenes* inoculation (Trypsteen *et al.*, 1991). List of plant species successfully transformed to hairy root induction, strains involved and explants used are shown in the Table 2.

Karmarkar (2001) used hypocotyls and shoot buds for transformation in *Holostemma ada-kodien* using *A. rhizogenes* strains A_4, ATCC 15834 and pCA_4. Leaf segments and shoot tips showed efficient transformation in *Withania somnifera* using *A. rhizogenes* (Varghese, 2006).

Table 2. Plant species, strains involved and the explants used for successful transformation

Sl No.	Plant	Strains	Explants	Secondary metabolite	Reference
1.	*Atropa belladonna*	A4 ATCC15834 MAFF-03-01724	Stem,Leaf segment	Atropine 5.3mg/l Atropine (5.3mg/l)	Jung and Tepfer, 1987; Jaziri *et al.*, 1994
2.	*Azadirachta indica*	LBA 9402	Leaf	Azadirachtin (0.007%)	Allan *et al.*, 2002
3.	*Calystegia sepium*	8196	Stem	Cusohyringone (2.3mg/l)	Jung and Tepfer, 1987
4.	*Catharanthus roseus*	15834	Hypocotyl	Catharanthin (1.5mg/l)	Jung *et al.*, 1995
5.	*Cichorium intybus*	LBA 9402	Leaf	Sesquiterpene lactone (0.03g/l)	Malarz *et al.*, 2002
6.	*Gentiana acaulis*	ATCC15834 A4M70GUS	Shoot bud	Geotiopicrin	Momocilovic *et al.*, 1997
7.	*Hyoscyamus albus*	A4	Leaf disc	Hyoscyamine (5.42g/Kg)	Sauerwein *et al.*, 1992
8.	*Isatis indigotica*	A4 R1601 ATCC15834	Cotyledon, Hypocotyl	3.6 Carboxy phenol 43 M-quinazolenone	Xu *et al.*, 2004
9.	*Glycyrrhiza uralensis*	ATCC15834	Stem	Glycyrrhizin (4.7%)	Koe *et al.*, 1989
10	*Holostemma ada-kodien*	A4, ATCC15834 pCA_4	Shoot buds hypocotyls	Terpenoid sugars and amino acids	Karmarkar, 2001
11	*Perzia cuernavacana*	AR12	Internodal segment	Perzone (1.3%)	Arellano *et al.*, 1996
12	*Panax ginseng*	ATCC15834	Stem	Ginsenoide (0.82-0.47%)	Koe *et al.*, 1989
13	*Plumbago rosea*	ATCC15834	Shoots	Plumbagin (2.53mg/g)	Komaraiah *et al.*, 2002
14	*Pueraria phaseolides*	ATCC15834	Leaf	Puerarin	Shi and Kintzios, 2003
15	*Salvia miltiorrhiza*	ATCC15834 A4	Leaf	Tanshinones	Hu and Alfermann, 1993
16	*Withania Somnifera*	A4, ATCC15834	Leaf shoot tips	Withanolide	Varghese, 2006

2.4.11 Pre culturing of explants

Yoshikawa and Furuya (1987) used the co-culture method to obtain hairy roots. Cultured cells of ginseng were partially digested with cellulose and pectinase and incubated with *A. rhizogenes*. Momocilovic *et al.* (1997) reported that shoots of *Gentiana acualis* were elongated on basal media with 34.6mg l^{-1} GA_3 for 4 weeks prior to inoculation with *A. rhizogenes*.

Shi and Kintzios (2003) reported preculturing of *Pueraria phaseolides* leaf. Leaves were cut in to pieces with or without petiole and were precultured on MS solid media with out growth regulator for 24 hours.

Xu *et al.* (2004) reported that leaves (0.5 cm^3 blocks) and hypocotyls (1cm long) explants of *Isatis indigolica* were pre cultured for 2 days in 12 hour light-dark period. Leaves from one month old *in vitro* grown plants of *Catharanthus roseus* were incubated on half strength Gamborg's B_5 medium (Gamborg *et al.*, 1968) for 24 hrs (Batra *et al.*, 2004).

2.4.12 Wounding of explants

Wounding is a pre requisite for *Agrobacterium* infection. According to Hilderbrand (1934), *A. rhizogenes* has to enter a wound deep enough to reach the phloem region to induce hairy roots on apple trees.

Moore *et al.* (1974) showed that most hairy roots emerged from the pericycle tissue of carrot vascular cylinder. Stems of potato shoots were punctured with a drawn out broken Pasteur pipette and the wound sites were infected with *A. rhizogenes* (Ooms *et al.*, 1985). Leaves of *Salvia miltiorrhiza* were wounded with forceps (Hu and Alfermann, 1993).

Only cells containing high levels of auxin and sucrose are able to act as root meristem initials and are ideal targets for *A. rhizogenes* infection (Nilsson and Olsson, 1997). Pawar and Maheswari (2004) purposely wounded leaf discs of *Withania somnifera* using a scalpel.

Embryos at cotyledonary stage 10 to 20 mm long of *Aesculus hippocastanum* were wounded by puncturing with a hypodermic needle to a depth of 1 to 2 mm, which was found to be more efficient than mere scratching. Since ray cells and phloem cells are positioned in the region with high amount of sucrose and considerable amount of IAA, they could be convenient targets for *A. rhizogenes* (Zdravkovic-Korac *et al.*, 2004).

Leaf segments of *Holostemma ada-koden* were prepared by cutting the leaf margins and ten pricks were made on the lower side of leaf on the leaf lamina and mid rib, 15 pricks were made at shoot buds and inter nodal segments (Karmarkar and Keshavachandran, 2005).

2.4.13 Inoculation and co- culturing of explants with *A. rhizogenes*

1. Direct inoculation of bacterial colonies

The bacterial inoculum used influences the transformation. A loopful of bacterial culture was applied to decapitated stem surface of *Solanum sp* (Davey *et al.*, 1987).

Stem segments of *Atropa belladonna* and *Calystegia sepium* were inoculated with varying amounts of bacteria scraped from the surface of fresh culture on agar (Jung and Tepfer, 1987). Patena *et al.* (1988) reported that bacterial colonies were superior to suspension cultures for inducing hairy roots in carrot, kalanchoe and apple.

Koe *et al.* (1989) infected the ginseng plants directly with *A. rhizogenes* strain ATCC 15834 at the several wounded sites on stem. Leaf discs of *Hyoscyamus albus* were induced to produce hairy roots by co-culture with *A. rhizogenes* strains A_4, 15834 and MAFF 03-01724 (Sauerwein *et al.*, 1992). The leaves of *Hyoscyamus albus* were wounded with a sterile needle containing *A. rhizogenes* strain ATCC 15834 from solid media on the midrib of leaves (Vanhala *et al.*, 1995). The bacteria were harvested with spatula from agar plates and spread on the top of stem internodes of potato (Dobigny *et al.*, 1995).

A_4 strain produced transformation by direct inoculation method and suspension cultures where as ATCC 15834 produced transformation by suspension cultures alone in *Withania somnifera* (Varghese, 2006). Transformation frequency of different bacterial strain was influenced by the co- culture period. Leaf segments of *W. somnifera* showed maximum response to hairy roots in one day co-culture period with A_4 and ATCC 15834 where as shoot tips produced maximum response by two day culture (Varghese, 2006).

2. Inoculation and co-culturing of explants using bacterial suspension

Hawes *et al.* (1988) reported that motile strains of *Agrobacterium* exhibited virulence only in liquid medium but mutant strains (non motile) exhibited virulence when inoculated directly on wounds.

Hu and Alfermann (1993) reported that leaf explants of *Salvia miltiorrhiza* were infected with small droplets of bacterial suspension. Elongated shoots of *Gentiana acaulis* were inoculated by puncturing internodes or central leaf nodes with a hypodermic needle dipped in bacterial suspension or bacterial suspension was smeared on cut surface of decapitated shoot (Momocilovic *et al.*, 1997) and kept for incubation for 48 hrs.

Azadirachta indica leaves were infected by scratching with a sterile needle dipped in dense *Agrobacterium* suspension and incubated in MS medium in dark at 25°C (Allan *et al.*, 2002). Leaf explants of *Pueraria* were infected by dipping them into *Agrobacterium* suspension in MS medium for 20 min (Shi and Kintzios, 2003) and were co-cultivated at 28°C for 2 days. Cotyledon and hypocotyls explants of *Isatis indigotica* were immersed in bacterial suspension at exponential growth phase (OD_{600} ~ 0.7) for ten min and co-cultivated in dark for two days on MS basal medium (Xu *et al.*, 2004).

The wounded embryos of *Aesculus hippocastanum* were immersed in bacterium suspensions for 5 min and they were transferred to MS medium with or without 50 μM acetosyringone and co-cultivated for 72 hrs (Zdravkovia- Korac *et al.*,

2004). The wounded explants *H. ada-kodien* were inoculated with 48 hrs old cultures of *A. rhizogenes* directly by applying a drop of bacterial suspension on each wound. The explants were then co-cultured for 24 hrs (Kamarkar and Keshavachandran, 2005).

Tylophora explants were wounded at different sites with a hypodermic needle loaded with 10ml of bacterial suspension. Excised leaf explants were wounded either at the leaf midrib or petiole. Excised stem segment (without leaf) were wounded at node or internode and incubated for 72 hrs (Chaudhury *et al.*, 2005).

Pre incubated leaves of *Catharanthus roseus* were infected with bacterial culture (OD_{600} ~ 0.8-1) by multiple pricks with a syringe and the infected leaves were incubated in the dark (Batra *et al.*, 2004).

2.4.14 Influence of acetosyringone in hairy root induction

A phenolic substance, acetosyringone, released by plants induce expression of *vir* region of T-DNA. Successful infection of some plant species with *A. rhizogenes* can be achieved by addition of acetosyringone (Tepfer, 1984).

It has been reported that the presence of acetosyringone at co-cultivation enhaced the virulence of *Agrobacterium* mediated transformation frequencies in *Salvia miltiorrhiza* (Hu and Alfermann, 1993). Vanhala *et al.* (1995) reported that acetosyringone has inhibitory effect on hairy root transformation.

Zdravkovic-Korac *et al.* (2004) reported that the presence of 50 μM acetosyringone during co culturing of *Aesculus hippocastanum* embryo with *A. rhizogenes* A_4GUS significantly increased the number of putative transformants.

Chaudhuri *et al.* (2005) added acetosyringone (10 mM) to the bacterial suspensions (A4 and LBA 9402) one hour before inoculation to increase the transformation efficiency. Tsuro *et al.* (2005) soaked the leaf explants of *Dendranthema grandiflorum* in YEB medium that contained suspended *Agrobacterium* supplemented with 100 μM acetosyringone for 10 min so as to

increase the transformation efficiency. Presence of 100 μM acetosyringone in the co culturing media increased the number of transformants with A_4 strain in *Withania somnifera* (Varghese, 2006).

2.4.15 Co- culture and conditions for hairy root induction

The leaf explants of *Salvia miltiorrhiza* after infection with bacteria were cultured on MS medium with $1gl^{-1}$ casamino acids, 2 per cent sucrose and 0.8 per cent agar containing 500 mg l^{-1} cefotaxime. Cultures were maintained at 25°C in dark (Hu and Alfermann, 1993).

Medium had a significant effect on root formation in *Hyoscyamus muticus*. LS medium favoured root formation when compared to B_5. But hairy root formation was faster on B_5 than on LS medium, particularly in case of LBA 9402. The low salt concentration in medium (B_5) favoured all the bacteria to spread over the medium. The wounded leaves of *Hyoscyamus muticus* were transferred to basal medium containing 500 mg l^{-1} carbenecellin (Vanhala *et al.*, 1995). The affected potato explants were subcultured on MS basal medium with 500 mg l^{-1} cefotaxime (Dobigny *et al.*, 1995). The infected explants of *Gentiana acaulis* after 48 hrs were transferred to basal medium supplemented with 200 mg l^{-1} cefotaxime (Momocilovic *et al.*, 1997).

The infected leaflets of *Azadirachta indica* after 3 to 7 days co-cultivation were transferred to fresh MS medium containing 400 mg l^{-1}ampicillin (Allan *et al.*, 2002). The leaf explants of *Pueraria phaseloides* after cocultivation were placed on MS medium containing carbenecellin 500 mg l^{-1} and kept in unairconditioned chamber at 25°C under 14hrs light photo period to induce hairy roots (Shi and Kintzios, 2003).

The *Aesculus hippocastanum* embyos infected with *A. rhizogenes* A_4 were co-cultured for 72 hrs and were transferred to MS medium with 500 mg l^{-1} cefotaxime (Zdravkovic-Korac *et al.*, 2004). After co-cultivation infected explants of *Isatis indigotica* were rinsed with sterile distilled water five times dried thoroughly on sterile filtered paper and transferred to MS basal medium containing 300 mg l^{-1} cefotaxime (Xu *et al.*, 2004). Lorence *et al.* (2004) transferred the infected explants of

Campotheca acuminata after cocultivation on B_5 solid medium containing timentin 300 mg l^{-1} and plants were incubated at 25°C under 16 hrs photoperiod.

The *Agrobacterium* infected *Tylophora* explants were washed in 1000 mg l^{-1} ampicillin for 30 min and co-cultured with 500 mg l^{-1} ampicillin (Chaudhuri *et al.*, 2005). *A. rhizogenes* strains A_4, ATCC15834 and MTCC 2364 were killed by using 500 mg l^{-1} cefotaxime (Varghese, 2006).

2.4.16 Efficiency of *A. rhizogenes* inducing hairy root

The plant species which were shown to be insusceptible to one *A. rhizogenes* strain have been successfully transformed with other strains (Jung and Tepfer, 1987).

It has been reported that virulence of *Agrobacterium* strains varies among plant hosts (Hobbs *et al.*, 1989; Bush and Pueppke, 1991) and that transformation efficiency of host species vary among different bacterial strain (Godwin *et al.*, 1991; Hu and Alfermann 1993). Significant difference was observed between transformation ability of differential strains of *Agrobacterium* (Vanhala *et al.*, 1995).

Jung and Tepfer (1987) reported that *A. rhizogenes* strain 8196 is better adapted to root initiation in *Calystegia sepium* and A_4 is better suited to *Atropa belladonna*. Sauerwein *et al.* (1992) reported that maximum yield of alkaloid *Hyoscyamus albus* transformed with A_4 when compared to 15834 and MAFF 03.01724.

Hu and Alfermann (1993) reported LBA 9402 strain caused 85 per cent rooting in leaf explants of *Salvia miltiorrhiza* when compared to A_4 (10%), ATCC 15834 (20%). Mannopine and cucumopine strains were unable to induce root formation in potato. So NAA pretreatment was done to stem fragments. The strain 15834 was poor in inducing root formation (Dobigny *et al.*, 1993).

Xu *et al.* (2004) reported that the strain A_4 was better when compared to R1601 and ATCC 15834 and root induction efficiency from cotyledons was higher than from hypocotyls. Lorence *et al.* (2004) tested the ability of two different strains of *A. rhizogenes*, ATCC 15834 and R-1000 on inducing hairy roots in *Camptotheca acuminata*. They found that the strain 15834 infected more than 40 to 45 per cent of explants, but in contrast, strain R-1000 infected only 20 to 24 per cent of exposed tissue. Chaudhuri *et al.* (2005) reported that *Tylophora indica* is more susceptible to strain A_4 than to strain LBA 9402.

Among the five *A. rhizogenes* strains evaluated, PcA_4, 15834 and A_4 induced hairy roots in *Holostemma ada-kodien* whereas 8196, 2659 did not induce hairy roots at all (Karmarkar and Keshavachandran, 2005). Of the three strains A_4, ATCC 15834 and MTCC 2364, ATCC 15834 and A_4 were able to induce hairy roots in *Withania somnifera* (Varghese, 2006).

2.4.17 Establishment of hairy root cultures

In most cases hairy roots emerge within 1 to 4 weeks. The transformed roots of potato were excised and grown on agar solidified MS media with 2 per cent sucrose and 200 $\mu g ml^{-1}$ cefotaxime or carbenecellin (Ooms *et al.*, 1985). Roots obtained from *Solanum sp.* transformed with A_4 strain were excised and individual root tips were transferred to petridishes containing MS medium with 500μg cm^{-3} cefotaxime (Davey *et al.*, 1987).

Sauerwein *et al.* (1992) reported that the axenic hairy roots obtained from leaf discs of *Hyoscyamus albus* were subcultured in hormone free WP (Lloyd and Mc Cown, 1980) liquid medium and cultures were maintained on a rotary shaker at 100 rpm in dark or under continuous light at 25°C. Root tips of 1 to 2 cm length were inoculated in 25 ml media in 100 ml flasks and cultivated on gyratory shaker at 120 rpm in darkness at 25°C (Hu and Alfermann, 1993). The hairy roots emerged from *Acmella oppositifolia* were transferred to basal solid media containing 100 to 200 mg l^{-1} carbenecillin (Flores *et al.*, 1993).

Segments of hairy roots of *Perezia cuernavacana* were excised and cultured on hormone free MS liquid medium with 30 gl^{-1}sucrose and 300 mg l^{-1} cefotaxime, at 80 rpm (Arellano *et al.*, 1996). The adventitious roots of *Gentiana acaulis* 1.5 cm long were excised and about 5 tips were transferred to petridish containing 30 ml of basal medium with antibiotic (Momocilovic *et al.*, 1997).

Single roots of 2 to 3 cm long, produced by leaf explants of *Azadirachta indica* transformed by *A. rhizogenes* strain LBA 9402 were transferred to MS liquid medium containing 100 mg l^{-1} ampicillin. Later ampicillin content was decreased by 50 per cent upon each weekly transfer of whole roots (Allan *et al.*, 2002).

The transformed roots of *Cichorium intybus* were cultivated in a modified liquid MS contining half strength macronutrients and 3 per cent sucrose supplemented with 500 mg l^{-1} cefotaxime. Cultures were kept in gyratory shaker at 25°C, 110 rpm. The roots were subcultured every four weeks by inoculating 0.7 g fresh weight of roots in 250 ml Erlenmeyer flask with 30 ml of nutrient medium (Malarz *et al.*, 2002).

Roots of *Tylophora* that formed at the wounded sites of infected explants were excised and cultured in dark in basal media with 500 mg l^{-1} ampicillin. Each excised primary root was propagated as a separated clone and sub cultured at 4 week interval. Hairy roots turned white to yellowish white then to reddish brown in 6 to 8 weeks (Chaudhuri *et al.*, 2003)

Hairy roots of *Aesculus hippocastanum* were isolated and placed in MS plant growth regulator free liquid medium supplemented with 500 mg l^{-1} cefotaxime to inhibit bacterial growth shaken at 85 rpm and transferred biweekly. The concentration of cefotaxime was gradually lowered (Zdravkovic-Korac *et al.*, 2004).

2.4.18 Applications of hairy roots

Production of higher levels of secondary metabolites compared to normal root has been reported in hairy roots of many medicinal plants. The ease of transformation and cultivation of hairy roots makes this system suitable for expressing

many recombinant protein including functional antibodies and vaccines (Bapat and Ganapathi, 2005).

The hairy roots can regenerate into plants and the altered phenotype is useful and can be exploited to increase the ornamental value of plants. Hairy roots have been widely exploited for the introduction of agronomically useful traits. The cryopreservation of hairy roots cultures offer an important aspect of plant biotechnology in conserving *Agrobacterium* mediated transformed roots of commercial value. Metabolic engineering is another key area where hairy root will play a significant role and may become a best choice for genetic manipulation. Production of shikonin and betacyanins in hairy roots cultures of *Lithospermum erythrorhizon* and *Beta vulgaris* have been scaled up in bioreactors (Shimomura *et al.*, 1991).

2.4.19 Effect of culture media and conditions on growth of hairy roots

Several physical and chemical factors have been found influencing growth and productivity of hairy root cultures. Toivonen *et al.* (1991) found that low nutrient levels enhanced alkaloid production, but biomass yield was maximum in media containing high sucrose and ammonia. Factors such as carbon source and its concentration, ionic concentration of medium, light (Christen *et al.*, 1992) phytohormones (Rhodes *et al.*, 1994) temperature (Toivonen *et al.*, 1992) and inoculum (Mano *et al.*, 1986) are known to influence the growth and alkaloid production of hairy roots. *A. rhizogenes* strains also have effect on biomass and alkaloid productivity of hairy roots.

Sauerwin *et al.* (1992) reported that light and phytohormones influence the alkaloid production in transformed root cultures of *Hyoscyamus albus*. The normal as well as hairy roots changed to callus like structures upon addition of phytohormones. Growth rate of hairy roots produced by A_4 cultured in light was enhanced by addition of low concentration of phytohormones whereas addition of phytohormones reduced the growth of hairy roots transformed by *A. rhizogenes* MAFF-03-01724.

Supplementation of heavy metal ions such as Cu^{2+} has been shown to stimulate alkaloid production (Christen *et al.*, 1992). Specific extra cellular productivity cannot be significantly increased by varying either the temperature or the relative nutrient levels of sucrose and minerals in media (Christen *et al.*, 1992). The hairy roots of different species behave differently in the same culture conditions. Individual hairy root clones can also have different optimum concentrations of sucrose or mineral ions (Oksman – Caldentey *et al.*, 1994).

Gamborg's B_5 medium is the most widely used medium for the hairy roots of many species (Hilton and Wilson, 1995). The roots of *Hyosyamus muticus* were measured by transferring roots to B_5 medium containing 3 per cent sucrose (Vanhala *et al.*, 1995).

Growth rate variation observed in different hairy roots of a plant species or different species of a genus is given in Table 3. The list of plants in which specific secondary metabolites are synthesized in hairy roots at higher levels than in corresponding normal root is given in Table 4. Hairy roots are capable of synthesing novel metabolites. The list is given in Table 5.

Xu *et al.* (2004) found that the culture medium have a significant effect on *Isatis indigotica* hairy root growth. Among four liquid media (MS, 1/2MS, B_5 and White's) tested, MS and half MS were found to be significantly superior to the other two and on comparison between B_5 and white's medium B_5 medium was significantly better than White's medium on hairy root growth. Half MS media with 3 per cent sucrose was found to be superior for promoting hairy roots in *W. somnifera* (Varghese, 2006).

2.4.20 Growth characteristics and morphology of hairy roots

Hairy roots are capable of fast growth in hormone free medium. The precise mechanism involved in lateral root formation is still not clearly understood, but roots transformed by *A. rhizogenes* are characterized by the spontaneous formation

Table 3. Growth rate variation observed in hairy roots of different plant species

Plant	*Agrobacterium rhizogenes* strain	Culture medium	Culture period(days)	Increase in hairy root biomass/ biomass yield
Atropa belladona	A_4	Normal MS	28	285 fold
Atropa belladona	A_4	Normal MS without NH_4	30	24 folds
Atropa belladona	15834	Normal MS	30	60 fold
Cathranthus roseus	15834	½ B_5 salts with full sucrose	26	One fold
Cathranthus roseus	15834	¼ B_5 salts	27	2 fold
Panax ginseng	A_4	Normal MS medium	21	2 fold

Table 4. Hairy roots producing specific secondary metabolites

Sl No.	Plant species	*A.rhizogenes* strains used	Secondary metabolites	Quantity in hairy root/ quantity in normal root
1.	*Atropa belladona*	15834	Scopolamine	5 fold
2.	*Datura innoxia*	15834	Hyoscyamine and Scopolamine	6 fold
3.	*Datura quercifolia*	LBA 9402	Hyoscyamine and Scopolamine	20 fold
4.	*Hyoscyamus niger*	15834	Hyoscyamine and Scopolamine	2 fold
5.	*Rubia tinctoria*	15834	Anthraquinone	19 fold
6.	*Tagetes patula*	LBA 9365	Thiophene	25 fold

(Kukreja *et al.*, 1997)

Table 5. Hairy roots synthesizing novel secondary metabolites

Plant	*A. rhizogenes* strain	Secondary metabolite
Fagopyrum esculentum	ATCC 15834	Procyanidins (Flavanoid)
Lobelia inflata	ATCC 15834	Robetylonin (polyacetylene)
Rauwolfia serpentine	A_4	Hydroxy ajmaline
Swertia japonica	ATCC 15834	8–O–Primeverosyl bellidifolin

(Kukreja *et al.*, 1997)

of numerous laterals, an important factor contributing to their high biomass productivity (Tepfer, 1984).

Significant differences were observed in the transformation ability of different strains of *Agrobacterium* in *Hyosyamus muticus*. The roots were formed mainly on the midrib of leaf and they differed morphologically depending on the bacteria. The A_4 roots were white, thin and long. The LBA 9402 roots were thin and white turning green. The 15834 roots were long, green in colour and it was difficult to remove bacterial growth (Vanhala *et al.*, 1995).

Many variations in the morphology of transformed hairy roots have been reported. The correlation studies on the integration of genes of T_L- DNA and T_R regions with morphology of hairy roots and regenerated plants have been carried out (Hanishten -Cate *et al.*, 1990; Limani *et al.*, 1998; Christey, 2001).

Hairy roots show morphological variation depending upon the interaction, nature of plant cell genotype and strain of the bacterium and show differences in root thickness, degree of branching and amount of hairy root production (Bapat and Ganapathi, 2005). A4, 15834 and PcA4 induced hairy roots of *H. ada-kodien* were whitish in colour and showed negative geotropic growth (Karmarkar and Keshavachandran, 2005). The roots induced by ATCC 15834 were relatively thick with high root hairs compared to that of A4 strain, which produced relatively thin roots with less root hair in *W. somineifera* (Varghese, 2006).

2.4.21 Confirmation of transformation

2.4.21.1 Opine detection

Transformation of plant tissues by *A. rhizogenes* could be confirmed by biochemical detection of the production of opine in plant tissue (Petit *et al.*, 1983). The genes in T-DNA synthesizing opine could be transferred and intergrated into genome of host plant. These genes when expressed in plants produced opine synthesizing enzyme and catalysed the production of opine (Xu *et al.*, 2004). Hundred mg of *Isatis indigotica* hairy roots was ground homogenously in 100µl of 0.1 *M* HCl

left at 4°C for 2 hrs and then centrifuged (9000g) for 5 min and the standard samples of mannopine were blotted on Whatmann 3mm filter paper and HVPE were conducted (Xu *et al.*, 2004).

Transformed nature of hairy roots of *Cichorium intybus* were proved by opine assay (Malarz *et al.*, 2002). Opine production can however be unstable in hairy roots and may disappear after few passages (Godwin *et al.*, 1991). For this reason, detection of T-DNA by Southern hybridization is often necessary to confirm the genetic transformation (Tepfer, 1984). The polymerase chain reaction simplifies the detection of transformation (Hamill *et al.*, 1991).

Transformation in *H.ada-kodien* was confirmed by opine analysis. The roots induced by all strains A4, 15834, PcA4 showed presence of agropine (Karmarkar and Keshavachandran, 2005). Confirmation of transformation by opine detection using high voltage electrophoresis was found to be unsuccessful in *W.sominifera* because of existence of interfering substances (Varghese, 2006).

2.4.21.2 PCR analysis of hairy roots

Transformation of *Azadirachta indica* by LBA 9402 was confirmed using polymerase chain reaction analysis (Allan *et al.*, 2002). For PCR analysis of transformed roots of *Aesculus hippocastanum* using *A.rhizogenes* A4 GUS Zdravkovic-Korac *et al.* (2004) used primers for the *rol* c amplification. They are 5'-TAC GTC GAC TGC CCG ACG ATG ATG-3' and 5'-AAA.CTT GCA CTC GCC ATG CCT CAC-3'.

Chaudhuri *et al.* (2005) used pLJ plasmid covering the Ri TL-DNA as positive control and PCR was performed with primer 5'-ATG GAA TTA GCC GGA CTA AAC G-3' complimentary to 5' coding sequence of the *rol* A gene and primer 5' ATG GAT CCC AAA TTG CTA TTC C-3' complementary to the 3' coding sequence of the *rol* B gene. The PCR identification of rooting locus gene *rol* B and *rol* C was performed using DNAs from the hairy roots template and non transformed roots as control. The primers used were *rol* B-1 (5' GCT CTT GCA CTG CTA GAT TT-3'),

rol B-2 (5' GAA GGT GCA AGC TAC CTC TC-3'), *rol* C-1 (5' CTC CTG ACA TCA AAC TCG TC-3') and *rol* C-2 (5' TGC TTC GAG TTA TGG GTA CA-3') (Shi and Kintzios, 2003).

The PCR confirmed the presence of *rol* B and *rol* C genes of TL-DNA in the hairy roots of *W. somnifera* induced by A4 and ATCC 15834 (Varghese, 2006).

2.4.21.3 Southern hybridization

The DNA probes used for Southern hybridization were 1.23 kb Bam H1 fragment of pLJ 85, that covers T_R with a part of *aux* 1 gene of pHRi and the 1.52 kb Bam H1 fragment of pLJ, cosmid comprising the T_L DNA with a fragment of the *rol* C gene of pHR_1 (Jouanin, 1984; Momocilovic *et al.*, 1997).

The transformed nature of *Perzia cuernavacana* tissue was confirmed by PCR and slot blot hybridization and expression of *gus* gene (Arellano *et al.*, 1992). Southern blot analysis of transformed roots of *Cephaelis ipecacuanha* revealed that only the T_L DNA was integrated into the plant genome without incorporation of the T_R DNA (Yoshimatsu, 2003). Lorence *et al.* (2004) obtained the probe by PCR using *A. rhizogenes* 15834 DNA as template with gene specific primers for *rol* A and *rol* B genes.

2.5 GENETIC TRANSFORMATION IN *Artemesia annua*

Due to native resources and difficulty of total chemical synthtesis, attempts at increasing the production of artemisinin in *A. annua* cells by means of biotechnology have been made (Van-Geldre *et al.*, 1997). However production of artemisinin at significant level in *A. annua* cells and tissue cultures has not been successful. It would be therefore be interest to develop transgenic *A. annua* plants or organs with increased accumulation of artemisinin (Chen *et al.*, 1999).

Few transformation studies have been conducted on members of Anthemideae, which include several important plants like *Artemesia sp.* and Chrysanthemum. In Anthemideae, main focus has been given to use hairy roots in

bioreactor systems to improve yield of economically and pharmacologically important compounds such as artemisinin (Da Silva, 2003).

In *Achillea millifolium*, hairy root cultures were established (Lorenco *et al.*, 1999). The biggest drawback was the low yield of terpenes (Figueiredo *et al.*, 1995). A_4 strain induced hairy roots of *Matricaria recutita* (Maday *et al.*, 1999).

Production of antimalarial compound from transgenic hairy roots of related species *Artemesia absinthum* has also been reported, however the potent drug artemisinin could not be produced in these transformants (Nin *et al.*, 1997).

A few studies have been carried out for induction of hairy roots in *A. annua.* Tansformation of *A. annua* plants with *A. rhizogenes* results in the formation of transformed roots that can produce high levels (0.001-0.45%) artemisinin (Jaziri *et al.*, 1995; Weathers *et al.*, 1994).

Vergauwe *et al.* (1996) and Chen *et al.* (1999) respectively reported transferring a GUS and GFP reporter gene into *A. annua* with *A. tumefaciens* and regenerating transgenic plants. Ghosh *et al.* (1997) found that for wild type strains, the transformation frequency in *A. annua* was significantly affected by parameters like explant type, strain type and age of plant source for explants.

2.5.1 *Agrobacterium rhizogenes* strains used for hairy root induction in *A. annua*.

Various strains were used to induce hairy roots. Transformation efficiency is known to differ with different bacterial strains. Hairy root cultures of *A. annua* were established using *A. rhizogenes* strains MAFF03-01724 or NCIB 8196 (Jaziri *et al.*, 1995).

A. rhizogenes strains LBA 9402, 8196 and A_4 were used for transformation in *A. annua* (Mukherjee *et al.*, 1995). Strain R-1601 was used for infecting *A. annua* (Cai *et al.*, 1995). ATCC-15834 induced hairy roots in *A. annua* (Liu *et al.*, 1998; Chen *et al.*, 1999). Primary roots were visible within 7-21 days depending on the

A. rhizogenes strain. Percentage of explants with primary hairy roots observed after 3 weeks of transformation with different strains of *A.rhizogenes* is represented in Table 6.

Table 6. Efficiency of different strains in inducing hairy roots

Agrobacterium rhizogenes strain	No of days for primary roots to appear	Leaf bits showing hairyroots (%)	Phenotype of primary hairy roots
LBA 9402	10 – 15	100	No callus, abundant formation of branched hairy roots at wound sites
8196	18 -21	30	Wound callusing with or without rooting, roots thicker less hairy rooting at leaf apex or base, less along midrib
A_4	7 - 10	100	Profuse formation of branched or unbranched hairy roots, no callus.

(Mukhrjee *et al.*, 1995).

A. rhizogenes strains A_4, 15834, K_{599}, LBA-9402, 9365 and 9340 were used for induction of hairy roots in *A. annua*. The best root formation response as transformation frequency was achieved with *A. rhizogenes* strains was in the following order LBA 9402, 9340, 9365, 15834 and A_4. Bacterial cultures induced with acetosyringone and explants co-cultivated on MS basal medium with acetosyringone reduced the time of hairy root induction by a week (Giri *et al.*, 2001).

2.5.2 Explants used for transformation in *Artemesia annua*

Leaves from *in vitro* grown plants (Jaziri *et al.*, 1995), from 6 to 8 week old (Mukherjee *et al.*, 1995) or from 3 week old seedlings (Chen *et al.*, 1999; Wang *et al.*, 2004) were used as explant as they normally did not develop roots spontaneously in control experiments

Leaf, stem and root explants from 12 to 18 week old plants and cotyledons and hypocotyls from 8 day old seedling were used for transformation using *A. rhizogenes* in *A. annua* (Veragauwe *et al.*, 1998). Leaf, petiole sections and shoot

tips from *in vitro* grown *A. annua* shoot cultures were used as explants for transformation (Giri *et al.*, 2001).

2.5.3 Transformation method

Bacterial colony in solid medium or bacterial suspension can be used for transformation. Hairy root cultures were established by co-culture method using leaf discs of *Artemesia annua* (Jaziri *et al.*, 1995). Mukherjee *et al.* (1995) produced hairy root cultures by infecting leaves of 6 to 8 week old plants as described by Ooms *et al.* (1985).

Leaves from 2 to 3 week old aseptic seedling were floated in 8 ml liquid MS containing 2 ml of a late log phase *A. rhizogenes* in 50 ml Erlenmeyer flask. After 20 min of infection, explants were blotted with sterile filter paper and subcultured on MS solid medium and kept at 25°C for 2 days. The explants were transferred to MS medium with 500 mg l^{-1} cefotaxime (Chen *et al.*, 1999).

Explants were incubated for 20 min in actively growing bacterial culture of different *A. rhizogenes* strain on nutrient broth with or without acetosyringone. They were co-cultivated for 48 hrs (Giri *et al.*, 2001).

Artemesia annua was grown overnight at 20°C in liquid YEB media and pelleted by centrifugation. This was resuspended in liquid YEB and diluted 10 fold with MS liquid media and explants were incubated in this for 20 min (Wang *et al.*, 2004).

2.5.4 Establishment of *Artemesia annua* hairy root cultures

Mukherjee *et al.* (1995) reported that hairy roots appeared at the point of inoculation within 2 weeks and these were transferred to liquid MS medium without growth regulators containing 500 mg l^{-1} ampicillin. The cultures were maintained at 25 ± 1°C at 70 rpm. Control roots were maintained in MS medium with 0.2 mg l^{-1} IBA.

The transformed roots of *A. annua* were cultured in ½ MS medium and maintained at 25°C in dark on rotatory shaker (100 rpm) (Jaziri *et al.*, 1995). The fastest hairy root growth was observed in one fourth MS (Giri *et al.*, 2001). Hormone free MS medium with 3 per cent sucrose were used for culturing hairy roots (Wang and Tan, 2002). Gonzalez and Weathers (2003) used B_5 media with 3 per cent sucrose for sub culturing of hairy roots.

2.5.5 Artemisinin content in *Artemesia annua* hairy roots

No artemisinin was detected in different root clones of *A. annua* hairy root cultures. Transformed rhizogenic calli or calli derived from hairy roots are incapable of synthesizing artemisinin (Mukherjee *et al.*, 1995). Artemisinin in hairy roots transformed with *A. annua* was low and unstable ranging from zero to 1.9 mg g^{-1} (Weathers *et al.*, 1994; Cai *et al.*, 1995).

2.5.6 Factors affecting artemisinin production in hairy root culture

The productivity of artemisinin depended on the root clones, basal medium and cultural conditions. A number of studies have focused on altering the growth conditions to optimize the production of artemisinin in hairy roots of *A. annua* (Paniego and Giulietti., 1994; Smith *et al.*, 1997; Weathers *et al.*, 1996).

2.5.6.1 Basal medium

MS medium characterized with higher nitrogen concentration has been selected as basal medium by most of the investigators (Qin *et al.*, 1994, Cai *et al.*, 1995., Liu *et al.*, 1999).

Superior growth of hairy roots was obtained from cultures of *A. annua* growth in MS and B_5 media, but the highest artemisinin content per flask was observed in MS medium (Jaziri *et al.*, 1995) and it was 8 times higher than in half MS medium. MS medium with 30 gl^{-1} sucrose was used for culture of hairy roots (Wang *et al.*, 2001).

Weathers and Kim (2001) used B_5 medium with half the normal calcium concentration for hairy root cultures of *A. annua*. Single roots grown in B_5 medium with 0.01 mg l^{-1} GA_3 produced the highest number of lateral roots, length of primary roots, lateral root tip density, total lateral root length and total root length (Weathers *et al.*, 2004).

2.5.6.2 Sucrose

Fast growth of hairy roots and maximal production of artemisinin was observed in presence of 3 per cent sucrose (Cai *et al.*, 1995). Liu *et al.* (1997) analysed the effect of sucrose concentration on growth and artemisinin accumulation in hairy roots and found that optimum sucrose concentration for both growth and artemisinin production based on dry weight was 70 gl^{-1}. Growth of hairy roots in sucrose was equivalent to that in fructose and significantly better than in glucose. Growth of hairy root and artemisinin production varies in autoclaved medium (Weathers *et al.*, 2004).

2.5.6.3 pH

Cai *et al.* (1995) reported that optimum pH value of medium was found to be 5.4. Ammonium may suppress the activity of glutamine synthetase or glutamate synthetase and also decrease the medium pH (Kaul and Hoffman, 1993). Liu *et al.* (1998) reported that MS liquid media become more acidic during the cultivation of *A. annua* hairy roots. Lower pH is detrimental to accumulation and biosynthesis.

2.5.6.4 Type of reactor

Mist reactor yielded roots with more artemisinin than roots grown in shake fast or bubble column reactor (Kim *et al.*, 2001).

2.5.6.5 Nitrogen source

Accumulation of ammonium in medium has repressive effect on artemisinin biosynthesis (Crawford, 1995). Liu *et al.* (1997) reported optimum nitrate concentration 30 m*M* for highest artemisinin content. Nitrogen source is an essential factor for the growth and biosynthesis of artemesisnin in *A. annua* hairy roots though

high ammonium itself inhibited root growth and artemisinin production. Optimum concentration of total initial nitrogen was 24 μ*M* and ratio was 1:5(NH_4^+: NO_3^-) (Wang and Tan, 2002).

2.5.6.6 Growth regulators

Cai *et al.* (1995) reported that low concentration of NAA 0.025 mg l^{-1} enhanced growth of hairy roots but inhibited artemisinin production. Optimum concentration of GA_3 (4.8 mg l^{-1}) greatly promoted artemisinin production in hairy roots.

The growth regulators 2,4-D, IAA, NAA, IBA in hairy root cultures reduced biomass and artemisinin content. Biomass and artemisinin were increased by adding GA_3 (5 ppm). Benzyl adenine at 0.1 to 0.5 ppm increased artemisinin content (Liu *et al.*, 1997).

Hairy root cultures provided with ABA yielded highest amount of biomass. Both 6.benzyl aminopurine and 2-isopentenyl adenine inhibited root growth. Isopentenyl adenine stimulated artemisinin production (Weathers *et al.*, 2004).

2.5.6.7 Light

Jaziri *et al.* (1995) suggested a regulatory role for light in artemisinin production. Artemisinin was detected in hairy roots grown under light condition (Cai *et al.*, 1995) and no artemisinin was reported in shoots cultured in dark (Liu *et al.*, 1999). Red light at 660 nm gave the highest mass of hairy roots and artemisinin content in *A. annua* hairy roots (Wang *et al.*, 2004).

2.5.6.8 Temperature

Chen *et al.* (2004) reported that maximum hairy root growth was found at 25°C, however the highest artemisinin production was found at 30°C.

2.5.7 Relation between artemisinin content and growth of hairy root

Artemisinin content in transformed roots of *A. annua* varies during growth and ageing of *in vitro* cultures (Weathers *et al.*, 1996). Liu *et al.* (1998) reported that the difference of growth rates and artemisinin contents among hairy roots, untransformed roots and calli were significant. The artemisinin content decreased slowly during the exponential phase increased while growth rate slowed down and remained constant after the growth rate slowed down and remained constant after the growth stopped. Optimum culture time for hairy roots of *A. annua* was 21 days. Artemisinin content was closely related to growth (Giri *et al.*, 2001).

2.5.8 Morphology of hairy roots

Hairy roots clones of *A. annua* that were induced by *A. rhizogenes* NCIB 8196 turned green (Jaziri *et al.*, 1995) and by ATCC 15834, LBA 9402, turned green when exposed to light (Giri *et al.*, 2001). The hairy roots of *A. annua* turned brown if not subcultured and there was no spontaneous shoot regeneration from hairy roots (Mukherjee *et al.*, 1995).

2.5.9 Confirmation of transformation

2.5.9.1 Opine assay

The genetic transformation of cultures was proved by opine assay (Jaziri *et al.*, 1995; Giri *et al.*, 2001).

2.5.9.2 PCR and Southern hybridization

Total DNA from hairy roots was isolated by using CTAB method and PCRs were carried out by 30 cycles of 94°C for 40 sec, 47°C for 60 sec, 72°C for 30 sec and final extension step of 72°C for 5 min (Chen *et al.*, 1999).

DNA extracted from hairy root cultures was digested with Bam H1 electrophoresed on 0.8 per cent agarose gel using Tris borate buffer (pH 8) and Southern blotting was carried out as described by Sambrook *et al.* (1989). Bam H1

fragment 8a isolated from pLJ1 was used as probe to detect T_L-T-DNA (Mukherjee *et al.*, 1995).

2.5.10 Biomass analysis

Fresh weight of hairy roots was measured after the roots were washed with deionised water and blotted dry. Dry weight was measured after oven drying at 60°C for atleast 24 hrs (Gonzalez and Weathers, 2003).

2.5.11 Estimation of artemisinin

Artemisinin lacks a UV or fluorescent chromophore as well as functional groups for direct derivatisation hence sensitive and specific analytical method for detection and determination of artemisinin is desirable (Gupta *et al.*, 1996).

A variety of procedures for the estimation of sesquiterpenes of *A.annua* have been reported. A number of techniques have been applied for the estimation of artemisinin in plant material including TLC densitometry. (Gupta *et al.*, 1996) HPLC (Charles *et al.*, 1990) electrochemical methods (Acton *et al.*, 1985) gas chromatography (Sipahimalani *et al.*, 1991) and ELISA for detection of artemisinin (Jaziri *et al.*, 1993).

2.5.11.1 Extraction of artemisinin

Air dried leaves (200g) were extracted with boiling petroleum ether for 48 hrs. Removal of solvent *in vacuo* gave dark brown syrup that was dissolved in 20 ml of $CHCl_3$ and to this solution CH_3CN 180 ml was added. Insoluble material was removed and filtrate was evaporated under reduced pressure to give gummy residue, which was used for TLC (Klayman *et al.*, 1984).

Leaves, shoot, root or seeds of *A. annua* were extracted with petroleum ether (30-60°C) and reflexed for 48 hrs at 45°C. After filtering, the solvent was evaporated and the residue was treated with 10-15 ml CH_3CN and again filtered and used for HPLC. Cells from suspension cultures of *A. annua* were filtered and freeze

dried and extracted successively with $CHCl_3$ and MeOH. The extracts were used for TLC detection of artemisinin (Liu *et al.*, 1992).

Hairy root cultures in MS liquid medium were extracted with methanol and artemisinin was estimated using TLC (Jaziri *et al.*, 1995). Oven dried (35-50°C) 0.2g plant material of *A. annua* was extracted with n-hexane (3×10ml) concentrated under vacuum, dissolved in methanol and made up to 1 ml in methanol and used for HPLC (Gupta *et al.*, 1997).

One gram fresh plant material was ground in 5 ml n-hexane in mortar for 5 min, filtered evaporated and redissolved in 0.5 ml n-hexane. Standard artemisinin was dissolved in n-hexane. Artemisinin was estimated by using TLC (Gupta *et al.*, 1996, Kawamato *et al.*, 1999).

One gram of hairy roots was extracted with 3 ml of toluene in an ultrasonic bath to 30 min in ice cold water followed by 10 min centrifugation at 4,390 g, the supernatant was saved and pellets were extracted again in toluene. Supernatants were decanted, pooled dried under liquid nitrogen and stored at -20°C until HPLC analysis (Smith *et al.*, 1997).

Leaves of *A. annua* were dried by lyophilization and ground to powder. This was extracted with methanol in an ultrasonic bath for 30 min followed by centrifugation at 10000 rpm and the supernatant was used for analysis (Wallart *et al.*, 2000).

Dry tissue (100 mg) was powdered and extracted with petroleum ether (20 ml) by 30 min ultrasonication. Extract was evaporated and dissolved in 1 ml ethanol and centrifuged at 10,000g. Supernatant was used for HPLC analysis (Tang *et al.*, 2000).

Transformed roots were dried ground and extracted with hexane in soxhlet apparatus at 60°C for 8 hrs. The extract was concentrated by evaporation. Artemisinin was quantified by HPLC (Giri *et al.*, 2001). Hairy roots of *A. annua* were rinsed with

distilled water and extracted with toluene and analysis was done by HPLC (Weathers *et al.*, 2004).

2.5.11.2 HPLC (High Pressure Liquid Chromatography)

Analysis of artemisinin and its precursors were done by HPLC. HPLC with UV detector 260 nm and C18 water column were used. Mobile phase was phosphate buffer: methanol 6:4 (pH: 7.9) (Gupta *et al.*, 1996) 55:45 v/v pH: 7.0 at flow rate of 1 ml/min and retention time was 12 min (Smith *et al.*, 1997). The mobile phase used was aqueous 0.1 *M* NH_4OAc, CH_3CN (80:20) and flow rate was 1.5 ml/min (Charles *et al.*, 1990).

The 0.1 ml extract of plant was mixed with 0.2 per cent NaOH (0.4 ml) and warmed in water bath at 45°C for alkalization. After cooling mixture was diluted to 1 ml with 0.1 *M* acetic acid in 20 per cent ethanol (neutralization). Mobile phase was 0.01 *M* Na_2HPO_4 –NaH_2PO_4 buffer. The absorbance at 260 nm was detected. Flow rate was 1ml/min (Kawamoto *et al.*, 1999; Tang *et al.*,2000; Kim *et al.*, 2001).

Wallart *et al.* (2000) used H_2O, 0.1 *M* H_3PO_4, CH_3CN (49:1:50 V/V) as mobile phase with 0.75 ml/min flow rate and artemisinin was detected at 210 nm. Mobile phase used for HPLC was 1 per cent TFA in water: acetonitrile (30:70) at flow rate of 1 ml/min. Artemisinin was monitored at 220 nm with UV detector (Giri *et al.*, 2001).

An accurate reverse phase HPLC with UV detection was described for determination of artemisinin where artemisinin in extract was converted to UV absorbing compound Q_{260} by being treated with 0.2 *N* NaOH and 0.08 *M* acetic acid. Mobile phase was methanol: acetonitrile: 0.9 m*M* Na_2HPO_4 buffer (45:10:45). Flow rate was 0.5 ml/min (Qian *et al.*, 2005).

2.5.11.3 Thin Layer chromatography (TLC)

Silica Gel G_{25} UV 254 plates with $CHCl_3$: iso propanol (9:1) as solvent were used (Tawfiq *et al.*, 1989). The developing system for TLC was 5 to 7.5 per cent

EtOAc/$CHCl_3$ solution. Visulisation of TLC plates was performed using anisaldehyde/H_2SO_4 (6%) spray reagent (El-Markby, *et al.*, 1987; Tawfiq *et al.*, 1989; El-Sohly, 1990).

Among the reported procedures for estimation of artemisinin HPLC is commonly utilized. However due to simplicity, accuracy and low cost than HPLC. TLC densitometry is now utilized frequently. Mobile phase was n- hexane diethyl ether (1:1). Spots were visualized by immersing the plates in freshly prepared mixture of glacial acetic acid: concentrated H_2SO_4: anisaldehyde (50:1:0.5) followed by heating the plates at 110°C for 15 min to visualize a pink colour of artemisinin. For quantification, TLC spot corresponding to artemisinin was scanned at 540 nm and 610 nm using dual wavelength mode with background sustraction and using a light spot of 1 x 2 cm (Gupta *et al.*, 1996).

A TLC method was developed to analyze artemisinin and its derivatives artemether and arteether using a silica gel plate with a mobile phase containing pure chloroform. Methoxybenzaldehyde (1%) in an acidic solution of 98 per cent v/v sulphuric acid and 96 -98 per cent v/v acetic acid (respectively 2% and 10% v/v in alcohol water 60:30, v/v) was used as the spray reagent. It presents purple colour against a slightly colored background. Activation was done at 110°C (Gabriel and Plaizier - Vercammen, 2003).

2.6 ENHANCEMENT OF ARTEMISININ IN HAIRY ROOT CULTURES

Attempts were made to improve artemisinin production in cultures by various regulators such as gibberlic acid, casein hydrolysate, feeding precursors, mutagenic compounds and fungal elicitors (Dhingra *et al.*, 2000). Casein hydrolysate enhaced the production of artemisinin (Abdin *et al.*, 2003). Addition of 22S, 23S homobrassinolide (steroidal lactone) to hairy root cultures of *A. annua* increased artemisinin production (Wang *et al.*, 2002).

Various research efforts such as precursor feeding, elicitation, incorporation of specific genes of seconday metabolite pathway within the Ri T- DNA

are now being directed towards comprehending and manipulating the secondary production of hairy root culture (Kukreja *et al.*, 1997).

2.6.1 Addition of precursors

A wide range of potentially valuable compounds have been produced by adding precursors to various culture species. Often the precursor undergoes more than one bioconversion, resulting in a complex mixture of products (Pras and Woerdenbag, 2000). Feeding of coniferin to cultures of *Podophyllum hexandrum* increased accumulation of podophyllotoxin (Van-Uden *et al.*, 1990).

Balvanoys *et al.* (2002) reported that presence of phenylalanine or lysine increased the production of lobeline in *Lobelia inflata* hairy root cultures. Morgan and Shanks (2000) observed that feeding higher levels of auxin or tryptophan resulted in increased branching and thickening of *Catharanthus roseus* hairy root cultures. Varghese (2006) reported that addition of precursor methionine was ineffective in increasing the withanolide production.

2.6.1.1 Precursors of artemisinin

Two probable biosynthetic precursors of artemisinin are arteannuin B and qinghao acid (Jeremic *et al.*, 1973). The biosynthetic sequence suggested for artemisinin is as follows.

Farnesyl pyrophosphate ⟶ dihydro costonolide ⟶
cadinanolide ⟶ arteannuinB ⟶ artemisinin (Akhila *et al.*, 1987).

Biosynthesis of sesquiterpenoids proceeds via mevalonic acid. But the addition of mevalonic acid in form of its lactone has negatively influenced growth and artemisinin content (Woerdenbag *et al.*, 1991). Feeding of ^{14}C labelled arteannuic acid into call free systems of *A.annua* resulted in formation of arteannuin B and artemisinin. This result suggested that arteannuic acid might be a common precursor for arteannuin B and artemisinin (Sangwan *et al.*, 1993).

Abdin *et al.* (2003) reported that artemsinin can be produced from its precursors artemsinic acid and arteannuin B which are easy to be sythasised. Biosynthetic pathway of artemisinin is given in Fig. 1.

2.6.2 Addition of osmoregulants

Stress may act quantitatively and qualitatively as regulator of secondary product biosynthesis (Frischknecht and Baumann, 1985). The osmoregulant polyethylene glycol at 2 per cent elicited a positive response in leaf calli of *Sida cordifolia* on biosynthesis of ephedrine (Sankar, 1998).

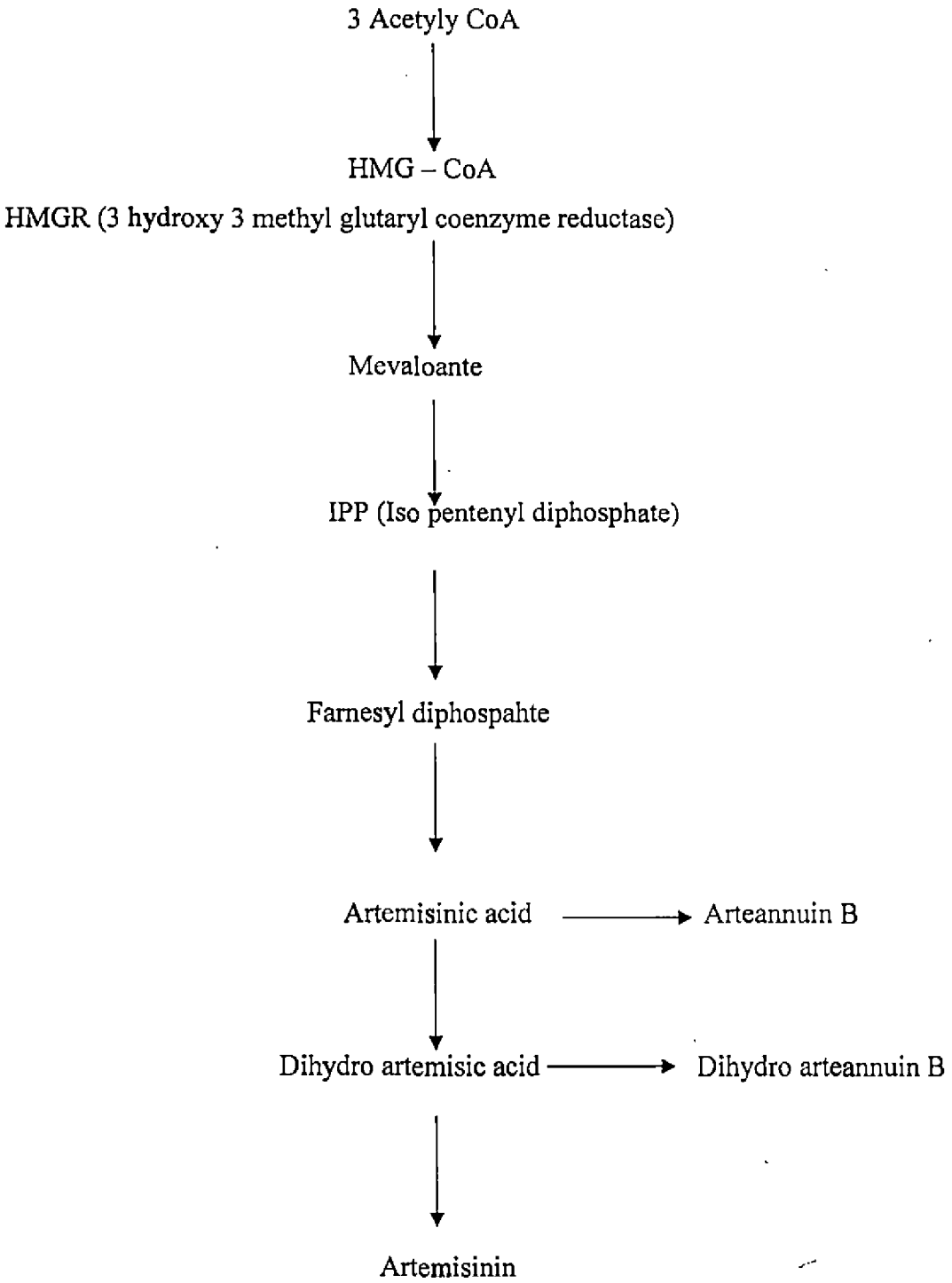
Osmoregulants like sorbitol and mannitol failed to enhance metabolite production in *Sida spp.* (Sankar, 1998) and *Nicotiana* (Gangopadhay *et al.*, 1997). Addition of PEG (9%) molecular weight 3350 increased ajmalicine production capacity of *Catharanthus roseus* (Akcam-Oluk *et al.*, 2003). The addition of osmoregulant PEG (2.5 and 5%) failed to elicit a positive influence in the biosynthesis of Withaferin A in root cultures (Varghese, 2006).

2.6.3 Addition of elicitors

Elicitation is one of the methods that have been used to enhance secondary metabolites of cell cultures. Different factors like specificity and concentration of elicitor, duration of exposure and timing of elicitor determine the product yield (Vander- Heijden *et al.*, 1989). Chitosan has been used as effective elicitor. It induces pore formation in plasmalemma which cause faster secondary product release (Brodelius *et al.*, 1989).

Experimental results show that a wide variety of substances (biotic and abiotic) are able to act as metabolites, which trigger production of secondary metabolites in plants. Biotic elicitors originate either from the host plant or from plant pathogens. Macromolecules from fungi such as glycoproteins, oligosaccharides, peptides and phospholipids have been identified as biotic elicitors. UV irradiation, light, salinity, heavy metals, antibiotic, metabolic inhibitors, carbohydrates and several synthetic products have been identified as abiotic inhibitors (De, 2001). Elicitation by

Fig. 1. Biosynthetic pathway of artemisinin production



(Wallart *et al.*, 2000; Croteau *et al.*,2000)

Aspergillus homogenate (250 µl/125 ml) was found to induce a positive response on biosynthesis of withaferin (Varghese, 2006) whereas addition of yeast extract showed reduction in biosynthesis. The biosynthesis of sesquiterpenic phytoalexin capsidol was investigated using *in vitro* root cultures of chilli elicited with cellulose. Maximum production was achieved at 24 hrs after elicitation (Patricia *et al.*, 1996).

The elicitation of *Hyoscyamus muticus* root and cell suspension cultures by fungal elicitor *Rhizoctonia solani* caused dramatic changes in growth, respiration and nutrient yield (Carvaleho and Curtis, 2002).

Ajmalicine accumulation increased by about three fold when callus was treated with *Aspergillus niger, Fusarium moniliforme,* and *Trichoderma viride.* The maximum ajmalicine production was observed in cells treated with *T. viridae* (Nomadeo *et al.*, 2002).

The treatment of cells of *Plumbago rosea* L. with *A. niger, Rhizopus oryzae* and yeast resulted in two to three fold more plumbagin over control cells (Komaraiah *et al.*, 2002). Dong and Zhang (2002) reported that methyl jasmonate feeding will improve taxuyunnaine production in *Taxus chinensis* cell culture. A dramatic increase in cell growth and hypericin production was observed in *Hypericum perforatum* callus culture. Other elicitors such as jasmonic acid and fungal elicitors failed to show any stimulating effect (Walker *et al.*, 2002). Cusido *et al.* (1999) reported that Tween 20 treatment encouraged both growth and alkaloid productivity of hairy roots of *Datura metel* L.

Elicitors are compounds which defend the plant against any foreign organism such as fungus. Elicitors do not have any direct effect but it decreases cell permeability and it may increase the formation of secondary metabolites because of feed back inhibition and intracellular degradation (Brodelius *et al.*, 1989).

Biotic and abiotic elicitors including solvents and detergents have been reported to release the products from from hairy roots into medium without any loss of

viability and production capacity of hairy roots (Dutta-Alvarez *et al.*, 2000). Effect of some biotic and abiotic elicitors on secondary metabolite is given in Table 7.

Liu *et al.* (1997) found that maximum artemisinin accumulation was obtained at 48 hrs of elicitation with 5 ml homogenate of *Aspergillus oryzae* cultures. It was found that microbial transformation sesquiterpene lactone arteannuinB from *A. annua* using *Aspergillus flavipes* produced dihydroarteannuin using, *Beauveria bassiana* produced 3 β-hydroxy arteannun B and 13-hydroxyl 11-epi dihydro arteannuin B (El-Markby *et al.*, 1987). Blocking sterol biosynthesis by using enzyme squalene synthase resulted in increased production of artemisinin (Woerdenbag *et al.*, 1993).

2.6.4 Immobilization

Towler and Weather (2003) reported that poly L-lysine coated polypropylene was used to immobilize transformed roots of *A. annua* in bioreactor. Attachment process was found to be enhanced by sheep serum.

Table 7. Summary of studies involving elicitation of hairy roots in medicinal plants

Elicitor	Metabolite eluted	Plant material	Reference
Rhodototula rubra	Acridone epoxide	*Ruta graveolens* cell culture	Bohlman and Eilert, 1994
Aspergillus niger	Total alkaloid	*Catharathus roseus* cell cuture	Hernandez and Vargas, 1991
Phytophthora parasitica	Sesquiterpenoids	*Nicotina tabaccum* cell culture	Chapell *et al.*, 1989
Fungal cell culture filtrate	Shikonin	*Lithospermum erythrorhizon* cell culture	Kim and Chang,1990
Yeast crude polysaccharides	Modified composition of oil	*Anthemis nobilis* tissue culture	Fauconnier *et al.*, 1993
Alginate	Enzyme	*Catharathus roseus* cell cuture	Aoyagi *et al.*, 1998

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled "Genetic transformation in annual worm wood (*Artemesia annua* L.) for hairy root induction and enhancement of secondary metabolites" was carried out at the Centre for Plant Biotechnology and Molecular Biology of the College of Horticulture, Vellanikkara from September 2005 to December 2006.

3.1 CULTURE MEDIA

3.1.1 Chemicals

The major and minor nutrients for the preparation of media were of analytical grade and procured from M/s Sisco Research Laboratories (SRL), HIMEDIA and M/s Merck India Ltd. The aminoacids, vitamins and plant growth regulators were obtained from M/s Merck India Ltd, SRL, Lobachemie, HIMEDIA and Sigma Chemicals, USA. Artemisinin standard for biochemical analysis was procured from Sigma Chemicals. The primers and restriction enzymes were obtained from Imperial Bio Medics, Chandigarh.

3.1.2 Glass and Plastic wares

Borosilicate glasswares of Corning/Borosil brand and disposable petridishes of Axygen and Tarson were used for the study. The glasswares were initially subjected to steam and cleaned with detergent solution and then with tap water. The glasswares were then soaked in a solution containing potassium dichromate, water and sulphuric acid (8: 80: 250) followed by thorough washing with jets of tap water and finally with distilled water. Test tubes were kept in racks and dried in open air. Conical flasks were dried in hot air oven. Jam bottles were further sterilized in an autoclave and dried in hot air oven. The centrifuge tubes were first washed in soap solution then with distilled water. The tubes were filled with distilled water and sterilized in autoclave and kept in hot air oven. Glass petriplates were washed dried in open air, wiped with alcohol. It is then autoclaved and dried in hot air oven.

3.1.3 Composition of media

Murashige and Skoog (1962) medium (MS), SH medium and B_5 medium were tried to find the best basal medium for micropropagation of *Artemesia annua*. Half strength MS was also used. In the development of transformed root cultures MS, half MS and B_5 medium were used. The composition of the media are given in Appendix I.

The different *Agrobacterium rhizogenes* strains were cultured in Yeast Extract Broth (YEB), Yeast Extract Peptone, Yeast Extract Mannitol (YEM), Nutrient Agar (NA), Luria Bretani Agar (LBA) and *Xanthomonas* media. *E. coli* were cultured in LB medium only. The basal compositions of these media are given in Appendix II.

3.1.4 Preparation of tissue culture medium

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of plant tissue culture media. Stock solutions of major and minor nutrients were prepared and stored in pre-cleaned glass bottles in refrigerated conditions. Stock 3 was stored in amber coloured bottles.

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. For preparing media of full strength, 20 ml was pipetted from 50X stocks and 10 ml from 100X stocks. A small volume of distilled water was added to it and later on, required quantities of sucrose 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 to 5.7 using 0.1 *N* NaOH.

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 absorbent cotton. For solid media, test tubes (15 cm x 2.5 cm) were used whereas for liquid media conical flasks or Erlenmeyer flasks (100, 250 and 500ml) were used as culture vessels. Fifteen ml medium was poured in each test tube, 30 ml medium in 100 ml conical flask, 120

ml in 250 ml conical flask and 250 ml in 500 ml conical flask. Vessels containing media were sterilized in an autoclave at 121°C in 15 psi for 20 min. The medium was allowed to cool to room temperature and stored in culture room until used.

3.1.5 Growth regulators

Auxins (NAA, IBA) and cytokinins (BAP, KN) were incorporated in the media at various stages of culture for culture establishment, direct organogenesis, multiplication and rooting.

GA_3 was added to the media for shoot elongation. Since GA_3 is thermolabile, it was filter sterilized using micro filter before addition to the medium. For this the nitrocellulose filter was placed in the filter assembly and sterilized in an autoclave and dried in hot air oven. The required quantity of filter sterilized GA_3 was pipetted out using micropipette and added to the sterilized, melted and cooled medium (40-50°C) under the hood of a laminar airflow cabinet.

3.1.6 Organic supplements

Casein hydrolysate (0.1%) was tried for their effect on regeneration of inflorescence bits.

3.1.7 Carbon source

Sucrose (2 - 3%) was used as the main source of carbon in this study.

3.1.8 Preparation of YEB, YEP, YEM, NA, *Xanthomonas* media and LBA 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. A small volume 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 was adjusted to 7.0 for NA, YEP, YEM and LBA and 7.2 for YEB and

Xanthomonas medium using a standard pH meter by adding either 0.1 *N* NaOH or HCl.

For solid media, agar was added at the rate of 20 g l^{-1}. The media were stirred and heated, to melt the agar and were poured when hot, into conical flasks and jam bottles. Conical flasks were plugged with non-absorbent cotton and jam bottles were sealed tightly using cello tape after placing the lid. Autoclaving was done at 121°C at 15 psi for 20 min to sterilize the medium and they were further kept in the culture room until used.

3.1.9 Antibiotics

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

Different antibiotics (ampicillin, cefotaxime, carbenicillin and kanamycin) were used in MS medium, YEB medium, Nutrient Agar medium and LB medium for killing the bacteria, for testing the resistance of bacteria to antibiotics and also for studying the sensitivity of explants to various antibiotics.

3.2 TRANSFER AREA AND ASEPTIC MANIPULATIONS

All the aseptic manipulations such as surface sterilization of explants, preparation and inoculation of explants, subsequent sub culturing, preparation of antibiotic media and transformation works were all carried out under a laminar airflow cabinet.

3.3 CULTURE ROOM

The cultures were incubated at 26 ± 2°C in an air-conditioned culture room with 16 hrs light photoperiod (1000 lux) from fluorescent tubes. Humidity in the culture room varied from 60 to 80 per cent according to the climate prevailing. Dark

condition for culturing bacteria was provided by black cotton cloth fixed on culture racks.

3.4 SOURCE OF EXPLANTS

Stock plants were brought from GKVK College, University of Agricultural Sciences, Bangalore. They were planted in pots and were placed in the shade house giving daily irrigation. Established plants were sprayed with contact fungicide Fytolan (copper oxychloride) at 0.25 per cent concentration fortnightly. The explants for *in vitro* regeneration studies were taken from these plants

3.5 STANDARDISATION OF *IN VITRO* REGENERATION

3.5.1 Explants used for micropropagation

Various explants like leaf segments, petiole segments, shoot tips, nodal segments, inflorescence bits and roots were used for the study.

3.5.2 Collection and preparation of explants

3.5.2.1 *In vivo* plants.

The explants were collected from the net house. They were washed in tap water to remove dust. The explants were put in detergent solution for 10 min. They were washed thoroughly with distilled water to remove detergent solution. The explants were then treated with Bavistin 0.1 per cent solution for 3-5 min depending on age of explants. The explants were then thoroughly washed with distilled water and dried on blotting paper. The explants were then wiped with 50 per cent ethanol. Further, surface sterilization was carried out under the hood of the laminar airflow cabinet.

3.5.2.2 *In vitro* plants

Different parts of *in vitro* plantlets namely shoot tip, roots, nodal segments and petioles were used for the study.

3.5.3 Standardization of surface sterilization of explants

Surface sterilization of explants was done in order to make the explants free of microorganisms. Surface sterilization was carried out under aseptic conditions in laminar airflow cabinet. The explants were sterilized with mercuric chloride ($HgCl_2$) at varying times and at the concentrations of 0.05, 0.1, and 0.2 per cent. Observations were made on percentage of survival without contamination after one week and percentage of culture establishment after two weeks. For this purpose, MS medium containing 3 per cent sucrose and optimum level of growth regulators were used.

In all treatments, explants were submerged in sterilant for the required period and with frequent agitation. After the surface sterilization solution was drained off, explants were washed free of the chemical sterilant using sterile water. Then the explants were dried carefully by transferring them onto filter paper pieces on a sterile petridish.

The leaf pieces were trimmed on the four sides and reduced to 0.5 cm^2. The end portions from both sides of inflorescence segments were removed and made to 0.5 cm long pieces. From the *in vitro* grown shoots, petiole (~1.0 cm) nodal segments (~1.5 cm), shoot tips (~3.0 cm) and root bits (~1.0 cm) were cut and separated out. Nodal segments, leaf, petiole and shoot tips were taken from two month old seedlings. Nodal segments were cut with sterile blade such that each segment carried one to two nodes.

3.5.4 Standardization of explants

Leaf segments, petiole, shoot tip, nodal segments, inflorescence bits and roots were cultured in MS media supplemented with different combinations of auxins and cytokinins. The cultures were incubated in the culture room. The regeneration response and the number of shoot buds obtained were recorded after 25 days.

3.5.4.1 Leaf explants

The leaf pieces were kept in media with the abaxial side facing upwards. The following growth regulator combinations were tried for regeneration. Each treatment consisted of ten replicates.

1) MS + NAA (0.05, 0.1,0.5, 1,2,3 mg l^{-1})
2) MS + BAP (0.5,1,2,3,4 mg l^{-1})
3) MS + NAA (0.05,0.1,0.2,0.5,1,2,3 mg l^{-1}) + BAP (0.05,0.1,0.2,0.5,1,2,3 mg l^{-1})

Observations regarding the number of multiple shoots produced per shoot bud were recorded after 25 days.

3.5.4.1.1 Multiplication

Regenerated shoot buds from the leaf segments were multiplied in MS medium supplemented with BAP (0.5, 1, 2 mg l^{-1}), NAA (0.5 mg l^{-1}) and BAP (0.5, 1, 2 mg l^{-1}) + NAA (0.5 mg l^{-1}). Data regarding the number of shoot buds produced were recorded after 25 days.

3.5.4.1.2 Elongation

The shoots produced from leaves of length 0.5 to 2.0 cm were separated and tried for elongation in the following media

1. MS + BAP 0.1 mg l^{-1} + GA_3 (0.1,0.2,0.5mg l^{-1})
2. MS + GA_3 (0.01,0.05,0.1,0.2 mg l^{-1})
3. MS + Sucrose 3 per cent
4. MS + Sucrose 2 per cent
5. ½ MS + Sucrose 3 per cent
6. MS + NAA (0.5 mg l^{-1})

Observations were recorded after 25 days regarding the elongation obtained, number of internodes and number of leaves.

3.5.4.1.3 Rooting

The shoots which were elongated in MS media supplemented with GA_3 and also shoots obtained from multiplication media were excised using a sterile blade and inoculated in the following media for rooting.

1. MS basal
2. MS + NAA (0.5 mg l^{-1})
3. ½ MS + NAA (0.5 mg l^{-1})
4. MS + IBA (0.05, 0.1, 0.5, 1, 2, 5 mg l^{-1})
5. ½ MS + IBA (0.5 mg l^{-1}) + sucrose 2 per cent
6. ½ MS + IBA (0.5 mg l^{-1}) + sucrose 2 per cent + activated charcoal (0.05 , 0.1 , 0.2 per cent)

Observations on days taken for rooting, nature of roots, mean number of roots and mean number of shoots were recorded.

3.5.4.1.4 Hardening and planting out

The *in vitro* rooted plantlets obtained, were taken out of the test tubes using forceps, after soaking the media in the test tubes in water for five min. The solidified media from the plantlets was washed out under running tap water. The plantlets were then planted in small earthern pots filled with sterilized sand. They were transferred to the green house. After 2 months the plants were transferred to large pots containing potting mixture.

3.5.4.2 Regeneration from callus

The friable callus obtained from leaf explants were kept in MS media with different growth regulator combinations. Observations regarding regeneration and colour of callus were recorded after 25 days.

3.5.4.3 Nodal segments and shoot tip explants

The shoot tips and nodal segments taken from the *in vitro* grown plants were inoculated on media containing various growth regulator combinations. Observations regarding number of shoot buds produced per explants were recorded after 25 days.

3.5.4.3.1 Elongation

The regenerated shoot buds along with the basal callus were further elongated in MS media supplemented with GA_3 (0.01, 0.05, 0.1, 0.2 mg l^{-1}).

3.5.4.3.2 Rooting

The elongated shoots were rooted on rooting media (half MS + IBA 0.5 mg l^{-1} + sucrose 2 per cent). Observations regarding the elongation rate, number of internodes, leaves, roots, days taken for root induction and rooting percentage were recorded after 20 days of inoculation.

3.5.4.4 Inflorescence bits and petiole segment explants

The mature and immature inflorescence segments were taken from the net house and after surface sterilization they were cut into pieces of ~1.0 cm. The petioles were taken from *in vitro* plants. The explants were inoculated in varying combinations of growth regulators. Observations regarding the number of shoot buds produced were recorded after 25 days.

3.5.4.4.1 Multiplication

The shoot buds obtained were multiplied in the following media.

1) MS + BAP (0.5, 1, 2 mg l^{-1})
2) MS + NAA (0.5,1,2 mg l^{-1})
3) MS + NAA (0.5 mg l^{-1}) + BAP (0.5, 1, 2 mg l^{-1})

The number of multiple shoot buds obtained was recorded after three weeks of culture.

3.5.4.4.2 Elongation and rooting

The regenerated shoot buds along with the basal callus were further elongated in MS media supplemented with GA_3 (0.01, 0.05, 0.1, 0.2 mg l^{-1}). The elongated shoots were rooted on rooting media (half MS + IBA 0.5 mg l^{-1} + sucrose 2 per cent).

3.5.4.5 Root explants

Root tips of approximately 1.0 cm were taken from *in vitro* rooted plantlets and were inoculated in MS medium with varying growth regulator combinations. Observations were taken after 25 days.

3.6 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

Three strains of *Agrobacterium rhizogenes* A4, MTCC 2364 and ATCC 15834 of agropine family were used for the present study.

3.6.1 Culturing of *A. rhizogenes* strains

The bacterial strains were cultured on four different media such as Luria Bretani Agar (LBA), Yeast Extract Peptone, Yeast Extract Mannitol (YEM), Yeast Extract Broth (YEB), *Xanthomonas* media and Nutrient Agar (NA) to select a suitable growth medium. The solid media was melted and cooled to 40-50°C and poured into sterilized petriplates. Each strain was streaked on plates containing the respective media. The growth rate of bacteria on each medium was observed.

3.6.2 Screening of *A. rhizogenes* strains for antibiotic sensitivity

The *A. rhizogenes* strains used for the study were tested for resistance to antibiotics cefotaxime, ampicillin and carbenicillin. Nutrient agar media supplemented with 50, 100, 200, 300, 400 and 500 mg l^{-1} of each antibiotic separately were used for the study. Bacterial strains were also streaked in nutrient agar medium without any antibiotics, to be used as control.

3.6.3 Explants for transformation

Explants such as shoot tips, leaf segments and nodal segments were used for transformation studies.

3.6.3.1 Pre-culturing of explants

The explants were taken from *in vitro* rooted plantlets. The leaf margins were cut at all sides. Nodal segments were dissected such that each segment carried one or two nodes. The explants were cultured on MS solid medium in petriplates for two days prior to their infection with bacteria. Explants which were not inoculated with bacteria were also placed on growth regulator free MS media as control.

3.6.3.2 Wounding of explants

Wounds were made on the explants using a sterile blade and an injection needle. The leaf margins were cut on all sides with a blade and pricks were made at base of shoot tip with blade. A fresh cut was made at the base of shoot tip with blade. The shoot tips and were pricked with sterile needle. About ten pricks were made on each segment using the hypodermic injection needle.

3.6.4 Evaluation of the sensitivity of explants to various antibiotics

Different explants like shoot tip, nodal segments and leaf segments were tested for their sensitivity to various concentrations of the antibiotic cefotaxime (100, 250, 500 and 1000 mg l^{-1}). As control, the explants were cultured in MS solid medium without antibiotics.

3.6.5 Standardization of inoculation method

The bacterium from isolated single cell colonies (hereafter referred to as Direct Inoculation Method or DIM) and bacterial suspension were used as the inoculum (hereafter referred to as the Suspension culture inoculation Method or SM).

3.6.5.1 Direct Inoculation Method

In this method, bacterium from isolated single cell colonies was used as the inoculum. Explants were wounded by using a sterile blade and the injection needle dipped in the inoculum. The explants were then blotted with a sterile blotting paper and placed on solid MS medium without growth regulators contained in the petriplates. Uninfected explants were placed on growth regulator free MS medium as control.

3.6.5.2 Suspension culture inoculation Method

The pre-cultured explants were wounded first using sterile blade and injection needle. The *Agrobacterium* suspension prepared ($O.D_{600}$ ~1.0) was transferred to sterile filter paper in sterile petriplates. The wounded explants were immersed in the suspension for 20 min with intermittent gentle agitation. The explants were then blotted dry using sterile blotting paper and placed on solid MS medium without growth regulators taken in the petriplates. Uninfected explants were placed on growth regulator free MS medium as control.

3.6.6 Co-cultivation of explants with *Agrobacterium*

The infected explants co-cultured in dark at 26 ± 2°C for 1 to 4 days. Transformation percentage obtained and response of different explants under different preculture and different co-cultivation period were recorded.

3.6.6.1 Influence of acetosyringone in hairy root induction

Two methods were used to study the influence of acetosyringone in hairy root induction. The infected explants were co-cultured in acetosyringone containing media. Acetosyringone (3', 5'-dimethoxy-4'-hydroxyacetophenone) dissolved in dimethyl sulfoxide (DMSO) was used as the stock. Autoclaved MS solid medium prepared in the conical flask was melted and cooled to 40-50°C. From the stock, acetosyringone at the rate of 100 μM was added to the medium aseptically, shaken well and poured to sterile petriplates and solidified.

Explants were infected by DIM or SM and then co-cultured in dark for two days in solid MS media containing 100 μ*M* acetosyringone (hereafter referred as Direct Inoculation and Co-cultivation with Acetosyringone or DICA method and Suspension culture and Co-cultivation with Acetosyringone or SCA method respectively). As control, one set of explants inoculated by DIM and SM were placed in co-culturing media without acetosyringone under dark photoperiod for two days.

3.6.7 Culture media and conditions for hairy root induction

The infected explants after co-cultivation were washed thoroughly with MS liquid medium containing 500 mg l^{-1} cefotaxime. After washing, the explants were blotted dry using sterile blotting paper. The explants were then transferred to solid MS medium containing 500 mg l^{-1} cefotaxime. The explants were further cultured at 26 ± 2°C under diffused light.

The number of hairy roots per transformed explant, the transformation percentage and the number of normal roots per explant were recorded 25 days after infection.

3.6.8 Standardization of explants for efficient transformation

Different explants like leaf segments, shoot tips and nodal segments were infected by all the three strains of *Agrobacterium rhizogenes* (A4, ATCC 15834 and MTCC 2364). The infection was carried out using different inoculation methods and co-cultured for different durations under diffused light. Transformation percentage obtained in the case of each explants was recorded. The mean number of hairy roots per transformed explant and the mean number of normal roots per infected explant under each condition were recorded after 20 days.

3.6.9 Efficiency of strains in inducing hairy roots

To study the efficiency of strains in inducing hairy roots, the three strains, A4, ATCC 15834 and MTCC 2364 were inoculated on different explants using different inoculation methods and co-cultured for different durations (1-3 days).

Transformation percentage obtained by using each strain in different explants was calculated. The number of days taken for induction of roots from infected explants by using different strains under different inoculation methods was recorded.

3.6.10 Establishment of hairy root cultures

The adventitious roots emerged from the explants within 1 to 4 weeks after infection and those having hairy nature and diffused geotropism were excised out using a sterile blade. The roots were washed in liquid MS medium containing 250 mg l^{-1} of cefotaxime and blotted dry. The individual root tips were separated and transferred to MS solid medium containing 250 mg l^{-1} cefotaxime. The roots were incubated in the culture room at 26 ± 2°C under diffused light.

3.6.11 Rapid culturing of hairy roots

About 25 days after the establishment of root cultures, the roots were washed in liquid MS medium, blotted dry and then randomly cut into small pieces. Both the root tips and root segments were transferred to MS liquid medium without antibiotics. The cultures were incubated in orbital shaker at 110 rpm under diffused light and dark condition for rapid multiplication (referred to as shake flask cultures). The normal roots obtained from control explants were also cultured similarly. Cultures were also maintained in solid MS medium with 100 mg l^{-1} cefotaxime in petriplates. The shake flask cultures were harvested 25 days after incubation, washed in double distilled water and the fresh weight was measured.

3.6.12 Effect of culture media and conditions on the growth of hairy roots

To study the effect of culture media and conditions on the growth of hairy roots, the root cultures were initiated in MS and half MS with 3.0 per cent sucrose and B_5 with 3.0 and 2.0 per cent sucrose. The hairy roots cultured in the conical flask were collected, randomly cut to small pieces of 2.0 to 4.0 cm length and inoculated in 100 ml conical flasks containing 50 ml half MS with 3.0 per cent sucrose, MS with 3.0 per cent sucrose, B_5 with 3.0 per cent sucrose and 2.0 per cent sucrose without antibiotics.

The cultures were incubated in rotary shaker at 110 rpm under dark photoperiod. The fresh weight of the roots was recorded 25 days after inoculation.

3.7 CONFIRMATION OF TRANSFORMATION

The confirmation of transformation was done on the basis of,

1) Morphological features
2) Opine analysis
3) PCR analysis of *rol* B genes
4) Dot blot analysis
5) Southern hybridization

3.7.1 Morphological features

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

3.7.2 Opine analysis

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

3.7.2.1 Preparation of reagents

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

1) 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.
2) Solution II - 2.0 per cent NaOH in 90 per cent ethanol in water was prepared.

3) Solution III -

a) Reducer A Concentrate: Saturated potassium ferricyanide

b) Reducer B Concentrate: Saturated sodium thiosulfate

c) Reducer C Concentrate: 25 percent sodium carbonate

One ml of reducer A concentrate was mixed with 2.0 ml of reducer B concentrate and 0.35 ml reducer C concentrate was added to the mixture. The mixture was diluted to 150 ml using distilled water.

3.7.2.2 Extraction of opines

Three hundred mg fresh root tissue was taken in an Eppendorf tube. Distilled water (3 ml/g of the 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 g at room temperature. The supernatant was collected and used for the detection of opines.

3.7.2.3 Separation of opines

Ten µl of root extract was spotted on Whatman No. 1 chromatography paper strip. Standard agropinic acid, mannopine, and mannopinic acid were dissolved in autoclaved double distilled water and used for spotting. The spots were made at a distance of 1.5 cm. Small volumes of samples were applied successively using micropipette and in between a current of warm air from a hair drier was used to concentrate the spots. The paper strip was moistened with buffer excluding 0.5 cm area on both sides of the spots. The moistened paper strip was placed on the support of horizontal electrophoresis unit (BIORAD, SUB CELL GT) containing equal volumes of buffer in both wells, such that both ends of paper touched 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.

3.7.2.4 Detection of opines

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

3.7.3 Confirmation by PCR analysis and Southern hybridization

3.7.3.1.1 Isolation of DNA from roots

For PCR analysis and Southern hybridization, DNA was isolated from hairy roots obtained using A4, ATCC 15834 and MTCC 2364 strains roots produced from control explants, following modified procedure reported by Rogers and Bendich (1994).

3.7.3.1.2 Reagents

1. 2X CTAB extraction buffer

   2 per cent CTAB (w/v), 100 m*M* Tris (pH 8), 20 m*M* EDTA (pH 8), 1.4 *M* NaCl, 1 per cent PVP

2. 10 per cent CTAB solution

   10 per cent CTAB (w/v), 0.7*M* NaCl

3. TE Buffer

   10 m*M* Tris pH 8, 1 m*M* EDTA pH 8

4. Iso-propanol

5. Chloroform: Isoamyl alcohol mixture (24:1, v/v)
6. Ethanol 100 per cent and 70 per cent
7. RNAse
8. β-mercaptoethanol

3.7.3.1.3 Procedure

One gram root sample was weighed accurately and ground using a pre-chilled mortar and pestle in the presence of liquid nitrogen. β-mercaptoethanol 50µl was added. The ground tissue was transferred into a 50 ml Oakridge tube containing 4 ml pre-warmed 2X CTAB extraction buffer. The contents were mixed well and incubated at 65°C for 15-20 min. Then equal volume of chloroform: isoamyl alcohol mixture was added, mixed gently by inversion and centrifuged at 10,000 rpm for 10 minutes at 4°C. The mixture separated into three distinct phases from which the upper aqueous phase containing DNA was pipetted out into a fresh 50 ml Oakridge tube. To this, $1/10^{th}$ volume 10 per cent CTAB was added and mixed gently by inversion. Equal volume of chloroform: isoamyl alcohol mixture was added, mixed gently to form an emulsion and centrifuged at 10,000 rpm for 10 min at 4°C. The aqueous phase was collected in a fresh Oakridge tube and 0.6 volumes of chilled isopropanol was added and mixed gently to precipitate the DNA. It was incubated at -20°C for 20 min. The contents were then centrifuged at 10,000 rpm for 15 min at 4°C to pellet the DNA. The isopropanol was poured off retaining the DNA pellet that was later washed with 70 per cent alcohol. The DNA pellet was air dried to remove the alcohol and then dissolved in 100µl of autoclaved milli-Q water.

3.7.3.1.1 RNAse treatment of DNA

Hundred µl of DNA suspended in autoclaved distilled water was treated with 2 µl of RNAse solution and incubated at 37°C for 1 hour. The total volume was made up to 500 µl with distilled water and equal volume of phenol: chloroform mixture (1:1) was added. It was centrifuged at 10000 rpm for 10 min at 4°C. The top layer was transferred to a fresh Eppendorf tube and equal volume of chloroform - isoamyl alcohol (24:1) mixture was added. The final aqueous phase was collected into a fresh Eppendorf tube and 0.6 volume of chilled isopropanol was added, mixed gently and incubated at -20°C for 30 min to precipitate the DNA. It was centrifuged at 10000 rpm for 10 min at 4°C. The DNA pellet was retained and washed first with 70 per cent alcohol. It was then air dried and dissolved in 25 µl autoclaved distilled water.

3.7.3.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed based on the method described by Sambrook *et al.* (1989) to check the quality of DNA, to separate the amplified products and also to separate the DNA after restriction for doing Southern blotting.

3.7.3.2.1 Materials

1. Agarose
2. 50X TAE buffer

Tris Base - 242g, 0.5*M* EDTA (pH 8) - 100ml, glacial acetic acid - 57.1ml

3. Tracking dye (6X)

Bromophenol blue 0.25 per cent, Xylene cyanol FF 0.25 per cent, Glycerol in water 30 per cent.

4. Ethidium bromide

3.7.3.2.2 Procedure for casting, loading and running the gel

Four hundred ml of electrophoresis buffer (1X TAE) was prepared to fill the electrophoresis tank and to prepare the gel. The open ends of the gel-casting tray were sealed with a cellophane tape and placed in a perfectly horizontal levelled platform and the comb was set properly. Agarose (1.0 per cent (w/v) for genomic DNA and 0.7 per cent (w/v) for PCR) was added to 1X TAE, boiled till the agarose dissolved completely and then cooled to luke warm temperature. Ethidium bromide was added to a final concentration of 0.5 μgml^{-1} as an intercalating agent of DNA, which will help in its visualization in UV rays. It was then poured into the gel-casting tray with comb and allowed to solidify. After the solidification of the gel (30 min at room temperature), the comb and parafilm were removed carefully. The gel was placed in the electrophoresis tank with the wells near the cathode and submerged in 1X TAE to a depth of 1.0 cm.

A piece of parafilm was placed on a solid surface and 2.0 µl of 6X loading buffer was dispensed in small quantity on the tape. A quantity of 5 to 8 µl of DNA was added to each slot (in the case of PCR products, 10.0-15.0 µl) and mixed well by pipetting in and out for two to three times. Then the mixture was loaded into the wells with the help of a micropipette. Appropriate molecular weight marker (Lambda DNA – *Hind* III/ *Eco* R1 double digest) was also loaded in one of the wells. After closing the tank, the cathode and anode were connected to the power pack and the gel was run at a constant voltage of 100 Volts and 50 A current. The power was turned off when the tracking dye reached at about two third length of the gel. The gel was observed in UV trans illuminator and documented in gel documentation system.

3.7.3.3 Isolation of cosmid from E. coli

E. coli containing pLJ1 and pLJ85 were obtained from INRA, France. Cosmids, pLJ1 and pLJ85 were isolated from *E. coli* using alkaline mini-prep procedure as given by Birnboim and Doly (1979). *E. coli* strains containing pLJ1 cosmid with kanamycin resistance were used as positive control in PCR analysis and Southern hybridization. The cosmid contains *rol* B gene.

3.7.3.3.1 Reagents

Solution I (Resuspension buffer)

Solution II (Lysis buffer)

Solution III (Neutralisation buffer)

The composition of the reagents is given in Appendix V.

3.7.3.3.2 Procedure for cosmid Isolation

1. A single bacterial colony was transferred in to 2.0 ml LB medium containing kanamycin and the culture was incubated overnight at 37°C with vigorous shaking.
2. 1.5 ml of the culture was poured in to an Eppendorf tube and the cells pelleted by centrifugation at 12,000 rpm for one minute at 4°C.

3. The supernatant was discarded and the bacterial pellet resuspended in 100 µl ice-cold Solution I by vigorous shaking.
4. To the above, 200 µl of freshly prepared Solution II was added and mixed gently by inverting the tube five times.
5. Ice-cold Solution III (150 µl) was added to the tube, vortexed gently and kept on ice for 5 min.
6. The contents were centrifuged at 12,000 g for five minutes at 4°C and the pellet was discarded.
7. To the supernatant, 0.6 volume of ice-cold isopropanol was added to precipitate the DNA and kept at room temperature for two minutes.
8. The contents were centrifuged at 12,000 g for five minutes at 4°C and the supernatant was discarded.
9. The pellet was rinsed with 1.0 ml of 70 per cent (v/v) ethanol at 4°C.
10. The supernatant was discarded and the pellet was air dried and dissolved in 30 µl autoclaved double distilled water.
11. The cosmids isolated were observed in agarose gel electrophoresis and documented.

3.7.3.4 Quantification of DNA and cosmids

The quality of DNA was further evaluated using nanodrop spectrophotometer ND-1000. Distilled water was used as the blank (1µl). The blank was set as zero nanogram per microlitre. Then the loading point was wiped with tissue paper. Sample DNA 1µl was loaded. The concentration of DNA was given as nanogram/microlitre. The purity of DNA was assessed from the ratio Optical Density (OD) value at 260 nm and 280 nm. A ratio of 1.8 indicates good quality DNA.

3.7.3.5 PCR analysis of rol B genes

The primer sets used for amplifying *rol* B gene were Rol BF1R1 and Rol BF2R2. Details of primer are given in Table.8. The PCR analysis was carried out using DNA isolated from the hairy roots induced by A4, ATCC 15834 and MTCC 2364. The DNA isolated from roots produced by control explants was used as the negative control. The cosmid pLJ1 was used as the positive control, since it contains *rol* B gene. A blank (without DNA) was also set.

Table 8. Details of different combinations of primer

Sl No.	Primer combination	Amplicon size (bp)	Annealing temperature (°C)
1	Rol BF1R1	740	54
2	Rol BF2R2	205	56

3.7.3.5.1 Composition of the reaction mixture for PCR

The reaction mixture was set in 200 µl microfuge tubes chilled over ice flakes.

a) Root DNA	- 1.0 µl (1:10 dilution)
or	
Cosmid	- 5.0 µl (1:5 dilution)
b) 10X Taq assay buffer	- 2.5 µl
c) d NTPmix (1 m*M*)	- 1.0 µl
d) Forward primer	- 1.0 µl (1:10 dilution)
e) Reverse primer	- 1.0 µl (1:10 dilution)
f) Taq DNA polymerase (0.3u)	- 2.0 µl
g) Autoclaved distilled water	- 16.5 µl (12.5 µl for cosmid)
Total	25.0 µl

A momentary spin was given to the reaction mixture for thorough mixing of the cocktail components. The tubes were then placed in a thermal cycler (Eppendorf master cycler gradient) for polymerase chain reaction under suitable programme with a heated lid condition.

3.7.3.5.2 Thermal Cycler Program

The following program was set to amplify *rol* B genes from template DNA.

1. 94 °C for 2.0 min - Initial denaturation
2. 94 °C for 45 sec - Denaturation

3. 54 °C and 56 °C for 1.0 min - Annealing
4. 72 °C or 2.0 min - Extension
5. Go to 2, 29 times
6. 72 °C for 10 min - final extension
7. 4 °C for 5.0 min - to hold the sample

The annealing temperature was changed based on the primer combinations used. The PCR product was loaded on 0.7 per cent agarose gel and finally documented.

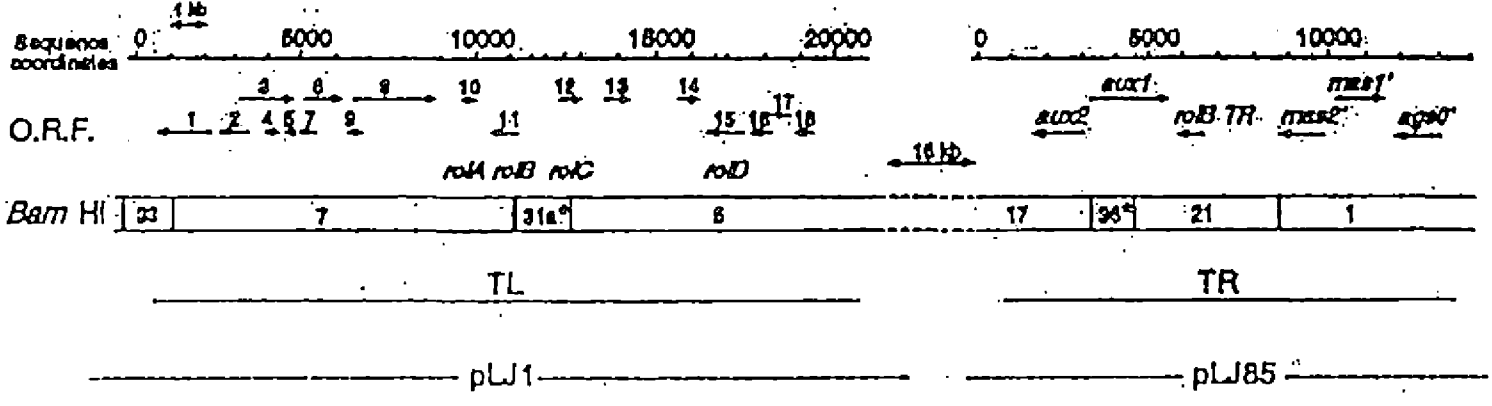
3.7.3.6 Southern hybridization

Southern hybridization was carried out for the confirmation of transformation. The PCR product obtained from pLJ1 cosmid, using Rol BF2R2 primer set (205 bp) was used as probe in Southern hybridization. Restriction map of the TL and TR regions of pRiA4 showing the inserts of pLJ1 and pLJ85 cosmids (Jouanin, 1984) is given in the Fig. 2. To confirm the presence of TL-DNA, pLJ1 cosmid was used as the positive control and the DNA isolated from the roots produced by control explants was used as the negative control.

3.7.3.6.1 Restriction digestion of DNA

The DNA isolated from hairy roots obtained using A4 and ATCC 15834 strains, roots produced from MTCC 2364 infected explants and roots produced by control explants were restricted using *Bam* H1 restriction enzyme. The cosmid pLJ1 was also restricted with the same enzyme. The DNA for restriction was taken in such a way that each reaction mix contained 30µg DNA. Enzyme was taken such that there were 4 unit enzyme for 1 µg DNA (120 units for 30µg DNA). Enzyme buffer was taken at the rate of 2 µl per 20µl reaction mix. The bottom of the microfuge tube was gently tapped to mix the reaction components. The tubes were incubated at 37°C for 16 hrs. After the required time, the reaction was arrested by adding 0.5 *M* EDTA. Then the reaction mix was precipitated by the following procedure.

Fig. 2. Restriction map of the TL and TR regions of pRiA4 showing the inserts of pLJ1 and pLJ85 cosmids (Jouanin, 1984).



Sodium acetate 0.1volume and isopropanol 0.7volume was added. It was incubated for 30 min at 4°C, spun for 10 min at 10,000 rpm and washed with 70 per cent ethanol. The precipitate was air dried and dissolved in Milli Q water (15µl). The restriction digestion was confirmed by running the digest in 0.8 per cent (w/v) agarose gel and documented.

3.7.3.6.2 Southern Blotting

1. DNA sample was digested with restriction enzyme.
2. The restricted products were electrophoresed on 0.8 per cent agarose gel.
3. The gel was trimmed to exact size, cutting off the left hand corner for identification.
4. The gel was initially soaked in depurination solution (0.2 *N* HCl) for 10 min, followed by rinsing in deionized water.
5. The DNA contained in the gel was denatured by soaking in denaturation solution (1.5 *M* NaCl, 0.5 *N* NaOH) for 45 min with gentle agitation. The gel was further rinsed with deionized water.
6. The gel was neutralized using neutralization solution (1.0 *M* Tris pH 7.4 and 1.5 *M* NaCl), with gentle agitation for 30 min.
7. The neutralization solution was replaced with fresh neutralization solution and the gel was gently agitated in it for another 15 min. Now the gel was ready for blotting.
8. A transfer assembly (plastic tray and transfer buffer 10X SSC) was set up and a glass plate was placed above the tray.
9. A piece of Whatman No 1 filter paper wetted with 10X SSC was wrapped around the glass plate such that the two ends of Whatman filter paper touched the buffer (filter paper wick).
10. The gel was placed in an inverted position on the top of the filter paper wick and the air bubbles formed between the gel and filter paper were squeezed out.
11. A piece of nitrocellulose filter slightly larger than the gel was placed above the gel after cutting the left hand corner of the membrane and carefully aligned.

12. Three pieces of wet Whatman filter paper were aligned on the top of the nitrocellulose filter.
13. A 10 cm stack of dry filter paper towels cut to the size was aligned above the filter papers and on the top, a glass plate was placed. Finally an evenly distributed weight of 0.5 kg was placed on the top of the glass plate.
14. Strips of parafilm were placed around the gel to prevent direct contact between the stack of paper towels and the wick, which can cause a short circuit in the flow of the transfer solution.
15. The transfer was allowed to proceed for 12-18 hours at room temperature.
16. After the transfer, nitrocellulose filter was taken out after marking the wells and washed in 6X SSC for five minutes at room temperature. The gel was stained in ethidium bromide to ensure complete transfer of bands from the gel.
17. The membrane was placed flat on a paper towel and dried for 30 min. After drying, the membrane was sandwiched between filter papers and baked for one hour at 80°C in vacuum oven. The reagents for Southern hybridization are given in Appendix VII.

3.7.3.6.3 Preparation of radio labelled probe

The probe was radio labelled using random labeling kit.

1. 10 µl of DNA (probe) was taken.
2. The double stranded probe was denatured by boiling the contents in a boiling water bath for five minutes.
3. The cap of the microfuge tube containing DNA was pierced two or three times with a needle before boiling so as to release pressure.
4. The denatured sample was chilled for five to ten minutes.
5. The sample was spun at 8000 rpm for 30 seconds.
6. 2.5 µl of 10 X labelling buffer was added to sample followed by 1 µl of 100 µg/µl random primer.
7. To the reaction mix, 2.5 µl of 20 m*M* DTT solution was added followed by 2.0 µl of d NTP mix (2.5 m*M*- dCTP, dGTP, dTTP)

8. 3.0 µl of α'32 P dATP was added to the reaction mix (specific activity > 3000 Curie).
9. 1.0 µl of (3-units/ µl) klenow fragment polymerase was added, mixed gently and incubated at room temperature for two hours. The reaction mix was boiled for 5 min before adding to the hybridization solution.

3.7.3.6.4 Procedure for pre hybridization, hybridization and washing

1. The blotted nitrocellulose filter was washed with 6X SSC for 2 min.
2. The filter was transferred to individual box containing 20 ml pre hybridization solution and incubated in hybridization shaker/ oven set at 68°C for 4 hrs with gentle shaking.
3. The pre hybridization solution was removed and replaced with hybridization solution to which radiolabelled probe was added and further incubated overnight at 68°C with gentle shaking in hybridization oven.
4. After the hybridization, the filter was washed in several hundred ml of 2X SSC with 0.5 per cent SDS at room temperature for 5 min.
5. It was then washed with 2X SSC with 0.1 per cent SDS for 15 min at room temperature followed by washing in 0.1X SSC with 0.5 per cent SDS for 1 hr at 37°C in hybridization oven/ shaker.
6. After 1 hour, the solution was replaced with fresh 0.1X SSC with 0.5 per cent SDS and incubated at 68°C for one hour in hybridization oven/ shaker.
7. Finally the filter was washed with 0.1X SSC at room temperature and air-dried.
8. The nitrocellulose filter was covered with cling film, and a sheet of X-ray film (Photographic emulsion) was placed over and kept in intensifying cassette in dark. The cassette was covered and incubated at -20°C.

3.7.3.6.5 Autoradiography

From the cassette, the X-ray film was taken out after the required exposure time (based on the activity) in a dark room where only a red light was available for vision. The X-ray film was first washed in developer solution for 2-5 min and then

washed in distilled water. The image developed was fixed by washing in fixer solution for two minutes. The film was taken out and drip-dried.

3.7.3.7 Dot blot Analysis

Dot blot analysis was carried out as a part of confirmation of transformation. The PCR products obtained from pLJ1 cosmid, using Rol BF2R2 primer set (205 bp) was used as probe in dot blot analysis.

3.7.3.7.1 Procedure

1. A piece of nitrocellulose filter of required size based on the number of samples to be spotted was taken.
2. 5.0 µl of DNA, isolated from hairy roots induced by A4 and ATCC 15834 and MTCC 2364 strains, control roots, pLJ1 cosmid (positive control) and pLJ85 cosmid were spotted separately on the nitrocellulose membrane.
3. Each spot was labelled using pencil and a round pencil mark was made around the spot.
4. The air-dried membrane was sandwiched between two filter papers and baked for 15 min at 80°C in vacuum oven.
5. The blotted nitrocellulose membrane was soaked in depurination solution (0.2 *N* HCl) for 5 min.
6. The DNA contained in the membrane was denatured by soaking in denaturation solution (1.5 *M* NaCl, 0.5 *N* NaOH) for 15 min with gentle agitation.
7. It was then neutralized by soaking in neutralization solution (1 *M* Tris - pH 7.4, 1.5 *M* NaCl) for 15 min with gentle agitation.
8. The membrane was washed in 6XSSC and then placed flat on a paper towel and dried for 30 min.
9. After drying, the membrane was sandwiched between filter papers and baked for one hour at 80°C in vacuum oven.
10. It was followed by pre-hybridization, hybridization, washing and developing, same as that of Southern hybridization.

3.8 ESTIMATION OF ARTEMISININ

A quantitative thin layer chromatography (TLC) method modified from Gupta *et al.* (1996) was used for the estimation of artemisinin present in the roots of *A. annua*. Artemisinin was estimated from roots, shoots, leaves and inflorescence of field-grown plants, *in vitro* roots (non- transformed), *in vitro* shoots, shoots of transformed plants and hairy roots (transformed roots). Enhancement of artemisinin through precursor feeding, elicitation and addition of osmoregulants were also studied.

3.8.1 Extraction and estimation

3.8.1.1 Preparation of standard

Standard artemisinin procured from Sigma Chemicals, USA was used as standard. A 1000 ppm solution of artemisinin prepared by dissolving 1 mg reference in 1 ml n-hexane was used for spotting on TLC plate.

3.8.2 Extraction of artemisinin from plant samples

The samples (0.5-10.0 g) were ground in a mortar and pestle with 5ml n-hexane and it was centrifuged at 10,000 rpm for 10minutes. The supernatant was taken and evaporated to dryness. The concentrated form was redissolved in 0.5ml n-hexane and used for the TLC estimation of artemisinin.

The media was extracted with equal quantity of hexane. The root grown media was taken in a separating funnel to which equal quantity of hexane was added and shaken vigorously so as to extract the artemisinin. The lower clear hexane fraction was collected and solvent evaporated off. The residue was redissolved in 500 µl n-hexane and used for TLC analysis.

3.8.3 Application of sample on TLC plate

For the TLC analysis, Silica gel 60 F_{254} (Merck) plate was used. TLC plates of 5cm width and 10cm height were used for the analysis. A straight line was drawn at a distance of 2.0 cm from the lower edge of the plate. On the plate, samples were spotted at a distance of 1.0 cm leaving a margin of 0.7 cm on either side. Using a

micropipette, different volumes (0.25-1.0 µl) of standard solution of artemisinin were spotted. Known volumes of samples (5.0-25.0 µl) were applied successively and solvent was removed between additions with a current of warm air from a hair drier.

3.8.4 Development of chromatographic plates

The mobile phase n- hexane: diethyl ether (1:1) was used to develop the chromatographic plates.

3.8.4.1 Procedure

Developing solvent (mobile phase) was mixed well and poured into the developing chamber to a depth of 0.5 cm and the chamber allowed to saturate with the vapour of the solvent. Spout less beaker was used as the developing chamber. The spotted TLC plate was placed in the chamber, the chamber lid placed correctly and the chromatogram was developed to two third distance. The plate was then removed and dried.

3.8.4.2 Visualization of spots

After development, the plates were taken out from the developing chamber and solvent removed under a stream of hot air. The spots were visualized by immersing the plate in a pool of freshly prepared glacial acetic acid: conc. sulphuric acid: anisaldehyde (50:1:0.5) followed by drying in a chromatographic oven at 110°C for 15 minutes. The plates were immediately documented in Alfa Imager ™ 1200 documentation system, Herolab/ Biorad, Gel Doc XR under white light.

3.8.5 Quantification of artemisinin

A TLC-densitometry technique was used for the quantification of artemisinin. The image of the TLC plates stored in the Alfa Imager was analysed using the SPOT DENSO tool present in the Tool box 3 of the Alfa Imager. The spots of artemisinin in the samples and reference standard on the plate were initially selected by delineating the boundaries. A background correction was made using AUTO BACKGROUND and the INVERT option was selected so that the spots appeared as

dark on a light background. The spots of artemisinin in the reference standard was selected and the corresponding values of artemisinin in micrograms were entered. The quantity of artemisinin in unknown samples appeared automatically in the spot denso results.

3.8.6 Enhancement of secondary metabolite production

The hairy roots derived from ATCC 15834 derived root clones were subjected to enhancement studies.

3.8.6.1 Addition of osmoregulants

The hairy root cultures were cultured in half MS medium supplemented with polyethylene glycol (PEG) of molecular weight 6000 g at 2.0 per cent and 5.0 per cent. The hairy roots were grown for twenty days on half MS media. On the 20th day the media was replaced with new growth regulator free half MS media with 2.0 per cent and 5.0 per cent polyethylene glycol.

The media was prepared by dissolving 20 g l^{-1} and 50 g l^{-1} PEG 6000 in half MS media. The pH of the media was adjusted to 5.7 before autoclaving. Approximately 2.0 g of hairy roots were inoculated in 125 ml of stress media in 250 ml conical flask. The cultures were grown for 12 days at room temperature under dark photoperiod on rotary shaker (110 rpm) at room temperature. Then the whole roots and media were collected and artemisinin content was analysed.

3.8.6.2 Addition of precursors

The effect of precursor feeding on artemisinin production in hairy roots was studied. Hairy roots were inoculated in half MS media supplemented with 1.0 m*M* methionine. Methionine was filter sterilized and added to 125 ml sterilized half MS liquid media in 250 ml conical flask. Methionine was dissolved in small quantity of sterilized liquid half MS so as to facilitate filter sterilization. Twenty five day old root cultures were inoculated in the media and further grown for five days. Then the whole roots and media were collected and artemisinin content was analysed.

3.8.6.3 Addition of Elicitors

Two types of biotic elicitors, *Aspergillus niger* homogenate and yeast extract were used in the elicitation studies, for enhancing the production of artemisinin.

3.8.6.3.1 Elicitation by Aspergillus homogenate

The spores of *Aspergillus niger* was inoculated in 50 ml LB broth and the culture was grown in a rotary shaker at 140 rpm for two days at 30°C. The culture was further grown for five more days without shaking wherein mycelial mass had spread over the culture. On the seventh day of incubation, the culture was filtered through muslin cloth and the mycelial mass was collected. It was dispersed in 40 ml distilled water and was homogenized in the Polytron homogenizer. The *Aspergillus* homogenate was autoclaved and added to half MS liquid medium at the rate of 250 µl per 125 ml in 250 ml conical flask. Twenty day old root culture was inoculated in the above media and the culture was incubated in rotary shaker at 110 rpm for 72 hrs. The root culture and the media were collected and the artemisinin content was analysed by TLC.

3.8.6.3.2 Elicitation by Yeast Extract

Yeast extract at two concentrations, 2.5 g l^{-1} was used to elicit the cultures. Half MS liquid medium was supplemented with 2.5 g l^{-1} yeast extract and the pH was adjusted to 5.7 before autoclaving. Twenty days old culture was inoculated in the media and the culture was incubated on a rotary shaker at 110 rpm for 72 hrs. TLC analysis was carried out to estimate the artemisinin content.

3.9 STATISTICAL ANALYSIS

Statistical analysis was carried out wherever necessary as per Panse and Sukhatme (1985)

Results

4. RESULTS

The results of the study on "Genetic transformation in *Artemesia annua* L. for hairy root induction and enhancement of secondary metabolites are presented in this chapter.

4.1 STANDARDIZATION OF *IN VITRO* REGENERATION

4.1.1 Standardization of surface sterilization

Leaf segments and inflorescence bits were taken from the plants grown in the net house. To standardize surface sterilization of these explants, $HgCl_2$ at varying concentration (0.05, 0.1 and 0.2%) were tried at varying time intervals. Effect of various concentrations of $HgCl_2$ on surface sterilization are represented in Table 9.

For leaf explants 0.05 per cent $HgCl_2$ for 10 min and 0.1 per cent $HgCl_2$ for 1 min proved to be best. Higher concentration of $HgCl_2$ resulted in yellowing of the explant. Survival and establishment of cultures from inflorescence bit explants was more at 0.1 per cent $HgCl_2$ for 1 min treatment.

4.1.2 Leaf explants

4.1.2.1 Regeneration

4.1.2.1.1 Effect of NAA on leaf explants

NAA induced callusing and rooting. Leaves produced callus only at sides in low concentration of the auxin where as at high concentration, calli were formed from all sides of leaves and from the midribs. Calli were cream coloured. Roots were produced directly from the leaf as well as from the callus. Roots were thin and white. Short and thick roots were produced in 0.5 mg l^{-1} NAA.

Effect of NAA in leaf explant is shown in Table 10.

Table 9. Effect of various concentration of $HgCl_2$ and time of sterilization on culture establishment of *Artemesia annua*

$HgCl_2$ (%)	Time of treatment	Survival after one week (%)		Culture establishment after15 days (%)	
		Leaf	Inflorescence	Leaf	Inflorescence
0.05	30 seconds	39.00	29.80	30.08	19.00
0.05	1 min	39.80	32.10	36.25	19.25
0.05	2 min	40.00	48.00	38.43	33.35
0.05	5 min	60.00	64.00	35.00	56.00
0.05	10 min	90.00	75.00	80.00	63.00
0.1	30 seconds	62.00	68.00	54.50	55.00
0.1	1 min	84.00	80.05	80.00	74.63
0.1	2 min	52.00	80.00	50.00	60.00
0.1	5 min	40.00	62.00	38.00	53.00
0.1	10 min	30.00	25.00	00.00	00.00
0.2	30 seconds	65.00	45.00	49.00	10.00
0.2	1 min	75.00	23.00	40.00	06.00
0.2	2 min	35.00	00.00	30.00	00.00
0.2	5 min	30.00	00.00	18.00	00.00
0.2	10 min	00.00	00.00	00.00	00.00

Each treatment consisted of 10 replicates.

Table 10. Effect of NAA on leaf explant

MS media + NAA (mg l^{-1})	Per cent response	Response
0.05	20	Callusing + rooting
0.1	25	Callusing + rooting
0.2	84	Callusing + rooting
0. 5	100	Callusing + rooting
1 .0	83	Callusing + rooting
2 .0	80	Callusing + rooting
3 .0	78	Callusing

Each treatment consisted of 10 replicates, observations after 25 days

4.1.2.1.2 Effect of BAP and NAA on leaf explants

The effect of varying concentrations of BAP and NAA on the leaf explants is represented in Table 11. Leaf segments took 15 to 20 days for regeneration whereas callus proliferation started within one week. Regeneration of shoot buds occurred directly from explant as well as from proliferated callus. In all treatments cultures callused. Calli were formed from all sides of leaf and from the midrib. In low BAP and high NAA calli formed were hard and green. In other combinations calli were cream coloured and friable. BAP alone was found to induce regeneration from the leaf explant. In MS+ 0.5 mg l^{-1} BAP shoot initials of light green colour were formed from the explant and from the callus (Plate1). Ninety per cent regeneration was found in this media. A maximum of 6 shoots were formed per explant.

4.1.2.1.3 Effect of basal media on regeneration

To study the effect of basal media on regeneration, the leaf explants were inoculated to MS, SH and B_5 media containing 0.5 mg l^{-1} BAP. The observations are given in Table 12.

Table 12. Effect of basal media on regeneration from leaf explant

Media	No. of shoot buds/explant (Mean)	Regeneration (%)
MS + 0.5 BAP	3.22	90
SH + 0.5 BAP	2.20	70
B5 + 0.5 BAP	0.52	66
½ MS + 0.5B BAP	2.87	82

Each treatment consisted of 10 replicates, observations after 25 days.

Regeneration of leaf explant was observed in all basal media but MS media was found to be the best.

Table 11. Effect of different concentrations of NAA and BAP on shoot bud regeneration from leaf explant

NAA + BAP (mgl^{-1})	Shoot bud regeneration (%)	No. of shoot buds/explant (Mean ± SD)	Response
0 + 0.5	90	3.22 ± 2. 04	C + R
0 + 1	75	2.22 ± 0.83	C+ R
0 + 2	70	1.98 ± 0.76	C + R
0 + 3	30	0.33 ± 0.50	C + R
0 + 4	10	0.11 ± 0.33	C+ R
0.05 + 1, 2, 3	Nil	0.00 ± 0.00	C
0.1 + 0.05	30	0.30 ± 0.48	C+ R
0.1 + 0.1	80	1.70 ± 1.17	C+ R
0.1 + 0.2	56	0.22 ± 0.50	C+ R
0.1 + 0.5	42	0.44 ± 0.46	C + R
0.1+ 1	40	0.70 ± 0.69	C + R
0.1 + 2	66	2.25 ± 2.20	C + R
0.1 + 3	20	0.40 ± 0.96	C + R
0.2 + 0.05, 0.1, 0.2, 0.5	Nil	0.00 ± 0.00	C
0.2 + 1	60	0.90 ± 0.87	C + R
0.2 + 2	Nil	0.00 ± 0.00	C
0.2 + 3	Nil	0.00 ± 0.00	C
0.5 + 0.05, 0.1, 0.2	Nil	0.00 ± 0.00	C + R
0.5 + 0.5	10	0.11 ± 0.33	C + R
0.5 + 1	11	0.25 ± 0.46	C + R
0.5 + 2	20	0.44 ± 0.69	C+ R
0.5 + 3	Nil	0.00 ± 0.00	C
1 + 0.05, 0.1, 0.2	Nil	0.00 ± 0.00	C + R
1 + 0.5	20	0.25 ± 0.46	C+ R
1 + 1	40	0.70 ± 0.95	C+ R
1 + 2	30	0.40 ± 0.69	C+ R
1 + 3	10	0.10 ± 0.30	C
2 + 0.05 to 2.0	Nil	0.00 ± 0.00	C
2 + 3	10	0.10 ± 0.30	C + R
3 + 0.05 to 2.0	Nil	0.00 ± 0.00	C

Each treatment consisted of 10 replicates, observations after 25 days

C: Callusing; R: Regeneration of shoot buds

4.1.2.2 Multiplication

The shoot buds obtained from leaf explants were multiplied in various media and the observations are given in Table 13. MS media supplemented with 0.5 mg l^{-1} BAP produced 3.6 shoots per each shoot buds. Number of shoot buds was less in MS media supplemented with NAA, but elongated shoots were produced. Rooting was also observed. Elongation of shoots was less in media containing BAP alone. However BAP supplemented media produced multiple shoots (Plate 1).

4.1.2.3 Elongation

Elongation of leaf derived shoot tips were studied and results are represented in Table 14. Maximum elongation was obtained on MS with 0.2 GA_3 followed by ½ MS with 3 per cent sucrose. Number of internodes as well as number of leaves was also more in MS with 0.2 mg l^{-1} GA_3. These shoots were also healthier than in medium at half strength.

4.1.2.4 Rooting

The effect of various treatments on rooting of elongated shoots are presented in Table 15. Rooting occurred within 14 days in all treatments. Short, thick roots were formed in MS with 0.5 or 1 mg l^{-1} IBA. Mean number of roots, mean root length and mean shoot length were more in MS media supplemented with 0.5 mg l^{-1} IBA than compared to 1 mg l^{-1} IBA. The best basal media for rooting was ½ MS. Mean shoot length, mean leaf number, and mean root length was more and callusing of shoot less in ½ MS. Callusing of shoots were absent in half strength MS media supplemented with 0.5 mg l^{-1} IBA and 2 per cent sucrose. Addition of activated charcoal produced no positive response. The rooted plants were hardened and planted out. The rooted plants with out callus survived better than with callus.

4.1.3 Regeneration from callus

4.1.3.1 Effect of NAA on callus

NAA at low concentration (0.05 to 0.2 mg l^{-1}) induced callus proliferation where as at higher concentration (0.5 to 3 mg l^{-1}) rooting was induced.

Table 13. Effect of growth regulators on multiplication of regenerated shoot buds from leaf explant in MS medium

MS + growth regulator (mgl^{-1})		Mean number of multiple shoots
NAA	BAP	
0.00	0.5	3.6
0.00	1.0	1.5
0.00	2.0	1.5
0.50	0.5	2.0
0.50	1.0	2.3
0.50	2.0	2.7

Each treatment consisted of 10 replicates, observations after 25 days

Table 14. Effect of growth regulator combinations on elongation of leaf derived shoots of *A. annua*

Media	Mean length of shoot ± (S.D)	Mean no. of internodes/shoots ± (S.D)	Mean no. of leaves / shoots ± (S.D)
MS+ 3% sucrose	3.10 ± 1.40	2.20 ± 2.80	5.10 ± 2.25
MS + 2% sucrose	2.33 ± 0.35	2.00 ± 0.45	4.20 ± 1.60
½ MS + 3% sucrose	6.33 ± 0.65	2.30 ± 0.48	8.20 ± 2.02
MS + 0.5 mg l^{-1} NAA	5.50 ± 0.76	1.22 ± 0.44	6.50 ± 2.25
MS + 0.1 mg l^{-1} BAP + 0.1 mg l^{-1} GA_3	1.76 ± 0.34	2.00 ± 0.12	6.00 ± 5.60
MS + 0.1 mg l^{-1} BAP + 0.2 mg l^{-1} GA_3	1.63 ± 0.95	2.00 ± 0.85	5.00 ± 0.52
MS + 0.1 mg l^{-1} BAP + 0.5 mg l^{-1} GA_3	2.48 ± 1.46	2.20 ± 0.44	5.00 ± 0.56
MS + 0.01 mg l^{-1} GA_3	4.15 ± 1.20	2.30 ± 0.52	5.10 ± 0.73
MS + 0.05 mg l^{-1} GA_3	4.25 ± 0.95	2.00 ± 0.45	6.00 ± 0.83
MS + 0.1 mg l^{-1} GA_3	4.35 ± 1.85	3.10 ± 0.46	6.00 ± 0.45
MS + 0.2 mg l^{-1} GA_3	6.00 ± 1.20	4.50 ± 0.22	6.20 ± 1.74

Each treatment consisted of 10 replicates, observations after 20 days

Table 15. Effect of various growth regulators on rooting of shoots derived from leaf segments of *A. annua*

Media	Mean length of shoot ±S.D	Mean no. of internodes /shoots ±S.D	Mean no. of leaves /shoots ±S.D	Length of root (cm)	Mean number of roots	Rooting (%)	Morphology of roots
MS	2.33 ± 0.52	1.28 ± 0.75	4.00 ± 0.63	2.04 ± 0.49	07.33 ± 01.75	70	Elongated very thin roots
½ MS + 0.5 mg l^{-1} NAA	5.38 ± 0.69	1.22 ± 0.44	6.55 ± 1.23	2.72 ± 0.45	13.11 ± 04.01	100	Roots thick at base
MS+ 0.5 mg l^{-1} NAA	2.96 ± 1.08	2.11 ± 0.33	4.11 ± 0.33	2.90 ± 0.44	13.63± 07.13	100	Elongated thin roots, callusing of shoots
MS + 0.05 mg l^{-1} IBA	2.73 ± 0.04	2.22 ± 0.44	5.67 ± 0.87	2.73 ± 0.51	13.11 ± 04.01	100	Elongated thin roots
MS + 0.1 mg l^{-1} IBA	2.84 ± 0.78	2.40 ± 0.96	9.30 ± 2.50	3.07 ± 0.53	23.67± 09.77	100	Elongated thin roots
MS + 0.5 mg l^{-1} IBA	4.50 ± 0.81	2.89 ± 0.60	6.80 ± 2.45	1.52 ± 0.99	21.60 ± 02.49	100	Short thick root, callusing
MS + 1 mg l^{-1} IBA	4.48 ± 1.14	3.20 ± 0.79	7.00 ± 1.25	0.74 ± 0.79	12.20 ± 03.49	100	Short thick root, callusing
MS + 2 mg l^{-1} IBA	4.57 ± 0.79	3.50 ± 1.18	10.50 ± 02.46	1.31 ± 1.05	23.10 ± 05.20	100	Elongated thin roots
MS + 5 mg l^{-1} IBA	2.35 ± 0.69	2.30 ± 0.48	6.60 ± 1.70	1.40 ± 0.68	09.20 ± 05.50	100	Elongated thin roots
½ MS + 0.5 mg l^{-1} IBA	5.73 ± 2.22	2.30 ± 0.67	9.00 ± 1.10	4.28 ± 0.52	19.20 ± 10.55	100	Short thick root, callusing
½ MS + 0.5 mg l^{-1} IBA + 2% sucrose	5.30 ± 1.11	1.20 ± 0.63	8.75 ± 1.04	3.50 ± 0.74	17.25 ± 07.09	100	Short thick root
½ MS + 0.5 mg l^{-1} IBA + 2% sucrose +0.05% AC	4.10 ± 1.57	1.30 ± 0.50	7.00 ± 1.85	3.19 ± 0.96	02.75 ± 00.46	100	Very thin roots
½ MS + 0.5 mg l^{-1} IBA + 2% sucrose + 0.1% AC	3.46 ± 0.22	2.14 ± 0.89	7.40 ± 1.13	3.80 ± 0.25	2.43 ± 0.79	100	Very thin roots
½ MS + 0.5 mg l^{-1} IBA + 2% sucrose + 0.2% AC	2.80 ± 0.76	1.40 ± 0.55	8.00 ± 1.40	3.46 ± 0.84	3.40 ± 0.89	100	Very thin roots

Each treatment consisted of 10 replicates, observations after 25 days

Leaf explant

Rooting from leaf
(MS + 2 mg l^{-1} NAA)

Callus mediated
shoot regeneration
(MS + 0.5 mg l^{-1} BAP)

Direct schizogenesis
(MS + 0.5 mg l^{-1} BAP)

Multiplication
(MS + 0.5 mg l^{-1} BAP)

Elongation
(MS + 0.2 mg l^{-1} GA_3)

Plate 1. *In vitro* **regeneration from leaf explant**

MS

MS + 0.5 mg l^{-1} NAA

MS + 0.5 mg l^{-1} IBA

1/2 MS + 0.5 mg l^{-1} IBA

1/2 MS + 0.5 mg l^{-1} IBA
+ 0.05% AC

1/2 MS + 0.5 mg l^{-1} IBA
+ 0.1% AC

Plate 2. Effect of growth regulators on rooting of shoots produced from leaf

4.1.3.2 Effect of BAP and NAA on callus

The calli from leaf explants were cultured on MS with BAP alone (0.5, 1,2,3,4 mg l^{-1}), NAA (0.05, 0.1, 0.2, 0.5, 1, 2, 3 mg l^{-1}) and BAP (0.5, 1,2,3,4 mg l^{-1}). Regeneration of leaf originated callus was observed in only a few combinations (Plate 3). The observations on regeneration from callus is given in Table 16.

Maximum regeneration was observed in MS media supplemented with 3 mg l^{-1} BAP and MS media supplemented with 0.2 mg l^{-1} NAA and 2 mg l^{-1} BAP. Calli from leaf explants kept in MS media supplemented with 0.5 mg l^{-1}BAP were used for the study. It was observed that shoots formed in MS media supplemented with 0.1 mg l^{-1} NAA and 2 mg l^{-1}BAP were clustered together, dark green in colour and deformed in appearance. Calli produced rooting on media containining NAA (0.5-3 mg l^{-1}) and shoots with roots were formed on media containing 0.5 mg l^{-1} NAA and 2 mg l^{-1}BAP.

4.1.4 Shoot tip and nodal segment as explant

4.1.4.1 Effect of NAA on shoot tip and nodal segment

NAA (0.5 to 3 mg l^{-1}) produced white thick callus on the base of nodal segment. Elongation of nodal segment occurred in 0.5 to 1 mg l^{-1} NAA. In shoot tips, NAA produced callusing, rooting and elongation.

4.1.4.2 Effect of NAA and BAP on regeneration from shoot tip and nodal segments

The effect of NAA and BAP on regeneration from shoot tip and nodal segment are given in Table 17.

Shoot tips recorded 100 per cent regeneration in MS media containing 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} BAP where the mean number of shoots per explant was 1.8. Mean number of shoots per explant was maximum in MS media containing 0.05 mg l^{-1} NAA and 0.2 mg l^{-1} BAP (Plate 4).

Table 16. Effect of various growth regulators on regeneration from callus

MS Media + NAA + BAP (mgl^{-1})	Regeneration (%)	Mean no. of shoot buds ± S.D
0.0 + 0.5	4.63	1 ± 0.46
0.0 + 1.0	5.89	1 ± 0.58
0.0 + 2.0	16.08	1 ± 0.00
0.0 + 3.0	20.00	1 ± 0.00
0.0 + 4.0	10.00	1 ± 0.00
0.05 + 0.5	10.00	2 ± 0.00
0.05 + 1.0	10.00	2 ± 0.00
0.05 + 2.0	10.00	2 ± 0.00
0.1 + 0.5	00.25	1 ± 0.00
0.1 + 1.0	00.50	1 ± 0.55
0.1 + 2.0	10.00	2 ± 0.00
0.2 + 0.5	10.00	2 ± 0.00
0.2+ 1.0	16.00	2 ± 0.00
0.2 + 2.0	20.00	2 ± 0.00
0.5 + 0.5	00.00	0 ± 0.00
0.5 + 1.0	10.00	1 ± 0.00
0.5 + 2.0	10.00	1 ± 0.00

Each treatment consisted of 10 replicates, observations after 30 days

Callus explant

Callus proliferation
(MS + 0.2 mg l^{-1} NAA)

Schizogenesis
(MS + 0.5 mg l^{-1} BAP)

Rhizogenesis
(MS + 3 mg l^{-1} NAA)

Organogenesis
(MS + 0.5 mg l^{-1} NAA + 2 mg l^{-1} BAP)

Plate 3. *In vitro* regeneration from leaf derived calli

Table 17. Effect of different concentrations of NAA and BAP on shoot bud regeneration from shoot tips and nodal segments of *A. annua*

MS Media with NAA + BAP	Shoot tip			Nodal segment		
	Response	Regeneration (%)	No. of shoot buds/explant (Mean ± SD)	Response	Regener-ation (%)	No. of shoot buds/explant (Mean ± SD)
0 + 0.5	C + R	60	1.00 ± 1.00	C + R	30	0.33 ± 0.50
0 + 1	C + R	90	0.90 ± 0.32	C + R	70	0.70 ± 0.48
0 + 2	C + R	80	1.50 ± 1.27	C	0	0.00 ± 0.00
0 + 3	C	0	0.00 ± 0.00	C	0	0.00 ± 0.00
0 + 4	C	0	0.00 ± 0.00	C	0	0.00 ± 0.00
0.05 + 0.05	C	0	0.00 ± 0.00	C	0	0.00 ± 0.00
0.05 + 0.1	C	0	0.00 ± 0.00	C	0	0.00 ± 0.00
0.05 + 0.2	C + R	90	4.00 ± 1.22	C+ R	10	0.11 ± 0.33
0.05 + 0.5	C + R	80	2.80 ± 0.33	C + R	10	0.11 ± 0.33
0.05 + 1	C + R	80	2.30 ± 0.44	C + R	10	0.22 ± 0.44
0.05 + 2	C + R	75	0.55 ± 0.52	C + R	50	0.57 ± 0.52
0.05 + 3	C + R	50	1.30 ± 1.42	C + R	10	0.11 ± 0.33
0.1 + 0.05	C	0	0.00 ± 0.00	C + R	70	1.44 ± 1.24
0.1 + 0.1	C + R	40	1.00 ± 1.40	C + R	80	2.22 ± 1.20
0.1 + 0.2	C + R	60	2.55 ± 2.35	C + R	90	2.55 ± 1.06
0.1 + 0.5	C + R	30	0.90 ± 1.45	C + R	60	0.60 ± 0.52
0.1+ 1	C + R	30	0.60 ± 0.97	C + R	50	0.50 ± 0.53
0.1 + 2	C + R	60	1.00 ± 1.05	C + R	50	1.00 ± 1.05
0.1 + 3	C + R	50	1.50 ± 1.58	C + R	50	2.10 ± 2.77
0.2 + 0.05	C + R	40	0.80 ± 1.04	C + R	50	0.70 ± 0.95
0.2 + 0.1	C + R	60	1.00 ± 1.15	C + R	50	0.70 ± 0.87
0.2 + 0.2	C + R	90	2.30 ± 1.34	C + R	90	0.90 ± 0.32
0.2 + 0.5	C + R	100	1.80 ± 1.14	C + R	90	1.70 ± 1.25
0.2 + 1	C + R	90	1.00 ± 0.47	C + R	90	1.40 ± 0.84
0.2 + 2	C + R	70	0.70 ± 0.48	C + R	70	0.70 ± 0.48
0.2 + 3	C + R	40	0.55 ± 0.45	C	0	0.00 ± 0.00
0.5 + 0.05	C + Rt	0	0.00 ± 0.00	C + Rt	0	0.00 ± 0.00
0.5 + 0.1	C + Rt	0	0.00 ± 0.00	C + Rt	0	0.00 ± 0.00
0.5 + 0.2	C + Rt	20	0.20 ± 0.42	C + Rt	0	0.00 ± 0.00
0.5 + 0.5	C + R	60	0.60 ± 0.52	C + R	60	0.70 ± 0.67
0.5 + 1	C + R	50	0.50 ± 0.53	C + R	50	0.50 ± 0.44
0.5 + 2	C + R	40	0.60 ± 0.84	C + R	40	0.40 ± 0.52
0.5 + 3	C + R	40	1.00 ± 1.33	C + R	20	0.20 ± 0.42
1 + 0.05	C + Rt	0	0.00 ± 0.00	C + Rt	0	0.00 ± 0.00
1 + 0.1	C + R	10	0.20 ± 0.63	C + R	0	0.00 ± 0.00
1 + 0.2	C + R	10	0.20 ± 0.44	C + R	10	0.10 ± 0.32
1 + 0.5	C + R	60	0.70 ± 0.67	C + R	10	0.10 ± 0.32
1 + 1	C + R	70	2.00 ± 1.83	C + R	10	0.10 ± 0.42
1 + 2	C + R	40	1.00 ± 0.67	C	0	0.00 ± 0.00
1 + 3	C	0	0.00 ± 0.00	C	0	0.00 ± 0.00

C: Callusing; C + R: Callusing + Shoot bud regeneration; C+ Rt: Callusing + Rooting
Each treatment consisted of 10 replicates, observations after 25 days

Maximum regeneration of nodal segment was found in MS media supplemented with 0.1 mg l^{-1} NAA and 0.2 mg l^{-1}BAP, 0.2 mg l^{-1} NAA and BAP (0.2, 0.5,1 mg l^{-1}). Mean number of shoots were maximum in media with 0.1mg l^{-1} NAA and 0.2 mg l^{-1} BAP. At high concentration of NAA (2-3mg l^{-1}) along with BAP (0.05-2 mg l^{-1}), shoot tip and nodal segment explants failed to regenerate. They produced green hard callus which regenerated roots. In MS media supplemented with 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} BAP regeneration, multiplication, elongation and rooting of shoot tip and nodal segment explants were observed.

4.1.4.3 Elongation

All treatments resulted in elongation. Maximum elongation of both shoot tip and nodal segment derived shoots were found in MS media supplemented with 0.2 mg l^{-1} GA_3 (Plate 4, 5). Data regarding the elongation of shoot tip and nodal segment derived shoots in different culture media are given in Table 20.

4.1.4.4 Rooting

The elongated shoots were rooted in ½ MS supplemented with 0.5 mg l^{-1} IBA and 2 per cent sucrose. Observations are given in Table 21. Hundred per cent rooting was obtained from shoot tip originated shoots. Roots of plants originated from nodal segment and shoot tip derived shoots were thick and healthy.

4.1.5 Inflorescence and petiole as explants

4.1.5.1 Effect of NAA

NAA (0.5-3 mg l^{-1}) produced white hard callus with inflorescence explant where as in petiole, callusing and rooting were observed. Calli were formed at both ends of petiole touching the media. Short, white and thick roots were formed from callus and also directly from petiole.

4.1.5.2 Effect of gibberellic acid

Inflorescence bits produced no regeneration in MS medium containing GA_3 (0.05, 0.1, 0.2, 0.5 mg l^{-1}). Immature inflorescence was found to be better for

Shoot tip explant

Shoot bud regeneration (MS + 0.2 mg l^{-1} NAA + 0.2 mg l^{-1} BAP)

Elongation (MS + 0.2 mg l^{-1} GA_3)

Rooting (1/2 MS + 0.5 mg l^{-1} IBA)

Nodal segment as explant

Callusing of nodal segment

Shoot bud regeneration

Shoot multiplication

Shoot elongation

Rooting of shoots

Plate 5. *In vitro* regeneration from nodal segments

regeneration compared to the mature. Premature germination of embryos was observed when mature inflorescences were used.

4.1.5.3 Effect of organic supplement

The inflorescence explant when placed in MS media with 1g casein hydrolysate and BAP (1, 2, 3, 4 g l^{-1}) produced only 10 per cent regeneration producing single shoot per explant.

4.1.5.3 Effect of BAP and NAA

The combination of 0.5 mg l^{-1} NAA along with kinetin (0.5, 1.5,2, mg l^{-1}) produced only callusing in petiole and inflorescence explants. The effect of growth regulator combinations on the petiole and inflorescence are given in Table 18.

In media containing combinations of NAA (1, 2, 3 mg l^{-1}) and BAP (0.05, 0.1, 0.2, 0.5, 1, 2, 3 mg l^{-1}) petiole and inflorescence bits produced green hard calli only. Inflorescence bits produced maximum number of shoots in MS media supplemented with 0.2 mg l^{-1} NAA and 1 mg l^{-1} BAP (Plate 6). Maximum regeneration from petiole explants was in MS media containing 0.1 mg l^{-1} NAA and 0.1 or 0.2 mg l^{-1} BAP (40 per cent) (Plate 7).

4.1.5.4 Multiplication

The results of multiplication from shoot buds from inflorescence bits and petiole are presented in Table 19. Maximum number of multiple shoots was formed from inflorescence originated shoot buds in MS media supplemented with 0.5 mg l^{-1} BAP whereas petiole originated shoot buds produced maximum shoot buds in MS media containing 2 mg l^{-1}BAP.

4.1.5.5 Elongation

Observations recorded on elongation of shoots derived from petiole and inflorescence bits are given in Table 20. Maximum elongation of both petiole shoots

Table 18. Effect of different concentrations of NAA and BAP on regeneration of shoot bud from petiole and inflorescence segments of *A. annua*

MS Media with NAA + BAP	Petiole			Inflorescence		
	Response	Regener-ation (%)	No. of shoot buds/explant (Mean ± SD)	Response	Regener-ation (%)	No. of shoot buds/explant (Mean ± SD)
0 + 0.5	C	0	0.0 ± 0.00	C +R	40	0.40 ± 0.52
0 + 1.0	C	0	0.0 ± 0.00	C + R	60	1.00 ± 0.52
0 + 2.0	C	0	0.0 ± 0.00	C + R	40	0.80 ± 0.52
0 + 3.0	C	0	0.0 ± 0.00	C	0	0.00 ± 0.00
0 + 4.0	C	0	0.0 ± 0.00	C	0	0.00 ± 0.00
0.05 + 0.05	C	0	0.0 ± 0.00	C	0	0.00 ± 0.00
0.05 + 0.1	C	0	0.0 ± 0.00	C	0	0.00 ± 0.00
0.05 + 0.2	C	0	0.0 ± 0.00	C	0	0.00 ± 0.00
0.05 + 0.5	C+ R	10	0.1 ± 0.32	C	0	0.00 ± 0.00
0.05 + 1.0	C	0	0.0 ± 0.00	C	0	0.00 ± 0.00
0.05 + 2.0	C	0	0.0 ± 0.00	C	0	0.00 ± 0.00
0.05 + 3.0	C	0	0.0 ± 0.00	C	0	0.00 ± 0.00
0.1 + 0.05	C +R	30	0.3 ± 0.48	C	0	0.00 ± 0.00
0.1 + 0.1	C +R	40	0.4 ± 0.52	C	0	0.00 ± 0.00
0.1 + 0.2	C +R	40	0.4 ± 0.52	C	0	0.00 ± 0.00
0.1 + 0.5	C +R	30	0.3 ± 0.48	C +R	10	0.10 ± 0.32
0.1+ 1.0	C +R	20	0.2 ± 0.40	C +R	40	0.40 ± 0.52
0.1 + 2.0	C +R	10	0.1 ± 0.32	C +R	50	0.50 ± 0.53
0.1 + 3.0	C +R	10	0.1 ± 0.32	C +R	50	0.50 ± 0.53
0.2 + 0.05	C	0	0.0 ± 0.00	C	0	0.00 ± 0.00
0.2 + 0.1	C +R	10	0.1 ± 0.32	C	0	0.00 ± 0.00
0.2 + 0.2	C +R	10	0.3 ± 0.95	C +R	60	0.60 ± 0.52
0.2 + 0.5	C +R	30	0.3 ± 0.48	C +R	70	0.80 ± 0.63
0.2 + 1.0	C +R	10	0.3 ± 0.95	C +R	70	1.40 ± 1.17
0.2 + 2.0	C +R	10	0.4 ± 0.97	C +R	40	0.60 ± 1.45
0.2 + 3.0	C	0	0.0 ± 0.00	C	0	0.00 ± 0.00
0.5 + 0.05	C	0	0.0 ± 0.00	C +Rt	0	0.00 ± 0.00
0.5 + 0.1	C	0	0.0 ± 0.00	C	0	0.00 ± 0.00
0.5 + 0.2	C	0	0.0 ± 0.00	C +R	10	0.10 ± 0.32
0.5 + 0.5	C +Rt	0	0.0 ± 0.00	C +R	50	0.70 ± 0.82
0.5 + 1.0	C	0	0.0 ± 0.00	C	0	0.00 ± 0.00

C: Callusing
C + R: Callusing + Shot bud regeneration
C+ Rt: Callusing + Rooting
Each treatment consisted of 10 replicates, observations after 25 days

Table 19. Effect of growth regulator on multiplication of regenerated shoot buds from inflorescence and petiole

Growth regulators mg l^{-1}		No. of shoot buds/explant (Mean ± SD)	
NAA	BAP	inflorescence	petiole
0.0	0.5	2.70	2.00
0.0	1.0	1.80	2.40
0.0	2.0	1.75	2.80
0.0	0.5	1.80	1.63
0.5	1.0	1.65	1.80
0.5	2.0	1.60	1.83

Each treatment consisted of 10 replicates, observations after 20 days

Table 21. Rooting of shoots derived from different explants of *A. annua*

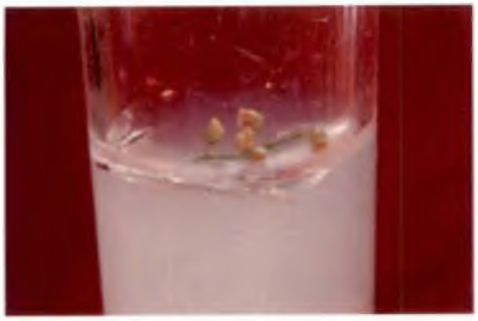
Explant	Mean length of shoot (±S.D)	Mean no. of internodes/ shoots (±S.D)	Mean no. of leaves /shoot (±S.D)	Length of root (cm)	Mean no of roots	Rooting (%)	Morphology of roots
Shoot tip	2.53 ± 0.52	1.20 ± 0.75	4.00 ± 0.62	2.04 ± 0.44	5.33 ± 1.75	100	White roots thick at base
Nodal segment	3.53 ± 0.32	2.00 ± 0.52	5.00 ± 0.44	1.80 ± 0.22	3.35 ± 2.50	80	Thick and short healthy
Petiole	2.90 ± 0.85	3.00 ± 0.45	5.20 ± 0.11	2.10 ± 0.64	3.50 ± 0.34	60	White thin roots
Inflorescence	2.20 ± 0.42	1.50 ± 0.34	4.00 ± 0.82	2.08 ± 0.32	2.75 ± 1.80	75	White roots thick at base

Each treatment consisted of 10 replicates, observations after 20 days

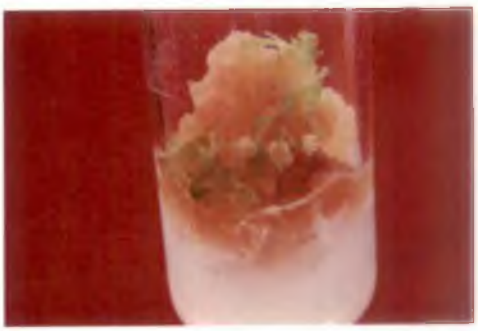
Table 20. Effect of GA_3 on elongation of shoot buds derived from various explants of *A. annua*

Media	Explants											
	Shoot tip			Nodal segment			Petiole			Inflorescence bits		
	Length of shoot (Mean ±S.D)	No. of internodes/ shoot (Mean ±S.D)	No. of leaves/ shoot (Mean ±S.D)	Length of shoot (Mean ±S.D)	No. of internodes/ shoot (Mean ±S.D)	No. of leaves/ shoot (Mean ±S.D)	Mean length of shoot (±S.D)	Mean no. of internodes/ shoots (±S.D)	Mean no. of leaves / shoots (±S.D)	Mean length of shoot (±S.D)	Mean no. of internodes/ shoots (±S.D)	Mean no. of leaves /shoots (±S.D)
MS + 0.01 mg l^{-1} GA3	4.30 ± 1.43	2.00 ± 0.00	3.00 ± 0.46	4.00 ± 0.57	2.00 ± 0.00	3.50 ± 0.22	3.00 ± 1.00	2.00 ± 0.32	5.00 ± 0.42	2.14 ± 0.12	2.00 ± 0.24	4.00 ± 0.32
MS + 0.05 mg l^{-1} GA3	6.83 ± 1.44	2.86 ± 0.32	4.00 ± 0.00	5.50 ± 0.50	2.00 ± 0.00	3.80 ± 0.58	4.71 ± 0.91	2.00 ± 0.56	5.00 ± 0.56	2.50 ± 0.44	2.00 ± 0.87	4.00 ± 0.86
MS+0.1 mg l^{-1} GA_3	7.50 ± 0.86	3.00 ± 0.00	4.00 ± 0.00	7.33 ± 0.58	3.33 ± 0.58	4.00 ± 0.00	4.90 ± 0.94	3.00 ± 0.45	6.00 ± 1.16	3.86 ± 0.46	3.00 ± 0.22	6.00 ± 0.32
MS + 0.2 mg $l^{-1}$$GA_3$	8.50 ± 1.32	4.00 ± 0.00	6.67 ± 1.46	8.33 ± 2.64	4.30 ±1.52	5.30 ± 2.30	5.20 ± 0.76	3.00 ± 0.65	7.00 ± 1.04	5.03 ± 0.32	3.00 ± 1.06	6.00 ± 0.95

Each treatment consisted of 10 replicates, observations after 20 days



Inflorescence explant

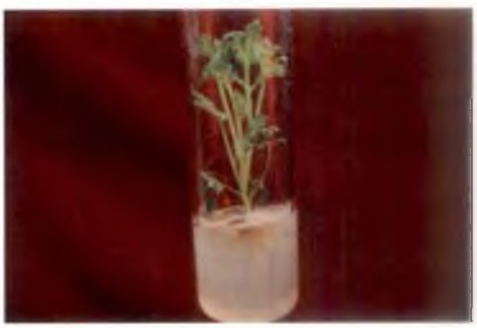


Callusing and
shoot bud initiation
(MS + 0.5 mg l^{-1} BAP)

Shoot regeneration
(MS + 0.2 mg l^{-1} NAA
+ 0.2 mg l^{-1} BAP)

Multiplication
(MS + 0.5 mg l^{-1} BAP)

Elongation
(MS + 0.2 mg l^{-1} GA_3)



Rooting
(1/2 MS + 0.5 mg l-1 IBA)

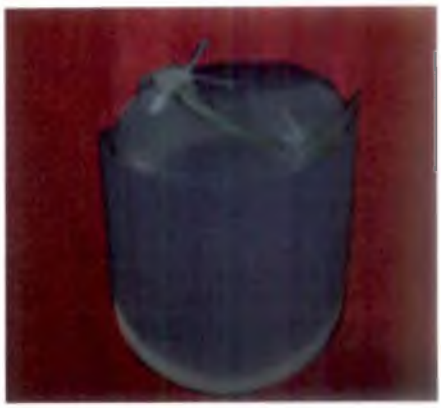
Premature germination of embryo from mature inflorescence
(MS + 0.5 mg l^{-1} BAP)

Plate 6. *In vitro* regeneration from inflorescence explant

Petiole explant

Callusing (MS + 1 mg l^{-1} BAP)

Schizogenesis (MS + 0.1 mg l^{-1} + NAA 0.2 mg l^{-1} BAP)



Rhizogenesis (MS + 1 mg l^{-1} NAA)

Multiplication (MS + 2 mg l^{-1} BAP)

Elongation (MS + 0.2 mg l^{-1} GA_3)

Rooting (1/2 MS + 0.5 mg l^{-1} IBA)

and inflorescence shoots were observed in MS media supplemented with 0.2 mg l^{-1} GA_3.

4.1.5.6 Rooting

The elongated shoots from inflorescence bits and petiole were rooted in ½ MS with 0. 5 mg l^{-1} IBA. Observations are given in Table 21. Only 60 per cent rooting was observed in shoots derived from the petiole and roots formed from such shoots were white and thin. Rooting in shoots derived from inflorescence was much better and those roots were also white in colour.

4.1.6 Roots as explants

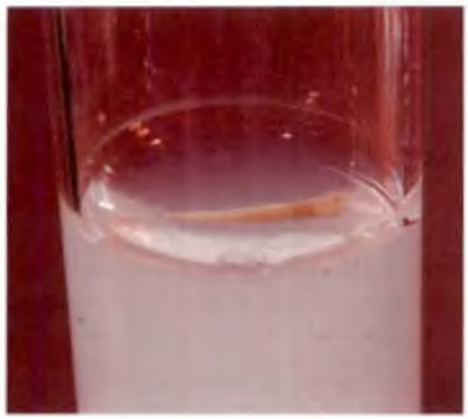
NAA (0.05, 0.1, 0.2 0.5, 1, 2, 3 mg l^{-1}) when added to basal media produced small friable cream coloured callus from the roots. In combinations of NAA (0.05, 0.1, 0.2 0.5, 1, 2, 3 mg l^{-1}) and BAP (0.05, 0.1, 0.2 0.5, 1, 2, 3 mg l^{-1}), roots produced cream friable callus. A green structure was seen at the top of the cream friable callus formed from root bits in MS media containing 0.1 mg l^{-1} NAA and 3 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 1 mg l^{-1} BAP and in 0.5 mg l^{-1} NAA and 2 mg l^{-1} BAP (Plate 8). These green structures were further sub cultured to the same media and there was no regeneration.

4.2 CULTURING AND SENSITIVITY SCREENING OF *AGROBACTERIUM* AND EXPLANTS

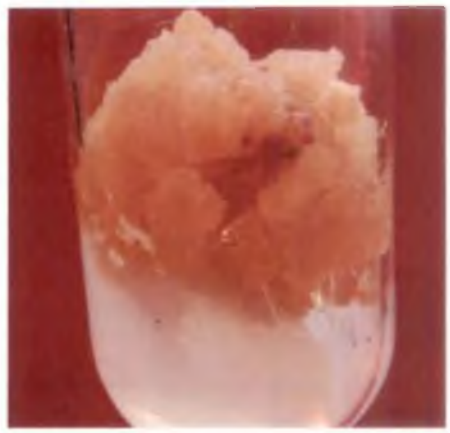
4.2.1 Culturing of *A. rhizogenes* strains

The *A. rhizogenes* strains were cultured on YEB, YEM, YEP, NA, Xanthomonas and LBA media. The strains differed in their growth on the media tested. The influence of culture media on the growth of *A. rhizogenes* strains are given in Table 22.

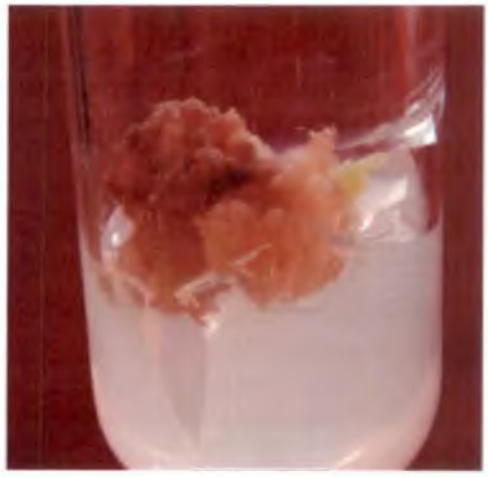
All the culture media favoured the growth of *A. rhizogenes* strain. Strain MTCC 2364 showed very fast growth in all the media tested except YEM. So YEM was selected for growing MTCC 2364, so as to obtain single cell colonies. Both NA



Root Explant



Callusing



Green structure from callus

Plate 8. *In vitro* regeneration from root

Hardening

Flowering in *in vitro* derived plants

Plate 9. Hardening of tissue culture derived plant

Table 22. Influence of culture media on the growth of *A. rhizogenes* strains

Media	A4	ATCC 15834	MTCC 2364
YEB	+ +	+ +	+ + +
YEM	+ +	+ +	+ +
YEP	+	+	+ + +
XM	+	+	+ + + +
NA	+ +	+ +	+ + +
LBA	+ + +	+ + +	+ + +

+ Slow growth, + + fast growth, + + + very fast growth, + + + + excessive growth

and YEB were found best for growing ATCC 15834 and A4 (Plate 10). The optimum temperature for the growth of all the strains was observed to be 26 ± 2°C.

4.2.2 Cultural characteristics of *Agrobacterium*

The colonies of strain A_4 appeared within two days after streaking. Colonies were round with smooth margin, convex, whitish coloured and mucoid in nature (Plate 10). The bacterial colonies of MTCC 2364 appeared within one day and the colonies were of large size with serrated margin and were whitish in colour (Plate 10). The strain ATCC 15834 produced smooth round small colonies at closer spacing two day after streaking and they were whitish and mucoid in nature (Plate 10).

4.2.3 Screening of *A. rhizogenes* strains for antibiotic sensitivity

The response of *A. rhizogenes* strains to different concentrations of antibiotics such as ampicillin, cefotaxime and carbenicillin is given in the Table 23. The strain A4 showed resistance to ampicillin whereas the strain MTCC 2364 showed resistance to both ampicillin and carbenicillin. ATCC 15834 was found to be sensitive to ampicillin, cefotaxime and carbenicillin. All the three strains were sensitive to cefotaxime. The strain MTCC 2364 survived up to 400 mg l^{-1} cefotaxime. Cefotaxime at 500 mg l^{-1} killed all the three strains of *A. rhizogenes*. So cefotaxime at 500 mg l^{-1} was identified as the optimum concentration of the antibiotic to kill *A. rhizogenes* strains under study.

4.2.4 Sensitivity of explants to antibiotics

Sensitivity of explants to cefotaxime at different concentrations is shown in Table 24. Different explants like shoot tip, nodal segment and leaf segments were found to be healthy in cefotaxime up to 500 mg l^{-1}. All explants were pale at 1000 mg l^{-1} cefotaxime (Plate 11). The explants rooted in presence as well as absence of antibiotics.

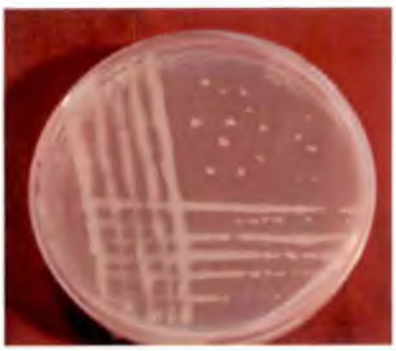
Table 23. Response of *A. rhizogenes* strains to different concentrations of antibiotics

Antibiotic concentration ($mg\ l^{-1}$)		Response of *Agrobacterium* strains		
		A4	MTCC 2364	ATCC 15834
Ampicillin	50	growth	growth	growth
	100	growth	growth	poor growth
	200	growth	growth	no growth
	300	growth	growth	no growth
	400	growth	growth	no growth
	500	growth	growth	no growth
Cefotaxime	50	growth	growth	growth
	100	growth	growth	poor growth
	200	growth	growth	no growth
	300	no growth	growth	no growth
	400	no growth	growth	no growth
	500	no growth	poor growth	no growth
Carbenecellin	50	growth	growth	growth
	100	poor growth	growth	no growth
	200	poor growth	growth	no growth
	300	poor growth	growth	no growth
	400	no growth	growth	no growth
	500	no growth	growth	no growth

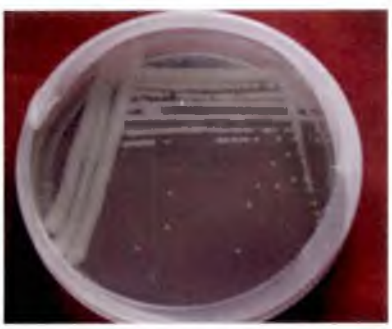
Table 24. Screening of sensitivity of explants to cefotaxime

Concentration of cefotaxime $mg\ l^{-1}$	Response			Remarks
	Shoot tip	Nodal segment	Leaf	
0	Healthy	Healthy	Healthy	Resistant
100	Healthy	Healthy	Healthy	Resistant
250	Healthy	Healthy	Healthy	Resistant
500	Healthy	Healthy	Healthy	Resistant
1000	Pale	Pale	Pale	Sensitive

ATCC 15834



A4



MTCC 2364

Plate 10. ***Agrobacterium rhizogenes*** **strains employed in the study**

0

100

250

500

1000

Plate 11. Sensitivity of leaf explant to cefotaxime (mgl^{-1})

4.2.5 Preculturing and wounding of explants

Wounded explants remained healthy in growth regulator free media when they were precultured for two days (Plate 12).

4.3 STANDARDISATION OF TRANSFORMATION TECHNIQUES

4.3.1 Standardisation of inoculation methods

4.3.1.2 Direct inoculation and suspension culture method

4.3.1.2.1 Influence of bacterial inoculum

The influence of bacterial inoculum on transformation is represented in Table 25. Among the three explants used, shoot tip and leaf segments responded better to transformation as compared to nodal segments. Similarly the different *A. rhizogenes* strains used for infection differed in their transformation ability. Both the single cell colonies and bacterial suspension produced transformation. The strain ATCC 15834 showed highest transformation (70 per cent) by both the methods with shoot tip followed by A4 with shoot tip as the explant. A4 and ATCC 15834 produced maximum transformation by SM where as MTCC 2364 produced maximum transformation by DIM. MTCC 2364 by SM did not induce hairy roots in leaf and nodal segment (Plate 13, 14). Co-cultivation of shoot tip with MTCC 2364 suspension for more than 2 days led to yellowing. Nodal segments could not produce hairy roots by SM of both A4 and ATCC 15834.

Table 25. Influence of bacterial inoculum on transformation

A. rhizogenes strains	Inoculum	Percentage of transformation		
		Leaf	Shoot tip	Nodal segment
A_4	DIM	20.00	50.00	10.16
	SM	16.38	30.00	0.00
ATCC 15834	DIM	17.00	50.00	2.38
	SM	19.85	70.00	0.00
MTCC 2364	DIM	12.00	22.00	0.00
	SM	0.00	10.00	0.00

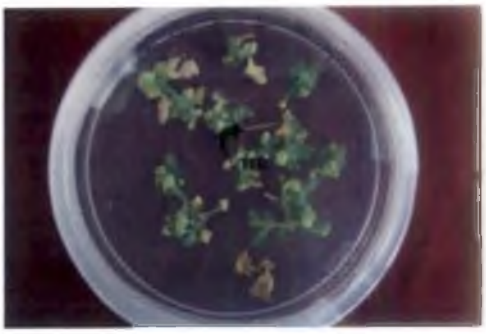
Shoot tip

Leaf segments

Nodal segments

Plate 12. Preculturing of explants for induction of hairy roots

A4

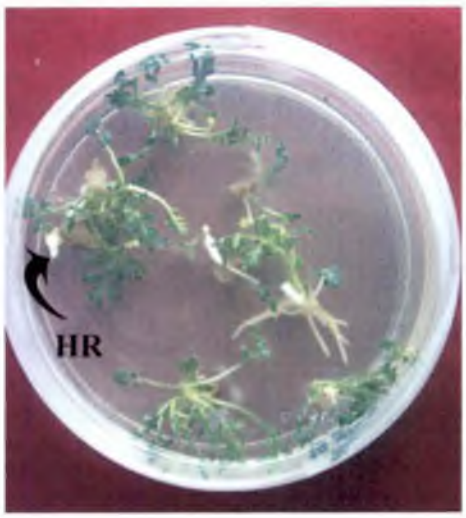


ATCC 15834

MTCC 2364

Control Leaves

Plate 13. Hairy roots from leaf explant



A4

ATCC 15834

MTCC 2364

Control shoot tips

Plate 14. Hairy roots from shoot tip explants

4.3.1.2.2 Influence of co-culture period on transformation

The transformation frequency was influenced by co-culture period. The different *A.rhizogenes* strains also differed in their transformation ability. The influence of co-culture period under DIM and SM on transformation is given in the Table 26.

All strains showed maximum transformation in all explants co-cultivated for 1 or 2 days. A co-cultivation of more than 2 days reduced transformation efficiency. Co-cultivation for 4 days resulted in yellowing of explants. The maximum transformation (70 per cent) was in shoot tip co-cultivated with ATCC 15834 for 2 days by DIM and for 1 day by SM.

In MS media, all explants produced rooting. Callusing was less in shoot tip when compared to leaf and nodal segments. In case of leaf explant, A4 produced maximum transformation by DIM (20 per cent) co-cultivated for 2 days followed by ATCC 15834. The strain ATCC 15834 produced 19.85 per cent transformation in leaf explant co-cultivated for 2 days.

One day co-cultivation of leaf with A4 could not produce transformation where as ATCC 15834 produced transformation by DIM and SM when co-cultivated for 1, 2 or 3 days. Only the single colony of MTCC 2364 produced transformation in leaf co-cultivated for 1 or 2 days. The bacterial suspension of MTCC 2364 failed to produce transformation in leaf explants even if it was co-cultivated for 3 days. A greater response of leaf segments to induce hairy roots was seen when they were co-cultivated for two days (Fig. 3).

A4 produced 50 per cent transformation in shoot tips by DIM irrespective of co-cultivation period (1-3 days). Co-cultivation for 4 days resulted in yellowing of the explant. A4 produced better transformation by SM when co-cultivated for 2 days than when co-cultivated for 1 or 3 days. In case of MTCC 2364, excess bacterial growth was observed when explants were co-cultivated for more than two days.

Table 26. Influence of co-culture period on transformation

A. rhizogenes strains	Explant	Inoculation method	Co-cultivation period	Response	Transformation per cent
A4	Leaf	DIM	1	R	00.00
			2	R	20.00
			3	R	00.00
			4	Y	00.00
		SM	1	R	00.00
			2	R	16.38
			3	R	16.00
			4	Y	00.00
	Shoot tip	DIM	1	R	50.00
			2	R	50.00
			3	R	50.00
			4	Y	00.00
		SM	1	R	20.00
			2	R	30.00
			3	R	20.00
			4	Y	00.00
	Nodal segment	DIM	1	R	05.08
			2	R	06.00
			3	R	04.38
			4	Y	00.00
		SM	1	R + C	00.00
			2	R + C	00.00
			3	R + C	00.00
			4	Y	00.00
ATCC 15834	Leaf	DIM	1	R+ C	15.12
			2	R + C	17.00
			3	R + C	14.35
			4	Y	00.00
		SM	1	R+ C	10.16
			2	R+ C	19.85
			3	R+ C	14.00
			4	Y	00.00
	Shoot tip	DIM	1	R	50.00
			2	R	70.00
			3	R	10.00
			4	Y	00.00
		SM	1	R	50.00
			2	R	70.00
			3	R	10.00
			4	Y	00.00
	Nodal segment	DIM	1	R+ C	02.38
			2	R+ C	01.06
			3	R+ C	00.00
			4	Y	00.00
		SM	1	R + C	00.00
			2	R + C	00.00
			3	R + C	00.00
			4	Y	00.00

R- Rooting, Y-Yellowing, R+C- Rooting + Callusing

Table 26 continued.

A. rhizogenes strains	Explant	Inoculation method	Co-cultivation period	Response	Transformation per cent
MTCC 2364	Leaf	DIM	1	R + C	10.68
			2	R+ C	12.00
			3	R + C	00.00
			4	Y	00.00
		SM	1	R+ C	00.00
			2	R+ C	00.00
			3	R+ C	00.00
			4	Y	00.00
	Shoot tip	DIM	1	R+ C	18.08
			2	R+ C	22.35
			3	R+ C	13.39
			4	Y	00.00
		SM	1	R+ C	03.00
			2	R+ C	10.00
			3	R+ C	00.00
			4	Y	00.00
	Nodal segment	DIM	1	R+ C	00.00
			2	R+ C	10.00
			3	R+C	00.00
			4	Y	00.00
		SM	1	R + C	00.00
			2	R + C	00.00
			3	R + C	00.00
			4	Y	00.00

R- Rooting, Y-Yellowing, R+C- Rooting + Callusing

ATCC 15834 and MTCC 2364 produced maximum transformation in shoot tips co-cultivated for 2 days with bacterial colony or suspension (Fig. 4).

4.3.1.2.2 Influence of acetosyringone in co-culturing media for hairy root induction

The influence of acetosyringone in hairy root induction was studied using DICA and SCA methods. All explants responded to transformation using 100μ*M* acetosyringone. The strain A4 and ATCC15834 produced successful transformation by the addition of acetosyringone following DICA and SCA in leaf and shoot tip. Both strains produced transformation in nodal segments only by DICA. Results are given in Table 27.

There was no transformation in nodal segment by the suspension method using the strains A4 and ATCC15834 in presence as well as absence of acetosyringone. MTCC2364 strain failed to produce any successful transformation following, SCA method in leaf and nodal segment.

The presence of 100μ*M* acetosyringone in the co-culturing media significantly increased the number of putative transformants produced by shoot tips with all the strains. Following DICA method, A4 strain produced 24 per cent transformation with a mean of 2.5 hairy roots per transformed shoot tip explant. In the absence of acetosyringone (DIM) only 22.35 per cent transformation was obtained with a mean of 1.25 hairy roots per transformed explant. The strain gave 14 per cent transformation by SCA method, which produces a mean of 1.20 hairy roots.

The strain ATCC 15834 produced 23 per cent transformation in presence of 100 μ*M* acetosyringone in leaf by DICA when compared to 17 per cent transformation by DIM. By SCA, the strain gave 22.95 per cent transformation in leaf where as in the absence of acetosyringone it produced 19.85 per cent transformation. In shoot tip, ATCC 15834 could increase the transformation from 70.00 per cent (DIM) to 72.50 per cent (DICA) with 1.35 hairy roots per transformed explant.

Table 27. Influence of acetosyringone in hairy root induction

A. rhizogenes strains	Explant	Inoculation method	Mean no of hairy roots per explant	Mean no of normal roots per explant	Transformation (%)
A4	Leaf	DIM	0.45	0.38	12.00
		DICA	0.50	0.36	15.00
		SM	0.00	0.26	00.00
		SCA	0.46	0.58	01.00
	Shoot tip	DIM	1.25	1.60	22.35
		DICA	2.50	0.36	24.00
		SM	0.60	1.68	10.00
		SCA	1.20	1.25	14.00
	Nodal segment	DIM	0.95	0.66	06.00
		DICA	0.98	0.58	08.00
		SM	0.00	0.50	00.00
		SCA	0.00	0.48	00.00
ATCC 15834	Leaf	DIM	0.22	0.00	17.00
		DICA	0.98	0.11	23.00
		SM	0.50	0.11	19.85
		SCA	0.65	0.20	22.95
	Shoot tip	DIM	0.50	0.25	70.00
		DICA	1.35	0.25	72.50
		SM	0.58	0.36	70.00
		SCA	0.98	0.33	71.25
	Nodal segment	DIM	0.32	0.00	01.06
		DICA	0.68	0.00	04.38
		SM	0.00	0.25	00.00
		SCA	0.00	0.20	00.00
MTCC 2364	Leaf	DIM	0.22	0.18	12.00
		DICA	0.65	0.20	14.00
		SM	0.00	0.15	00.00
		SCA	0.00	0.10	00.00
	Shoot tip	DIM	0.98	0.40	22.35
		DICA	1.32	0.46	25.00
		SM	0.20	0.40	10.00
		SCA	0.95	0.33	13.85
	Nodal segment	DIM	0.66	0.20	10.00
		DICA	0.75	0.15	12.50
		SM	0.00	0.22	00.00
		SCA	0.00	0.25	00.00

Fig. 3. Response of leaf segments to different *A.rhizogenes* on two day co-culture using SM and DIM

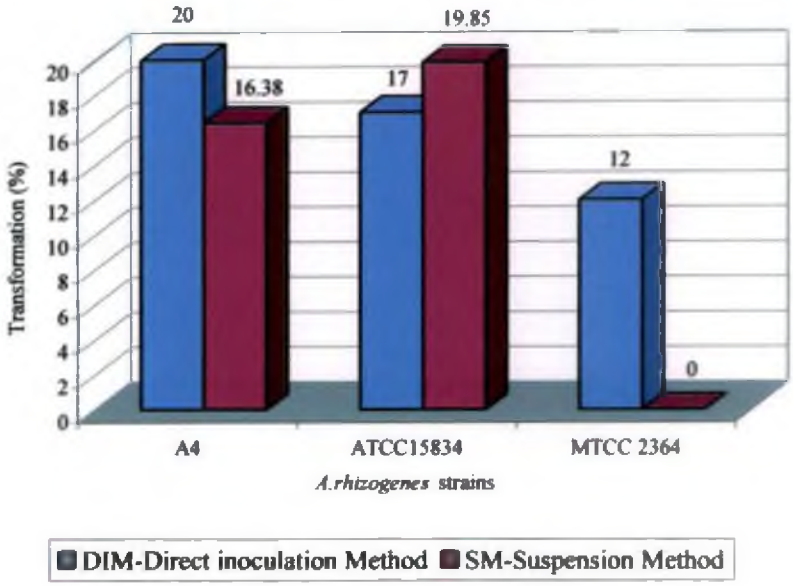
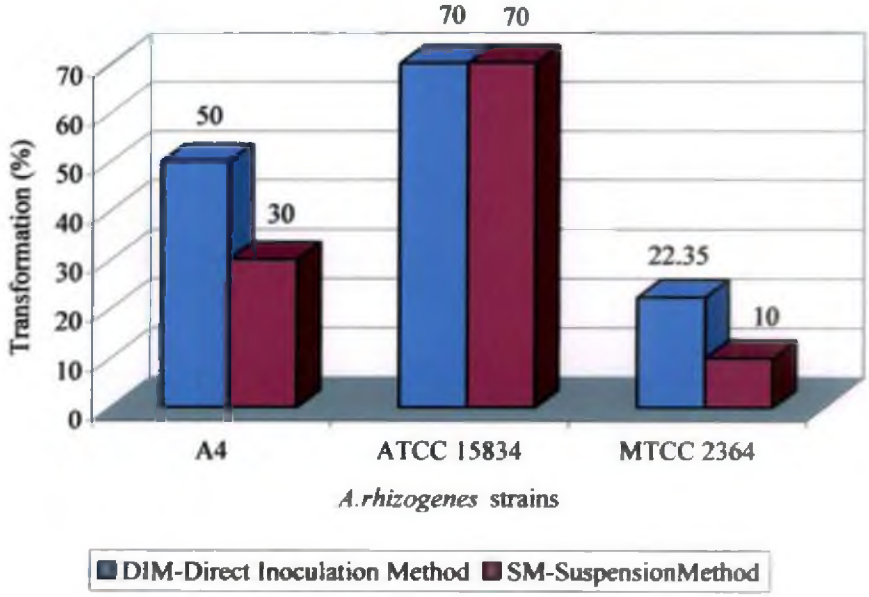


Fig. 4. Response of shoot tip to different *A.rhizogenes* on two day coculturing using SM and DIM



MTCC 2364 produced 25 per cent transformation and 1.32 hairy roots per transformed shoot tip explant by DICA when compared to 22.35 per cent transformation by DIM. In leaf and nodal segment transformation per cent by DICA method was more than by DIM (Plate 15). The effect of acetosyringone in transformation of leaf and shoot tip explant with the *A. rhizogenes* strains are given in Fig. 5 and Fig. 6 respectively.

4.3.2 Standardization of explants for efficient transformation

Response of various explants to different *A. rhizogenes* strains are given in Table 28. Among different explants tested, shoot tip was found to be the best explant for efficient transformation followed by leaf segment. Nodal segment responded poorly to transformation. The hairy roots were induced from basal portion of the shoot tip. No roots developed from the leaves attached to shoots. From the leaves, hairy roots were produced from the petiolar end. In the nodal segment also hairy roots were produced from basal portion.

Maximum of seven hairy roots were produced per single shoot tip by ATCC 15834. The infected explants produced normal roots in addition to hairy roots. Rooting and callusing occurred from most of the explants, however shoot tip showed least tendency for callusing. Maximum transformation percentage was by ATCC 15834 (72.50%) but the mean number of hairy roots was maximum in shoot tips transformed by A4 (1.25). The control explants also showed normal rooting and callusing. Elongation of both shoot tip and nodal segment was also observed in control and infected explants.

4.3.3 Efficiency of strains in inducing hairy roots

The strain ATCC 15834 showed highest efficiency (70 per cent) in transforming plant tissues followed by A4 strain (50 per cent). MTCC 2364 produced transformation in leaf and nodal segment by DIM where as in shoot tip it produced hairy roots by DIM and SM. Excess bacterial growth was also observed in explants transformed with MTCC 2364. The number of hairy roots per explant was more in A4 transformed shoot tip. ATCC 15834 was able to induce a maximum of seven hairy

ATCC 15834 infected shoot tip

Control

MTCC 2364 infected leaf segments

Control

Plate.15 Influence of acetosyringone in hairy root induction

Fig. 5. Influence of acetosyringone on transformation percentage of leaf segment using DIM and SM

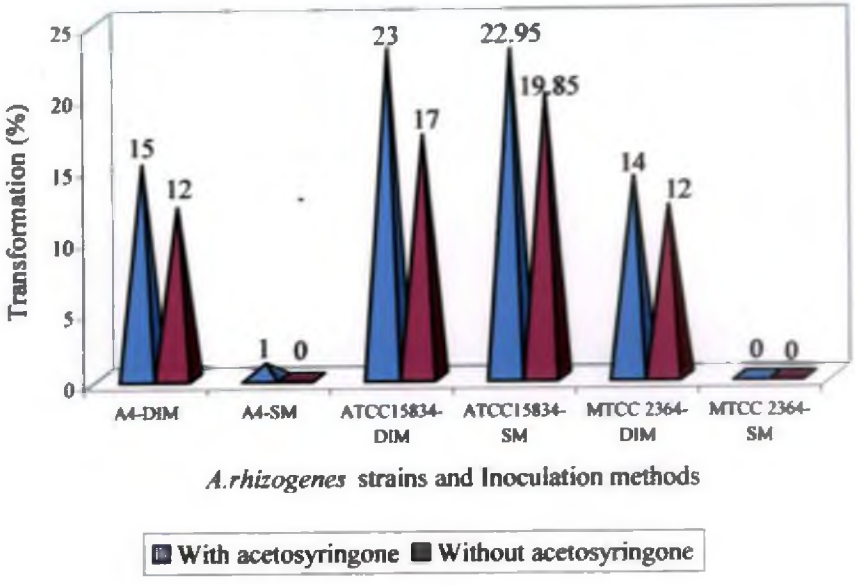


Fig. 6. Influence of acetosyringone on transformation percentage of shoot tips using DIM and SM

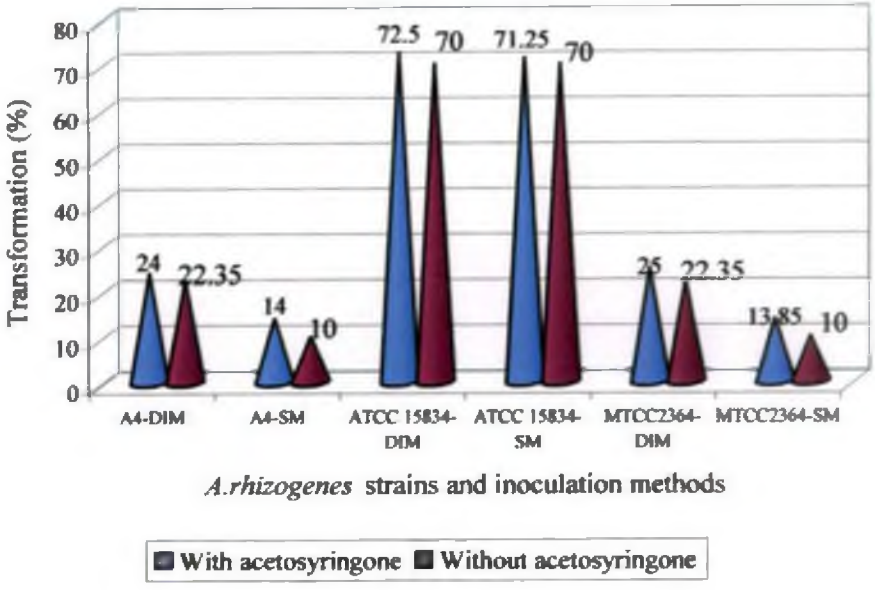


Table 28. Standardization of explants for efficient transformation

A.rhizogenes strains	Explant	Inoculation method	Co-cultivation period	Mean number of hairy roots per explant	Mean number of normal roots per explant	Transfor-mation (%)	Response
A4	Leaf	DIM	1	0.00	0.11	00.00	R
			2	0.45	0.38	12.00	R
			3	0.00	0.45	00.00	R
		SM	1	0.00	0.18	00.00	R
			2	0.00	0.26	00.00	R
			3	0.00	0.42	00.00	R
	Shoot tip	DIM	1	0.98	0.75	18.08	R
			2	1.25	1.60	22.35	R
			3	1.14	1.20	13.39	R
		SM	1	0.50	1.57	03.00	R
			2	0.60	1.68	10.00	R
			3	0.00	1.88	00.00	R
	Nodal segment	DIM	1	0.68	0.33	05.08	R
			2	0.95	0.66	06.00	R
			3	0.50	0.93	04.38	R
		SM	1	0.00	0.44	00.00	R + C
			2	0.00	0.50	00.00	R + C
			3	0.00	0.67	00.00	R + C
ATCC 15834	Leaf	DIM	1	0.20	0.00	15.12	R+ C
			2	0.22	0.00	17.00	R+ C
			3	0.18	0.50	14.35	R + C
		SM	1	0.40	0.11	10.16	R+ C
			2	0.50	0.11	19.85	R+ C
			3	0.35	0.25	14.00	R+ C
	Shoot tip	DIM	1	0.22	0.00	50.00	R
			2	0.50	0.25	70.00	R
			3	0.66	0.33	10.00	R
		SM	1	0.44	0.00	50.00	R
			2	0.58	0.36	70.00	R
			3	0.11	0.50	10.00	R
	Nodal segment	DIM	1	0.12	0.25	02.38	R+ C
			2	0.32	0.00	01.06	R+ C
			3	0.00	0.58	00.00	R+ C
		SM	1	0.00	0.25	00.00	R+ C
			2	0.00	0.33	00.00	R+ C
			3	0.00	0.48	00.00	R+ C

Table 28. continued

A.rhizogenes strains	Explant	Inoculation method	Co-cultivation period	Mean number of hairy roots per explant	Mean number of normal roots per explant	Transformation (%)	Response
MTCC 2364	Leaf	DIM	1	0.11	0.00	10.68	R+C
			2	0.22	0.18	12.00	R+C
			3	0.00	0.22	00.00	R+C
		SM	1	0.00	0.11	00.00	R+C
			2	0.00	0.15	00.00	R+C
			3	0.00	0.40	00.00	R+C
	Shoot tip	DIM	1	0.25	0.40	18.08	R+C
			2	0.98	0.40	22.35	R+C
			3	0.66	0.50	13.39	R+C
		SM	1	0.10	0.40	03.00	R+C
			2	0.20	0.40	10.00	R+C
			3	0.00	0.00	00.00	R+C
	Nodal segment	DIM	1	0.00	0.11	00.00	R+C
			2	0.66	0.20	10.00	R+C
			3	0.00	0.20	00.00	R+C
		SM	1	0.00	0.20	00.00	R+C
			2	0.00	0.22	00.00	R+C
			3	0.00	0.25	00.00	R+C

R: Rooting
R + C: Rooting + Callusing

roots per single shoot tip. The number of days for hairy root induction from infected explants using different strains under different inoculation method is shown in Table 29. The number of days for hairy root induction differed under different inoculation methods. In general 6 to 28 days were taken to induce hairy roots.

4.3.4 Establishment of hairy root cultures

Some hairy root cultures showed faster growth with high lateral branching where as some showed only slower growth (Plate 16). Some roots failed to grow and turned brown. Control roots were non hairy and were positively geotropic in nature. It showed no lateral branching. Normal roots produced fresh weight of 5g after two months of culturing in growth regulator free MS media.

4.3.5 Rapid culturing of hairy roots

The final fresh weight of hairy root clones of different *A. rhizogenes* strains are shown in Table 30. Compared to control roots and hairy roots induced by other experimental strains, A4 induced hairy roots showed faster growth producing more biomass. The growth of hairy roots liquid medium without growth regulator was rapid when compared to growth in solid medium (Plate 17). The colour of hairy roots in liquid media turned brown within three weeks of culture.

4.3.6 Effect of culture media and conditions on the growth of hairy roots

The effect of culture media on the growth of hairy roots is shown in Fig. 8. Among the liquid medias tested (MS, ½ MS, B_5 with 2 per cent sucrose and B_5 with 3 per cent sucrose). B_5 supplemented with 3.0 per cent sucrose was found to be superior for promoting hairy root growth followed by B_5 with 2.0 per cent sucrose MS and ½ MS. In all media, the newly growing regions of hairy roots were creamy white in colour, whereas the initial roots incubated, turned brown.

Growth pattern of hairy roots produced by ATCC 15834 was observed. No change was noticed in first week. Root growth started after 10 to 12 days and it increased later. After 4 weeks, all roots turned brown.

Table 29. Number of days for hairy root induction

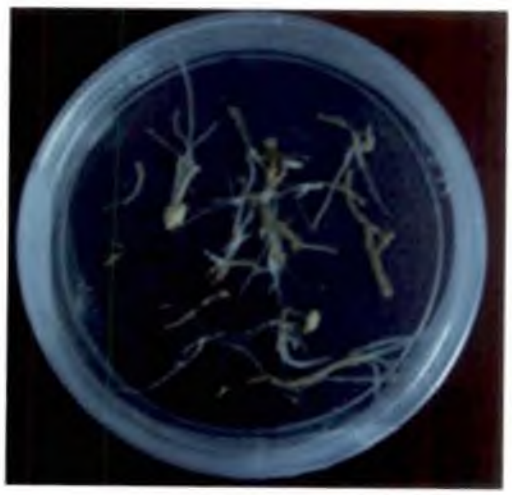
A. rhizogenes strains	Inoculation method	Explant	Number of days taken for root induction
A4	DIM	Leaf	12-17
		Shoot tip	06-20
		Nodal segment	06-14
	DICA	Leaf	10-15
		Shoot tip	06-15
		Nodal segment	06-10
	SM	Leaf	25-28
		Shoot tip	20-24
	SCA	Leaf	20-24
		Shoot tip	18-21
ATCC 15834	DIM	Leaf	07-10
		Shoot tip	07-19
		Nodal segment	10-19
	DICA	Leaf	07-10
		Shoot tip	07-15
		Nodal segment	07-12
	SM	Leaf	15-20
		Shoot tip	14-18
	SCA	Leaf	12-20
		Shoot tip	14-18
MTCC 2364	DIM	Leaf	10-15
		Shoot tip	10-18
		Nodal segment	21-28
	DICA	Leaf	10-12
		Shoot tip	07-15
		Nodal segment	17-21
	SM	Shoot tip	15-20
	SCA	Shoot tip	14-18

Table 30. Variation in fresh weight of hairy roots of different *A.rhizogenes* strains

A.rhizogenes strains	Fresh weight of hairy roots after 25 days(g)
A4	2.75
ATCC 15834	1.156
MTCC 2364	1.162
Control roots	1.735

A4

ATCC 15834



MTCC 2364

Plate 16. Establishment of hairy root culture

A4

ATCC 15834

MTCC 2364

Control roots

Plate 17. Rapid culture of hairy roots in liquid media

Fig. 7. Efficiency of *A.rhizogenes* strains in inducing hairy roots

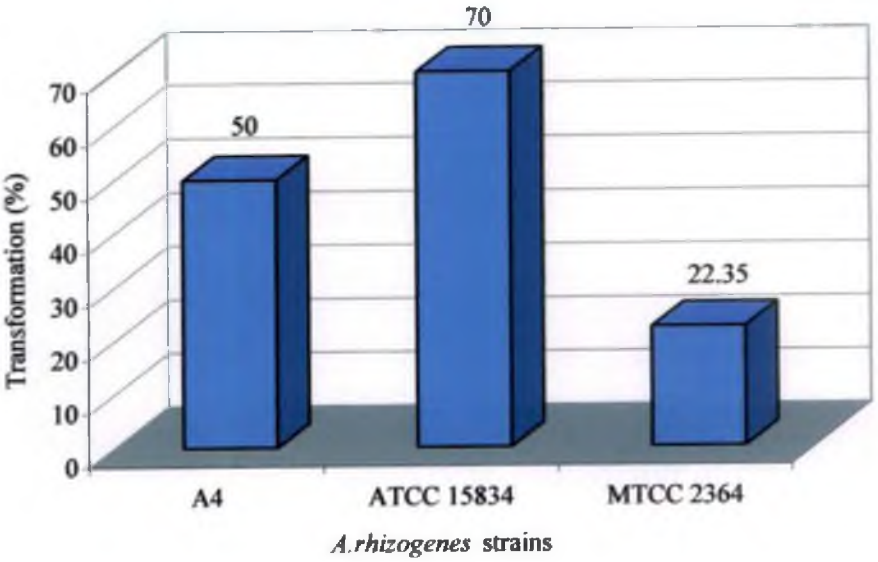
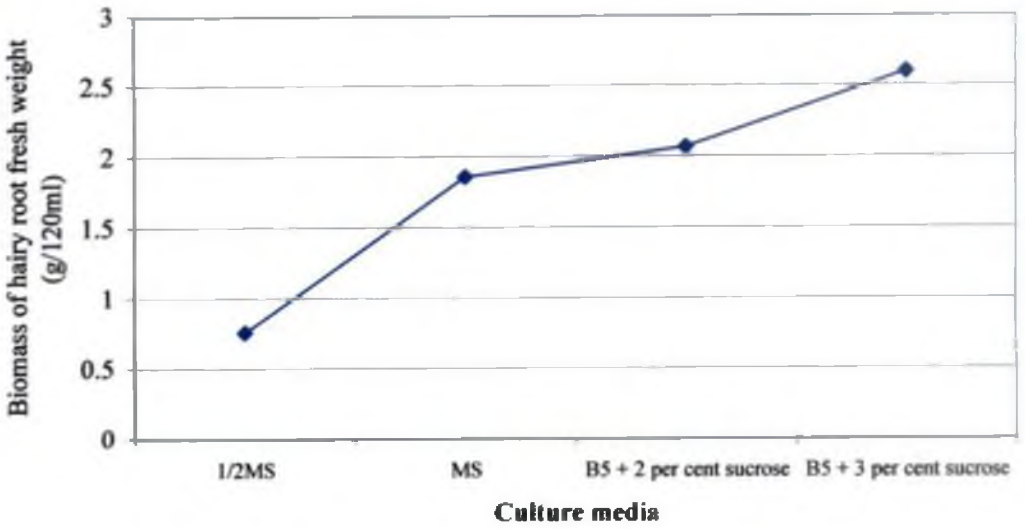


Fig. 8. Effect of culture media on growth of hairy roots of ATCC 15834



4.4 CONFIRMATION OF TRANSFORMATION

4.4.1 Morphology of hairy roots

The roots induced by the strain A4 produced calli in MS media. Most of the roots were positively geotropic. However some showed negative geotropism. Lateral roots were more in hairy roots. There was no callusing and lateral branching in normal roots. Hairy nature of roots was observed during initial stages of culturing (Plate 16).

4.4.2 Opine analysis

Opines extracted from A4, ATCC 15834 and MTCC 2364 induced hairy roots produced spots corresponding to agropine. No spot was produced by control roots. Also no spot was produced at positions corresponding to mannopine and mannopinic acid. The response of transformed and normal roots to the presence of opines is given in the Plate 18.

The intensity of the spots showed variation. The opines extracted from roots produced from MTCC 2364 showed only less intense spots as compared to opines from other transformed roots. Confirmation of the transformation by detection of opines using high-voltage paper electrophoresis was found to be successful. Further confirmation was done by PCR.

4.4.3 Confirmation by PCR analysis

PCR analysis of hairy roots was carried out for confirming the genetic transformation. Polymerase Chain Reaction was used to demonstrate the presence of TL-DNA with *rol* B and genes in the transformed roots.

4.4.3.1 Isolation of genomic DNA

Upon electropohoresis on one per cent agarose gel, intact DNA was observed in all the wells (Plate 19).

M-Mannopine

MA-Mannopinic acid

AA- Agropinic acid

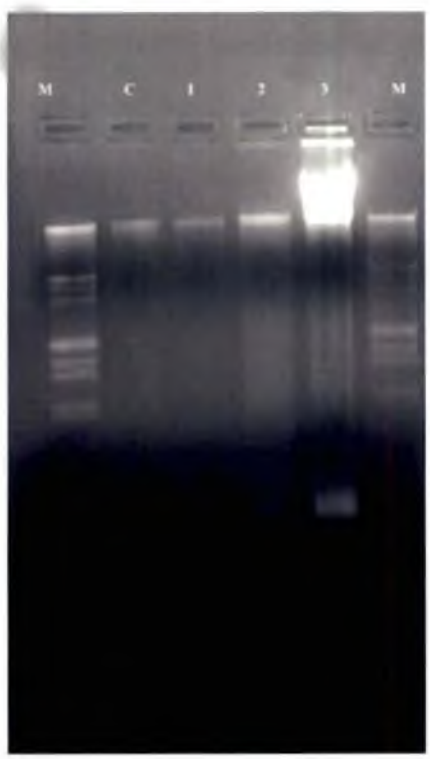
1- A4 derived hairy root clone

2- ATCC 15834 hairy root clone

3- MTCC 2364 derived hairy root clone

C-Control roots

Plate 18. Detection of opines by High Voltage Paper Electrophoresis



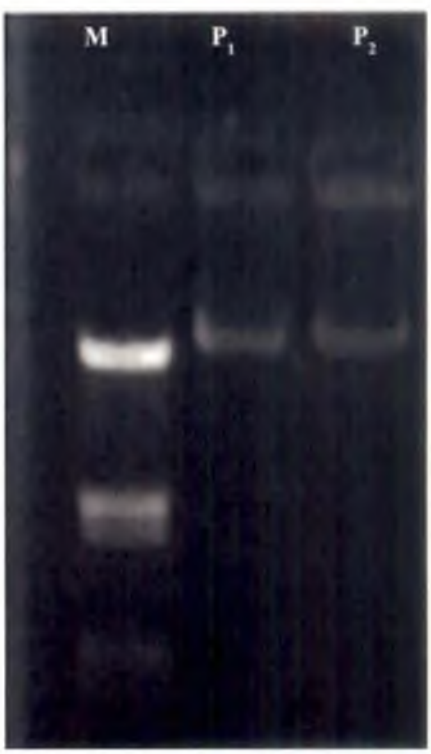
C-Control root

1-A4derived hairy root clone

2-ATCC 15834 derived hairy root clone

3-MTCC 2364 derived hairy root clone

Plate 19. Isolation of genomic DNA



M-Molecular weight marker

P1,P2- pLJ1 cosmids

Plate 20. Isolation of cosmids

4.4.3.1 Isolation of cosmid from E. coli

The cosmid pLJ1was isolated from *E.coli* and cosmid had high molecular size (Plate 20).

4.4.3.3 PCR analysis of rol B and genes

PCR analysis was carried out using two sets of primers, Rol BF1R1 and Rol BF2R2. The amplified DNA samples electrophoresed on 0.7 per cent agarose gel is given in Plate 21.

Amplification was obtained with Rol BF2R2 primer sets which confirmed the presence of *rol* B gene in the transformed roots as well as positive control. Amplification with Rol BF2R2 primer set corresponding to *rol* B gene produced a 205 bp band for the transformed roots and for the cosmid pLJ1. No amplification occurred in negative control .The transgenic nature of the hairy roots was thus confirmed by PCR analysis.

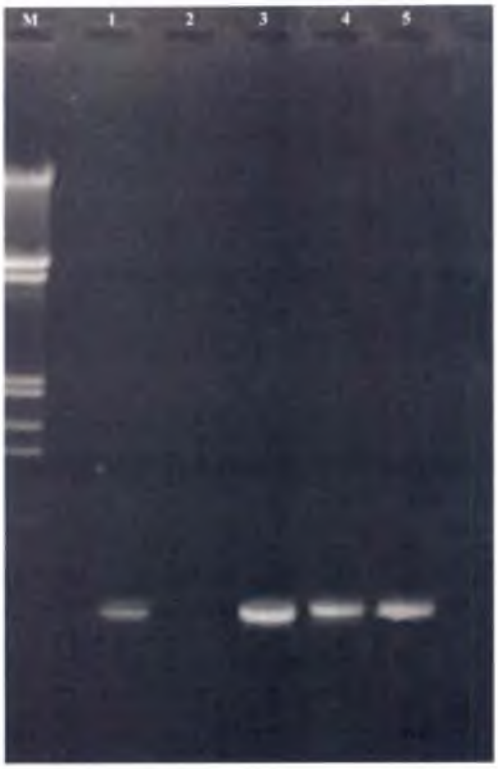
4.4.4 Southern hybridization

On viewing the gel using UV transilluminator, it was observed that the entire DNA after restriction digestion with *Bam* H1, appeared as a smear and no separate intact bands were visible (Plate 22.). After blotting the staining of gel with ehidium bromide showed no bands in the gel. This indicates that all bands are transferred to the membrane.

Southern hybridization was carried out further for confirming the presence of *rol* B gene of TL-DNA in the transformed roots. The regions of hybridization were detected by autoradiograpy. On developing the film after the required exposure time there was no signal detected.

4.4.5 Dot blot analysis

In dot blot analysis DNA was directly loaded on to nitrocellulose membrane. The cosmid pLJ1 amplified with Rol BF2R2 primer was used as the probe. There was no signal upon exposure to x-ray film.



M-Molecular weight marker

(lDNA ECoRI/HindIII double digest)

1-Positive control (pLJ1 cosmid)

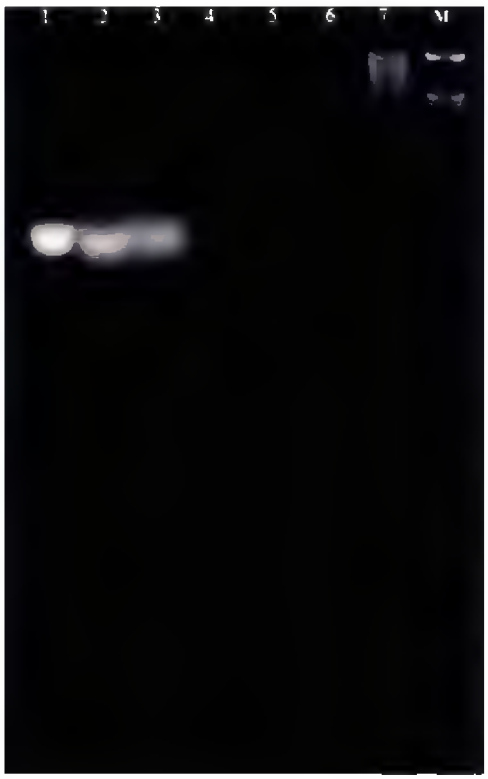
2- Control roots

3-A4 derived hairy root clone

4-ATCC 15834 derived hairy root clone

5- MTCC 2364 derived root clone

Plate 21. PCR analysis of hairy roots for rol B gene



M- Molecular weight marker

1- unrestricted pLJ1

2,3- Restricted pLJ1

4- Control roots

5- A4 derived root clone

6- ATCC 15834 derived root clone

7- MTCC 2364 derived root clone

Plate 22. Restriction digestion of genomic DNA and pLJ1 cosmid

4.5 ARTEMISININ CONTENT OF EXPERIMENTAL SAMPLES

Thin Layer Chromatographic plates used for quantitative analysis of artemisinin in various plant samples are shown in Fig. 23a and 23b. Extract of samples were spotted on the base line beside standard solution of artemisinin. The spot of artemisinin developed at an R_f of 0.6. Artemisinin spot in samples were identified by the coincidence of their R_f values into that of standard artemisinin.

Quantification of artemisinin was done with the help of densitometric assay carried out with the help of SPOT DENSO analytical tool. Artemisinin content of different experimental samples is given in Table 31.

Maximum artemisinin was found in inflorescence from field grown plants followed by rooted *in vitro* shoots and *in vitro* shoots of A4 transformed plant. The artemisinin content in ATCC 15834 transformed shoot and MTCC 2364 shoot was also comparable with artemisinin content in untransformed *in vitro* shoot. The artemisinin content in transformed shoots (The shoot tip from which hairy root was dissected out and cultured in hormone free MS media) was almost equal or more than that in *in vitro* shoots. Among hairy roots, the root induced by ATCC 15834 produced more artemisinin.

4.6 ENHANCEMENT OF SECONDARY METABOLITE PRODUCTION

4.6.1 Addition of osmoregulants

The addition of osmoregulant PEG (6000g) was found in no way affecting the hairy root growth. There was no change in the artemisinin content of hairy roots grown in stress media. The TLC analysis of media also showed absence of artemisinin.

4.6.2 Addition of precursors

The hairy root cultures were grown in media supplemented with 1.0 m*M* methionine. There was no change in growth of hairy roots in the media. There was no change in artemisinin content. The hairy roots of ATCC 15834 were used for the

study. The artemisinin content of control hairy roots and hairy roots grown in media containing methionine was found to be 0.0039 per cent FW and 0.00387 per cent FW respectively. On TLC analysis of the culture medium, no artemisinin was detected.

4.6.2 Addition of elicitors

4.6.2.1 Elicitation by Aspergillus homogenate

The *Aspergillus niger* homogenate was added to media. There was no change in growth of hairy roots. But there was increase in artemisinin content of ATCC 15834 induced hairy roots grown in media elicited with the homogenate (from 0.0039 per cent FW to 0.0042 per cent FW). But there was no artemisinin in the culture media. The *Aspergillus* homogenate at the rate of 250 µl/ 125ml was added to the medium.

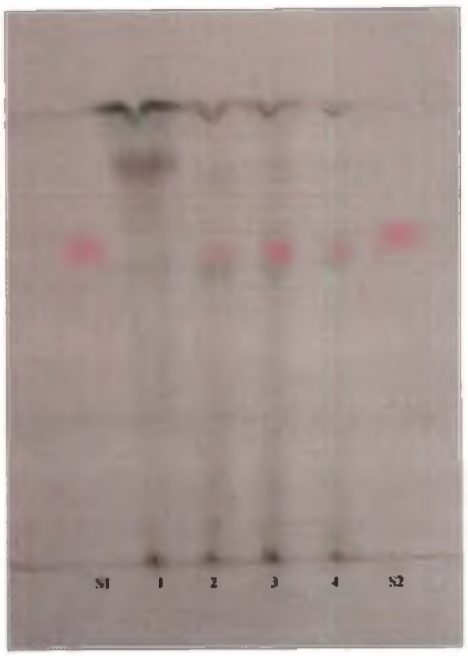
4.6.2.2 Elicitation by yeast extract

Addition of yeast extract to media resulted in no specific change in growth and artemisinin content of ATCC 15834 induced hairy roots. There was no artemisinin in the media also.

Table 31. Artemisinin content of experimental samples

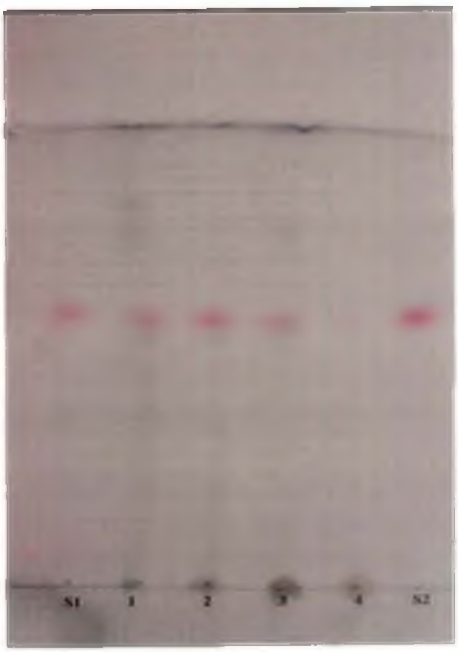
Sl. No.	Sample	Artemisinin content (µg/g) ± SD
A	Field grown plant	
1	Root	00.00 ± 0.00
2	Shoot	79.50 ± 0.01
3	Inflorescence	94.50 ± 0.04
4	Leaves	10.00 ± 0.32
B	*In vitro* plant	
1	Shoots	20.00 ± 0.22
2	Roots	00.00 ± 0.00
C	Transformed shoot	
1	A4	36.00 ± 0.69
2	ATCC 15834	19.00 ± 0.01
3	MTCC 2364	18.00 ± 0.99
D	Transformed root	
1	A4	17.00 ± 0.22
2	MTCC 2364	00.00 ± 0.00
3	ATCC 15834	39.00 ± 0.14
E	Rooted *in vitro* shoot	88.00 ± 0.32

A. Field grown plants



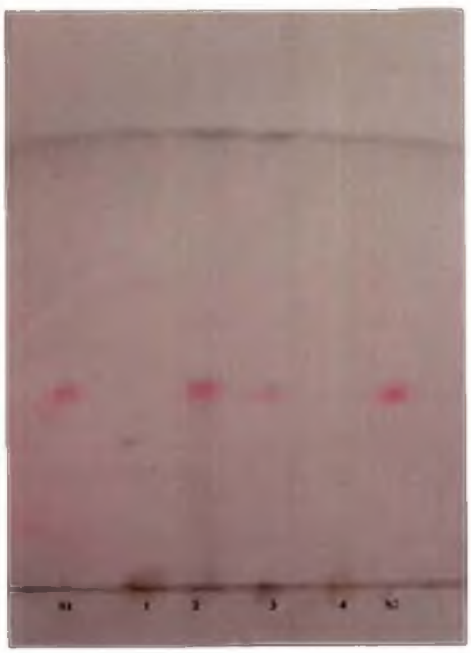
1-Roots, 2-Shoots
3-Inflorescence
4 -Leaves
S1, S2 - Standards

B. *In vitro* plants



1 - Shoot
2 - A4 transformed shoot
3 - ATCC 15834 transformed shoot
4 - MTCC 2364 transformed shoot
S1, S2 - Standards

C. *In vitro* root and hairy roots



1 - *In vitro* root
2 - A4 derived root
3 - ATCC 15834 derived root
4 - MTCC 2364 derived root
S1, S2 - Standards

D. Influence of rooting in increasing artemisinin content

1- *In vitro* shoot
2- Rooted *in vitro* shoot
S1, S2 - Standards

E. Artemisinin content in *in vitro* root, hairy root and roots from plants grown in field

S1, S2 - Standards
1-Root from the plants grown in the field
2- *In vitro* root
3- ATCC 15834 derived root clone

F. Influence of enhancement media on artemisinin content in hairy roots

S1, S2- Standards
1-Control hairy roots
2-HR in *Aspergillus* homogenate media
3- HR in methionine media
4- HR in YE media
5- HR in PEG media

Plate 23b. Thin Layer Chromatograms of various experimental samples (continued

Discussion

5. DISCUSSION

Artemesia annua (L.) commonly known as annual wormwood is a Chinese medicinal plant used for centuries against fever and malaria and is now among the top ten pharmaceutical crops which are the sources of compounds that are being developed as life saving antimalarial drugs (Gupta *et al.*, 1996). Artemisinin, the antimalarial principle isolated from *Artemesia annua* is a sesquiterpenoid.

Artemisinin must be produced from cultivated or collected plants, as its synthesis is complex and commercially unviable. Next to selection of *Artemesia annua* plants, a biotechnological approach has been considered to be an alternative for the production of artemisinin (Pras *et al.*, 1991).

Artemisinin is produced by differentiated shoot cultures and the presence of roots enhanced the production of artemisinin in shoot cultures (Ferreira and Janick, 1996). Hairy root culture systems are more efficient than normal root cultures, because of their genetic and biochemical stability.

The results obtained in the study on "Genetic transformation in *Artemesia annua* L. for hairy root induction and enhancement of secondary metabolites" are discussed in this chapter.

5.1 STANDARDIZATION OF *IN VITRO* REGENERATION

5.1.1 Standardization of surface sterilization

As per the present study, treatment of 0.05 per cent $HgCl_2$ for 10 min or 0.1 per cent $HgCl_2$ for 1 min was found to be optimum for surface sterilization of leaf and inflorescence explants. Increase in treatment time with 0.1 per cent $HgCl_2$ resulted in reduction of establishment of cultures.

This result differs with the findings of Gulati *et al.* (1996) who reported a 30 min treatment of leaves and inflorescence bits with 10 per cent sodium hypochlorite followed by 7 minute treatment of 0.1 per cent $HgCl_2$. This may be due to difference in age and vigour of explants.

5.1.2 Leaf as explants

5.1.2.1 Regeneration

Leaf explant produced callusing and rooting in media with NAA at the concentration of 0.05 to 3 mg l^{-1}. The result is in confirmity with the findings of Nair *et al.* (1986) who reported rooting of leaf segments on modified MS or B_5 media supplemented with IBA or NAA (0.05-2 mg l^{-1}).

Jha *et al.* (1988) reported that the auxins IAA, IBA and NAA (1-2 mg l^{-1}) when used alone induced roots from cut ends of leaves of *A.annua* and did not induce callus formation. Paniego and Giulietti (1994) reported best callusing of leaf explants of *A.annua* in media containing 5.4μM NAA.

In the present study, maximum shoot bud differentiation of leaf explants was obtained on MS with BAP (0.5 mg l^{-1}) alone and in combination of NAA (0.1 mg l^{-1}) with BAP (2 mg l^{-1}).

Leaf segments produced callusing in media containing NAA (0.05 mg l^{-1}) with BAP (0.05-3 mg l^{-1}). This result is in agreement with that obtained by Nair *et al.* (1986) who reported callusing of leaf explant cultured in MS or B_5 media containing 0.05 mg l^{-1}NAA and 0.1 to 0.2 mg l^{-1}BAP.

Martinez and Staba (1988) also reported callusing of leaf explants on modified MS or B_5 media containing 0.02 to 0.05 mg l^{-1} NAA and 0.1 to 0.5 mg l^{-1} BAP. Woerdenbag *et al.* (1993) observed shoot cultures from *A. annua* leaf cultured on medium with 0 to 0.5 mg l^{-1} NAA and 0 to 2 mg l^{-1} BAP.

In the present study, shoot buds were formed from leaf explants cultured on MS media containing 0.1 mg l^{-1} NAA and 0.05-3 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 1 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and 0.5-3 mg l^{-1} BAP, 1 mg l^{-1} NAA and 0.5-2 mg l^{-1} BAP, 2 mg l^{-1} NAA and 3 mg l^{-1} BAP.

5.1.2.1.1 Effect of basal media on regeneration

Among the four basal media (MS, SH, B_5 and 1/2MS) tested, MS medium was the best. Many workers suggested MS nutrient media for *in vitro* micro propagation of *A. annua* (Nair *et al.*, 1986; Martinez and Staba, 1988; Fulzele *et al.*, 1995; Usha and Swamy, 1998).

5.1.2.2 Multiplication

In media containing BAP (0.5 mg l^{-1}) alone 3.6 shoot buds were produced per explant (Table 13). Multiplication of shoot buds was less in media with only NAA though elongation and rooting of shoot buds were observed. Elongation and rooting of shoot buds were absent in media containing BAP alone. This is in agreement with the findings of Ferreira and Janick (1996) who reported that BAP had greatest effect in increasing shoot proliferation but reduced rooting in *A.annua*. According to Muthuvel *et al.* (2005), the increase and decrease in regeneration frequency is attributed to the cytokinin concentration.

5.1.2.3 Elongation

The shoots from leaf explant produced maximum elongation on MS with GA_3 0.2 mg l^{-1}. This is in accordance with the result obtained by Gulati *et al.* (1996) who reported elongation of *A. annua* shoots on a medium supplemented with 0.3μM GA_3.

In the present study, elongation of shoots of *A. annua* was also observed in MS with 0.1 mg l^{-1} BAP and GA_3 (0.2-0.5 mg l^{-1}). Reddy *et al.* (2001) reported elongation of shoots of *Glossocardia bosvallia* on media with GA_3 (0.2-0.5 mg l^{-1}) with BAP (0.1-0.5 mg l^{-1}).

5.1.2.4 Rooting

Rooting of leaf originated shoots was observed to be best in ½ MS with 0.5 mg l^{-1} IBA with 2 per cent sucrose. Medium with low sugar content is used in most cases for rooting of shoots (Roy *et al.*, 1990). Watanabe *et al.* (1991) reported

that root formation in *Asparagus* cultures was reduced with increasing sugar levels. Reddy *et al.* (2001) reported successful rooting of *A. annua* shoots in half strength MS medium without phytohormones. Elongation and rooting of *in vitro* regenerated shoots of *W. somnifera* was reported in half MS with 2.5 per cent sucrose (Varghese, 2006).

In the present study, 70 per cent rooting occurred in MS basal media within 2 weeks. But the roots formed were elongated and very thin. Nair *et al.* (1986) reported that roots were developed from shoots kept on basal media without any growth regulator for more than 3 months. Martinez and Staba (1988) also reported rooting of *A. annua* in MS media without growth regulator and with NAA (3 mg l^{-1}). Jha *et al.* (1988) and Usha and Swamy (1998) reported that the shoots excised from proliferating shoot cultures were rooted on basal medium with IBA (2 mg l^{-1}). In the present study, 100 per cent rooting was observed in MS with IBA (0.1-5 mg l^{-1}). The low concentration of IBA (0.5 or 1 mg l^{-1}) was found to be best for inducing short, thick roots from shoot.

In the present study, addition of activated charcoal in the rooting media induced no positive response. Roots formed were thin and unhealthy. However Varghese (2006) had reported that 0.25 per cent activated charcoal had a promoting effect in rooting of *in vitro* shoots of *W. somnifera*.

The capacity of cultured shoots to develop roots was affected by the culture conditions before, during and after shoot production and selection of correct multiplication medium with a low BAP concentration was important to avoid inhibition of rooting (Patience and Alderson, 1987).

5.1.3 Regeneration from leaf originated callus

Regeneration was obtained from leaf originated callus in media containing BAP alone (0.5-4 mg l^{-1}) with maximum regeneration in MS with 0.5 mg l^{-1} BAP. This is in agreement with the result obtained by Ferreira and Janick (1996) who reported that shoots were induced from callus on media with BAP (4.4 and 44.4 μM).

Paneigo and Giulietti (1994) reported abundant shoots from callus cultured on medium supplemented with 13.32μM BAP alone or in combination with 1.08μM NAA. In the present study also, shoots were produced from callus on medium supplemented with 0.2 mg l^{-1} NAA and 0.5-2 mg l^{-1} BAP.

Jha *et al.* (1988) reported rooting from callus on medium with BAP (0.5 mg l^{-1}) and NAA (2 mg l^{-1}). Callus showed profuse shoot regeneration in 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA. Results of the present study also showed regeneration from callus on media containing 0.5 mg l^{-1} NAA and 1 to 2 mg l^{-1} BAP. Shoot regeneration and root formation from callus was observed in media with 0.5 mg l^{-1} NAA and 2 mg l^{-1} BAP. Rooting of calli was observed in media with NAA (0.5-3 mg l^{-1}). But there was no rooting on medium with NAA (2 mg l^{-1}) and BAP (0.5 mg l^{-1})

5.1.4 Shoot tips and nodal segments as explants

Regeneration was obtained from *in vitro* shoot tips on media containing BAP (0.5-2 mg l^{-1}). This result was in accordance with Jha *et al* (1988) who reported regeneration of shoot tip in media with 2 mg l^{-1} BAP. Regeneration from apical meristem was obtained in media with BAP (2.5 mg l^{-1}).

In the present study, 100 per cent shoot bud regeneration from shoot tip was observed in 0.2 mg l^{-1}BAP. Mean number of shoots per explant was maximum on 0.05 mg l^{-1}NAA and 0.2 mg l^{-1}BAP. Regeneration of shoot tip was also observed in media with 0.05 mg l^{-1} NAA and 0.2 to 3 mg l^{-1} BAP, 0.01 mg l^{-1} NAA and 0.1 to 3 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 0.05 to 3 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and 0.2 to 3 mg l^{-1} BAP, 1 mg l^{-1} NAA and 0.1 to 2 mg l^{-1} BAP.

Inn vitro nodal segments of *A. annua* plants produced regeneration in media with NAA (0.05 mg l^{-1}) and BAP (0.2-3 mg l^{-1}), 0.1 mg l^{-1} NAA and 0.05 to 3 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 0.05 to 2 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and 0.5 to 3 mg l^{-1} BAP, 1mg l^{-1} NAA and 0.2 to 1 mg l^{-1} BAP. Da Silva (2003) reported shoot regeneration from nodal segment of *Artemesia asplenifolia* in media containing BAP (1 mg l^{-1}), IAA (0.1 mg l^{-1}) and GA_3 (0.025 mg l^{-1}).

5.1.4.1 Elongation

Elongation of both shoot tip and nodal segment originated shoots were obtained in MS with GA_3. Varghese (2006) reported elongation of shoot tip and nodal segment originated shoots of *Withania somnifera* in MS with GA_3 (0. 5 mg l^{-1}).

5.1.4.2 Rooting

Shoot tip originated shoots showed 100 per cent rooting where as only 80 per cent rooting were observed in nodal segment originated shoots. Callusing was observed in both explants. Varghese (2006) also reported variation in rooting of shoot buds originated from different explants

5.1.5 Inflorescence and petiole as explants

Shoot regeneration of petiole was observed in media with 0.1 to 0.2 mg l^{-1} NAA and 0.05 to 2 mg l^{-1} BAP. BAP alone (0.05-4 mg l^{-1}) had no effect on regeneration from petiole. In combinations of NAA (1, 2, 3 mg l^{-1}) and BAP (0.05-3 mg l^{-1}) petioles produced green hard callus only. This result was in confirmation with results of Jha *et al.* (1988) who reported callusing of petiole of *A. annua* plants in NAA (1.2mg l^{-1}) and BAP (1 mg l^{-1}).

Gulati *et al.* (1996) reported that petiole of *A. annua* plants produced callus on MS media with 2.5 μM NAA and 9 μM kinetin. In the present study also petiole produced callusing on media with 0.5 mg l^{-1}NAA and kinetin (0.5, 1, 2 mg l^{-1}).

Inflorescence bits produced maximum regeneration in 0.2 mg l^{-1} NAA and 0.5 mg l^{-1}BAP. BAP alone (0.5-2 mg l^{-1}) also produced regeneration. Casein hydrolysate along with BAP (1, 2, 3, 4 mg l^{-1}) in media produced only 10 per cent regeneration and single shoot per explant in inflorescence bits. This is in conformity with the findings of Mathur (1998) who reported that shoot cultures were established from inflorescence segments on MS medium with 1g l^{-1} casein hydrolysate and BAP (1 mg l^{-1}).

As per the results of present study, immature inflorescence is suitable for regeneration. The inflorescence bits failed to regenerate in MS with GA_3 alone (0.05, 0.1, 0.2, 0.5 mg l^{-1}). Gulati *et al.* (1996) reported that immature inflorescence developed shoots from MS media with 0.5 μ*M* NAA, 0.3 μ*M* GA_3 and 13 μ*M* BAP or in MS with 1 μ*M* NAA, 13μ*M* BAP and 2 per cent coconut milk.

In the present study, mature inflorescence failed to produce callus or shoot buds however premature germination of embryo was observed in MS media supplemented with varying concentrations of NAA and BAP. Gulati *et al.* (1996) reported callusing of mature inflorescence on MS media with NAA (0.5 μ*M* NAA), BAP (9 μ*M*) and 0.3 μ*M* GA_3.

5.1.5.1 Multiplication

Shoot buds produced from inflorescence bits and petioles were multiplied in media with BAP alone. Multiplication of shoots was less with NAA and BAP. Increasing the concentration of BAP increased the multiplication of shoot buds from the shoots produced by the petiole whereas multiplication of shoot buds from shoots produced by inflorescence was less in high BAP media. The observations were in accordance with result obtained by Martinez and Staba (1988) who reported that multiple shoots further proliferated in 2.5 mg l^{-1} BAP. Muthuvel *et al.* (2005) reported that increase and decrease in regeneration frequency is attributed to cytokinin concentration.

Maximum elongation of shoots produced from inflorescence and petiole were observed in MS with 0.2mg l^{-1} GA_3. With increase in concentration of GA_3, elongation of shoot buds was also increased. The shoots produced from petiole and inflorescence were rooted on ½ MS with 0.5 mg l^{-1} IBA and 2 per cent sucrose. The shoots derived from petiole rooted but the roots were white and thin. Percentage of rooting varied among explants. This may be due to difference in vigour of shoots formed from different explants.

5.1.6 Roots as explants

The roots of *in vitro* rooted plantlets produced good callusing in all media tested but failed to regenerate shoots. Roots produced cream colured friable callus in NAA (0.05-3 mg l^{-1}) and in combination of NAA (0.05-3mg l^{-1}) and BAP (0.05-3 mg l^{-1}). This result confirmed results of Mukherjee *et al.* (1995) who reported green compact callus from roots of *A. annua* with BAP (1 mg l^{-1}) and NAA (1 mg l^{-1}). Roots of *A. annua* produced callus in media containing 1 mg l^{-1} NAA and BAP (2 mg l^{-1}). Media with 5.4 μ*M* NAA or NAA - BA (5.4 μ*M*-0.22 μ*M*, 5.4 μ*M* - 0.66 μ*M*) produced shoot regeneration from roots (Paniego and Giulietti, 1994).

In the present study green structures were seen at top of cream coloured friable callus formed from root in MS with 0.1 mg l^{-1} NAA and 3 mg l^{-1} BAP. Shoots regeneration from roots of *A. annua* was observed in media with BAP (3 mg l^{-1}) and NAA (0.2 mg l^{-1}) (Da Silva, 2003).

5.2 CULTURING AND SENSITIVITY SCREENING OF *AGROBACTERIUM* AND EXPLANTS

5.2.1 Culturing of *A. rhizogenes* strains

Nutrient agar media and YEB media was found to be optimum for culturing ATCC 15834 and A4 strain and YEM media for MTCC 2364. Same result was obtained by Varghese (2006) in *W. somnifera*. YEB medium was suggested for A4 and ATCC 15834 (Sauerwein *et al.*, 1992; Jaziri *et al.*, 1994; Momocilovic *et al.*, 1997; Chen *et al.*, 1999; Shi and Kintzios, 2003; Yoshimatsu *et al.*, 2003; Xu *et al.*, 2004; Zdravkovic-Korac *et al.*, 2004).

5.2.2 Screening of *A. rhizogenes* strains and explants for antibiotic sensitivity

The strains A4 and ATCC 15834 were susceptible to ampicillin, carbenecillin and cefotaxime. MTCC 2364 was resistant to ampicillin and carbenecillin. Cefotaxime 500 mg l^{-1} killed all strains of bacteria. Same concentration of cefotaxime was used for eliminating bacteria by Koike *et al.* (2003) and Varghese (2006).

All the explants used in the transformation event were found to be healthy at this concentration (500 mg l^{-1}) of cefotaxime. Slight callusing occurred at the basal cut ends of nodal segments, shoot tips and leaf segments in the presence and absence of cefotaxime which may be due to the action of the endogenous auxin at the basal cut ends, which stimulate cell proliferation. Cefotaxime at 500 mg l^{-1} was selected as the antibiotic concentration to kill *A. rhizogenes* strains in several reports (Koike *et al.*, 2003; Zdravkovic-Korac *et al.*, 2004).

5.2.3 Preculturing of explants

The explants were cultured for two days on MS medium without antibiotics prior to transformation thereby preparing the explants for transformation. Yu *et al.* (2001) reported that hairy roots emerged 3-4 days earlier than with usual treatment, if leaf explants of *Pueraria lobata* were precultivated for 2-3 days before transformation with *A. rhizogenes* strain, R 1601.

5.2.4 Wounding of explants

Wounding is a prerequisite for *Agrobacterium* infection. According to Hilderbrand (1934), *A. rhizogenes* has to enter a wound deep enough to reach the phloem region to induce hairy roots on apple trees. A plant cell becomes susceptible to *Agrobacterium* when it is wounded. The wounded cells release phenolic compounds, such as acetosyringone that activate the *vir* region of the bacterial plasmid (Binns and Thomashow, 1988).

In the present study, the hairy roots were induced in and around the wounded regions, which are the sites of *Agrobacterium* infection. The hairy roots were produced from basal region of shoot tips and nodal segments and from petiolar end of leaf.

Sevon and Oksman-Caldentey (2002) reported that the transferred T-DNA derived from the Ri plasmid causes the plant cells to proliferate adventitious roots at or near the site of infection. Zdravkovic-Korac *et al.* (2004) found that hairy roots appeared from the deep wounds of androgenic embryos of *Aesculus hippocastanum*.

5.3 STANDARDISATION OF TRANSFORMATION TECHNIQUES

5.3.1 Influence of bacterial inoculum

The bacterial inoculum used affects the transformation frequencies. In the present study, when the bacterial colonies were used as the inoculum, transformation resulted with all the strains. But when bacterial suspension was used as the inoculum, both A4 strain and ATCC 15834 showed successful transformation in leaf and shoot tip. The strain MTCC 2364 failed to produce transformation in leaf by the suspension method. Transformation was not successful with nodal segment by any strain when bacterial suspension was used.

In general, transformation using bacterial colony was found to be effective than bacterial suspension. Patena *et al.* (1988) reported that bacterial colonies were more effective than bacterial suspension on transformation of Kalanchoe leaves, carrot discs and apple shoot buds.

Jaziri *et al.* (1995) have reported that the hairy root cultures of *A. annua* were established either using bacterial colonies or bacterial suspension. Hairy roots of *A. annua* were produced by infecting leaves with bacterial colony (Mukherjee *et al.*, 1995).

Chen *et al.* (1999) reported successful transformation in *A. annua* using bacterial suspension of A4 strain. They used entire seedling as the explant. Actively growing cultures of A4, ATCC 15834 strains were used for transformation in *A. annua* (Giri *et al.*, 2001).

In the present study, performance of ATCC 15834 by both the method was almost similar. A4 strain showed better transformation when bacterial colony was used. Similar result was obtained by Varghese (2006) who reported better performance of A4 strain when used as single colony for inducing hairy roots in *W. somnifera* compared to bacterial suspension. In practice, the influence of bacterial inoculum on transformation may change among strains, explant used, genotype and co-cultivation period given.

5.3.2 Influence of co-culture period on hairy root induction

The transformation frequency was influenced by the co-culture period. A co- cultivation of more than 2 days reduced transformation efficiency. Co-cultivation for 4 days resulted in yellowing of explant. Sarmento *et al.* (1992) reported that increasing the co-cultivation period might lead to the death of the explant due to the hypersensitive response of the tissue.

The optimum quantity of bacteria was available for transformation after 24 hrs and hence a higher transformation was achieved at this stage. After 36 hrs, the level of bacterial cells reached supra optimum level and competitive inhibition of competent bacterial cells resulted in inhibition of transformation (Karmarkar *et al.*, 2001).

The decrease in transformation rate after three days of co-cultivation may not reflect a decrease in the virulence of *A. rhizogenes* strains, but indicated an overgrowth of bacteria, which killed the explant tissues (Varghese, 2006). Callusing and rooting of the explants may be attributed to the endogenous auxin, as well as to the auxin synthesis directed by T-DNA. Alteration of auxin metabolism in transformed cells has been reported to play an important role in expression of the hairy root phenotype (Gelvin, 1990). The induction of wound callus and transformed roots were correlated with bacterial strain, explant type and wound site. In the present study callusing was less in shoot tip when compared to the leaf and nodal segment. This is in conformity with the findings of Chaudhuri *et al.* (2005). They reported that scanty yellow-green wound callus appeared in some of the explants, infected with either of the bacterial strains (A4 and LBA 9402) as well as in some of the uninfected controls. Callusing was also observed in *W. somnifera* transformed with A4 and ATCC 15834 (Varghese, 2006).

5.3.3 Influence of acetosyringone in hairy root induction

The accumulation of phenolic compounds, such as acetosyringone or those released by cultured cells activate the *vir* genes of Ti plasmid and stimulate

transformation (Binns and Thomashow, 1988). In the present study, acetosyringone at a concentration of 100 μ*M* during co-cultivation in MS media enhanced the transformation efficiency of all strains. Giri *et al.* (2001) reported that bacterial cultures induced with acetosyringone and explants co-cultivated on MS basal media with acetosyringone reduced the time of hairy root induction by a week.

In the present study, acetosyringone enhanced the transformation percentages with A4 strain in leaf, shoot tip and nodal segment. This is in conformity with the findings of Zdravkovic -Korac *et al.* (2004) who reported that the presence of 50 μ*M* acetosyringone in the co-cultivation media significantly increased the number of putative transformants using A4GUS in *Aesculus hippocastanum*. The addition of acetosyringone in co-cultivation media might have resulted in the activation of *vir* genes and thereby increased the virulence of A4 strain. Transformation by A4 was increased in the presence of acetosyringone in *W. somnifera* (Varghese, 2006).

In the present study, transformation per cent by ATCC 15834 was also increased in the presence of acetosyringone. This is contradictory to the findings of Varghese (2006) who reported reduction in transformation of *W. somnifera* using ATCC 15834 in presence of acetosyringone. Pawlicki (1992) also reported reduction in transformation in the presence of acetosyringone.

Addition of acetosyringone in the bacterial suspension was found to have a positive effect on transformation response. This is in agreement with the result of Chaudhuri *et al.* (2005) and Tsuro *et al.* (2005) who reported the addition of acetosyringone in the bacterial suspension A4 and A13 respectively for improving the transformation efficiency. The observation of present study was contradictory to findings of Varghese (2006) who reported negative effect on transformation by bacterial suspension in the presence of acetosyringone.

5.3.4 Standardization of explants for efficient transformation

In the present study, shoot tip of *A. annua* was found to be the best explant for efficient transformation followed by leaf segment. Leaf of *A.annua* had been used

by many workers for inducing hairy roots (Mukherjee *et al.*, 1995; Jaziri *et al.*, 1995; Chen *et al.*, 1999; Wang *et al.*, 2004).

Giri *et al.* (2001) used leaf, petiole and shoot tip of *A.annua* for hairy root induction. Shoot tip was used as explant in *Panax ginseng* (Koe *et al.*, 1989) and in *Plumbago rosea* (Komaraiah *et al.*, 2002) using ATCC 15834 strain. Root induction efficiency was influenced by type of explant and *Agrobacterium* strains (Xu *et al.*, 2004).

The age, differentiation status of plant tissue and plant type can also affect the chances of successful transformation. The level of tissue differentiation also determines the ability to give rise to transformed roots after *A. rhizogenes* inoculation (Trypsteen *et al.*, 1991). The leaf segement and shoot tips of *W. somnifera* showed efficient transformaion (Varghese, 2006).

The explant cells differ in their DNA synthesis and cell division ability due to the difference in the physiological maturity of the cells (Karmarkar, 2001). Proliferation of hairy roots without intervening callus was reported in *Camptotheca acuminata* with *A. rhizogenes* strains ATCC 15834 and R-1000 from wounded hypocotyls and true leaves (Lorence *et al.*, 2004). Similar results have been reported in *Isatis indigotica* with A4, R 1601 and ATCC 15834 strains from cotyledon and hypocotyl explants (Xu *et al.*, 2004).

In the present study, hairy roots were induced directly from the explant and proliferation of hairy roots via callus was rare. Normal rooting and callusing was also observed from explants. Giri *et al.* (2001) reported that leaf segment of *A. annua* is best suited for hairy root induction since chance of normal root production from leaf is less.

Normal rooting and callusing of the control explants in growth regulator free MS medium indicated the presence of endogenous auxins in the cells even after preculturing. The transformed explants also produced normal roots along with hairy roots in *W. somnifera* (Varghese, 2006). The occurrence of normal rooting from

infected explants was also reported by Karmarkar (2001) in *Holostemma ada-kodien*, whereas Chaudhuri *et al.* (2005) reported that roots never appeared on the control explants inoculated with LBA 9402 in *Tylophora indica*.

5.3.5 Efficiency of strains in inducing hairy roots

A4, ATCC 15834 and MTCC 2364 produced transformation in *A.annua*. Efficiency of strains in inducing hairy roots is in the order of ATCC 15834, A4 and MTCC 2364. The best root formation frequency in *A. annua* was achieved with *A. rhizogenes* in the following order LBA 9402, 9340, 9365, 15834 and A4 (Giri *et al.*2001). ATCC 15834 induced hairy roots in *A. annua* (Liu *et al.*, 1998 and Chen *et al.*, 1999). A4 and 8196 strains were used to induce transformation in *A. annua* (Cai *et al.*, 1993; Mukhrjee *et al.*, 1995 and Jaziri *et al.*, 1995).

Significant differences were observed between the transformation ability of different strains of *Agrobacterium*. The agropine-type Ri plasmids are considered to be the most virulent and therefore more often used in the establishment of hairy root cultures (Sevon and Oksman-Caldentey, 2002).

In *W. somnifera*, ATCC 15834 showed the highest efficiency in transforming plant tissues, followed by A4. The strain MTCC 2364 however failed to produce any successful transformation (Varghese, 2006). In the present study also ATCC 15834 showed highest efficiency than A4. MTCC 2364 also produced transformation. Pawar and Maheshwari (2004) reported transformation in *W. somnifera* using MTCC 2364 and ATCC 15834.

Agrobacterium rhizogenes strain A4 was significantly better than strains R1601 and ATCC 15834 for inducing roots in *Isatis indigotica* (Xu *et al.*, 2004). Rhodes *et al.* (1989) reported that the agropine strains (15834, A4, TR-7 etc.) have wide host range that is attributed to the presence of TR-DNA fragment of the T-DNA. Spencer and Towers (1989) reported that different wide host range *A. rhizogenes* strains like 15834 and A4 are more sensitive to the wound induced compounds than the limited host range strains like 8196 and 2659.

The strain ATCC 15834 induced 40 to 45 per cent transformation of *Campotheca acuminata* when compared to R-100 (Lorence *et al.*, 2004). Maximum hyoscyamine was detected in hairy roots of *Hyoscyamus albus* transformed with A4 when compared to ATCC 15834 (Sauerwein *et al.*, 1992). Hu and Alfermann (1993)reported LBA 9402 strain caused 85 per cent rooting in the leaf explants of *Salvia miltiorrhiza* when compared to A_4 (10%) and ATCC 15834 (20%). Xu *et al.* (2004) reported that the strain A_4 was better when compared to R1601 and ATCC 15834 in root induction efficiency from *Isatis indigotica* explants.

It has been reported that virulence of *Agrobacterium* strains varies among plants (Hobbs *et al.*, 1989; Bush and Pueppke, 1991) and that transformation efficiency of host species varies among different bacterial strain (Godwin *et al.*, 1991; Hu and Alfermann 1993). Among the five *A.rhizogenes* strains evaluated PcA4, 15834 and A4 induced hairy roots in *Holostemma adkodien* whereas 8196, 2659 did not induce hairy roots at all (Karmarkar and Keshavachandran, 2005).

5.3.6 Number of days for root induction

Hairy root induction was achieved in a time period of 1 to 4 weeks in majority of the plant species. In the present study almost 6 to 28 days (1-4 weeks) was taken for hairy root induction from leaf and shoot tip explants irrespective of the method of inoculation. The hairy roots developed 1 to 3 weeks after in *W. somnifera* (Varghese, 2006), and 4 to 10 weeks after infection in *Camptotheca acuminata* (Lorence *et al.*, 2004). With acetosyringone, number of days for hairy root induction was reduced to 1 to 2 weeks. In the present study also, acetosyringone treatment reduced the number of days for hairy root induction.

5.3.7 Establishment of hairy root cultures

The hairy roots showed fast growth and reduced geotropism. In the present study some hairy root clones showed faster growth while some showed slow growth.

Sevon and Oksman- Caldentey (2002) had reported that there is difference in growth of hairy roots among different species and between different root clones of the same species. Xu *et al.* (2004) reported that high level of lateral branching is due to the presence of many lateral root tips resulting in high growth rate. Hairy roots are having high degree of cell division (Bapat and Ganapathi, 2005).

In the present study, control roots showed no lateral branching. This is in agreement with Shi and Kintzios (2003) who reported that control roots did not branch and perished after 2 to 3 subcultures. Chaudhuri *et al.* (2005) and Varghese (2006) also reported absence of lateral branching in control roots.

5.3.8 Rapid culturing of hairy roots

Compared to normal roots and hairy roots induced by other strains, hairy roots of A4 strain showed faster growth producing more biomass. The growth of hairy roots was influenced by the strain used in hairy root induction. This is in agreement with Oksman-Caldenty (2002) who reported that *A. rhizogenes* strain could also have effect on biomass and alkaloid productivity of hairy roots.

Growth of hairy roots in liquid culture was faster than in solid media. The same result was obtained by Shi and Kintzios (2003), Chaudhuri *et al.* (2005) and Varghese (2006). Hairy roots score over normal roots in their ability to grow without hormones (Bapat and Ganapathi, 2005).

5.3.9 Effect of culture media and conditions on the growth of hairy roots

In the present study, B_5 with 3.0 per cent sucrose was found to be superior for promoting hairy root growth followed by B_5 with 2.0 per cent sucrose, MS and half MS. Culture medium, thus has a significant effect on hairy root growth. This is in conformity with the findings of Xu *et al.* (2004) who reported that MS and half MS are superior to B_5 and White's media for the growth of hairy roots of *Isatis indigotica.*

Kukreja *et al.* (1997) reported that biomass and growth of hairy root depend on the culture medium. The hairy roots of different species behave differently in the same culture conditions. Individual hairy root clones can also have different optimum concentration of sucrose or mineral ions (Oksman -Caldenty *et al.*, 1994). Half MS medium with 3 per cent sucrose was found to be superior for promoting hairy roots in *Withania somnifera* (Varghese, 2006).

In the present study, B_5 was found to be superior for inducing hairy roots of *A. annua*. Jaziri *et al.* (1995) reported that growth of hairy roots of *A. annua* was superior in both MS and B_5 media but highest artemisinin content/flask was observed in MS medium, which was 8 times higher than ½ MS medium. MS medium wns selected as the basal medium by many investigators for culturing *A. annua* hairy roots (Qin *et al.*, 1994; Cai *et al.*, 1995; Liu *et al.*, 1999 and Wang *et al.*, 2001).

The result obtained in the present study was in conformity with the findings of Weathers and Kim (2001) and Weathers *et al.* (2004) who reported B_5 media for the rapid growth of *A. annua* hairy root. Giri *et al.* (2001) reported fastest growth of *A. annua* hairy root in ¼ MS medium.

In the present study, 3 per cent sucrose was found optimum for hairy root growth than 2 per cent. Similar results were reported by Cai *et al.* (1995).

The addition of growth regulators 2, 4-D, IAA, NAA and IBA in hairy root culture reduced the biomass and artemisinin content where as GA_3 increased the biomass and artemisinin content (Liu *et al.*, 1997). Weathers *et al.* (2004) reported the highest biomass of *A. annua* hairy root cultures in media supplemented with ABA. 6-benzyl amine and 2-isopentenyl adenine inhibited root growth. Temperature is also having an influence on root growth. Maximum growth of *A. annua* hairy root cultures was found at 25°C (Chen *et al.*, 2004).

5.3.10 Growth pattern of hairy roots

The growth of hairy roots induced by ATCC 15834 showed stationary phase for one week. Then the growth increased at a faster rate for 2 to 3 weeks. After 25 days there was no growth and all roots turned brown.

Liu *et al.* (1998) reported that optimum time for culture of hairy roots was 21 days. The result of present study is in accordance with the findings of Babaoglu *et al.* (2004) who reported that the hairy roots induced in *Lupinus mutabilis* exhibited a sigmoid growth pattern with lag (0 to 5 days), exponential (5 to 15 days) and stationary phases.

The hairy roots of *Isatis indigotica* grew fast and showed a S shape growth curve and reached its peak on the 24th day (Xu *et al.*, 2004). Varghese (2006) reported a sigmoid growth pattern in the hairy root growth of *W. somnifera*. The hairy roots originally white, gradually changed colour to brown and the biomass began to decrease, owing to depletion of nutrients in the liquid medium and lysis of cells. A similar phenomenon was also observed in the hairy root cultures of *Isatis indigotica* (Xu *et al.*, 2004).

5.4 CONFIRMATION OF TRANSFORMATION

5.4.1 Morphology of hairy roots

Callusing and lateral branching was observed in hairy roots. Hairy nature of root was also observed during initial stages. The hairy roots were creamy white in colour and later turned brown. No shoot regeneration was found from hairy roots. These observations were in accordance with the findings of Mukherjee *et al.* (1995) who reported that the hairy roots of *A. annua* turned brown if not sub cultured and there was no shoot regeneration.

Liu *et al.* (2002) reported that original white hairy roots of *A. annua* turned brown due to decrease in nutrients of media. According to Giri *et al.* (2001) the hairy roots of *A. annua* induced by A4 and ATCC 15834 turned green when exposed to light. No such change was observed in the present study.

The hairy roots induced by A4 showed callusing. Lateral branching and hairy nature of root were also observed. The roots were thin and white initially. The 15834 roots were greenish and long and it was difficult to remove the bacterial growth (Vanhala *et al.*, 1995). But in the present study, the hairy roots induced by ATCC 15834 were white in colour. It was difficult to remove the bacterial growth from hairy roots induced by MTCC 2364.

The hairy roots show morphological variation depending upon the interaction nature of plant cell phenotype and strain of the bacterium and show difference in root thickness, degree of branching and amount of hairy root production (Bapat and Ganapathi, 2005). The strains A4, ATCC 15834 and pCA4 induced hairy roots of *Holostemma ada-kodien,* that were whitish in colour and showed negative geotropic growth (Karmarkar and Keshavachandran, 2005).

Callusing was very less in hairy roots of MTCC 2364. A4 roots produced more callusing and laterals than roots of other strains. Varghese (2006) reported that hairy roots induced by ATCC 15834 were thick with high root hairs compared to that of A4 root. The difference in expression of hairy root may be due to presence of the TR and / or TL-DNA in a different copy number. There may be differences in the length of the T-DNA of Ri plasmid that get stably integrated into the plant genome bringing out differences in the morphological features (Merlo *et al.*, 1980).

5.4.2 Opine analysis

The induction of rhizogenesis and opine formation following infection is a consequence of transfer of DNA from bacterial plasmid into host cell and its integration into the plant genome (Rhodes *et al.*, 1990). In the present study, opines extracted from A4, ATCC 15834 and MTCC 2364 induced hairy roots produced spots corresponding to agropine. Control roots produced no spot. Also no spot was produced at positions corresponding to mannopine and mannopinic acid. Jaziri *et al.* (1995) and Giri *et al.* (2001) confirmed genetic transformation in *A.annua*. In the present study A4, ATCC 15834 and MTCC 2364 opines produced spots corresponding to agropine. Transformation in *H. ada-kodien* was confirmed by opine analysis. The roots induced

by all strains A4, ATCC 15834 and pCA4 showed the presence of agropine (Karmarkar, 2001).

Opine production can however be unstable in hairy roots and may disappear after few passages (Godwin *et al.*, 1991). For this reason, detection of T-DNA by PCR or Southern hybridization is necessary to confirm genetic transformation. Varghese (2006) reported the presence of spots corresponding to agropine in both transformed and control roots. Confirmation was unsuccessful in this case due to interfering substances that show positive reaction to silver staining.

5.4.3 Confirmation by PCR analysis

PCR analysis of hairy roots was carried out for the confirmation of transformation. The genomic DNA isolated from hairy roots and normal roots as well as the cosmid was amplified with *rol B* primer. The fragment of length 205bp was amplified only from hairy roots and cosmid pLJ_1 and not from untransformed roots. These results indicated that *rol B* gene from the Ri plasmid of *A. rhizogenes* were successfully integrated into the genome of *A. annua* hairy roots.

The *rol B* gene plays a major role in root induction and the *rol C* gene product confers optimal growth capacity to newly transformed roots (Lee *et al.*, 2004). The confirmation of transformation using PCR analysis for detecting the presence of *rol* genes was reported in many species like *Pueraria phaseoloides* (Shi and Kintzios *et al.*, 2003), *Tylophora indica* (Chaudhuri *et al.*, 2005) and *Withania somnifera* (Varghese, 2006).

5.4.4 Confirmation by Southern hybridization

Southern (1975) reported the method for detecting DNA fragments in an agarose gel by blotting on a nylon or nitrocellulose membrane followed by detection with a probe of complementary DNA or RNA sequence.

The DNA from hairy roots induced by A4, ATCC 15834 , MTCC 2364 strains and control roots were subjected to restriction digestion using *Bam* HI

restriction endonuclease prior to blotting. The amplified products corresponding to *rol* B gene obtained by PCR reaction using Rol BF2R2 primer was used as the probe.

There was no signal detected after development of film. Since Paper electrophoresis and PCR amplification gave the confirmation, the absence of signal would not be due to non transformed nature. After blotting the gel was stained and there were no bands in the gel. Hence there was complete transfer of restricted bands to the membrane. The absence of signal may be due to low activity or may be due to low DNA binding capacity of the nitrocellulose filter.

James (2001) also reported absence of signal in Southern hybridization. Varghese (2006) obtained bands of in Southern hybridization even though PCR amplification was obtained. Southern hybridization by detecting the presence of T-DNA for confirming the transformation was carried out in *Aesculus hippocastanum* (Zdravkovic-Korac *et al.*, 2004) *Gentiana species* (Momocilovic *et al.*, 1997) *Taraxacum platycarpum* (Lee *et al.*, 2004).

5.4.5 Dot blot analysis

The major applications of this technique are (i) rapid detection of specific sequences, and (ii) determination of the relative amounts of any given species or sequences of RNA or DNA in a complex sample. As the sample is normally applied in a circular form, on exposure of the membrane filter to detection procedure, it will be visualized as a dot (Chawla, 2002).

The double stranded DNA does not bind efficiently to the filters, hence the denaturation treatment facilitated the efficient binding of DNA to the membrane in single stranded form. There was no spot produced in the photographic plate. It may be due to low activity or low DNA binding ability of nitrocellulose membrane. Varghese (2006) reported a dark spot corresponding to transformed hairy root DNA and to the probe which confirmed the presence of *rol* B genes (TL- DNA) in hairy roots transformed by A4 and ATCC 15834.

5.5 ARTEMISININ CONTENT OF PLANT MATERIALS

Artemisinin is a sesquiterpenoid lactone present in shoots of *A. annua* plant. No other organism is known to synthesise artemisinin.

A variety of procedures for the estimation of sesquiterpenes of *A.annua* have been reported. Various techniques have been applied for the estimation of artemisinin in plant material including TLC densitometry (Gupta *et al.*, 1996), HPLC (Charles *et al.*, 1990), electrochemical methods (Acton *et al.*, 1985) gas chromatography (Sipahimalani *et al.*, 1991) and ELISA (Jaziri *et al.*, 1993).

In the present study, thin layer chromatographic method was employed for the estimation of artemisinin present in the different samples due to its simplicity, accuracy and low cost over HPLC.

Artemisinin in samples were extracted with n-hexane. The use of this extractant for the extraction of artemisinin from *A. annua* are reported by several workers (Gupta *et al.*, 1996; Gupta *et al.*, 1997 and Kawamato *et al.*, 1999). Transformed roots of *A.annua* were extracted with n- hexane (Giri *et al.*, 2001). Alternatively petroleum ether was also used for extraction (Klayman *et al.*, 1984 and Tang *et al.*, 2000). Jaziri *et al.* (1995). Wallart *et al.* (2000) used methanol for extraction from hairy roots whereas toluene was used by Smith *et al.* (1997) and Weathers *et al.* (2004).

In the present study, culture media of hairy roots were also extracted with n-hexane. Liu *et al.* (1992) extracted culture medium with n-hexane for TLC detection of artemisinin.

Silica gel $_{60}$ F254 plates were used for the present study together with n-hexane: diethyl ether (1:1) as solvent system as reported by Gupta *et al.* (1996).

In the present study, artemisinin was detected in the inflorescence, leaves and shoots. No artemisinin was detected in roots. Liu *et al.* (1996) isolated artemisinin from leaves and inflorescence of *A. annua*. Artemisinin has been reported in green

stems, buds, leaves, flowers and seeds (Ferreira *et al.*, 1995; Leirsch *et al.*, 1986). Artemisinin was not detected in roots (Ferreira *et al.*, 1995).

From the results obtained in the present study, the artemisinin content in field plant vary from 0 to 0.009 per cent on fresh weight basis. The highest artemisinin content was found in inflorescence. Artemisinin production by *A.annua* is usually in the range of 0.01 to 0.4 per cent (Acton *et al.*, 1985). Although some authors have reported artemisinin content being highest during preflowering stages (Acton *et al.*, 1985; Leirsch *et al.*, 1986; El-Sohly 1990; Woerdenbag *et al.*, 1991; Woerdenbag *et al.*, 1994), others have reported artemisinin reaching its peak during full flowering (Singh *et al.*, 1988; Pras *et al.*, 1991; Morales *et al.*, 1993; Ferreira *et al.*, 1995; Laughlin, 1995).

Reports on distribution of artemisinin throughout the plant are inconsistent. Artemisinin is reported to be higher at the top of the plant in some clones (Charles *et al.*, 1990; Laughlin, 1995) and equally distributed in others (Laughlin, 1995). Artemisinin content in full bloom flowers were 4 to 5 times higher than in leaves (Ferreira *et al.*, 1995).

In temperate climate of the Kashmir valley, the average artemisinin content in leaves was reported to be 0.1 per cent DW while the leaves collected from subtropical climate of Lucknow was 0.06 per cent DW (Singh *et al.*, 1986). In the present study content of artemisinin in leaf is 0.001 per cent FW.

In the study, artemisinin was detected in *in vitro* shoot. Rooting of *in vitro* shoots enhanced the artemisinin production in shoot (from 0.002 to 0.0088 per cent FW). This is in conformity with the findings of Ferreira and Janick (1996) who presented that artemisinin production in shoots was enhanced by the presence of roots. The highest level of artemisinin (0.287 %DW) was obtained in hormone free medium when root production was maximized. Martinez and Staba (1988) reported an increase in artemisinin content *in vitro* when plants developed a root system.

Most workers (Martinez and Staba, 1988; Tawfiq *et al.*, 1989, Fulzele *et al.*, 1995; Kim *et al.*, 1992) did not detect artemisinin in roots, except Nair *et al.* (1986) and Jha *et al.* (1988) who detected trace amounts. It was generalized that low yield of artemisinin are produced from roots and rooted plantlets derived from leaf explants or callus cultures (Nair *et al.*, 1986). In the present study also artemisinin was not detected in *in vitro* as well as field roots.

The role of roots in increasing artemisinin in shoot is unclear. The possibility that roots may provide a precursor or promoter of artemisinin synthesis that is transported to shoots warrants study. Ferreira and Janick (1996) reported that specialized glands require roots to reach their full potential for artemisinin biosynthesis. Martinez and Staba (1988) reported presence of artemisinin in various *A. annua* cultures but its concentration is more than five hundred times lower than in plants grown in the field.

Artemisinin content in transformed roots of *A. annua* varies during growth and ageing of *in vitro* cultures (Weathers *et al.*, 1996). No artemisinin was detected in different root clones of *A. annua* hairy root cultures or transformed rhizogenic calli. Calli derived from hairy roots are incapable of synthesizing artemisinin (Mukherjee *et al.*, 1995). Its level in hairy roots transformed with *A. annua* was low and unstable ranging from zero to 1.9 mg/g (Weathers *et al.*, 1994; Cai *et al.*, 1995; Paneigo and Giulietti, 1994).

Giri *et al.* (2001) reported that artemisinin content was closely related to the growth of hairy root. Jaziri *et al.* (1995) observed artemesinin content 0.001 per cent DW in hairy roots transformed with *A. rhizogenes* MAFF 03-01724 or NCIB 8196. In the present study, artemisinin was detected from hairy roots induced by A4 and ATCC 15834 strains.

5.6 ENHANCEMENT OF SECONDARY METABOLITE PRODUCTION

5.6.1 Addition of osmoregulants

The addition of osmoregulant PEG (2.0 % and 5.0 %) failed to elicit a positive influence in the growth of *A. annua* hairy root and artemisinin biosynthesis. The result was in accordance with the findings of Varghese (2006) who reported that PEG did not affect the biosynthesis of withaferin in *W. somnifera*. Sindhu (1999) also reported that none of osmoregulant added to the basal growth media such as PEG, mannitol and sorbitol (1.5-3%) could sustain callus growth and berberine synthesis. Contrary to these results, Sankar (1998) reported that the osmoregulant PEG at 2.0 per cent elicited a positive response in leaf calli of *Sida cordifolia* on biosynthesis of ephedrine.

5.6.2 Addition of precursors

In the present study, the addition of precursor, methionine at 1.0 m*M* was found to be ineffective in influencing the growth of hairy root and artemisinin production. Varghese (2006) reported that the addition of 1.0 m*M* and 2.0 m*M* methionine was found to be ineffective in increasing the withanolide production of hairy root cultures. However, the biomass of the roots increased progressively in this culture medium. The low incorporation obtained with methionine may be explained considering that this precursor is involved in a large number of biosynthetic pathways many of which are probably more active than those leading to withanolides (Veleiro *et al.*, 1985).

The addition of casein hydrolysate, a source of amino acids to shoot cultures of *A.annua* increased the artemisinin content (Abdin *et al.*, 2003). Wordenbag *et al.* (1993) reported that the addition of mevalonic acid lactone negatively influenced the growth and artemisinin content. The precursors of artemisinin when added to tissue culture medium resulted in four fold increase of artemisinin in tissue and eleven fold increase of artemisinin in spent medium (Weathers *et al.*, 1994).

5.6.3 Addition of elicitors

5.6.3.1 Addition of Aspergillus homogenate

In the present study, *Aspergillus* homogenate at the rate of 250 µl/ 125ml elicited a positive response on biosynthesis of artemisinin in ATCC 15834 hairy root. No artemisinin was detected in elicited culture media. This result was in conformity with the findings of Vazquez-Flota *et al.* (1994) who reported that the use of *Aspergillus* homogenate produced an increase in both the accumulation and yield of ajmalicine in *Catharanthus roseus* hairy root cultures.

The response of plant cells and tissues to elicitation with fungal homogenates is in direct relation with the composition of the cell wall of the fungi, where their different components are the true elicitors. Some types of cellulose-glucans are better elicitors than chitin-glucan (Kombrink and Hahlbrock, 1986).

The elicitors may act as hormones (in that they trigger a metabolic cascade) regulating secondary metabolism in those plant cells which are able to receive, decode and further modulate the molecular signal. The treatment of cultured plant cells with fungal elicitors can alter gene expression for secondary metabolite synthesis (DiCosmo and Misawa, 1985).

Sevon and Oksman-Caldentey (2002) reported that the elicitors increase the cell permeability. Enhancement of cell permeability may increase the formation of secondary products, because feed back inhibition and intracellular degradation of the products decrease.In the present study, no artemisinin was released to medium. Contrary to this, Varghese (2006) reported release of withaferin to culture medium.

Biotic elicitors have been reported to release the products from hairy roots into the medium without any loss of viability and production capacity of the hairy roots (Sevon and Oksman-Caldentey, 2002). Nef *et al.* (1991) reported that low concentration of the fungal homogenates induced the liberation of 90 per cent of the total ajmalicine into the medium.

The hairy root cultures were exposed to *Aspergillus* homogenate for 72 hrs to study the influence on secondary metabolite production. The maximum artemisinin accumulation was obtained at 48 hrs of elicitation with 5 ml homogenate of *Aspergillus oryzae* cultures (Liu *et al.*, 1997). DiCosmo and Misawa (1985) reported that in elicitor: plant cell culture systems, the elicitation (induction) and accumulation of antibiotic secondary metabolites shows rapid kinetics, with maximum levels often elicited within 48-72 hrs after exposure to elicitor.

Liu *et al.* (1997) reported that, homogenate of *Aspergillus oryzae* (5 ml per flask) elicited artemisinin accumulation after 48 hrs. Wordenbag *et al.* (1993) reported that the addition of elicitors such as cellulose, chitosan and nigeran from *Aspergillus japonicus* negatively influenced tha artemisinin content in shoot cultures of *A. annua.*

5.6.3.2 Addition of Yeast extract

Addition of yeast extract had no effect in hairy roots induced by ATCC 15834. There was no artemisinin in media and the growth of hairy root was not at all influenced. The hairy root cultures showed reduction in the accumulation of withaferin A content on adding yeast extract at the rate of 2.5 and 5.0 g l^{-1}(Varghese, 2006).

Linsafors *et al.* (1989) observed that treatment of *Panax ginseng* tissue cultures with yeast glucans and chitins did not stimulate accumulation of ginsenoides. The use of yeast (*Rhodotorula marina*) failed to elicit the accumulation and yield of alkaloids in *Catharanthus roseus* hairy root cultures (Vazquez- Flota *et al.*, 1994).

DiCosmo and Misawa (1985) reported that low-molecular weight products from certain fungi could inhibit elicitation activity by a process, which may involve competition for membrane receptor sites. Specific fungal molecules can therefore suppress plant cell metabolism to synthesize antibiotic secondary metabolite. Wibberley *et al.* (1994) reported that with the use of yeast extract elicited the accumulation of sesquiterpene phytoalexins (capsidol and debneyol) in *Nicotiana tabacum.*

Summary

SUMMARY

The present study entitled "Genetic transformation in *Artemesia annua* L. for hairy root induction and enhancement of secondary metabolites was carried out at the Centre for Plant Biotechnology and Molecular Biology of the College of Horticulture, Vellanikkara. The salient findings of the study are stated below:

1. Surface sterilization of leaf and inflorescence segment with 0.1 per cent $HgCl_2$ for 1 min was found to be optimum for culture establishment.
2. Maximum regeneration of leaf segments (90%) was obtained on MS medium with 0.5 mg l^{-1} BAP alone or on 0.1 mg l^{-1} NAA and 2 mg l^{-1} BAP. Shoot buds were produced directly from leaf as well as from callus. NAA alone produced direct rooting from leaf. Among the different basal media tested, regeneration of shoot buds were more on MS basal medium than compared to SH or B_5.
3. Among the different combinations tested, multiplication of leaf derived shoot buds were more on MS medium supplemented with 0.5 mg l^{-1} BAP and elongation of differentiated shoots were found to be very less.
4. Maximum elongation of leaf derived shoot was observed on MS medium supplemented with GA_3 0.2 mg l^{-1}.
5. Rooting of leaf originated shoots were found optimum in 1/2 MS media with 0.5 mg l^{-1} IBA and 2 per cent sucrose.
6. Leaf originated callus produced regeneration in MS with zero to 4 mg l^{-1} BAP alone and maximum regeneration was in MS with 3 mg l^{-1} BAP. Callus produced both shoot and root in MS media with 0.5 mg l^{-1} NAA and 2 mg l^{-1} BAP.
7. Maximum regeneration from shoot tips was observed in MS with 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} BAP. Shoot tips produced regeneration in media with BAP alone (0.5-2 mg l^{-1}).
8. Nodal segments produced shoot buds in almost all media where shoot tips were regenerated. Maximum regeneration of nodal segments was found in 0.1 mg l^{-1} NAA and 0.2 mg l^{-1} BAP.

9. Shoot buds originated from shoot tips and nodal segments were elongated in GA_3 media. Maximum elongation was found in MS with 0.2 mg l^{-1} GA_3. Elongation of shoot buds originated from shoot tips were more than that of shoot buds originated from nodal segments.
10. Regeneration was also obtained from inflorescence bits and petiole. Petiole produced maximum regeneration in media with 0.1 mg l^{-1} NAA and 0.1 or 0.2 mg l^{-1} BAP. Inflorescence bits showed maximum regeneration in media with 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} BAP. Inflorescence bits also produced regeneration in MS media containing BAP whereas shoot buds were not regenerated from petiole bits placed in media with BAP alone.
11. Shoot buds originated from inflorescence and petiole were multiplied in BAP (0.5-2 mgl^{-1}). Increasing the concentration of BAP increased the multiplication of shoot buds from the shoots produced by the petiole whereas multiplication of shoot buds from shoots produced by inflorescence was less in high BAP media.
12. Maximum elongation of shoot buds originated from petiole and inflorescence was in MS media with 0.2 mg l^{-1} GA_3.
13. Shoot buds originated from petiole and inflorescence were rooted in 1/2MS with 0.5 mg l^{-1} IBA and 2 per cent sucrose.
14. The *in vitro* root showed good callusing tendency but very less regeneration tendency. However, green coloured structures were obtained from root callus in MS + BAP 3mg l^{-1} and NAA 0.1 mg l^{-1}.
15. Among the different antibiotics tested, cefotaxime (500 mg l^{-1}) was found to be effective for the elimination of *A. rhizogenes* strains from the explant tissues.
16. Among the three *Agrobacterium rhizogenes* strains tested, all strains produced hairy roots in *A. annua*. ATCC 15834 was the most efficient in inducing hairy roots compared to other two.
17. Among the various explants tested, the shoot tips showed maximum efficient transformation (70%).

18. The bacterial concentration or the type of inoculum affected the transformation frequencies. The bacterial cells from single cell colonies was found to be more effective than using the bacterial suspension.
19. The transformation frequency was influenced by the co-culture period. Co-cultivation for more than 2 days reduced transformation. All explants showed yellowing when they were co-cultivated for 4 days.
20. Callusing and normal rooting also occurred from transformed explants along with hairy root induction. Callusing was less in shoot tip.
21. The presence of 100 μM acetosyringone in the co-culturing media, increased the number of putative transformants with all strains.
22. A maximum of four hairy roots were produced per single leaf segment and the shoot tips produced a maximum of seven hairy roots per single explant.
23. More hairy roots were induced from the petiolar region of leaf segments and they were produced directly from the leaf. From the shoot tips, hairy roots were produced from and around the basal portion only and no roots developed from the leaves attached to shoots.
24. Among the root cultures, some root lines showed faster growth with high lateral branching whereas some hairy roots showed only slower growth. The hairy roots normally showed a reduced geotropism. The colour of the hairy roots gradually changed from white to cream and later to brown almost 25 to 28 days after incubation.
25. Control roots grew slowly compared to transformed roots and they showed no lateral branching.
26. Compared to normal roots and hairy roots induced by other strains, A4 induced hairy roots showed faster growth producing more biomass.
27. In comparison with roots in solid cultures, hairy roots cultured in liquid growth regulator free medium grew rapidly with high lateral branching.
28. Among the four liquid media tested, B_5 with 3.0 per cent sucrose was found to be superior for promoting hairy roots in *A.annua* followed by B_5 with 2.0 per cent sucrose, MS and half MS and respectively.
29. The hairy roots exhibited a sigmoid growth pattern.

30. The roots induced by ATCC 15834 and A4 were thin and white. A4 roots showed callusing. It was difficult to remove the excess bacteria from the hairy roots induced by MTCC 2364.
31. Confirmation of transformation was done by opine detection using high-voltage electrophoresis. Opines from all hairy roots showed spots corresponding to agropine.
32. Genomic DNA could be isolated from *A. annua* using modified CTAB method.
33. The Polymerase Chain Reaction confirmed the presence of *rol* B gene of TL-DNA in the hairy roots.
34. Confirmation of transformation by Southern hybridization and Dot blot analysis was found unsuccessful.
35. Thin Layer Chromatography method can be employed for the estimation of artemisinin.
36. Silica gel_{60} F_{254} plates with n-hexane-diethyl ether (1:1) solvent system can be used for TLC analysis of artemisinin.
37. The pink spot corresponding to artemisinin was detected by immersing the plates in in a pool of freshly prepared glacial acetic acid: conc. sulphuric acid: anisaldehyde (50:1:0.5) followed by drying in a chromatographic oven at 110°C for 15 minutes.
38. Artemisinin was detected in shoot, inflorescence, leaves from the plants grown in the field, *in vitro* shoots, transformed shoots and hairy roots induced by A4 and ATCC 15834.
39. No artemisinin was detected in roots from plants grown in the field, *in vitro* root and hairy root induced by MTCC 2364. Rooting of *in vitro* shoots enhanced the artemisinin content.
40. Artemisinin was maximum in the inflorescence followed by *in vitro* rooted shoots.
41. Addition of osmoregulant PEG (2.0 and 5.0 %) failed to elicit a positive influence in the biosynthesis of artemisinin in root cultures.

42. Addition of precursor methionine at 1.0 mM was found to be ineffective in increasing the artemisinin production of hairy root cultures.
43. Elicitation by *Aspergillus* homogenate at the rate of 250 µl/ 125ml elicited a positive response on biosynthesis of artemisinin content in hairy root induced by ATCC 15834.
44. Hairy root cultures showed no change in artemisinin content as well as growth upon addition of yeast extract at the rate of 2.5 g l^{-1}.

References

REFERENCES

Abdin, M.Z., Israr, M., Rehman, R and Jain, S.K. 2003. Artemisinin, a novel antimalarial drug biochemical and molecular approaches for enhanced production. *Planta Med.* 69: 289-299.

*Ackermann, C.1977. Pflanzen aus *Agrobacterium rhizogenes* Tumoren an *Nicotiana tabacum. Pl. Sci. Lett.*8: 23-30.

Acton, N., Klayman, D.L and Rollman, I.J. 1985. Reductive electrochemical HPLC assay for artemisinin (qinghaosu). *Planta Med.* 51: 445-446.

Akcam-Oluk, E., Demisay, H. and Gurel, E. 2003. Alkaloid production from cell suspension cultures obtained from osmotic stressed callus lines of *Catharanthus roseus. Pl. Cell Biotech. Mol. Biol.* 4: 91-94

Akhila, A., Thakur, R.S and Popli, S.P. 1987. Biosynthesis of artemisinin in *Artemesia annua. Phytochemistry.* 26: 1927-1930

Ali, M. and Siddiqui, N.A. 2000. Volatile oil constituents of *Artemesia annua* leaves. *J. Medicinal Aromatic Pl. Sci.* 22: 568-571

Allan, E.J., Eeswara, J.P., Jarvis, A.P., Mordue, A.J., Morgan, E.D and Stuchbury, T. 2002. Induction of hairy root cultures of *Azadirachta indica* A. Juss and their production of azadirachtin and other important insect bioactive metabolites. *Pl. Cell Rep.* 21: 374-379

Aoki, S. and Syono, K. 1999. Synergetic function of *rol*B, *rol*C, ORF13 and ORF14 of TL-DNA of *Agrobacterium rhizogenes* in hairy root induction in *Nicotiana tabacum. Pl. Cell Physiol.* 40: 252-256

Aoyagi, H., Yasasuhira, J. and Tanaka, H. 1998. Alginates promote production of various enzymes by *Cathranthus roseus* cells. *Pl. Cell Rep.* 17: 243-247

Arellano, J., Vazquez, F., Villegas, T. and Hernandez, G. 1996. Establishment of transformed root cultures of *Perzia cuernavacana. Pl. Cell Rep.* 15: 455-458

Asolkar, L.V., Kakkar, K.V. and Chakre, O.J. 1992. *Second supplement to glossary of Indian medicinal plants with active principles*. National Institute of Science communication. New Delhi. 414p.

Babaoglu, M., Davey, M.R., Power, J.B., Sporer, F. and Wink, M. 2004. Transformed roots of *Lupinus mutabilis*: induction, culture and isoflavone biosynthesis. *Pl. Cell Tissue Organ. Cult.* 78: 29-36

Bagchi, G.D., Ram, M., Sharma, S. and Kumar, S. 1997. Effect of planting date on growth and development of *Artemesia annua* under subtropical climatic conditions. *J. Medicinal Aromatic Pl. Sci.* 19: 387-394

Balachandran, S., Viswakarma, R.A. and Popli, S.P.1987. Chemical investigation of some *Artemesia annua* species: Search for artemisinin or other related sesquiterpene lactones with a peroxide bridge. *Indian.J.Pharm.Sci.* 49: 152-154.

Balvanyos, I., Szoke, E. and Kursinszki, L. 2002. The influence of aminoacids on the lobeline production of *Lobelia inflata* L. hairy root cultures. *Pl. Growth Regulation.* 36: 241-244

Bapat, V.A. and Ganapathi, T.R. 2005. Hairy roots- a novel source for plant products and improvement. *Natl. Acad. Sci. Lett.* 28: 61-68.

Batra, J., Dutta, A., Singh, D., Kumar, S. and Sen, J. 2004. Growth and terpenoid production in *Catharanthus roseus* hairy root clones in relation to left and right termini linked Ri T-DNA gene integration. *Pl. Cell Rep.* 23: 148-154.

Benjamin, B.D., Roja, G. and Heble, M.R. 1993. *Agrobacterium rhizogenes* mediated transformation of *Rauwolfia serpentina:* Regeneration and alkaloid synthesis. *Pl. Cell Tissue Organ Cult.* 35: 253-257.

Benjamin, B.D., Sipahimalani, A.T. and Heble, M.R. 1990. Tissue culture of *Artemesia pallens.* Organogenesis, terpenoid production. *Pl. Cell Tissue Organ Cult.* 21:159-164.

Bhadra, R., Vani, S. and Shanks, J.V. 1993. Production of indole alkaloids by selected hairy root lines of *Catharanthus roseus. Biotech. Bioeng.* 41:581-592.

Bhakuni, R.S., Jain, D.C., Sharma, R.P. and Kumar, S. 2001. Secondary metabolites of *Artemesia annua* and their biological activity. *Current Sci.* 80:35-48.

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

Bohlmann, J. and Eilert, U. 1994. Elicitor induced secondary metabolism in *Ruta graveolens*. L. Role of chitinase utilizing enzymes. *Pl. Cell Tissue Organ Cult.* 38: 189-195

*Brevet, J. and Tempe, J. 1988. Homology mapping of T-DNA regions of three *Agrobacterium rhizogenes* Ri plasmids by electron microscope hetero-duplex studies. *Plasmid.* 19:75-83.

Brodelius, P., Funk, C., Haner, A. and Villegas, M. 1989. A procedure for the determination of optimal chitosan concentration for elicitation of cultured plant cells. *Phytochemistry.* 28:2651-2654

Bush, A.L and Pueppke, S.G. 1991. Cultivar strain specificity between *Chrysanthemum morifolium* and *Agrobacterium tumefaciens. Physiol. Mol. Pl. Pathol.* 39: 309-323

Cai, G.Q., Li, G.Z., Ye, H.C. and Li, G.F. 1995. Hairy root culture of *Artemesia annua* L. by Ri plasmid transformation and biosynthesis of artemesinin. *Chinese J. Biotech.* 1: 315-320

Cardarelli, M., Mariotti, D., Pomponi, M., Spano, L., Capone, I. and Constantino, P. 1987. *Agrobacterium rhizogenes* T-DNA genes capable of inducing hairy root phenotype. *Mol. Gen. Genet.* 209: 475-480.

Carvaleho, E.B. and Curtis, W.R. 2002. Effect of elicitors on growth, respiration and nutrient uptake of root and cell suspension cultures of *Hyoscyamus muticus*, *Biotech Progress.* 18:282-289

Celma, C.R., Palazon, J., Cusido, R.M., Pinol, M.T. and Keil, M. 2001. Decreased scopalamine yield in field grown *Dubosia* plants regenerated from hairy roots. *Planta Med.* 67: 249-253.

Chapell, J., Lanken, C.V., Vogeli, V. and Bhatt, P. 1989. Sterol and sesquiterpenoid biosynthesis during a growth cycle of tobacco cell suspension cultures. *Pl. Cell Rep.* 8:48-55

Charles, D.J., Simon, J.E., Wood, K.V. and Heinstein, P. 1990. Germplasm variation in artemisinin content of *Artemesia annua* using an alternative method of artemisinin analysis from crude plant extracts. *J. Nat. Prod.* 53: 157-160.

Chaudhuri, K.N., Ghosh, B., Tepfer, D. and Jha, S. 2005. Genetic transformation of *Tylophora indica* with *Agrobacterium rhizogenes* A4: growth and tylophorine productivity in different transformed root clones. *Pl. Cell Rep.* 24: 25-35.

Chawla, H.S. 2003. *Introduction to Plant Biotechnology*. Second edition. Oxford and IBH Publishing Co. Pvt. Ltd, New Delhi, 538 p.

Chen, D.H., Ye, H.C., Li, G.F. and Liu, B.Y. 1999. Expression of green fluorescent protein gene in transgenic shoots of *Artemesia annua*. *Acta. Bot. Sin.*19:1097-1106

Chen, F.T. and Zhang, G.H. 1987. Studies on several physiological factors on artemisinin synthesis in *Artemesia annua*. *Pl. Physiol.* 5:26-30.

Chen, G., Chen, L., Ye, C. and Feng, L. 2004. Effect of temperature on growth and artemesinin in hairy root cultures of *Artemesia annua*. *Act. Bot. Sin.* 24: 1828-1831

Christen, P., Aoki. T. and Shimomura, K. 1992. Characteristics of growth and tropane alkaloid production in *Hyoscyamus albus* hairy roots transformed with *Agrobacterium rhizogenes* A4. *Pl. Cell rep.* 11:597-600

Christey, M.C. 2001. Use of Ri- mediated transformation for production of transgenic plants. *In Vitro Cell Dev. Biol. Pl.* 37: 687-700.

Combard, A. and Baucher, M.F. 1988. A common organization of T-DNA genes expressed in plant hairy root induced by different plasmids of *Agrobacterium rhizogenes*. *Pl. Mol. Biol.*10: 499-509

Crawford, N.M. 1995. Nitrate: nutrient and signal for plant growth. *Pl. Cell.* 7: 859-868

Croteau, R., Kutchan, T. and Lewis, N. 2000. Natural products (secondary metabolites) Buchnan, B., Gruissem, W. and Jones, R. (eds). *Boichemistry and molecular biology of plants.* American Society of Plant Physiologists, Rock ville. pp. 1250-1318

Cusido, R.M., Palanzon, J., Pinol, M.T., Bonfill, M. and Morales, C. 1999. *Datura metel*: *in vitro* production of tropane alkaloid. *Planta Med.* 65: 144-48

Da Silva, J.A. 2003. Anthemideae: advances in tissue culture, genetics and transgenic biotechnology. *African J. Biotech.* 2: 547-556

Davey, M.R., Mulligan, B.J., Gaitland, K.M.A., Peel, E., Saegent, A.W. and Morgan, A.J. 1987. Transformation of *Solanum* and *Nicotiana* species using an Ri plasmid vector. *J. Exp. Bot.* 38: 1507-1516

De, B. 2001. Cell signaling and elicitation of secondary plant metabolite production. *J. Medicinal Aromatic Pl. Sci.* 23: 413-428

Dehio, C., Grossman, K., Schell. J. and Schmilling, T. 1993. Phenotype and hormonal status of transgenic tobacco plants over expressing the *rol A* genes of *Agrobacterium rhizogenes* T-DNA. *Pl. Mol. Biol.* 23: 1199-1210

Delabays, N., Benakis, A. and Gollet, G. 1993. Selection and breeding of high artemisinin (quinghaosu) yielding strains of *Artemesia annua*. *Acta Hort.* 330: 203-206

Dessaux, Y., Petit, A. and Tempe, J. 1991. Opines in *Agrobacterium* Biology. Verma, D.P.S. (ed.) *Molecular signals in Plant-Microbe communications.* CRC Press, London. p.109-136

Dhingra, V., Pakki, S.R. and Narasu, M.L. 2000. Antimicrobial activity of artemisinin and its precursors. *Current Sci.* 78: 709-713

Dhingra, V., Rao, K.V. and Narasu, M.L. 2000. Current status of artemisinin and its derivatives as antimalarial drugs. *Life Sci.* 66: 279-300

DiCosmo, F. and Misawa, M. 1985. Eliciting secondary metabolism in plant cell cultures. *Trends Biotech.* 3: 318-322

Dobigny, A., Ambroise, A., Haicour, R., David, C., Rossignol, L. and Sihachakr, D. 1995. Transformation of potato using mannopine and cucmopine strains of *Agrobacterium rhizogenes*. *Pl. Cell Tissue Organ Cult.* 40: 225-230

Dong, H.D. and Zhang, J.J. 2002. Enhanced taxane productivity in bioreactor cultivation of *Taxus chinensis*. *Enzyme Microbial Tech.* 31: 116-121

*Doron, P.M. 1989. Prospects for the production of plant chemicals from genetically transformed roots. *Aust. J. Biotech.* 3: 270-275

Duke, M.V., Paul, R.N., El Sohly, H.N., Sturtz, G. and Duke, S.O. 1987. Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemesia annua* L. *Intl. J. Pl. Sci.* 155: 365-372

Duke, S.O. and Paul, R.N. 1993. Development and fine structure of glandular trichomes of *Artemesia annua* L. *Intl. J. Pl. Sci.* 154: 107-118

Durand-Tardif, M., Broglie, R., Slightom, J. and Tepfer, D. 1985. Structure and expression of TR-DNA from *Agrobacterium rhizogenes* in *Nicotiana tabacum* organ and phenotypic specificity. *J. Mol. Biol.* 186: 557-564

Dutta-Alvarez, S.J., Spollansky, T.C and Giulietti, A.M. 2000. The influence of different biotic and abiotic elicitors on the production and profit of tropane alkaloid in hairy root cultures of *Brugmonsia candida. Enzyme Microb. Tech.* 26: 252-8

Echeverrigaray, S., Fracaro, F., Andrade, L.B., Biasio, S. and Atti-Serafini, L. 2000. *In vitro* shoot regeneration from leaf explants of Roman Chamomile. *Pl. Cell Tissue Organ Cult.* 60: 1-4.

El-markby, S.A., El-Feraly, S.F., El-Sohly, H.N., Croom, E.M. and Hufford, C.D. 1987. Microbial transformation studies on arteannuin B. *J. Nat. Prod.* 55: 903-909

El-Sohly, H.M., Croom, E.M., El-Feraly, F.S. and El-Sherei, M.M. 1990. A large scale extraction technique of artemisinin from *Artemisia annua. J.Nat.Prod.* 53: 1560-1564

Fauconnier, M.L., Jajiri, M., Marlier, M., Roggemans, J., Wathelet, J.P., Lognay, G., Severin, M., Homes, J and Shimomura, K. 1993. Essential Oil production by *Anthemis nobilis* L. tissue culture. *J. Pl. Physiol.* 12:1141-1148

Ferreira, J.F.S. and Janick, J. 1996. Roots as an enhancing factor for the production of artemisinin in shoot cultures of *Artemesia annua. Pl.Cell Tissue Organ Cult.* 44:211-217.

Ferreira, J.F.S., Janick, J.E. and Janick, J. 1995. Developmental studies of *Artemesia annua*: Flowering and artemisinin production under green house and field conditions. *Planta Med.* 61: 351-355.

Figueiredo, A.C., Pais, M.S.S. and Scheffer, J.J.C. 1995. Composition of the essential oil from cell suspension cultures of *Achillea millefolium* ssp *millefolium. Pl. Cell Tissue Organ Cult.* 40:113-118

Flores, H. and Filner, P. 1985. Metabolic relationship of putresine, GAVA and alkaloids in cell and root cultures of *Solanaceae.* Barz, W., Newmann, K. and Reinhard, E. (eds) *Primary and Secondary metabolism of plant cell cultures.* Springer Verlag, Berlin. p.174-175.

Flores, H.E., Dai, Y., Cuello, J.L., Ignacio, E., Mendoza, M and Loyola-Vargas, V.M. 1993. Green roots:Photosynthesis and photoautotrophy in an undergroumd plant organ. *Pl. Physiol.* 11: 363-371

Flores, H.E., Hoy, M.W. and Pickard, J.J. 1987. Secondary metabolites from root cultures. *Trends Biotech.* 5: 64-68.

Frischknecht, P.M. and Baumann, T.W. 1985. Stress induced formation of purine alkaloids in plant tissue culture of *Coffea arabica. Phytochemistry* 24: 2255-2257

Fu, C.X., Xu, Y.J., Zhao, D.X and Ma, F.S. 2006. A comparison between hairy root cultures and wild plants of *Saussurea involcurata* in phenyl propanoid production. *Pl. Cell Rep.* 24: 750-754.

Fulzele, D.P., Sipahimalani, A.T and Heble, M.R. 1995. Tissue cultures of *Artemesia annua*: Organogenesis and artemisinin production. *Phytother Res.* 5:149-153.

Gabriel, M. and Plaizer-Vercammen, J.A. 2003. Densitometric Thin layer Chromatographic determination of artemisinin and its lipophilic derivatives, artemether and arteether. *J. Chromatographic Sci.* 41:359-366

Gamborg, O.L., Miller, R.A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell. Res.* 50: 151-158

Gangopadhyay, G., Basu, A.S., Mukherjee, B.R. and Gupta, S. 1997. Effects of salt and osmotic shocks on unadapted and adapted callus lines of tobacco. *Pl. Cell Tissue Organ Cult.* 49: 45-52

Gelvin, S.B. 1990. Crown gall disease and hairy root disease- a sledge hammer and a track hammer. *Pl. physiol.* 92: 281-285.

Ghosh, B., Mukherjee, S. and Jha, S. 1997. Genetic transformation of *Artemesia annua* by *Agrobacterium tumefaciens* and artemisinin synthesis in transformed cultures. *Pl. Sci.* 122:193-199

Giri, A. and Narasu, M.L. 2000. Transgenic hairy roots: recent trends and applications. *Biotech. Adv.* 18: 1-22.

Giri, A., Ravindra, S.T., Dhingra, V. and Narasu, M.L. 2001.Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and artemisinin production in *Artemesia annua*. *Current Sci.* 81:378-382

Godwin, I., Todd, G., Ford-Lloyd, B. and John-Newbury, H. 1991. The effect of acetosyringone and pH on *Agrobacterium* mediated transformation vary according to plant species. *Pl. Cell Rep.* 9: 671-673

Gonzales, D.L. and Weathers, P.J. 2003. Tetraploid *Artemesia annua* hairy roots produced more artemisinin than diploids. *Pl. Cell Rep.* 21: 809-813

Grummer, G. 1961. The role of toxic substances in the inter relationships between plants. Mithorpe, F.L. (ed) *Mechanism in biological competition.* Academic press, Newyork. p. 219-228.

Gulati, A., Bharel, S., Jain, S.K., Abdin, M.Z. and Srivastava, P.S. 1996. *In vitro* micropropagation and flowering in *Artemesia annua*. *J. Pl. Biochem. Biotech.* 5: 31-35.

Gupta, M.M., Jain, D.C., Verma, R.K. and Gupta, A.P. 1996. A rapid analytical method for the estimation of artemisinin in *Artemesia annua*. *J. Medicinal Arom Pl. Sci.* 18:5-6

Gupta, M.M., Verma, R.K., Gupta, A.P., Bhartariya, K.G. and Kumar, S. 1997. Simultaneous determination of artemisinin, arteannuic B and artemisinic acid in *Artemesia annua* by high performance liquid chromatography with the combination of ODS and CN columns. *J. Medicinal Aromatic Pl. Sci.* 19: 968-972.

Halligan, J.P. 1975. Toxic substances from *Artemesia californica*. *Ecology*. 56: 999-1003.

Hamill, J.D., Parr, A.J., Robins, R.J. and Rhodes, M.J.C. 1986. Secondary products formation by the cultures of *Beta vulgaris* and *Nicotiana rustica* transformed with *Agrobacterium rhizogenes*. *Pl. Cell Rep.* 5: 111-114.

Hamill, J.D., Rounstey, S., Spencer, A., Todd, G. and Rhodes, M.J.C. 1991. The use of the polymerase chain reaction in plant transformation studies. *Pl. Cell Rep.* 10: 221-224

Hanischten-Cate, C.H., Loonen, A.E., Ottavani, M.P., Enniklvn-Eldik, G and Stiekema, W.J. 1990. Frequent spontaneous deletions of Ri-T-DNA in *Agrobacterium rhizogenes* transformed potato roots and regenerated plants. *Pl. Mol. Biol.* 14: 735-741

*Hawes, M.C., Smith, L.Y. and Howarth, A. J. 1988. *Agrobacterium tumefaciens* mutants deficient in chemotaxis to root exudates. *Mol. Pl. Microb. Interaction.* 1: 182-190

He, X.C., Zeng, M.Y., Li, G.F. and Liang, Z. 1983. Callus induction and regeneration of plantlets from *Artemesia annua* and changes of qinghaosu contents. *Acta Bot. Sin.* 25: 87-90

Hernandez, G.G. and Vargas, L.V.M. 1991. Effect of fungal homogenate, enzyme inhibitors and osmotic stress on alkaloid content of *Catharanthus roseus* cell suspension cultures. *Pl. Cell Rep.* 16: 287

Hildebrand, E.M. 1934. Life history of hairy root organism in relation to its pathogenesis on nursery apple trees. *J. Agric. Sci.* 48: 857-885

Hilton, M.G. and Wilson, P.D.G. 1995. Growth and uptake of sucrose and mineral ions by transformed root cultures of *Datura stramomium, Datura candida, Datura wrightii, Hyoscyamus muticus* and *Atropa belladonna. Planta Med.* 61: 345-350

Hobbs, S.L.A., Jackson, J.A. and Mahon, J.D. 1989. Specificity of strain and genotype in the susceptibility of pea to *Agrobacterium tumefaciens. Pl. Cell Rep.* 8: 55-58

Hu, Z.B. and Alfermann, A.W. 1993. Diterpenoid production in hairy root cultures of *Salvia miltiorrhiza. Phytochemistry.* 32: 699-703

Huffman, G.A., White, F.F., Gordon, M.P. and Nester, E.W. 1984. Hairy root inducing plasmid: physical map and homology to tumour inducing plasmid. *J. Bacteriol.* 157: 269-276

James, D. 2001. Molecular characterization of *Ralstonia solanacearum* Smith, (Yabuchi *et al.*) causing bacterial wilt in solanaceous vegetables. M.Sc. (Ag.) thesis. Kerala Agricultural University, Trichur, 107p.

Jaziri, M., Diallo, B., Vanharlen, M., Homes, J., Yoshimatsu, K. and Shimomura, K.1993. Immunodetection of artemisinin in *Artemesia annua* L. cultivated in hydroponic conditions. *Phytochemistry.* 33: 821-826

Jaziri, M., Shimomura, K., Yoshimatsu, K. and Fauconnier, M.L. 1995. Establishment of normal and transformed root cultures of *Artemesia annua* L. for artemisinin production. *J. Pl. Physiol.* 144: 175-177

Jaziri, M., Yoshimatsu, K., Homes, J. and Shimomura, K. 1994. Traits of *Atropa belladona* doubly transformed with different *Agrobacterium rhizogenes* strains. *Pl. Cell Tissue Organ Cult.* 38: 257-262

Jeremic, D., Jokic, A., Behbud, A. and Stefanovic, M. 1973. A new type of sesquiterpene lactone isolated from *Artemesia annua* L. Arteannuin B. *Tetrahedron Lett.* 32: 3039-3042

Jha, S. 2000. Genetic transformation for the production of Secondary Metabolites. Ramawat, K.G. and Merillon, J.M. (eds.) *Biotechnology Secondary Metabolites.* Oxford and IBH, New Delhi. pp.305-329

Jha, S., Jha, T.B. and Mahato, S.B. 1988. Tissue culture of *Artemesia annua* L. a potential source of antimalarial drug. *Current Sci.* 57: 344-346

*Jouanin, L. 1984. Restriction map of an agropine type Ri plasmid and its homologies with Ti plasmids. *Plasmid.* 12: 91-102

Jouanin, L., Bouchez, D., Drong, R.F. Tepfer, D. and Slightom, J.L. 1989. Analysis of TR-DNA / plant junctions in the genome of a *Convolvulus arvensis* clone transformed by *Agrobacterium rhizogenes* strain A4. *Pl. Mol. Biol.* 12: 75-85

Jung, G. and Tepfer, D. 1987. Use of genetic transformation by the Ri T-DNA of *Agrobacterium rhizogenes* to stimulate biomass and tropane alkaloid production in *Atropa belladonna* and *Calystegia sepium* roots. *Pl. Sci.* 50: 145-151

Jung, K.H., Kwak, S.S., Choi, C.Y. and Liu, J.R. 1995. An interchangeable system for hairy root and suspension cultures of *Catharanthus roseus* for indole alkaloid production. *Pl. Cell Rep.* 15: 51-54

Jung, M. and Schinazi, R.F. 1994. Synthesis and *in vitro* antihuman deficiency virus activity of artemisinin (Qinghaosu). *Biorg. Med. Chem. Lett.* 4: 931-935

Juteau, F., Jerkovic, I., Masotti, V., Milos, M., Mastelic, J., Bessiere, J.M. and Viano, J. 2002. Composition and antimicrobial activity of the essential oil of *Artemesia absinthium* from Croatia and France. *Planta Med.* 69: 158-161

Kamada, H., Okamura, N., Satake, M., Hasada, H. and Shimomura, K. 1986. Alkaloid production by hairy root cultures in *Atropa belladonna. Pl. Cell Rep.* 5: 239-242

Karmarkar, S.H. 2001. Genetic tranformation and hairy root culture in ada-kodien (*Holostemma ada-kodien* K. Schum). M.Sc. (Horticulture) thesis, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, 137 p.

Karmarkar, S.H. and Keshavachandran, R. 2005. Hairy root induction in *Holostemma ada-kodien* K. schum with *A. rhizogenes.* Proceedings of ICAR National Symposium on Biotechnological interventions for improvement of Horticultural crops: Issues and strategies; 10-12 Jan. 2005; Thrissur, Kerala. pp.350-351

Karmarkar, S.H., Keshavachandran, R., Nazeem, P.A. and Girija, D. 2001. Hairy root induction in Adapathiyan (*Holostemma ada-kodien* K. Schum.). *J. trop. Agric.* 39: 102-107

Kaul, K. and Hoffman, S.A 1993. Ammoniun ion inhibition of *Pinus strobes* L. callus growth. *Pl. Sci.* 88:169-173

Kawamoto, H., Sckine, H. and Furuya, T. 1999. Production of artemisinin and related sesquiterpenes in Japanese *Artemesia annua* during a vegetation period. *Planta Med.* 65: 88-89

Kim, D.J. and Chang, H.N. 1990. Increased shikonin production in *Lithospermum erthrorhizon* suspension cultures with *in situ* extraction antifungal cell treatment (elicitor). *Biotech. Lett.* 12: 443-448

Kim, N.C., Kim, J.G., Lim, H.J., Hahn, T.R. and Kim, S.U. 1992. Production of secondary metabolites by tissue culture of *Artemesia annua* L. *J. Korean Agr. Chem. Soc.* 35: 99-105

Kim,Y., Wyslouzil, B.E. and Weathers, P.J. 2001. A comparative study of mist and bubble column reactors in the *in vitro* production of artemisinin *Pl. Cell Rep.* 20: 451-455

Klayman, D.L. 1985. Qinghaosu (Artemisinin): An antimalarial drug from China. *Science*. 228: 1049-1055

Klayman, D.L., Lin, A.J., Acton, N., Scovill, J.P., Hock, J.M. and Milhous, W.K. 1984. Isolation of artemisinin (Qinghaosu) from *Artemesia annua* growing in the United States. *J. Nat. Prod.* 47: 715-717

Koe, S., Noguchi, H., Ebizuka, Y. and Sankawa, U. 1989. Oligoside production by hairy root cultures transformed by Ri plasmids. *Chem. Pharm. Bull.* 37: 245-248

Koike, Y., Hoshino, Y., Mii, M. and Nakano, M. 2003. Horticultural characterisation of *Angelonia salicarifolia* plants transformed with wild type strains of *Agrobacterium rhizogenes*. *Pl. Cell Rep.* 21: 981-987

Komaraiah, P., Amruth, R.N., Jogeswar, G., Ramakrishna, S.V. and Kishor, K.P.B. 2002. Production of plumbagin from hairy root cultures of *Plumbago rosea*, L. *Pl. Cell Biotech. Mol. Biol.* 3: 65-68

Kombrink, E. and Hahlbrock, K. 1986. Responses of cultured parsley cells to elicitors from phytopathogenic fungi. Timing and dose dependency of elicitor-induced reactions. *Pl. Physiol.* 81: 216-221

Krizkova, L. and Hrouda, M. 1998. Direct repeats of T-DNA *integrated* in tobacco chromosome: characterization of junction regions. *Pl. J.* 16: 673-680

Kudakasseril, G.J., Lam, L. and Staba, E.J. 1987. Effect of sterol inhibitors on incorporation of ^{14}C-isopentenyl pyrophosphate into artemisinin by cell free system for *Artemesia annua* tissue cultures and plants. *Planta Med.* 28: 280-284

Kukreja, A.K., Zehra, M., Sultana, A. and Banerjee, S. 1997. Hairy roots of medicinal plants: Potential and prospects. Ravisankar, G.A. and Venkataraman, L.V. (eds.) *Biotechnological applications of plant tissue and cell culture.* Oxford and IBH, New Delhi. pp.75-89

Kumar, S. and Srivastava, S. 2005. Establishment of artemisinin combination therapy as a first line treatment for combating malaria: *Artemesia annua* cultivation in India needed for providing sustainable supply chain of artemisinin. *Current Sci.* 89: 1097-1102

Laughlin, J.C. 1995. The influence of distribution of antimalarial constituents in *Artemesia annua* L. on time and method of harvest. *Acta Hort.* 390: 67-73

Le, W.J., You, J.Q., Yang, Y.Q., Mei, J.Y., Guo, H., Yang, H.Z. and Zheng, C.W. 1982. Studies on the efficacy of artemether in experimental Schistosomiasis. *Acta Pharmacol Sin.* 17: 187-193

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

Leirsch, R., Soicke, H., Stehr, C and Tullner, H.U. 1986. Formation of artemisinin in *Artemisia annua* during one vegetation period. *Planta Med.* 52: 387-390

Li, W., Asada, Y. and Yoshikawa, T. 2000. Flavanoid constituents from *Glycyrrhiza glabra* hairy root cultures. *Phytochemistry.* 55: 447-456

Li, Y., Shan, F., Wu, J.M., Wu, G.S., Ding, J., Xiao, D., Yang, W.Y., Attasi, G., Leonce, S., Caignard, D.H. and Renard, P. 2001. Novel antitumour artemisinin derivatives targeting G1 phase of cell cycle. *Biorg. Med. Chem. Lett.* 11: 5-8

Limani, M.A., Sun, L.Y., Douat, C., Helgeson, J. and Tepfer, D. 1998. Natural genetic transformation by *Agrobacterium rhizogenes*. *Pl. Physiol.* 118: 543-550

Linsafors, J., Bijort, L. and Mosback, K. 1989. Influence of elicitors and mevalonic acid on biosynthesis of ginsenoides in tissue cultures of *Panax ginseng*. *Biochem. Physiol. Pl.* 184: 413-418

Liu, B., Ye, H., Li, G., Chen, B., Geng, S., Zhang, Y., Chen, J. and Gao, J. 1998. Studies on dynamics of growth and biosynthesis of artemisinin in hairy roots of *Artemesia annua*. L. *Chineese J. Biotech.* 14: 249-254

Liu, C.Z., Wang, Y.C., Ouyang, F., Ye, H.C. and Li, G.F. 1997. Production of artemisinin by hairy root cultures of *Artemesia annua* L. *Biotech. Lett.* 19: 927-929

Liu, C.Z., Wang,Y.C., Kang, X.Z. and Ouyang, F. 1999. Investigation of light. temperature and cultivated modes on growth and artemisinin synthesis of *Artemesia annua* L. shoots. *Acta Phytophy. Sci.* 25: 105-109

Liu, K.C., Yan, S.L., Roberts, M.F., Elford, B.C. and Phillipson, J.D. 1992. Antimalarial activity of *Artemesia annua* flavanoids from whole plants and cell cultures. *Pl. Cell Rep.* 11: 637-640

Lloyd, G.B. and McCown, B.H. 1980. Commercially feasible micropropagation of mountain laurel *Kalmia latifolia* by use of shoot culture. *Int. Pl. Prop. Soc.* 30: 421-427

Lorence, A., Bolivar, M.F. and Nessler, C.L. 2004. Camptothecin and 10-hydroxy camptothecin from *Camptotheca acuminata* hairy roots. *Pl. Cell Rep.* 22: 437-441

Lorenco, P.M.L., Figueiredo, A.C., Barroso, J,G., Pedro, L.G., Oliverira, M.M., Deans, S.G. and Scheffer, J.J.C. 1999. Essential oils from hairy root cultures and plant roots of *Achillea millefolium*. *Phytochemistry*. 51: 637-642

Luo, X.D. and Shen, C.C. 1987. The chemistry, pharmacology and clinical applications of qinghaosu (Artemesinin) and its derivatives. *Med. Res. Rev.* 7: 29-52

Maday, E., Szoke, E., Muskath, Z. and Lemberkovics, E. 1999. A study of the production of essential oils in chamomile hairy root cultures. *Europ. J. Drug Metaolism Pharmacokin.* 24: 303-308

Malarz, J., Stojakowska, A. and Kisiel, W. 2002. Sesquiterpene lactones in hairy root cultures of *Cichorium intybus*. *Naturforsch.* 57: 994-997

Mano, Y., Ohkawa, H. and Yamada, Y. 1986. Production of tropane alkaloids by hairy root cultures of *Dubosia leichardtii* transformed by *Agrobacterium rhizogenes*. *Pl. Sci.* 59: 191-201

Martinez, B.C. and Staba, J. 1988. The production of artemesinin in *Artemisia annua* L. tissue cultures. *Adv. Cell Cult.* 6: 69-87

Mathur, A.K. and Kumar, S. 1998. Micropropagation of *Artemesia annua* via inflorescence. *J. Herbs Spices Medicinal Pl.* 4: 61-71.

Mercury, A., Bruna, S., De-Benedetti, L., Burchi, G. and Schiva, T. 2001. Modification of plant architecture in *Limoneum spp.* induced by *rol* genes. *Pl. Cell Tissue Organ Cult.* 65: 247-253.

Merlo, D.J., Nutter, R.C., Montaya, A.L., Gafinkel, D.J., Drummond, M.H., Chilton, M.D., Gordon, M.P. and Nester, E.W. 1980. The boundaries and copy numbers of Ti plasmid T-DNA vary in crown gall tumours. *Mol. Gen. Genet.* 177: 637-643

Momocilovic, I., Grubisic, D., Kojic, M. and Neskovic, M. 1997. *Agrobacterium rhizogenes* mediated transformation and plant regeneration of four *Gentiana* species. *Pl. Cell Tissue and Organ Cult.* 50: 1-6.

Moore, L., Warren, G. and Strobel, G. 1974. Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes. Plasmid.* 2: 617-626.

Morales, M.R., Charles, D.J. and Simon, J.E. 1993. Seasonal accumulation of artemisinin in *Artemesia annua* L. *Acta Hort.* 344: 416-420.

Morgan, J.A. and Shanks, J.V. 2000. Determination of metabolic rate-limitations by precursor feeding in *Catharanthus roseus* hairy root cultures. *J. Biotech.* 79: 137-145

Moyano, E., Fornale, S., Valazon, J., Cusido, R.M., Bonfill, M. and Morales, C. 1999. Effect of *Agrobacterium rhizogenes* T-DNA on alkaloid production in *Solanaceae* plants. *Phytochemistry.* 52: 1287-1292.

Mukherjee, S., Ghosh, S.B., Jha, T.B. and Jha, S. 1995. Genetic transformation of *Artemesia annua* by *Agrobacterium rhizogenes. Indian J. Exp. Biol.* 33:868-871.

*Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Pl.* 15: 473 - 497

Muthuvel, M., Jawahar, M., Rajendran, A., and Jayabalan, M., 2005. An efficient protocol for organogenic callus induction and plant regeneration in tomato (*Lycopersicon esculentum* Mill.). *Pl. Cell Biotech. Mol. Biol.* 6: 41-46.

Nair, M.S.R., Acton, N., Klayman, D.L., Kendrick, K., Basile, D.V. and Mante, S. 1986. Production of artemisinin in tissue cultures of *Artemesia annua*. *J. Nat. Prod.* 49:504-507.

Nef, C., Rio, B. and Christin, H. 1991. Induction of catharanthine synthesis and stimulation of major alkaloids production by *Catharanthus roseus* cells under non-growth altering treatment with *Pythium vexans* extracts. *Pl. Cell Rep.* 10: 26-29

Nilsson, O and Olsson, O. 1997. Getting to the root: the role of the *Agrobacterium rhizogenes* rol genes in the formation of hairy roots. *Physiol. Pl.* 100: 463-473.

Nin, S., Bennii, A., Roselli, G., Mariotti, D., Schiff, S. and Magherini, R. 1997. *Agrobacterium* mediated transformation of *Artemesia absinthium* L. (wormwood) and secondary metabolites. *Pl. Cell Rep.* 16: 725-730

Noda, T., Tanaka, N., Mano, Y., Nabeshima, S., Ohkawa, H. and Matsui, C. 1987. Regeneration of horse radish hairy roots incited by *Agrobacterium rhizogenes*. *Pl. Cell Rep.* 6: 283-286.

Nomadeo, A., Patel, S. and Fulzele, D.P. 2002. Influence of fungal elicitors on production of ajmalicine by cell culture of *Catharanthus roseus*. *Biotech. Progress*. 18: 159-162

Oh, H.G., Youn, H.J., Noh, J.W., Jang, D.H. and Kang, Y.B. 1997. Anticoccidial effects of an extract of *Artemesia annua* on *Eimeria tenella*. *Korean J. Veterinary Res.* 35: 115-121.

Oksman-Caldentey, K.M., Sevon, N., Vanhala, L. and Hiltunen, R. 1994. Effect of nitrogen and sucrose on the primary and secondary metabolism of transformed root cultures of *Hyoscyamus muticus*. *Pl. Cell Tissue Organ Cult.* 38: 263-72

Oksman-Caldentey, M. and Hiltunen, R. 1996. Transgenic crops for improved pharmaceutical products. *Field Crops Res.* 45: 57-69.

Ooms, G., Karp, A., Burrell, M.M., Twell, D. and Roberts, J. 1985. Genetic modification of potato development using Ri T-DNA. *Theor. Appl. Genet.* 70: 440-446.

Ottaviani, M.P., Schel, J.H.N. and Hanischten-Cate. 1990. Variation in structure and plant regeneration of *Agrobacterium rhizogenes* transformed and control roots of potato. *Pl. Cell Tissue Organ. Cult.* 20: 25-34.

Paniego, N.B. and Giuliette, A.M. 1994. *Artemisia annua* L.: dedifferentiated and differentiated cultures. *Pl. Cell Tissue Organ Cult.* 36:163-168.

Panse, V.V. and Sukhathme, P.B. 1985. Statistical methods for Agricultural workers. Forth Edition. ICAR, New Delhi. p. 131-143

Patena, L., Sutter, E.G. and Dandekar, A.M. 1988. Root induction by *Agrobacterium rhizogenes* in a difficult to root woody species. *Acta Hort.* 227: 324-329.

Patience, P.A. and Alderson, P.C. 1987. Development of a system to study peroxidases during the rooting of lilac shoots *in vitro*. *Acta Hort.* 212: 267-272

Patricia, M., Moctezuma, C. and Gloria, L.E. 1996. Biosynthesis of sesquiterpenic phytoalexin capidol in elicited root cultures of chilli pepper *Capsicum annuum*. *Pl. Cell Rep.* 15: 360-366

Pawar, P.K. and Maheshwari, V.L. 2004. *Agrobacterium rhizogenes* mediated hairy root induction in two medicinally important members of family Solanaceae. *Indian J. Biotech.* 3: 414-417.

Pawlicki, N., Sangwan, R.S. and Norrel, B.S. 1992. Factors influencing the *Agrobacterium tumefaciens*-mediated transformation of carrot (*Daucus carota* L). *Pl. Cell Rep.* 31: 129-139

Payne, G.F., Bringi, V., Prince, Culture and Shuler, M.L. 1992. Root cultures. Shuler, M.L (ed). *Hanser series in biotechnology. Pant cell and tissue culture in liquid systems*. Hanser, Munich. p.225-279.

Petit, A., Berkaloff, A. and Tempe, J. 1986. Multiple transformations of plant cells by *Agrobacterium* may be responsible for the complex organization of T-DNA in crown gall and hairy root. *Mol. Gen. Genet.* 202: 388-393.

Petit, A., David, C., Dahl, G.A., Ellis, J.G., Guyon, P., Casse-Delhart, F. and Tempe, J. 1983. Further extension of the opine concept: plasmids in *Agrobacterium rhizogenes* co-operate for opine degradation. *Mol. Gen. Genet.* 190: 204-214.

Pras, N. and Woerdenbag, H.J. 2000. Production of secondary metabolites by bioconversion. Ramawat, K.G. and Merrilon, J.M. (eds). *Biotechnology Secondary Metabolites*. Oxford and IBH, New Delhi. p. 265-303

Pras, N., Visser, J.F., Batterman, S., Woerdenbag, H.J., Malingre, T.M. and Lugt, C.B. 1991. Laboratory selection of *Artemesia annua* L. for high artemisinin yielding types. *Phytochem. Anal.* 2:80-83.

Qian, P., Yan, Y.W. and Ren, Q.L. 2005. Determination of artemisinin in *Artemesia annua* by reverse phase HPLC. *J. Liquid Chromatography. Tech.* 28: 705-712

Qin, M. B., Liu, Z., Yun, Y., Ye, H.C. and Li, G.F. 1994. Induction of hairy root from *Artemesia annua* with *Agrobacterium rhizogenes* and its cultures *in vitro*. *Acta. Bot. Sin.* 36:165-170

Ram, M., Jain, D.C. and Kumar, S. 1997. *Cultivation of Quinghaosu Artemesia annua L.* Farm bulletin No: 003. CIMAP, Lucknow. 12 p.

Ramachandra, R.S. and Ravishankar, G.A. 2002. Plant Cell cultures: Chemical factories of secondary metabolites. *Biotech. Adv.* 20: 101-153.

Reddy, U.P., Reddy, K.B., Acharya, B.M., Reddy, R.P. and Vaidyanath, K. 2001. *In vitro* plant regeneration and analysis of oil profiles in *Glossocardia bosvallia* Roxb. *Pl. Cell Biotech. Mol. Biol.* 2: 89-96.

Rhodes, M.J.C., Parr, A.J., Giulietti, A. and Aird, E.L.H. 1994. Influence of exogenous hormones on the growth and secondary metabolite formation in transformed root cultures. *Pl. Cell Tissue Organ Cult.* 38: 143-51

Rhodes, M.J.C., Robins, R.J., Hilton, M., Hamill, J.D. and Parr, A.J. 1986. Nicotene production by hairy root cultures of *Nicotiana rustica,* fermentation and product recovery. *Biotech. Lett.* 8: 415-420.

Rhodes, M.J.C., Robins, R.J., Hilton, M., Hamill, J.D., Parr, A.J. and Walton, N.J. 1987. Secondary product formation using *Agrobacterium rhizogenes* transformed hairy root cultures. *TCA Newlett.* 53: 2-15.

* Rhodes, M.J.C., Robins, R.J., Hilton, M., Hamill, J.D., Parr, A.J. and Walton, N.J. 1990. Properties of transformed root cultures. Charlwood, B.V. and Rhodes, M.J.C. (eds). *Secondary products from plant tissue cultures.* Clarendon Press, Oxford. p. 201-225.

Rhodes, M.J.C., Robins, R.J., Lindsay, E., Arid, M., Payne, J., Pare, A.J and Walton, N.J. 1989. *Primary and Secondary Metabolism of Plant Cell Cultures.* Springer-verlag, New York, p. 58-73

Ricker, A. J., Banfield, W.M., Wright, W.H., Keit, G.W. and Sage, H.E. 1930. Studies on infectious hairy roots of nursery apple trees. *J. Agric. Res.* 41: 507 - 540.

Roth, R.J and Acton, N. 1989. The isolation of sesquiterpenes from *Artemesia annua. J. Chem. Edu.* 66: 349-350.

Roy, S.K., Rahman, S.L. and Majumdar, R. 1990. *In vitro* propagation of jack fruit (*Artocarpus heterophyllus* Lam.). *J. Hort. Sci.* 65: 355-358

Rutenan, T. 1995. Alkaloid biosynthesis - basis for metabolic engineering of medicinal plants. *Pl. Cell.* 7: 1059-1070.

Saito, K., Yamazaki, M and Murakoshi, I. 1992. Transgenic Medicinal Plants: *Agrobacterium* mediated foreign gene transfer and production of secondary metabolites. *J. Nat. Prod.* 55: 149-162.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: A Laboratory Manual. Cold Spring Harbor, New York.

Sangwan, R.S., Agarwal, K., Luthra, R., Thakur, R.S., Sangwan, N.S. 1993. Biotransformation of arteannuic acid into arteannuic B and artemisinin in *Artemesia annua. Phytochemistry.* 34: 1301-1302

Sankar, A.M. 1998. *In vivo* and *in vitro* screening of *Sida spp.* for ephedrine content. Ph.D thesis, Kerala Agricultural University, Trichur, 207p.

Sarmento, G.G., Alpert, K., Tang, F.A and Punja, Z.K. 1992. Factors influencing *Agrobacterium tumefaciens* mediated transformation and expression of kanamycin resistance in pickling cucumber. *Pl. Cell. Tissue Organ Cult.* 31: 185-193

Sauerwein, M., Wink, M. and Shimomura, K.1992. Influence of light and phytohormones on alkaloid production in transformed root cultures of *Hyoscyamus albus. J. Pl. Physiol.* 140: 147-152.

Sevon, N. and Oksman-Caldentey, K. 2002. *Agrobacterium rhizogenes* – mediated transformation: Root cultures as a source of alkaloids. *Planta Med.* 68: 859-868.

Sharma, A., Bindra, R.L ane Tewari, R. 1991. *Artemesia annua*: Cultivation, utilization and chemical studies. *Current Res. Medicinal Aromatic Pl.* 13: 46-60.

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

Shimomura, K., Sudo, H., Saga, H. and Kamada, H. 1991. Shikonin production and secretion by hairy roots of *Lithospermum erythrorhizon*. *Pl. Cell Rep.* 10: 282-285

Shukla, A., Farooqi, A.A.H., Shukla, Y.N. and Sharma, S. 1992. Effect of triacontanol and chloremequat on growth, plant hormones and artemisinin yield in *Artemesia annua*. L. *Pl. Growth Regulation*. 11:165-171

Sindhu, M. 1999. *In vitro* callus induction and its exploitation in *Coscinium fenestratum* (Gaertn.) Colebr. M.Sc. (Hort.) thesis. Kerala Agricultural University, Thrissur, 130 p.

Singh, A., Kaul, V.K., Mahajan, V.P., Misra, L.N. and Thakur, R.S. 1986. Introduction of *Artemesia annua* in India and isolation of artemisinin a promising antimalarial drug. *Indian J. Pharmaceut. Sci.* 48: 137-138.

Singh, A., Vishwakarma, R.A. and Husain, A. 1988. Evaluation of *Artemisia annua* strains for higher artemisinin production. *Planta Med.* 54:475-476.

Singh, N.P. and Lai, H. 2001. Selective toxicity of dihydroartemisinin and holotransferrin towards human breast cancer cells. *Life Sci.* 70: 49-56.

Sinkar, V., Pythoud, F., White, F., Neste, E. and Gordon, M. 1988. *Rol* A locus of the Ri plasmid directs developmental abnormalities in transgenic plants. *Genes Dev.* 2: 688-698.

Sipahimalani, A.T., Fuzele, D.P. and Heble, M.R. 1991. Analytical method for the detection and determination of artemesinin by gas chromatography. *J. Chromatography.* 538: 452-454

Skoog, F. and Miller, C.O. 1957. Chemical regulation of growth and organ formation in plant tissue cultured *in vitro*. *Sym. Soc. Exp. Biol.* 11: 118-130.

Slightom, J.L., Durand-Tardif, M., Jouanin, L and Tepfer, D. 1986. Nucleotide sequence analysis of the TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. *J. Biol. Chem.* 216: 108-121.

Smith, T.C., Weathers, P.J. and Cheetham, R.D. 1997. Effect of gibberellic acid on hairy root cultures of *Artemesia annua* growth and artemisinin production. *In Vitro Cellular Dev. Biol. Pl.* 33: 75-79

Southern, E.M. 1975. Detection of specific sequence among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98: 503-517

Spena, A., Schmulling, T., Koncz, C. and Schell, J.S. 1987. Independent and synergistic activity of *rol* A, B, C loci in stimulating abnormal growth in plants. *EMBO.* 6: 3891-3899.

*Spencer, P.A and Towers, G.H.N. 1989. Virulence-inducing phenolic compounds directed by *Agrobacterium tumefaciens*. Lewis, N. G. and Paice, M.G. (eds.). *Plant Cell Wall Polymers:Biogenesis and Biodegradation.* American Chemical Society, Washington, p.383

Tan, R.X., Zheng, W.F. and Tang, H.Q. 1998. Biologically active substances from the genus *Artemesia. Planta Med.* 64:295-302.

Tang, H.Q., Hu, J., Yang, L. and Tan, R.X. 2000. Terpenoids and flavanoids from *Artemesia* species. *Planta Med.* 66: 391-393

Tawfiq, N.K., Anderson, L.A., Roberts, M.F., Phillipson, J.D., Bray, D.H. and Warhurst, D.C. 1989. Antiplasmodial activity of *Artemisia annua. Pl.Cell Rep.* 8: 425-428

Taylor, B., Amasino, R., White, F., Nester, E. and Shahin, E. 1985. Ri plasmid as a helper for introducing vector DNA into alfalfa plants. *Pl. Mol. Biol.* 8: 206-216

Tepfer, D. 1984. Genetic transformation of several species of higher plants by *Agrobacterium rhizogenes*: Phenotypic consequences and sexual transmission of the transformed genotype and phenotype. *Cell.* 37: 959-967

Tepfer, D. 1990. Genetic transformation using *Agrobacterium rhizogenes. Physiol. Pl.* 79: 140-146

Tiwari, S., Shah, P. and Singh, K. 2003. Efficient *in vitro* clonal propagation of *Rauvolfia serpentina* L.: An important medicinal plant. *Pl. Cell Biotech. Mol. Biol.* 4: 157-162.

Toivonen, L., Ojala, M. and Kauppinen, W. 1991. Studies on optimization of growth and indole alkaloid production by hairy root cultures of *Catharanthus roseus*. *Biotech. Bioeng.* 37: 673-680.

Torres, K.C. 1989. *Tissue culture Techniques for Horticultural crops*. Chapman and Hall, Newyork. 285p.

Towler, M.J. and Weathers, P.J. 2003. Adhesion of plant roots to poly-L-lysine coated polypropylene substrates. *J. Biotech.* 101:147-155

Trigg, P.I. 1989. Qinghaosu (Artemisinin) as an antimalarial drug (ed. Wagner, H., Norman, R.F and Hiroshi, H.). *Economic and medicinal plant research: Vol.3*. Academic Press, London. p.20-51.

Trypsteen, M., Van-Lijsebettens, M., Van-Severen, R. and Van-Montagu, M. 1991. *Agrobacterium rhizogenes* mediated transformation of *Echinacea purpurea*. *Pl. Cell Rep.* 10: 85-89.

Tsuro, M., Kubo, T., Shizukawa, Y., Takemoto, T. and Inaba, K. 2005. *Agrobacterium rhizogenes* is a useful transporter for introducing T-DNA of the binary plasmid into the chrysanthemum *Dendranthema grandiflorum* Kitamura genome. *Pl. Cell Tissue Organ Cult.* 81: 175-181.

Usha, R. and Swamy, P.M. 1998. *In vitro* micropropagation of sweet wormwood (*Artemesia annua* L). *Phytomorphology.* 48: 149-154.

Vadawale, A.V., Mehta-Bhatt, P. and Dave, A.M. 2004. Rapid *in vitro* propagation of Ashwagandha (*Withania somnifera*) through axillary bud multiplication and indirect organogenesis. *Phytomorphology* 54:59-64.

Vander-Heijden, R., Verpoorte, R., Ten-Hoopen, H.J.G. 1989. Cell and tissue cultures of *Catharanthus roseus* (L.) G. Donn : a literature Survey. *Pl. Cell Tissue Organ Cult.* 18:231

Van-Geldre, E., Vergauwe, A. and Van-Den-Eechout, E. 1997. Agrobacterium tumefaciens mediated transformation of *Artemesia annua* L. and regeneration of transgenic plants. *Pl. Mol Biol.* 33:199-20

Vanhala, L., Hiltunen, R and Oksman-Caldenty, M.1995. Virulence of different *Agrobacterium* strains on hairy root formation of *Hyscyamus muticus*. *Pl. Cell Rep.* 14: 236-240.

Van-Uden, W., Pras, N. and Maling, T.M. 1990. On the improvement of the podophyllotoxin production by phenyl propanoid precursor feeding to cell cultures of *Podophyllum hexandrum*. *Pl. Cell Tissue Organ Cult.* 23:217-224

Van-Wordragen, M.F., Pieter, B.F., Ouwerkerk, C.M. and Dons, A.J.M. 1992. *Agrobacterium rhizogenes* mediated induction of apparently untransformed roots and callus in Chrysanthemum. *Pl. Cell Tissue Organ. Cult.* 30:149-157.

Varghese, S. 2006. Genetic transformation for hairy root induction and enhancement of secondary metabolites in Aswagandha (*Withania somnifera* L. Dunal.). M.Sc.(Ag) thesis, Kerala Agricultural University, Trichur, 199p.

Vazquez-Flota, F., Valenzuela, O., Ham, M.M.L. and Coello-Coello, J. 1994. Catharanthine and ajmalicine synthesis in *Catharanthus roseus* hairy root cultures. *Pl. Cell Tissue Organ Cult.* 38: 273-279

Veleiro, A.S., Burton, G. and Gros, E.G. 1985. Biosynthesis of withanolides in *Acnistus breviflorus*: Biogenetic relationships among the main withanolides. *Phytochemistry* 24: 2573-2575

Vergauwe, A., Cammaert, R., Vanberghe, D., Gentello, C., Montgu, V.M. and Vanden-Eeckout, E. 1996. *Agrobacterium tumefaciens* mediated transformation of *Artemesia annua*. *Pl. Cell Rep.* 15: 929-933

Vergauwe, A., Van-Geldre, E., Inze, D., Van-Montagu, M. and Vanden-Eeckhout, E. 1998. Factors influencing *Agrobacterium tumefaciens* mediated transformation of *Artemesia annua* L. *Pl. Cell Rep.* 18: 105-110

Vialine, F and Casse-Delbart, F.1987. Independent induction of transformed roots by the TL and TR regions of the Ri plasmid of agropine type *Agrobacterium rhizogenes*. *Mol. Gen. Genet.* 206: 17-23.

Walker, T.S., Bais, H.P. and Vivanco, J.M. 2002. Jasmonic acid induced hypericin production in cell suspension cultures of *Hypericum perforatum*. *Phytochemistry*. 60:289-293

Wallaart, T.E., Pras, N., Quax, W.J. and Beekmun, A.C. 2000. Seasonal variation of artemisinin and its biosynthetic precursors in plants by *Artemesia annua* of different geographical origin: proof for the existence of chemotypes. *Planta Med.* 66: 57-62

Walwalkar, M.G. 2004. A new antimalarial candidate. *Current Sci.* 87: 1035-1037.

Wang, H., Ge, L., Ye, H.C., Chong, K., Liu, B.Y. and Li, G.F. 2004. Studies on the effect of *fpf1* gene on *Artemesia annua* flowering time and linkage between flowering and artemisinin biosynthesis. *Planta Med.* 70: 347-352

Wang, J. W. and Tan, R.X. 2002. Artemisinin production in *Artemesia annua* hairy root cultures with improved growth by altering the nitrogen source in medium. *Biotech Lett.* 24: 1153-1156

Wang, J. W., Kong, F.X. and Tan, R.X. 2002. Improved artemesinin accumulation in hairy root cultures of *Artemisia annua* by (22S, 23S) homobrassinolide. *Biotech Lett.* 24: 1573-1577

Wang, Y., Zhang, H., Zhao, B. and Yuan, X. 2001. Improved growth of *Artemesia annua* L. hairy roots and artemisinin production under red light condition. *Biotech. Lett.* 23:1971-1973

Watanabe, S., Imakawa, S. and Yakuwa, T. 1991. Conditions of rooting from shoot apices for mass propagation in asparagus. *J. Fac. Agric. Hokkaido Univ.* 64: 292-303

Weathers, P.J. and Kim, Y. J. 2001. Transformed roots of *Artemesia annua* exhibits an unusual pattern of border cell release. *In Vitro Cell. Dev. Biol.Pl.* 37: 440-445

Weathers, P.J., Cheetham, R.D., Follansbee, E. and Teoh, K. 1994. Artemisinin production by transformed roots of *Artemesia annua. Biotech. Lett* 16:1281-1286

Weathers, P.J., Gonzales, D.J., Kim, Y.J., Souret, F.F. and Towler, M.J. 2004. Alteration of biomass and artemisinin production in *Artemesia annua* hairy roots by media sterilization method and sugars. *Pl. Cell Rep.* 3:414-418

Weathers, P.J., Hemavanh, D.D., Walcerz, D.B. and Cheetham, R.D. 1997. Interactive effects of nitrate and phosphate salts, sucrose, and inoculum culture age on growth and sesquiterpene prodution in *Artemesia annua* hairy root cultures. *In Vitro Cell. Dev. Biol. Pl.* 33: 309-312.

Weathers, P.J., Smith, T., Hemmavahh, D., Follansbee, E., Ryan, J. and Cheetham, R. 1996. Production of the antimalarial artemisinin by transformed roots of *Artimesia annua*. *Acta Hort.* 426:157-163

Wei-Chen, S., Jia-Xin, H., Wei-Yiy, Y., Dungan, D. and Xiu-Fang, J. 1992. Antitumour activites of artemisinic acid and arteannuin B *in vitro*. *Acta Pharmacol. Sin.* 13: 541-543.

Whipkey, A., Simon, J.E. Charles, D.J. and Janick, J. 1992. *In vitro* production of artemisinin from *Artemisia annua* L. *J. Herbs Spices Medicinal Pl.* 1:15-25.

White, F.F. and Nester, E.W. 1980. Hairy root: Plasmid encode virulence trait in *Agrobacterium rhizogenes*. *J. Bacetriol.* 41: 1134-1141.

White, F.F., Taylor, B.H., Huffman, G.A., Gordon, M.P. and Nester, E.W. 1985. Molecular and genetic analysis of transferred DNA regions of root inducing plasmid of *Agrobacterium rhizogenes*. *J. Bacteriol.* 164: 33-44.

Wibberley, M.S., Lenton, J.R. and Neil, S.J. 1994. Sesquiterpenoid phytoalexins produced by hairy roots of *Nicotiana tabaccum*. *Phytochemistry* 37: 349-351

Woerdenbag, H.J., Luers, J.F.J., Van-Uden, W., Pras, N., Malingre, T.M. and Alfermann, A.W. 1993. Production of the new antimalarial drug artemisinin in shoot cultures of *Artemisia annua* L. *Pl. Cell Tissue Organ Cult.* 32:247-257.

Woerdenbag, H.J., Lugt, C.B. and Pras, N. 1990. *Artemesia annua* L.: a source of antimalarial drugs. *Pharm Week Sci.* 12: 169-181.

Woerdenbag, H.J., Pras, N., Bos, R., Visser, J.F., Hendriks, H. and Malingre, T.M. 1991. Analysis of artemisinin and related sesquiterpenoids from *Artemisia annua* L. by combined gas chromatography/mass spectrometry. *Phytochem. Anal.* 2:215-219.

Woerdenbag, H.J., Pras, N., Chan, N.G., Bang, B.T., Bos, R., Van Uden, W., Van Y.P., Boi, N.V., Batterman, S. and Lugt, C.B. 1994. Artemisinin, related sesquiterpenes, and essential oil in *Artemisia annua* during a vegetation period in Vietnam. *Planta Med.* 60:272-275.

Wysokinska, H and Chmiel, A. 1997. Transformed root cultures for biotechnology. *Acta biotech.* 17: 131-159

Xu, T., Zhang, L., Sun, X., Zhang, H. and Tang, K. 2004. Production and analysis of organic acids in hairy root cultures of *Isatis indigotica* Fort. (Indigo wood). *Biotech Appl. Biochem.* 39: 123 – 128.

Yoshikawa, T. and Furuya, T. 1987. Saponin production by cultures of *Panax ginseng* transformed with *Agrobacterium rhizogenes. Pl. Cell Rep.* 6: 449-453.

Yoshimatsu, K., Shimomura, K., Yamazaki, M., Saito, K. and Kivchi, F. 2003. Transformation of Ipecac (*Cephaelis ipecacuanha*) with *Agrobacterium rhizogenes. Planta Med.* 69: 1018–1023.

You, T.Y., Yun, N.M., Yu-rong, Z., Lan-na, L., Shu-lian, C., Mu-quan, Z., Xiu-zhen, W., Zheng, J. and Xiao-tian, L. 1982. Studies on the constituents of *Artemesia annua* Part 2. *Planta Med.* 44: 143-145.

Yu, S.H., Lu, C.F., Li, L. and Pan, R.C. 2001. Factors affecting genetic transformation of *Pueraria lobata* by *Agrobacterium rhizogenes. Chinese J. appl. Environ. Biol.* 7: 474-477

Zambryski, P. 1988. Basic processes underlying *Agrobacterium* mediated DNA transfer to plant cells. *A. Rev. Genet.* 22: 1-30.

Zambryski, P., Tempe, J. and Schell, J. 1989. Transfer and function of T-DNA genes from *Agrobacterium* Ti and Ri plasmid in plants. *Cell.* 56: 193-201.

Zdravkovic-Korac, S., Muhovski, Y., Druart, P., Calic, D. and Radojevic, L. 2004. *Agrobacterium rhizogenes* mediated DNA transfer to *Aesculus hippocastanum* L. and the regeneration of transformed plants. *Pl. Cell Rep.* 22: 698-704

*Originals not seen

Appendices

APPENDICES

Appendix 1. Composition of different tissue culture media

Chemical	MS (mg l^{-1})	SH (mg l^{-1})	B5 (mg l^{-1})
Inorganic constituents			
$(NH_4)\ NO_3$	1650	-	600
KNO_3	1900	2500	2100
KH_2PO_4	170	-	250
$MgSO_4.7H_2O$	370	400	400
$(NH_4)\ H_2PO_4$	-	300	-
$CaCl_2.2H_2O$	440	200	450
Na_2 EDTA	37.3	15	-
$FeSO_4.7H_2O$	27.8	20	-
EDTA Na ferric salt	-	-	43
$MnSO_4.H_2O$	22.3	10	10
$ZnSO_4.7H_2O$	8.6	1.0	2.0
H_3BO_3	6.2	5.0	3.0
KI	0.83	1.0	0.8
$Na_2MoO_4.2H_2O$	0.25	0.1	0.25
$CuSO_4.5H_2O$	0.025	0.2	0.025
$CoCl_2.6H_2O$	0.025	0.1	-
Organic constituents			
Glycine	2.0	-	-
Nicotinic acid	0.5	5.0	1.0
Pyridoxine acid HCl	0.5	0.5	1.0
Thiamine HCl	0.1	5	10.0
Sucrose	30000	30000	30000
Myoinositol	100	1000	250
P^H	5.8	5.8	5.8

APPENDIX II

Composition of bacterial culture media

Constituent	NA ($g\ l^{-1}$)	YEM ($g\ l^{-1}$)	YEB ($g\ l^{-1}$)	LBA ($g\ l^{-1}$)	YEP ($g\ l^{-1}$)	XM ($g\ l^{-1}$)
Beef extract	1.0	-	5.0	-	-	-
K_2HPO_4	-	0.5	-	-	-	-
Yeast extract	2.0	1.0	1.0	5.0	1	10
$MgSO_4.7H_2O$	-	0.2	0.5	-	-	-
Peptone/Trypton	5.0	-	5.0	10.0	10	-
Mannitol	-	10.0	-	-	-	-
NaCl	5.0	0.1	-	10.0	5	-
Sucrose		-	5.0	-	-	-
Galactose	-	-	-	-	-	20
Agar	15.0	20	20	20	20	20
Adjust pH to 7.0			7.2			7.2

APPENDIX III

Reagents used for DNA isolation

1) Extraction buffer (2X)

   a) 2 per cent CTAB - 4 g/200 ml
   b) 10 m*M* Tris pH 8 - 2.422 g/200 ml
   c) 20 m*M* EDTA pH 8 - 1.48896 g/200 ml
   d) 1.4*M* NaCl - 29.7792 g/400 ml
   e) 1 per cent PVP - 4 g/200 ml

2) 10 per cent CTAB - 10g /100ml

3) TE buffer

   (Tris HCl –10.0 m*M*; EDTA - 1.0 m*M*)
   Tris HCl 1.0 *M* (pH 8.0) - 1.0 ml
   EDTA 0.25 *M* (pH 8.0) - 0.4 ml
   Distilled water - 98.6 ml

   Autoclaved and stored at room temperature.

4) Ice-cold Isopropanol
5) Chloroform- isoamyl alcohol (24:1 v/v)
6) Ethanol (70 per cent)

APPENDIX IV

Buffer and dyes used in gel electrophoresis

1) 6X Loading / Tracking dye

Bromophenol blue	-	0.25 per cent
Xylene xyamol	-	0.25 per cent
Glycerol	-	30 per cent

The dye was prepared and kept in fridge at 4 °C.

2) Ethidium bromide (intercalating dye)

The dye was prepared as a stock solution of 10 mg ml^{-1} in water and was stored at room temperature in a dark bottle.

3) 50X TAE buffer (pH8.0)

Tris base	-	242.0 g
Glacial acetic acid	-	57.1 ml
0.5 *M* EDTA (pH 8.0)	-	100 ml
Distilled water	-	1000 ml

The solution was prepared and stored at room temperature.

APPENDIX V

Reagents used for cosmid isolation

1) Solution I (Resuspension buffer)

Glucose	-	50 m*M*
Tris	-	25 m*M*
EDTA	-	10 m*M*
pH	-	8.0

2) Solution II (Lysis buffer)

NaOH	-	0.2 *M*
SDS	-	0.1 per cent

3) Solution III (Neutralization buffer)

CH_3COOK	-	5*M*
pH	-	5.5

Appendix VI

Reagents for Southern hybridization

1) Pre hybridization solution
   - 6X SSC
   - 5X Denhardts reagent
   - 0.5 per cent SDS
   - Denatured salmon sperm DNA- 10 mg ml^{-1}

2) Hybridisation solution
   - 6X SSC
   - 5X Denhardts reagent
   - 0.5 per cent SDS
   - Radiolabelled probe

   a) 20X SSC

   Sodium chloride - 175.3 g
   Sodium citrate (trisodium citrate 2-hydrate) - 88.2 g
   The pH was adjusted to 7.0 and the final volume was made up to 1000 ml with distilled water.

   b) Denhardts reagent (50X)

   Ficoll (type 400) - 5.0 g
   Poly vinyl pyrolidone - 5.0 g
   Bovine sperm albumin (fraction V) - 5.0 g
   Finally made up to 500 ml with distilled water, filter sterilized and stored at –20 °C.

   c) Salmon sperm DNA

   Stock solution 10 mg ml^{-1} was boiled for 10 minutes and stored at –20°C as aliquots. Just before use it was heated in water bath for 5 minutes and quickly chilled on ice.

3) Developer solution (For developing X- ray film)

   Metol - 4 g
   Sodium sulfite (anhydrous) - 300 g
   Hydroquinone - 16 g
   Potassium bromide - 10 g
   Sodium carbonate (anhydrous) - 200 g
   The chemicals were dissolved in distilled water and the final volume was made up to 2.0 litre.

4) Fixer

   Hypo (Sodium thiosulfate) - 200 g/ 2 litre

GENETIC TRANSFORMATION IN *ARTEMESIA ANNUA* L. FOR HAIRY ROOT INDUCTION AND ENHANCEMENT OF SECONDARY METABOLITES

By

SHANEEJA. V. M.

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(Plant Biotechnology)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2007

ABSTRACT

The present study entitled "Genetic transformation in *Artemesia annua* L. for hairy root induction and enhancement of secondary metabolites" was carried out at the Centre for Plant Biotechnology and Molecular Biology of the College of Horticulture, Vellanikkara. The study was undertaken to standardize the *in vitro* regeneration protocol in *Artemesia annua* from different explants, to standardize the genetic transformation using *A. rhizogenes*, to standardize the biochemical techniques for the estimation of secondary metabolites in *Artemesia annua* and also to enhance the secondary metabolite production in the hairy root cultures of *Artemesia annua*.

An efficient method for *in vitro* plant regeneration was developed in *Artemesia annua*. Different explants such as leaves, petiole, shoot tip, nodal segments, inflorescence segments and roots of *Artemesia annua* were used for the study.

Maximum regeneration from leaf explants was in MS with 0.5 mg l^{-1} BAP. Shoot buds produced from leaf explant showed good multiplication in the same media. MS was found to be the best basal media for regeneration from leaf explants.

The leaf originated callus produced regeneration in MS with 3 mg l^{-1} BAP. The shoot tip and inflorescence bits showed maximum regeneration in 0.2mg l^{-1} NAA and 0.5 mg l^{-1} BAP. Nodal segments showed maximum regeneration in MS with 0.1 mg l^{-1} with NAA and 0.2 mg l^{-1} BAP.

The roots taken from *in vitro* rooted plantlets showed high callusing but failed to regenerate by direct organogenisis. However, a green coloured structure was obtained from root callus in MS media supplemented with BAP 3 mg l^{-1} and NAA 0.1 mg l^{-1}.

The shoot/ shoot buds showed good elongation in MS media with GA_3 0.2 mg l^{-1}. The shoots were successfully rooted in half MS with 0.5 mg l^{-1} IBA and 2 per

cent sucrose. The plants were successfully hardened and transferred to large pots in the green house.

Genetic transformation was carried out in *A. annua.* using three different *A. rhizogenes* strains like A4, ATCC 15834 and MTCC 2364 for inducing hairy roots. The explants such as leaf segments shoot tips and nodal segments were used for genetic transformation. Here the influence of different parameters such as type of explants, type of bacterial inoculum, co-cultivation periods and acetosyringone effects on transformation frequencies were studied. Among the three *A. rhizogenes* strains used, ATCC 15834 produced the highest transformation efficiency. Acetosyringone (100 μM) enhanced the transformation percentages in all the strains.

The hairy root cultured in hormone free basal media showed high lateral branching. Among the four liquid media tested, B_5 with 3.0 per cent sucrose was found to be superior in promoting hairy root growth followed by B_5 with 2.0 per cent sucrose, MS and half MS.

The confirmation of transformation was obtained by opine detection and PCR.

A Thin Layer Chromatographic method was employed for artemisinin estimation. Artemisinin obtained from Sigma Chemicals, USA was used as the standard in estimation studies. Silica gel_{60} F_{254} plate with hexane- diethyl ether (1:1) as the solvent system was used. The pink spot was observed by immersing the plates in a pool of freshly prepared glacial acetic acid: conc. sulphuric acid: anisaldehyde (50:1:0.5) followed by drying in a chromatographic oven at 110°C for 15 min. No artemisinin was detected in roots taken from plants grown in the field, *in vitro* roots and hairy roots induced by MTCC 2364. Rooting of *in vitro* shoots enhanced the artemisinin content.

Enhancement of secondary metabolite production was studied using techniques such as addition of osmoregulants, precursor feeding and elicitation. The artemisinin content in the hairy root biomass and the culture medium were estimated. The

osmoregulant PEG (2.0 % and 5.0 %) and the precursor methionine (1 m*M*) and yeast extract (2.5g l^{-1}) failed to enhance the artemisinin content. However, the biotic elicitor *Aspergillus* homogenate (250µl /125 ml) elicited a positive influence on the biosynthesis of artemisinin in the hairy root cultures.