GENETIC TRANSFORMATION IN SARPAGANDHA (*Rauvolfia serpentina* (L.) Benth.) FOR ENHANCEMENT OF SECONDARY METABOLITE PRODUCTION

By

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THESIS

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DECLARATION

I, S. Thanga Suja (2007-11-118) hereby declare that this thesis entitled "Genetic transformation in sarpagandha (*Rauvolfia serpentina* (L.) Benth.) for enhancement of secondary metabolite production**"** is a bonafide record of research work done by me during the course of research and this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled **"**Genetic transformation in sarpagandha (*Rauvolfia serpentina* (L.) Benth.) for enhancement of secondary metabolite production**"** is a record of research work done independently by **Miss S. Thanga Suja** 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.

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Dedicated to my
Daddu and Mom

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S.T. Suja

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ABBREVIATIONS

%	Per cent
µg	Micro gram
µl	Micro litre
µ*M*	Micro molar
2,4-D	2, 4-dichlorophenoxyacetic acid
A	Ampere
A. rhizogenes	*Agrobacterium rhizogenes*
$AgNO_3$	Silver nitrate
ATCC	American Type Culture Collection
BA	Benzyl amino purine
bp	base pair
^0C	Degree Celsius
C_6H_6	Benzene
$CaCl_2$	Calcium chloride
CCC	Cycocel
CH_3CN	Methyl cyanide
CH_3COOH	Acetic acid
CH_3OH	Methanol
$CHCL_3$	Chloroform
cm	Centi metre
CTAB	Cetyl Trimethyl Ammonium Bromide
$CuSO_4$	Copper sulfate
DIM	Direct Inoculation Method
DMSO	Dimethyl Sulphoxide
DNA	Deoxy ribo Nucleic acid
dNTP	deoxy ribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
$FeCl_3$	Ferric Chloride
FW	Fresh weight
g l^{-1}	Grams per litre
GA	Gibberellic acid
GFP	Green Flourescent protein
H_2SO_4	Sulphuric acid
H_3PO_4	Phosphoric acid
ha	Hectare

HCl	Hydrochloric acid
$HgCl_2$	Mercuric Chloride
HPLC	High Pressure Liquid Chromatography
hr(s)	Hour (s)
HVPE	High Voltage Paper Electrophoresis
IAA	Indole acetic acid
IBA	Indole butyric acid
kD	Kilo Dalton
Kg	Kilo gram
KN	Kinetin
LBA	Luria Bretani Agar
Lux	Lux
M	Molar
MeOH	Methanol
mg l^{-1}	Milligrams per litre
min	Minute
ml	Milli litre
mm	Milli metre
m*M*	Milli Molar
MS	Murashige and Skoog`s medium
MTCC	Microbial Type Culture Collection
N	Normality
NA	Nutrient Agar
Na_2HPO_4	Disodium phosphate
NA_2SO4	Sodium sulfate
NAA	Naphthalene acetic acid
NaH_2PO_4	Monosodium phosphate
NaOH	Sodium hydroxide
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
ppm	Parts per million
psi	pounds per square inch

rpm	revolutions per minute
S	second (s)
SDS	Sodium Dodecyl Sulphate
SM	Suspension culture method
TAE	Tris acetate EDTA buffer
T-DNA	Transfer DNA
TDZ	Thidiazuron
TE	Tris EDTA buffer
T_L	Transfer (left) DNA
TLC	Thin layer Chromatography
T_R	Transfer (Right) DNA
UV	Ultra violet
V	Volume
V/ cm	Volt/ cm
w/v	Weight/ Volume
WPM	Woody Plant Medium
YE	Yeast extract
YEB	Yeast Extract Broth
YEM	Yeast Extract Mannitol
α	Alpha
2,iP	2, isopentenyl adenine

Introduction

1. INTRODUCTION

Mankind is dependent on plants as the source of carbohydrates, proteins and fats for food and shelter since centuries. In addition, plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colors, biopesticides and food additives.

The use of plants as medicine has an ancient background and almost every civilization has a history of medicinal plant use. Approximately 80 per cent of the people in the world rely on traditional medicine for their primary healthy care needs, and about 85 per cent of traditional medicine involves the use of plant extracts. Worldwide, 121 clinically useful prescription drugs are derived from plants (Payne *et al*., 1991)

A wide range of such plant derived molecules of pharmaceutical interest accumulates in plant roots as secondary metabolites. One such important medicinal shrub of family Apocynaceae is *Rauvolfia serpentina* (L.) Benth. known as Sarpagandha. This versatile medicinal species is distributed in India in the foot hills of Himalayas from Punjab eastwards to Assam, lower hills of Gangetic plains, Eastern and Western ghats, parts of central India and in Andamans.

The dried root extracts of *Rauvolfia serpentina* is used in ayurvedic and unani systems of medicine for treating various mental and physical aliments, epilepsy, asthma, high blood pressure etc. In traditional herbal medicine, the root was brewed as a tea. The purified alkaloid, reserpine was isolated in 1952 and was the first modern drug used for the treatment of hypertension. Reserpine was found to interfere with the human sympathetic nervous system, thereby initiating the pharmacotherapeutic control of hypertension.

More than 200 alkaloids are known to be present in *Rauvolfia* roots. The reserpine, ajmaline and serpentine groups of alkaloids identified in *Rauvolfia serpentina* are the major constituents responsible for its biomedical properties.

The roots contain 0.15 per cent reserpine- rescinnamine group of alkaloid and has immense hypotensive properties (Panda, 2003). The world requirement of dried *Rauvolfia* roots is around 25,000 tonnes per annum and according to a rough estimate more than 630 tonnes of *Rauvolfia* are utilized in our country to extract the active ingredient from the root (Farooqi and Sreeramu, 2004).

Huge demand for this alkaloid in the world pharmaceutical industry resulted in depletion of wild sources. Export of *Rauvolfia* roots from India was banned from 1969 and presently this species is in one of the appendices included in the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) (Panda, 2003).

Tissue and cell cultures such as callus and cell suspension culture systems have been established for the production of secondary metabolites. Compared to cell cultures, hairy root culture has been used to produce secondary metabolites at comparable, or greater levels than those of the intact plant (Sevon and Oksman-Caldentey, 2002).

Hairy roots can be obtained by genetic transformation of the wounded plant tissue by a pathogenic soil bacterium, *Agrobacterium rhizogenes*. This bacterium is capable of infecting a wide range of plants, causing roots to proliferate at the infection site. More importantly, hairy roots grow rapidly on auxin free medium and are capable of sustaining biochemical and genetic stability. Therefore, hairy root cultures hold greater promise for viable industrial application and also stand as an alternative to plant cultures for the production of secondary metabolites.

The need for *Rauvolfia* root alkaloid prompted the investigation of induction of hairy root culture in *Rauvolfia serpentina*. This study reports the establishment and analysis of hairy roots of *Rauvolfia serpentina* with the following objective:

- To genetically transform *Rauvolfia serpentina* (L.) Benth. using *Agrobacterium rhizogenes* by inducing hairy roots so as to enhance the secondary metabolite production

Review of Literature

2. REVIEW OF LITERATURE

Plants are the traditional source of many chemicals used as pharmaceuticals. Most valuable phytochemicals are products of plant secondary metabolism. Medicines in common use such as aspirin, digitalis are derived from plants. Many of these natural products cannot be easily synthesized artificially and must be extracted from plants.

Various strategies have been employed for improving the secondary plant products in *in vitro* systems. However the *in vitro* culture system tends to be unstable and in most cases the compounds were undetectable or were accumulated at low levels in the cultures (Verpoorte *et al*., 2002).

In vitro genetic transformation of plant material with *Agrobacterium rhizogenes* strains helped to overcome some of the difficulties of *in vitro* plant organ cultures (Nader *et al*., 2006). Hairy roots, transformed with *A. rhizogenes,* have been found to be suitable for the production of secondary metabolites because of their stable and high productivity. A large number of plant species including medicinal plants have been successfully transformed with *A. rhizogenes. Rauvolfia serpentina* is an important medicinal plant with reserpine and rescinnamine alkaloids which have been used in the allopathic system for the treatment of hypertension, cardiovascular diseases and as a sedative, tranquilizing agent. Since the natural production in the plant is rather low, different approaches have already been made to increase the production of the alkaloid of which, one is genetic transformation using *A. rhizogenes*.

Literature pertaining to these aspects are presented in this chapter.

2.1 *RAUVOLFIA SERPENTINA*

Rauvolfia serpentina (L.) Benth commonly known as Sarpagandha is an important medicinal shrub which has been used in the Indian sub continent for more than 4000 years. *R. serpentina* was pronounced as the "Wonder drug of India" in 1949, when a British journal reported the plant to be clinically effective in treating high blood pressure. Its roots are used as a valuable remedy for high blood pressure, insomnia, anxiety, excitement, schizophrenia, insanity, epilepsy, hypochondria and other disorders of the central nervous system (Kirtikar and Basu, 1993).

2.1.1 Occurrence and distribution

2.1.1.1 World

Monachino (1954) reported that, *Rauvolfia* has an Indo-Malayan origin, widely distributed in India, Sri Lanka, Andaman Islands, Burma, Zaire, Java and Sumatra. CSIR (1969) reported that, *Rauvolfia* is a large genus of shrubs or trees distributed in the Tropical Asia, Africa, and America and five species were reported in India. Sahu (1979) enlisted the names of 155 species of *Rauvolfia* found in different parts of the world, mainly Tropical Asia, Africa and America. Gauniyal *et al*. (1988) reported 175 species found in tropical and subtropical regions of the world. They also reported that *Rauvolfia* was generally found in the moist regions, with annual rainfall of 150-375 cm and altitude up to 1200 m. Thailand is the chief exporter of *Rauvolfia* alkaloids followed by Zaire, Bangladesh, Sri Lanka, Indonesia and Nepal.

2.1.1.2 India

Hooker (1882) described seven species of *Rauvolfia* found in India. They are *Rauvolfia serpentina, R. penguana, R. densiflora, R. micrantha, R. beddomei, R. microcarpa*, and *R. decurva.* It is naturally distributed in the foothills of Himalayan range and South West coast in Kerala. The species is also distributed sporadically in

Andhra Pradesh, Bastar forest of Madhya Pradesh, Orissa and Chota Nagpur of Bihar. At present, it is cultivated in small areas, scattered far and wide in states of Uttar Pradesh and Uttarakhand.

R. serpentina is one among the species protected under CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) which regulates international trade in species threatened with extinction (Ved *et al*., 1998). According to Ravikumar and Ved (2002) *R. serpentina* is an endangered species of conservation concern in Southern India mainly due to the decline in area of occupancy, extent of occurrence and quality of habitat.

2.2 BOTANICAL DESCRIPTION

R. serpentina is an erect, evergreen perennial under shrub reaching a height of 75 to 100 cm. Leaves are simple in whorls of three, opposite or even alternate with a short petiole, glandular at the base; elliptical, lanceolate and bright green when young. Inflorescence is terminal or sometimes axillary, usually dense crowded, many flowered cymes forming a hemispheric head with a long peduncle. The flowers are small, pedicalate and hermaphrodite. Fruit is an ovoid drupe, purplish black in color at the stage of maturity. Seeds are wrinkled and ovoid in nature. Roots are prominent, tuberous, usually branched, 0.5-2-5 cm in diameter. Tap root grows 40-60 cm deep in soil. Outer bark of the root is corky with irregular longitudinal fissures. The fresh roots emit a characteristic acrid aroma, and are bitter to taste (Kurian and Sankar, 2007)

2.3 CYTOLOGY

Raghavan (1957) and Tapadar (1963) confirmed that *Rauvolfia serpentina* is a diploid with a basic chromosome number 2n=22. Rajkhora (1964) reported a series of polyploidy in the genus *Rauvolfia*. *R. serpentina* is diploid with 2n=22, *R. densiflora* is tetraploid with 2n=44 and *R. tetraphylla* is hexaploid with 2n=66.

Banerjee and Sharma (1989) reported that *R. serpentina* and *R. vomitoria* are diploids with 2n=22 while *R. canescens* is hexaploid with 2n=66.

2.4 CHEMICAL COMPOSITION AND PHARMACOLOGICAL ACTIVITY

More than 204 alkaloids were isolated from different species of *Rauvolfia* of which reserpine, rescinnamine, deserpidine, ajmalicine, neoajmaline, serpentine and α-yohimbine are pharmacologically important alkaloids. Alkaloids are classified into 3 groups viz. reserpine, ajmaline and serpentine groups (Shimolina *et al*., 1984). Reserpine group comprising reserpine, rescinnamine and deserpine act as hypotensive, sedative and tranquilizing agents. Overdose causes diarrhoea, bradycordia and drowsiness. Ajmaline group comprising ajmaline, ajmalicine, ajmalinine and iso-ajmaline stimulates central nervous system, respiration and intestinal movement with slight hypotensive activity. A number of new derivatives of ajmaline and isoajmaline have been prepared. Serpentine group comprising, serpentine, serpentinine and alstonine are mostly antihypertensive. Value of sarpagandha roots depends on total alkaloid content, and proportion of reserpine and ajmalicine alkaloids present in it. The pharmacological activity of *Rauvolfia* roots is due to the presence of several alkaloids of which reserpine is the most important. The stem and leaves have a low alkaloid content. Alkaloids abound mainly in the bark of the root which constitutes 40-50 per cent of the whole root and contains 90 per cent of the total alkaloids. The fibrous roots are more active than the interior of the main tap root. The seed contains 0.2-0.3 per cent alkaloid content. The total alkaloid content of the *Rauvolfia* varies from 1.49-2.38 per cent.

Cook (1905) reported that total alkaloid content of *Rauvolfia* differs with location and season. The root of *R. serpentina*, particularly its bark is used entirely for the extraction of pure alkaloids. The average total yield of alkaloids is 2.4 per cent in the root bark as compared to 0.4 per cent in the root wood. The fibrous roots contain an average of 2.52 per cent of the total alkaloids, while stem and leaves contain alkaloids in smaller amount ranging between 0.45-0.54 per cent.

2.5 MICROPROPAGATION

The best commercial application of tissue culture is in the production of true to type plants at a very rapid rate compared to the conventional methods (Levy, 1981). Murashige (1974) advocated the possibility of three routes of *in vitro* propagule production, which included enhanced release of axillary buds, production of adventitious shoots through organogenesis and somatic embryogenesis. Vasil and Vasil (1980) reported that the tissue culture derived plantlets grow faster and mature earlier than seed propagated plants.

2.5.1 *Rauvolfia serpentina*

In vitro multiplication and regeneration of cytologically stable plants of *R. serpentina* through shoot tip culture was attempted by Mukhopadhyay *et al*. (1991). They observed that rate of multiplication and growth of individual shoot tip was better in the MS medium containing NAA (0.5 mg l^{-1}) and BAP (2.0 mg l^{-1}). For root regeneration, the best response was obtained in the medium containing NAA (3.0 mg l^{-1}) and kinetin (1.0 mg l^{-1}) where tap root with numerous lateral roots originated.

Roja and Heble (1996) reported that plantlets were successfully regenerated from shoot cultures of *R. serpentina* initiated from axillary meristems on media containing BA (4.44 μ*M*) + NAA (0.54 μ*M*). Rooting was initiated in White's basal medium supplemented with NAA (0.54 μ*M*). They observed that tissue culture derived plants of *R. serpentina* were similar to normal plants in their morphological characteristics and chemical constitution. The roots of tissue cultured derived plants were thick and tuberous where as the roots of the plants established from stem cutting were long and slender with some lateral branches.

Sarker *et al*. (1996) reported that multiple shoots were induced from nodal segments and shoot apices of *R. serpentina* and MS medium containing 1.0 mg l^{-1} IBA and 0.1 mg l^{-1}NAA was found to give the best shoot proliferation rate. Callus

formed at cut bases of the explants produced shoots when sub cultured on media containing low concentration of BA (0.5 or 0.1 mg l^{-1}) and NAA (0.1 mg l^{-1}). The *in vitro* proliferated shoots were rooted and later transferred to soil.

According to Baksha *et al*. (2007), multiple shoots (eight shoots per explant) of *R. serpentina* have been induced on MS medium containing 4.0 mg l^{-1} BAP with 0.5 mg l^{-1} NAA within 10-15days. The elongated shoots rooted well in half strength MS medium with 0.5 mg l^{-1} NAA.

Sehrawat *et al*. (2002) reported induction of multiple shoots (2 to 4) from shoot apices and nodal segments on MS medium supplemented with benzyladenine (1.5 mg l^{-1}) and NAA (0.5 mg l^{-1}) in *R. serpentina.*

Ahmad *et al*. (2002) reported that the shoots of *R. serpentina* cultured on MS medium supplemented with 0.5 mg l^{-1} BA and 0.2 mg l^{-1} NAA as the best combination for induction of callus and shoot bud formation but it failed to support elongation of shoots. The cultured shoots elongated on MS medium with 2.0 mg l^{-1} BA and 0.05 mg l^{-1} NAA and the regenerated shoots rooted well on 0.2 mg l^{-1} IBA and 0.2 mg l^{-1} NAA combination

2.5.2 Other *Rauvolfia* species

Sudha and Seeni (1996) induced high frequency formation of shoots (77 per cent) from nodal explants of *R. micrantha* in MS medium. Shoot formation was maximum when node explant was cultured in a combination of 13.2 μ*M* BA and 2.68 μ*M* NAA. Repeated sub-culturing of the shoot tips and single nodes at 6-week intervals for over a year in a combination with 4.4 μ*M* BA and 0.27 μ*M* NAA enabled mass multiplication of shoots without any evidence of decline.

Patil and Jayanthi (1997) reported proliferation of shoots (75 per cent in *R. tetraphylla* and 82 per cent in *R. micrantha*) from axillary bud on MS medium supplemented with BA (2.0 mg l^{-1}) within 15 days of inoculation. Further

multiplication of the shoots was best achieved when the medium contained adenine sulphate (0.005 mg l^{-1}) and lower BA level (0.05 mg l^{-1}). A high rate of multiplication rate (1: 9) in both species (*R. tetraphylla* and *R. micrantha*) and the successful weaning (*ex vitro*) of 60 per cent of such shoots was also obtained.

Sharma *et al*. (1999) reported that MS medium containing kinetin (0.1-0.2 mg l^{-1}) + BAP (0.4-0.5 mg l^{-1}) induced high frequency of multiple shoot development in *R. tetraphylla*. Root development occurred from the base of *in vitro* shoots after 20-25 days of culture on NAA (0.2- 0.6 mg l^{-1}) containing media.

Ghosh and Banerjee (2003) reported that maximum callus formation from the base of nodal explants *(R. tetraphylla)* was recorded on NAA (1.0 mg l^{-1}) and BA (5.0 mg l^{-1}). Higher level of BA with NAA suppressed shoot elongation but increased the shoot multiplication rate. Best shoot bud multiplication was recorded in IAA (1.0 mg l^{-1}) and BA (5.0 mg l^{-1}) where approximately 15.5 shoots could be counted per explant. Among the other plant growth regulators used, 2, 4-D was found effective in callus formation, but the growth of the callus ceased and it gradually turned brown within 60 days. Shoot apices cultured in MS containing IBA (0.5 - 2.0 mg l^{-1}) or NAA (0.5 - 2.0 mg l^{-1}) induced roots with in 60 days.

Faisal *et al*. (2005) observed that in *R. tetraphylla*, the highest shoot regeneration frequency (90 per cent) and mean number (18.50) of shoots per explant were achieved from nodal segment cultured on MS medium supplemented with 5.0 μ*M* TDZ for 4 weeks prior to transfer to MS medium without TDZ for 8 weeks. The regenerated shoots rooted best on MS medium containing 0.5 μ*M* IBA. Micropropagated plantlets were hardened to survive *ex-vitro* conditions and were then established into soil.

2.6 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

Many plant secondary products are exploited commercially and many of these natural compounds cannot be artificially synthesized and must be extracted from plants. As the production of the chemicals by isolation from field-grown plants is associated with problems, an alternative would be the exploitation of plant cell cultures for the production of secondary metabolites. The application of *in vitro* culture system in commercial production of secondary metabolites has been achieved in shikonin production with *Lithospermum erythrorhizon* cell cultures by Mitsui Petroleum Co., Japan (Curtin, 1983). The major problem associated with *in vitro* culture is the slow growth and the cultures have a great tendency to be genetically and biochemically unstable and thereby tend to synthesize very low levels of secondary metabolites. *In vitro* transformation of plant material with *Agrobacterium rhizogenes* strains allowed to overcome some of the difficulties of *in vitro* plant organ culture and led to fast growing organs, exhibiting extensive branching and capable of producing the main metabolites of the mother plant or even new metabolites undetected in the mother plant (Nader *et al*., 2006). *Agrobacterium* mediated hairy root induction offers a promising technology for secondary metabolite production (Hamill *et al*., 1987) such as tropane alkaloids (Oksman-Caldentey and Arroo, 2000) and many other metabolites (Giri and Narasu, 2000).

2.6.1 Hairy root

The soil bacterium *A. rhizogenes* generates the “hairy root” syndrome in infected plants and is characterized by the neoplastic outgrowth of roots (Riker *et al.*, 1930). The molecular basis of this phenomenon is the transfer and integration of a specific part of the root inducing (Ri) plasmid of *A. rhizogenes*, called “Transfer DNA” (T-DNA) into the genome of plant cells (Chilton *et al.*, 1982; White *et al.*, 1982). The first directed transformation of higher plants using *A. rhizogenes* was made by Ackermann (1977).

2.6.2 Induction of hairy roots

Hairy roots are obtained by genetic transformation of wounded plant tissue with *A. rhizogenes*. This bacterium is capable of infecting a wide range of dicotyledonous plants, causing roots to proliferate rapidly at the infection site (White and Sinkar, 1987). The process of infection starts with attachment of bacterium to the wounded plant cell. The wounded cells release phenolic substances such as acetosyringone to induce the expression of *vir* genes of plasmid (Binns and Thomashow, 1988). Integration and expression of T-DNA genes in host plant lead to the development of hairy roots (Vanhala *et al.*, 1995; Tepfer, 1984). The bacterium transfers the T-DNA from its large root-inducing Ri plasmid. The plasmid contains *vir* genes that are responsible for virulence of bacteria together with chromosomal genes and cause transfer of T-DNA. The border sequences (25 bp) in the T-DNA region determine the mobility of T-DNA (Sevon and Oksman-Caldentey, 2002).

2.6.2.1 Steps involved in transfer of T-DNA 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)

2.6.3 Characterization of *Agrobacterium* based on opine utilization

A. rhizogenes are characterized by production of novel compounds called opines. These compounds are synthesized by enzymes which are products of the inserted plasmid DNA (Mansouri *et al.*, 2002). Opines are unusual aminoacid derivatives that are produced in transformed tissues and catabolized by the *Agrobacterium*. The Ri plasmids are classified into four groups based on the type of opine produced namely, agropine, mannopine, cucumopine and mikimopine (Van de Velde *et al.*, 2003) and the latter being the stereo-isomer of cucumopine (Suzuki *et al.*, 2001; Petit *et al.*, 1983). Characterization of *Agrobacterium* plasmids on the basis of opine catabolism has been shown in Table 1.

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

Sl. No.	Ri plasmid type	*A. rhizogenes* strains	References
1.	Agropine	A4, ATCC 15834, HRI, NCPPB 1855	Petit *et al.*, (1983); Filetici *et al.*, (1987)
2.	Cucumopine	NCPPB 2659, K 599	Filetici *et al.*, (1987)
3.	Mannopine	LMG 63, LMG 150, TR7, TR 101, TR 105	Trypsteen *et al.*, (1991) Petit *et al.*, (1983)
4.	Mikimopine	MAFF 30 1724, MAFF-02-10266 A13	Shiomi *et al.*, (1987) Daimon *et al.* (1990)

2.6.4 Genes responsible for hairy root induction

A. rhizogenes contain a T-DNA region located on the Ri plasmid that carries genes involved in root initiation and development (*rol* genes), genes concerned with opine biosynthesis and genes of unknown function (Hansen *et al.*, 1994). The Ri plasmid of cucumopine and mannopine type strains consists of only one T-DNA region, whereas that of agropine strains are a split T-DNA, consisting of two T-DNA regions, T_L and T_R each ranging in size from 15 to 20 kb. Both T_L and T_R DNA are transferred and integrated independently into the host plant genome, the transfer of

T_L-DNA is essential for the induction of the hairy-root syndrome (Sevon and Oksman-Caldentey, 2002).

2.6.4.1 rol genes

Several loci on the T_L-DNA of Ri plasmids are essential for hairy root induction. The T_L-DNA of the agropine-type Ri plasmid consists of four loci, *rol* A, B, C and D (White *et al.*, 1985). The T_R-DNA of agropine-type Ri plasmids consists of genes that control opine and auxin biosynthesis (Christey, 2001). The T-DNA of cucumopine and mannopine type strains do not possess *aux* genes but only *rol* genes and are sufficient for producing hairy root phenotype (Veena and Taylor, 2007).

The sequence analysis of the T_R-DNA revealed 18 ORF (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). The *rol* B gene plays a central role in hairy root induction while *rol* B and C and some other ORF`s act in synergy to promote root induction (Aoki and Syono, 1999).

ORF8, ORF13 and ORF14 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). Christey (2001) reported that integration of Ri T_L DNA and T_R DNA leads to the alternations 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).

Bonhomme *et al.* (2000) confirmed that the *rol* A, B, C genes together had synergistic effect upon the growth of the hairy root cultures of *Atropa belladonna* and the growth rate was found dependent on the gene combination.

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 *rol* A gene has been implicated in changes in hormone physiology including interference with gibberellin (Dehio and Schell, 1993).

2.6.5 *Agrobacterium rhizogenes* mediated genetic transformation

A. rhizogenes and *A. tumefaciens* are naturally occurring 'genetic engineers' of plants (Zupan and Zambryski, 1997) and have been exploited for plant transformation (Davey *et al.*, 1994). *A. rhizogenes* is able to induce hairy root symptoms in a large variety of dicot species (Bourgaud *et al.*, 1997). Transgenic root system by introducing additional genes along with the Ri plasmid offers a valuable tool for studying the biochemical properties and the gene expression profile of metabolic pathways (Veena and Taylor, 2007).

A set of morphological markers is encoded by Ri T_L-DNA in regenerated plants (Ackermann, 1977; Sinkar *et al.*, 1988). The 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.

2.6.6 Characteristics of hairy roots

2.6.6.1 Rapid growth

Hairy roots have more number of apical zones with high degree of cell division (Bapat and Ganapathi, 2005). Expression of Ri T-DNA results in uncontrolled growth and abundant branching (Van de Velde *et al.*, 2003). The characteristic of hairy root cultures is the fast hormone independent growth (Bensaddek *et al.*, 2008) producing the secondary metabolites at levels similar to or even exceeding those of the normal roots. 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).

2.6.6.2 Secondary metabolite production

High stability of the production of secondary metabolite is a characteristic of hairy root cultures (Guillon *et al.*, 2006). The hairy roots excrete secondary

metabolites into growth medium and the extent of release varies between species (Hamill *et al*., 1986). Some of the important medicinal plants in which hairy roots have been successfully induced for secondary metabolite production are listed on Table 2.

Table 2. Valuable metabolites produced by hairy root cultures

Sl.No	Plant species	Metabolites	Properties	References
1	*Camptotheca acuminate*	Camptothecin	Anti-cancer, Antiviral	Lorence *et al*., 2004
2	*Gingko biloba*	Ginkgolides	Aging disorders	Ayadi and Tremouillaux- Guiller, 2003
3	*Gmelina arborea*	Verbascoside	Stomach disorders, fever	Dhakulkar *et al*., 2005
4	*Linum flavum*	Coniferin	Anti-cancer	Lin *et al*., 2003
6	*Rauvolfia micrantha*	Ajmalicine, ajmaline	Anti-hypertensive	Sudha *et al*., 2003
7	*Artemesia annua*	Artemesin	Anti-malarial	Shaneeja, 2007
8	*Withania somnifera*	Withanolide	Ergostane type steroid	Varghese, 2006

2.6.7 Culture medium for *A. rhizogenes* strains

Hooykass *et al.* (1977) reported the use of liquid YEM medium for culturing A4, ATCC 11325, ATCC 15834 & LBA 9402 strains. Benjamin *et al.* (1993) reported the use of AB minimal media for culturing 15834 strain. LB medium was used for culturing *A. rhizogenes* strains 15834 (Lee *et al.*, 2004) AR-12 (Arellano *et*

al.,1996). Kang *et al.* (2006) suggested YEP medium for culturing ATCC 15834 and A4 strains. `

YEM 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 A4 and LBA 9402 (Li and Leung, 2003), TR 105 (Lonkova and Fuss, 2009) ATCC 15834 (Hu and Alfermann, 1993; Vanhala *et al.*, 1995).

YEB medium was found to be the best for the growth of MAFF 03-01724 (Sauerwein *et al.*, 1992; Jaziri *et al.*, 1994; Shi and Kintzios, 2003; Xu *et al.*, 2004).

2.6.8 Culture conditions for *A. rhizogenes* strains

Temperature has influence on the growth of *A. rhizogenes* strains. Sanita di Toppi *et al.* (1997) suggested that *Agrobacterium* cultures are grown at temperatures below 28-30°C because plasmid loss occurs at elevated temperatures.

Optimum temperature for growth of *A. rhizogenes* strains like LBA 9402, 8196 and R 1000 was 24°C for two days (Kovalenko and Maliuta, 2003). Xu *et al.* (2004) reported that A4, R1601 and ATCC 15834 were cultured at 27°C and the culture media was shaken at 150 rpm.

2.6.9 Explants used for hairy root induction

The susceptibility of plant species to *Agrobacterium* infection varies greatly (Hamill *et al.*, 1989) with the species and the age of the plant tissue particularly, with the younger ones being more sensitive to bacterial infection (Sevon and Oksman-Caldentey, 2002). Different explants such as shoot buds, leaf segments, seedlings, stem segments, root segments and callus are used for hairy root induction.

Trypsteen *et al.* (1991) reported that successful responses to *Agrobacterium* infections were obtained with young etiolated seedlings and not from older plantlets. Sarma *et al.* (1997) used leaf segments of *R. serpentina* for inducing hairy roots. Shoot cultures of *R. serpentina* inoculated with *A. rhizogenes* resulted in hairy root induction (Benjamin *et al.*, 1993). List of plant species successfully transformed for hairy root induction, strains involved and the explants used are shown in Table 3.

Table 3. Plant species, strains, and the explants used for successful transformation

Sl. No.	Plant spp.	Strains used	Explants	References
1.	*Aralia elata*	ATCC 15834	Root and petiole	Kang *et al.*, (2006)
2.	*Azadirachta indica*	LBA 9402	Leaf	Allan *et al.*, (2002)
3.	*Clitoria ternatea*	A 13	Leaf and leaf derived callus	Malabadi and Nataraja (2003)
4.	*Gmelina arborea*	ATCC 15834	Leaf, stem, root, hypocotyls, cotyledonary nodal segments and embryo axis	Dhakulkar *et al.*, 2005
5.	*Arachis hypogaca L.*	ATCC 15834	Hypocotyl segments	Karthikeyan *et al.*, 2007
6.	*Glycyrrhiza glabra*	K 599	Leaves	Mehrotra *et al.*, 2008
7.	*Lens culinaris*	ATCC 15834	Epicotyl	Dogan *et al.*, 2005
8.	*Rubia tinctorum*	ATCC 15834, R 1000, 2628 and 9365	Cotyledon	Ercan *et al.*, 1999
9.	*Holostemma adakodien*	P_CA_4, A4, ATCC 15834	Hypocotyl shoot buds	Karmarkar *et al.*, 2001
10.	*Withania somnifera*	A4, ATCC 15834	Leaf, shoot tips	Varghese, 2006
11.	*Artemesia annua*	A4, ATCC 15834, MTCC 2364	Leaf, shoot tips, nodal segments	Shaneeja, 2007
12.	*Plumbago rosea*	ATCC 15834	Shoots	Komaraiah *et al.*, 2002

2.6.9 Pre-culturing of explants

Momocilovic *et al.* (1997) reported that shoots of *Gentiana acualis* were elongated on basal media with 34.6 mg l^{-1} GA_3 for 4 weeks prior to inoculation with *A. rhizogenes*.

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, R1601.

Leaves from one month old *in vitro* grown plants of *Catharanthus roseus* were incubated on half strength Gamborg's B5 medium (Gamborg *et al.*, 1968) for 24 hours (Batra *et al.*, 2004). Different seedling explants like root, stem, leaf, hypocotyls, cotyledons, cotyledonary nodal segments and embryo axis of *in vitro* grown *Gmelina arborea* Roxb. were pre-cultured on MS basal medium for two days for better transformation (Dhakulkar *et al*., 2005).

2.6.10 Wounding of explants

Wounding is a prerequisite for *Agrobacterium* infection. Wounding triggers the secretion of *vir* gene inducing compounds (Stachel *et al.*, 1985) and can be done either with a sterile scalpel dipped in bacterial inoculation or with a syringe, injecting a small amount of inoculum.

According to Hildebrand (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.

Mano *et al.* (1989) wounded leaf disks and stem segments of *Duboisia leichhardtii* with a sharp scalpel. Leaves of *Salvia miltiorrhiza* were wounded with forceps (Hu and Alfermann, 1993).

Leaf segments of *Artemesia annua* were wounded by cutting the leaf margins and ten pricks were made using the hypodermic injection needle on the leaf lamina and shoot tips (Shaneeja, 2007).

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

2.6.11.1. Direct inoculation method

This method is particularly used for inoculation of stem segments, seedlings and leaf explants with *Agrobacterium*. The bacterial inoculum used affects the transformation frequencies. Patena *et al.* (1988) reported that colonies were superior to suspension cultures for inducing hairy roots in carrot and apple. They attributed the superior activity of bacterial cell colonies to the greater concentration of bacteria in the colonies as compared to suspensions.

Benjamin *et al.* (1993) smeared bacteria from single cell colony on wounds of shoot explants of *Rauvolfia serpentina*.

In *Medicago truncatula*, *A. rhizogenes* was coated on the cotyledon surface after removing the radicle tip (Chabaud *et al.*, 2006).

Satdive *et al.* (2007) smeared the bacteria on the wounded areas of three-week-old germinated seedlings.

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). Dobigny *et al.* (1995) harvested the bacteria from agar plates using spatula and smeared on the top of stem internodes of potato.

MTCC 2364 produced maximum transformation by direct inoculation whereas A4 and ATCC 15834 produced maximum transformation by suspension method (Shaneeja, 2007).

2.6.11.2. Suspension method of inoculation

A. rhizogenes infection consists of incubating wounded explants for a limited time between 5 and 30 min in a diluted bacterial suspension of OD_{600} = 01-1.0,

followed by blotting off excess bacteria on sterile paper before the co-cultivation step (Karimi *et al.*, 1999).

Sudha *et al.* (2003) smeared the wounded explants with bacterial culture by dipping a sterile scalpel into the bacterial culture. Hypocotyls of non-aseptic intact seedlings were injected with approximately 10 ml of *Agrobacterium* suspension at the site of about 1.5-2.0 cm above the hypocotyl-root junction using a syringe (Li and Leung, 2003).

Bacterial cultures of different *A. rhizogenes* strains on nutrient broth (cell density adjusted to 5 x 10^9 cells/ml) were used for infecting the *A. annua* explants by incubating them for 20 mins (Giri *et al.*, 2001). Kumar *et al.* (2006) reported that leaf discs pricked manually with a metal needle dipped in *A. rhizogenes* culture resulted in hairy root induction from wounded regions.

The bacterial suspension re-suspended in liquid MS medium at a density of 10^8 cells/ml was used for infection of the hypocotyl explants of *Arachis hypogaea* which were soaked in the bacterial suspension for 15 min and co-cultivated for two days (Karthikeyan *et al.*, 2007).

The wounded explants of *H. ada-kodien* were inoculated with 48 hrs age old culture 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, 2007). The wounded embryos of *Aesculus hippocastanum* were immersed in bacterial suspensions for 5 mins and they were transferred to MS medium with or without 50 µ*M* acetosyringone and co-cultivated for 72 hrs (Zdravkovic-Korac *et al.*, 2004).

2.6.12 Influence of acetosyringone in hairy root induction

Certain phenolic compounds, e.g. acetosyringone, α-hydroxy acetosyringone, etc. enhance T-DNA integration by inducing transcription of *Vir* region (Stachel *et al.*, 1985).

Giri *et al.* (2001) reported that 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 in *A. annua*, as compared to explants infected with *A. rhizogenes* without *Vir* gene inducers.

Acetosyringone of 100 µ*M* concentration in the co-culturing media increased the number of transformants with A_4 strain in *W. somnifera* (Varghese, 2006).

Kumar *et al.* (2006) found that sonication along with 100 µ*M* acetosyringone treatment resulted in 4.1 fold increase with 86 per cent transformation frequency in tobacco leaf explants.

2.6.13 Co-culturing of explants with bacteria

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

Hairy roots were successfully established in *Clitoria ternatea* (L.) using *A. rhizogenes* strain A13 with a co-cultivation period of five days (Malabadi and Nataraja, 2003).

Yoshikawa and Furuya (1987) induced hairy roots from calli of *Panax ginseng* by co-culture of cellulase and mannitol treated calli with *A. rhizogenes* suspension for 3 days.

Pawar and Maheshwari (2004) transformed *W. somnifera* by treating the stem, leaf and hypocotyl explants on the co-cultivation medium with a 10 µ*l* aliquot of *A. rhizogenes* suspension for two days.

2.6.14 Elimination of bacteria

Sarma *et al.* (1997) cultured the infected leaves of *R. serpentina* on MS solid medium. On occurrence of whitish bacterial growth on media, the leaves were transferred on MS basal medium supplemented with 400 mg l^{-1} ampicillin for elimination of bacteria.

After incubation for three days, Benjamin *et al.* (1993) transferred the infected shoots to a hormone free MS basal medium containing 3 per cent (w/v) sucrose and 1 gm l^{-1} carbenicillin.

After co-cultivation period, Karthikeyan *et al.* (2007) washed the infected hypocotyl segments with MS basal medium containing cefotaxime (250 mg l^{-1}) to remove overgrowth of *A. rhizogenes* on the surface of the explant.

Lonkova and Fuss (2009) transferred the co-cultured leaf explants to MS medium containing 500 mg l^{-1} sodium cefotaxime and incubated in dark at 25°C.

The infected explants of *A. annua* 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 and were transferred to solid MS medium containing 500 mg l^{-1} cefotaxime and were then cultured at 26 ± 2°C under diffused light (Shaneeja, 2007).

2.6.15 Efficiency of *A. rhizogenes* in inducing hairy roots

Different strains of *A. rhizogenes* vary in their efficiency to bring about successful transformation. Five strains of *A. rhizogenes* were tested for their transformation ability. Among the different *A. rhizogenes* strains tested, the strains PcA4, 15834 and A4 induced hairy roots whereas 8196 and 2659 strains did not induce hairy roots (Karmarkar and Keshavachandran, 2007)

Benjamin *et al.* (1993) reported that *A. rhizogenes* 15834 was an effective vector for specific genetic transformation in *R. serpentina*.

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 per cent) and ATCC 15834 (20 per cent).

Among the three *A. rhizogenes* strains used for hairy root induction in *A. annua*, ATCC 15834 strain caused 70 per cent transformation followed by A_4 50 per cent and MTCC 2364, 22 per cent (Shaneeja, 2007).

2.6.16 Establishment of hairy root cultures

Hairy root induction was achieved in a time period of 1-4 weeks in majority of the plant species, and is further maintained and propagated by sub-culturing.

Giri *et al.* (2001) reported that the axenic hairy roots obtained from shoot tip explants were inoculated into different strengths (full, half, one-fourth) of MS liquid medium and cultures were maintained on a gyratory shaker at 120 rpm at 25 ± 2°C under continuous light.

Hairy roots, which arose from the cut surface of the explants of *Gmelina arborea* were separated, when they attained a length of 4-5 cm and were maintained by sub-culturing 3-4 cm long pieces on B5 solid medium for four weeks (Dhakulkar *et al.*, 2005).

The hairy roots emerged 4-10 weeks after infection in *Camptotheca accuminata*. The established hairy root cultures were sub-cultured every two weeks in liquid B5 medium (35 ml per 125 ml Erlenmeyer flask) with three per cent sucrose on a rotary shaker at 90-100 rpm (Lorence *et al.*, 2004).

The transformed roots of *Tylophora* were excised and cultured in dark in basal media with 500 mg l^{-1} ampicillin. Each excised primary root was propagated as a separate clone and sub-cultured at 4 week interval (Chaudhuri *et al.*, 2005).

2.6.17 Effect of media components on the growth and metabolite accumulation of hairy roots

The composition of medium and culture conditions influences the growth and metabolite accumulation of hairy roots.

Xu *et al.* (2004) found that the culture medium has a significant effect on *Isatis indigotica* hairy root growth. Among the four liquid media (MS, ½ MS, B5 and White's) tested, MS and half MS media were found to be significantly superior to the other two and on comparison between B5 and White's medium, B5 medium was significantly better than White's medium in hairy root growth.

Bensaddek *et al.* (2001) studied the effect of nitrate and ammonium concentrations on growth and alkaloid accumulation of *Atropa belladonna* hairy roots and found increase of ammonium concentration in the culture medium resulted in lowering the growth rate while an increase of the nitrate concentration had a deleterious effect on the alkaloid biosynthesis and accumulation.

Wheathers *et al.* (2005) evaluated the response of cultures of *A. annua* to five types of hormones: auxins, cytokinins, ethylene, gibberellins and abscissic acid. The highest biomass was obtained when 1-5 mg l^{-1} ABA was supplied in the medium whereas cytokinin stimulated the production of artemisinin more than 2 fold.

Gamborg's B5 medium is the most widely used medium for the hairy roots of many species (Hilton and Wilson, 1995). Mehrotra *et al.* (2008) reported that NB modified medium composition supported best growth of hairy roots followed by MS, B5 and WP medium.

2.6.18 Application of hairy roots

Hairy root cultures offer promise for high production and productivity of valuable secondary metabolites in many plants (Smita and Ashok, 2007). 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).

Compared with suspension cell culture, transformed hairy root can produce plant materials that are rich in secondary metabolites. Geerlings *et al.* (1999) reported that cell suspension cultures of *Cinchona officinalis* produced quinoline alkaloids less than 1 µg g^{-1} dry weight, while hairy root of the same plant produced upto 50 µg g^{-1} fresh weight quinoline alkaloids. The levels of the steroidal alkaloid solasodine were significantly higher in hairy root cultures than either callus or cell suspensions (Kittipong-Patana *et al.*, 1998).

Hairy root metabolic engineering was applied successfully to improve alkaloid production in *Catharanthus rosesus* and in various solanaceae plants (Palazon *et al.*, 2003; Moyano *et al.*, 2003).

Shimomura *et al.* (1991) reported the production of Shikonin and betacyanins in hairy root cultures of *Lithospermum erythrorhizon* and *Beta vulgaris* which have been scaled up in bioreactors.

Another key area is phytoremediation wherein, hairy roots from hyper-accumulator plants are able to uptake cadmium, nickel or uranium (Boominathan *et al.*, 2004; Eapen *et al.*, 2003; Boominathan and Doran, 2003). Hairy roots of *Helianthus annuus* and *Brassica napus* can detoxify the pesticide DDT (dichlorodiphenyl trichloroethane), tetracycline and oxytetracycline antibiotics and 2,4-dichlorodiphenyl (2,4-DPC) from industrial effluents (Agostini *et al*., 2003; Gujarathi *et al*., 2005).

2.6.19 Confirmation of transformation

2.6.19.1 Growth characteristics and morphology of hairy roots

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

Hairy roots can be screened roughly based on their intrinsic characteristics that distinguish them from non-transformed roots. These characteristics include high degree of lateral branching, enhanced growth rate, profusion of root hairs and negative geotropism.

A key characteristic of hairy roots is their ability to grow quickly in the absence of exogenous plant growth regulators (Veena and Taylor, 2007). They are genetically stable and produce high contents of secondary metabolites characteristic to the host plant (Sevon and Oksman-Caldentey, 2002).

The average growth rate of hairy roots varies from 0.1 to 2.0 g dry weight/litre/day. The greatest advantage of hairy roots compared to conventional roots is their ability to form several new growing points and consequently, lateral branches (Oksman-Caldentey and Hiltunen, 1996). The growth rate of hairy roots varies greatly between species, but differences were also observed between different root clones of the same species (Mano *et al.*, 1989).

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).

The hairy roots obtained from *H. ada-kodien* using A_4, 15834 and PcA4 strains were whitish in colour and showed negative geotropic growth (Karmarkar and Keshavachandran, 2007). 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. somnifera* (Varghese, 2006).

2.6.19.2 Opine detection

Another frequently used approach to confirm transformation is to check for the presence of opines. Upon integration of the Ri T-DNA into the plant cell genome, opine synthesis will be initiated through expression of the opine biosynthetic genes. Opines (agropine and mannopine) were detected by paper electrophoresis of alcoholic extracts (Petit *et al.*, 1983) using Whatman No.3, formic acid, acetic acid, water (30:60: 910 v/v/v) at 10 vcm^{-1} and alkaline sliver nitrate reagent detects agropine and mannopine by producing spots.

Transformed nature of *H. ada-kodien* was proved by opine assay (Karmarkar and Keshavachandran, 2007).

Opines extracted from A_4, ATCC 15834 and MTCC 2364 induced hairy roots in *A. annua* produced spots corresponding to agropine (Shaneeja, 2007).

Opine production can however be available 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).

2.6.19.3 PCR analysis of hairy roots

The Polymerase chain reaction can be used to detect the Ri T-DNA integration in hairy roots (Flem-Bonhomme *et al*., 2004).

Transformation of *R. micrantha* by ATCC 15834 was confirmed using polymerase chain reaction analysis (Sudha *et al.*, 2003).

Kumar *et al.* (2006) used *rol* A specific primers for PCR analysis of transformed roots of tobacco produced by infection with A4 strain. A 308 bp rol A gene fragment was amplified using the forward 5'-AGAATGGAATTAGCCGGACTA-3' and reverse 5'-GTATTAATCCCGTAGGTTTGTTT-3' primer. Similarly PCR analysis confirmed the presence of *rol* B genes in the hairy roots of *A. annua* (Shaneeja, 2007).

2.6.19.4 Southern hybridization

Detection of T-DNA integration by Southern hybridization is often necessary for confirmation of transformation. Sudha *et al.* (2003) used the PCR amplified fragment of *rol* A gene as probe for southern hybridization after labeling with digoxigenin.

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 *et al.*, 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.

2.7 ENHANCEMENT OF SECONDARY METABOLITES AND QUANTIFICATION

2.7.1 Enhancement of secondary metabolites

Attempts have been made to increase the productivity of cell cultures of *Catharanthus roseus* by selecting high yielding strains, adding the precursors of desired compounds into the medium, eliciting the cultures with fungal extracts or stimulating stress conditions in the culture medium (Moreno *et al*., 1993).

2.7.1.1 Elicitation

Elicitation is one of the methods to increase the production of secondary metabolites in cell cultures. The procedure consists of treating the cultures with a physical or a chemical agent that causes phytoalexin production leading to defence mechanisms in the plant cells. The eliciting agents are classified into two large categories: abiotic elicitors (physical, mineral and chemical factors) and biotic elicitors which are factors of plant or pathogen origin (Yoshikawa, 1978). Effect of elicitors on hairy root induction of different plant species has been shown in Table 4.

Table 4. Various elicitors used for enhanced metabolite production in hairy root cultures of different plant species

Sl.No	Plant species	Elicitors	Metabolites
1	*Ammi majus*	BION, *Enterobacter sakazaki*	Coumarine, furocoumarine
2	*Oxalis tuberose*	*Phytosphtora cinnamoni*	Harmaline, harmine
3	*Panax ginseng*	Chitosan, MeJA, Vanadyl sulfate	Ginsenoside
4	*Pharbitis nil*	$CuSO_4$, MeJA	Umbelliferone, scopoletin
5	*Salvia miltiorrhiza*	Yeast elictor, Ag	Transhinone

6	*Scopolia parviflora*	*Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus*	Scopolamine
7	*Solanum tuberosum*	*Rhizoctonia bataticola*, *Bacillus cyclodextrin*, MeJA	Sesquiterpene lypooxygenase

(Guillon *et al.*, 2006)

BION-Benzo (1,2,3)-thiadiazole-7-carbothionic acid S-methyl ester

Abiotic elicitors such as $NiSO_4$ (20 µ*M*), selenium (0.5 m*M*) and NaCl (0.1 per cent) supplemented in transformed root cultures of *Panax ginseng* increased the saponin content 1.15-1.33 times compared to control (Jeong and Park, 2006). Sodium acetate (10.2 m*M*), added for 24 hrs to the culture medium of *Arachis hypogaea* hairy roots lead to a 60-fold induction and secretion of *trans*-resveratrol into the culture medium (Medina-Bolivar *et al.*, 2007).

In transformed roots of *P. ginseng*, plant derived oligosaccharides from *Paris polyphylla* var yunanensis increased the saponin content by more than 3 times (Zhou *et al.*, 2007). Fungus derived oligosaccharides from the fungal endophyte *Colletotrichum gloeosporoides*, yeast elicitor and chitosan increased artemisinin production in *A. annua* 1-5, 3 and 6 fold respectively (Wang *et al.*, 2006, Putalun *et al.*, 2007).

Combination of bioextract from endophyte mycorrhizal fungus *Acremonium sp.* and yeast extract treatment resulted in higher yields of flavonoids and triterpenoids in licorice cell cultures (Kovalenko and Maliuta, 2003).

The biotic elicitor *Aspergillus* homogenate elicited a positive influence on the biosynthesis of artemisinin in the hairy root cultures of *A. annua* (Shaneeja, 2007).

The secondary plant messengers methyl jasmonate and salicylic acid can also induce secondary metabolism without affecting the growth rate of the cultures (Palazo *et al.*, 2003 and Komaraiah *et al.*, 2003). The addition of 100 m*M* jasmonic acid and salicylic acid showed a 6-9 fold enhancement of azadirachtin in the hairy root cultures of *Azadirachta indica* as compared to control (Satdive *et al.*, 2007).

2.7.1.2 Addition of osmoregulants

Stress may act quantitatively and qualitatively as regulator of secondary product biosynthesis (Frischknecht and Baumann, 1985).

Sorbitol added as an osmoticum increased the tanshinone yield in *Salvia miltiorrhiza* hairy roots by 4.5 fold as compared to the control (Shi *et al.*, 2007). Addition of PEG (9 per cent) of molecular weight 3350 increased ajmalicine production capacity of *Catharanthus roseus* (Akcam-Oluk *et al.*, 2003). The addition of osmoregulant PEG, failed to elicit a positive influence in the biosynthesis of withaferin A and artemisinin in hairy root cultures of *W. somnifera* and *A. annua* respectively (Varghese, 2006 and Shaneeja, 2007).

2.7.1.3 Addition of precursor

Precursor feeding is another approach for enhanced production of secondary metabolites from plant cells grown *in vitro*. Higher concentrations of ajmalicine were produced by addition of tryptophan as a precursor in culture medium of *Catharanthus roseus* (Fulzele and Heble, 1994).

Addition of amino acids to cell suspension culture media enhanced the production of tropane alkaloids, indole alkaloids etc. (Namdeo *et al.*, 2007). Addition of phenylalanine to *Salvia officinalis* cell suspension cultures stimulated the production of rosmarinic acid (Ellis and Towers, 1970). Effect of various precursors on secondary metabolite production has been shown on Table 5.

Table 5. Effect of precursor feeding on the production of secondary metabolites in various medicinal plants

Sl. No.	Culture	Precursor	Product	References
1.	*Atropa belladonna*	Tropinone, tropic acid, and tropanol tropic acid	Tropane alkaloid	Simola *et al.*, 1990
2.	*Azadirachta indica*	Sodium acetate, squaline	Azadirachtin	Balaji, 2001
3.	*Catharanthus roseus*	Secologanin, loganin	Ajmalicine and strictosidine	Moreno *et al.*, 1993
4.	*Digitalis purpurea*	Glucose	Digitoxose	Franz and Hassid, 1997
5.	*Nicotiana tabacum*	Ornithine	Nicotine	Zenk *et al.*, 1975
6.	*Ricinus communis*	Glycerol, succinic acid	Ricinine	Robinson, 1978
7.	*Rauvolfia tetraphylla*	Tryptophan	Reserpine	Anitha and Kumari, 2006

2.7.2 Extraction of alkaloid – Reserpine

Hairy root cultures of *A. annua* were ground in a mortar and pestle with 5 ml n-hexane and was centrifuged at 10,000 rpm for 10 minutes. The supernatant was evaporated to dryness and the concentrated form was redissolved in 0.5 ml n-hexane and used for TLC analysis (Shaneeja, 2007).

Dried powdered material (20 mg) of *Azadirachta indica* was homogenized with 1 ml of methanol by sonication for 20 min at room temperature. After sonication, the samples were centrifuged at 12000 x g for 5 min and extracted with chloroform three times. The chloroform fraction was dried, filtered, evaporated, concentrated and used for HPLC analysis (Satdive *et al.*, 2007).

The regenerated plants of *R. serpentina* were defatted with petroleum ether and extracted with methanol: ammonia and extract used for TLC and HPLC (98: 2 v/v) (Benjamin *et al.*, 1993).

The dried plant materials of *R. serpentina* were extracted with rectified spirit for 7 days. The solvent was evaporated under reduced pressure at 40°C in a rotary evaporator under reduced pressure at 40°C in a rotary evaporator. The crude extract was suspended into water and extracted with petroleum ether and evaporated under reduced pressure. The aqueous layer was separated and extracted with chloroform and subjected to TLC (Ahmad *et al.*, 2002).

2.7.2.1 Thin Layer Chromatography (TLC)

Solvent systems, benzene/ethyl acetate/ammonium hydroxide (70: 27:3 v/v) were used for TLC and sprayed with Dragendorff reagent (Waldi, 1962). TLC plates were eluted with ethyl acetate: methanol (4:1) sprayed with Dragendorff's reagent and examined under UV light for the detection of alkaloid spots (Ahmad *et al.*, 2002).

Karmarkar *et al.* (2001) reported the use of chloroform: methanol (3:4) running solvent system for the elution of sterols in *H. ada-kodien*. The mobile phase n-hexane: Diethyl ether (1:1) were used to develop the chromatographic plates of *A. annua* (Shaneeja, 2007).

2.7.2.2 High Pressure Liquid Chromatography (HPLC)

Analysis of alkaloids of *R. serpentina* were done by HPLC. Solvent system was MeOH: CH_3CN: 1 m*M* $(NH_4)_2$ HPO_4 45: 30: 25 at flow rate of 1 ml/min (Roja *et al.*, 1984).

Sudha *et al.* (2003) used 0.5 per cent (v/v) triethyl amine-water: acetonitrile (50:50 v/v) as mobile phase with flow rate 1 ml min^{-1} and ajmalicine and ajmaline detected at 285 nm.

HPLC with UV detector 227 nm were used for azadirachtin detection. Mobile phase was acetonitrile: water (60:40) at a flow rate of 1 ml/min (Satdive *et al.*, 2007).

HPLC with a chromolith performance RP – 18 e column (100 x 4.6 mm i.d.) and a binary gradient mobile phase composed of 0.01 M CpH 3.5) phosphate buffer containing 0.5 per cent glacial acetic acid and acetonitrile at a flow rate of 1.0 ml/ min with the detector operated at a wavelength of 254 nm for the quantitative determination of *Rauvolfia* root alkaloids (Srivastava *et al.*, 2006).

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled "Genetic transformation in sarpagandha (*Rauvolfia serpentina* (L.) Benth.) for enhancement of secondary metabolite production" was conducted at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 2008 to 2009.

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. Reserpine and nicotine standards for biochemical analyses and the primers for PCR reaction were procured from Sigma Chemicals.

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 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 and jam bottles were dried in hot air oven.

3.1.3 Composition of media

Murashige and Skoog (1962) medium (MS) was used for micropropagation of *Rauvolfia serpentina*. In the development of transformed root cultures MS, half MS and B_5 medium were used. The compositions of the media are given in Appendix I.

The different *Agrobacterium rhizogenes* strains were cultured in Yeast Extract Mannitol (YEM), Yeast Extract Broth (YEB), Nutrient Agar (NA), and Luria Bretani Agar (LBA) media. 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 under refrigerated conditions. Stock III was stored in amber coloured bottle.

A clean plastic beaker, 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 in microwave oven 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 (100, 250 and 500ml) were used as culture vessels. Fifteen ml of medium was poured in test tubes, 30 ml medium in 100 ml conical flask, 120 ml in 250 ml conical flask and 250 ml in 500 ml conical flask. Culture 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 media store room until used.

3.1.5 Growth regulators

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

3.1.6 Carbon source

Sucrose (3 per cent) was used as the main source of carbon.

3.1.7 Preparation of YEM, YEB, NA and LBA medium

A clean plastic beaker rinsed with distilled water was used to prepare the media. The ingredients were weighed on electronic balance and were added into the beaker. A small volume of distilled water was added to dissolve the ingredients. The desired volume was made up by adding distilled water. The pH of the media was adjusted to 7.0 for NA, YEM and LBA and 7.2 for YEB 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 absorbent cotton and jam bottles were sealed tightly using cello tapes after placing the caps. Autoclaving was done at 121°C at 15 psi for 20 min to sterilize the medium and they were further kept in the media store room until used.

3.1.8 Antibiotics

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

Different antibiotics (ampicillin, cefotaxime, and carbenicillin) were used in MS medium and YEM medium for killing the bacteria, 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, streaking of bacterial cultures, preparation of antibiotic media and transformation works were 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 photoperiod (1000 lux) from fluorescent tubes. Humidity in the culture room varied from 60 to 80 per cent according to the climate prevailing. Bacterial cultures were incubated at 28 ± 2°C in an incubator.

3.4 SOURCE OF EXPLANTS

Stock plants were brought from Central Nursery, Mannuthy, Thrissur. They were planted in pots and placed in the shade house giving daily irrigation. Established plants were sprayed with the systemic fungicide carbendazin and the contact insecticide carbaryl, at 0.3 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

Shoot tips and nodal segments were used as explants 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 immersed 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 maturity of explant. They were washed thoroughly with distilled water and treated

with streptocyclin 0.15 per cent for 3-5min. The explants were then again thoroughly washed with distilled water and dried on blotting paper. They were then wiped with 70 per cent ethanol for 1min. Further, surface sterilization was carried out under the hood of the laminar airflow cabinet.

3.5.3 Standardization of surface sterilization of explants

Surface sterilization of explants was carried out under aseptic conditions in laminar airflow cabinet to make them free of microorganisms. The explants were prepared as described in *3.5.2.1* and were sterilized with mercuric chloride ($HgCl_2$) for varying period at concentrations of 0.05 and 0.1 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 surface sterilization treatment, explants were submerged in sterilant for the required period and with frequent agitation. Then explants were washed free of the chemical sterilant using sterile water. Then they were dried carefully by transferring onto filter paper pieces on a sterile petridish.

3.5.4 Explant regeneration

3.5.4.1 Establishment of shoot tip and nodal segment explants

The explants after surface sterilization were inoculated on media containing various combinations of growth regulators.

Different concentrations of BA & NAA were tried for regeneration from shoot tip and nodal segment explants

1) MS + 1 mg l^{-1} BA + 0.1 mg l^{-1} NAA
2) MS + 1 mg l^{-1} BA + 0.2 mg l^{-1} NAA
3) MS + 1 mg l^{-1} BA + 0.5 mg l^{-1} NAA
4) MS + 1.5 mg l^{-1} BA + 0.1 mg l^{-1} NAA
5) MS + 1.5 mg l^{-1} BA + 0.2 mg l^{-1} NAA
6) MS + 1.5 mg l^{-1} BA + 0.2 mg l^{-1} NAA

7) MS + 2 mg l^{-1} BA + 0.1 mg l^{-1} NAA

8) MS + 2 mg l^{-1} BA + 0.2 mg l^{-1} NAA

9) MS + 2 mg l^{-1} BA + 0.5 mg l^{-1} NAA

Observations on the establishment of explants were recorded after 25 days.

3.5.4.2. Multiplication

The regenerated shoots from nodal segment and shoot tip explants were multiplied in MS medium supplemented with BA and NAA at varying concentrations.

The regenerated shoots were sub-cultured in the following media for multiplication.

1) MS + 1 mg l^{-1} BA +0.1 mg l^{-1} NAA
2) MS +1 mg l^{-1} BA +0.2 mg l^{-1} NAA
3) MS + 1 mg l^{-1} BA + 0.4 mg l^{-1} NAA
4) MS + 2 mg l^{-1} BA + 0.1 mg l^{-1} NAA
5) MS + 2 mg l^{-1} BA + 0.2 mg l^{-1} NAA
6) MS + 2 mg l^{-1} BA + 0.4 mg l^{-1} NAA

Observations on the number of shoot buds produced per explant were recorded after 25 days.

3.5.4.3 Rooting

3.5.4.3.1 In vitro rooting

The shoots obtained from multiplication media were excised using a sterile blade and were inoculated in the following rooting media for induction of roots

1) Basal MS medium
2) MS +0.5 mg l^{-1} NAA
3) ½ MS + 0.5 mg l^{-1} NAA
4) MS +0.5 mg l^{-1} IBA
5) ½ MS +0.5 mg l^{-1} IBA
6) MS + 0.1 mg l^{-1} IBA + 0.1 mg l^{-1} NAA
7) ½MS + 0.1 mg l^{-1} IBA + 0.1 mg l^{-1} NAA

8) MS + 0.1 mg l^{-1} IBA + 0.2 mg l^{-1} NAA
9) ½MS + 0.1 mg l^{-1} IBA + 0.2 mg l^{-1} NAA
10) MS + 0.2 mg l^{-1} IBA + 0.1 mg l^{-1} NAA
11) ½MS + 0.2 mg l^{-1} IBA + 0.1 mg l^{-1} NAA
12) MS + 0.2 mg l^{-1} IBA + 0.2 mg l^{-1} NAA
13) ½ MS + 0.2 mg l^{-1} IBA + 0.2 mg l^{-1} NAA

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

3.5.4.3.2 Hardening and planting out

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

3.6 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

Six strains of *Agrobacterium rhizogenes* ATCC 15834, ATCC 11325, A4, TR107, TR 7 and MTCC 532 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 Yeast Extract Mannitol (YEM), Yeast Extract Broth (YEB), Luria Bretani Agar (LBA) 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. The strains were streaked on plates containing the media and incubated at 26 ± 2°C. The growth rate of bacteria on each medium was observed after 48 hrs.

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, ampicillin, cefotaxime, and carbenicillin. Yeast Extract Mannitol 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 spotted in Yeast Extract Mannitol agar medium without any antibiotics, to be used as control.

3.6.3 Evaluation of the sensitivity of explants to various antibiotics

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

3.6.4 Standardization of transformation techniques in *R. serpentina*

3.6.4.1 Explants for transformation

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

3.6.4.1.1 Pre-culturing of explants

The explants were taken from *in vitro* grown 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 infection with bacteria.

3.6.4.1.2 Wounding of explants

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

3.6.4.1.3 Standardization of explants for efficient transformation

Explants like shoot tips, nodal segments and leaf segments were infected by all the six strains of *Agrobacterium rhizogenes* (ATCC 15834, ATCC 11325, A4, TR107, TR7 and MTCC 532). The infection was carried out using bacterial suspension culture and co-cultured for two days. The response of explants to different *Agrobacterium* strains was recorded.

3.6.4.2 Standardization of inoculation method

The bacterial isolates from single cell colonies (referred to as Direct Inoculation Method or DIM) and bacterial suspension (referred to as Suspension Culture Inoculation Method or SIM) were used as the inoculum. The response of different explants to different inoculation method was recorded.

3.6.4.2.1 Direct Inoculation Method

In this method, single cell bacterial colony was used as the inoculum. The pre-cultured explants were wounded by using an injection needle. A loopful of single cell bacterial colony was taken and swabbed on the wounded explants. The explants were then placed on solid MS medium without growth regulators. Uninfected explants were placed on growth regulator free MS medium as control.

3.6.4.2.2 Suspension culture inoculation method

Bacterial suspension of 48 hrs old was used as the inoculum. The pre-cultured explants were wounded first using an injection needle dipped in the suspension. The wounded explants were then immersed in the bacterial 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. Uninfected explants were placed on growth regulator free MS medium as control.

3.6.4.3 Standardization of different methods for hairy root induction

3.6.4.3.1 Influence of co-culture period on transformation

The infected explants were co-cultured at 26 ± 2°C for 1 to 3 days. Transformation percentage obtained and response of different explants under different inoculation method and different co-cultivation period was recorded.

3.6.4.3.2 Influence of acetosyringone on transformation

Three methods were used to study the influence of acetosyringone in hairy root induction. Acetosyringone was added to the bacterial medium before inoculation, and in another method the infected explants were co-cultured in acetosyringone containing MS media. In the third method acetosyringone was added to both the bacterial medium and MS basal medium. Acetosyringone (3', 5'-dimethoxy-4'-hydroxyacetophenone) dissolved in dimethyl sulfoxide (DMSO) was used as the stock. From the stock solution, acetosyringone at the rate of 100 µ*M* was taken and added aseptically.

As control, one set of explants without acetosyringone treatment was placed on solid MS media for two days. Response of cultures to acteosyringone treatment was recorded.

3.6.4.3.3 Influence of etiolated cultures as explant on transformation

The *in vitro* nodal segments of *R. serpentina* was inoculated on MS media with 1 mg l^{-1} BA + 0.1 mg l^{-1} NAA and kept in dark for one week which favored etiolation. The etiolated shoot tip was used as the explant for transformation under suspension method. The response of etiolated shoot tip cultures to transformation using different *Agrobacterium* strains was recorded.

Recalcitrance of *R. serpentina* explants to *Agrobacterium* infection was observed. So the further part of the research work such as checking the virulence of the strains and viability of the methodology followed, confirmation of transformation

and enhancement of secondary metabolite present in the hairy roots was done in *Nicotiana tabacum* which was taken as a model plant.

3.6.5 Standardization of transformation techniques in *Nicotiana tabacum*

3.6.5.1 Standardization of explant

Different explants like leaf segments, shoot tips and nodal segments were infected by all the six strains of *A. rhizogenes* (ATCC 15834, ATCC 11325, TR7, A4, TR107 and MTCC 532). The infection was carried out using suspension culture method and co-cultured for two days under diffused light. Transformation percentage and the response obtained in case of each explant were recorded. The mean number of hairy roots per transformed explant was recorded after 20 days.

3.6.5.2 Standardization of inoculation method

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

3.6.5.2.1 Direct Inoculation Method

In this method, single cell bacterial colony was used as the inoculum. Explants were wounded by using a sterile blade and swabbed with a loopful of inoculum. The explants were then blotted with a sterile blotting paper and placed on solid MS medium without growth regulators in petriplates. Uninfected explants were placed on growth regulator free MS medium as control. The transformation percentage obtained in case of each type of explant was recorded.

3.6.5.2.2 Suspension culture inoculation Method

The explants were wounded using injection needle dipped in the bacterial suspension. The wounded explants were then immersed in the suspension for 20 min with intermittent, gentle agitation. The explants were blotted dry, using sterile blotting paper and placed on solid MS medium without growth regulators. Uninfected explants

were placed on growth regulator free MS medium as control. The transformation percentage obtained in case of each type of explant was recorded.

3.6.5.3 Co-cultivation of explants with Agrobacterium

The infected explants co-cultured at 26 ± 2°C for 1 to 3 days. Transformation percentage obtained and response of different explants under different inoculation methods and different co-cultivation periods were recorded.

3.6.6 Elimination of bacteria

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 and were further cultured at 26 ± 2°C.

3.6.7 Efficiency of strains in inducing hairy roots

Six strains of *A. rhizogenes* (ATCC 15834, ATCC 11325, A4, TR107, TR 7 and MTCC 532) were inoculated on different explants using different inoculation methods and co-cultured for two days. Transformation percentage obtained using different strains in different explants was calculated. Days taken for induction of hairy roots from infected explants by using different strains under different inoculation methods were recorded.

3.6.8 Establishment of hairy root cultures

The adventitious roots emerged from the infected explants within one week 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 tip was 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. The concentration of cefotaxime antibiotic was gradually reduced week after week.

3.6.9 Rapid culturing of hairy roots

After the establishment of hairy 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 rotary shaker at 100 rpm under diffused light and dark condition for rapid multiplication. The normal roots obtained from control explants 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 taken.

3.6.10 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, half MS and B5 with 3.0 per cent sucrose. The hairy roots cultured in the conical flask were collected, randomly cut to small pieces and inoculated in 250 ml conical flasks containing 100 ml each half MS, MS and B_5 with 3.0 per cent sucrose without antibiotics. The cultures were incubated in rotary shaker at 100 rpm under dark photoperiod. The fresh weight of the roots was recorded after 15, 30 and 60 days of inoculation.

3.7 CONFIRMATION OF TRANSFORMATION

The confirmation of transformation was done on the following basis:

1) Morphological features
2) Opine analysis
3) PCR analysis for *rol* B and *rol* C genes

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 growth rate.

3.7.2 Opine analysis

Opine analysis in hairy roots 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 a black colored bottle under 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 per cent sodium carbonate

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

3.7.2.2 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 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 horizontal electrophoresis unit (Genei) 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

3.7.3.1 Isolation of DNA from roots

For PCR analysis, DNA was isolated from hairy roots obtained using ATCC 15834, ATCC 11325, TR7, A4, TR107, and MTCC 532 strains in *N. tabacum*, TR107 induced roots in *R. serpentina*, non-transformed (control) explants of *N.*

tabacum and from *Artemesia annua* hairy root culture which served as positive control for PCR analysis, following the modified procedure reported by Rogers and Bendich (1994).

3.7.3.1.1 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. β-mercaptoethanol

3.7.3.1.2 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 100 µl of autoclaved milli-Q 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.

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 (0.7 per cent (w/v) for genomic DNA and 1.0 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 µg l^{-1} as an intercalating agent, which helps in visualization of DNA. It was then poured into the gel-casting tray with comb and allowed to solidify. After the solidification of the gel (20 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 – *Hin*d III/ *Eco* R1 double digest for total DNA and 100 bp ladder for PCR product) was also loaded in 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 transilluminator and documented in gel documentation system.

3.7.3.3 Isolation of plasmid from A4 strain

A4 plasmid DNA was isolated using alkaline mini-prep procedure as given by Birnboim and Doly (1979). A4 strain containing *pRiA4* plasmid was used as positive control in PCR analysis.

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 plasmid Isolation

1. A single bacterial colony was transferred in to 2.0 ml YEM medium and the culture was incubated overnight at 28°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 plasmid isolated was observed in agarose gel electrophoresis and documented.

3.7.3.4 Quantification of DNA and plasmids

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 of 1 µl 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 for rol **B** *and* **C** *genes*

The primer sets used for amplifying *rol* B and C gene were Rol BF1R1 and Rol CF1R1. Details of primer sets are given in Table 6. PCR analysis was carried out using DNA isolated from the hairy roots of *N. tabacum* induced by ATCC 15834, ATCC 11325, TR7, A4, TR107 and MTCC 532 and also from TR107 induced roots of *R. serpentina*. The DNA isolated from roots produced by non-transformed (control) explants of *N. tabacum* was used as the negative control. The plasmid *pRiA4* and *A.*

annua hairy root DNA was used as the positive control. A blank (without DNA) was also set.

Table 6. Details of different combinations of primer

Sl No.	Primer combination	Amplicon size (bp)	Annealing temperature (°C)
1	Rol BF1R1	740	54
2	Rol CF1R1	520	60

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	
Plasmid	- 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 plasmid)
	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 and C genes from template DNA.

1.	94 °C for 2.0 min	- Initial denaturation
2.	94 °C for 45 sec	- Denaturation
3.	54 °C and 60 °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 1.0 per cent agarose gel and finally documented.

3.8 ESTIMATION OF ALKALOID- NICOTINE

HPLC method was used for the estimation of nicotine present in the roots of *N. tabacum.* Nicotine was estimated from roots, shoots, and leaves of field-grown plants, *in vitro* roots and shoots (non- transformed), transformed shoots and hairy roots (transformed roots). Enhancement of nicotine through precursor feeding, elicitation and addition of osmoregulants was also attempted.

3.8.1 Extraction and estimation

3.8.1.1 Preparation of standard

Standard nicotine procured from Sigma Chemicals, USA was used as standard.

3.8.1.2 Extraction of nicotine from samples

The hairy root along with the medium was filtered through Whatman No.1 filter paper and the filtered hairy root culture was blotted dried and weighed. The hairy

root culture was then macerated in a pestle and mortar using water and made up to 10 ml. The homogenized extract was filtered through cellulose nitrate filter of pore size (0.45 µm) and was used for HPLC analysis.

The medium collected was filtered through cellulose nitrate filter and was directly used for HPLC analysis.

3.8.1.3 HPLC assay

The separation was performed using Shimadzu HPLC system equipped with a SPD-10A UV vis detector, an FCV-130 AL pump (Shimadzu, Japan) and a Rheodyne injector with 20 µl loop. Aluspher column RP select B (5 m) was used together with a Lichro cart 4-4 guard column. The mobile phase consisted of methanol: water (1:1), at a flow rate of 1.0 ml/min. The total run time was 20 min. The samples were injected using Rheodyne injector with 20 µl loop. The peak areas of samples were integrated at the wavelengths of 260 and 232 nm. They were initially assigned by comparing retention times with the standards and confirmed with characteristic spectra obtained from the UV detector.

Calibration curve was prepared based on peak area of six concentrations of 0-10 ppm. Linearity was obtained in the concentration range of 2-10 ppm. All data were processed using class LC10 software (Shimadzu, Japan).

3.9 ENHANCEMENT OF SECONDARY METABOLITE PRODUCTION

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

3.9.1 Addition of osmoregulants

3.9.1.1 Addition of PEG

The hairy root cultures were cultured in half MS medium supplemented with Polyethylene Glycol (PEG) of molecular weight 6000 g at 1.0 per cent, 2.0 per cent and 5.0 per cent.

The media was prepared by dissolving 10 g l^{-1}, 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 0.25 g of hairy roots was inoculated in 75 ml of stress media in 250 ml conical flask. The cultures were grown for 15 days at room temperature under dark photoperiod on a rotary shaker (110 rpm) at room temperature. The whole roots and media were collected and nicotine content was analysed as described in *3.8.1.3*.

3.9.1.2 Addition of sorbitol

The hairy root cultures were cultured in half MS medium supplemented with sorbitol at 1.0 per cent, 2.0 per cent and 5.0 per cent.

The media was prepared by dissolving 10 g l^{-1}, 20 g l^{-1} and 50 g l^{-1} sorbitol in half MS media. The pH of the media was adjusted to 5.7 before autoclaving. Approximately 0.25 g of hairy roots was inoculated in 75 ml of stress media in 250 ml conical flask. The cultures were grown for 15 days at room temperature under dark photoperiod on a rotary shaker (110 rpm) at room temperature. The whole roots and media were collected and nicotine content was analysed as described in *3.8.1.3*.

3.9.2 Addition of precursors

The effect of precursor feeding on nicotine production in hairy roots was studied. Hairy roots were inoculated in half MS media supplemented with 50 ppm, 100 ppm and 150 ppm of L- arginine. L- arginine was filter sterilized and added to 75 ml sterilized half MS liquid media in 250 ml conical flask. Twenty five day old root cultures were inoculated in the media and further grown for seven days. The whole roots and media were collected and nicotine content was analysed as described in *3.8.1.3*.

3.9.3 Addition of Elicitors

3.9.3.1 Elicitation by Yeast Extract

Yeast Extract at two concentrations, 2 and 5 per cent was used to elicit the cultures. Half MS liquid medium was supplemented with 20 g l^{-1} and 50 g l^{-1} of 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 7 days. The whole roots and media were collected and nicotine content was analysed as described in *3.8.1.3*.

Results

4. RESULTS

The results of the study on "Genetic transformation in sarpagandha (*Rauvolfia serpentina* (L.) Benth.) for enhancement of secondary metabolite production" are presented in this chapter.

4.1 STANDARDIZATION OF *IN VITRO* REGENERATION

4.1.1 Standardization of surface sterilization

Effect of different concentrations of $HgCl_2$ and duration of treatment on culture establishment and survival in *Rauvolfia serpentina* are presented in Table 7.

In shoot tip and nodal segment explants, $HgCl_2$ at 0.1 per cent for 1 min proved to be the best. Higher concentration of $HgCl_2$ resulted in drying of the explant. Survival and establishment of cultures from shoot tip explant was 72 and 61 per cent and from nodal segment explant was 84 and 80 per cent respectively at 0.1 per cent $HgCl_2$ for 1 min.

4.1.2 Explant regeneration

4.1.2.1 Establishment of shoot tip and nodal segment explants

Effect of different concentrations of growth regulators on shoot bud growth from shoot tip and nodal segments of *R. serpentina* are shown in Table 8 and 9.

Maximum regeneration response from shoot tip and nodal segment were obtained on MS medium, supplemented with 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA (Plate 1). Bud burst occurred within 15 to 20 days in shoot tip and nodal segment explants.

Initially, shoot tip explant showed fast growth whereas after 15 days, the growth remained stagnant but nodal segment continued to grow with an average shoot length of 2.5 to 4.0 cm after 30 days of culturing.

Shoot tip explant showed 85 per cent regeneration and 3.2 mean number of shoots/explant within four weeks of culturing in MS medium supplemented with 1 mg l^{-1}

BA and 0.1 mg l^{-1} NAA. In the case of nodal segment, the regeneration response was slightly higher (92 per cent) with 4.5 mean number of shoots within four weeks in the same medium, MS + 1 mg l^{-1}BA + 0.1 mg l^{-1} NAA.

In combinations of BA and NAA, higher NAA concentration favored callusing on the lower or basal portion of the explants, whereas lower concentrations of NAA favored callusing along with rooting of cultures. However, increased concentration of BA resulted in low percentage of regeneration and shortening of internodes.

4.1.2.2 Multiplication

The shoot buds obtained were inoculated on MS medium containing different combinations of BA and NAA for leaf proliferation and multiplication. Data regarding the influence of growth regulators on the multiplication of regenerated shoot explants is given in Table 10.

The regenerated shoots gave out numerous differentiated shoots on the establishment media MS + 1 mg l^{-1} BA + 0.1 mg l^{-1} NAA (Plate 2). An average of 4.5 shoots was produced per shoot bud, of which 2-3 shoots showed faster elongation.

4.1.2.3 Rooting

Data regarding the days taken for rooting, mean number of roots, percentage of rooting and nature of roots is given in the Table 11. Rooting occurred within 22 days in all the treatments.

Cent per cent rooting was obtained on ½ MS + 0.2 mg l^{-1} IBA + 0.2 mg l^{-1} NAA with 7.5 mean number of roots/explant (Plate 3a and 3b). The best basal media for rooting was ½ MS. Mean number of roots and mean root length was more and callusing of shoots was less. Roots were branched, elongated with cent percent rooting in ½ MS + 0.2 mg l^{-1} IBA + 0.2 mg l^{-1} NAA combination. The rooted plants were hardened and planted out which showed 95 per cent survival (Plate 4).

Table 7. Effect of different concentrations of $HgCl_2$ and time of sterilization on culture establishment of *R. serpentina*

$HgCl_2$ (%)	Time of treatment	Survival after one week (%)		Culture establishment after 15 days (%)	
		Shoot tip	Nodal segment	Shoot tip	Nodal segment
0.05	1 min	32.10	39.00	26.08	30.00
	3 min	35.00	40.00	28.48	38.43
	5 min	35.50	60.00	28.61	45.00
0.1	1 min	72.00	84.00	61.60	80.00
	3 min	35.90	40.00	24.80	38.00
	5 min	25.62	35.00	23.10	30.00

Table 8. Effect of different concentrations of BA and NAA on shoot bud growth from shoot tip explants

Media composition (mg l^{-1})	Explant response			
	Bud burst (%)	Days taken for bud initiation	Mean number of shoot buds/ explant	Response
1 BA + 0.1 NAA	85	15	3.2	C+R
1 BA + 0.2 NAA	82	17	2.7	C+R
1 BA + 0.5 NAA	82	17	2.1	C
1.5 BA + 0.1 NAA	74	19	1.6	C+R
1.5 BA + 0.2 NAA	62	20	1.1	C
1.5 BA + 0.5 NAA	55	21	1	C
2 BA + 0.1 NAA	72	20	1.25	C+R
2 BA + 0.2 NAA	69	19	0.7	C+R
2 BA + 0.5 NAA	65	21	0.4	C

C- Callusing; C+R- Callusing along with rooting

Table 9. Effect of different concentrations of BA and NAA on shoot bud growth from nodal segment explants

Media composition (mg l^{-1})	Explant response			
	Bud burst (%)	Days for bud initiation	Mean number of shoot buds/ explant	Response
1 BA + 0.1 NAA	92	18	4.5	C+R
1 BA + 0.2 NAA	86	18	3.4	C+R
1 BA + 0.5 NAA	82	20	2.5	C
1.5 BA + 0.1 NAA	74	19	2.6	C+R
1.5 BA + 0.2 NAA	72	19	1.5	C+R
1.5 BA + 0.5 NAA	65	20	1.5	C
2 BA + 0.1 NAA	82	19	1.25	C+R
2 BA + 0.2 NAA	75	19	1	C+R
2 BA + 0.5 NAA	70	20	1	C

C- Callusing; C+R- Callusing along with rooting

Table 10. Effect of different concentrations of growth regulators on multiple shoot induction and proliferation from regenerated shoot explants

Media composition (mg l^{-1})	Mean no of shoots /explant
1 BA + 0.1 NAA	4.5
1 BA + 0.2 NAA	3.4
1 BA + 0.4 NAA	2.5
2 BA + 0.1 NAA	1.25
2 BA + 0.2 NAA	1
2 BA + 0.4 NAA	1

Table 11. Effect of various growth regulators on rooting of shoots of *R. serpentina*

Media composition (mg l^{-1})	Days taken for rooting	Mean number of roots/ explant	Rooting (%)	Nature of roots
MS Basal	22	1.3	76	Very thin roots
MS + 0.5 NAA	15	2.6	98	Thin elongated roots
½MS + 0.5 NAA	15	3.5	100	Thin elongated roots
MS + 0.5 IBA	20	2	66	Thick roots
½MS + 0.5 IBA	20	1.1	20	Moderately thick roots
MS + 0.1 IBA + 0.1 NAA	19	6.2	66..6	Thin roots
½MS + 0.1 IBA + 0.1 NAA	20	5.8	67	Thin roots
MS + 0.1 IBA + 0.2 NAA	20	5.6	65.2	Thin elongated roots
½ MS + 0.1 IBA + 0.2 NAA	20	5.6	63	Elongated roots
MS + 0.2 IBA + 0.1 NAA	20	2.7	43.3	Moderately thick roots
½ MS + 0.2 IBA + 0.1 NAA	20	3.7	83	Thick roots
MS + 0.2 IBA + 0.2 NAA	12	7.3	100	Branched elongated roots
½ MS + 0.2 IBA + 0.2 NAA	10	7.5	100	Branched, elongated roots
½MS + 0.1 IBA + 0.1 NAA	20	5.8	67	Thin roots
MS + 0.1 IBA + 0.2 NAA	20	5.6	65.2	Thin elongated roots
½ MS + 0.1 IBA + 0.2 NAA	20	5.6	63	Elongated roots
MS + 0.2 IBA + 0.1 NAA	20	2.7	43.3	Moderately thick roots
½ MS + 0.2 IBA + 0.1 NAA	20	3.7	83	Thick roots
MS + 0.2 IBA + 0.2 NAA	12	7.3	100	Branched elongated roots
½ MS + 0.2 IBA + 0.2 NAA	10	7.5	100	Branched, elongated roots

Nodal segment inoculated in
1 BA + 0.1 NAA

Bud burst of nodal explant

1.5 BA + 0.5 NAA
Callusing at the base of explants

Shoot growth in
1.5 BA + 0.1 NAA

Shoot growth in
2 BA + 0.1 NAA

Shoot growth in
1 BA + 0.1 NAA

Plate 1. ***In vitro*** **regeneration of** ***R. serpentina***

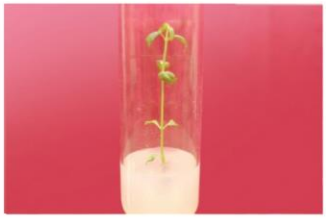
Shoot growth in
1 BA + 0.2 NAA

Callusing around the base of
explant in 1 BA + 0.4 NAA

1 BA + 0.1 NAA
Multiple shoot induction

Plate 2. Multiplication of established cultures of *R. serpentina*

Rooting in
MS basal

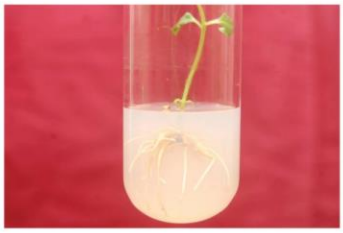


Rooting in
MS + 0.5 IBA

Rooting in
½ MS + 0.5 IBA

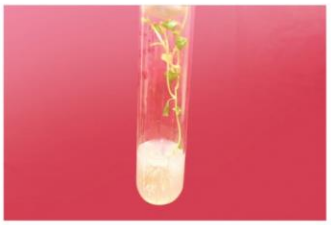
Rooting along with callusing of explants
MS + 0.5 NAA

Plate 3a. Effect of various growth regulators on rooting of *R. serpentina*



Rooting in ½MS + 0.5 IBA

Rooting in ½MS + 0.1 IBA + 0.1 NAA



Rooting in ½ MS + 0.2 IBA + 0.1 NAA

Rooting in ½ MS + 0.2 IBA + 0.2 NAA

Plate 3b. Effect of various growth regulators on rooting of *R. serpentina*

Plate 4. Hardening and planting out of *R. serpentina*

4.2 CULTURING AND SENSITIVITY SCREENING OF *AGROBACTERIUM RHIZOGENES* AND EXPLANTS

4.2.1 Culturing of *Agrobacterium rhizogenes* strains

The growth pattern of *A. rhizogenes* strains on different culture media are given in Table 12.

All the culture media favored the growth of *A. rhizogenes* strains. Strain MTCC 532 showed very fast growth in all the media tested, except YEM. The other strains showed uniform growth with single cell colonies in YEM. In LBA medium, ATCC 15834 strain showed excessive growth, whereas ATCC 11325 showed very poor growth in NA and LBA media. Hence YEM was selected for growing six strains of *A. rhizogenes.* Optimum temperature for the growth of all the strains was observed to be 26 ± 2°C.

4.2.2 Cultural characteristics of *A. rhizogenes* strains

The bacterial colonies of ATCC 15834 and TR 7 appeared within two days of streaking and the colonies were smooth, round and mucoid (Plate 5a). The colonies of strain A4 appeared within a day of streaking. The colonies were round with smooth margin, convex, whitish coloured and mucoid in nature. The colonies of TR 107 were creamy yellow, closely spaced and appeared two days after streaking (Plate 5b).

The colonies of ATCC 11325 appeared after two days of streaking at slightly lower temperature of 24°C and were small, round, whitish, closely spaced and mucoid in nature (Plate 5a). In MTCC 532, colonies appeared two days after streaking and were serrated, closely spaced and yellowish in colour (Plate 5b).

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 Table 13. The strain A4 was resistant to ampicillin and carbenicillin but sensitive to cefotaxime at 500 mg l^{-1} whereas

Table 12. Growth pattern of *Agrobacterium rhizogenes* strains in different media

Media	ATCC 15834	ATCC 11325	A4	TR107	TR 7	MTCC 532
YEB	++	++	++	++	++	+++
YEM	++	++	++	++	++	++
NA	++	+	++	++	++	+++
LBA	+++	+	++	++	++	+++

+ Slow growth; ++fast growth; +++ very fast growth; ++++ excessive growth

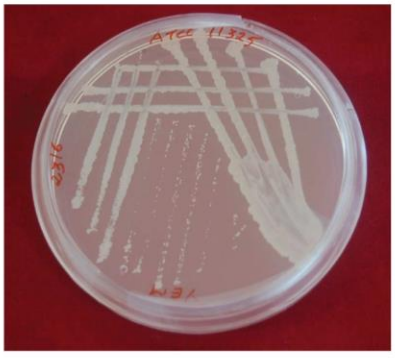
YEB-Yeast Extract, YEM-Yeast Extract Mannitol
NA-Nutrient Agar, LBA-Luria Bertani Agar

Table 13. Sensitivity of *Agrobacterium rhizogenes* strains to different antibiotics

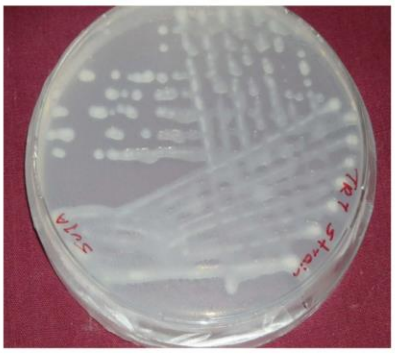
Antibiotic concentration (mg l^{-1})		Response of strains					
		ATCC 15834	ATCC	A4	TR 107	TR 7	MTCC 532
Ampicillin	50	+	+	+	-	+	+
	100	+/-	+	+	-	+	-
	200	-	+	+	-	+	-
	300	-	+	+	-	+	-
	400	-	+	+	-	+	-
	500	-	+/-	+	-	+	-
Cefotaxime	50	+	-	+	+	+	+
	100	+	-	+	-	-	-
	200	-	-	+	-	-	-
	300	-	-	+	-	-	-
	400	-	-	+/-	-	-	-
	500	-	-	-	-	-	-
Carbenicillin	50	+	-	+	+	+	+
	100	-	-	+	-	-	-
	200	-	-	+	-	-	-
	300	-	-	+	-	-	-
	400	-	-	+	-	-	-
	500	-	-	+/-	-	-	-

+ Growth; +/- Slow growth; - no growth

ATCC 15834

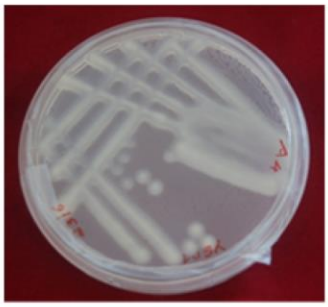


TR 107

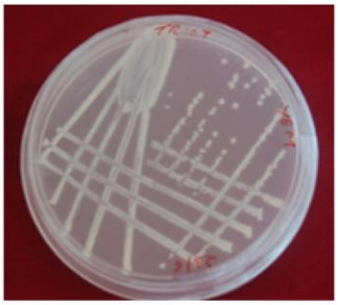


TR 7

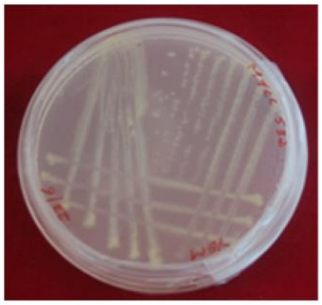
Plate 5a. ***Agrobacterium rhizogenes*** **strains used for the study**



A4



TR107



MTCC532

Plate 5b. ***Agrobacterium rhizogenes*** **strains used for the study**

TR7 and ATCC 11325 were sensitive to cefotaxime and carbenicillin but resistant to ampicillin at 500 mg l^{-1}. The strains ATCC 15834, MTCC 532 and TR107 were sensitive to ampicillin, carbenicillin and cefotaxime. Cefotaxime at 500 mg l^{-1} killed all the six strains of *A. rhizogenes* (Plate 6). Hence, cefotaxime at 500 mg l^{-1} was identified as the optimum antibiotic concentration to kill the *A. rhizogenes* strains under study

4.2.4 Screening of explants to antibiotics

Sensitivity of explants to cefotaxime at different concentrations is shown in Table 14. Different types of explants like shoot tip, nodal segment and leaf segments were found to be healthy in cefotaxime up to 500 mg l^{-1}. At 1000 mg l^{-1} concentration, the explants were pale and started yellowing (Plate 7). Shoot tips rooted in the presence of antibiotics.

4.2.5 Pre-culturing and wounding of explants

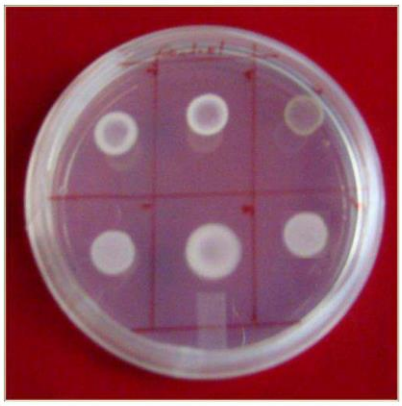
Explants were pre-cultured on MS basal medium for two days prior to infection. The pre-cultured explants remained healthy in growth regulator free media which were then wounded for transformation (Plate 8).

4.3 STANDARDISATION OF TRANSFORMATION TECHNIQUES IN *RAUVOLFIA SERPENTINA*

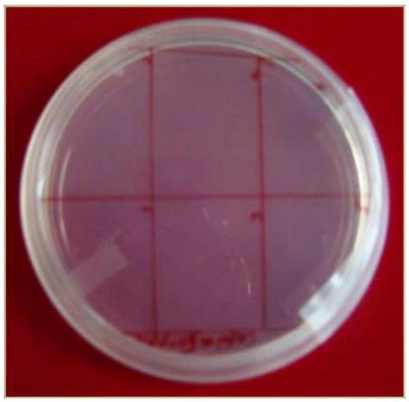
4.3.1 Standardization of explants

The response of explants to different *Agrobacterium* strains is represented in Table 15.

Among the three explants used, shoot tip responded better to infection with *A. rhizogenes* than leaf and nodal segments. Nodal segments infected with ATCC 15834, A4, TR7 and MTCC 532 recorded yellowing within 15 days. Leaf segments in all the six strains showed inward curling and yellowing (Plate 9) within 15 days. Shoot tip recorded bud burst and elongation in all the six strains (Plate 10). Nodal segments showed bud burst and yellowing of nodal explants was also noticed (Plate 9 and 11) after 15 days.



Control



Cefotaxime 500 mg l^{-1}

Plate 6. Sensitivity of *Agrobacterium rhizogenes* strains to cefotaxime

Table 14. Screening of explant sensitivity 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

Table 15. Standardization of explants for efficient transformation in *R. serpentina*

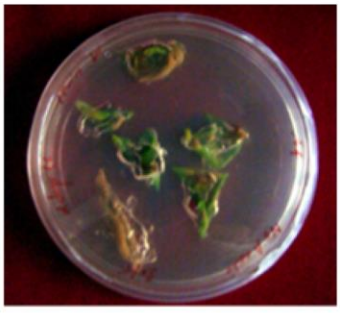
A. rhizogenes strains	Explant	Inoculation method	Response
ATCC 15834	Leaf	SM	Y
	Shoot tip	SM	EL
	Nodal segment	SM	Y
ATCC 11325	Leaf	SM	Y
	Shoot tip	SM	EL
	Nodal segment	SM	B
A 4	Leaf	SM	Y
	Shoot tip	SM	EL
	Nodal segment	SM	Y
TR 107	Leaf	SM	Y
	Shoot tip	SM	EL
	Nodal segment	SM	B
TR 7	Leaf	SM	Y
	Shoot tip	SM	EL
	Nodal segment	SM	Y
MTCC 532	Leaf	SM	Y
	Shoot tip	SM	EL
	Nodal segment	SM	Y

SM – Suspension inoculation method; B –Bud burst; EL – Elongation; Y- Yellowing

Leaf segments (500 mg l^{-1})

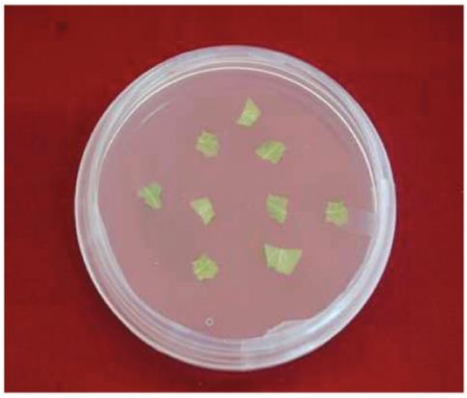
Shoot tips (500 mg l^{-1})

Nodal segments (500 mg l^{-1})

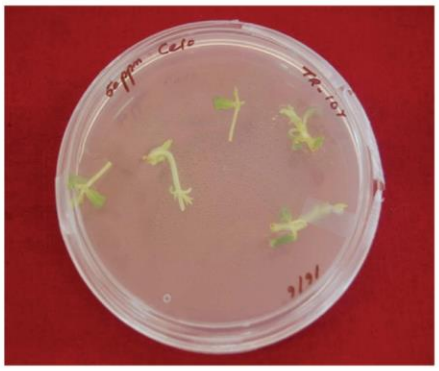


Leaf segments (1000 mg l^{-1})

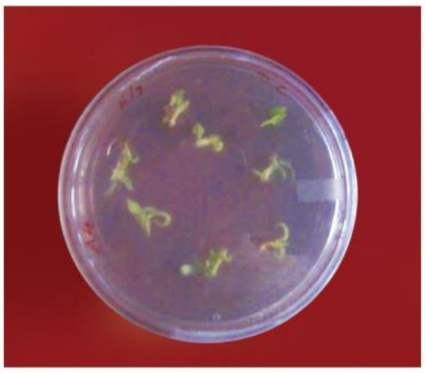
Plate 7. Sensitivity of explants to cefotaxime



Leaf segments

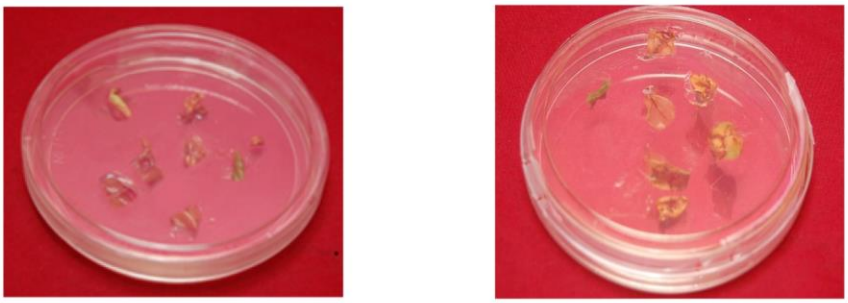


Shoot tips

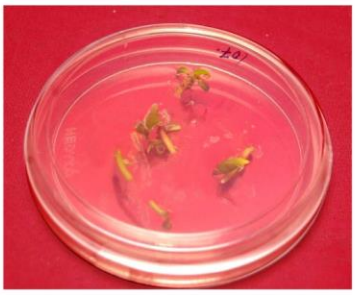


Nodal segments

Plate 8. Pre-culturing of explants of *R. serpentina*

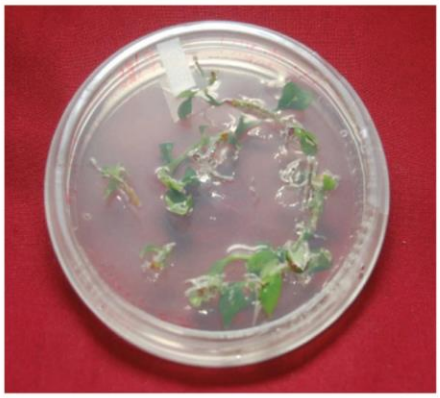


Leaf segments



Nodal segments

Plate 9. Yellowing of *R. serpentina* explants four weeks after infection with *A. rhizogenes*



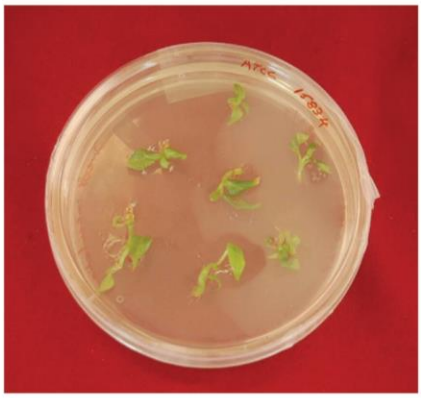


Plate 10. Shoot tip explants of *R. serpentina* showing elongation four weeks after infection with *A. rhizogenes*

Plate 11. Nodal segments of *R. serpentina* showing bud burst four weeks after infection with *A. rhizogenes*

4.3.2 Standardization of inoculation method

4.3.2.2 Influence of inoculation method on hairy root induction in R. serpentina

The influence of inoculation method on transformation in *R. serpentina* is represented in Table 16. In suspension culture method, yellowing of leaf explant, bud burst and elongation of shoot tip and nodal segment explant were observed with all the strains except A4, where yellowing of three types of explant used was observed. In case of direct inoculation method, nodal segments showed yellowing in most of the strains experimented.

4.3.3 Standardization of different methods for hairy root induction

4.3.3.1 Effect of co-culture period on hairy root induction

The effect of co-culture period on induction of hairy roots in *R. serpentina* using different types of explants is given in Table 17, 18 and 19.

Leaf explants co-cultivated for 1, 2, or 3 days showed inward curling and yellowing. Co-cultivation of nodal segment for 1 or 2 days resulted in bud burst and for 3 days resulted in yellowing of explants with ATCC 15834, ATCC 11325, TR 107, TR 7 and MTCC 532 strains. Co-cultivation for more than one day resulted in yellowing of nodal segments with A4 strain.

Co-cultivation of leaf segments with *A. rhizogenes* resulted in yellowing of explant. Co-cultivation of shoot tip for 1, 2 or 3 days resulted in elongation of shoots, in all the strains except A4. Co-cultivation period of more than one day with A4 strain resulted in yellowing of explants. Co-cultivation of nodal segments for more than 2 days with *A. rhizogenes* showed yellowing of explant.

Root induction along with elongation of shoot tip explant was observed with TR 107 strain co-cultivated for 2 days. The induced roots were excised and cultured on MS basal without antibiotics but instead of rapid proliferation, drying of induced root was noticed (Plate 12).

4.3.3.2 Effect of acetosyringone on hairy root induction

4.3.3.2.1 Acetosyringone (100 µM) added to the bacterial medium

The effect of acetosyringone added to the bacterial media on hairy root induction under SM is represented in Table 20.

There was no positive response of hairy root induction using acetosyringone added to the bacterial media. Shoot tips showed shoot elongation with all the six strains. Nodal segment showed bud burst with ATCC 11325, TR107 and control. Yellowing of nodal segments occurred with ATCC 15834, A4, TR7 and MTCC 532.

4.3.3.2.2 Acetosyringone (100 µM) added to the co-cultivation medium

The effect of acetosyringone added to MS basal co-cultivation media on hairy root induction under SM is shown in Table 21.

There was no positive response of hairy root induction using acetosyringone added to the co-cultivation media. Shoot tips showed shoot elongation with all the six strains and control. Nodal segment showed yellowing with ATCC 15834, A4, MTCC 532 and TR 7 strains whereas ATCC 11325, TR 107 strains and control showed bud burst.

Table 16. Influence of inoculation method on transformation in *Rauvolfia serpentina*

A. rhizogenes strains	Inoculum	Response		
		Leaf	Shoot tip	Nodal segment
ATCC 15834	DIM	Y	EL	Y
	SM	Y	EL	B
ATCC 11325	DIM	Y	EL	B
	SM	Y	EL	B
TR 7	DIM	Y	EL	Y
	SM	Y	EL	B
A 4	DIM	Y	EL	Y
	SM	Y	Y	Y
TR 107	DIM	Y	EL	B
	SM	Y	EL	B
MTCC 532	DIM	Y	EL	Y
	SM	Y	EL	B
Control	DIM	N	EL	B
	SM	N	EL	B

Y-Yellowing; EL-Elongation; B- Bud burst; N –No response

DIM – Direct inoculation method, SM – Suspension culture method

Table 17. Effect of co-culture period on hairy root induction in *R. serpentina* using leaf explant

A. rhizogenes strains	Inoculation method	Co-cultivation period (days)	Response
ATCC 15834	SM	1	Y
		2	Y
		3	Y
ATCC 11325	SM	1	Y
		2	Y
		3	Y
A 4	SM	1	Y
		2	Y
		3	Y

TR 107	SM	1	Y
		2	Y
		3	Y
TR 7	SM	1	Y
		2	Y
		3	Y
MTCC 532	SM	1	Y
		2	Y
		3	Y

Y-Yellowing; SM – Suspension culture method

Table 18. Effect of co-culture period on hairy root induction in *R. serpentina* using shoot tip explant

A. rhizogenes strains	Inoculation method	Co-cultivation period (days)	Response
ATCC 15834	SM	1	EL
		2	EL
		3	EL
ATCC 11325	SM	1	EL
		2	EL
		3	EL
A 4	SM	1	EL
		2	Y
		3	Y
TR 107	SM	1	EL
		2	EL+R
		3	EL
TR 7	SM	1	EL
		2	EL
		3	EL
MTCC 532	SM	1	EL
		2	EL
		3	EL

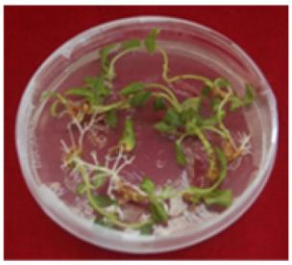
EL – Elongation; EL+ R – Elongation along with rooting

SM – Suspension culture method

Table 19. Effect of co-culture period on hairy root induction in *R. serpentina* using nodal segment as explant

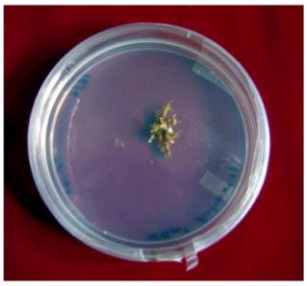
A. rhizogenes strains	Inoculation method	Co-cultivation period (days)	Response
ATCC 15834	SM	1	B
		2	B
		3	Y
ATCC 11325	SM	1	B
		2	B
		3	Y
A 4	SM	1	B
		2	Y
		3	Y
TR 107	SM	1	B
		2	B
		3	Y
TR 7	SM	1	B
		2	B
		3	Y
MTCC 532	SM	1	B
		2	B
		3	Y

B-Bud burst; N-No response; Y-Yellowing; SM -Suspension culture method



Root induction by TR107

Culturing of induced roots on MS basal medium



Drying of induced roots

Plate 12. Root induction in *R. serpentina* by TR107 strain

Table 20. Effect of acetosyringone (100 µ*M*) added to the bacterial media on hairy root induction in *R. serpentina*

***A. rhizogenes* strains**	**Explant**	**Response**
ATCC 15834	Shoot tip	EL
	Nodal segment	Y
ATCC 11325	Shoot tip	EL
	Nodal segment	B
A 4	Shoot tip	EL
	Nodal segment	Y
TR 107	Shoot tip	EL
	Nodal segment	B
TR 7	Shoot tip	EL
	Nodal segment	Y
MTCC 532	Shoot tip	EL
	Nodal segment	Y
Control	Shoot tip	EL
	Nodal segment	B

B – Bud burst; EL –Elongation; Y-Yellowing

Table 21. Effect of acetosyringone (100 µ*M*) added to MS basal co-cultivation media on hairy root induction in *R. serpentina*

A. rhizogenes strains	Explant	Response
ATCC 15834	Shoot tip	EL
	Nodal segment	Y
ATCC 11325	Shoot tip	EL
	Nodal segment	B
A 4	Shoot tip	EL
	Nodal segment	Y
TR 107	Shoot tip	EL
	Nodal segment	B
TR 7	Shoot tip	EL
	Nodal segment	Y
MTCC 532	Shoot tip	EL
	Nodal segment	Y
Control	Shoot tip	EL
	Nodal segment	B

B –Bud burst; EL – Elongation; Y-Yellowing

4.3.3.2.3 Acetosyringone added to the co-cultivation medium and bacterial medium

The effect of acetosyringone added to the co-cultivation medium and bacterial medium on hairy root induction is shown in Table 22.

There was no positive response of hairy root induction using acetosyringone added to the co-cultivation as well as bacterial medium. Shoot tips showed shoot elongation in all the six strains and control. In the case of nodal segment, yellowing was seen with ATCC 15834, A4 and TR7 strain. Bud burst of nodal explant was observed with ATCC 11325, TR 107, MTCC 532 and control.

4.3.3.3 Effect of etiolated shoot tip explants on hairy root induction

Effect of etiolated shoot tip cultures on hairy root induction under SM is shown in Table 23.

There was no hairy root induction using etiolated shoot tip cultures. Etiolated cultures of one week old after infection with *Agrobacterium* showed shoot elongation in all the six strains and control (Plate 13).

The general procedure for induction of hairy roots in plants was followed in *R. serpentina*. Different parameters like variation in co-culture period, addition of phenolic compounds and use of etiolated shoot cultures for induction of hairy roots in *R. serpentina* were tried. However there was paucity in induction of hairy roots in *R. serpentina*. Hence the hairy roots induced from *Nicotiana tabacum* which is a model plant were used for the remaining part of the research work which included the checking of the virulence of the strains and viability of the methodology, confirmation of transformation and enhancement of secondary metabolite present in the hairy roots.

4.4 STANDARDIZATION OF TRANSFORMATION TECHNIQUES IN *NICOTIANA TABACUM*

In vitro raised *Nicotiana tabacum* was used as a source explant for transformation works (Plate 14)

4.4.1 Standardization of explants for hairy root induction in *N. tabacum*

The response of explants to different *A. rhizogenes* strains is represented in Table 24 and Plate 15. Among the three explants used, leaf segments responded better to transformation when compared to nodal segments and shoot tips. Leaf segments infected with the six strains of *Agrobacterium* showed transformation with the highest transformation per cent (92.1) and 4.5 mean number of hairy roots per explant with ATCC 15834 strain.

Shoot tip and nodal segments also showed transformation except with A4 strain. Nodal segment co-cultivated with A4 strain for two days resulted in yellowing of explant.

Table 22. Effect of acetosyringone (100µ*M*) added to the co-cultivation medium and bacterial medium on hairy root induction in *R. serpentina*

***A. rhizogenes* strains**	**Explant**	**Response**
ATCC 15834	Shoot tip	EL
	Nodal segment	Y
ATCC 11325	Shoot tip	EL
	Nodal segment	B
A 4	Shoot tip	EL
	Nodal segment	Y
TR 107	Shoot tip	EL
	Nodal segment	B
TR 7	Shoot tip	EL
	Nodal segment	Y
MTCC 532	Shoot tip	EL
	Nodal segment	B
Control	Shoot tip	EL
	Nodal segment	B

B –Bud burst; EL – Elongation; Y-Yellowing

Table 23. Effect of etiolated shoot tip cultures on hairy root induction in *R. serpentina*

***A. rhizogenes* strains**	**Inoculation method**	**Co-cultivation period (days)**	**Response**
ATCC 15834	SM	3	EL
ATCC 11325	SM	3	EL
A 4	SM	3	EL
TR 107	SM	3	EL
TR 7	SM	3	EL
MTCC 532	SM	3	EL
Control	SM	3	EL

EL – Elongation; SM- Suspension culture method

Plate 13. Etiolated shoot cultures of *R. serpentina* showing elongation after infection with *A. rhizogenes*

Plate 14. ***In vitro*** **derived** ***Nicotiana tabacum*** **plant**

Table 24. Standardization of explants for transformation in *N. tabacum*

A. rhizogenes strains	Explant	Average number of hairy roots/explant	Response	Transformation (%)
ATCC 15834	Leaf	4.52	HR+C	92.10
	Shoot tip	2.30	HR	66.00
	Nodal segment	2.91	HR+C	71.20
ATCC 11325	Leaf	2.10	HR+C	66.00
	Shoot tip	0.72	HR	19.80
	Nodal segment	1.05	HR+C	24.20
TR 7	Leaf	3.60	HR+C	79.40
	Shoot tip	0.98	HR	40.60
	Nodal segment	1.66	HR+C	52.80
A 4	Leaf	1.70	HR+C	61.50
	Shoot tip	0.60	HR	30.80
	Nodal segment	0.00	Y	0.00
TR 107	Leaf	1.14	HR+C	30.10
	Shoot tip	0.32	HR	20.30
	Nodal segment	0.58	HR+C	26.70
MTC 532	Leaf	0.36	HR+C	14.10
	Shoot tip	0.12	HR	4.40
	Nodal segment	0.27	HR+C	11.50

HR- Hairy root; HR+C- Hairy root induction along with callusing

$$\text{Number of hairy roots/ explant} = \frac{\text{Total number of hairy roots formed}}{\text{Number of explants}}$$

$$\text{Transformation percentage} = \frac{\text{Number of explants showing hairy roots}}{\text{Total number of explants inoculated with } \textit{A. rhizogenes}} \times 100$$

Leaf segment

Shoot tip

Nodal segment

Plate 15. Response of different explants of *N. tabacum* for hairy root induction

4.4.2 Standardization of inoculation method for hairy root induction in *N. tabacum*

4.4.2.1 Direct inoculation and suspension culture method

4.4.2.1.1 Influence of inoculation method on hairy root induction in N. tabacum

The influence of bacterial inoculum on transformation in *N. tabacum* is represented in Table 25. 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 percentage of transformation by both the methods followed by TR 7, with leaf as the explant. ATCC 15834, ATCC 11325 and TR 7 produced maximum transformation by SM whereas A4, TR 107, and MTCC 532 produced maximum transformation by DIM. Co-cultivation of nodal segment with A4 suspension for more than one day led to yellowing of explant.

4.4.2.1.2 Influence of co-culture period on hairy root induction in N. tabacum

The influence of co-culture period under DIM and SM on transformation in *N. tabacum* using different types of explants is given in Table 26, 27 and 28. The transformation frequency was influenced by the co-culture period.

All the strains experimented showed maximum transformation in all the explants co-cultivated for 1 or 2 days. However, co-cultivation period of more than 2 days reduced the transformation efficiency. Co-cultivation for more than 3 days resulted in yellowing of the explants. Maximum transformation (94 per cent) was in leaf explant co-cultivated with ATCC 15834 for 2 days by DIM and SM.

The explants infected with different *A. rhizogenes* strains showed callusing along with hairy root induction (Plate 16) except shoot tip explant. Callusing was absent in shoot tip. The strain TR 7 recorded 80 per cent transformation by SM with leaf explant co-cultivated for 2 days.

Table 25. Influence of inoculation method on transformation in *N. tabacum*

A. rhizogenes strains	Inoculum	Percentage of transformation		
		Leaf	Shoot tip	Nodal segment
ATCC 15834	DIM	95.0	63.0	72.0
	SM	98.0	68.0	75.0
ATCC 11325	DIM	66.0	22.0	25.0
	SM	75.0	29.0	27.0
TR 7	DIM	66.6	32.0	42.0
	SM	81.3	40.3	50.0
A 4	DIM	70.0	40.0	19.0
	SM	62.0	36.6	0.0
TR 107	DIM	55.0	20.0	25.0
	SM	22.0	17.8	22.0
MTCC 532	DIM	40.0	18.6	19.0
	SM	26.0	12.0	9.0
Control	DIM	20.0	19.3	53.0
	SM	25.0	15.6	48.0

DIM- Direct inoculation method; SM- suspension culture method

Table 26. Influence of co-culture period on hairy root induction in *N. tabacum* using leaf explant

A. rhizogenes strains	Inoculation method	Co-cultivation period	Response	Transformation (%)
ATCC 15834	DIM	1	HR+C	82.60
		2	HR+C	93.20
		3	HR+C	73.20
	SM	1	HR+C	83.30
		2	HR+C	95.00
		3	HR+C	77.00
ATCC 11325	DIM	1	HR+C	58.00
		2	HR+C	63.00
		3	HR+C	46.60
	SM	1	HR+C	70.50
		2	HR+C	74.20
		3	HR+C	54.20
TR 7	DIM	1	HR+C	56.10
		2	HR+C	66.30
		3	HR+C	46.80
	SM	1	HR+C	70.20
		2	HR+C	80.10
		3	HR+C	60.10
A 4	DIM	1	HR+C	31.80
		2	HR+C	66.00
		3	HR	32.60
	SM	1	HR+C	32.30
		2	HR+C	57.00
		3	Y	0.00
		3	Y	0.00
TR 107	DIM	1	HR+C	23.60
		2	HR+C	35.60
		3	HR+C	18.40
	SM	1	HR+C	21.00
		2	HR+C	33.10
		3	HR+C	11.20
MTCC 532	DIM	1	HR	9.80
		2	HR+C	12.30
		3	HR+C	1.60
	SM	1	HR+C	11.10
		2	HR+C	11.60
		3	HR+C	0.80

DIM- Direct inoculation method; SM- Suspension culture method; HR- Hairy roots; HR+C- Hairy root induction along with callusing; Y- Yellowing

Table 27. Influence of co-culture period on hairy root induction in *N. tabacum* using shoot tip as explant

A. rhizogenes strains	Inoculation method	Co-cultivation period	Response	Transformation (%)
ATCC 15834	DIM	1	HR	48.9
		2	HR	62.0
		3	HR	43.0
	SM	1	HR	58.0
		2	HR	66.0
		3	HR+C	48.0
ATCC 11325	DIM	1	HR	16.1
		2	HR	22.3
		3	HR	8.6
	SM	1	HR	17.0
		2	HR	20.6
		3	HR	10.1
TR 7	DIM	1	HR	27.9
		2	HR	37.2
		3	HR	22.6
	SM	1	HR	40.1
		2	HR	46.0
		3	HR	31.2
A 4	DIM	1	HR	18.9
		2	HR	35.0
		3	HR	17.3
	SM	1	HR	27.7
		2	HR	33.7
		3	HR	09.0
TR 107	DIM	1	HR	21.0
		2	HR+C	22.2
		3	HR	10.9
	SM	1	HR	13.3
		2	HR	18.6
		3	HR	12.2
MTCC 532	DIM	1	HR	3.3
		2	HR	6.9
		3	HR	0.8
	SM	1	HR	2.9
		2	HR	5.1
		3	HR+C	0.6

DIM- Direct inoculation method; SM- Suspension culture method; HR- Hairy roots; HR+C- Hairy root induction along with callusing

Table 28. Influence of co-culture period on hairy root induction in *N. tabacum* using nodal segment as explant

A. rhizogenes strains	Inoculation method	Co-cultivation period (days)	Response	Transformation (%)
ATCC 15834	DIM	1	HR+C	65.0
		2	HR+C	72.1
		3	HR+C	53.2
	SM	1	HR+C	70.1
		2	HR+C	71.2
		3	HR+C	51.6
ATCC 11325	DIM	1	HR+C	21.3
		2	HR+C	21.0
		3	HR+C	15.1
	SM	1	HR+C	26.2
		2	HR+C	25.0
		3	HR+C	17.0
TR 7	DIM	1	HR+C	39.0
		2	HR+C	39.6
		3	HR+C	30.1
	SM	1	HR+C	48.9
		2	HR+C	49.2
		3	HR+C	36.0
A 4	DIM	1	HR	7.6
		2	HR	18.1
		3	HR	6.0
	SM	1	HR	8.7
		2	Y	0.0
		3	Y	0.0
TR 107	DIM	1	HR	19.3
		2	HR+C	18.7
		3	HR+C	09.6
	SM	1	HR+C	16.5
		2	HR+C	13.2
		3	HR+C	10.1
MTCC 532	DIM	1	HR+C	8.6
		2	HR+C	13.5
		3	HR+C	7.7
	SM	1	HR+C	8.1
		2	HR+C	10.4
		3	HR+C	4.2

DIM- Direct inoculation method; SM- Suspension culture method; HR- Hairy roots; HR+C- Hairy root induction along with callusing; Y- Yellowing

Plate 16. Callusing along with rooting of leaf explants in *N. tabacum*

In the case of A4 strain, 66 per cent transformation was obtained by DIM with leaf explant co-cultivated for 2 days. Co-cultivation of nodal segments by SM with A4 strain for 2 days resulted in yellowing of the explant. A4 produced better transformation by DIM when co-cultivated for 2 days than when co-cultivated for 1 or 3 days.

DIM of MTCC 532 and TR107 produced highest transformation in leaf co-cultivated for 1 or 2 days. In the case of ATCC 11325 and A4, excess bacterial growth was observed when explants were co-cultivated for more than two days.

4.4.3 Efficiency of strains in inducing hairy roots

The strain ATCC 15834 showed highest efficiency (94 per cent) in transforming plant tissues followed by TR 7 strain and ATCC 11325 (Plate 17a and b). A4, TR 107 and MTCC 532 produced maximum transformation in leaf by DIM. The number of hairy roots per explant was more in ATCC 15834 transformed leaf explant. ATCC 15834 was able to induce a maximum of 5 hairy roots per explant.

The efficiency of different strains and number of days taken for hairy root induction under different inoculation method is shown in Table 29. The number of days for hairy root induction differed with the *A. rhizogenes* strain and inoculation method. ATCC 15834 was able to produce hairy roots within 3 days of infection in leaf explant whereas MTCC 532 produced hairy roots in leaf explant only after 8 days. In general 3 to 10 days were taken to induce hairy roots in *N. tabacum*.

4.4.4 Establishment of hairy root cultures

Hairy root cultures show rapid growth with high lateral branching. ATCC 15834 and TR7 induced hairy roots showed faster growth with high lateral branching in comparing with other strains (Plate 17a). Control roots showed slow growth and were positively geotropic in nature.

Table 29. Efficiency of different strains and duration for induction of hairy roots in *N. tabacum*

A. rhizogenes strains	Inoculation method	Explant	Days taken for root induction	Transformation %
ATCC 15834	DIM	Leaf	3	95.00
		Shoot tip	5	63.00
		Nodal segment	5	72.00
	SM	Leaf	3	98.00
		Shoot tip	3	68.00
		Nodal segment	3	75.00
ATCC 11325	DIM	Leaf	6	66.00
		Shoot tip	8	22.00
		Nodal segment	7	22.00
	SM	Leaf	5	75.00
		Shoot tip	7	29.00
		Nodal segment	6	27.00
TR7	DIM	Leaf	4	66.00
		Shoot tip	5	32.00
		Nodal segment	5	45.00
	SM	Leaf	4	81.00
		Shoot tip	5	40.00
		Nodal segment	4	50.00
A4	DIM	Leaf	4	70.00
		Shoot tip	4	40.00
		Nodal segment	4	19.00
	SM	Leaf	4	62.00
		Shoot tip	5	36.00
		Nodal segment	5	0.0
TR 107	DIM	Leaf	7	0.0
		Shoot tip	8	0.0
		Nodal segment	9	0.0
	SM	Leaf	8	0.0
		Shoot tip	9	0.0
		Nodal segment	10	0.0
MTCC 532	DIM	Leaf	8	0.0
		Shoot tip	10	0.0
		Nodal segment	9	0.0
	SM	Leaf	9	0.0
		Shoot tip	10	0.0
		Nodal segment	10	0.0

DM- Direct inoculation method; SM- Suspension culture method

ATCC 15834

ATCC 11325

TR 7

A4

Plate 17a. Hairy roots induced by different *A. rhizogenes* strains in *N. tabacum*

MTCC 532

TR 107

Control

Plate 17b. Hairy roots induced by different *A. rhizogenes* strains in *N. tabacum*

4.4.5 Rapid culturing of hairy roots

The fresh weight of hairy roots induced using different *A. rhizogenes* strains after 25 days of inoculation are shown in the Table 30. On comparing control roots and hairy roots induced by experimental strains, ATCC 15834 induced hairy roots showed faster growth producing more biomass. The growth of hairy roots in liquid medium without growth regulators was rapid when compared to growth in solid media (Plate 18a and b). The hairy roots in liquid media after 3 weeks of inoculation, turned brown.

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

The effect of different basal media on the growth of hairy roots at different time intervals is shown in Table 31. Among the liquid medium tested, ½MS with 3 per cent sucrose was found to be superior to MS and B5 with 3 per cent sucrose.

In the media tested, the newly emerging hairy roots were creamy white in color whereas the initially inoculated roots turned brown. The maximum fresh weight of culture was between 15–30 days of inoculation. After 30 days the fresh weight remained more or less static.

Observations of the growth pattern of hairy roots induced by ATCC 15834 and TR7 revealed that no growth was observed in the initial first week of inoculation. Root growth started after 10 to 12 days and the growth rate was rapid after 15-20 days of inoculation. After 4 weeks, all the roots started to turn brown.

Table 30. Variation in fresh weight of hairy roots of *N. tabacum* induced by different *A. rhizogenes* strains

A. rhizogenes strains	Fresh weight of hairy roots after 25 days (g/100 ml)
ATCC 15834	2.065
ATCC 11325	1.230
TR 7	1.786
A 4	1.076
TR 107	0.989
MTCC 532	1.001
Control	1.389

Grown on ½MS basal medium (100 ml)

Initial inoculum ~ 0.5g

Table 31. Growth of transformed roots of *N. tabacum* in different basal media

A. rhizogenes	Medium	Fresh weight (g)/100 ml		
		15 days	30 days	60 days
ATCC 15834	MS Basal	0.53	1.33	1.36
	½MS Basal	0.80	2.22	2.59
	B5 Basal	0.30	1.06	1.21
TR 7	MS Basal	0.35	1.18	1.20
	½MS Basal	0.67	1.38	1.42
	B5 Basal	0.32	0.91	1.10
Control	MS Basal	0.36	1.06	1.10
	½MS Basal	0.35	1.07	1.09
	B5 Basal	0.22	0.76	0.90

Initial inoculum ~ 0.25- 0.5g

ATCC 15834

TR 7

A4

Plate 18a. Proliferation of hairy roots of *N. tabacum* in liquid media

ATCC 11325

TR 107

MTCC 532

Control roots

Plate 18b. Proliferation of hairy roots of *N. tabacum* in liquid media

4.5 CONFIRMATION OF TRANSFORMATION

4.5.1 Morphology of hairy roots

The hairy roots were induced from the wounded sites and the roots induced by all the experimental strains produced calli in MS media. Negative geotropism was seen in the root s induced by ATCC 15834, and TR7. Lateral branching were more in hairy roots. Rapid growth of the hairy root was noted on comparison with the control.

4.5.2 Opine analysis

Opines extracted from ATCC 15834 and A4 induced hairy roots in *Nicotiana tabacum* produced spots corresponding to agropinic acid. Opine extracted from TR7 induced hairy root in *N. tabacum* showed two spots, one corresponding to agropinic acid and the other to mannopinic acid. No spot was produced by TR 107 induced root in *Rauvolfia serpentina*. The response of transformed and normal control roots to the presence of opines is given in Plate 19.The opines extracted from roots induced using TR 107, ATCC 11325 and MTCC 532 in *N. tabacum* showed no spots.

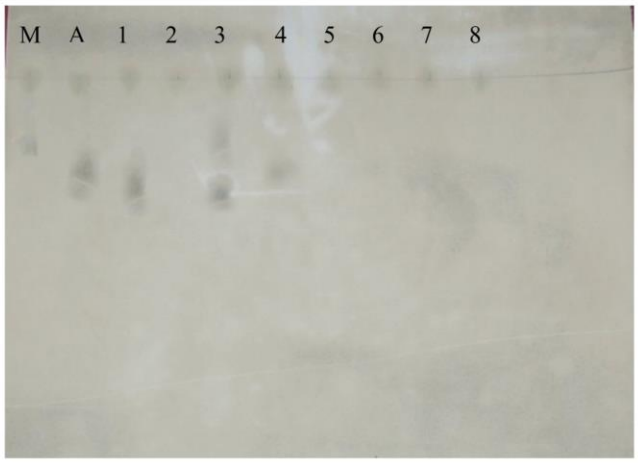
Confirmation of transformation by detection of opines using high voltage paper electrophoresis was successful in hairy roots induced using ATCC 15834, TR 7 and A4 in *N. tabacum*. Further confirmation was done by PCR.

4.5.3 Confirmation by PCR analysis

PCR analysis of hairy roots was carried out for confirmation of genetic transformation. Polymerase Chain Reaction was used to demonstrate the presence of *rol* B and C genes in the transformed roots.

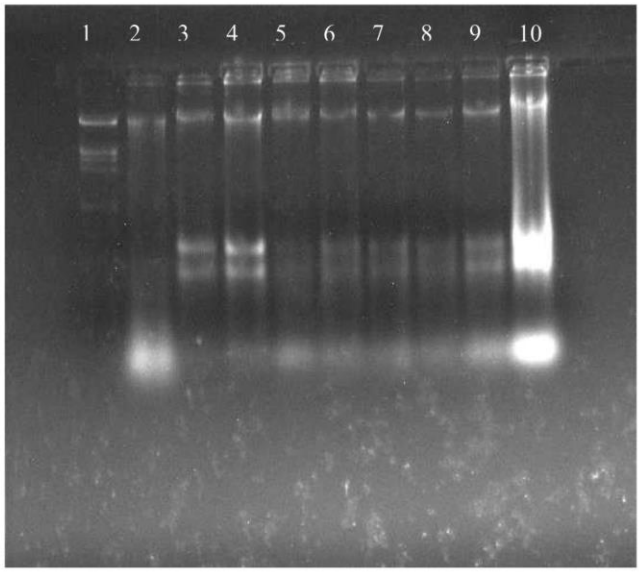
4.5.3.1 Isolation of genomic DNA

Upon electrophoresis on 0.7 per cent agarose gel, intact DNA was observed (Plate 20).



M- Mannopinic acid (Standard)
A- Agropinic acid (Standard)
1- ATCC 15834
2- ATCC 11325
3- TR7
4- A4
5- TR107
6- MTCC532
N. tabacum
7- Non-transformed *N. tabacum* (Control)
8- TR107 – *R. serpentina*

Plate 19. Detection of opines by High Voltage Paper Electrophoresis



1- Marker
2- ATCC 15834
3- ATCC 11325
4- TR7
5- A4
6- TR107
7- MTCC532
N. tabacum
8- Non-transformed *N. tabacum (control)*
9- TR107 - *R. serpentina*
10- ATCC 15834 - *Artemesia annua*

Plate 20. Isolation of Genomic DNA

4.5.3.2 Isolation of plasmid DNA

The plasmid DNA was isolated from *A. rhizogenes* strain A4 and was electrophoresed on 0.7 per cent agarose gel. The plasmid DNA had high molecular size (Plate 21).

4.5.3.3 PCR analysis of rol B and C genes

PCR analysis was carried out using two sets of primers, Rol BF1R1 and Rol CF1R1. ATCC 15834, ATCC 11325, TR7 and A4 transformed roots of *N. tabacum* and A4 plasmid and *A. annua* hairy root DNA (positive control) showed amplification of amplicon size 740 and 520bp corresponding to *rol* B and C gene.

TR107 induced roots of *R. serpentina* and non-transformed roots of *N. tabacum* (negative control) showed no amplication. The amplified DNA samples were electrophoresed on 1 per cent agarose gel (Plate 22).

4.6 ENHANCEMENT OF SECONDARY METABOLITE AND QUANTIFICATION

4.6.1 Alkaloid estimation- Nicotine

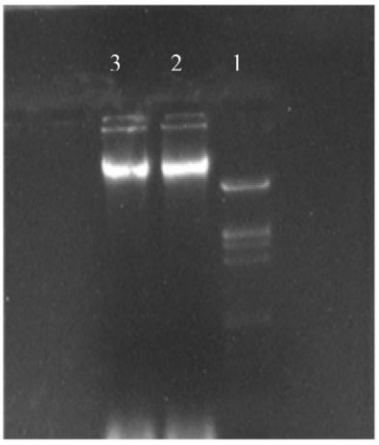
HPLC technique was used for the quantitative analysis of nicotine in various samples. Quantification of nicotine was done with the help of class LC10 software (Shimadzu, Japan).

Fig 1. represents HPLC profile of the nicotine standard when separated on an Aluspher column RP select B (5µm) column using a mobile phase of methanol: water (1:1). Retention time at a flow rate of 1ml/min for nicotine standard was.

The calibration curve for nicotine is as follows,

$Y=30704*x$, with $R^2=0.9986$

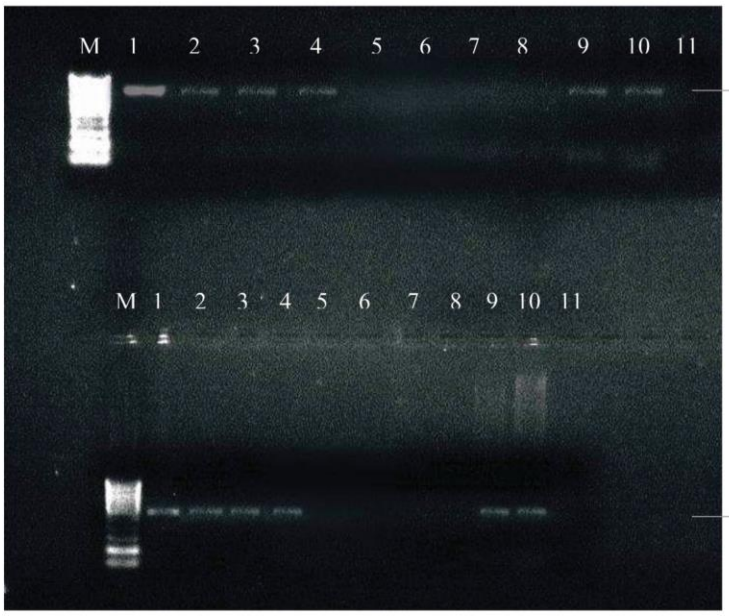
In the calibration curve, X represents concentration (ppm) and Y stands for peak area.



1- Marker

2, 3 - A4 plasmid DNA

Plate 21. Isolation of Plasmid DNA



M- Marker

1-ATCC 15834
2-ATCC 11325
3-TR7
4-A4
5-TR107
6-MTCC532
} *N. tabacum*

7-Non-transformed *N. tabacum* - Negative control

8- TR 107 *R. serpentina*

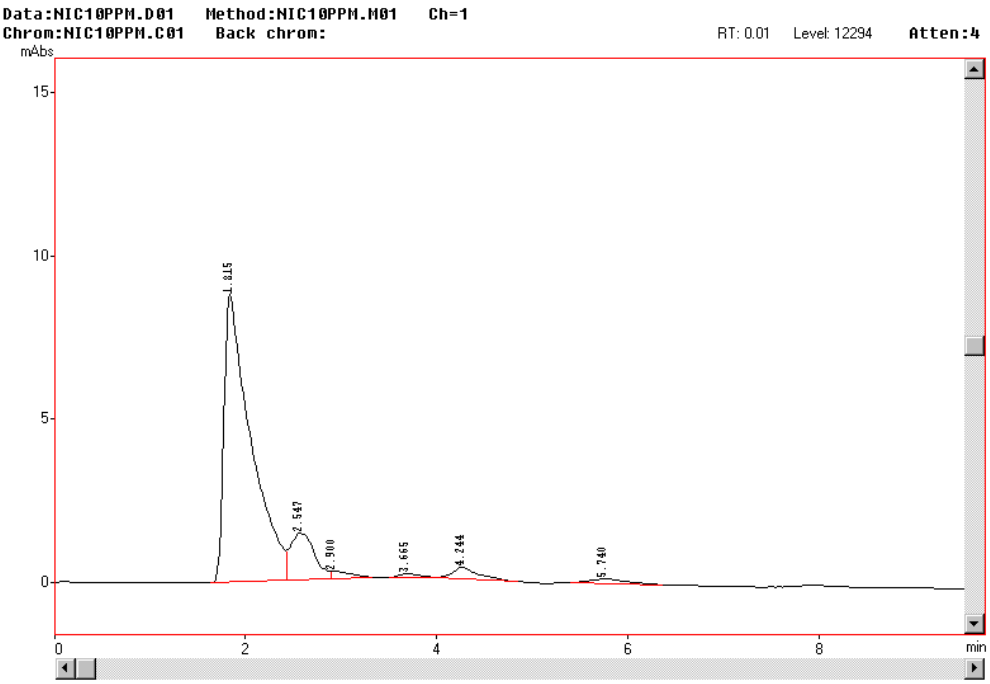
9-ATCC 15834 *A. annua*
10- A4 plasmid
} Positive control

11-Blank

Plate 22. PCR analysis of hairy roots for *rol* B and C gene

Fig 1. HPLC profile of the nicotine standard

CPBMB nicotine 2009
Col: Alumina RPSB
SS: Methanol-water 50:50
Flow: 1 ml/min
Sample: nicotine 10ppm in methanol
WL: 260 nm
20 ul



** Peak Report ** NIC10PPM.D01 09/11/10 13:54:54

PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	1.815	171035	8818			81.3430	Nicotine
2	2.547	24529	1410	V		11.6659	
3	2.900	3043	236	V		1.4471	
4	3.665	1409	108			0.6699	
5	4.244	6640	349			3.1579	
6	5.740	3609	141			1.7162	
TOTAL		210264	11062			100.0000	

4.6.1.1 Nicotine content of plant samples

Nicotine content of plant samples analyzed by HPLC is given in Table 32. Analysis of field grown and *in vitro* plants as well as hairy root cultures showed that field grown roots had the highest nicotine content followed by hairy roots. It was also observed that field grown plants of three leaf stage, showed a uniform distribution of nicotine alkaloid throughout the plant.

4.6.1.2 Effect of media components on alkaloid accumulation

The effect of culture media on alkaloid accumulation in *N. tabacum* is shown in Table 33.

Hairy roots cultured in half MS medium showed the highest content of nicotine. Nicotine yield as well as growth rate of hairy roots in B5 was the lowest but the concentration was not significantly different from that of full MS. In B5 media nicotine exudation into the culture medium was about 7 fold higher than that of full MS.

Table 32. Nicotine content of *in vivo*, *in vitro* and transformed samples of *N. tabacum*

Sl.No	Sample		Nicotine concentration (µg/g)
1	Field grown plant	Shoot tip	119.32
		Leaf	111.70
		Root	110.23
2.	*In vitro* plant	Shoot tip	48.76
		Leaf	48.68
		Root	79.3
3.	Transformed plant	Shoot tip	88.87
		Leaf	74.35
		Root	104.74

Table 33. Effect of culture media on nicotine accumulation in *N. tabacum*

Sl.No	Media	Fresh weight of hairy roots (gm)	Nicotine concentration (µg/g)	Quantity of nicotine in the culture (µg)	Quantity of nicotine in the medium (µg)
1.	Full MS	0.2346	166.40	39.04	1.27
2.	Half MS	0.6913	234.03	161.79	8.08
3.	B5	0.016	122.93	1.97	8.65

4.6.2 Enhancement of secondary metabolite production

Response of hairy root cultures to different treatments for enhancement of nicotine production is shown in Table 34.

4.6.2.1 Addition of osmoregulants

4.6.2.1.1 Addition of osmoregulant- sorbitol

Addition of 1 per cent sorbitol increased the biomass along with an increase of nicotine content by 2.2 fold than that of control. With further increase in the concentration, biomass production as well as nicotine content decreased. Exudation of nicotine into the culture medium was noticed with the addition of sorbitol.

4.6.2.1.1 Addition of osmoregulant- PEG

Poly Ethylene Glycol (PEG) at 5 per cent, increased the nicotine concentration but there was no significant difference when compared with control. Also the biomass yield with 5 per cent PEG was the least. PEG at 2 per cent, increased the biomass but failed to increase the nicotine concentration. Exudation of nicotine into the culture medium was noticed in all the three concentrations with 2 per cent PEG showing the highest.

4.6.2.2 Addition of Yeast Extract

Yeast Extract of 2 per cent and 5 per cent elicited a positive response on enhancement of nicotine but failed to increase the biomass. Increase in percentage of yeast extract augmented the nicotine yield. Exudation of nicotine into the culture medium was not noticed.

4.6.2.3 Addition of precursor

The precursor L-arginine, increased the nicotine concentration at 50 and 100 ppm after which, increase in precursor concentration reduced the yield of nicotine. Increase in fresh weight of cultures was noticed at 50 ppm along with an increase of nicotine yield by

2.6 fold than control. Exudation of nicotine into the culture medium was noticed in the three concentrations tried, with 100 ppm L-arginine showing the highest.

Table 34. Response of hairy root cultures of *N. tabacum* to different treatments for enhancement of secondary metabolite- nicotine

Sl. No	Compound		Fresh weight of hairy roots (gm)	Nicotine concentration (µg/gm)	Quantity of nicotine in the culture(µg)	Quantity of nicotine in the medium (µg)	Fold increase
1	Control		0.6039	104.74	63.25	0.83	0
2	Sorbitol	1%	0.9173	149.27	136.93	17.56	2.2
		2%	0.4054	85.22	34.55	4.41	0.5
		5%	0.4102	79.01	32.41	7.77	0.5
3	PEG	1%	0.3694	13.9	5.14	6.88	0.08
		2%	1.0185	10.41	10.6	9.45	0.2
		5%	0.129	111.57	14.39	3.35	0.23
4	Yeast Extract	2%	0.1992	154.32	30.74	0	0.49
		5%	0.2437	238.31	58.08	0	0.92
5	L-arginine	50 ppm	1.0002	161.55	162.39	8.65	2.6
		100 ppm	0.5881	233.67	125.74	9.69	1.99
		150 ppm	0.899	130.1	110.53	8.14	1.75

Initial inoculum ~ 0.25-0.5 g

Discussion

5. DISCUSSION

Rauvolfia serpentina (L.) Benth. commonly known as sarpagandha is an important medicinal shrub of Apocynaceae family. The roots of *Rauvolfia* are used by the ayurvedic and unani systems of medicine in India. The drug reserpine isolated from the dried roots of *Rauvolfia* proved to be very effective in treating high blood pressure, insanity and schizophrenia. The indiscriminate extraction of the drug has rendered it as an extremely rare and vulnerable species.

Hairy roots induced by *Agrobacterium rhizogenes* offer a promising system for production of valuable compounds from medicinal plants. Optimization of hairy root culture would be highly suitable for the sustainable production of alkaloids and increased production of biomass (Sudha *et al.*, 2003).

The results obtained in the study on "Genetic transformation in sarpagandha (*Rauvolfia serpentina* (L.) Benth.) for enhancement of secondary metabolite production are discussed in this chapter.

5.1 STANDARDIZATION OF *IN VITRO* REGENERATION

5.1.1. Standardization of surface sterilization

A variety of chemicals are used for sterilization purposes, which includes halogens, heavy metals, phenolic compounds, alcohol etc. Heavy metals cause inactivation of cells, one such effective disinfectant is mercuric chloride which is used commonly as a surface sterilization agent. Its mode of action involves the inactivation of enzymes by coupling to the sulphydryl groups of protein (Tauro *et al.*, 2006).

In the present study, $HgCl_2$ at 0.1 per cent for 1 minute was found to be optimum for surface sterilization of shoot tip and nodal segment explants. Increase in time of treatment resulted in drying of the explant and thereby reduction in culture establishment.

The result of the study is in accordance with the findings of Shaneeja (2007) who has reported that 0.05 per cent $HgCl_2$ for 10 min or 0.1 per cent $HgCl_2$ for 1 min was optimum for surface sterilization of leaf and inflorescence explant in *Artemesia annua*.

5.1.2 Explant regeneration

5.1.2.1 Establishment of shoot tip and nodal segment explants

The ratio of auxin to cytokinin is required to elicit a morphogenetic response which varies with the plant species, the plant part used and the media composition. A high concentration of auxin and a low concentration of cytokinin in the medium promotes callus formation whereas low auxin and high cytokinin results in induction of shoot morphogenesis (Nazeem and Smitha, 2007).

Maximum bud burst from shoot tip and nodal segment was elicited on MS medium supplemented with BA and NAA combinations. Effectiveness of BA and NAA combination for *in vitro* shoot regeneration and multiplication from shoot tip and nodal segment explants were also reported by Sarker *et al*. (1996) and Salma *et al*. (2008).

Shoot regeneration from shoot tip and nodal segment explants of *R. serpentina* was observed in MS media with 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA. This result is in accordance with the findings of Sarker *et al.* (1996) who reported shoot regeneration from nodal segment and shoot tip explants of *R. serpentina* in MS medium containing 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA.

The use of NAA and BA combinations at varying concentrations in establishment of shoot cultures from shoot tip and nodal segment explants stimulated callus formation at the base of the explants. Similar results were also observed by Sarker *et al.* (1996) and Ghosh and Banerjee (2003) in *Rauvolfia serpentina*, where α–naphthaleneacetic acid and 6-benzyladenine stimulated callus formation at the base of nodal segment explant. This may be because auxins normally induce callusing and rooting up to certain optimum concentrations. Pant and Joshi (2008) reported that higher NAA concentration (1ppm) was effective for callus induction in *R. serpentina*.

Increased concentration of BA resulted in low percentage of response and shortening of internodes. Baksha *et al*. (2007) reported that increased rate of cytokinin in medium resulted in poor response, over growth of leaves and shortening of internodes.

5.1.2.2 Multiplication

MS media supplemented with BA and NAA at varying levels, gave maximum shoot morphogenesis. The established shoot cultures gave numerous differentiated shoots on the establishment media, MS + 1 mg l^{-1} BA + 0.1 mg l^{-1} NAA. This is in confirmity with the findings of Sarker *et al.* (1996) who reported that MS media supplemented with 1 mg l^{-1} BA + 0.1 mg l^{-1} NAA promoted induction of multiple shoots from shoot tip and nodal segment explants of *R. sepentina*.

Higher concentration of NAA with BA resulted in reduction of number of shoots per explant. This was in accordance with the findings of Skoog and Miller (1957) where they reported the need to reduce the auxin level in the medium to promote shoot formation.

5.1.2.3. Rooting

Rooting of *in vitro* shoots was observed to be best in ½ MS supplemented with 0.2 mg l^{-1} IBA and 0.2 mg l^{-1} NAA. Half strength MS medium favored better rooting than full MS. This could be corroborated with the findings of Khan *et al.* (1999) who reported that root growth was better when MS medium concentration was reduced.

In the present study, 76 per cent rooting occurred in MS basal media within three weeks and the roots formed were very thin. Pant and Joshi (2008) reported that MS hormone free medium showed positive response for induction of roots in *R. serpentina.*

In media containing 0.5 mg l^{-1} NAA, cent percent rooting was observed but the roots were thin and elongated. This is contradictory with the findings of Baksha *et al.* (2007) who has reported NAA (0.5 mg l^{-1}) as the ideal growth regulator concentration for rooting in *R. serpentina.*

In the present study ½ MS media with 0.2 mg l^{-1} IBA and 0.2 mg l^{-1} NAA showed cent per cent rooting within 10 days and the roots were branched and elongated. This is in confirmity with the findings of Ahmad *et al.* (2002) who has identified 0.2 mg l^{-1} IBA and 0.2 mg l^{-1} NAA as the best auxin combination for proper rooting in *R. serpentina.*

5.2 CULTURING AND ANTIBIOTIC SENSITIVITY SCREENING OF *AGROBACTERIUM RHIZOGENES* AND EXPLANTS

5.2.1 Culturing of *A. rhizogenes* strains

YEM medium was found to be optimum for culturing ATCC 15834, ATCC 11325, TR 7, A4, TR107 and MTCC 532. YEM medium was used for culturing A4 and LBA 9402 (Li and Leung, 2003), TR105 (Lonkova and Fuss, 2009), ATCC 15834 (Hu and Alfermann, 1993, Vanhala *et al.,* 1995), A4, ATCC 11325, ATCC 15834 and LBA 9402 (Hooykass *et al.*, 1977).

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

For effective *Agrobacterium*-mediated transformation, the antibiotic selected should control bacterial overgrowth without inhibiting the regeneration of the plant cells. Also it is necessary to identify the antibiotic that eliminates *Agrobacterium* with minimum phytotoxic effects.

In the present study, A4 strain was resistant to ampicillin and carbenicillin but sensitive to cefotaxime whereas TR 7 and ATCC 11325 were sensitive to cefotaxime and carbenicillin but resistant to ampicillin. ATCC 15834, TR 107 and MTCC 532 were sensitive to ampicillin, carbenicillin and cefotaxime. Cefotaxime at 500 mg l^{-1} killed all the experimental strains. Cefotaxime 500 mg l^{-1} was used for eliminating bacteria by Varghese (2006) and Shaneeja (2007) as well.

The explants used in the transformation studies remained healthy up to 500 mg l^{-1} of cefotaxime concentration. At 1000 mg l^{-1} of cefotaxime concentration, the explants exhibited yellowing and browning. Leaf sections were more sensitive than shoot tip and nodal segment to cefotaxime at 1000 mg l^{-1}.

Rooting of shoot tip explants was observed in the MS basal medium with antibiotics but at 250 and 500 mg l^{-1} of cefotaxime concentration, the roots produced were thick in nature.

The explants were healthy at 500 mg l^{-1} of cefotaxime concentration. At the same time 500 mg l^{-1} of cefotaxime concentration was able to kill all the six experimental strains.

Cefotaxime at 500 mg l^{-1} has been selected as the antibiotic concentration for transformation work and this has been reported by several workers (Lonkova and Fuss, 2009; Sudha *et al.,* 2003; Satdive *et al.,* 2007 and Kumar *et al.,* 2006).

5.2.3 Pre-culturing of explants

The explants were pre-cultured for two days on MS basal medium without antibiotics prior to transformation. This is mainly to make the explants acclimatize to the new culture condition and also to make use of the endogenous hormones thereby making the explants ready for transformation studies. Yu *et al.* (2001) reported that hairy roots emerged 3-4 days earlier, if leaf explants of *Pueraria lobata* were precultured for 2-3 days before transformation with *A. rhizogenes* strain R 1601.

5.3. STANDARDIZATION OF TRANSFORMATION TECHNIQUES IN *RAUVOLFIA SERPENTINA*

In the present study on transformation in *R. serpentina*, the six strains studied failed to induce hairy roots. Several methods like co-culture period, addition of phenolic compounds like acetosyringone and etiolation of shoot tip explant were tried to induce hairy roots but there was no positive response. Shoot elongation and yellowing of explant was noticed in most of the cases.

Various plant species differ greatly in their susceptibility to *Agrobacterium* infection (Anderson and Moore, 1979; Porter, 1991). Even within a species, different cultivars or ecotypes may show different degrees of susceptibility by particular *Agrobacterium* strains and the difference in the susceptibility of genotypes to *Agrobacterium* could be due to the presence of inhibition system in the *Agrobacterium* sensory machinery (Karami *et al.,* 2009).

In the present study, the plant defence mechanism would have responded to the *A. rhizogenes* infection thereby preventing integration of T-DNA into the plant cell. Karmarkar *et al.* (2001) reported that certain inhibitory compounds are induced from the wounds and not all the *A. rhizogenes* strains are capable of degrading the inhibitory compounds. Parrott *et al.* (2002) suggested that production of reactive oxygen species is one of the earliest defense mechanism in plants on pathogen infection. The reactive oxygen species produced kills the *Agrobacterium*,

thereby preventing *Agrobacterium* from infecting plant cells and delivering T-DNA into plants (Dan, 2008).

Franklin *et al.* (2008) reported the recalcitrance of *Hypericum perforatum* plant cells to *Agrobacterium* infection by an intense oxidative burst thereby reducing the viability of infection. Fukai *et al.* (1991) reported that tea leaves are recalcitrant to *Agrobacterium* mediated genetic transformation, due to high content of bactericidal polyphenols. Resistance of plants to *in vitro* regeneration and transformation were reported by several workers in different crops like sunflower (Mohamed *et al*., 2006), jute (Sarker *et al*., 2007), legumes (Sarker *et al*., 2005).

Nin *et al.* (1997) have reported that specificity of *Agrobacterium* transformation is closely connected with the age and hormonal balance of the host tissue.

The shoot tip explants showed bud burst whereas leaf and nodal segments showed yellowing followed by death of explants. Ghosh and Chatterjee (1990) reported the regeneration of multiple shoot buds from the *Agrobacterium* infected cotyledonary petioles of *Corchorus_capsularis.* Yellowing and death of the explants may be due to the damage caused by the *Agrobacterium* infection. Kuta and Tripathi (2005) stated that exposure of plant tissues to *Agrobacterium* during plant transformation leads to browning and necrosis of targeted cells which affect transformation efficiency.

Co-cultivation of shoot tip explant with TR107 strain showed bud burst and elongation along with induction of roots, but this response was not reproducible. The roots were excised and placed on MS basal medium without antibiotics but drying of root was noticed.

Islam *et al.* (2009) reported that Tossa jute infected with *Agrobacterium* showed abnormal morphology including adventitious root formation. Li and Leung (2003) reported that adventitious roots induced using various *A. rhizogenes* treatments were not able to survive on hormone free medium.

The present results indicate the non integration of T-DNA and the induction of roots from infected explant may be due to the presence of endogenous auxin in the cells after pre-culturing. Li and Leung (2003) reported that hypocotyl segments of *Pinus radiata* inoculated with *A. rhizogenes* in IBA medium resulted in better rooting of cultures but there was no integration of

T-DNA. The induction of normal roots from infected explants was also reported by Karmarkar *et al*. (2001) in *Holostemma ada-kodien*.

Further detailed study is needed to know the mechanism hindering the transformation using *A. rhizogenes* in *R. serpentina*. Since poor response in induction of hairy roots in *R. serpentina* was noticed, the remaining part of the research work such as checking the virulence of the strains and viability of the methodology followed, confirmation of transformation and enhancement of secondary metabolite present in the hairy roots was done with the hairy roots induced in *Nicotiana tabacum* which was taken as a model plant.

5.4. STANDARDISATION OF TRANSFORMATION TECHNIQUES IN *NICOTIANA TABACUM*

5.4.1 Standardization of explant

In the present study on *Nicotiana tabacum*, leaf segment was found to be the best explant for efficient transformation followed by shoot tip and nodal segment (Fig 2). Suitability of leaf segments for inducing hairy roots in *N. tabacum* was also reported by Kumar *et al*. (2006).

Leaf as explant for inducing hairy root was also reported in different plants by several workers (Allan *et al.*, 2002; Malabadi and Nataraja, 2003; Mehrotra *et al.*, 2008). The leaf segment and shoot tips of *Withania somnifera* showed efficient transformation (Varghese, 2006). Shoot tip followed by leaf segment showed efficient transformation in *Artemesia annua* (Shaneeja, 2007).

Various tissues, organs and cell types within a plant differ in their susceptibility to *Agrobacterium* transformation (Repellin *et al.,* 2001). The age of the explant is a crucial factor with juvenile material being optimum for transformation (Hu and Du, 2006). The level of tissue differentiation also determines the ability to give rise to transformed roots after *A. rhizogenes* inoculation (Trypsteen *et al.,* 1991).

In the present study, callusing of infected explants as well as callusing of non-transformed (control) explants was noticed. This may be due to the endogenous auxin present and also may be due to T-DNA directed auxin synthesis. Callusing of shoot tip explant was less when compared to leaf and nodal segment. Similarly, the amount of callus induced varied with

the strain. So it could be said that induction of hairy roots followed by callus formation is dependent on the bacterial strain, explant type, type of infection and site infected. Sudha *et al.* (2003) reported that tendency for callusing and rooting varied depending on the type of bacterial strain and explant.

The leaf explants induced hairy roots directly from the wounded sites of the explant and proliferation of hairy roots was seen along with callusing. Wounding of leaf explant resulted in induction of hairy roots from petiole and midvein regions. Similar results have been reported in *N. tabacum* where manual wounding resulted in induction of hairy roots from the midvein region of leaf explant (Kumar *et al*., 2006). Giri *et al.* (2001) reported that hairy roots were induced from the site of infection of explants on MS basal medium. Sudha *et al.* (2003) reported hairy root induction and callus formation from the wounded sites of the explants. This indicates that phenolic from the wounded sites induce *vir* gene expression and thereby integration of T-DNA and T-DNA directed auxin synthesis.

5.4.2. Influence of inoculation method on transformation

The inoculation method (DI and SM) used affects the transformation frequency. In the present study, transformation was achieved by both the methods with all the six strains. But the percentage of transformation varied with the methodology.

ATCC 15834, ATCC 11325 and TR7 showed highest transformation percentage by the Suspension Method (SM) with leaf as explant whereas A4, TR107 and MTCC 532 showed maximum transformation by the Direct Inoculation Method (DIM) with leaf as explant (Fig 2). Similar result was reported by Varghese (2006) and Shaneeja (2007) where A4 showed better transformation when used as single cell colony.

In general, transformation with single cell bacterial colony as well as bacterial suspension was effective in *N. tabacum*. Jaziri *et al*. (1995) have reported that the hairy root cultures of *A. annua* were established using either bacterial colonies or bacterial suspension.

5.4.3. Influence of co-culture period on transformation

Transformation frequency was influenced by the co-culture period. Co-cultivation of explants for more than 2 days reduced the percentage of transformation. This may not be

attributed to the virulence property of the strain but the reduction in percentage may be due to the over growth of bacteria thereby killing the explant tissue.

Karmarkar *et al.* (2001) reported that after 36 hrs, the bacterial cells reached supra optimum level and competitive inhibition for competent plant cells resulted in reduction of transformation.

5.4.4 Efficiency of strains in inducing hairy roots

ATCC 15834, ATCC 11325, TR7, TR107 and MTCC 532 produced transformation in *N. tabacum.* Efficiency of strains in inducing hairy roots is in the order of ATCC 15834, TR7, ATCC 11325, A4, TR107 and MTCC 532 (Fig 3). Shaneeja (2007) reported induction of hairy roots in *A. annua* with *A. rhizogenes* in the following order, ATCC 15834, A4 and MTCC 532.

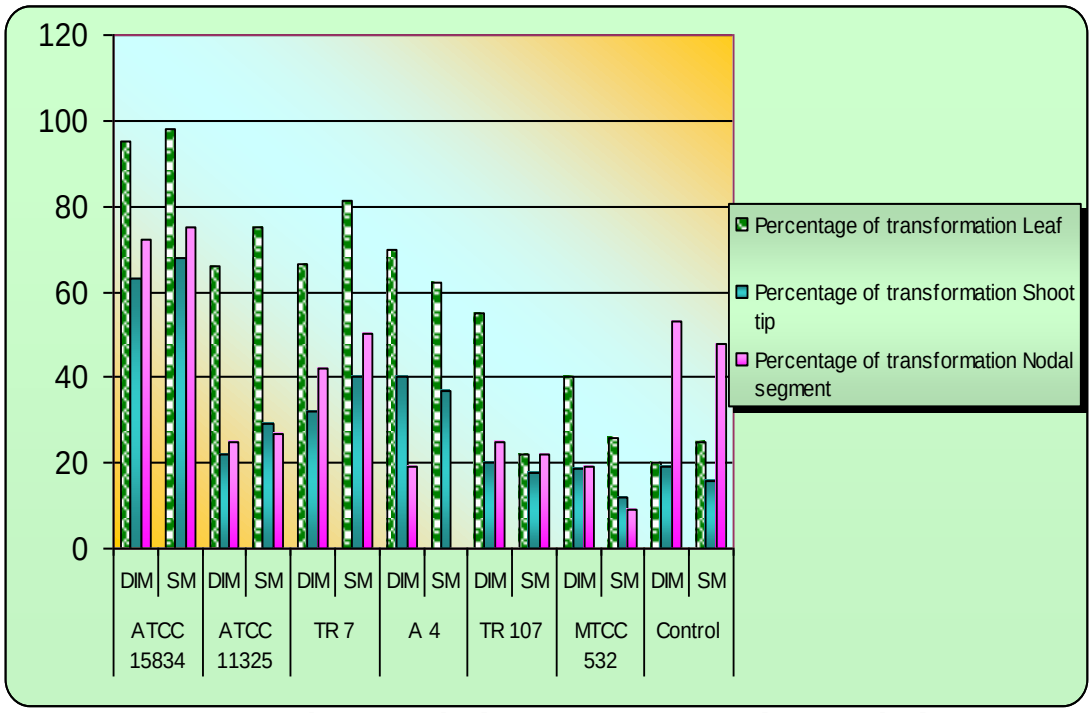
The different *A. rhizogenes* strains varied greatly in their efficiency for successful transformation. The agropine – type Ri plasmids are considered to be the most virulent and therefore more often used in the establishment of hairy root cultures (Sevon and Oksman-Caldentey, 2002). Rhodes *et al.* (1989) reported that the agropine strains (15834, A4, TR7) have a wide host range that is attributed to the presence of T_R-DNA fragment of the T-DNA.

The bacterial strains exhibit different levels of virulence to different plant species. In *Linum tauricum*, 32 per cent transformation occurred with ATCC 15834, whereas TR105 showed 55 per cent of transformation (Lonkova and Fuss, 2009). Hu and Alfermann (1993) reported that LBA 9402 strain caused 85 per cent rooting in the leaf explants of *Salvia miltiorrhiza* when compared to A4 (10 per cent) and ATCC 15834 (20 per cent).

5.4.5 Number of days for hairy root induction

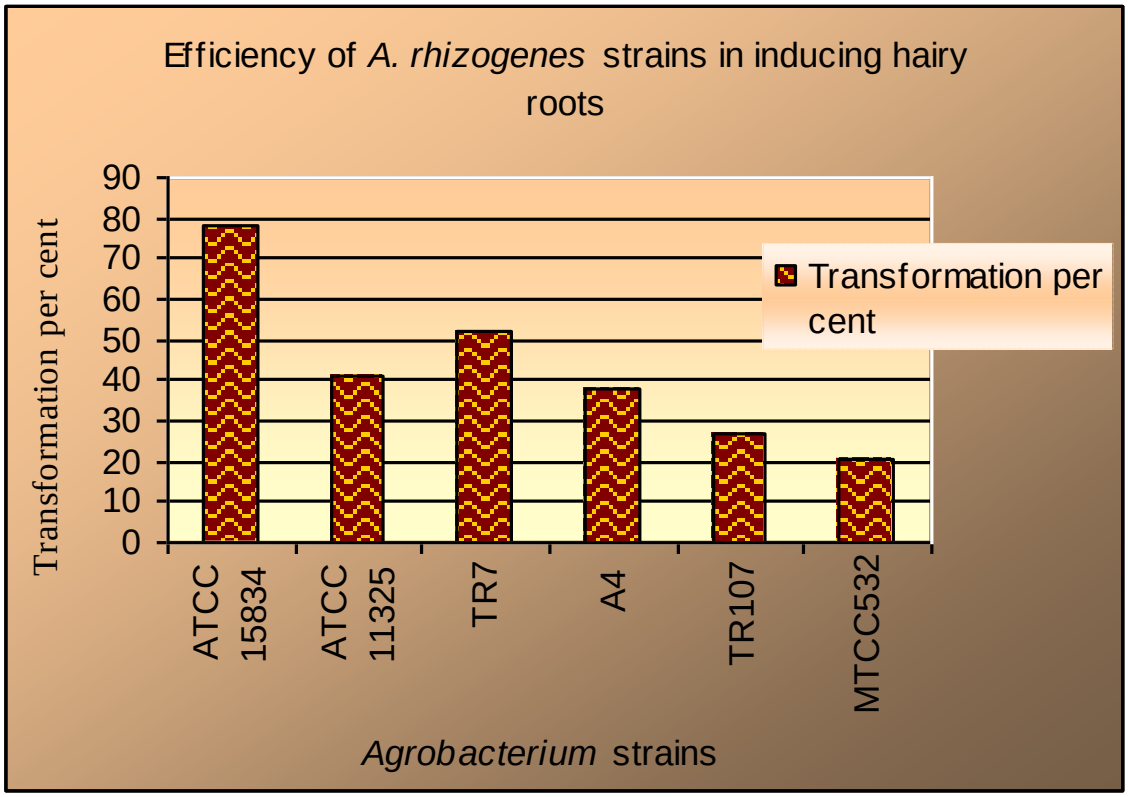
In the present study on *N. tabacum*, hairy root induction was achieved in a time period of 3-10 days from leaf, shoot tip and nodal segment explant irrespective of the method of inoculation. Different plant species vary with the time period for hairy root induction from one week to a month (Hu and Du, 2006).

Fig 2. Influence of bacterial inoculation method and explant on transformation in *N. tabacum*



DIM- Direct Inoculation Method, SM- Suspension Culture Method

Fig 3. Efficiency of *A. rhizogenes* strains in inducing hairy roots in *N. tabacum*



Satdive *et al*. (2007) reported the induction of roots at the end of third week in *Azadirachta indica* while Varghese (2006) reported the induction of hairy roots 1 to 3 weeks after infection in *W. somnifera*.

5.4.6 Establishment of hairy root cultures

The hairy roots induced showed negative geotropism, fast growth rate, high degree of lateral branching and were able to grow in the absence of growth regulators. This is in agreement with the statement of Veena and Taylor (2007) that hairy roots grow quickly in the absence of exogenous plant growth regulators.

5.4.7 Rapid culturing of hairy roots

On comparison of hairy roots induced by the *A. rhizogenes* strains, with roots produced in control, hairy roots showed rapid growth with lateral branching. Among the hairy roots induced, ATCC 15834 induced hairy roots showed faster growth producing more biomass. This may be because the growth of hairy roots and the biomass produced may be influenced by the strain experimented. Oksman -Caldentey and Hiltunen (1996) reported that *A. rhizogenes* strains 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. Peng *et al*. (2008) reported hairy roots grown in liquid media had many branches and also grew fast.

5.4.8 Effect of culture media and condition on the growth of hairy roots.

In the present study, ½ MS with 3.0 per cent sucrose was found to be superior for promoting hairy root growth followed by MS and B5 with 3.0 per cent sucrose. This shows that the culture medium and conditions have significant influence on the growth of hairy roots. This is in accordance with the findings of Xu *et al*. (2004) who reported that MS and half MS are superior to B5 and White`s medium for the growth of hairy roots. MS medium was the best culture medium compared to B5 and Woody plant medium (WPM) and the difference in the ionic strength of media may be the factor for the differential growth rate of hairy roots in the different media (Wu, 2007). Half MS medium with 3 per cent sucrose was found to be superior for promoting hairy roots in *W. somnifera* (Varghese, 2006).

5.4.9 Growth pattern of hairy roots

The growth of hairy roots induced by ATCC 15834 and TR7 showed a sigmoid `S` shaped curve with an initial lag phase of one week. Then the growth increased at a faster rate for 15 to 30 days, that is, the exponential phase. After 30 days, there was a steady state that is, the stationary phase in which the cultures started to turn brown (Fig 4).

This is in confirmity with the findings of Tiefeng *et al*. (2004) who reported that the hairy roots of *Isatis indigotica* grew fast and showed an S-shaped growth curve that reached its apex on the 24th day of culture. Varghese (2006) reported a sigmoid growth pattern in the hairy root cultures of *W. somnifera*.

5.1 CONFIRMATION OF TRANSFORMATION

Confirmation of the transformed nature of hairy roots and the integration of T-DNA from *A. rhizogenes* into the hairy root genome was done by examining the morphological features and by performing opine and PCR analysis. Sawada *et al.* (1995) reported that PCR technique could be used for the detection of Ti and Ri plasmids from phytopathogenic *Agrobacterium* strains.

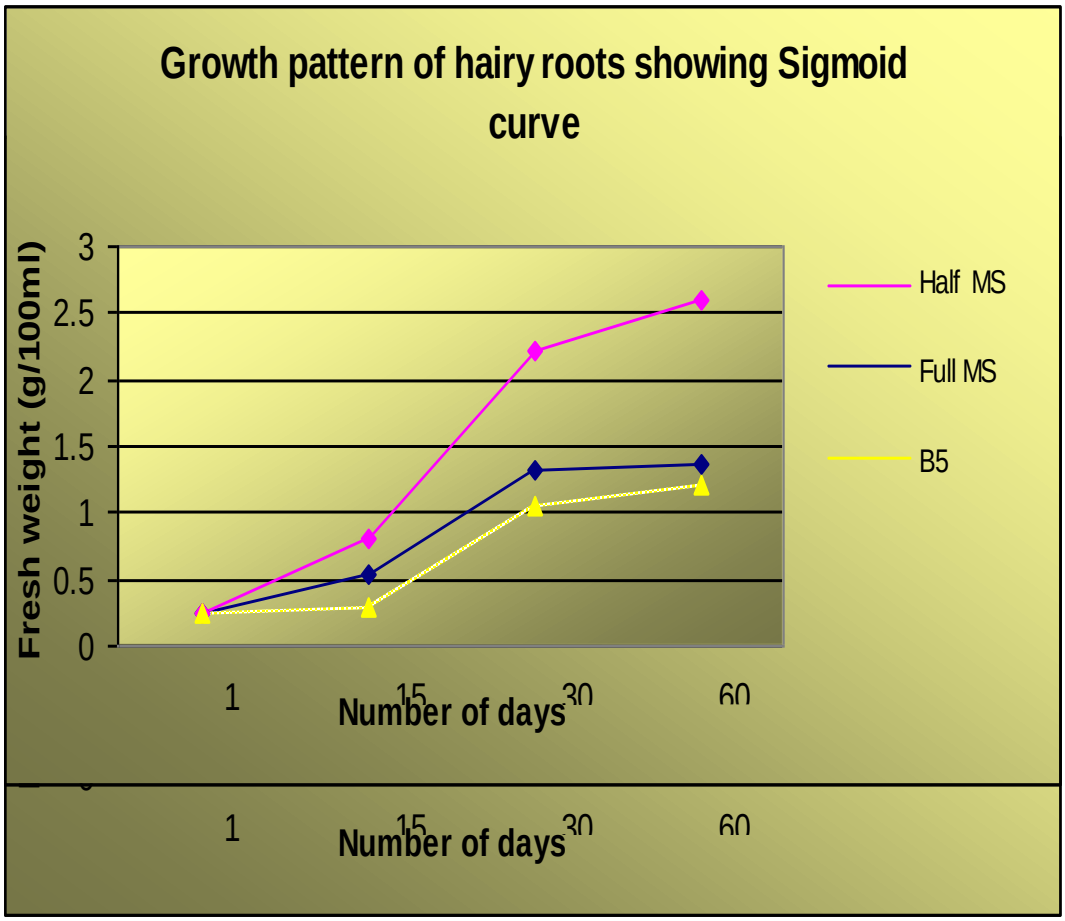
5.1.1 Morphology of hairy roots

The hairy roots induced showed fast growth with lateral branching. The young roots were white in colour with more root hairs during initial stages. The roots after 3 to 4 weeks of culturing turned brown, which may be mainly due to the depletion of nutrients in the culture media. Liu *et al*. (1998) had also reported that the initial inoculated hairy roots turned brown after 3 weeks mainly due to the decrease in nutrients in the media.

The hairy roots induced by ATCC 15834 and TR7 showed high lateral branching. The roots were thick with the younger root tips showing negative geotropism. Similary the hairy roots induced by A4 and ATCC 11325 were long and white in colour showing lateral branching. The roots induced by TR107 and MTCC 532 showed slow growth with less lateral branches.

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 hairs in *W. sonnifera* (Varghese, 2006).

Fig 4. Growth pattern of ATCC 15834 induced hairy roots of *N. tabacum*



5.1.2 Opine analysis

Upon integration of the Ri T-DNA into the plant cell genome, opine synthesis will be initiated through expression of the opine biosynthetic gene. In the present study, opines extracted from ATCC 15834, TR7 and A4 produced spots corresponding to agropinic acid. The roots induced by A4, ATCC 15834, pCA4 showed the presence of agropine (Karmarkar *et al*., 2001).

Non- transformed (control) tobacco roots produced no spot. A faint spot corresponding to mannopinic acid was observed in TR7 alone. Petit *et al*. (1983) reported that roots produced by agropine-type strains (A4, 15834, HRI) contain agropine, mannopine, mannopinic acid, and agropinic acid, whereas roots produced by mannopine-type strains (8196, TR7, TR101) contain mannopine, mannopinic acid and agropinic acid. A faint spot corresponding to mannopinic acid observed in TR7 lane may be because TR7 is a mannopine- type strain and could have produced mannopinic acid in trace amounts.

No spot was detected from ATCC 11325, TR107, and MTCC 532 induced roots in *N. tabacum* and also TR107 induced roots in *R. serpentina*. Porter (1991) suggested that *A. rhizogenes* strain ATCC 11325 should be placed with *Agrobacterium tumefaciens* due to nopaline production in transformed plants. This may be the reason for the absence of a spot corresponding to agropinic acid and mannopinic acid from the transformed roots of ATCC 11325.

Opine production can be unstable in hairy roots and may disappear after a few passages (Godwin *et al*., 1991). Varghese (2006) was unable to confirm transformation through opine analysis because of interfering substances. Further confirmation was carried out with PCR. Detection of integrated T-DNA by PCR or Southern hybridization is often necessary to confirm genetic transformation.

5.1.3 PCR analysis for confirmation

To confirm the integration of T-DNA from the soil bacteria into the hairy root genomic DNA, DNA from hairy roots of *N. tabacum* and TR107 induced roots of *R. serpentina* were subjected to PCR.

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 *N. tabacum* (Kumar *et al*., 2006), *Atropa belladonna* (Bonhomme *et al.,* 2000), *Gmelina arborea* (Dhakulkar *et al*., 2005), *Linum tauricum* (Lonkova and Fuss, 2009), *W. somnifera* (Varghese, 2006) and *A. annua* (Shaneeja, 2007).

The PCR amplification was carried out using *rol* B and C primers. Normal non-transformed roots of *N. tabacum* served as negative control whereas *pRiA4* plasmid and DNA from *Artemesia annua* hairy roots served as positive control.

The PCR results showed that the hairy root lines of *N. tabacum* induced by ATCC 15834, ATCC 11325, TR7 and A4 contained *rol* B and C gene which are part of T-DNA of Ri plasmid of *A. rhizogenes*. The fragment of length 740bp and 520bp corresponding to *rol* B and C respectively was amplified from hairy roots of *N. tabacum* induced by ATCC 15834, ATCC 11325, TR7 and A4. Also amplification was seen in ATCC 15834 induced hairy roots of *Artemesia annua* and A4 plasmid DNA which served as the positive control. In non- transformed *N. tabacum* (negative control) and blank there was no amplification.

TR107 induced roots of *R. serpentina* showed no amplification of *rol* B and C. The transfer of T-DNA was not detected in the induced roots of *R. serpentina*. It could be said that no T-DNA transfer had occured or the T-DNA was present at a level lower to be detected by the PCR procedure. The roots induced by TR 107 strain in *R. serpentina* may be due to the endogenous auxin in plant cell, also may be due to the modification of culture medium by addition of antibiotics thereby creating stress for the plant cell.

5.5 ENHANCEMENT OF SECONDARY METABOLITE AND QUANTIFICATION

5.5.1 Alkaloid estimation- Nicotine

5.5.1.1 Nicotine content of plant materials

Nicotine is an alkaloid, which only exists in tobacco plants, and accounts for approximately 95 per cent of its total alkaloid content (Baldwin, 1989). Nicotine concentration in the cultivated tobacco species, *N. tabacum* and *N. rustica* ranges between 0.5 to 8.0 percent

(Murthy *et al.*, 1986). Nicotine biosynthesis occurs primarily in the tobacco roots, from where it is transported through the xylem to aerial parts of the plant, and accumulated in the leaves (Baldwin, 1989; Dawson, 1941). Akira *et al.* (2005) reported that nicotine alkaloid synthesis occurs in the root in response to insect damage and is then transported to the aerial parts of tobacco plants. The concentration of nicotine increases with the age of the plant. Tobacco leaves contain 2 to 8 per cent of nicotine combined as malate or citrate. The distribution of nicotine in the mature plant is widely variable, 64 per cent of the total nicotine exists in the leaves, 18 per cent in the stem, 13 per cent in the root, and 5 per cent in the flowers (Landoni, 1990). In undamaged tobacco plants, the nicotine concentration is 0.1–1 per cent of its dry mass (Baldwin, 1989).

In the present study, analysis of field grown seedlings of three leaf stage showed 0.119 mg g^{-1} of nicotine in shoot tip, 0.112 mg g^{-1} in leaf and 0.110 mg g^{-1} in roots. Analysis of *in vitro* cultures at three leaf stage, showed 0.048 mg g^{-1} of nicotine in shoot tip, 0.048 mg g^{-1}in leaf and 0.079 mg g^{-1} in roots. Solt (1957) reported that limited nicotine production occurs in the tobacco shoot and the alkaloid accumulation pattern is related to the growth of the plant. In field grown plant, the alkaloid content of shoot, leaf and root had no significant difference whereas in *in vitro* plant, roots possessed the highest alkaloid content. This partly explains the known fact that nicotine biosynthesis occurs primarily in the root and from root, it is being translocated to the leaves.

Nicotine has been analyzed by different methods such as steam distillation and spectroscopic method, TLC and densitometry and automated procedures using gas chromatography techniques (Griffith, 1957; Harvey *et al*., 1969 and Bush, 1972). In the present study, High performance Liquid Chromatography was employed for the estimation of nicotine present in the different experimental samples due to its reliability and speed.

5.5.2 Enhancement of secondary metabolite

Attempts have been made to increase the productivity of cell cultures of *Catharanthus roseus* by selecting high yielding strains, adding the precursors of desired compounds into the medium, eliciting the cultures with fungal extracts or stimulating stress conditions in the culture medium (Moreno *et al*., 1993).

5.5.2.1 Addition of osmoregulants

Sorbitol at 1 per cent added as an osmoticum increased the nicotine concentration as well as biomass production by 2.2 fold as compared to control. Accumulation of nicotine in the culture medium was also noticed. Cultures grown in 2 and 5 per cent sorbitol and 1, 2, and 5 per cent PEG did not have significant effect on nicotine production. It could be said that the stress induced by sorbitol at low concentration increased the yield and further increase in concentration resulted in low yield of nicotine. This may be due to the toxic effects of osmoregulants resulting in loss of cell viability. Thus it can be inferred that the use of appropriate amount of osmoregulants can increase the production of nicotine along with an increase in biomass production of hairy root cultures of *N. tabacum*.

The findings were in line to the work of Shi *et al*. (2007) who reported that the addition of sorbitol increased the tanshinone yield of *Salvia miltiorrhiza* hairy roots by 4.5 fold as compared to the control.

5.5.2.2 Addition of Yeast Extract

In the present study, it was found that addition of Yeast Extract augmented the nicotine yield but failed to increase the biomass.

Bensaddek *et al*. (2008) reported that elicitors elicit positive response on alkaloid accumulation by stimulating biosynthetic pathways. Wang *et al*. (2006) reported that addition of yeast elicitor increased artemisinin production in *A. annua* by three fold.

5.5.2.3 Addition of precursor

The addition of L-arginine as precursor had an impact on biomass production as well as yield of nicotine. Addition of the precursor, L-arginine at 50 ppm increased the nicotine yield by 2.6 fold but as the L-arginine concentration increased to 150 ppm, the nicotine production of hairy root cultures dropped down. Namdeo *et al.* (2007) reported that addition of amino acids to cell suspension culture media enhanced the production of tropane alkaloids, indole alkaloids etc. Ellis and Towers (1970) reported that addition of phenylalanine to cell suspension cultures stimulated the production of rosmarinic acid in *Salvia officinalis.*

Summary

SUMMARY

The present study entitled "Genetic transformation in sarpagandha (*Rauvolfia serpentina* (L.) Benth.) for enhancement of secondary metabolite production" 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 shoot tip and nodal segment explant with 0.1 per cent $HgCl_2$ for 1 min was found to be optimum for culture establishment.

2. The maximum regeneration response from shoot tip and nodal segment explant was obtained on MS medium supplemented with 1 BA mg l^{-1} and 0.1 NAA mg l^{-1}. 3. The established shoots gave out numerous differentiated shoots on the MS medium supplemented with 1 BA mg $l^{-1}$and 0.1 NAA mg l^{-1}.

4. Cent per cent rooting was obtained in ½ MS medium with 0.1 IBA mg l^{-1} and 0.2 NAA mg l^{-1}.

5. *Agrobacterium rhizogenes* strains used showed uniform growth on YEM producing single cell colonies.

6. 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.

7. Explants of *R. serpentina* were found healthy at cefotaxime 500 mg l^{-1} concentration.

8. Among the various explants tested for efficient transformation in *R. serpentina*, leaf segments showed inward curling and yellowing, shoot tips showed elongation and nodal segments showed bud burst and elongation.

9. The influence of various parameters like co-culture period, addition of phenolic compounds like acetosyringone and etiolated cultures as explants on transformation in *R. serpentina* were studied, but there was no positive response.

10. Shoot tip explants of *R. serpentina* co-cultivated for two days using TR 107 strain showed root induction.

11. Sub-culturing of induced roots on MS basal medium resulted in drying of induced roots.

12. Virulence of the *Agrobacterium* strains as well as viability of the methodology was checked in *Nicotiana tabacum*

13. Transformation in *Nicotiana tabacum* showed leaf segment as the best explant for efficient transformation followed by nodal segment and shoot tip explant.

14. Among the six *A. rhizogenes* strains tried in *N. tabacum*, the efficiency of strains in inducing hairy roots is in the order of ATCC 15834, TR7, ATCC 11325, A4, TR107 and MTCC 532

15. Transformation with single cell bacterial colony as well as bacterial suspension was found effective in *N. tabacum*. ATCC 15834, ATCC 11325 and TR7 showed highest transformation percentage by SM with leaf as explant whereas A4, TR107 and MTCC 532 showed maximum transformation by DIM with leaf as explant

16. The transformation frequency was influenced by the co-culture period. Co-cultivation of explants for more than 2 days reduced the percentage of transformation. The explants showed yellowing when they co-cultivated for more than three days.

17. Callusing along with hairy root induction of transformed explant in *N. tabacum* was seen and the amount of callus induced varied with the strain. Callusing of shoot tip explant was less when compared to leaf and nodal segment.

18. Hairy roots were induced directly from the wounded sites and proliferation of hairy roots was seen along with callusing. Wounding of leaf explant resulted in induction of hairy roots from petiole and mid vein region.

19. A maximum of 5 hairy roots per leaf explant was produced by ATCC 15834 strain followed by TR7 strain producing 3 hairy roots per leaf explant.

20. The number of days for hairy root induction differed with the *A. rhizogenes* strain and inoculation method. ATCC 15834 was able to produce hairy roots within 3 days of infection whereas MTCC 532 produced roots only after 8 days.

21. The hairy roots induced showed negative geotropism, fast growth rate, high degree of lateral branching and were able to grow in the absence of growth regulators.

22. Control roots showed slow growth and were positively geotropic in nature.

23. On comparing control roots and hairy roots induced by the experimental strains, ATCC 15834 induced hairy roots showed faster growth producing more biomass.

24. The growth of hairy roots in liquid medium without growth regulator was rapid when compared to growth in solid media .and the newly emerging hairy roots in liquid media were creamy white in color whereas the initial inoculated roots turned brown.

25. Among the liquid media tested, ½MS with 3 per cent sucrose was found to be superior for promoting hairy roots in *N. tabacum* followed by MS and B5 with 3 per cent sucrose respectively.

26. The growth pattern of hairy roots exhibited a sigmoid `S` shaped curve with an initial lag phase followed by an exponential phase and a stationary phase.

27. Confirmation of transformation was done by opine detection using high voltage paper electrophoresis. Opines from hairy roots induced by ATCC 15834, TR7 and A4 in *N. tabacum* showed spots corresponding to agropinic acid.

28. TR7 induced hairy roots in *N. tabacum* showed a faint spot corresponding to mannopinic acid. No spot was observed from the roots induced by TR107 in *R. serpentina*.

29. Genomic DNA could be isolated from *N. tabacum* and *R. serpentina* using modified CTAB method.

30. The Polymerase Chain Reaction confirmed the presence of *rol* B and C gene in the hairy roots of ATCC 15834, ATCC 11325, TR7 and A4 in *N. tabacum*. No amplication was observed with TR107 induced roots of *R. serpentina*.

31. HPLC could be employed for the estimation of nicotine.

32. Hairy roots cultured in half MS medium produced the highest content of nicotine than that of hairy roots cultured in MS and B5.

33. Nicotine was detected in root, shoot and leaves of field grown plants, *in vitro* plants and transformed plants.

34. In *in vitro* plants, nicotine was found maximum in the roots, whereas in field grown plants the amount of nicotine was equally distributed through out the plant.

35. The addition of 1 per cent sorbitol increased the biomass along with an increase of nicotine content by 2.2 fold than that of control.

36. Increase in the concentration of sorbitol resulted in reduced biomass production as well as the nicotine content.

37. Addition of 5 per cent PEG increased the nicotine concentration but there was no increase in biomass production.

38. The addition of yeast extract augmented the nicotine yield but failed to increase the biomass

39. The addition of L-arginine 50 ppm increased the biomass along with an increase in nicotine content by 2.6 fold.

40. Increase in L-arginine concentration increased the nicotine yield up to 100 ppm after which reduction in nicotine yield was noted.

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* Originals not seen

Appendices

APPENDICES

Appendix I. Composition of different tissue culture media

Chemical	MS (mg l^{-1})	B5 (mg l^{-1})
Inorganic constituents		
$(NH_4)NO_3$	1650	600
KNO_3	1900	2100
$MgSO_4 .7H_2O$	170	250
$(NH_4) H_2PO_4$	370	400
$CaCl_2. 2H_2O$		-
Na_2 EDTA	440	450
$FeSO_4 . 7H_2O$	37.3	-
EDTA Na Ferric salt	27.8	-
$MnSO_4. H_2O$		43
$ZnSO_4. 7H_2O$	22.3	10
H_3BO_3	8.6	2
KI	6.2	3
$Na_2MoO_4. 2H_2O$	0.83	0.8
$CuSO_4. 5H_2O$	0.25	0.25
$CoCl_2. 6H_2O$	0.025	0.025
Organic constituents		
Glycine	2	-
Nicotinic acid	0.5	1
Pyridoxine acid HCl	0.5	1
Thiamine HCl	0.1	10
Sucrose	30000	30000
Myoinositol	100	250
P^H	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})
Beef extract	1	-	5	-
K_2HPO_4	-	0.5	-	-
Yeast extract	2	1	1	5
$MgSO_4$. $7H_2O$	-	0.2	0.5	-
Peptone/Trypton	5	-	5	10
Mannitol	-	10	-	-
NaCl	5	0.1	-	10
Sucrose	-	-	5	-
Galactose	-	-	-	-
Agar	15	20	20	20
pH	7	7	7.2	7

APPENDIX III

Reagents used for DNA isolation

1) Extraction buffer (2X)

   a) 2 per cent CTAB - 4g/200ml

   b) 10 mM Tris pH 8 - 2.422g/200ml

   c) 20 mM EDTA pH 8 - 1.48896 g/400ml

   d) 1.4 M NaCl - 29.7792 g/400ml

   e) 1 per cent PVP - 4g/200ml

2) 10 per cent CTAB - 10g /100ml

3) TE buffer

   Tris HCl 1.0 M (p^H 8.0) - 1.0 ml

   EDTA 0.25 M (p^H 8.0) - 1.0 ml

   Distilled water - 98.6 ml

Autoclaved and stored at room temperature

4) Ice cold propanol

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 xyanol	-	0.25 per cent
Glycerol	-	30 per cent

The dye was prepared and kept in fridge at 4 ^{0}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 (pH- 8.0)

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 plasmid 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

Abstract

GENETIC TRANSFORMATION IN SARPAGANDHA (*Rauvolfia serpentina* (L.) Benth.) FOR ENHANCEMENT OF SECONDARY METABOLITE PRODUCTION

By

S. THANGA SUJA
(2007-11-118)

ABSTRACT OF THE THESIS

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ABSTRACT

The present study entitled "Genetic transformation in sarpagandha (*Rauvolfia serpentina* (L.) Benth.) for enhancement of secondary metabolite production" was carried out at the Centre for Plant Biotechnology and Molecular Biology of the College of Horticulture, Vellanikkara. The objective of the study is to genetically transform *Rauvolfia serpentina* (L.) Benth. using *Agrobacterium rhizogenes* by inducing hairy roots so as to enhance the secondary metabolite production.

A viable protocol for *in vitro* propagation of *Rauvolfia serpentina* was developed. Shoot tip and nodal segment explants from net house grown plants were used for the study. MS media supplemented with BA 1 mg $l^{-1}$and NAA 0.1 mg l^{-1} gave maximum regeneration response and also induced multiple shoots (4 shoots/explant) from shoot tip and nodal segment explants. The shoots obtained were best rooted on half strength MS medium supplemented with IBA 0.2 mg $l^{-1}$and NAA 0.2 mg l^{-1}. The *in vitro* rooted plantlets were successfully hardened and planted out with 95 per cent survival.

Genetic transformation for induction of hairy roots in *R. serpentina* was attempted with six strains of *Agrobacterium rhizogenes*, namely ATCC 15834, ATCC 11325, TR 7, A4, TR 107 and MTCC 532. Cefotaxime (500 mg l^{-1}) was found effective for elimination of *A. rhizogenes* strains from the explant tissues. Different explants such as leaf segments, shoot tips and nodal segments were used for the genetic transformation. The influence of various parameters like co-culture period, acetosyringone treatment and etiolation of shoot tip explant on transformation were studied but none of the treatments showed positive response on induction of hairy roots in *R. serpentina*.

Co-cultivation of shoot tip explant with TR107 strain for two days showed root induction, but the induced roots failed to further proliferate on MS basal medium.

Virulence of the different strains and viability of the methodology were checked by inducing hairy roots in *Nicotiana tabacum*. Among the various explants used for transformation in *N. tabacum*, leaf segments showed a greater percentage of transformation followed by nodal segment and shoot tip explant. Suspension method of inoculation showed higher percentage of transformation with leaf as explant in ATCC 15834, ATCC 11325 and TR7 strain whereas direct inoculation method showed higher

percentage of transformation with leaf as explant in A4, TR 107 and MTCC 532 strain. Among the six strains used for transformation, ATCC 15834 showed the highest frequency of transformation followed by TR7, while TR107 and MTCC 532 failed to infect the explants.

The hairy roots induced showed high degree of lateral branching, negative geotropism, fast growth rate and were able to grow in the absence of growth regulators. Opine analysis confirmed the transformation in ATCC 15834, TR7 and A4 hairy root cultures of *N. tabacum*. Molecular detection through PCR further confirmed the integration of T-DNA from the soil bacteria into hairy root genomes. TR107 induced roots of *R serpentina* elicited a negative result in PCR amplification as well as opine analysis.

Growth rate of hairy roots in liquid culture was faster than in solid media. Half MS with 3.0 per cent sucrose was found to be superior for promoting hairy root growth followed by MS with 3.0 per cent sucrose and B5 with 3.0 per cent sucrose. ATCC 15834 induced hairy roots, showed faster growth producing more biomass as compared to other strains and control.

HPLC technique was used for the quantitative analysis of nicotine with the help of class LC10 software (Shimadzu, Japan). Analysis of roots of field grown and *in vitro* plants as well as hairy root cultures showed that field grown roots had the highest nicotine content followed by hairy roots and attempts were made to enhance the secondary metabolite in hairy root cultures.

Enhancement of secondary metabolite production was studied using techniques such as addition of osmoregulants, elicitation and precursor feeding. Addition of 1 per cent sorbitol increased the biomass along with an increase in nicotine content by 2 fold and with further increase in the concentration, the growth rate as well as the nicotine content decreased. Polyethylene glycol at 5 per cent increased the nicotine concentration but there was no increase in biomass production. Yeast Extract at 2 and 5 per cent augmented the nicotine yield but failed to increase the biomass. Addition of the precursor, L-arginine at 50 ppm increased the biomass production along with an increase in nicotine content by 2.6 fold, and with further increase in L- arginine concentration, the nicotine yield dropped down.