GENETIC TRANSFORMATION FOR HAIRY ROOT INDUCTION AND ENHANCEMENT OF SECONDARY METABOLITES IN *ASWAGANDHA* (*Withania somnifera* (L.) Dunal)

By

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THESIS

submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that this thesis entitled "Genetic transformation for hairy root induction and enhancement of secondary metabolites in *Aswagandha* (*Withania somnifera* (L.) Dunal)" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis, entitled "Genetic transformation for hairy root induction and enhancement of secondary metabolites in *Aswagandha* (*Withania somnifera* (L.) Dunal)" is a record of research work done independently by Ms. Smini Varghese 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 Loving Family

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ABBREVIATIONS

%	Per cent
°C	Degree Celcius
µg	Micro gram
µl	Micro litre
µ*M*	Micro molar
A	Ampere
$AgNO_3$	Silver nitrate
A. rhizogenes	*Agrobacterium rhizogenes*
AH	*Aspergillus* homogenate
ASCA	Activated Suspension culture and Co-cultivation with acetosyringone
ATCC	American Type Culture Collection
BAP	Benzyl amino purine
$CaCl_2$	Calcium chloride
$CdCl_2$	Cadmium chloride
$CHCl_2$	Methyl chloride
C_6H_6	Benzene
CH_3COOH	Acetic acid
CH_3OH	Methanol
$CHCl_3$	Chloroform
cm	Centi metre
CS	Cotyledonary segment
$CuSO_4$	Copper sulfate
CW	Coconut Water
DICA	Direct Inoculation and Co-cultivation with acetosyringone
DIM	Direct Inoculation Method
DNA	Deoxy ribo Nucleic Acid
dNTP	Deoxy ribo Nucleoside Triphosphate
EDTA	Ethylene diamine tetra acetic acid
EtOAc	Ethyl Acetate
$FeCl_3$	Ferric Chloride
FW	Fresh weight
$g\ l^{-1}$	Grams per litre
GA_3	Gibberellic acid
hrs	Hour (s)
H_2SO_4	Sulphuric acid
ha	Hectare
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
HPLC	High Pressure Liquid Chromatography
HS	Hypocotyl segment
IAA	Indole acetic acid

IBA	Indole butyric acid
IMTECH	Institute of Microbial Technology
IPA	Iso pentenyl adenine
kD	Kilo Dalton
KN	Kinetin
LBA	Luria Bretani Agar
LS	Leaf segment
lux	lux
M	Molar
MeOH	Methanol
mg l^{-1}	Milligrams per litre
min	Minute (s)
ml	Milli litre
mm	Milli metre
MS	Murashige and Skoog's medium
MTCC	Microbial Type Culture Collection
N	Normal
NA	Nutrient Agar
NAA	Naphthalene acetic acid
Na_2SO_4	Sodium sulfate
NaOH	Sodium hydroxide
Nm	Nano metre
NS	Nodal segment
OD	Optical Density
PCR	Polymerase Chain Reaction
PEG	Poly ethylene glycol
pH	Hydrogen ion Concentration
psi	pounds per square inch
rpm	revolutions per minute
$SbCl_3$	Antimony chloride
SCA	Suspension culture and Co-cultivation with acetosyringone
SDS	Sodium dodecyl sulphate
sec	second (s)
SM	Suspension culture Method
SSC	Sodium chloride and Sodium citrate
ST	Shoot tip
TAE	Tris acetate EDTA buffer
T-DNA	Transfer DNA
TDZ	Thidiazuron
TE	Tris EDTA buffer
TLC	Thin Layer Chromatography
TL-DNA	Transfer (Left) DNA
TR-DNA	Transfer (Right) DNA
UV	Ultra violet
v	Volume

V/ cm	Volt/ cm
W. somnifera	*Withania somnifera*
YE	Yeast extract
YEB	Yeast Extract Broth
YEM	Yeast Extract Mannitol
α	Alpha

Introduction

1. INTRODUCTION

Plants produce an array of secondary metabolites that find application as pharmaceuticals, agrochemicals, flavours and fragrances. Advancements in genetic engineering have opened up new avenues to understand and produce precious products from the plants.

Withania somnifera (L.) Dunal (Solanaceae) commonly known as aswagandha or Indian ginseng is a highly valuable medicinal plant of the Indian system of medicine with a wide spectrum of biological activities to its credit. Classically known for rejuvenative benefits, it is the subject of considerable modern scientific attention. It is a component of nearly hundred different preparations/ formulations of Ayurveda/ Unani of which many are readily available commercially (Tripathi *et al.*, 1996). The medicinal properties of *Ashwagandha* have been attributed to its chemical constituents mainly alkaloids and steroidal lactones (primarily of the withanolide class). It has been reported to have remarkable antitumour, immunosuppressive, anti-inflammatory and antimicrobial activities (Furmanowa *et al.*, 2001).

Although the leaves and fruit of aswagandha are therapeutic, most of the herbal medicines available are derived from the roots. According to an estimate, India produces about 3500 tonnes of dried roots annually, as against an estimated annual demand of about of 7000 tonnes (Singh *et al.*, 2003). To meet the great demand for its raw material in the market, it is necessary to develop protocols for rapid and efficient multiplication of selected superior genotypes where tissue culture offers great potential. Another major alternative to meet the increasing demand is to produce the active chemicals in *W. somnifera* through *in vitro* techniques.

Recent developments leading to the production of rapidly growing, genetically and biosynthetically stable organized 'hairy' root cultures following the

genetic transformation of plants with *Agrobacterium rhizogenes* strains may revolutionize certain areas of plant cell biotechnology.

The 'hairy roots' are capable of fast growth on a hormone free medium. This system has the following wide-ranging applications: from production of secondary metabolites to foreign proteins, from restructuring of plant phenotype to the study of rhizosphere biology, from phytoremediation to molecular farming, ecology and evolution (Eapen and Mitra, 2001).

The inherent problem of slow growth rate of conventional root cultures can be overcome successfully by hairy root induction. Moreover, elicitation and modification of the culture conditions offer an interesting option to enhance secondary metabolite production. It would be a significant contribution to mankind if the bioactive compounds in aswagandha could be successfully obtained through 'hairy root cultures'.

In this context, this study was undertaken to genetically transform *Withania somnifera* (L.) Dunal using *Agrobacterium rhizogenes* by inducing hairy roots so as to enhance the secondary metabolite production. The objectives of the study were,

- To standardize the *in vitro* regeneration protocol in *W. somnifera* from different explants.
- To standardize the genetic transformation in *W. somnifera* using *Agrobacterium rhizogenes.*
- To standardize the biochemical techniques for the estimation of secondary metabolites in the roots of *W. somnifera.*
- To enhance the secondary metabolite production in hairy root cultures of *W. somnifera* employing different techniques.

Review of Literature

2. REVIEW OF LITERATURE

In the past few decades, considerable interest has been shown in the production of secondary products by plant cell cultures (Oksman-Caldentey and Hiltumen, 1996). The success, however, has been moderate mainly because the biosynthesis pathways of the secondary metabolites, including the enzymes and the regulatory mechanism governing expression and function of the pathways, are poorly understood (Kutchan, 1995). Secondary product formation is often low and unstable in undifferentiated callus and suspension cultures; the metabolism of secondary products seems to correlate with the degree of organization of cell structures. Therefore, the roots are capable of accumulating a large range of secondary metabolites reflecting biosynthetic capacity (Doran, 1989).

The major problem associated with the *in vitro* culture of conventional roots is usually the slow growth rate. In contrast, *Agrobacterium* mediated hairy roots are fast growing and genetically stable which can also be successfully cultured in large scale bioreactors (Wilson, 1997). Transformed roots are able to produce secondary metabolites at levels that are often comparable to, or greater than that of the intact plants (Giri and Narasu, 2000).

A number of plant species including many medicinal plants have been successfully transformed with *Agrobacterium rhizogenes*. Transformed root cultures have also been found to be a potential source of high-value pharmaceuticals. The selection of high productive root lines based on somaclonal variation offers an interesting option to enhance the productivity. Elicitors and modification of culture conditions have been shown to increase the growth and the alkaloid production in some cases (Sevon and Oksman-Caldentey, 2002).

Withania somnifera (L.) Dunal is a plant of the Indian system of medicine commonly known as aswagandha which means smells like a horse. The medicinal properties of aswagandha have been attributed to its chemical constituents, mainly

alkaloids and steroidal lactones (primarily of the withanolide class) (Thiagarajan *et al.*, 2003).

2.1 *Withania somnifera* (L.) Dunal

Withania somnifera is a dicotyledonous plant belonging to the family Solanaceae. Its roots are compared with the restorative properties of ginseng roots and have been given the name Indian ginseng (Thiagarajan *et al.*, 2003). In each language it has a specific name, indicating the wide spread recognition of the medicinal properties of the plant. It is called '*Asgand*' in Hindi, '*Amukkilang kizhangu*' in Tamil, '*Amangura*' in Kannada, '*Asvagandha*' in Bengali, '*Ashvagandha*' in Sanskrit, '*Asundha*' in Gujarathi and '*Winter Cherry*' in English (Navdanya Organisation, 2005).

The plant native to India, Northern Africa and the Middle East, is now cultivated around the world, including the temperate climates of United States (Chopra and Simon, 2000). In India 5000 ha are under cultivation in Rajasthan, Madhya Pradesh, Andhra Pradesh and Uttar Pradesh (Kothari *et al.*, 2003). Six-month-old plants had the highest withanolide content (0.61%). Among the different plant organs, roots had the highest withanolide content (0.30%), followed by leaves (0.15%) and stems (0.08%) (Muthumanickam and Balakrishnamurthy, 1999).

2.1.1 Botany of *Withania somnifera* (L.) Dunal

It is an erect, slightly hairy plant with fairly long tuberous roots. Leaves are 5-10 cm long and 2.5-7 cm wide. The flowers are small, greenish, single or in small clusters in the leaf axils. The fruits are smooth, round, fleshy, many seeded, orange-red when ripe and enclosed in a membraneous covering. The flowering and fruiting is from September to November and then March to May (Navdanya Organisation, 2005)

2.1.2 Chemical constituents of *Withania somnifera* roots

Withania somnifera is characterized by the presence of steroidal lactones, alkaloids and flavanoids. The steroidal lactones have been given the name withanolides. Withanolides in root includes Withanolide A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, Q, R, T, U, Y, Δ^{16}, steroidal lactone, Withaferin I, II, III, Sito indoside VII, VIII, IX and X, Withaferin A and Withaferinil (Tripathi *et al.*, 1996)

Many biochemical heterogenous alkaloids have been isolated from roots. The important alkaloids are cuscohygrine (pyridine derivative), anahygrine having a pyrrolidine and piperidine moieties, tropine, pseudotropine, anaferine, isopellatierine and 3-troplyltigloate.

Flavanoids are present in the leaves. The aminoacids detected were cysteine, glycine, glutamic acid, alanine, proline and tryptophan (Bhakuni and Jain, 1995)

2.1.3 Medicinal properties of *Withania somnifera* (L.) Dunal

Withania somnifera has been reported to have a wide range of biological activities. *Withania somnifera* extract, is used for a variety of illness such as asthma, rheumatic pain, inflammation of joints, nervous disorders, epilepsy, as a uterine sedative, antispasmodic, sedative, hypnotic and against eye diseases (Thiagarajan *et al.*, 2003). It shows antimicrobial activity (Sethi *et al.*, 1974), anti-tumour and radiosensitizing effects, anti-inflammatory, immunomodulatory, anti stress adaptogenic activity, anti-convulsive hemopoetic and rejuvenating properties (Thiagarajan *et al.*, 2003). Recent literature suggests its efficacy as a cardioprotective agent (Dhuley, 2000), inhibitor of drug-induced urotoxicity, enhancer of white blood cell and platelet counts (Agarwal *et al.*, 1999), an agent that enhances immunoprotection, cytokine production and stem cell proliferation (Davis and Kuttan, 2000) and an antioxidant (Bhattacharya *et al.*, 1997).

2.1.4 Applications of *in vitro* plant regeneration in *Withania somnifera* (L.) Dunal

Plant tissue culture has long been recognized as an efficient tool for rapid clonal propagation (George and Sherrington, 1984). The plant regeneration systems with high efficiency are desirable for application of current practical studies and genetic transformation (Muthuvel *et al.*, 2005).

The conventional propagation is achieved through seeds but the seed viability is limited to one year and the success rate of vegetative propagation is very low (Kulkarni *et al.*, 2000). As aswagandha is one of the endangered medicinal plants of North-Eastern Karnataka (Seetharam *et al.*, 1998), it is necessary to conserve it both *in situ* and *ex situ* methods. Owing to the great demand for its raw material in the market and to preserve the germplasm it is necessary to develop protocols for rapid and efficient multiplication of selected superior chemotypes where, tissue culture offers great potential (Govindaraju *et al.*, 2003).

Standardisation of regeneration protocol in *W. somnifera* facilitates the rapid propagation of this valuable medicinal plant; it can be used to provide quality-planting material for its cultivation program and can also be of use for genetic transformation studies using *Agrobacterium rhizogenes* (Vadawale *et al.*, 2004).

2.1.5 *In vitro* regeneration studies

2.1.5.1 Explant Selection

Several explants can be harvested from a plant for *in vitro* propagation, but there are differences between the explants in terms of their regeneration competence. In addition, there are other parameters that are taken into account which include the uninterrupted availability of explants, its dormancy status, polyphenol exudation level, uniformity and ease of decontamination (Tiwari, *et al.*, 2003).

Sen and Sharma (1991) reported that seeds of *Withania somnifera* showed eighty per cent germination when incubated under a dark photoperiod, whereas only sixty per cent germination was obtained under a 16-h photoperiod.

Roja *et al.* (1991) studied multiple shoot formation and callus formation from axillary meristem explants of *W. somnifera.* Shoot multiplication was achieved *in vitro* from shoot tips and germinating seeds of *W. somnifera* (Sen and Sharma, 1991)

Baburaj and Gunashekaran (1995) and Abhyankar and Chinchanikar (1996) obtained shoot differentiation in *W. somnifera* from leaf explants. Multiple shoots were produced from callus of various explants like axillary leaves, axillary shoots, hypocotyls and root segments through indirect organogenesis (Rani and Grover, 1999). Kulkarni *et al.* (2000) developed direct regeneration protocol from node, internode, hypocotyl and embryo explants of *W. somnifera.*

An efficient and rapid regeneraion protocol via callus and directly from various explants like leaves, internodes, shoot tips, nodal segments and petiole of *W. somnifera* was reported by Govindaraju *et al.* (2003). A rapid *in vitro* propagation of *W. somnifera* was achieved through axillary bud multiplication and indirect organogenesis (Vadawale *et al.*, 2004).

Rani and Grover (1999), Kulkarni *et al.* (2000), Govindaraju *et al.* (2003) reported that nodal segments produced maximum number of shoots in *W. somnifera* among various explants tested.

2.1.5.2 Basal Media

Murashige and Skoog (1962) medium is found to be the most popular one and has been successfully reported in many medicinal plants. Earlier reports indicate comprehensive use of MS nutrient medium for *in vitro* propagation of *Withania somnifera* (Roja *et al.*, 1991; Sen and Sharma, 1991; Baburaj and

Gunashekaran, 1995; Abhyankar and Chinchanikar, 1996; Pawar *et al.*, 2001; Kulkarni *et al.*, 2000; Govindaraju *et al.*, 2003 and Vadawale *et al.*, 2004).

In other *Solanaceous* crops like *Lycopersicon esculentum*, (Muthuvel *et al.*, 2005); *Solanum surattense* (Seetharam *et al.*, 2003); and *Capsicum annuum* (Sandhu *et al.*, 2003), Murashige and Skoog (MS) medium was used as the basal medium.

2.1.5.3 Addition of growth regulators

The most important factor in successful tissue culture is the addition of growth regulators. The growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium and the growth substances produced endogenously by cultured cells (Krikorian, 1982).

For axillary shoot proliferation, cytokinin has been utilized to overcome the apical dominance of shoots and to enhance the branching of lateral buds from the leaf axis (Murashige, 1974). He observed that a variety of auxins including IAA, NAA, IBA, and 2,4-D were used either alone or in combination, but among those auxins, IAA was the weakest, but showed minimum harmful effect on explant tissue. 2, 4-D was the most potent and it stimulated callus cultures. Among the various cytokinins like zeatin, kinetin and BAP, the latter is commonly used. Lo *et al.* (1980) reported that high cytokinin content was deleterious to the initiation and elongation of roots.

For axillary bud proliferation, exogenous auxin was not always needed. Although exogenous auxins do not promote axillary shoot proliferation, culture growth has been improved by their presence (Wang and Hu, 1980). One of the possible roles of auxin at the elongation stage is to nullify the suppressive effect of high cytokinin concentration, thereby restoring normal shoot growth (Lundergam and Janick, 1980).

Hempel (1979) concluded that in majority of cases, callus growth was supported by auxin. Hesegawa (1980) also reported that high concentration of auxin might not only inhibit axillary bud break, but also induce callus formation.

2.1.5.4 In vitro regeneration from hypocotyls and cotyledon segments

Several workers have attempted plant regeneration from cotyledon and hypocotyl segments of *Capsicum annuum*. Valero-Montero and Ochoa-Alejo (1992) reported adventitious bud formation when rooted hypocotyls were placed upside down in medium. Hypocotyl segments generally produced roots and only the acropetal part differentiated shoot buds, where as roots are produced from both acropetal and medium parts (Ramage and Leung, 1996). An increased gradient of growth substances from the base of hypocotyl could be responsible for these differences (Fari and Czako, 1981).

In *Capsicum annuum* variety California Wonder, high regeneration was obtained from hypocotyl segments, when placed upside down in MS medium supplemented with IAA 0.3 mg l^{-1} + BAP 5.0mg l^{-1} and IAA 0.2 mg l^{-1} + BAP 3.5 mg l^{-1} (Girija *et al.*, 2004)

Mathew (2002) reported that cotyledon and hypocotyl explants of *Capsicum annuum* L.cvs Byadagi Dabbi and Arka Lohit showed best shoot morphogenesis in MS medium supplemented with BAP 2.0 mg $l^{-1}$and IAA 1.0 mg l^{-1} and also observed that regeneration potential of cotyledon was superior over hypocotyl.

In *Capsicum annuum* cvs Pico and Piquillo, hypocotyl explants showed higher organogenesis when compared to cotyledon (Arroyo and Revilla, 1991).

Ohki *et al.* (1978) analysed the *in vitro* shoot forming capacity in two lines of tomato (*Lycopersicon esculentum* Mill.) and their hybrids. Through a comparative experiment with several cytokinins and auxins, it was concluded that IPA combined with IAA gave the best results for shoot formation from hypocotyl.

Histological studies of hypocotyl indicated that shoots were differentiated from the callus originating from the superficial tissues.

Subbaiah and Minocha (1990) obtained multiple shoots in large numbers from excised hypocotyls of young *Eucalyptus terretecornis* seedlings on media containing BAP alone.

Kulkarni *et al.* (2000) reported that hypocotyls of *W. somnifera* gave rise to multiple shoots on MS with 0.5 mg l^{-1} BAP. Isolated embryos on MS with 0.1- 0.3 mg l^{-1} TDZ produced multiple shoot buds.

Muthuvel *et al.* (2005) found that in *Lycopersicon esculentum* Mill. MS medium containing IAA 2.0 mg l^{-1} and KN 1.0 mg l^{-1} produced high regeneration from cotyledon calli. Higher concentrations of auxin reduced the shoot regeneration frequency. The increase and decrease in regeneration frequency is attributed to the cytokinin concentration.

MS media containing NAA 1.0 mg l^{-1}, BAP 2.0 mg l^{-1} and KN 2.0 mg l^{-1} proved to be the best for callus induction and shoot regeneration from cotyledonary explants of *W. somnifera* (Gaikwad and Prasad, 2003).

2.1.5.5 In vitro shoot multiplication from other explants

Sen and Sharma (1991) reported that maximum shoot multiplication was achieved from *in vitro* shoot tips, when 2, 4-D or IBA 2.5 μM was added to MS medium containing BAP 4.4 μM during initiation of shoot multiplication in *W. somnifera*. They obtained direct shoot initiation from germinating seeds in the presence of BAP alone.

Nitsch and Nitsch media containing BAP 1.0 mg l^{-1} and IBA 1.0 mg l^{-1} produced maximum shoot multiplication from shoot tips of asceptically-germinated seedlings of *W. somnifera* (Furmanova *et al.*, 2001).

Direct shoot regeneration was obtained from nodal and internodal explants of *W. somnifera* on MS medium with BAP (0.1- 0.5 mg l^{-1}) (Kulkarni *et al.*, 2000).

Vadawale *et al.* (2004) reported that axillary buds from nodal explants of *W. somnifera* showed highest multiplication potential when BAP was used in combination with KN and produced an average of five shoots. Dekta *et al.* (1999) achieved *in vitro* regeneration in *W. somnifera* from axillary buds and shoot tips on MS medium supplemented with BAP 0.1-1.0 mg l^{-1} and KN 0.1-0.4 mg l^{-1}.

Kulkarni *et al.* (1996) reported direct regeneration in *W. somnifera,* with sixteen shoots on an average from leaf explant. Abhyankar and Chinchanikar (1996) found that shoot buds developed on leaf margins of *W. somnifera* after subculturing on MS medium containing IAA 0.4 mg l^{-1}, KN 0.2 mg l^{-1} and BAP 1.0 mg l^{-1}.

Direct regeneration of multiple shoots from leaves, shoot tips and nodal segments were obtained on MS medium supplemented with different concentrations of BAP (1.0-3.0 mg l^{-1}) in combination with IAA 0.5 mg l^{-1} and 10 per cent coconut milk. Maximum number of shoots (up to 24) was produced from nodal segments on MS medium fortified with BAP 2.5 mg l^{-1} and IAA 0.5 mg l^{-1}. (Govindaraju *et al.*, 2003).

The synergetic effect of BAP and KN in promoting the shoot initiation has been reported by Seetharam *et al.* (2003) in *Solanum surattense.* They reported that MS medium containing BAP 4.0 mg l^{-1} and KN 1.0 mg l^{-1} elicited maximum number of shoots per leaf explant, whereas BAP 2.0 mg l^{-1} and KN 1.0 mg l^{-1} elicited maximum number of shoots per nodal explant.

Vadawale *et al.* (2004) observed callus initiation at the bases of *in vitro* generated shoots on MS medium with BAP 3.32 μM, KN 1.16 μM and IBA 0.98 μM. Rani and Grover (1999) initiated callus cultures from axillary leaves, axillary

shoots, hypocotyls and root segments on MS medium supplemented with 2,4-D 2.0 mg l^{-1} and KN 0.2 mg l^{-1}.

Callusing was obtained from leaves and shoots of *Coscinium fenestratum* on half MS medium supplemented with IAA 2.0 mg l^{-1} and BAP 1.0 mg l^{-1} (Sindhu and Keshavachandran, 2000). According to Marks and Simpson (1994) callus formation may be due to the action of accumulated auxin at the basal cut ends, which stimulate cell proliferation, especially in the presence of cytokinin. Callus developed on MS medium with BAP and KN was hard and nodular in texture and dark brown in colour. Callus formation also occurred on medium fortified with cytokinin alone (Vadawale *et al.*, 2004). The potential of a medium supplemented with only a cytokinin for the induction of callus has been reported in *Tylophora indica* (Manjula *et al.*, 2000)

The node callus of *W. somnifera* induced maximum number of shoots from callus on MS medium with BAP 4.43 μM and IBA 0.98 μM (Vadawale *et al.*, 2004). Rani and Grower (1999) reported that shoots differentiated best from axillary shoot base callus of *W. somnifera* on MS medium containing BAP 2.0 mgl^{-1}.

Manickam *et al.* (2000) achieved regeneration of multiple shoots from stem callus of *W. somnifera* cultured on MS medium with BAP 4.4 μM and IAA 0.57 μM. Regenerated shoots were excised and multiplied on MS medium with BAP 4.44 μM. Govindaraju *et al.* (2003) found that callus isolated from explants such as internode, leaf and petiole except roots of *W. somnifera* formed large number of shoots in MS medium supplemented with BAP alone or along with IAA and 10 per cent coconut milk. Padmanabhan *et al.* (1973) succeeded in the induction of shoot buds from cultured *Lycopersicon esculentum* leaf callus using IAA and KN combinations. With the increase in KN concentration a corresponding increase in shoot regeneration was envisaged. However, it gradually showed decrease at higher concentrations.

2.1.5.6 Response of roots of W. somnifera

Govindaraju *et al.* (2003) reported that though good callusing response was obtained from roots of *W. somnifera* under light and dark conditions, the callus failed to regenerate shoots at different combinations and concentrations of media tested. Rani and Grover (1999) also reported similar results.

2.1.5.7 Influence of GA_3 on elongation

The relative concentration of GA_3 plays a vital role in organogenesis (Thorpe, 1980). The stimulatory effect of GA_3 on the growth of stem and its promotory effect on shoot elongation were reported by Seetharam *et al.* (2003). They observed maximum shoot elongation at BAP 1.0 mg l^{-1} and GA_3 1.0 mg l^{-1} from nodal and leaf explants in *Solanum surattense.*

GA_3 alone at low concentrations had a promoting effect on shoot regeneration and shoot elongation of *in vitro* shoots of *W. somnifera* (Govindaraju *et al.*, 2003). Such a shoot elongation response was reported in *Vigna sublobata* (Bhadra *et* al., 1994) and *Phaseolus vulgaris* (Martins and Sondaohl, 1989), where NAA and BAP were used besides GA_3.

2.1.5.8 Rooting of in vitro shoots

In rhizogenesis there are three phases that is, induction, initiation and elongation (Hu and Wang, 1983). Among the auxins, IBA and NAA are found to be most effective for root induction in artichoke (Ancora *et al.*, 1981).

In vitro rooting can successfully be achieved when the salt concentration in the media was reduced to one-half, one-third or one-forth of the standard strength (Lane, 1979; Skirvin and Chu, 1979). During rooting stage, sugar content of the medium was also lowered in jack fruit (Roy *et al.*, 1990).

In *W. somnifera*, shoots generated were rooted successfully in half strength liquid and solid MS medium with IBA (0.5-1.0 mg l^{-1}) alone or along with IAA (0.5 mg l^{-1}). Addition of IAA however, did not show much effect on the root length

of *W. somnifera*. Compared to IAA, IBA was found to be more effective for rhizogenesis. Here, the roots obtained were thick and long with callus at base in case of solidified medium and thin, long in case of liquid medium (Govindaraju *et al.*, 2003).

Siddique *et al.* (2004) obtained highest rooting percentage from nodal segments derived shoots of *W. somnifera* in MS medium supplemented with IBA and KN. Rani and Grover (1999) reported that regenerated shoots of *W. somnifera* rooted best on MS medium containing IBA 2.0 mg l^{-1} alone and IBA 2.0 mg l^{-1} with IAA 2.0 mg l^{-1}.

Vadawale *et al.* (2004) used full strength MS solid and liquid medium with auxins IBA and NAA at different concentrations for inducing roots from *W. somnifera*. According to Ludwig-Muller (2000), transport velocity of IBA was markedly slower compared to that of IAA and NAA. The slow movement and slow degradation of IBA facilitates its localization near the site of application and thus its function in inducing roots (Nickell, 1982).

Sen and Sharma (1991) obtained rooting of excised shoots of *W. somnifera* in growth regulator free MS medium. The multiple shoots of *W. somnifera* showed good rooting on Nitsch and Nitsch medium with KN 0.1 mg l^{-1}, IBA 0.5 mg l^{-1} and adenine sulphate 10.0 mg l^{-1} (Furmanowa *et al.*, 2001)

The combination of IBA and phloroglucinol was found to be effective for rooting in *Solanum surattense* (Seetharam *et al.*, 2003).

Activated charcoal has a profound influence on rooting of shoots *in vitro*. It can absorb residual cytokinin from shoot and it also shades *in vitro* roots from light, which in high intensity may inhibit root growth (Hu and Wang, 1983).

Gaikwad and Prasad (2003) used half strength MS medium containing IBA 1.0 mg l^{-1}, BAP 2.0 mg l^{-1} with sucrose 40 g l^{-1} for rooting of regenerated shoots

obtained from cotyledonary explant of *W. somnifera*. Darkness was also found to be favourable for rooting in *W. somnifera*.

2.1.6 Hairy root disease

The plant neoplasy known as 'hairy root disease', caused by *Agrobacterium rhizogenes* was first demonstrated by Ricker (1930). The physiological basis of hairy root disease is not totally understood. Alteration of auxin metabolism in transformed cells has been supported to play an important role in the expression of hairy root phenotype (Gelvin, 1990).

2.1.7 Mechanism of *Agrobacterium*-plant cell interation

One of the earliest stages in the interaction between *Agrobacterium* and a plant is the attachment of the bacterium to the surface of the plant cell. A plant cell becomes susceptible to *Agrobacterium* when it is wounded. The wounded cells release phenolic compounds, such as acetosyringone, that activate the *vir*- region of the bacterial plasmid (Binns and Thomashow, 1988).

It has been shown that the *Agrobacterium* plasmid carries three genetic components that are required for plant cell transformation (Zambryski *et al.*, 1989). The first component, the T-DNA that is integrated into the plant cells is a mobile DNA element. The second one is a virulence area (*vir*), which contains several *vir* genes. These genes do not enter the plant cell but together with the chromosomal DNA (two loci); cause the transfer of T-DNA. The third component, the so-called border sequence (25 bp), resides in the *Agrobacterium* chromosome (Sevon and Oksman-Caldentey, 2002).

The mobility of T-DNA is largely determined by these sequences, and they are the only *cis* elements necessary for direct T-DNA processing. The early steps of the transfer are relatively well studied but the mechanics of integration are not completely understood. According to Zambryski, (1988) it is a multistep process involving recombination, replication and repair activities, most likely mediated by host cell enzymes.

2.1.8 Genes responsible for hairy root formation

The Ri plasmids are large (200 to greater than 800 kb) and contain one or two regions of T-DNA and a *vir* (virulence) region (Gelvin, 1990). The T-DNA of the agropine type Ri- plasmid consists of two separate T-DNA regions designed the TL-DNA and TR-DNA (White *et al.*, 1985). Each of the T-DNA fragments spans a 15-20 kb region, and they are separated from each other by atleast 15 kb of non integrated plasmid DNA.

The genes encoding auxin synthesis (tms 1 and tms 2) and agropine synthesis (ags) have been localized on the TR-DNA of the agropine type Ri plasmid (Cardarelli *et al.*, 1985). TL-DNA plays the major role in hairy root induction, and the genes encoding auxin synthesis have a somewhat accessory role (Palazon *et al.*, 1997). The role of TR-DNA *aux* genes of agropine T-DNA is limited to supplementing auxin when the endogenous auxin is insufficient (Binns and Costantino, 1998).

Mutation analysis of the TL-DNA has led to identification of four genetic loci, designed locus *rol* A, *rol* B, *rol* C and *rol* D, which affect hairy root induction (White *et al.*, 1985). The complete nucleotide sequence of the TL region revealed the presence of 18 open reading frames (ORFs), four of which, ORFs 10, 11, 12, and 15 correspond to the *rol* A, *rol* B, *rol* C and rol D loci respectively. It was also shown that *rol* A, *rol* B and *rol* C play the most important role in hairy root induction. In particular, *rol* B seems to be the most crucial in the differentiation process of transformed cells, while *rol* A and *rol* C provide with accessory functions (Palazon *et al.*, 1997).

2.1.9 Hairy roots: A potential source of pharmaceuticals

Hundreds of plant species have been successfully transformed to produce hairy roots (Tepfer, 1990). For the past ten years, hairy roots have also been investigated as a potential source of pharmaceuticals. Some of the important medicinal plants in which hairy roots have been successfully induced for secondary metabolite production are listed on Table (1).

Table 1. Alkaloid production of the hairy root cultures of some medicinal plants

Sl. No.	Plant	Alkaloid	Content mg g^{-1} d.w.*
1	*Atropa belladona*	Atropine, Scopalamine	3.7
2	*Catharanthus roseus*	Ajmalcine, Catharanthine	3.0
3	*Cinchona ledgerina*	Cinchonine, Cinchonidine	4.0
4	*Datura candida*	Scopalamine, l-hyoscyamine	1.1
5	*Dubosia leichhardtii*	Scopalamine	18.0
5	*Hyoscyamus niger*	l- hyoscyamine	12.5
6	*Scopolia japonica*	l-hyoscyamine	13.0
7	*Solanum tuberosum*	Steroidal alkaloids	0.1 mg g^{-1} f.w.**

(d.w.*- dry weight, f.w.** - fresh weight)

(Sevon and Oksman-Caldentey, 2002)

2.1.10 Characterization of *Agrobacterium* plasmids based on opine catabolism

The transformed plant tissues are directed by T-DNA genes to produce unusual metabolites called opines that serve as specific nutrients for the bacteria (Chilton *et al.*, 1985). Agropine type strains induce roots to synthesize agropine, mannopine and the related acids, and mannopine type strains induce roots to produce mannopine and the corresponding acids (Rhodes *et al.*, 1990).

Table (2) shows characterization of *Agrobacterium* plasmids on the basis of opines catabolism.

Significant differences were observed between the transformation ability of different strains of *Agrobacterium* (Vanhala *et al.*, 1995). The most studied Ri-plasminds are the agropine type strains, which are considered to be the most

virulent and therefore more often used in the establishment of hairy root cultures (Rhodes *et al.*, 1987)

Table 2. Characterization of *Agrobacterium* plasmids based on opine catabolism

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

(Dessaux *et al.*, 1991)

2.1.11 *Agrobacterium* mediated genetic transformation

2.1.11.1 Culture media for A. rhizogenes strains

Benjamin *et al.* (1993) reported the use of AB minimal media for culturing ATCC 15834 strain. Mano *et al.* (1986) suggested Nutrient broth as a good culturing medium for ATCC 15834, A4, NCPB 1855 and NCPB 2659. YEB medium was found to be the best for the growth of *A. rhizogenes* strains like A4, ATCC 15834 and MAFF-03-01724 (Jaziri *et al.*, 1994; Zdravkovic-Korac *et al.*, 2004; Xu *et al.*, 2004; Yoshimatsu *et al.*, 2003). LB medium was used for culturing *A. rhizogenes* strain 15834 (Lee *et al.*, 2004)

2.1.11.2 Culture conditions for A. rhizogenes strains

Temperature has a profound influence on the growth of *A. rhizogenes* strains. *A. rhizogenes* prefer a growth temperature of 25 ± 3°C. The optimum

temperature for the growth of *A. rhizogenes* strains like A4 and ATCC 15834 was reported to be 28°C (Momcilovic *et al.*, 1997; Celma *et al.*, 2001; Shi and Kintzios, 2003; Lee *et al.*, 2004). Xu *et al.* (2004) suggested 27°C for culturing strains like A4, R 1601and ATCC 15834. Jaziri *et al.* (1994) reported that *A. rhizogenes* strains like ATCC 15834 and MAFF 03-01724 were grown at 25°C.

For preparing liquid cultures of *A. rhizogenes* strains like A4, R 1601, and ATCC 15834 the culture media was shaken at 150 rpm (Xu *et al.*, 2004).

2.1.11.3 Explants used for hairy root induction

The susceptibility of plant species to *Agrobacterium* strains varies greatly (Hamill *et al.*, 1989). The age and differentiation status of plant tissue can affect the chances of successful transformation. Different explants such as leaf segments, hypocotyls, cotyledonary segments, shoot buds and stem segments were mainly used for hairy root induction.

List of plant species successfully transformed for hairy root induction, strains involved and the explants used are shown in the Table (3).

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

Sl no.	Plant spp.	Strains used	Explants	Reference
1	*Aesculus hippocastanum L.*	A4 GUS	Androgenic embryos	Zdrakovic-Korac *et al.*, 2004
2	*Atropa belladona*	ATCC 15834, MAFF 03-01724	Leaf segments	Jaziri *et al.*, 1994
3	*Cephaelis ipecacuanha*	ATCC 15834	Petiole segments	Yoshimatsu *et al.*, 2003
4	*Clitoria ternatea*	A13	Leaf, leaf derived callus	Malabadi and Nataraja, 2003
5	*Dubosia mycoporoides x D. leichhardtii*	A4	Leaf disc	Celma *et al.*, 2001
6	*Gentiana sp.*	ATCC 15834, A4M70GUS	Shoots	Momcilovic *et al.*, 1997
7.	*Holostemma adakodien*	PcA4, A4, ATCC 15834	Hypocotyl, shoot buds	Karmarkar, 2001
8	*Isatis indigotica Fort.*	A4, R1601, ATCC 15834	Cotyledons, hypocotyl	Xu *et al.*, 2004
9	*Lupinus mutabilis*	R1601	Hypocotyl, epicotyl, stem	Babaoglu *et al.*, 2004
10	*Papaver somniferum*	LBA 9402	Hypocotyl segments	Flem-Bonhomme *et al.*, 2004
11	*Plumbago rosea L.*	ATCC 15834	Shoots	Komaraiah *et al.*, 2002
12	*Pueraria*	ATCC 15834	Leaf	Shi and Kintzios,

	phaseoloides			2003
13	*Rauvolfia serpentina*	ATCC 15834	Shoots	Benjamin *et al.*, 1993
14	*Scopolia japonica*	A4, NCPB 1855, NCPB 2659	Root segments	Mano *et al.*, 1986
15	*Solanaceae spp.*	A4	Leaf	Knopp *et al.*, 1988
16	*Taraxacum platycarpum*	ATCC 15834	Cotyledon, stem, root	Lee *et al.*, 2004
17	*Withania somnifera*	LBA 9402	Shoots	Ray *et al.*, 1996

2.1.11.4 Pre culturing of explants

Yu *et al.* (2001) reported that hairy roots emerged 3-4 days earlier than with usual treatment, if leaf explants of *Pueraria lobata* were precultivated for 2-3 days before transformation with *A. rhizogenes* strain, R 1601.

2.1.11.5 Wounding of Explants

Wounding is a pre-requisite for *Agrobacterium* infection. Pawar and Maheshwari (2004) purposely wounded leaf discs of *W. somnifera* using surgical scalpel and placed ventral surface touching the medium. Kamada *et al.* (1986) wounded the shoot tips of *Atropa belladonna* by pricking with sterile needle.

Moore *et al.* (1979) showed that most hairy roots emerged from the pericycle tissue of the carrot vascular cylinder. 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.

Nilsson and Olsson (1997) hypothesized that only cells containing high levels of auxin and sucrose (which regulate *rol* B and *rol* C promoters, respectively) are able to act as root meristem initials and are also ideal targets for

A. rhizogenes infection. Since ray cells and phloem cells are positioned in the region with the highest amount of sucrose and considerable amount of IAA, they could be convenient targets for *A. rhizogenes* (Zdravkovic-Korac *et al.*, 2004).

2.1.11.6 Inoculation and co-culturing of explants with A. rhizogenes

Explants were inoculated with *A. rhizogenes* strains using different methods.

2.1.11.6.1 Direct inoculation of bacterial colonies

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

Davey *et al.* (1987) applied a loopful of bacterial culture on the decapitated stem of *Solanum* and *Nicotiana* species for hairy root induction. Benjamin *et al.* (1993) infected shoots of *Rauvolfia serpentina* by smearing bacteria on incision made at the basal end of shoot.

Subroto and Doran (1994) obtained hairy roots by wounding *Solanum aviculare* plantlets with a syringe needle containing *A. rhizogenes.*

2.1.11.6.2 Inoculation and co-culturing of explants using bacterial suspension

The co-culture time and the bacterial concentration affect the transformation frequencies (Mihaljevic *et al.*, 1996). Hawes *et al.* (1988) have reported that the motile strains of *Agrobacterium* exhibited virulence only in liquid medium but mutant strains (non- motile) exhibit virulence when inoculated directly on wounds.

Momcilovic *et al.* (1997) inoculated the elongated shoots of *Gentiana spp.* by puncturing internodes or central veins with a hypodermic needle dipped into

bacterial suspension. Bacterial suspension was also smeared on the cut surface of decapitated shoots and further incubated for 48 hrs.

Yoshimatsu *et al.* (2003) added 200 µl of overnight grown bacterial suspension to the petiole segments placed in a flask containing half MS liquid medium with two per cent sucrose, and co-cultured for two days at 25 °C in the dark on a rotary shaker at 100 rpm.

Cotyledon and hypocotyls explants of *Isatis indigotica* Fort was immersed in bacterial suspension at exponential growth phase ($O.D_{600}$: 0.7) for ten minutes and co-cultivated in dark for two days on MS basal medium (Xu *et al.*, 2004).

Koike *et al.* (2003) immersed the leaf segments of *Angelonia salicarifolia* in an *Agrobacterium* suspension ($O.D_{600}$: 1.0) for 20 seconds and then blotted on to a filter paper. It was then co-cultivated for three days on a filter paper placed on half strength MS basal salts medium containing sucrose 20g l^{-1} and gellan gum 2.0 gm l^{-1}.

The bacterial suspension ($O.D_{600}$: 1) was centrifuged at 4,000 rpm for five minutes and resuspended in liquid MS medium. The leaf explants of *Pueraria phaseoloides* were dipped in this bacterial suspension for 20 min. and co-cultivated at 28 °C in the dark for two days (Shi and Kintzios, 2003).

Cotyledon, stem and root explants of *Taraxacum platycarpum* were dipped in bacterial suspension for five min and co-cultivated on half strength MS medium with Gelrite 0.3 mg l^{-1} and sucrose 2.0 per cent for three days (Lee *et al.*, 2004).

Malabadi and Nataraja (2003) inoculated leaf segments and callus derived leaf segments of *Clitoria ternatea* with an overnight freshly grown *Agrobacterium* suspension (~10^9 cell ml^{-1}) using sterilized needle and co-cultivated on solid Gamborg B_5 with sucrose 30 g l^{-1} for five days.

The bacterial culture ($O.D_{600}$: 1) was harvested and centrifuged at 10,000 rpm for 10 min. and the pellet was resuspended in the same volume of MSO liquid medium. Leaf explants of *Lupinus mutabilis* were immersed in bacterial suspension for 30 min. and blotted dry with sterile filter paper. The bacterial suspension was used undiluted or after dilution 1:1, 1:5 or1:10 (v/v) with MS liquid medium (Babaoglu *et al.*, 2004)

2.1.11.6.3 Influence of acetosyringone in hairy root induction.

Zdravkovic-Korac *et al.* (2004) transferred the inoculated androgenic embryos of *Aesculus hippocastanum* to MS medium with and without acetosyringone (50μ*M*) and co-cultivated for 72 hrs. They found that the presence of acetosyringone 50μ*M* during co-culturing significantly increased the number of putative transformants.

Chaudhuri *et al.* (2005) added acetosyringone (10 m*M*) to the bacterial suspensions (A4 and LBA 9402) one hour before inoculation to increase the transformation efficiency.

Tsuro *et al.* (2005) soaked the leaf explants of *Dendranthema grandiflorum* in YEB medium that contained suspended *Agrobacterium* ($O.D_{600}$: 0.1) supplemented with 100 μ*M* acetosyringone for 10 min so as to increase the transformation efficiency.

2.1.11.7 Culture media and conditions for hairy root induction

After infection, the explants are placed in suitable culture media and essential conditions are provided for hairy root induction.

Lorence *et al.* (2004) transferred the infected explants of *Camptotheca acuminata* after co-cultivation on Gamborgs B_5 (Gamborg *et al.*, 1968) solid medium containing timentin 300mg l^{-1} and plates were incubated at 25 °C under a 16-hrs photoperiod.

After co-cultivation, the leaf segments of *Angelonia salicarifolia* were transferred on to half strength MS basal salts medium containing Cefotaxime 500 mg l^{-1}, sucrose 20 g l^{-1}, gellan gum 2.0 g l^{-1} (Koike *et al.*, 2003).

The leaf explants of *Pueraria phseoloides* after co-cultivation, were placed on MS medium containing carbenicillin 500mg l^{-1} and kept in an air conditioned chamber at 25 °C under 14 hrs light photoperiod to induce hairy roots (Shi and Kintzios, 2003).

After co-cultivation, Yoshimatsu *et al.* (2003) placed the petiole segments of *Cephaelis ipecacuanha* on half MS solid medium containing Claforan 500 mgl^{-1} and sucrose two per cent at 25 °C in the dark.

2.1.11.8 Efficiency of A.rhizogenes in inducing hairy roots

The type of *A. rhizogenes* strains used influenced the root induction efficiency.

Significant differences were observed between the transformation ability of different strains of *A. rhizogenes* in inducing hairy roots from leaf segments of *W. somnifera*. The best response in terms of transformation ability and growth of hairy roots was obtained with strain A4, followed by LBA 9402; LBA 9360 failed to induce a transformation event. (Banerjee *et al.*, 1994)

Agrobacterium rhizogenes strain A4 was significantly better than strains R1601 and ATCC 15834 for inducing roots in *Isatis indigotica* (Xu *et al.*, 2004). Benjamin *et al.* (1993) reported that *A. rhizogenes* 15834 was an effective vector for specific gene transformations in *Rauvolfia serpentina*.

The *A. rhizogenes* strain LBA 9402 showed high transformation frequency on *Hyscyamus muticus* (Vanhala *et al.*, 1995).

Two *A. rhizogenes* strains LBA 9402, 15834 and one *A. tumefaciens* strain GUS-2 were tested for the ability to induce the formation of hairy roots on *Papaver somniferum* hypocotyls. Hairy root development at the wound site of the hypocotyls occurred after five weeks of culture, only from the hypocotyls infected with the *A. rhizogenes* LBA 9402 strain and then transferred into the Linsmaier and Skoog hormone free liquid medium. This indicated that this strain is capable of high virulence in the particular condition used (Flem-Bonhomme *et al.*, 2004).

A. rhizogenes strain A4 obviously has the ability to induce well growing roots in a wide range of *Solanaceous* host plants compared to 8196 strain (Knopp *et al.*, 1988)

Lorence *et al.* (2004) tested the ability of two different strains of *A. rhizogenes*, ATCC 15834 and R-1000 on inducing hairy roots in *Camptotheca acuminata.* They found that strain 15834 infected more than 40-45 per cent of explants, but in contrast, strain R-1000 infected only 20-24 per cent of the exposed tissue.

Agrobacterium rhizogenes strains, 15834, A4, 1855 and 2659 were tested for their ability to induce hairy roots on rhizomes of *Scopolia japonica* and strain 15834 was found to be most active in inducing hairy roots (Mano *et al.*, 1986).*ous* host plant compared to 8196 strain (Knopp *et al.*, 1988).

2.1.11.9 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 subculturing.

Thirty days after infection with ATCC 15834, hairy roots induced in *Pueraria phaseoloides* were excised from leaf explants and cultured on growth regulator free MS agar (1%) medium with carbenicillin 500 mg l^{-1} to eliminate *Agrobacterium* (Shi and Kintzios, 2003).

Transformed roots of *Lupinus mutabilis* induced by *A. rhizogenes* strain R1601 were excised after 14-20 days and cultured on semisolid MS medium containing cefotaxime 300 mg l^{-1} and kanamycin sulfate 40 mg l^{-1} (Babaoglu *et al.*, 2004).

Adventitious roots that developed 2-4 weeks after co-cultivation were isolated from leaf segments of *Angelonia salicarifolia* and transferred on to half MS basal salts medium containing sucrose 20g l^{-1} and gellan gum 2.0 g l^{-1}, and maintained by subculturing root tips every four weeks on same medium at 25 °C in the dark (Koike *et al.*, 2003).

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 B_5 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).

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

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

Yoshimatsu *et al.* (2003) reported that transformed hairy roots of *Cephaelis ipecacuanha* showed best growth in B5 liquid medium compared to half MS and Full MS liquid medium. Addition of IBA (either 0.1 or 0.5 mg l^{-1}) was found to improve the growth of root material and the growth under dark was superior to that under light.

Xu *et al.* (2004) found that the culture medium have 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.

2.1.12 Confirmation of transformation

2.1.12.1 Growth characteristics and Morphology of Hairy roots

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

Hairy roots are fast growing and laterally branched, and are able to grow in hormone free medium. Moreover, these organs are not susceptible to geotropism any more. 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 in their ability to form several new growing points and consequently, lateral branches (Oksman-Caldentey and Hiltumen, 1996). The growth rate of hairy roots may vary greatly between species, but differences are also observed between different root clones of the same species (Mano *et al.*, 1989).

The hairy roots obtained from *Papaver somniferum* var. album were morphologically thin, highly branched and showed lot of root hairs with plagiotropic growth habit (Flem-Bonhomme *et al.*, 2004). In *Atropa belladonna* (Bonhomme *et al.*, 2000) and *Catharanthus roseus* (Palazon *et al.*, 1998), the hairy roots produced were thick in morphology.

2.1.12.2 Detection of opines

The genetic transformation can be confirmed by assaying the opines produced (Sevon and Oksman- Caldentey, 2002).

Opine synthase genes in principle constitute good reporter genes since no natural equivalent to their gene products has been found in plant cells (Chawla, 2002). Opines were detected by high voltage paper electrophoresis (Petit *et al.*, 1983). Alkaline silver nitrate reagent visualized agropine and mannopine.

Yoshimatsu *et al.* (2003) reported that confirmation of transformation by detection of opines (agropine and mannopine) using high voltage paper electrophoresis was unsuccessful, because of the existence of interfering substances, which produced spots near the positions of agropine and mannopine after silver staining.

Moreover, opine production can be unstable in hairy roots and may disappear after a few passages (Flores *et al.*, 1987). For this reason, detection of T-DNA by Southern blot hybridization is often necessary to confirm the genetic transformation (Tepfer, 1984).

2.1.12.3 PCR analysis of hairy roots

The polymerase chain reaction (PCR) often simplifies the detection of transformation (Hamill *et al.*, 1991). Flem-Bonhomme *et al.* (2004) reported that the hairy roots produced from *Papaver somniferum* showed that they contained the *rol* A, *rol* B and *rol* C genes.

For PCR analysis of transformed roots of *Taraxacum platycarpum* produced by infection with *A. rhizogenes* 15834, Lee *et al.* (2004) used *rol* C gene and *vir* G gene specific primers. The forward and reverse primers used for amplifying *rol* C gene were 5'- ATG GCT GAA GAC GAC CTG TGT T- 3' and 5'-TTA GCC GAT TGC AAA CTT GCT C-3' respectively.

Celma *et al.* (2001) used PCR for the identification of the rooting locus genes, *rol* A, *rol* B and *rol* C using DNAs from the hairy roots as template and the nontransformed roots as control. The primer sequences used were, 5'- TGG AAT TAG CCG GAC TAA AC- 3' (*rol* A-1), 5'- GCG TAC GTT GTA ATG TGT TG-3' (*rol* A-2), 5'- AGT TCA AGT CGG CTT TAG GC-3' (*rol* B-1), 5'- TCC ACG ATT TCA ACC AGT AG-3' (*rol* B-2), 5'- TAA CAT GGC TGA AGA CGA CC-3' (*rol* C-1), 5'- AAA CTT GCA CTC GCC ATG CC -3' (*rol* C-2).

2.1.12.4 Southern Hybridization

The detection of T-DNA by Southern hybridization is often necessary to confirm the genetic transformation.

The DNA probes used for Southern hybridization were a 1.25 kb *Bam* H1 fragment of pLJ 85, that covers TR with a part of *aux* 1 gene of pHRi, and the 1.52 kb *Bam* H1 fragment of pLJ1 cosmid, comprising the TL-DNA with a part of the *rol* C gene of pHRi (Jouanin, 1984).

Southern blot analysis of transformed roots of *Cephaelis ipecacuanha* revealed that only the TL-DNA was integrated into the plant genome without incorporation of the TR-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.

2.1.13 Estimation of withanolides

Withanolides are ergostane type steroids with atoms C-22 and C-26 bridged by a lactone functionality (Ganzera *et al.*, 2003). Withanolides are characterized by the presence of large number of oxygen-containing functional groups (hydroxyls, ketones, epoxides, cyclic ethers). Ninety per cent of all known withanolides possess a 1-oxo-group (e.g. withaferin A). Withanolides occur predominantly as aglycones, although glycosides are known from some sources. They occur mainly in the leaves of withanolide containing species (Dinan *et al.*, 2001).

Withanolides are predominantly associated with the members of the Solanaceae, and in particular for the genus *Withania* (Ganzera *et al.*, 2003). Recently, they are detected in *Tacca plantaginea* (Taccaceae), *Cassia simae* (Leguminosae) and *Ajuga parviflora* (Labiatae) (Dinan *et al.*, 2001).

Withanolide production by *in vitro* cultures like callus, shoots, leaves and roots transformed by *A. rhizogenes*, derived from the Italian *W. somnifera* was

investigated on MS media either supplemented with BAP or 2,4-D or free of plant growth regulators. Limited production was observed in shoot and callus cultures whereas, no withanolides were detected in hairy roots (Vitali *et al.*, 1996).

Banerjee *et al.* (1994) studied the production of withanolides, with special reference to withaferin A, in the A4 induced hairy root lines at different growth phases (4,10 and 24 weeks) using HPLC. Maximum levels of withaferin A were observed in the media and hairy roots of 10-week old cultures.

Mishra (1994) developed a colorimetric method for the estimation of total withanolides from the leaf of *Withania somnifera*. Gupta *et al.* (1996) used TLC densitometry method for the quantitative determination of withaferin A in different plant parts like leaves, root, stem, seed and persistant calyx of *W. somnifera*.

Mahadevan *et al.* (2003) developed a High Pressure Thin Layer Chromatography method to quantify withaferin A in herbal extract and polyherbal formulations. Precoated silica gel plates were used as stationary phase and toluene: ethyl acetate: formic acid (50:15:5) was used as the mobile phase.

Ganzera *et al.* (2003) developed a High Pressure Liquid Chromatography method for the quantitative estimation of withaferin A and withanolide D in *W. somnifera*. The analysis of root, stem and leaf confirmed the presence of withaferin A and withanolide D in all parts of the plant, but with significant differences in the ratio. In the roots, withanolide D was most dominant (0.193%). This compound was only minor in the leaves, where a rather high amount of withaferin A was found (0.238%).

Separation, identification and quantification of selected withanolides in plant extracts of *W. somnifera* by HPLC- UV (DAD) - Positive ion electroscopy ionization- mass spectrometry was studied by Khajuria *et al.* (2004).

2.1.13.1 Extraction of withanolides

Withanolides were generally extracted from plant material with methanol or ethanol, which was then mixed with water before being partitioned against hexane to remove plant pigments. The aqueous methanol phase was then partitioned against $CHCl_3$, $CHCl_2$ or diethyl ether to extract out the withanolides (Dinan *et al.*, 2001).

Khajuria *et al.* (2004) carried out the extraction of withanolides in ethanol: water in 1:1 ratio. Ganzera *et al.* (2003); Ray *et al.* (1996); Gupta *et al.* (1996) used methanol to extract out the withanolides from *W. somnifera.*

The extract was defatted with n-hexane and then extracted with 1.0 per cent H_2SO_4, basified with ammonia, extracted with $CHCl_3$ and dried over anhydrous Na_2SO_4, filtered and evaporated to separate the alkaloid. The H_2SO_4 insoluble fraction was extracted with diethyl ether, dried over anhydrous Na_2SO_4, filtered and evaporated to obtain the crude withanolide (Gupta *et al.*, 1996).

Stock solutions of withaferin A were prepared in $CHCl_3$ (Gupta *et al.*, 1996). Khajuria *et al.* (2004) prepared stock solutions in MeOH: H_2O (1: 1).

2.1.13.2 Thin Layer Chromatography

Thin Layer Chromatography (TLC) was convenient and suitable for the analysis of withanolides. It was often used to monitor fractionations for final purification of withanolides. The most frequently used solvent system for aglycones was $CHCl_3$: MeOH (95:5) and $CHCl_3$: MeOH (90: 10) for glycosides (Dinan *et al.*, 2001).

Karmarkar *et al.* (2001a) reported the use of chloroform: methanol (3: 4) running solvent system for the elution of sterols in *Holostemma ada-kodien.* Ray *et al.* (1996) analysed the withanolides in a solvent system of $CHCl_3$: EtOAc: CH_3OH: C_6H_6 (72: 4: 8: 16) and sprayed with Liebermann Burchard reagent.

Chromatography was carried out using chloroform: methanol (95:5) as the mobile phase and the spots were visualized by immersing the spots for one minute at 25 °C in a freshly prepared mixture of vanillin (0.5 g), boric acid (10 g), concentrated H_2SO_4 (20 ml) and MeOH (1000 ml), followed by heating at 110 °C for 15 minutes (Gupta *et al.*, 1996).

Withanolides could be detected after chromatography by (i) UV quenching (ii) spraying with Dragendorff's reagent (even though they are not N-containing), (iii) an epoxide reagent (4-[4-nitrobenzyl]-pyridine or (iv) spraying with a saturated $CHCl_3$ solution of $SbCl_3$ followed by heating (Dinan *et al.*, 2001).

2.1.14 Enhancement of secondary metabolite production

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 in to the medium, eliciting the cultures with fungal extracts or stimulating stress conditions in the culture medium (Moreno *et al.*, 1995).

2.1.15 Addition of precursors

Lockley *et al.* (1976) studied the biosynthesis of steroidal withanolides in *W. somnifera.* The incorporation of 3H radioactivity from 24-methylene-cholesterol-(28-^{3}H) into withaferin A and withanolide D demonstrated that 24-methylene cholesterol could serve as a biosynthetic precursor of these compounds in *W. somnifera.* The yield of withanolide was improved when 24-methylene cholesterol was incorporated in admixture with 3-(RS) mevalonolactone-(2-^{14}C).

According to Velde *et al.* (1983), 3 β hydroxy-2,3-dihydro-withanolide and ergoslate-5, 25-diene-3β, 24-diol could be considered as intermediate products in the biosynthetic sequence which produces the withanolides in *Withania coagulans.*

Veleiro *et al.* (1985) administered sodium acetate, mevalonolactone and methionine to the excised leaves and 15-day old seedlings of *Acnistus breviflorus.* From the results, it was deduced that mevalonolactone was a precursor of both

withaferin A and jabrosalactone D whereas; sodium acetate and methionine were poorer precursors in terms of absolute incorporation. The incorporation of methionine in to withanolides could involve the formation of a C_{28}-sterol that retains the C-28 methyl group upon conversion to the withanolides.

To enhance berberine production in established plant cell cultures of *Thalictrum minus subsp. saxatile*, Smolko and Peretti (1994) found that addition of 10^{-19} M-pectate lyase was beneficial, which also stimulated secretion of the alkaloid in the medium.

Maximum production of tropane alkaloids (3.97%) in *Hyoscyamus muticus* callus cultures was obtained by supplementing the medium with phenylalanine, while maximum growth was obtained using isoleucine (El-Bahr *et al.*, 1997).

Hashimoto and Yamada (1987) studied the effects of precursor feeding on tropane alkaloid formation in *Hyoscyamus niger* suspension cultures. N-Methyl putrescine, tropane, phenylalanine and tropic acid increased alkaloid formation, but there was considerable variation in the relative amounts there of between trials.

Balvanyos *et al.* (2002) studied the influence of aminoacids on the lobeline production of *Lobelia inflata* L.hairy root cultures. In hairy root cultures lobeline production increased in the presence of either phenylalanine or lysine compared with the control, but the addition of both greatly decreased the synthesis. In contrast, the addition of both aminoacid to media supplemented with IAA and KN increased lobeline production.

Hay *et al.* (1986) demonstrated that feeding of *Cinchona ledgerina* root organ suspension cultures with L-tryptophan at 500 mg l^{-1} gave a five fold increase in the alkaloid yield (quinine and quinidine) over the control, when fed during the lag phase of the growth cycle.

Morgan and Shanks (2000) observed that feeding higher levels of auxin or tryptophan resulted in increased branching and thickening of the *Catharanthus roseus* hairy root cultures, which also resulted in dramatic reduction in flux to the indole alkaloids.

2.1.14.2 Addition of osmoregulants

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

Osmotic stress stimulates secondary metabolism in cultured plant cells (Akcam-Oluk *et al.*, 2003). According to Smith *et al.* (1987) addition of sodium chloride, potassium chloride and sorbitol to 5-day-old cell suspension cultures of *Catharanthus roseus* stimulated the intracellular accumulation of catharanthine and other indole alkaloids within 48-72 hrs.

Shokubai (1991) reported that polyethylene glycol of high molecular weight, could be utilized for metabolite production without damaging cellular activity. The osmoregulant polyethylene glycol at 2.0 per cent elicited a positive response in leaf calli of *Sida cordifolia* on biosynthesis of ephedrine (Sankar, 1998). Osmoregulants like sorbitol and mannitol failed to enhance metabolite production in *Sida spp.* (Sankar, 1998) and *Nicotiana* (Gangopadhyay *et al.*, 1997).

Application of osmotic stress to *Catharanthus roseus* cell suspension cultures using 9.0 per cent PEG of molecular weight 3350 increased ajmalicine production capacity, although it did not trigger the production of other alkaloids involved (Akcam-Oluk *et al.*, 2003).

2.1.14.3 Addition of Elicitors

Elicitation is one of the methods that have been used to enhance the production of secondary metabolites of cell cultures (Eilert, 1987).

Table (4) gives the list of some medicinal plants where elicitors have been applied to hairy root cultures.

The biotic elicitors include glucan polymers, glycoproteins, low molecular weight organic acids and fungal cell wall material. Abiotic elicitors include ultraviolet radiation, salts of heavy metals and chemicals, some of which have affinity for double stranded DNA while others disturb cellular membrane integrity (DiCosmo and Misawa, 1985).

Table 4. Summary of studies involving elicitation of hairy roots in medicinal plants

Sl. No.	Host plant	Elicitor	Effect
1	*Brugmansia candida*	Salicylic acid, Yeast extract, $CaCl_2$, AgNO3, $CdCl_2$	Stimulation of hyoscyamine and scopalamine
2	*Catharanthus roseus*	Methyl jasmonate	Stimulation of catharanthine and ajamalicine
3	*Hyoscyamus muticus*	Purified chitosan	Stimulation of l-hyoscyamine
4	*Hyosyamus muticus*	Methyl jasmonate+ Wounding + *Rhizoctonia* solani	Solavetivone and lubimin accumulation
5	*Nicotiana tabacum*	Yeast extract, *Botrytis fabae* extract	Accumulation of sesquiterpene phytoalexins (capsidol and debneyol)

(Sevon and Oksman-Caldentey, 2002)

Those compounds, which defend the plants against micro-organisms, namely phytoalexins, are often easily formed in response to the elicitors, but the

accumulation of the alkaloids of interest has not usually been induced (Sevon and Oksman-caldentey, 2002).

Vazquez-Flota *et al.* (1994) used three fungal species *Aspergillus* spp., *Trichoderma viridae* and *T. reseii* and yeast *Rhodotorula marina* for biotic elicitation of *Catharanthus roseus* hairy root cultures. *Aspergillus* treatment and the use of macerocyme increased the accumulation of ajamalicine selectively, while the addition of methyljasmonate increased the yield of both ajamalicine and catharanthine.

Phytophthora infestans sonicate (20 µl ml^{-1}, cellulase from *Trichoderma viridae* (10 µg ml^{-1}), pectinase from *Aspergillus niger* (10 µg ml^{-1}), arachidonic acid (0.025 m*M*) and CuSO4 (1.0 m*M*) elicited the formation of sesquiterpenoid phytoalexins in cell suspension cultures of potato (cv. Kennebec), *Capsicum* (cv. New Ace), tobacco (cv. White Burley) and *Datura stramomium* (Threlfall and Whitehead, 1988).

Cusido *et al.* (1999) reported that tween 20 treatments encouraged both growth and alkaloid productivity of hairy roots of *Datura metal* L.

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled "Genetic transformation for hairy root induction and enhancement of secondary metabolites in *Aswagandha* (*Withania somnifera* (L.) Dunal)" was carried out at the Centre for Plant Biotechnology and Molecular Biology and Biochemistry Laboratory of the College of Horticulture, Vellanikkara and the Biochemistry Laboratory of Aromatic and Medicinal Plants Research Station, Odakkali from 2004 to 2006. The materials used and the methodologies adopted are described below

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, HIMEDIA and Sigma Chemicals, USA. Withaferin A, standard for biochemical analysis was procured from Laila Impex, Vijayawada. The primers and restriction enzymes were obtained from Imperial Bio Medics, Chandigarh.

3.1.2 Glass and Plastic wares

Borosilicate glasswares of Corning/ Borosil brand and disposable petridishes of Axygen and Tarson were used for the study. The glasswares were initially steam sterilized and cleaned with detergent solution and then with tap water. The glass wares were then soaked in a solution containing potassium dichromate, water and sulphuric acid followed by thorough washing with jets of tap water and finally with distilled water. Test tubes were kept in racks and dried in open air. Conical flasks were dried in hot air oven. Jam bottles were further sterilized in autoclave and dried in hot air oven.

3.1.3 Composition of media

Murashige and Skoog (1962) medium (MS) was reported to be the best basal medium for micropropagation of *Withania somnifera* and hence, was used as the basal medium in the tissue culture studies. Half strength MS was also used. In the development of transformed root cultures MS, half MS and B5 medium were used. The compositions of the media are given in Appendix I.

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

3.1.4 Preparation of tissue culture medium

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

A clean steel vessel, rinsed with distilled water was used to prepare the medium. Aliquots from all stock solutions were pipetted in proportionate volumes in the vessel. For preparing media of full strength 20 ml was pipetted from 50X stocks and 10ml 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 0.70 per cent (w/v) concentration, after adjusting the pH. The medium was stirred and heated to melt the agar, and was poured when hot, into culture vessels and were plugged with non-absorbent cotton. For solid media, test tubes (15 cm x 2.5 cm) were used whereas for liquid media conical flasks or Erlenmeyer flasks (100, 250 and 500ml) were used as culture vessels. Fifteen ml medium was poured in each test tube, 30 ml medium in

100 ml conical flask, 125 ml in 250 ml conical flask and 250 ml in 500 ml conical flask. Vessels containing media were sterilized in an autoclave at 121°C in 15psi for 20 min. The medium was allowed to cool to room temperature and stored in the culture room until used.

3.1.5 Growth regulators

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

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

3.1.6 Organic supplements

Coconut milk (10 %) was tried for its effect in regeneration and multiplication.

3.1.7 Carbon source

Sucrose was used as the main source of carbon (3.0 %) in this study

3.1.8 Preparation of YEB, YEM, NA and LBA medium

Clean steel vessels, rinsed with distilled water were used to prepare the media. The ingredients were weighed on electronic balance and were added into the vessels. A small volume of distilled water was added to it and the ingredients were dissolved. The desired volume was made up by adding distilled water. The pH of the media was adjusted to 7.0 using a standard pH meter by adding either 0.1*N* NaOH or HCl. For solid media agar was added at 2.0 per cent (w/v) concentration. The media were stirred and heated to melt the agar and were poured

when hot into conical flasks and jam bottles. Conical flasks were plugged with non-absorbent cotton and jam bottle were sealed tightly using cello tape after placing the lid. Autoclaving was done at 121 °C at 15 psi for 20 min to sterilize the medium and they were further kept in the culture room until used.

3.1.9 Antibiotics

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

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

3.2 TRANSFER AREA AND ASEPTIC MANIPULATIONS

All the aseptic manipulations such as surface sterilization of explants, preparation and inoculation of explants, subsequent sub culturing, preparation of antibiotic media and transformation studies were carried out under the hood of a laminar air flow cabinet fitted with UV lamp.

3.3 CULTURE ROOM

The cultures were incubated at 26 ± 2 °C in an air-conditioned culture room with 16 hr photoperiod (1000 lux) from fluorescent tubes. Humidity in the culture room varied from 60 to 80 per cent according to the climate prevailing. For incubating cultures in the dark, black cotton cloth was fixed in culture racks.

3.4 SOURCE OF EXPLANTS

Stock plants were brought from the Seed Farm, Mannuthy. They were planted in pots and placed in the shade house giving daily irrigation. Established plants were sprayed with contact fungicide Fytolan (copper oxychloride) at 0.3 per

cent concentration at fortnightly intervals. Seedlings were raised *in vitro* from fresh ripe berries and sun dried seeds obtained from the stock plants, which served as the source of explants for the present study.

3.5 STANDARDISATION OF *IN VITRO* REGENERATION

3.5.1 Surface sterilization and *in vitro* seed germination

Fresh ripe berries as well as sun-dried seeds were used for raising *in vitro* seedlings. They were first washed with liquid soap (Teepol) for a few minutes to remove surface adhered particular dirt and then washed under running tap water to remove the traces of soap. Further, surface sterilization was carried out under the hood of a laminar airflow cabinet. Fresh berries were treated with 50 per cent alcohol for five seconds and blotted dry. The berries were then disinfected with 0.1 per cent $HgCl_2$ for two minutes followed by four rinses with sterile water. They were then blotted dry using sterile blotting paper and cut open with sterile blade and forceps. Dried seeds were treated with 0.1 per cent $HgCl_2$ for only one minute.

Dried surface sterilized seeds were inoculated on half MS medium containing 2.5 per cent sucrose. Seeds were incubated at 26 ± 2°C under dark and 16 hr light photoperiod. Days taken for *in vitro* seed germination and percentage of germination were recorded.

3.5.2 Explants used for micro propagation

Various explants like hypocotyls, cotyledonary segments, leaf segments, shoot tips, nodal segments and roots were used for the study.

3.5.3 Preparation of explants

Ten to fifteen days old *in vitro* seedlings were taken out of the culture tubes in a laminar airflow cabinet on pre-sterilized steel plates. The seedlings were cut with a sterile blade to separate hypocotyls, cotyledons and roots. The hypocotyls were cut approximately 1.5 to 2.0 cm below the embryo axis and separated from the roots. The cotyledonary segments were cut on all sides so as to obtain discs of approximately 0.5 cm width and 1.0 cm length. Root tips (~2.0 cm) were also

separated out. Nodal segments, leaf and shoot tips were taken from two month old seedlings. Nodal segments were cut with a sterile blade such that each segment carries one to two nodes. Shoot tips were taken from the terminal portion of the seedlings of 1.5 to 2.0 cm length. Leaf segments (~ 1.0 cm^2) were prepared with a sterile blade by cutting the four sides.

3.5.4 Hypocotyls as explants

3.5.4.1 Regeneration

Hypocotyls taken from *in vitro* raised seedlings were cultured in MS media supplemented with different combinations of auxins and cytokinins. The hypocotyl segments (1.5- 2.0 cm) were placed both directly and also upside down in the following growth regulator combinations for regeneration. Each treatment consisted of ten replicates.

1) MS + IAA 0.5 mg l^{-1} + BAP 0.5 mg l^{-1}
2) MS + IAA 0.5 mg l^{-1} + BAP 1.0 mg l^{-1}
3) MS + IAA 0.5 mg l^{-1} + BAP 1.5 mg l^{-1}
4) MS + IAA 0.5 mg l^{-1} + BAP 2.0 mg l^{-1}
5) MS + IAA 0.5 mg l^{-1} + BAP 2.5 mg l^{-1}
6) MS + IAA 0.5 mg l^{-1} + BAP 1.0 mg l^{-1} + coconut milk 10 per cent

Observations on time taken for regeneration, percentage of regeneration and the number of shoot buds produced were recorded.

Multiplication

The clumps of shoot buds obtained, were cut into small pieces, each of 3-8 buds and were inoculated on MS with different growth regulator combinations for leaf differentiation and multiplication. The number of shoot buds per piece was recorded at the time of inoculation. The combinations tried are given below,

1. MS + IAA 0.5 mg l^{-1}
2. MS + IAA 0.5 mg l^{-1} + BAP 0.2 mg l^{-1}

3. MS + IAA 0.5 mg l^{-1} + BAP 0.4 mg l^{-1}
4. MS + IAA 0.5 mg l^{-1} + BAP 1.0 mg l^{-1}
5. MS + IAA 0.5 mg l^{-1} + BAP 2.0 mg l^{-1}
6. MS + BAP 1.0 mg l^{-1}
7. MS + BAP 2.0 mg l^{-1}

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

3.5.4.3 Elongation and rooting

The shoots produced from hypocotyls, of length 0.5-2.0 cm were separated and further elongated and rooted in the following growth regulator combinations

1) MS + sucrose 3.0 per cent
2) ½ MS + sucrose 2.5 per cent
3) MS + GA_3 0.5 mg l^{-1}
4) MS + GA_3 1.0 mg l^{-1}

The shoots which were elongated in MS + GA_3 0.5 mg l^{-1} and also the shoots obtained after multiplication were rooted on half MS by pulse treatment with IBA 1000 mg l^{-1} for varying periods (5sec, 15 sec and 30 sec).

Observations were recorded after 20 days, on the elongation obtained, number of internodes, leaves, roots, days taken for root induction and rooting percentage.

3.5.4.4 Hardening and planting out

The *in vitro* rooted plantlets obtained were taken out of the test tubes and the solidified media from the plantlets were washed out under running tap water. The plantlets were then planted in small earthern pots or polythene cover filled with sterilized sand. They were transferred to a green house provided with an extra shade net protection and sprinkler irrigation system for hardening the plantlets.

After one month, the hardened plants were transferred to large pots containing potting mixture sand, soil and water in 1:1:1 ratio and kept in the green house itself.

3.5.5 Cotyledonary segments as explants

3.5.5.1 Regeneration

Cotyledonary segments were inoculated such that the midrib portion in the lower side touched the MS basal medium with different growth regulator combinations. The combinations tried were,

1) MS + IAA 0.5 mg l^{-1} + BAP 0.5 mg l^{-1}
2) MS + IAA 0.5 mg l^{-1} + BAP 1.0 mg l^{-1}
3) MS + IAA 0.5 mg l^{-1} + BAP 1.5 mg l^{-1}
4) MS + IAA 0.5 mg l^{-1} + BAP 2.0 mg l^{-1}
5) MS + IAA 0.5 mg l^{-1} + BAP 2.5 mg l^{-1}
6) MS + IAA 0.5 mg l^{-1} + BAP 1.0 mg l^{-1} + coconut milk 10 per cent

The percentage of regeneration obtained and the number of shoot buds produced were recorded after 25 days.

3.5.5.2 Regeneration from callus

The regeneration of callus from cotyledonary segments were tried in the following combinations

1) MS + sucrose 3.0 per cent
2) MS + BAP 1.0 mg l^{-1}
3) MS + BAP 2.0 mg l^{-1}
4) MS + BAP 1.0 mg l^{-1} + KN 0.5 mg l^{-1}
5) MS + BAP 1.0 mg l^{-1} + KN 1.0 mg l^{-1}

The percentage regeneration and the mean number of shoot buds produced were recorded after 25 days.

3.5.5.3 Multiplication

Regenerated shoot buds from the cotyledonary segments were multiplied in MS medium supplemented with BAP 0.4 mg l^{-1} and IAA 0.5 mg l^{-1}. Observations regarding the number of shoot buds produced were recorded after 25 days.

3.5.5.4 Elongation and rooting

The shoots produced from cotyledonary segments were separated and further elongated in the following growth regulator combinations

1) MS + GA_3 0.5 mg l^{-1}
2) MS + GA_3 1.0 mg l^{-1}

The elongated shoots were rooted on half MS medium by pulse treatment with IBA, 1000 mg l^{-1}, for five seconds.

Observations regarding the elongation rate, number of internodes, leaves, roots, days taken for root induction and rooting percentage were recorded after 20 days of inoculation. The *in vitro* rooted plantlets obtained were hardened in small pots and later transferred to large pots in the green house.

3.5.6 Nodal segments and shoot tips as explants

3.5.6.1 ***Culture establishment***

Nodal segments and shoot tips taken from two months old seedlings were initially established in MS with BAP 1.0 mg l^{-1} and IAA 0.5 mg l^{-1}. The number of days taken for the establishment of cultures and percentage of establishment of cultures were recorded.

3.5.6.2 Multiplication

The established cultures were inoculated in the multiplication media after 10 days of establishment. The growth regulator combinations tried for multiplication are as follows,

1) MS + IAA 0.5 mg l^{-1} + BAP 1.0 mg l^{-1}
2) MS + IAA 0.5 mg l^{-1} + BAP 1.5 mg l^{-1}
3) MS + IAA 0.5 mg l^{-1} + BAP 2.0 mg l^{-1}
4) MS + IAA 0.5 mg l^{-1} + BAP 2.5 mg l^{-1}
5) MS + IAA 0.5 mg l^{-1} + BAP 3.0 mg l^{-1}
6) MS + IAA 0.5 mg l^{-1} + BAP 1.0 mg l^{-1} + coconut milk 10 per cent
7) MS + BAP 1.0 mg l^{-1} + KN 0.5 mg l^{-1}
8) MS + BAP 1.0 mg l^{-1} + KN 1.0 mg l^{-1}

The number of multiple shoot buds and the number of elongated shoots obtained were recorded separately after three weeks of culture.

3.5.6.3 Subculturing

The second subculture was tried in MS media supplemented with IAA 0.5 mg l^{-1} +BAP 1.5 mg l^{-1} and IAA 0.5 mg l^{-1} + BAP 2.5 mg l^{-1}.

The number of multiple shoot buds and the number of elongated shoots obtained were recorded separately after three weeks of culture.

3.5.6.4 Elongation and rooting

The clumps of shoot buds smaller than 1.5 cm were cut into small pieces and were elongated in MS + GA_3 0.5 mg l^{-1}. Elongation and rooting of the multiplied shoots were tried in the following combinations,

1) ½ MS + sucrose 3.0 per cent
2) ½ MS + sucrose 3.0 per cent + charcoal 0.25 per cent
3) ½ MS + IBA 0.5 mg l^{-1}
4) ½ MS + IBA 1.0 mg l^{-1}
5) ½ MS + IBA 0.5 mg l^{-1} + IAA 0.5 mg l^{-1}
6) ½ MS + IBA 1.0 mg l^{-1} + IAA 0.5 mg l^{-1}
7) ½ MS + IBA 1000 mg l^{-1} (pulse treatment)
8) ½ MS + IBA 1000 mg l^{-1} (pulse treatment) + charcoal 0.25 per cent

Observations regarding the elongation obtained, number of nodes, number of days for root induction, percentage of rooting, number of roots and the morphology of roots were recorded after three weeks. The *in vitro* rooted plantlets were hardened and established.

3.5.7 Leaf segments as explants

Leaf segments of approximately 1.0 cm^2 were placed in MS medium supplemented with BAP (1.0-3.0) mg l^{-1} and IAA 0.5 mg l^{-1} for regeneration. The regeneration response and the number of shoot buds obtained in each combination were recorded after 25 days.

The shoots were elongated and rooted in ½ MS + IBA 1000 mg l^{-1} (pulse treatment) for three seconds. A single shoot was also inoculated on half MS + sucrose 3.0 per cent + activated charcoal 0.25 per cent. The number of days for root induction, number of roots, nodes and length of shoot were recorded.

3.5.8 Roots as explants

Root tips of approximately 2.0 cm were taken from *in vitro* seedlings and *in vitro* rooted plantlets were inoculated in MS medium supplemented with BAP (1.0-2.5) mg l^{-1} and IAA 0.5 mg l^{-1} for regeneration. The somatic embryo like structures obtained from roots were inoculated in MS medium supplemented with BAP 1.5 mg l^{-1} and IAA 0.5 mg l^{-1} and the small shoots produced were further inoculated on half MS + IBA 0.5 mg l^{-1}. The regeneration from root callus was further tried on MS + BAP (2.0-6.0 mg l^{-1}) and IAA 0.2 mg l^{-1}.

3.6 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

3.6.1 Availability of *Agrobacterium rhizogenes* strains

Three *Agrobacterium rhizogenes* strains of agropine family were used for the present study. The strain A4 was obtained from Eugene W. Nester, University of Washington, USA. The strains MTCC 532 or ATCC 15834 and MTCC 2364 were procured from IMTECH, Chandigarh as freeze dried cultures. The details of the strains as given by IMTECH are given in the Table (5).

Table 5. Details of strains obtained from IMTECH

MTCC No	2364	532
Type	B	B
Genus Name	*Agrobacterium*	*Agrobacterium*
Species including Subspecies/ Variety	*rhizogenes*	*rhizogenes*
Strain Designation	30200	15834
Source of Isolation	Stem gall	Not mentioned
Culture Received From	DSM	ATCC
Equivalent Number of other Culture Collections	DSM 30200	ATCC 15834
Growth Medium	*Xanthomonas* medium	Nutrient Agar
Growth Condition	Aerobic	Aerobic
Growth Temperature	25 °C	25 °C
Special Features/ Applications	Not mentioned	Virulent

3.6.2 Culturing of *A. rhizogenes* strains

The bacterial strains were cultured on four different media, Luria Bretani Agar (LBA), Yeast Extract Mannitol (YEM), Yeast Extract Broth (YEB) and Nutrient Agar (NA) to select a suitable growth medium. The solid media was melted, cooled to 40-50°C and poured into sterilized petriplates. Each strain was streaked on plates containing the respective media. The growth rate of bacteria on each medium was observed. To study the influence of temperature on the growth of *A. rhizogenes* strains, one set of the culture was incubated at room temperature (30°C) and another set in the culture room at 26 ± 2 °C. The growth of bacteria in different culture conditions was observed.

3.6.3 Isolation of single cell colonies

The bacterial strains were streaked on appropriate culture medium so as to isolate single cell colonies. To streak the bacteria on to the plate, the transfer loop was flamed and cooled repeatedly, three times. The loop was then plunged into a well-grown bacterial colony. The lid of the petriplate containing sterilized, solid growth medium was lifted from one side and the loop loaded with bacteria was drawn gently on about one-third of the plate surface, to bring three lines close together, but separated from each other. The loop was again flamed, cooled and drawn across one end of the second streaked area and the remaining one-third area

of the plate was streaked. The third sector was also streaked similarly. The plate was closed, sealed with parafilm and kept in the culture room on a rack. Observations regarding growth of bacteria were documented.

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

The *A. rhizogenes* strains used for the study were tested for resistance to antibiotics. The antibiotics used for testing resistance were cefotaxime, ampicillin and carbenicillin. Nutrient agar medium was selected for the study. The sterilized media was melted and cooled to 40-50 °C and supplemented with 50, 100, 200, 300, 400 and 500 mg l^{-1} of each antibiotic separately. The medium was poured into petriplates and allowed to solidify. The petriplates containing solidified medium was divided by marker lines into three sectors. The bacteria from a single cell colony were streaked on the respective parts in each petriplate. Bacterial strains were also streaked in NA medium without any antibiotics, to be used as control.

3.6.5 Maintenance of strains

The strains were maintained as stabs and glycerol stocks.

3.6.5.1 Preparation of stabs

The best growth medium (YEB or NA) containing marker antibiotic for each strain was sterilized, poured into sterilized screw cap culture tubes and allowed to solidify. The transfer loop was flamed, cooled and plunged in an isolated single cell bacterial colony. The loop loaded with bacteria was used to stab the solid medium in the culture tube. Similarly, stabs for all the bacterial strains were prepared following the same procedure mentioned above, and allowed to stand in the culture room on racks for growth of bacteria in the medium. The stabs showing good growth of bacteria were further stored at 4-6 °C till further use.

3.6.5.2 Preparation of glycerol stocks

The strains were cultured in suitable liquid media (YEB or NA) on rotary shaker at 180 rpm until the optical density of culture reached approximately one ($O.D_{600}$ ~1.0). Then 800 µl of the culture was pipetted out into sterile Eppendorf

tubes aseptically, to which 200 µl autoclaved cooled glycerol was added and mixed thoroughly. The tubes were then stored at -20 °C.

3.6.6 Explants for transformation

The different explants such as hypocotyl segments, cotyledonary segments, shoot tips, leaf segments and nodal segments were used for transformation studies.

3.6.6.1 Preparation of explants

In vitro seedlings (10-15 days old) were taken out of the culture tube in a laminar airflow cabinet on pre-sterilized steel plates. The base of seedlings was cut out and similarly the tip carrying cotyledonary segments were also separated using sterile blade to obtain hypocotyls. Cotyledonary segments were separated and the distal end was removed retaining the petiolar end. Leaf segments, shoot tips and nodal segments were collected from 2- 2½ month grown *in vitro* seedlings. The leaf margins were cut from all sides, leaving a small petiole at the proximal end. Nodal segments were dissected such that each segment carried one or two nodes.

3.6.6.2 Pre-culturing of explants

The prepared explants were cultured on MS solid medium in petriplates for two days prior to their infection with bacteria. The medium was prepared in the required volume in conical flasks and kept in the culture room until use. On the day of use, the media was melted and then cooled to 40-50 °C. Twenty five millilitre media was transferred to each petriplate and allowed to solidify and attain room temperature. The explants were blotted dry and inoculated on media contained in the petriplate and were sealed with parafilm and further cultured for two days in the culture room.

3.6.6.3 Wounding of explants

Wounds were made on the explants using a sterile blade and an injection needle. The distal and proximal end of cotyledonary segments was freshly cut with the sterile blade leaving a small petiole at the proximal end. Ten pricks were made on each segment using the hypodermic injection needle.

Freshly cut hypocotyls were given only five pricks with sterile needle. The leaf margins were cut from all sides with a sterile blade leaving a small piece of the petiole at the proximal end. Alternatively, the leaf segments were pricked in the midrib and lamina with a sterile injection needle.

A fresh cut was made at the base of shoot tips with the sterile blade. The shoot tips were then pricked with sterile injection needle throughout. Both ends of the nodal segments were freshly cut and the segments were pricked with a sterile needle. Ten pricks were made on each explant.

3.6.7 Evaluation of the sensitivity of explants to various antibiotics

Different explants like hypocotyls segments, cotyledonary segments, shoot tips, nodal segments and leaf segments were tested for their sensitivity to various concentrations of antibiotics. The antibiotics used were cefotaxime and ampicillin. The wounded explants were cultured in solid MS medium containing 100, 250, 500 and 1000 mg l^{-1} concentrations of either ampicillin or cefotaxime. As control the explants were cultured in MS solid medium without antibiotics. Observations regarding the growth and the response of explants were recorded.

3.6.8 Standardization of inoculation method

Two types of bacterial inoculum were used for the study. In one method of inoculation the bacterium from isolated single cell colonies were used as the bacterial inoculum (hereafter referred to as Direct Inoculation Method or DIM). The bacterial suspension was used as the inoculum in the other method (hereafter referred to as the Suspension culture inoculation Method or SM).

3.6.8.1 Direct Inoculation Method

In this method, bacterium from isolated single cell colonies was used as the inoculum. Wounds were made on the explant using a sterile blade and the injection needle dipped in the inoculum. Cuts were made on all sides in the case of leaf segments and opposite end surface only in cotyledonary segments, nodal segments and hypocotyls using sterile blade loaded with inoculum. In the case of shoot tips,

cut was made only in basal portion. The pricks were made on the explant with a hypodermic injection needle dipped in a single bacterial colony. The explants were then blotted with a sterile blotting paper and placed on solid MS medium without growth regulators contained in the petriplates. As control, explants were wounded with a sterile blade and needle dipped in MS medium, blotted dry and placed on growth regulator free MS medium.

3.6.8.2 Suspension culture inoculation Method

The pre-cultured explants were wounded first using sterile blade and injection needle. The *Agrobacterium* suspension prepared ($O.D_{600}$ ~1.0) was transferred to sterile petriplates. The wounded explants were immersed in the suspension for 5 min. with intermittent gentle agitation. The explants were then blotted dry using sterile blotting paper and placed on solid MS medium without growth regulators taken in the petriplates. The control explants were wounded and dipped in liquid MS medium for 5 min and then cultured on solid MS contained in the petriplates.

3.6.9 Co-cultivation of explants with *Agrobacterium*

The infected explants were blotted dry using sterile blotting paper and were placed on growth regulator free MS solid medium in petriplates. The infected explants were then co-cultured in dark at 26 ± 2°C for 1-3 days in the culture room. Darkness was provided in the culture rack using black cloth. Observations regarding the growth of bacterium on media were noted.

3.6.9.1 Influence of co-culture period on hairy root induction

The explants infected by Direct inoculation method and Suspension culture inoculation method were co-cultured for 1-3 days at 26 ± 2.0 °C under dark photoperiod for efficient transformation. Transformation percentage obtained and the responses of different explants with respect to different co-culture period were recorded.

3.6.9.2 Influence of acetosyringone in hairy root induction

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

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

In the third method, *Agrobacterium* suspension was prepared. When $O.D_{600}$ of the bacterial suspension reached approximately one, the suspension was collected in an oak ridge centrifuge tube and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was discarded and the bacterial pellet was washed thrice with liquid MS. The pellet obtained was resuspended in 5.0 ml growth regulator free liquid MS medium. To the suspension 100µM acetosyringone was added and kept as such for two hours for activating the bacteria. After that, the suspension was made up to 30 ml and transferred to petriplates. Wounded explants were immersed in the suspension for five minutes with intermittent gentle agitation. The explants were blotted dry using sterile blotting paper and co-cultured in dark for two days on solid MS medium containing 100µM acetosyringone.

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

3.6.10 Culture media and conditions for hairy root induction

The explants after bacterial co-culture were washed three times successively with MS liquid medium containing 500 mg l^{-1} cefotaxime. To the sterilized MS liquid medium prepared in the jam bottles, 500 mg l^{-1} cefotaxime was added aseptically. The co-cultured explants were washed by immersing in the media containing antibiotics with intermittent shaking. After five minutes, the explants were transferred to new antibiotic containing MS media and the process of washing was repeated two more times.

After washing, the explants were blotted dry using sterile blotting paper. The explants were then transferred to solid MS medium containing bacteriostatic agent for the complete elimination of bacteria (MS + 500 mg l^{-1} cefotaxime). The explants were further cultured at 26 ± 2 °C under diffused light.

If the bacterial growth was seen after a few days, the explants were again washed with antibiotic containing liquid MS medium. This was repeated until no bacterial growth was seen on the media. The control explants were also treated similarly.

3.6.11 Standardization of explants for efficient transformation

Different explants like hypocotyl segments, cotyledonary segments, leaf segments, shoot tips and nodal segments were infected by all the three strains of *Agrobacterium rhizogenes* (A4, ATCC 15834 and MTCC 2364) available. The infection was carried out using different inoculation methods and co-cultured for different durations (1-3 days) under diffused light. Transformation percentage obtained in the case of each explants was recorded. The mean number of hairy roots per transformed explant and the mean number of normal roots per infected explant under each condition were recorded after 20 days.

3.6.12 Efficiency of strains in inducing hairy roots

To study the efficiency of strains in inducing hairy roots, the three strains, A4, ATCC 15834 and MTCC 2364 were inoculated on different explants using

different inoculation methods and co-cultured for different durations (1-3 days). Transformation percentage obtained by using each strain in different explants was calculated.

3.6.13 Days taken for hairy root induction

The number of days taken for induction of roots from infected explants by using different strains under different inoculation methods was recorded.

3.6.14 Establishment of hairy root cultures

The adventitious roots that emerged from the explants, 1-3 weeks after infection were excised out using sterile blade and cultured in solid MS medium containing low concentrations of cefotaxime compared to the co-culturing medium. Those roots showing morphological and growth characteristics almost similar to hairy roots were excised and cultured. The excised roots were washed in liquid MS medium containing 250 mg l^{-1} cefotaxime and blotted dry. The individual root tips (1-4 cm) were separated and transferred to MS solid medium containing 250 mg l^{-1} cefotaxime contained in the petriplate and incubated in the culture room at 26 ± 2 °C under diffused light. The normal roots obtained from control explants were also cultured similarly.

3.6.15 Rapid culturing of hairy roots

About 25 days after the establishment of root cultures, those roots that showed rapid growth, hairiness, lateral branching and plagiotropic growth habit were collected. The roots were washed in liquid MS medium, blotted dry and then randomly cut into small pieces of 2.0-4.0 cm length. Both the root tips and root segments were transferred to 100 ml and 250 ml conical flask containing 30 ml and 125 ml half MS liquid medium respectively without antibiotics. The cultures were incubated in orbital shaker at 110 rpm under diffused light and dark condition for rapid multiplication (referred as shake flask cultures). The normal roots obtained from control explants were also cultured similarly. Cultures were also maintained in solid MS medium with 100 mg l^{-1} cefotaxime in petriplates.

The shake flask cultures were harvested 25 days after incubation, washed in double distilled water and the fresh weight was measured. The initial and final fresh weight of hairy root clones obtained using A4 strain were recorded.

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

To study the effect of culture media and conditions on the growth of hairy roots, the root cultures were initiated in MS and half MS with 3.0 per cent sucrose and B_5 with 3.0 and 2.0 per cent sucrose. The hairy roots cultured in the conical flask were collected, randomly cut to small pieces of 2.0-4.0 cm length and approximately 0.5g was inoculated in 250 ml conical flasks containing 125 ml half MS with 3.0 per cent sucrose, MS with 3.0 per cent sucrose, B_5 with 3.0 per cent sucrose and 2.0 per cent sucrose without antibiotics. The cultures were incubated in rotary shaker at 110 rpm under dark photoperiod. To introduce darkness the conical flasks were covered with black polythene sheet. The fresh weight of the roots was recorded 25 days after inoculation.

3.6.17 Growth pattern of hairy roots

The growth pattern of hairy roots was studied using shake flask cultures. The root cultures were randomly cut into small pieces of 2.0-5.0 cm. About 0.5 g of the culture was inoculated in 125 ml half MS liquid medium in 250 ml conical flask and incubated in orbital shaker at 110 rpm under diffused light and dark photoperiod. The growth pattern of the hairy roots was recorded on alternate days.

3.6.18 Growth rate of hairy roots

Seven active root tips from a single root clone at the fifth subculture cycle were transferred to each 100 ml conical flask containing 30 ml B_5 medium with two per cent sucrose and half MS (3 % sucrose) and the growth rate of the roots was studied. The cultures were incubated in rotary shaker at 110 rpm under diffused light. The roots were harvested and the fresh weight was measured on 15 and 25 days after inoculation.

3.7 CONFIRMATION OF TRANSFORMATION

The confirmation of transformation was done on the basis of,

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

3.7.1 Morphological features

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

3.7.2 Opine analysis

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

3.7.2.1 Preparation of reagents

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

Various reagents used for the detection of opines, were prepared as follows,

1) Solution I - 0.4 per cent silver nitrate in 99: 1 acetone: water mixture was prepared. The reagent was stored in black coloured bottle in refrigerated conditions.
2) Solution II - 2.0 per cent NaOH in 90 per cent ethanol in water was prepared.
3) Solution III - a) Reducer A Concentrate: Saturated potassium ferricyanide
   b) Reducer B Concentrate: Saturated sodium thiosulfate

c) Reducer C Concentrate: 25 per cent sodium carbonate

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

3.7.2.2 Extraction of opines

Opines were extracted by two different methods

a) 300 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.

b) 500 mg of fresh root tissue was ground homogenously in 500µl of 0.1M HCl, and left at 4 °C for two hours. It was then centrifuged at 9000g for 5 min (Xu *et al.* 2004). The supernatant was collected and used for the detection of opines.

3.7.2.3 Separation of opines

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

GT) containing equal volumes of buffer in both wells, such that both ends of paper touched the buffer.

The spotted end of the strip was kept at the anode end of the electrophoresis unit and the extract was subjected to high voltage paper electrophoresis at 400 V/cm for 45 min. Following electrophoresis, the paper was dried in a stream of hot air using a hair drier.

3.7.2.4 Detection of opines

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

3.7.3 Confirmation by PCR analysis and Southern hybridization

3.7.3.1 Isolation of DNA from roots

For PCR analysis and Southern hybridization, DNA was isolated from hairy roots obtained using A4 and ATCC 15834 strains, roots produced from MTCC 2364 infected explants and roots produced from control explants, following modified Doyle and Doyle (1987) method.

3.7.3.1.1 Reagents

1. Extraction buffer (4X)
2. Lysis buffer
3. Sarcosine 5.0 %
4. TE Buffer
5. Ice cold isopropanol
6. Chloroform: Isoamyl alcolhol (24:1 v/v)
7. Ethanol 70% (v/v)

The details of reagents used for DNA isolation is given in Appendix III

3.7.3.1.2 ***Procedure***

1. Root sample weighing 0.5 g was freeze powdered in liquid nitrogen and ground with 6.0 ml of 1X extraction buffer, 50 µl β-mercaptoethanol and a pinch of sodium meta bisulphate, using an autoclaved mortar and pestle.
2. The homogenate was transferred into 30 ml oak ridge centrifuge tube containing 6.0 ml pre-warmed lysis buffer and 1.25 ml sarcosine was added to it.
3. The tubes were incubated in a water bath at 65 °C for 20 minutes, with intermitted stirring.
4. The tubes were taken out from the water bath, allowed to cool and an equal volume of chloroform: isoamyl alcohol mixture (24: 1 v/v) was added and mixed by gentle agitation.
5. The tubes were then centrifuged at 10,000 rpm for 15 min at 4 °C.
6. The upper aqueous phase was transferred to a fresh tube.
7. To this, 0.6v ice-cold isopropanol was added and mixed gently and then kept in a deep freezer for 30 min for complete precipitation of DNA.
8. The DNA was pelleted by centrifugation at 10,000 rpm for 15 min at 4 °C.
9. The supernatant was discarded and the pellet was washed with 70 per cent (v/v) ethanol by centrifuging at 10,000 rpm for 15 min at 4 °C.
10. The supernatant was discarded and the pellet was air-dried.
11. The dried pellet was dissolved in 200 µl of TE / sterile water and stored at -20°c

3.7.3.2 Agarose Gel Electrophoresis

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

3.7.3.2.1 Materials

a) Agarose (Genei, Low EEO)
b) 50X TAE buffer (pH 8.0)
c) Electrophoresis unit, power pack, casting tray, comb
d) 6X Loading buffer/ tracking dye
e) Ethidium bromide solution (stock 10 mg ml^{-1}; working concentration 0.5 $\mu g\ ml^{-1}$)
f) UV transilluminator (Herolab^{R})
g) Gel documentation system (Biorad, Gel Doc XR)

The buffer and dyes used in gel electrophoresis is given in Appendix IV

3.7.3.2.3 Procedure for casting, loading and running the gel

1) Four hundred ml of electrophoresis buffer (1X TAE) was prepared to fill the electrophoresis tank and to prepare the gel.
2) 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.
3) Agarose (1.0 per cent (w/v) for genomic DNA and 0.7 per cent (w/v) for PCR) was added to 1X TAE, boiled till the agarose dissolved completely and then cooled to luke warm temperature.
4) Ethidium bromide was added to a final concentration of 0.5 $\mu g\ ml^{-1}$ as an intercalating agent of DNA, which will help in its visualization in UV rays.
5) It was then poured into the gel-casting tray with comb and allowed to solidify.
6) After the solidification of the gel (30 min at room temperature), the comb and cellophane tape were removed carefully.
7) 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.
8) A piece of cellophane tape 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 5-8 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 micropipette. Appropriate molecular weight marker (Lambda DNA – *Hind* III/ *Eco* R1 double digest) was also loaded in one of the wells.

9) 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.
10) The power was turned off when the tracking dye reached at about two third length of the gel.
11) The gel was observed in UV transilluminator and documented in gel documentation system.

3.7.3.3 Isolation of Cosmid from E coli

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

3.7.3.3.1 Reagents

Solution I (Resuspension buffer)
Solution II (Lysis buffer)
Solution III (Neutralisation buffer)

The composition of the reagents is given in Appendix V.

3.7.3.3.2 Procedure for Cosmid Isolation

1. A single bacterial colony was transferred in to 2.0 ml LB medium containing kanamycin and the culture was incubated overnight at 37 °C with vigorous shaking.

2. 1.5 ml of the culture was poured in to an Eppendorf tube and the cells pelleted by centrifugation at 12,000 rpm for one minute at 4 °C.
3. The supernatant was discarded and the bacterial pellet resuspended in 100 µl ice-cold Solution I by vigorous shaking.
4. To the above, 200 µl of freshly prepared Solution II was added and mixed gently by inverting the tube five times.
5. Ice-cold Solution III (150 µl) was added to the tube, vortexed gently and kept on ice for 5 min.
6. The contents were centrifuged at 12,000 g for five minutes at 4 °C and the pellet was discarded.
7. To the supernatant, 0.6 volume of ice-cold isopropanol was added to precipitate the DNA and kept at room temperature for two minutes.
8. The contents were centrifuged at 12,000 g for five minutes at 4 °C and the supernatant was discarded.
9. The pellet was rinsed with 1.0 ml of 70 per cent (v/v) ethanol at 4 °C.
10. The supernatant was discarded and the pellet was air dried and dissolved in 30 µl autoclaved double distilled water.
11. The cosmids isolated were observed in agarose gel electrophoresis and documented.

3.7.3.4 PCR analysis of rol B and rol C genes

Three primer sets were designed for carrying out the Polymerase Chain Reaction. The primer sets used for amplifying *rol* B gene were Rol BF1R1 and Rol BF2R2. For amplifying *rol* C gene, the primer set used was Rol CF1R1. Rol BF2R2 primer set was designed based on the output of Primer 3 Software. The primer sets Rol BF1R1 and Rol CF1R1 were designed based on the available literature (Celma *et al.*, 2001; Lee *et al.*, 2004) only after cross checking the sequences for alignment with the original A4 sequence (>gi|38985|emb|X03433.1|ARTLROLB *A. rhizogenes* pRiA4 plasmid TL-DNA rolB-C genes) available in NCBI database (Appendix VI). Details of primer sets are shown in the Table (6) and (7).

The PCR analysis was carried out using DNA isolated from the hairy roots induced by A4 and ATCC 15834 and also the DNA isolated from the roots produced by MTCC 2364 infected explants. The DNA isolated from roots produced by control explants was used as the negative control. The cosmid pLJ1 was used as the positive control. A blank (without DNA) was also set.

Table 6. Details of gene specific primers designed

Primer	Type	Primer sequence	Length (bp)	Tm (°C)
Rol BF1	Forward	5'- TCCACgATTTCAACCAgTAg -3'	20	58
Rol BF2	Forward	5'- gAAgCCTgCTgCAgTAAACC-3'	20	62
Rol CF1	Forward	5'- TTAgCCgATTgCAAACTTgCTC-3'	22	64
Rol BR1	Reverse	5'- AgTTCAAgTCggCTTTAggC-3'	20	60
Rol BR2	Reverse	5'- TTCAgCAgCAggATCAACAC-3'	20	60
Rol CR1	Reverse	5'- ATggCTgAAgACgACCTgTgTT-3'	22	66

(Tm –Melting temperature)

Table 7. Details of different combinations of primer

Sl no.	Primer combination	Amplicon size (bp)	Annealing temperature (°C)
1	Rol BF1R1	740	54
2	Rol BF2R2	205	56
3	Rol CF1R1	520	60

3.7.3.4.1 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 (1:15 dilution)
or	
Cosmid	- 5.0 µl (1:50 dilution)
b) 10X Taq assay buffer	- 2.5 µl
c) d NTPmix (1 m*M*)	- 1.0 µl
d) Forward primer	- 1.0 µl
e) Reverse primer	- 1.0 µl
f) Taq DNA polymerase (0.3u)	- 2.0 µl
g) Autoclaved distilled water	- 16.5 µl (12.5 µl for cosmid)
	Total 25.0 µl

A momentary spin was given to the reaction mixture for thorough mixing of the cocktail components. The tubes were then placed in a thermal cycler (MJ Research, PTC-200, Peltier Thermal Cycler) for polymerase chain reaction under suitable programme with a heated lid condition.

3.7.3.4.2 Thermal Cycler Program

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

1.	94 °C for 2.0 min	- Initial denaturation
2.	94 °C for 45 sec	- Denaturation
3.	60 °C for 1.0 min	- Annealing (54 °C and 56 °C for *rol* B gene)
4.	72 °C or 2.0 min	- Extension
5.	Go to 2, 29 times	
6.	72 °C for 10 min	- final extension
7.	4 °C for 5.0 min	- to hold the sample

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

3.7.3.5 Southern hybridization

Southern hybridization was carried out for the confirmation of transformation. The PCR product obtained from pLJ1 cosmid, using Rol BF2R2 primer set (205 bp) and Rol CF1R1 primer set (520 bp) were used as probes separately in Southern hybridization. Restriction map of the TL and TR regions of pRiA4 showing the inserts of pLJ1 and pLJ85 cosmids (Jouanin, 1984) is given in the Figure (1). To confirm the presence of TL-DNA, pLJ1 cosmid was used as the positive control and the DNA isolated from the roots produced by control explants was used as the negative control.

3.7.3.5.1 Restriction digestion of DNA

The DNA isolated from hairy roots obtained using A4 and ATCC 15834 strains, roots produced from MTCC 2364 infected explants and roots produced by control explants were restricted using *Bam* H1 restriction enzyme. The cosmid pLJ1 was also restricted with the same enzyme.

1. The purity of the DNA was checked on 0.7 per cent (w/v) agarose gel before proceeding to restriction digestion.
2. The reaction mix was prepared as given below,

DNA	: 16 µl
Restriction enzyme	: 2.0 µl
Restriction buffer	: 2.0 µl
Total	20 µl

2. The bottom of the microfuge tube was gently tapped to mix the reaction components.
3. The tubes were incubated at 37 °C for 16 hours.

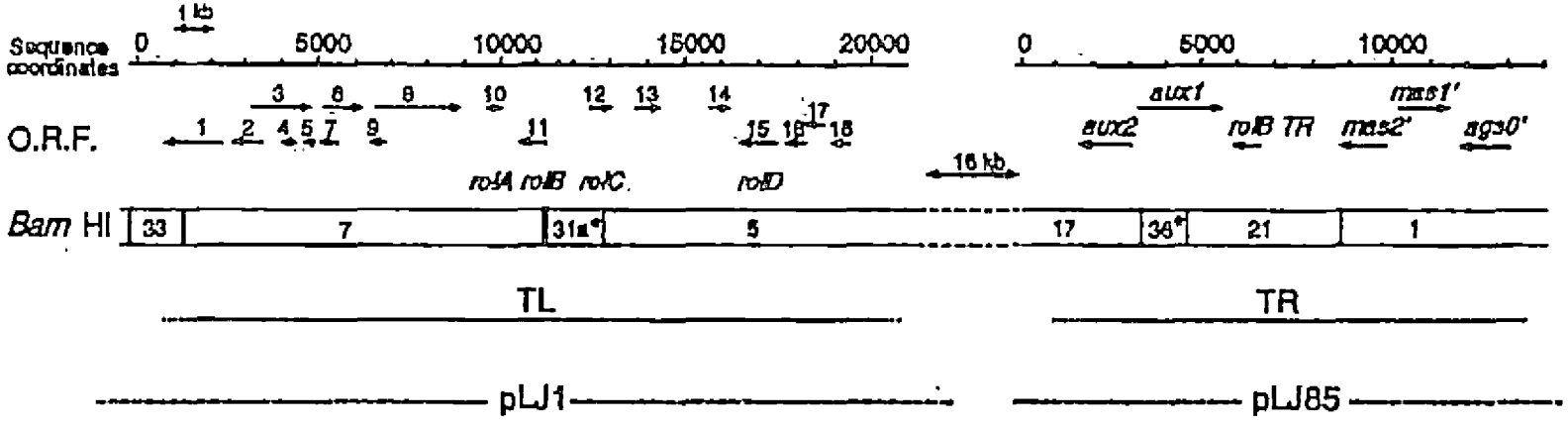


Fig. 1. Restriction map of the TL and TR regions of pRiA4 showing the inserts of pLJ1 and pLJ85 cosmids (Jouanin, 1984).

4. After the required time, the reaction was arrested by adding 0.5*M* EDTA.
5. The restriction digestion was confirmed by running the digest in 0.8 per cent (w/v) agarose gel and documented.

3.7.3.5.2 Southern Blotting

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

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

3.7.3.5.3 Preparation of radio labelled probe

The probe was radio labelled using random labeling kit.

1. 3.0 µl of DNA (probe) was made to 10 µl using autoclaved distilled water.
2. The double stranded probe was denatured by boiling the contents in a boiling water bath for five minutes.
3. The cap of the microfuge tube containing DNA was pierced two or three times with a needle before boiling so as to release pressure.
4. The denatured sample was chilled for five to ten minutes.
5. The sample was spun at 8000 rpm for 30 seconds.
6. 2.5 µl of 10 X labelling buffer was added to sample followed by 1 µl of 100 µg/µl random primer.
7. To the reaction mix, 2.5 µl of 20 mM DTT solution was added followed by 2.0 µl of d NTP mix (2.5 mM- dCTP, dGTP, dTTP)
8. 3.0 µl of α'32 P dATP was added to the reaction mix (specific activity > 3000 Curie)

9. 1.0 µl of (3-units/ µl) klenow fragment polymerase was added, mixed gently and incubated at room temperature for two hours. The reaction mix was boiled for 5 min before adding to the hybridization solution.

3.7.3.5.4 Procedure for pre hybridization, hybridization and washing

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

The composition of reagents is given in Appendix VII.

3.7.3.5.5 Autoradiography

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

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

3.7.3.6 Dot blot Analysis

Dot blot analysis was carried out as a part of confirmation of transformation. The PCR products obtained from pLJ1 cosmid, using Rol BF2R2 primer set (205 bp) and Rol CF1R1 primer set (520 bp), were used as probes separately in dot blot analysis.

3.7.3.6.1 Procedure

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

10. It was followed by pre-hybridization, hybridization, washing and developing, same as that of Southern hybridization.

3.8 ESTIMATION OF WITHANOLIDES

A quantitative thin layer chromatography (TLC) method was used for the estimation of withanolides present in the roots of *W. somnifera*. Among the different withanolides withaferin A, the most important withanolide (unsaturated steroidal lactone) was estimated form roots of field-grown plants, *in vitro* roots (non- transformed) and hairy roots (transformed roots). Enhancement of withanolide through precursor feeding, elicitation and addition of osmoregulants were also studied.

3.8.1 Standardization of extraction

3.8.1.1 Defatting

In this method, 10g root powder obtained from field grown plants was initially refluxed with 50 ml hexane for 10 min in a 250 ml refluxing flask attached with condenser. The extract was collected by filtration and tested for presence of withaferin A by TLC.

3.8.1.2 Separation of alkaloids and withanolides

The defatted material was then refluxed with 50 ml ethanol for one hour in a refluxing flask, attached with a condenser. It was then centrifuged and the extract was collected. After vapourising the solvent, the residue was acidified with 10 ml 0.02*N* HCl. It was then poured into a separating funnel followed by 25 ml ethyl acetate and shaken vigorously for five minutes. The stopper at the bottom end of the funnel was opened intermittently so as to release the gas pressure developed inside. A clear and a cloudy fraction get separated and each fraction was collected separately. The lower clear fraction containing withanolides was collected over anhydrous sodium sulphate. The solvent was evaporated and finally the concentrated sample was used for spotting. The extract obtained after refluxing with hexane was also analysed by TLC.

The solvent in the alkaloid containing fraction was removed over water bath. It was then made alkaline (pH 10) by the adding ammonium solution and then poured into a separating funnel to which chloroform was added followed by NaCl. The chloroform fraction was collected by passing through the funnel containing anhydrous sodium sulphate. The supernatant obtained was vapourised in a water bath. It was further dissolved in ethanol and spotted on a TLC plate.

3.8.1.3 Extraction of withanolides without defatting in the presence of alkaloids

Ten gram of the root powder was refluxed with 50 ml ethanol for one hour in a 250 ml round bottom flask. The extract was decanted and clarified by centrifugation. From the centrifugate 5.0 ml was taken to dryness and redissolved in 200 µl of ethanol. This extract was analysed by TLC.

3.8.1.4 Estimaton of withaferin A from fresh root samples

Withaferin A content of fresh roots from field grown plants, non-transformed *in vitro* roots and transformed hairy roots of *W. somnifera* were estimated. The fresh root sample (0.5 g) was weighed out and ground in a mortar after adding 2.0 ml absolute alcohol. The homogenate was transferred into a centrifuge tube and allowed to remain at room temperature for two hours so as to extract all the withanolides into ethanol. It was then centrifuged at 10,000 rpm for 10 min. The ethanol extract containing withanolides was analysed by TLC.

3.8.8.5 Estimation of withanolides released to the medium

The media was extracted with equal quantity of chloroform. The root grown media was taken in a separating funnel to which equal quantity of chloroform was added and shaken vigorously so as to extract the withanolides. The lower clear chloroform fraction was collected and dried over anhydrous sodium sulfate. Chloroform was further evaporated and the residue was dissolved in 5.0 ml ethanol, which was vapourised to 100 µl, and TLC analysis was carried out.

3.8.2 Preparation of standard

Standard withaferin A (Purity: more than 99 %) was procured from M/S Laila Impex, R & D Centre, Jawahar Autonagar, Vijayawada. Two milligram of the standard was dissolved in 1.5 ml ethanol and stored in Eppendorf tubes under refrigerated condition.

3.8.3 Application of sample on TLC plate

For the TLC analysis of root extracts, two types of pre coated (Merck) plates were used.

a) RP_{18} F_{254}

b) Silica gel 60 F_{254} (Merck)

Pre coated Aluminium backed TLC sheets of 7.0 cm length and 4.5 cm width were used for the analysis. A straight line was drawn at a distance of 2.0 cm from the lower edge of the plate. On the plate, samples were spotted at a distance of 1.0 cm leaving a margin of 0.7 cm on either side. Using a micropipette, 1.0 µl of standard was spotted so that each micro litre delivered 1.33µg of withaferin A. The volume of samples for spotting were selected such that it delivered an approximately similar quantity of withaferin A as that of standard. Samples were spotted in small circular spots of 4-5 mm diameter. Small volumes of samples were applied successively and solvent was removed between additions with a current of warm air from a hair drier.

3.8.4 Developing the Chromatographic plates

In the case of RP_{18} F_{254} plates, methanol: water (9: 1) was used as the developing solvent, whereas in the case of Silica gel 60 F_{254} plates chloroform: methanol (9.8: 0.2) was used to develop the chromatographic plates.

3.8.4.1 Procedure

Developing solvent (mobile phase) was mixed well and poured into the developing chamber to a depth of 0.5 cm and the chamber was shaken and allowed

to saturate with the vapour of the solvent. Spout less beaker was used as the developing chamber. The spotted TLC plate was placed in the chamber, the chamber lid placed correctly and the chromatogram was developed to a distance of 6.5 cm. The plate was then removed and dried with a stream of hot air from a drier. The plate was viewed under short wave UV (254 nm).

3.8.5 Preparation of spray reagent

To view the spots on this TLC plate, two types of spray reagents were tried. The compositions are as follows,

I	a) Vanillin	-	0.05 g
	b) Boric acid	-	1.0 g
	c) Conc. $H_2 SO_4$	-	2.0 ml
	d) Methanol	-	100 ml
II	a) FeCl3	-	50 mg
	b) Water	-	90 ml
	c) Acetic acid	-	5.0 ml
	d) Conc. $H_2 SO_4$	-	5.0 ml

3.8.6 Visualization of spots

After development, the plates were taken out from the developing chamber and solvent removed under a stream of hot air. The spots were visualized by immersing the plate in a pool of freshly prepared spray reagent followed by drying and charring. The plates were documented in Alfa Imager ^{TM} 1200 documentation system, Herolab/ Biorad, Gel Doc XR under white light.

3.8.7 Quantification of withaferin A

A TLC-densitometry technique was used for the quantification of withaferin A. The image of the TLC plates stored in the Alfa Imager was analysed

using the SPOT DENSO tool present in the Tool box 3 of the Alfa Imager. The spots of objects i.e., samples and reference standard on the plate were initially selected by delineating the boundaries. The background correction was made using AUTO BACKGROUND and the INVERT option was selected since the plates had dark spots on a light background. The standard curve option was used to draw the standard curve. The spot of reference standard was selected and the corresponding values of withaferin A in micrograms were entered. The value of unknown samples appeared automatically in the spot denso results. Also, a standard curve, smooth cubic spline was drawn which represented the Integrated Density Value (IDV) against withaferin A content in micrograms.

3.8.8 Enhancement of secondary metabolite production

The hairy roots derived from A4 derived root clones were subjected to enhancement studies.

3.8.8.1 Addition of osmoregulants

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

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

3.8.8.2 Addition of precursors

The effect of precursor feeding on withanolide production in hairy roots was studied. Hairy roots were inoculated in half MS media supplemented with 1.0

m*M* and 2.0 m*M* methionine. Methionine was filter sterilized and added to 125 ml sterilized half MS liquid media in 250 ml conical flask. Methionine was dissolved in small quantity of sterilized liquid half MS so as to facilitate filter sterilization. Twenty three day old root cultures were inoculated in the media and further grown for five days. Then the whole roots and media were collected and withaferin A content was analysed.

3.8.8.3 Addition of Elicitors

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

3.8.8.3.1 Elicitation by Aspergillus homogenate

The spores of *Aspergillus niger* was inoculated in 50 ml LB broth and the culture was grown in a rotary shaker at 140 rpm for two days at 30 °C. The culture was further grown for five more days without shaking wherein mycelial mass had spread over the culture. On the seventh day of incubation, the culture was filtered through muslin cloth and the mycelial mass was collected. It was dispersed in 40 ml distilled water and was homogenized in Polytron homogenizer. The *Aspergillus* homogenate was autoclaved and added to half MS liquid medium at the rate of 250 µl and 500 µl per 125 ml in 250 ml conical flask. Twenty five day old root culture was inoculated in the above media and the culture was incubated in rotary shaker at 110 rpm for 72 hrs. The root culture and the media were collected and the withaferin A content was analysed by TLC.

3.8.8.3.2 Elicitation by Yeast Extract

Yeast extract at two concentrations, 2.5 g l^{-1} and 5.0 g l^{-1} was used to elicit the cultures. Half MS liquid medium was supplemented with 2.5 g l^{-1} and 5.0 g l^{-1} yeast extract and the pH was adjusted to 5.7 before autoclaving. Seventeen days old culture was inoculated in the media and the culture was incubated on a rotary shaker at 110 rpm for 72 hrs. TLC analysis was carried out to estimate the withaferin A content.

Results

4. RESULTS

The results of the study on "Genetic transformation for hairy root induction and enhancement of secondary metabolites in *Aswagandha* (*Withania somnifera* (L.) Dunal)" are presented in this chapter under six major heads viz,

- Standardization of *in vitro* regeneration
- Culturing and sensitivity screening of *Agrobacterium* and explants
- Standardization of transformation techniques
- Confirmation of transformation
- Standardization of withanolide estimation
- Enhancement of secondary metabolite production

4.1 STANDARDIZATION OF *IN VITRO* REGENERATION

4.1.1 *In vitro* seed germination

The seeds were germinated *in vitro* on half MS medium with 2.5 per cent sucrose. Fresh berries and dried seeds were used to raise *in vitro* seedlings. Healthy seedlings were obtained on this culture medium. Eighty four per cent of the seeds germinated with in 6-15 days under dark photoperiod whereas seeds produced only 67 per cent germination with in 10-20 days under 16 hr (1000 lux) photoperiod. Dried seeds and seeds from fresh berries showed comparatively equal germination rate, but the contamination rate was more in the latter case.

4.1.2 Hypocotyls as explants

4.1.2.1 Regeneration

The effect of different concentrations of growth regulators on regeneration from hypocotyls is shown in the Table (8).

Regeneration of shoots from hypocotyls initiated within 10 days after inoculation. Swelling of the basal part of the hypocotyls occurred prior to regeneration. The maximum regeneration response (70%) from hypocotyls placed upside down was obtained on two combinations MS + BAP 1.5 mg l^{-1} + IAA 0.5

mg l^{-1} and MS + BAP 1.0 mg l^{-1} + IAA 0.5 mg l^{-1} + coconut milk 10 per cent which was followed by MS + BAP 1.0 mg l^{-1} + IAA 0.5 mg l^{-1} which produced 60 per cent regeneration. The mean number of shoot buds per explant was highest in the latter combination with 23.8 shoot buds. In all the combinations slight callusing was also noticed along with regeneration.

Table 8. Effect of different concentrations of growth regulators on shoot regeneration from hypocotyls

MS + Growth regulators (mg l^{-1})		Hypocotyls – upside down		Hypocotyls- direct	
BAP	IAA	Response (%)	No. of shoot buds/explant (Mean ± S.D)	Response (%)	No. of shoot buds/explant (Mean ± S.D)
0.5	0.5	20	2.5 ± 0.5	30	3.7 ± 1.2
1.0	0.5	60	23.8 ± 11.7	70	10.2 ± 4.6
1.5	0.5	70	10.8 ± 6.0	50	4.5 ± 0.5
2.0	0.5	10	4.0 ± 0.5	20	3.0 ± 1.0
2.5	0.5	10	3.0 ± 0.4	10	2.2 ± 0.8
1.0 + 10 % CM	0.5	70	8.8 ± 6.8	50	7.0 ± 3.6

Each treatment consisted of 10 replicates, observation after 25 days

When the hypocotyl explants were placed directly, the combination MS + BAP 1.0 mg l^{-1} + IAA 0.5 mg l^{-1} produced maximum regeneration response (70 %) followed by MS + BAP 1.5 mg l^{-1} + IAA 0.5 mg l^{-1} and MS + BAP 1.0 mg l^{-1} + IAA 0.5 mg l^{-1} + coconut milk 10 per cent with 50 per cent regeneration. The mean number of shoot buds per explant was highest in the combination MS + BAP 1.0 mg l^{-1} + IAA 0.5 mg l^{-1} with 10.2 shoot buds, which was less compared to mean number of shoot buds from hypocotyl segments that were placed upside down. Callusing occurred only on the lower or basal portion of the hypocotyls segments that were placed directly, and no callusing was observed on the upper portion.

In both the cases that is, hypocotyls placed directly or upside down, shoot buds were differentiated only from the upper portion of the hypocotyls (Plate 1)

4.1.2.2 Multiplication

The clump of shoot buds obtained was cut into small pieces of 3-8 buds, and was inoculated on MS medium with different growth regulator combinations for leaf proliferation and multiplication. Data regarding the multiplication of hypocotyl derived shoot buds is given in Table (9).

Table 9. Effect of growth regulators on multiplication of regenerated shoot buds from hypocotyl in MS medium

MS +Growth regulators (mg l^{-1})		No. of multiple shoots/ shoot buds (Mean)
BAP	IAA	
0.0	0.5	1.60
0.2	0.5	4.08
0.4	0.5	5.55
1.0	0.5	2.65
2.0	0.5	2.65
1.0	0.0	2.94
2.0	0.0	2.80

Each treatment consisted of 10 replicates, observations after 25 days

The combination MS + BAP 0.4 mg l^{-1}+ IAA 0.5 mg l^{-1} produced numerous differentiated shoots. An average of 5.55 shoots was produced per each shoot bud. Among the shoots, 1-2 shoots showed faster elongation. The combination MS + BAP 0.2 mg l^{-1}+ IAA 0.5 mg l^{-1} produced an average of 4.08 shoots, but more number of elongated shoots (2-4) were produced in this combination.

In the combination MS + IAA 0.5 mg l^{-1} without BAP, increased leaf size was obtained, rooting was observed and multiplication was nil or very less (1.6). The combinations MS + IAA 0.5 mg l^{-1} with BAP 1.0 (2.65) or 2.0 mg l^{-1} (2.65) produced good multiplication, but the elongation of the differentiated shoots were found to be very less, with fast proliferation of callus (Plate 1).

4.1.2.3 Elongation and rooting

Elongation as well as rooting occurred in all the media combinations tried. The initial length of the shoots, number of internodes and the number of leaves present at the time of inoculation were deducted from the final readings. Data regarding the elongation and rooting of hypocotyl derived shoots in different culture medium is given in the Table 10.

Maximum elongation was obtained on MS with GA_3 0.5 mg l^{-1}. Average length of shoots was 5.1 cm in this combination. It was followed by MS with GA_3 1.0 mg l^{-1} where, a mean length of 5.0 cm was obtained. The plantlets showed maximum health and vigour with dark green leaves in half MS with sucrose 2.5 per cent. The number of internodes, leaves and roots were also highest in this combination (Plate 1). The combination produced an average of 5.2 internodes and 4.9 leaves, which was followed by MS with GA_3 0.5 mg l^{-1} that produced an average of 5.0 internodes and 4.8 leaves.

Hundred percentage rooting occurred in MS + sucrose 3.0 per cent and half MS + sucrose 2.5 percent, whereas rooting percentage was low in the presence of GA_3. However MS + GA_3 1.0 mg l^{-1} produced 90 per cent rooting with a mean number of 3.6 roots per shoot. The maximum number of roots was obtained in half MS + sucrose 2.5 per cent with an average of 7.9 roots per shoot. Rooting was achieved within 9 days in half MS + sucrose 2.5 per cent, which was faster than other combinations. This combination produced both main and lateral roots with a greatest mean length (4.7 cm). In MS + GA_3 0.5 mg l^{-1}, elongation of the leaf petiole was observed, and also slight callusing occurred in the basal portion. The elongation of the internode was more in MS + GA_3 1.0 mg l^{-1}. The leaves were more larger and greenish in MS + GA_3 0.5 mg l^{-1} compared to GA_3 1.0 mg l^{-1}.

Table 10. Elongation and rooting of hypocotyl derived shoots of *W. somnifera* in different culture medium

Media	Length of shoot (Mean ± S.D)	No. of internodes/ shoot (Mean ± S.D)	No. of leaves/ shoot (Mean ± S.D)	No. of roots/ shoot (Mean ± S.D)	Length of root (cm)	Days for rooting	Root ing (%)
MS + 3 % sucrose	3.4 ± 1.6	4.2 ± 2.2	4.1 ± 2.0	6.9 ± 2.7	4.5	10.2	100
½ MS +2.5 % sucrose	4.8 ± 1.8	5.2 ± 1.5	4.9 ± 2.5	7.9 ± 3.2	4.7	9.0	100
MS+1.0 mg l^{-1} GA_3	5.0 ± 2.0	4.4 ± 1.6	3.7 ± 1.0	3.6 ± 2.0	4.0	12.5	90
MS + 0.5 mg $l^{-1}GA_3$	5.1 ± 2.1	5.0 ± 1.5	4.8 ± 1.8	3.2 ± 1.2	3.8	14.2	60

Each treatment consisted of 10 replicates, observations after 20 days

The shoots, were efficiently rooted by inoculating on half MS medium after pulse treatment with IBA 1000 mg l^{-1} that was carried out for varying time periods (5, 15 and 30 sec). The influence of variable duration pulse treatment with IBA 1000 mg l^{-1} on rooting is given in the Table 11.

There was cent percent rooting in all the treatments. Shoots inoculated after elongation on MS + GA3 0.5 mg l^{-1} showed efficient rooting with very less callusing tendency, whereas the shoots obtained after multiplication in BAP and IAA showed more tendency to callus along with efficient rooting. In all the treatments, shoots were rooted with in 7-8 days and the root length varied from 5.1- 5.8 cm. Even though the range of roots per shoot was more in pulse treatment with IBA 1000 mg l^{-1} for 30 s, five-second treatment was found to be ideal for rooting. The plantlets were healthier in this treatment compared to the other two (15 and 30 s treatments). In treatments with pulse treatment for 15 and 30 s there was good rooting, but roots were also induced from the upper portion of stem and sometimes from leaf, which affected the health and vigour of the plantlets.

Hypocotyl segments as explant

Regeneration from hypocotyl placed upside down

Regeneration from hypocotyl placed directly

Multiplication of shoots (MS + BAP 0.4 mg l^{-1} + IAA 0.5 mg l^{-1})

Multiplication of shoots (MS + BAP 1.0 mg l^{-1} + IAA 0.5 mg l^{-1})

Elongation and rooting (1/2 MS + 2.5 % sucrose)

Rooting by pulse treatment (1/2 MS + IBA 1000 mg l^{-1})

Plate 1. *In vitro* regeneration in *W. somnifera* from hypocotyl segments

Table 11. Influence of IBA pulse treatment on rooting of hypocotyl derived shoots of *W. somnifera*

Culture media (1000 mg l^{-1})	No. of roots/shoot	Root length (Mean-cm)	Rooting (%)	Days for rooting
½ MS + IBA 5 sec	20-30	5.8	100	7-8
½ MS + IBA 15sec	30-35	5.2	100	7-8
½ MS + IBA 30 sec	30-40	5.1	100	7-8

Each treatment consisted of 10 replicates, observations after 20 days

On an average almost 140 rooted plantlets could be produced from a single hypocotyl segment by tissue culture.

Seventy per cent of the *in vitro* rooted plantlets were hardened in green house. Flowering and fruiting occurred within two months after transfer, in plants, which were transferred to large pots containing potting mixture with sand, soil and cow dung in equal proportions.

4.1.3 Cotyledonary segments as explant

4.1.3.1 Regeneration

The effect of growth regulator combinations on regeneration from cotyledonary segments is shown in the Table 12.

Cotyledonary segments took 15 days for regeneration; where as callus proliferation occurred within ten days of inoculation. Regeneration of shoot buds occurred directly from the segments as well as from the proliferated callus. Ninety five per cent of the cultures showed callusing in all the combinations tried. Callusing was initiated mainly from the proximal or petiolar end. Later callus proliferation occurred on the distal end as well as on other sides. Friable creamish green coloured callus was produced and often with a brown tinge.

Table 12. Effect of growth regulator combinations on regeneration from cotyledonary segments of *W. somnifera*

MS+ Growth regulators (mg l^{-1})		Regeneration (%)	No.of shoot buds/explant (Mean ± S.D)
BAP	IAA		
0.5	0.5	0	0
1.0	0.5	22	8.0 ± 6.6
1.5	0.5	44	10.3 ± 6.0
2.0	0.5	0	0
2.5	0.5	0	0
1.0 + 10 % CM	0.5	0	0

Each treatment consisted of 9 replicates, observations after 25 days

Cotyledonary segments showed a maximum of 44 per cent regeneration response on MS + BAP 1.5 mg l^{-1} + IAA 0.5 mg l^{-1} (mean: 10.3 buds) which was followed by 22 per cent regeneration on MS + BAP 1.0 mg l^{-1} + IAA 0.5 mg l^{-1} (mean: 8 buds) (Plate 2). In all other combinations the regeneration response was nil. The shoot buds were mainly produced from the petiolar end. Presence of organic supplement i.e., coconut milk 10 per cent in MS + BAP 1.0 mg l^{-1} + IAA 0.5 mg l-1 supported only callusing and the regeneration response was nil in this combination.

4.1.3.2 Regeneration from callus

The regeneration of callus obtained from the cotyledonary segments was tried in basal medium alone or along with growth regulators BAP with or without KN. The effects of growth regulators on regeneration from callus obtained from cotyledonary segments are shown in the Table 13.

Table 13. Effect of growth regulators on regeneration of callus from cotyledonary segments of *W. somnifera*

Media composition	Regeneration (%)	No of shoot buds/explant (Mean ± S.D)
MS + 3 % sucrose	4	2.0 ± 0.0
MS + BAP 1.0 mg l^{-1}	16	11.0 ± 10.06
MS + BAP 2.0 mg l^{-1}	20	13.5 ± 10.4
MS + BAP 1.0 + KN 0.5 mg l^{-1}	0	0.0± 0.0
MS + BAP 1.0 + KN 1.0 mg l^{-1}	0	0.0± 0.0

Each treatment consisted of 25 replicates, observations after 25 days

Regeneration occurred within 10 days of inoculation on media supplemented with BAP alone. A maximum of 20 per cent regeneration was obtained on MS + BAP 2.0 mg l^{-1} with an average of 13.5 buds. It was followed by MS + BAP 1.0 mg l^{-1}, where 16 per cent regeneration was obtained with an average of 11 buds. The regenerated shoot buds showed good elongation response (5.0 cm) in MS + BAP 1.0 mg l^{-1} (Plate 2). In the above two combinations, creamish green callus gradually turned to greenish brown callus after 20 days of inoculation.

Regeneration was not obtained from callus on MS medium with BAP and KN. The combination did not promote further callusing and callus turned brown within twelve days of inoculation.

Four per cent regeneration was obtained on MS with 3 per cent sucrose with an average of 2.0 shoot buds.

4.1.3.3 Multiplication

The regenerated shoot buds from cotyledonary segments were multiplied on MS + BAP 0.4 mg l^{-1} + IAA 0.5 mg l^{-1}. The combination produced an average of 6.1 shoots per single shoot bud. The shoots showed good elongation (3.0-5.0 cm) within 25 days of inoculation (Plate 2).

4.1.3.4 Elongation and Rooting

The shoots obtained from the cotyledonary segment and its callus, of length 0.5-2.0 cm were separated and elongated on MS + GA_3 0.5 mg l^{-1} or 1.0 mg l^{-1}. The initial length of the shoots, internode number and leaf number recorded at the time of inoculation was deducted from the final readings. The influence of GA_3 on elongation and rooting of cotyledonary segment derived shoots is shown in the Table 14.

Table 14. Influence of GA_3 on elongation and rooting of cotyledonary segment derived shoots of *W. somnifera*

Culture medium (mg l^{-1})	Length of shoots Mean ± S.D (cm)	No. of internodes/ shoot (Mean ± S.D)	No. of leaves/shoot (Mean ± S.D)	No. of roots/shoot (Mean ± S.D)	Rooting (%)
MS + GA_3 0.5	4.1 ± 1.4	3.5± 1.8	6.1 ± 2.0	4.1 ± 3.0	80
MS + GA_3 1.0	3.5 ± 1.0	2.0 ± 0.4	5.0 ± 2.3	4.0 ± 1.7	100

Each treatment consisted of 10 replicates, observations after 20 days

Maximum elongation was obtained on MS + GA_3 0.5 mg l^{-1}, similar to hypocotyl derived shoots. An average height of 4.1 cm was obtained in this combination. The number of internodes, leaves and roots were also higher in this combination compared to MS + GA_3 1.0 mg l^{-1}. Shoots derived from hypocotyls showed faster elongation compared to shoots produced from cotyledonary segments. Hundred percentage rooting was obtained on MS + GA_3 1.0 mg l^{-1} with an average of 4.0 roots per shoot, whereas only 80 per cent rooting occurred in MS + GA3 0.5 mg l^{-1}. *In vitro* flowering was also obtained on MS + GA_3 0.5 mg l^{-1} in 10 per cent of the shoots.

The elongated shoots were rooted successively on half MS by pulse treatment with IBA 1000 mg l^{-1} for 5 sec. Hundred per cent rooting occurred by pulse treatment with an average of 20 roots per shoot. The roots were induced in 7-8 days and the mean length of the root was 5.1 cm. Sixty eight per cent of the *in vitro* rooted plantlets survived after hardening stage and got established.

Cotyledonary segment
used as explant

Formation of multiple shoots
(MS + BAP 1.0 mg l^{-1} + IAA 0.5 mg l^{-1})

Induction of callus
(MS + BAP 2.0 mg l^{-1} + IAA 0.5 mg l^{-1})

Regeneration from callus
(MS + BAP 1.0 mg l^{-1})

Further multiplication of shoots
(MS + BAP 0.4 mg l^{-1} + IAA 0.5 mg l^{-1})

In vitro rooting of shoots
(1/2 MS pulse treatment IBA 1000 mg l^{-1})

Plate 2. Response of cotyledonary segments of *W. somnifera* to *in vitro* regeneration

4.1.4 Shoot tips and nodal segments as explants

4.1.4.1 Culture establishment

Cultures of shoot tips and nodal segments were established on MS + BAP 1.0 mg l^{-1} + IAA 0.5 mg l^{-1} and cent percent establishment was obtained within 10 days.

4.1.4.2 Multiplication

The established cultures were placed for multiplication 10 days after establishment. The effect of different growth regulator combinations on multiplication of established shoot tips and nodal segments is shown in the Table15.

Table 15. Effect of different concentrations of growth regulators on multiplication of nodal segments and shoot tips of *W. somnifera*

Explant	MS + Growth regulators (mg l^{-1})		No. of shoots/ explant (Mean ± SD)	No. of shoot buds/ explant ± (Mean ± SD)
	BAP	IAA		
Nodal segment	1.0	0.5	3.3 ± 1.8	6.4 ± 2.8
	1.5	0.5	2.9 ± 1.2	11.1 ± 3.2
	2.0	0.5	1.5 ± 0.8	15.2 ± 5.9
	2.5	0.5	2.8 ± 0.8	15.7 ± 7.1
	3.0	0.5	1.9 ± 1.2	10.3 ± 3.2
Shoot tip	1.0	0.5	4.6 ± 2.0	6.2 ± 2.3
	1.5	0.5	4.4 ± 2.0	6.4 ± 2.1
	2.0	0.5	3.9 ± 0.9	7.4 ± 4.5
	2.5	0.5	2.5 ± 1.4	9.6 ± 4.1
	3.0	0.5	3.0 ± 1.2	6.0 ± 3.4

Each treatment consisted of 10 replicates; observations recorded after 25 days

Hundred percent regeneration responses were obtained from nodal segments and shoot tips in all the concentrations of BAP and IAA tested. The combination MS + BAP 1.0 mg l^{-1} + IAA 0.5 mg l^{-1} produced maximum number of shoots per nodal segment and shoot tip with a mean value of 3.3 and 4.6 respectively. The mean number of shoot buds was highest in MS + BAP 2.5 mg l^{-1} + IAA 0.5 mg l^{-1} (15.7 and 9.6). Taking the shoots and shoot buds together,

maximum multiplication was obtained from MS + BAP 2.5 mg l^{-1} + IAA 0.5 mg l^{-1} (Plate 3 & 5).

Compared to shoot tips, nodal segments produced more multiple shoots (up to 25). The number of elongated shoots was more at low concentration of BAP (1.0 mg l^{-1}). Callus formation was also noticed at the basal cut ends of nodal segments and shoot tips Regeneration of shoot buds from the basal callus was also obtained in the same culture cycle and was highest in MS + BAP 2.5 mg l^{-1} and IAA 0.5 mg l^{-1}. The number of differentiated shoot buds from basal callus increased with the increase in concentration of BAP (up to 2.5 mg l^{-1}) with respect to IAA.

4.1.4.3 Subculturing

The shoot buds were further subcultured and the influence of subculturing on shoot multiplication is shown in the Table 16.

Subculturing was found to be effective for multiplication of shoot buds obtained in the first culture cycle from nodal segments and shoot tips. The number of shoot buds was highest in MS + BAP 2.5 mg l^{-1} and IAA 0.5 mgl^{-1} with a mean value of 21.1 and 17.3 respectively from buds of shoot tips and nodal segments. The number of shoots per explant was more in MS + BAP 1.5 mg l^{-1} and IAA 0.5 mg l^{-1} with mean values of 4.4 and 3.6 from shoot tips and nodal segments respectively in the subculture. Taking both shoot buds and shoots together, shoot tip derived shoots produced a maximum of 35 numbers, as against nodal segment, which produced a maximum of 23 numbers. Subculturing was found to be highly promising for shoot tip derived shoots compared to the first culture cycle. The influence of subculturing was not so much pronounced in the case of nodal segments and the multiplication obtained was almost in par with the first culture cycle.

Table 16. Influence of subculturing of nodal segment and shoot tips derived shoots of *W. somnifera* on multiplication

MS + Growth regulator (mg l^{-1})		Nodal segments		Shoot tips	
BAP	IAA	No. of shoot buds/ explant (Mean ± S.D)	No.of shoots / explant (Mean ± S.D)	No. of shoot buds/ explant (Mean ± S.D)	No. of shoots / explant (Mean ± S.D)
1.5	0.5	15.7 ± 3.2	3.6 ± 1.8	14.3 ± 3.9	4.4 ± 2.6
2.5	0.5	17.3 ± 4.5	3.3 ± 1.8	21.1 ± 5.3	2.5 ± 1.0

4.1.4.4 Elongation and Rooting

The clump of shoot buds were elongated successfully on MS + GA_3 0.5 mg l^{-1}. Rooting occurred within 7-8 days in all the treatments with IBA. Pulse treatment with 1000 mg l^{-1} IBA for 5 sec and further inoculation on half MS + 0.25 per cent activated charcoal was found to be the best medium both for nodal segment and shoot tip derived shoots for rooting. In this combination, the roots were of medium thickness and 6.0-7.0 cm length. Callusing was less in this culture medium. The combination produced an average of 36.2 roots in the case of nodal segment derived shoots and 37.6 roots in the case of shoot tip derived shoots (Table 17).

Pulse treatment with 1000 mg l^{-1} IBA for 5 sec and further inoculation on half MS alone without activated charcoal produced numerous roots with a mean value of 50.2 from shoot tip and 48.4 from nodal segment derived shoots. But, the roots were short and stout with prominent callus on the shoot base.

Profuse laterals were produced on half MS + IBA 0.5 or 1.0 mg l^{-1}. On adding IAA along with IBA, roots became thicker and shorter and also the number of lateral roots produced decreased significantly. Proliferation of callus was also

Table 17. Effect of growth regulator combinations on rooting of shoots derived from nodal segments and shoot tips of *W. somnifera*

Explant	Culture medium	Mean length of shoots ± S.D	Mean no. of internodes/ shoot ± S.D	Mean no. or roots/shoot ± S.D	Mean root length ± S.D	Root morphology	Days for root induction
Shoot tip	½ MS	4.8 ± 0.8	4.3 ± 1.2	10.2 ± 2.0	4.2 ± 1.2	Thin, less laterals	10-11
	½ MS + 0.25 % AC	4.3 ± 0.8	5.0 ± 0.5	12.2 ± 2.4	5.2 ± 0.8	Thin, less laterals	9-10
	½ MS + 0.5 IBA	7.2 ± 0.9	4.5 ± 1.1	23.5 ± 4.9	6.2 ± 0.9	Meduim thick, profuse laterals	8-9
	½ MS + 1.0 IBA	5.9 ± 1.8	4.0 ± 0.7	18.9 ± 5.5	5.8 ± 1.1	Medium thick, profuse laterals	7-8
	½ MS + 0.5 IAA + 0.5 IBA	7.1 ± 2.2	6.0 ± 0.7	30.5 ± 2.3	2.5 ± 0.4	Thick, short, callus base, no laterals	7-8
	½ MS + 0.5 IAA + 1.0 IBA	5.8 ± 2.2	5.7 ± 1.2	36.4 ± 2.0	4.1 ± 0.5	Thick, short, callus base, less laterals	7-9
	½ Ms + IBA 1000 pulse treatment	7.8 ± 0.6	7.5 ± 0.5	50.2 ± 5.0	2.4 ± 0.7	Stout, short, prominent callus	7-8
	½ MS + IBA 1000 pulse treatmet + 0.25 % AC	6.0 ± 0.5	7.0 ± 0.5	37.6 ± 3.0	6.4 ± 1.0	Medium thick, long with laterals	7-8
Nodal segment	½ MS	5.5 ± 1.7	7.3± 0.9	9.3 ± 2.6	4.4 ± 1.5	Thin, less laterals	10-12
	½ MS + 0.25 % AC	5.3 ± 1.1	6.0 ± 1.4	13.4 ± 2.9	5.1 ± 0.9	Thin, less laterals	9-11

Table 17. contd

	½ MS + 0.5 IBA	10.0± 0.9	6.3 ± 1.3	14.4 ± 2.2	6.3 ± 0.9	Meduim thick, profuse laterals	8-9
	½ MS + 1.0 IBA	6.0 ± 2.9	4.5 ± 1.2	29.5 ± 2.0	5.6 ± 1.5	Medium thick, profuse laterals	7-9
	½ MS + 0.5 IAA + 0.5 IBA	7.6 ± 3.0	5.0 ± 1.3	14.2 ± 2.0	2.7 ± 0.4	Thick, short, callus base, no laterals	7-8
	½ MS + 0.5 IAA + 1.0 IBA	8.4 ± 2.2	5.6 ± 0.7	35.2 ± 2.0	4.3± 0.5	Thick, short, callus base, less laterals	7-8
	½ Ms + IBA 1000 pulse treatment	6.8 ± 0.3	7.5 ± 0.9	48.4 ± 4.1	2.6 ± 0.6	Stout, short, prominent callus	7-8
	½ MS + IBA 1000 pulse treatmet + 0.25 % AC	7.0 ± 0.5	8.5 ± 0.8	36.2 ± 3.0	6.6± 1.2	Medium thick, long with laterals	7-8

In vitro seedlings as a source of explants

Shoot tip

Formation of multiple shoots

Regeneration from basal callus

Elongation of shoots in media supplemented with GA_3

Plate 3. Different stages of *in vitro* regeneration in *W. somnifera* from shoot tip

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

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

(1/2 MS + IBA 1.0 mg l^{-1} + IAA 0.5mg l^{-1})

(1/2 MS + IBA 1000 mg l^{-1})

Plate 4. Effect of growth regulators on *in vitro* rooting of shoot tip derived shoots in *W. somnifera*

Nodal segment used as explant

Culture establishment

Formation of multiple shoots

Rooting of shoots

Hardening of *in vitro* plantlets

T. C derived plantlets

Flowering & friuiting in T. C derived plantlets

Plate 5. Different stages m*icropropagation* in *W. somnifera* from nodal segment

more at the cut ends on medium supplemented with IAA. On increasing the concentration of IBA from 0.5 to 1.0 mg l^{-1} in the presence of IAA the number of roots as well as the length of the roots increased.

Elongation was also obtained in all the combinations tried. Pulse treatment with IBA 1000 mg l^{-1} (half MS) produced maximum elongation from shoot tip derived shoots (mean of 7.8 cm) and the mean number of nodes (7.5) was also highest in this combination. Maximum elongation was obtained from nodal segment derived shoots on half MS + 0.5 IBA (mean of 10.0 cm). The internodes were longer (2-4 cm) in the middle portion of the stem in this combination. The number of internodes on nodal segment derived shoots was more (8.5) in half MS + 0.25 per cent activated charcoal + IBA (pulse treatment) (Plate 4).

4.1.5 Leaf as explant

The effect of growth regulator combinations on regeneration from leaf disc is shown in the Table 18.

A maximum of 30 per cent regeneration response was obtained on MS with BAP 1.0 mg l^{-1} and IAA 0.5 mg l^{-1}. The mean number of shoot buds was more on MS + BAP 2.0 mg l^{-1} and IAA 0.5 mg l^{-1} with 15 buds. Callusing occurred not only from the petiolar end but also from all sides touching the media. Proliferation of friable greenish brown callus or creamish green callus with brown tinge was observed on all the combinations tested (Plate 6).

Table 18. Effect of growth regulator combinations on regeneration from leaf segments of *W. somnifera*

MS + Growth regulators (mgl^{-1})		Regeneration (%)	No. of shoot buds /explant (Mean)
BAP	IAA		
1.0	0.5	30	8.5
1.5	0.5	10	2.0
2.0	0.5	10	15.0
2.5	0.5	10	4.0
3.0	0.5	20	3.0

Shoots were successfully rooted by pulse treatment with 1000 mg l^{-1} and an average of 30 roots was obtained. Shoots produced roots within seven days of inoculation. A mean shoot length of 7.0 cm elongation was obtained with an average of 7.0 nodes. The roots produced were small in this combination.

Only a single shoot was placed on half MS + 0.25 per cent activated charcoal. But instead of rooting flowering was obtained *in vitro*. Flowers were large and fully developed.

4.1.6 Roots as explants

Regeneration was tried from both *in vitro* seedling roots and also the roots taken from *in vitro* rooted plantlets.

In all the combinations (MS + BAP 1.0-2.5 mg l^{-1} + IAA 0.5 mg l^{-1}), both types of roots initially showed bulging, later friable callus having greenish brown colour with cream tinge proliferated. Compared to other combinations, callusing was low on MS with BAP 1.5 mg l^{-1} and IAA 0.5 mg l^{-1}. Among the 20 replicates, one of the seedling roots produced somatic embryo like structures. It was of globose shape, with green colour (5 in number) slightly separated from the root.

The somatic embryo like structures were further inoculated on MS with BAP 1.5 mg l^{-1} and IAA 0.5 mg l^{-1}. Callusing occurred and 12 buds got differentiated. The differentiated buds were inoculated on half MS with IBA 0.5 mg l^{-1} and later discarded due to contamination.

The root callus obtained from roots of *in vitro* seedlings and *in vitro* rooted plantlets were inoculated on MS with BAP 2.0-6.0 mg l^{-1} and IAA 0.2 mg l^{-1} for regeneration. But, only callusing was improved by this treatment. The proliferated callus was friable, creamish green in colour with brown tinge. Regeneration response was nil in all the combinations tested (Plate 7).

Leaf segment used as explant

Regeneration of multiple shoots

Callus formation

In vitro flowering (1/2 MS + 0.25 % activated charcoal)

Rooting of shoots

Plate 6. *In vitro* regeneration in *W. somnifera* from leaf segments

Root segments used as explants

Callus induced from root segments

Formation of somatic embryo like structures

Plate 7. Response of roots of *W. somnifera to in vitro* regeneration

4.2 CULTURING AND SENSITIVITY SCREENING OF *AGROBACTERIUM* AND EXPLANTS

4.2.1 Culturing of *A. rhizogenes* strains

The *A. rhizogenes* were cultured on YEB, YEM, NA and LBA media. The strains differed in their growth on the four media tested. The influence of culture media on the growth of *A. rhizogenes* strains is given in the Table 19.

Table 19. Influence of culture media on the growth of *A. rhizogenes* strains

A. rhizogenes strains	YEB	YEM	NA	LBA
A4	++	++	++	+++
ATCC 15834	++	++	++	++
MTCC 2364	+++	++	+++	+++

+ slow growth, ++ fast growth, +++ very fast growth

All the culture media favoured the growth of *A. rhizogenes* strain. Strain MTCC 2364 showed very fast growth in all the media tested except YEM. So YEM was selected for growing MTCC 2364, so as to obtain single cell colonies. Both NA and YEB were preferred for growing ATCC 15834 and A4. The optimum growth temperature for all the strains was observed to be 26 ± 2 °C.

4.2.2 Cultural characteristics of *Agrobacterium*

The colonies of strain A4 appeared within two days after streaking. Colonies were round with smooth margin, convex, whitish coloured and mucoid in nature. The bacterial colonies of MTCC 2364 appeared within one day and the colonies were of large size with serrated margin and were whitish in colour. The strain ATCC 15834 produced smooth round small colonies at closer spacing two day after streaking and they were whitish and mucoid in nature (Plate 8).

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

The response of *A. rhizogenes* strains to different concentrations of antibiotics such as ampicillin, cefotaxime and carbenicillin is given in the Table 20.

Table 20. Sensitivity of *Agrobacterium* strains to different antibiotics

Antibiotics	Concentration (mg l^{-1})	Response of *Agrobacterium rhizogenes* strains		
		A4	MTCC 2364	ATCC 15834
Ampicillin	0	++	++	++
	50	++	++	+
	100	++	++	+
	200	++	++	-
	300	++	++	-
	400	++	++	-
	500	++	++	-
Carbenicillin	0	++	++	++
	50	+	++	-
	100	-	++	-
	200	-	++	-
	300	-	++	-
	400	-	++	-
	500	-	++	-
Cefotaxime	0	++	++	++
	50	-	+	-
	100	-	+	-
	200	-	+	-
	300	-	+	-
	400	-	+	-
	500	-	-	-

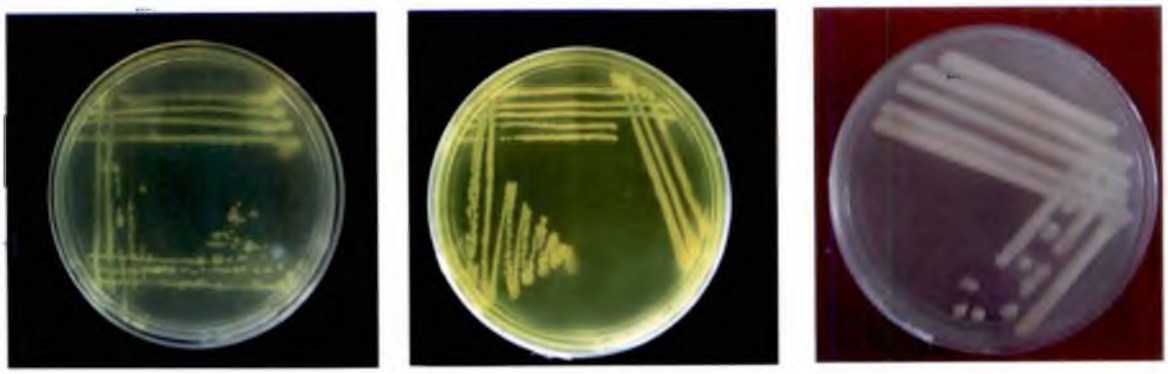
(- No growth, + Restricted growth, ++ Good growth)

The strain A4 showed resistance to ampicillin where as the strain MTCC 2364 showed resistance to both ampicillin and carbenicillin. ATCC 15834 was found to be sensitive to ampicillin, cefotaxime and carbenicillin. All the three strains were sensitive to cefotaxime (Plate 9).

The strain MTCC 2364 survived up to 400 mg l^{-1} cefotaxime. Cefotaxime at 500 mg l^{-1} killed all the three strains of *A. rhizogenes*. So 500 mg l^{-1} cefotaxime was taken as the optimum concentration of antibiotic to kill *A. rhizogenes* strains under study.

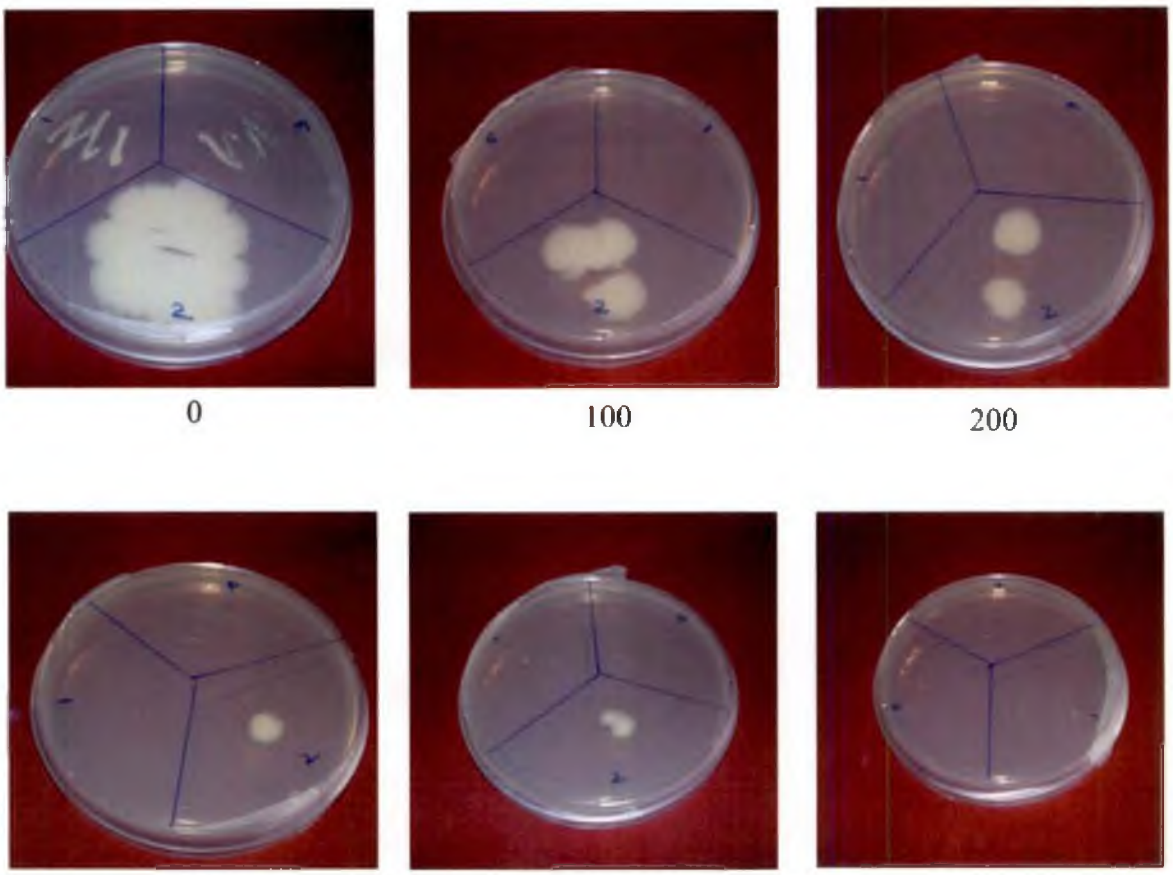
4.2.4 Sensitivity of explants to antibiotics

The sensitivity of explants to ampicillin and cefotaxime at different concentrations are shown in the Table 21.



MTCC 2364 ATCC 15834 A4

Plate 8. *Agrobacterium rhizogenes* strains



0 100 200

300 400 500

1- A4, 2- MTCC 2364 & 3- ATCC 15834

Plate 9. Sensitivity of *Agrobacterium rhizogenes* strains to different concentrations of Cefotaxime (mg l^{-1})

Table 21. Screening of explants sensitivity to different antibiotics

Antibiotics	Concentration (mg l^{-1})	Response					Remarks
	0	HS	CS	NS	ST	LS	Resistant
	100	H	H	H	H	H	Resistant
Cefotaxime	250	H	H	H	H	H	Resistant
	500	H	H	H	H	H	Resistant
	1000	P	H	H	H	H	Resistant (H- sensitive)
	0	H	H	H	H	H	Resistant
	100	H	H	H	H	H	Resistant
Ampicillin	250	H	H	H	H	H	Resistant
	500	H	H	H	H	H	Resistant
	1000	H	H	H	H	H	Resistant

(HS- Hypocotyl Segment, CS- Cotyledonary Segment, NS- Nodal Segment, ST- Shoot Tip, LS- Leaf Segment, H- Healthy, P-Pale)

The different explants like cotyledonary segments, shoot tips, nodal segments and leaf segments were found to be healthy in all the concentrations of cefotaxime tested (0, 100, 250, 500 and 1000 mg l^{-1}) except hypocotyl segments, which became pale at 1000 mg l^{-1} (even though it remained healthy at low concentrations). Slight callusing occurred at the basal cut ends of nodal segments, shoot tips and leaf segments both in the presence and absence of antibiotics (Plate 10).

All the explants, including hypocotyls showed good health and vigour in all the concentrations of ampicillin tested.

4.2.5 Pre culturing of explants

The explants were cultured for two days on MS medium without antibiotics prior to transformation so as to reduce the endogenous auxin level. The explants remained healthy in the pre culturing media.

4.2.5 Wounding of explants

Wounding is a prerequisite for *Agrobacterium* infection. In all the explants, the pricked region turned slightly brown within one week.

0

100

250

500

1000

Plate 10. Sensitivity of leaf segments to different concentrations of Cefotaxime (mg l^{-1})

4.3 STANDARDZATION OF TRANSFORMATION TECHNIQUES

4.3.1 Standardization of inoculation method

4.3.1.2 Direct Inoculation and Suspension culture inoculation Method

4.3.1.2.1 Influence of bacterial Inoculum

The bacterial concentration or the type of inoculum affects the transformation frequencies. The bacterium from isolated single cell colonies was used as the inoculum in Direct inoculation method (DIM) whereas, bacterial suspension (O.D 600 ~ 1) was used as the inoculum in Suspension culture inoculation method (SM).

Table 22. Influence of bacterial inoculum on transformation

A. rhizogenes strains	Inoculum	HS	CS	LS	ST	NS
A4	DIM	0.00	0.00	36.00	16.66	0.00
	SM	0.00	0.00	27.27	20.00	0.00
ATCC 15834	DIM	0.00	0.00	0.00	0.00	0.00
	SM	0.00	0.00	70.00	33.33	0.00
MTCC 2364	DIM	0.00	0.00	0.00	0.00	0.00
	SM	0.00	0.00	0.00	0.00	0.00

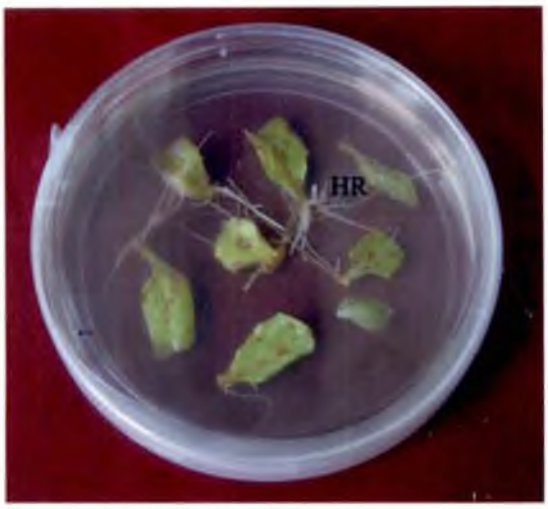
The maximum transformation percentage obtained in various explants by using different inoculums is given in the Table 22.

Among the various explants used only the leaf segments and shoot tips responded to transformation. Similarly, the different *A. rhizogenes* strains used for infection differed in their transformation ability. The bacterial cells from single cell colonies of A4 strain produced transformants, where as single cell colonies of ATCC 15834 and MTCC 2364 did not show any transformation. By DIM, A4 strain gave 36 per cent transformation in leaf and 16.66 per cent in shoot tip (Plate 11, 12A & 12B).

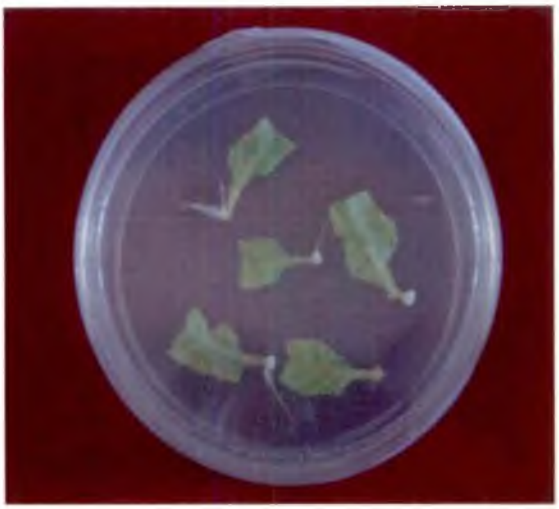
The *A. rhizogenes* strains A4 and ATCC 15834 responded in Suspension culture method of inoculation. The strain ATCC 15834 showed highest transformation (70 %) by SM in leaf and 33.33 per cent in shoot tip followed by

Hairy root initiation from leaf- Direct inoculation method

Hairy root initiation from leaf- Suspension culture method



Hairy root initiation from leaf- Direct inoculation with acetosyringone



Control

Hairy root initiation from shoot tip - Suspension culture method

Control

(HR - Hairy roots)

Plate 11. Genetic transformation in *W. somnifera* using *A. rhizogenes* strain A4

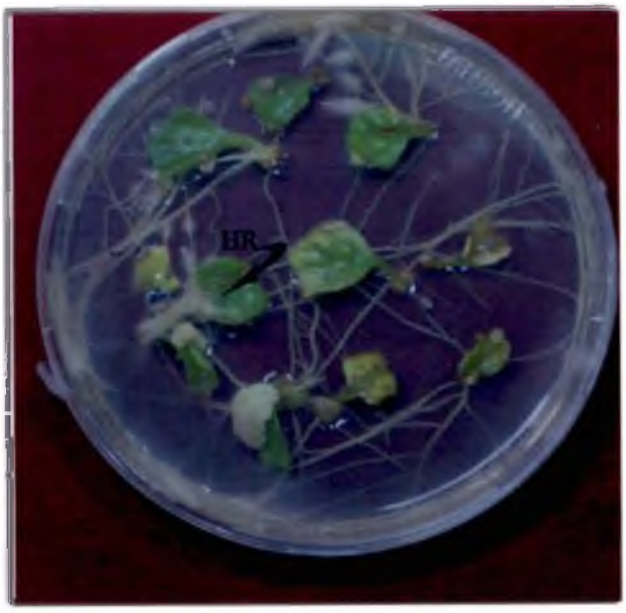
Hairy root initiation from leaf- Suspension culture method

(4 X)

Microscopic view of hairy roots

(6.3 X)

Plate 12A. Hairy root induction in *W. somnifera* using *Agrobacterium rhizogenes* strain ATCC 15834



Hairy root initiation from leaf - Suspension culture method with acetosyringone

Callus formation during suspension culture method

Hairy root initiation from shoot tip - Suspension culture method

Plate 12B. Hairy root induction in *W. somnifera* using *Agrobacterium rhizogenes* strain ATCC 15834

A4 strain which produced 27.27 per cent transformation by SM in leaf and 20 per cent in shoot tip.

The strain MTCC 2364 did not produced transformation in any of the methods (DIM or SM).

4.3.1.2.2 Influence of Co-culture periods on hairy root induction

The explants after infection were co-cultured for 1-3 days at 26 ± 2.0 °C under dark. Slight bacterial ooze or bacterial growth appeared around each explant on co-cultivation for 1-2 days. Normally bacterial overgrowth occurred on third day of co-cultivatioin, which can be removed by washing and further culturing the explants in antibiotics containing culture media. In due course of co-cultivation, no signs of explant necrosis were seen rather they remained healthy and green and leaf explants swelled a bit.

The transformation frequency was influenced by co-culture period. The different *A. rhizogenes* strains also differed in their transformation ability. The influence of co-culture period under DIM and SM on transformation is given in the Table 23.

Among the different explants tried, leaf segments and shoot tips responded to transformation. A greater response of leaf segments to induce hairy roots was seen when they were co-cultured for one day as compared to two-day co-culture (Fig. 2). The shoot tip showed maximum response in two-day co-culture period compared to one day (Fig. 3).

On comparison of the relative transformation percentage of individual bacterial strains in DIM and SM under different co-culture periods, it was observed that only A4 strain induced hairy roots by both the methods where as strain ATCC 15834 produced hairy roots only by SM.

The highest transformation percentage (70 %) was obtained when the leaf segments were co-cultured with strain 15834 for one day following SM, which was followed by 50 per cent response with respect to transformation by the two-day co-culture.

In the case of A4 strain, one-day co-culture produced 36 per cent transformation from leaf segments by DIM followed by 27.27 per cent by SM.

From the shoot tip, transformation frequencies were more after two-day co-culture period using either A4 or 15834 strains. A two day co-culture period showed a maximum of 33.33 per cent transformation response by infection with 15834 and 20 per cent response using A4 strain following Suspension culture method.

No transformation was obtained with strain MTCC 2364 under different co-culture period tested. On increasing the co-culture period, the strain 2364 was found to overgrow on the explants and media kept for hairy root induction (MS + cefotaxime 500 mg l^{-1}) and thereby affecting the tissues.

In the root inducing media (MS + cefotaxime 500 mg l^{-1}), callusing and normal rooting were also observed in almost all explants subjected to different co-culture period except hypocotyls. From hypocotyls, only rooting was obtained and the callusing tendency was found to be nil. Hypocotyl explants often showed necrosis, if co-cultivated for 2-3 days.

Callusing of explants was more with strain 15834 and the leaf segments produced more callus compared to other explants both from the petiolar end as well as from the wound sites present in the inner leaf lamina. Hairy roots were also produced from the proliferated callus and the size of the callus grew in the subsequent sub cultures. The proliferated callus was friable with creamish green colour. Non-transformed leaf segments produced callus only from the petiolar end.

Table 23. Influence of co-culture period on transformation of *W. somnifera*

A. rhizogenes strains	Explant	IM	Co-culture period					
			1 day		2 day		3 day	
			Transformation %	Response	Transformation %	Response	Transformation %	Response
A4	HS	DIM	0.00	Rooting	0.00	Rooting	0.00	-
		SM	0.00	Rooting	0.00	Rooting	0.00	-
	CS	DIM	0.00	Rooting + Callusing	0.00	Rooting	0.00	Rooting
		SM	0.00	Rooting	0.00	Rooting	0.00	Rooting
	LS	DIM	36.0	Rooting + Callusing	25.0	Rooting	20.0	Rooting + Callusing
		SM	27.27	Rooting + Callusing	18.18	Rooting	11.11	Rooting + Callusing
	ST	DIM	14.28	Rooting	16.66	Rooting	0.00	Rooting
		SM	0.00	Rooting	20.00	Rooting	0.00	Rooting
	NS	DIM	0.00	Rooting + Callusing	0.00	Rooting + Callusing	0.00	Rooting
		SM	0.00	Rooting + Callusing	0.00	Rooting + Callusing	0.00	Rooting
ATCC 15834	HS	DIM	0.00	Rooting + Callusing	0.00	Rooting	0.00	-
		SM	0.00	Rooting + Callusing	0.00	Rooting	0.00	Rooting
	CS	DIM	0.00	Rooting	0.00	Rooting	0.00	Rooting
		SM	0.00	Rooting	0.00	Rooting	0.00	-

Table 23. contd..

	LS	DIM	00.00	Rooting	00.00	Rooting + Callusing	0.00	Rooting + Callusing
		SM	70.00	Rooting	50.00	Rooting	21.43	Rooting + Callusing
	ST	DIM	0.00	Rooting + Callusing	0.00	Rooting	0.00	Rooting + Callusing
		SM	11.1	Rooting + Callusing	33.33	Rooting	0.00	Rooting
	NS	DIM	0.00	Rooting + Callusing	0.00	Rooting	0.00	Rooting
		SM	0.00	Rooting + Callusing	0.00	Rooting + Callusing	0.00	Rooting
MTCC 2364	HS	DIM	0.00	Rooting	0.00	BO	0.00	BO
		SM	0.00	Rooting	0.00	BO	0.00	BO
	CS	DIM	0.00	Rooting	0.00	BO	0.00	BO
		SM	0.00	Rooting	0.00	Rooting	0.00	BO
	LS	DIM	0.00	Rooting + Callusing	0.00	Rooting +	0.00	BO
		SM	0.00	Rooting + Callusing	0.00	Rooting + Callusing	0.00	BO
	ST	DIM	0.00	Rooting	0.00	Rooting	0.00	Rooting
		SM	0.00	Rooting	0.00	Rooting	0.00	BO
	NS	DIM	0.00	Rooring	0.00	BO	0.00	BO
		SM	0.00	Rooting	0.00	Rooting	0.00	
Control	HS	DIM	0.00	Rooting	0.00	Rooting	0.00	Rooting
		SM	0.00	Rooting	0.00	Rooting	0.00	Rooting
	CS	DIM	0.00	Rooting + Callusing	0.00	Rooting + Callusing	0.00	Rooting + Callusing

Table 23. contd.

		SM	0.00	Rooting + Callusing	0.00	Rooting + Callusing	0.00	Rooting + Callusing
	LS	DIM	0.00	Rooting+ Callusing	0.00	Rooting+ Callusing	0.00	Rooting+ Callusing
		SM	0.00	Rooting+ Callusing	0.00	Rooting+ Callusing	0.00	Rooting+ Callusing
	ST	DIM	0.00	Rooring	0.00	Rooring	0.00	Rooring
		SM	0.00	Rooting	0.00	Rooting	0.00	Rooting
	NS	DIM	0.00	Rooting+ Callusing	0.00	Rooting+ Callusing	0.00	Rooting+ Callusing
		SM	0.00	Rooting+ Callusing	0.00	Rooting+ Callusing	0.00	Rooting+ Callusing

(BO- Bacterial Overgrowth)

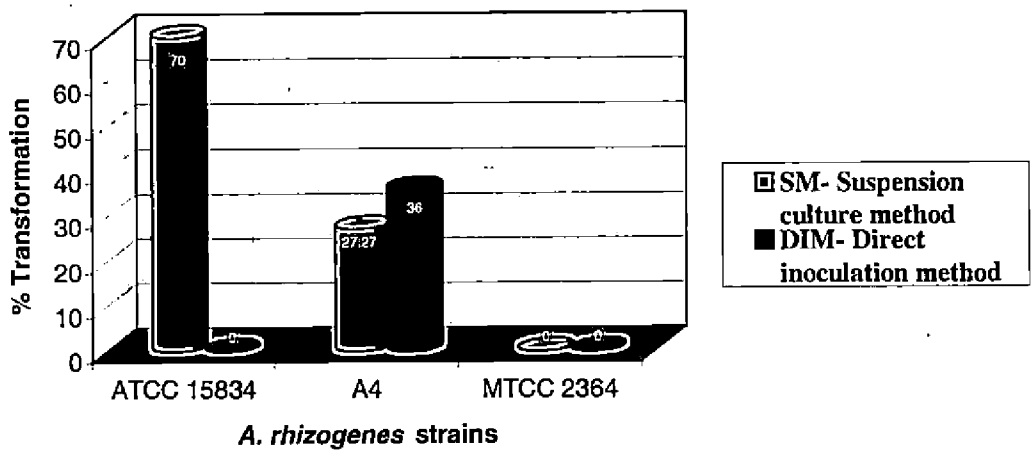


Fig. 2. Response of leaf segments to different *A. rhizogenes* strains on one day co-culture using SM and DIM

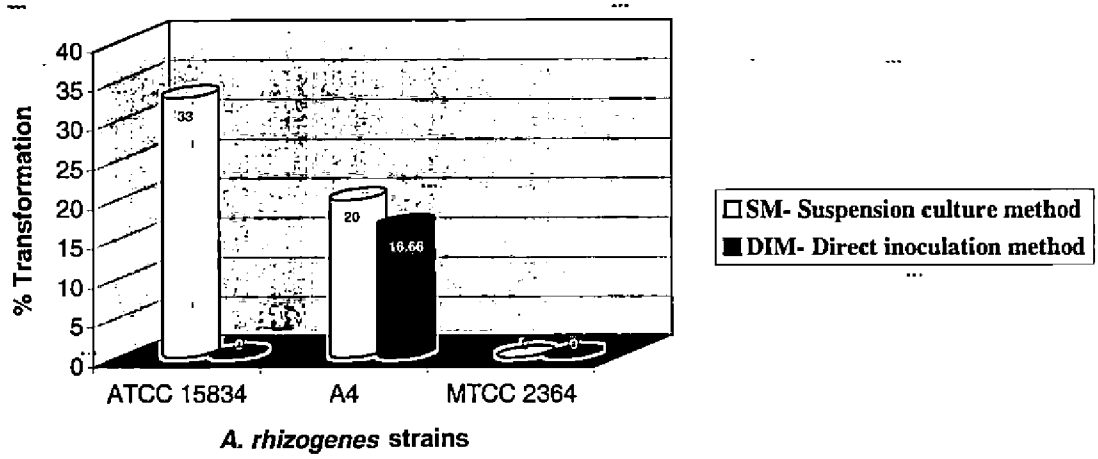


Fig. 3. Response of shoot tips to different *A. rhizogenes* strains on two day co-culture using SM and DIM

Explants other than leaf segments whether transformed or not, produced callus only from the basal end. No callus proliferation occurred from the inner wound sites.

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

The influence of acetosyringone in hairy root induction was studied following DICA, SCA and ASCA methods.

Among the different explants, only the leaf segments and shoot tips responded to transformation in all the three methods. The strain A4 and ATCC 15834 produced successful transformation by the addition of acetosyringone following SCA and ASCA method, where as only A4 strain responded positively in the DICA method. However, MTCC 2364 strain failed to produce any successful transformation following DICA, SCA and ASCA methods (Table 24).

The presence of 100 μM acetosyringone in the co-culturing media significantly increased the number of putative transformants with A4 strain. Following DICA method, A4 strain produced 28.57 per cent transformation with a mean of 2.25 hairy roots per transformed leaf explant. In the absence of acetosyringone (DIM) only 25 per cent transformation was obtained with a mean of 1.67 hairy roots per transformed explant (control) i.e., transformation per cent increase by 3.57 per cent by the addition of acetosyringone. The strain gave 25.0 per cent transformation by SCA method, which produces a mean of 1.0 hairy root. Only 18.18 per cent tranformation was obtained with a mean of 1.25 hairy roots in the absence of acetosyringone (SM) i.e., the increase in tranformation response was 6.82 per cent. In ASCA method, A4 strain showed only 14.29 per cent transformation, however it produced a mean of 3.0 hairy roots per transformed leaf segment.

Table 24. Influence of acetosyringone in hairy root induction in *W. somnifera*

A. rhizogenes strains	Explant	Inoculation & Co-cultivation method	Mean hairy roots/ transformed explant	Mean normal roots/ transformed explant	Transformation %
A4	HS	DICA	0.00	0.20	00.00
		SCA	0.00	0.10	00.00
		ASCA	0.00	0.00	00.00
	CS	DICA	0.00	0.22	00.00
		SCA	0.00	0.34	00.00
		ASCA	0.00	0.00	00.00
	LS	DICA	2.25	0.14	28.57
		SCA	1.00	0.25	25.00
		ASCA	3.00	0.00	14.29
	ST	DICA	1.00	0.25	20.00
		SCA	3.00	0.33	22.00
		ASCA	1.50	1.33	12.50
	NC	DICA	0.00	0.00	00.00
		SCA	0.00	1.12	00.00
		ASCA	0.00	1.00	00.00
ATCC 15834	HS	DICA	0.00	0.37	00.00
		SCA	0.00	0.20	00.00
		ASCA	0.00	0.11	00.00
	CS	DICA	0.00	0.20	00.00
		SCA	0.00	0.30	00.00
		ASCA	0.00	0.10	00.00
	LS	DICA	0.00	0.08	00.00
		SCA	2.50	0.09	36.36
		ASCA	1.50	1.00	20.00
	ST	DICA	0.00	0.33	00.00
		SCA	1.00	0.25	25.00
		ASCA	1.00	0.11	11.11
	NC	DICA	0.00	1.00	00.00
		SCA	0.00	0.25	00.00
		ASCA	0.00	0.83	00.00
MTCC 2364	HS	DICA	0.00	0.20	00.00
		SCA	0.00	0.00	00.00
		ASCA	0.00	0.00	00.00
	CS	DICA	0.00	0.20	00.00
		SCA	0.00	0.02	00.00
		ASCA	0.00	0.00	00.00
	LS	DICA	0.00	0.93	00.00
		SCA	0.00	1.75	00.00
		ASCA	0.00	0.80	00.00

Table 24. contd..

	ST	DICA	0.00	2.25	00.00
		SCA	0.00	0.33	00.00
		ASCA	0.00	1.16	00.00
	NC	DICA	0.00	0.36	00.00
		SCA	0.00	0.16	00.00
		ASCA	0.00	0.28	00.00
(Control) A4	LS	DIM	1.67	0.50	25.00
		SM	1.25	0.14	18.18
	ST	DIM	1.50	1.00	16.66
		SM	1.60	0.93	20.00
(Control) ATCC 15834	LS	DIM	0.00	1.25	00.00
		SM	3.33	0.58	50.00
	ST	DIM	0.00	0.00	00.00
		SM	2.66	0.55	33.33

(Control- No acetosyringone)

(DIM- Direct inoculation method, SM – Suspension culture inoculation method, DICA- Direct inoculation and co-cultivation in acetosyringone method, SCA- Suspension culture and co-cultivation in acetosyringone method, ASCA- Acivated suspension and co-cultivation in acetosyringone, Control- No acetosyringone)

With the strain ATCC 15834, the presence of acetosyringone was not found to positively influence transformation percentage. No transformations were obtained from leaf segments and shoot tips using ATCC 15834 both in the presence (DICA) and absence of acetosyringone (DIM) on using bacteria from single cell colonies.

The strain ATCC 15834 produced 50 per cent transformation with a mean of 3.33 hairy roots per transformed leaf segment in the absence of acetosyringone, whereas only 36.36 per cent transformation response with a mean of 2.5 hairy roots was obtained in the presence of acetosyringone by SCA method i.e., transformation percentage decreased by 13.64 per cent. Following ASCA method, the strain produced 20 per cent transformation giving a mean of 1.5 hairy roots.

Transformation percentage obtained from leaf segments using *A. rhizogenes* strains in the presence and absence of 100 μM acetosyringone in co-cultivation media by DIM and SM is shown in the Fig. (4).

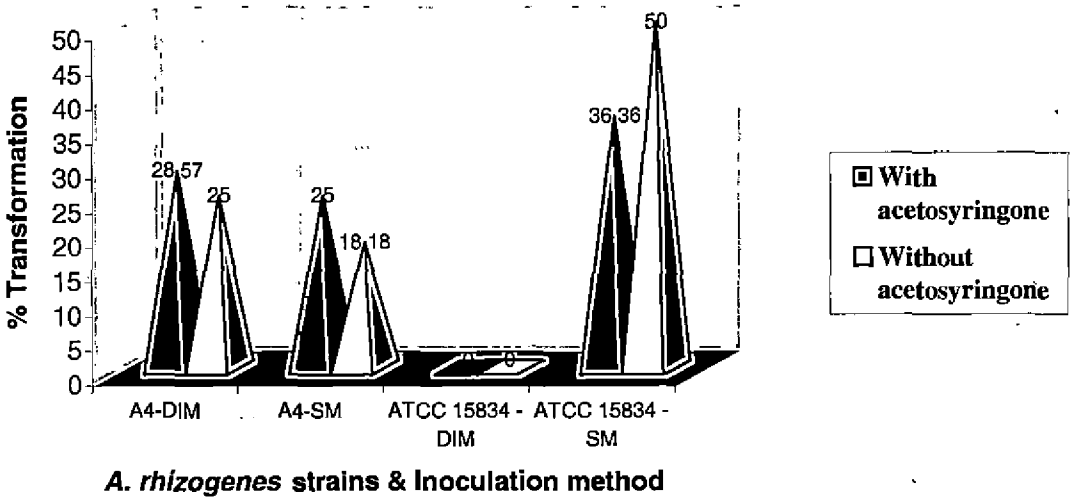


Fig. 4. Influence of acetosyringone on transformation percentage of leaf segments using DIM and SM

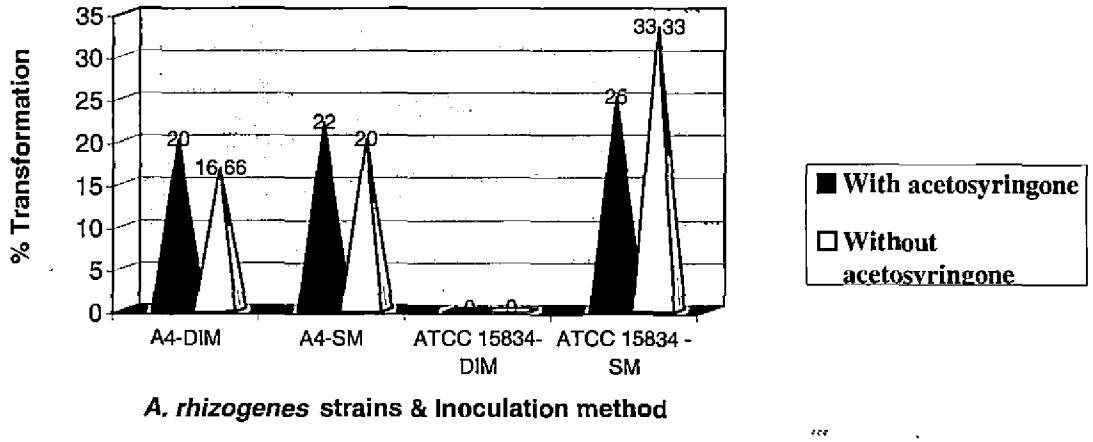


Fig. 5. Influence of acetosyringone on transformation percentage of shoot tips using DIM and SM

Figure (5) shows the transformation percentage obtained from shoot tips in the presence and absence of acetosyringone.

Shoot tip also responded positively producing 20 per cent transformation following DICA method with a mean of 1.0 root per transformed shoot tip with A4 strain. In the absence of acetosyringone (DIM), only 16.66 per cent transformation was produced with a mean of 1.5 hairy roots i.e., transformation response increased by 3.34 per cent. Twenty two per cent transformation was obtained by SCA method and 12.5 per cent, following ASCA method, with a mean of 3.0 and 1.5 hairy roots respectively from each transformed shoot tip. Twenty per cent transformation occurred with a mean value of 1.6 hairy roots in the absence of acetosyringone i.e., two per cent increase by the addition of acetosyringone.

The strain 15834 induced hairy roots from shoot tips by SCA and ASCA method. Twenty five per cent transformation was obtained with a mean of 1.0 hairy root in SCA method and 11.1 per cent transformation with a mean of 1.0 hairy root in ASCA method. In the absence of acetosyringone 33.33 per cent transformation was produced with a mean number of 2.66 hairy roots that is, 8.33 per cent decrease following the addition of acetosyringone.

The explants produced normal roots also in addition to hairy roots. Rooting and callusing occurred from most of the explants. However hypocotyls showed least tendency for callusing.

4.3.2 Standardization of explants for efficient transformation

Among the different explants tested, leaf segments of *W.somnifera* were found to be the best explant for efficient transformation under each variable followed by shoot tips. Other explants like hypocotyls segments, coyledonary segments and nodal segments failed to show any successful transformation (Table 25).

The hairy roots were induced more from the petiolar region in the case of leaf segments and in the absence of the petiole; hairy roots were produced from the proximal cut edges. From the shoot tips, hairy roots were produced from and around the basal portion only. No roots developed from the leaves attached to shoots.

Seventy per cent transformation response was shown by leaf segments with ATCC 15834 strain, while shoot tips gave a maximum of only 33.33 per cent transformation response. The mean number of hairy roots per transformed leaf segment was 3.33 and from the shoot tip the mean was 2.66.

In the inner laminar region of leaf segment, only one root was produced per wound site, whereas 2-3 roots were produced around the petiolar region in the case of infection with A4.

A maximum of ten hairy roots were produced per single leaf segment by ATCC 15834 infections, around the proximal end. Shoot tips produced a maximum of three hairy roots per single explant.

The infected explants also produced normal roots in addition to hairy roots. Rooting and callusing occurred from most of the explants, however hypocotyls showed least tendency for callusing.

On transformation of leaf segments with ATCC 15834, it was clearly visible that once the explant got transformed, the tendency for producing normal roots decreased and more number of hairy roots was induced in place of normal roots.

Table 25. Standardization of explants for efficient transformation

Strains	Explant	IM	Co-culture period	Mean no of hairy roots/ transformed explant	Mean no of normal roots/ explant	Transformation %	Response
A4	HS	DIM	1 day	0.00	0.75	00.00	Rooting
			2 day	0.00	0.66	00.00	Rooting
			3 day	0.00	0.00	00.00	-
		SM	1 day	0.00	0.42	00.00	Rooting
			2 day	0.00	0.00	00.00	-
			3 day	0.00	0.00	00.00	-
	CS	DIM	1 day	0.00	0.22	00.00	Rooting
			2 day	0.00	0.44	00.00	R + C
			3 day	0.00	0.33	00.00	Rooting
		SM	1 day	0.00	0.21	00.00	Rooting
			2 day	0.00	0.14	00.00	Rooting
			3 day	0.00	0.14	00.00	Rooting
	LS	DIM	1 day	1.88	0.38	36.00	R + C
			2 day	1.67	0.50	25.00	Rooting
			3 day	1.00	0.84	20.00	R + C
		SM	1 day	2.33	0.90	27.27	R + C
			2 day	1.25	0.14	18.18	Rooting
			3 day	3.00	0.00	11.11	R + C
	ST	DIM	1 day	1.00	1.00	14.28	Rooting
			2 day	1.50	1.00	16.66	Rooting
			3 day	0.00	0.14	00.00	Rooting
		SM	1 day	0.00	2.06	00.00	Rooting
			2 day	1.60	0.93	20.00	Rooting
			3 day	0.00	0.66	00.00	Rooting
	NC	DIM	1 day	0.00	0.43	00.00	R + C
			2 day	0.00	0.00	00.00	-
			3 day	0.00	0.33	00.00	-
		SM	1 day	0.00	1.00	00.00	R + C
			2 day	0.00	0.11	00.00	Rooting
			3 day	0.00	0.33	00.00	Rooting
ATCC 15834	HS	DIM	1 day	0.00	0.08	00.00	Rooting
			2 day	0.00	0.22	00.00	Rooting
			3 day	0.00	0.14	00.00	Rooting
		SM	1 day	0.00	0.11	00.00	Rooting
			2 day	0.00	0.28	00.00	Rooting
			3 day	0.00	0.00	00.00	-
	CS	DIM	1 day	0.00	0.08	00.00	R + C
			2 day	0.00	0.15	00.00	Rooting
			3 day	0.00	0.20	00.00	R + C

Table 25. contd..

		SM	1 day	0.00	0.64	00.00	R + C
			2 day	0.00	1.00	00.00	R + C
			3 day	0.00	0.20	00.00	R + C
	LS	DIM	1 day	0.00	1.50	00.00	R + C
			2 day	0.00	1.25	00.00	Rooting
			3 day	0.00	1.75	00.00	Rooting
		SM	1 day	1.75	0.80	70.00	R + C
			2 day	3.33	0.58	50.00	R + C
			3 day	1.60	0.85	21.43	R + C
	ST	DIM	1 day	0.00	0.00	00.00	Callus
			2 day	0.00	0.83	00.00	R + C
			3 day	0.00	0.33	00.00	Rooting
		SM	1 day	1.00	0.77	11.11	R + C
			2 day	2.66	0.55	33.33	R + C
			3 day	0.00	0.30	00.00	Rooting
	NC	DIM	1 day	0.00	0.82	00.00	Rooting
			2 day	0.00	0.53	00.00	Rooting
			3 day	0.00	0.00	00.00	-
		SM	1 day	0.00	1.00	00.00	R + C
			2 day	0.00	0.27	00.00	R + C
			3 day	0.00	0.00	00.00	R + C
MTCC 2364	HS	DIM	1 day	0.00	0.11	00.00	Rooting
			2 day	0.00	0.00	00.00	BO
			3 day	0.00	0.00	00.00	BO
		SM	1 day	0.00	0.25	00.00	Rooting
			2 day	0.00	0.00	00.00	BO
			3 day	0.00	0.00	00.00	BO
	CS	DIM	1 day	0.00	0.30	00.00	Rooting
			2 day	0.00	0.18	00.00	Rooting
			3 day	0.00	0.00	00.00	BO
		SM	1 day	0.00	0.44	00.00	Rooting
			2 day	0.00	0.00	00.00	BO
			3 day	0.00	0.00	00.00	BO
	LS	DIM	1 day	0.00	1.00	00.00	R + C
			2 day	0.00	0.83	00.00	R + C
			3 day	0.00	0.00	00.00	BO
		SM	1 day	0.00	0.85	00.00	R + C
			2 day	0.00	0.42	00.00	R + C
			3 day	0.00	0.00	00.00	BO
	ST	DIM	1 day	0.00	1.00	00.00	Rooting
			2 day	0.00	0.00	00.00	BO
			3 day	0.00	0.00	00.00	BO
		SM	1 day	0.00	1.07	00.00	R + C
			2 day	0.00	0.77	00.00	BO
			3 day	0.00	0.00	00.00	Rooting
	NC	DIM	1 day	0.00	0.42	00.00	R + C

Table 25. contd.

			2 day	0.00	0.66	00.00	Rooting
			3 day	0.00	0.00	00.00	BO
		SM	1 day	0.00	0.60	00.00	R + C
			2 day	0.00	0.50	00.00	R + C
			3 day	0.00	0.00	00.00	BO
Control	HS	DIM	1 day	0.00	0.25	00.00	Rooting
			2 day	0.00	0.11	00.00	Rooting
			3 day	0.00	0.13	00.00	Rooting
		SM	1 day	0.00	0.29	00.00	Rooting
			2 day	0.00	0.22	00.00	Rooting
			3 day	0.00	0.00	00.00	-
	CS	DIM	1 day	0.00	0.50	00.00	Rooting
			2 day	0.00	0.44	00.00	Rooting
			3 day	0.00	0.50	00.00	Rooting
		SM	1 day	0.00	0.25	00.00	R + C
			2 day	0.00	0.31	00.00	Rooting
			3 day	0.00	0.22	00.00	R + C
	LS	DIM	1 day	0.00	1.85	00.00	R + C
			2 day	0.00	2.00	00.00	R + C
			3 day	0.00	1.77	00.00	R + C
		SM	1 day	0.00	1.86	00.00	R + C
			2 day	0.00	2.60	00.00	R + C
			3 day	0.00	1.67	00.00	R + C
	ST	DIM	1 day	0.00	1.50	00.00	R + C
			2 day	0.00	1.66	00.00	R + C
			3 day	0.00	2.00	00.00	R + C
		SM	1 day	0.00	133	00.00	R + C
			2 day	0.00	1.71	00.00	Rooting
			3 day	0.00	2.00	00.00	Rooting
	NC	DIM	1 day	0.00	1.66	00.00	R + C
			2 day	0.00	1.63	00.00	R + C
			3 day	0.00	1.43	00.00	R + C
		SM	1 day	0.00	1.57	00.00	Rooting
			2 day	0.00	2.25	00.00	R + C
			3 day	0.00	1.40	00.00	Rooting

(IM- Inoculation method, R + C – Rooting and Callusing, BO- Bacterial Overgrowth, '-' indicate no rooting and callusing)

The number of normal roots was also more from leaf segments that failed to produce any successful transformation. The leaf segments showed more tendencies for callusing compared to other explants after infection with *A. rhizogenes* strains. Slight callusing occurred at the petiolar end in most of the

infected leaf segments. The callus proliferation was noticed from the pricked site only in the case of leaf segments. Initially a white friable callus slightly proliferated at the pricked portions that got further enlarged in the subsequent subcultures. The control explants also showed normal rooting and callusing from the basal ends.

4.3.3 Efficiency of strains in inducing hairy roots

The strain ATCC 15834 showed highest efficiency (70 %) in transforming the plant tissues, followed by A4 strain (36 %). The strain MTCC 2364 failed to produce any successful transformation. Similarly the strain ATCC 15834 produced more hairy roots per explant (3.33) compared to A4 strain, which produced a mean of 3.0 hairy roots per transformed explant (Fig. 6).

4.3.3.1 Number of days for root induction

The number of days taken for hairy root induction from infected explants using different strains under different inoculation methods is shown in the Table (26). The number of days for hairy root induction differed under different inoculation methods. In general 9-20 days was taken for hairy root induction.

Table 26. Number of days for hairy root induction

A. rhizogenes strains	Inoculation method	Explant	No of days for Root induction
A4	DIM	LS	8-18
		ST	9-20
	SM	LS	9-15
		ST	10-17
	DICA	LS	8-15
		ST	9-14
	SCA	LS	9-18
		ST	10-19
	ASCA	LS	9-20
		ST	8-18
ATCC 15834	SM	LS	10-18
		ST	10-16
	SCA	LS	9-19
		ST	9-16
	ASCA	LS	10-18
		ST	10-18

4.3.4 Establishment of hairy root cultures

The hairy roots were excised and the root tips were cultured on MS + 250 mg l^{-1} cefotaxime. Among the cultures, some hairy roots showed faster growth with high lateral branching whereas some hairy roots showed only slower growth. At the same time some roots failed to grow and later the colour turned brown (Plate 13).

The establishment of hairy root cultures induced by ATCC 15834 was assessed. It was noticed that among the 38 root clones inoculated, seven root clones showed fast growth, 20 root clones produced only slow growth, whereas 11 root clones showed no growth at all. The hairy roots, which showed faster growth produced on an average 29.5 root tips with in 25 days.

The hairy roots normally showed a plagiotropic growth habit while some showed reduced geotropism. Variation was also noticed in the number of root hairs among different root cultures. The hairy roots were white or creamy white in colour and the colour of the roots changed gradually from white to brown or reddish brown, almost 25-28 days after incubation.

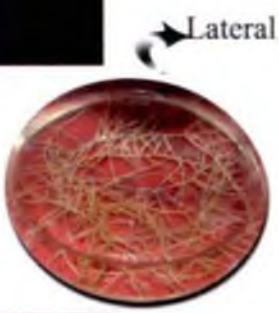
The control roots were non hairy or with least hairiness and are positively geotropic in nature. They grew slowly and the lateral branching was less compared to the transformed roots. They showed early browning and perished after 2-3 sub cultures in growth regulator free MS media.

4.3.5 Rapid culturing of hairy roots

Transformed hairy roots (~0.3 g) were cultured in shake flasks for further scaling up. The final fresh weight of hairy root clones of A4 strain in half MS 25 days after culturing is shown in the Fig. (7).

The growth of root clones differed in the same culture medium. The fresh weight of hairy root clones ranges from 2.12 to 3.52 with a mean of 3.03 g/125ml.

A4 derived hairy root clone



ATCC 15834 derived hairy root clone

Plate 13. Establishment of hairy root cultures in *W. somnifera*

The control roots showed an average of 1.75 g/125 ml in the same medium (Plate 14).

Compared to ATCC 15834 induced hairy roots, the hairy roots induced by A4 strain showed faster growth, producing more biomass.

In comparison with roots in the solid culture, hairy roots cultured in liquid growth regulator free half MS medium grew rapidly and had high lateral branching, but with increasing incubation time, the initial hairy roots incubated and the culture medium turned brown.

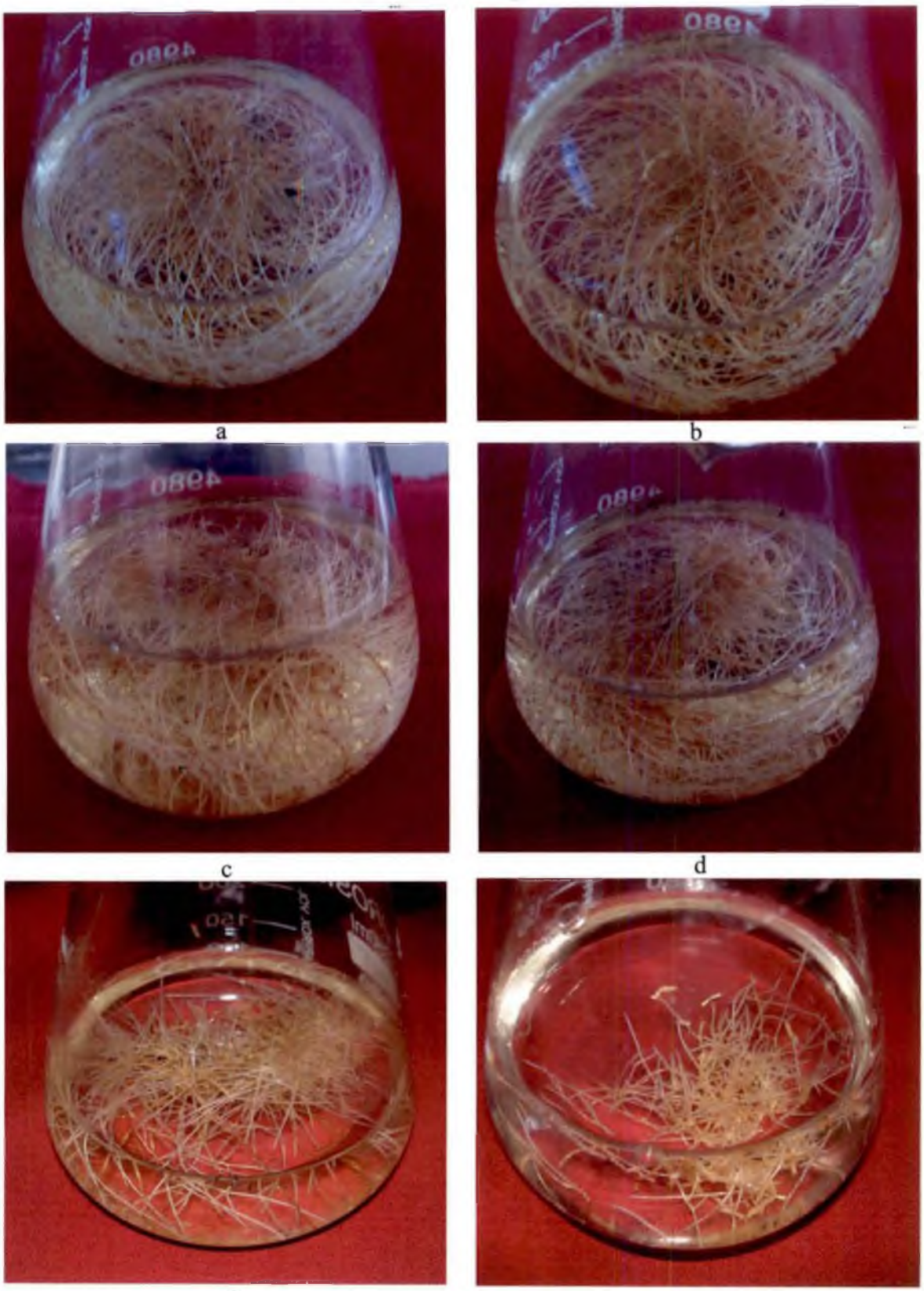
4.3.6 Effect of culture media and conditions on the growth of hairy roots

Among the four liquid media tested, half MS medium was found to be superior for promoting hairy root growth followed by MS, B5 + 2.0 per cent sucrose and B5 + 3.0 per cent sucrose respectively. In B5 media, bulging of the roots was observed. In B5 + 3.0 per cent sucrose, the roots showed callusing in addition to bulging and the growth was found to be very poor in this culture medium (Plate 15). In half MS and MS media, roots were of normal thickness, characterized by very fast growth and high lateral branching. In all media the newly growing regions of hairy roots were creamy white in colour, whereas the initial roots incubated turn brown.

The effect of culture media on the growth of hairy roots is shown in the Fig. 8.

4.3.7 Growth pattern of hairy roots

From the growth curve of hairy roots, it was observed that the root growth was nil in the first four days and was very slow up to 10 days. However hairy roots grew faster and subsequently lots of lateral branches were produced within 12-15 days of incubation and the growth was very faster in the next 15-20 days. After 20 days of culture, the growth rate of hairy roots began to slow down, but the biomass of hairy roots still increased until 25 days of culture. After 25 days, the hairy roots



a - e hairy root clones, f - normal root (control)

Plate 14. Further growth of A4 derived hairy root clones of *W. somnifera* in half MS media

B5 + 2 % sucrose

← ATCC 15834 derived hairy root clones

↓

B5 + 3 % sucrose

B5 + 2 % sucrose

↑

A4 derived hairy root clones →

B5 + 3 % sucrose

Plate 15. Effect of B5 basal media with different sucrose concentrations on the growth of hairy roots

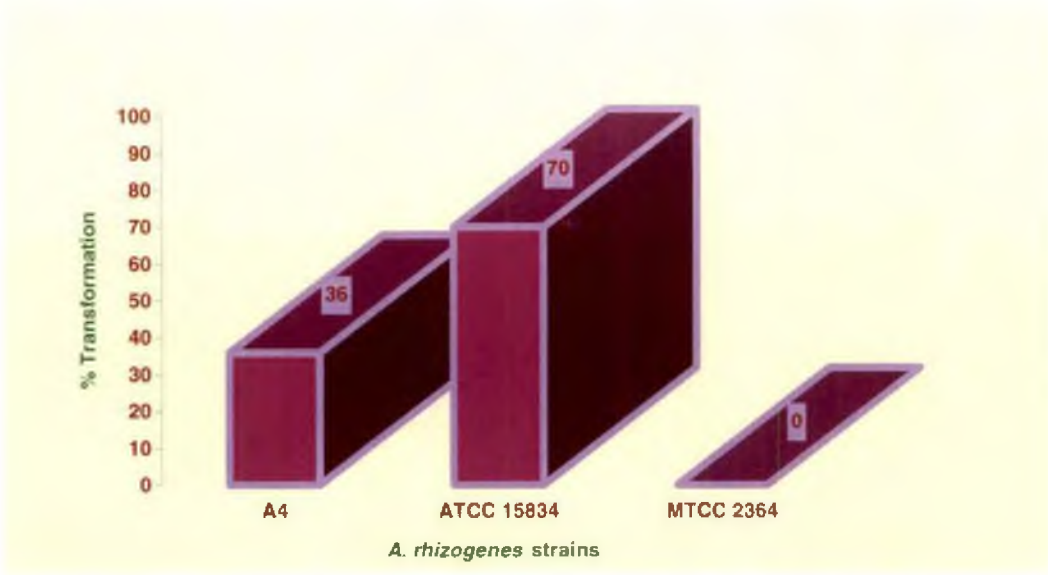


Fig. 6. Efficiency of *A. rhizogenes* strains in inducing hairy roots

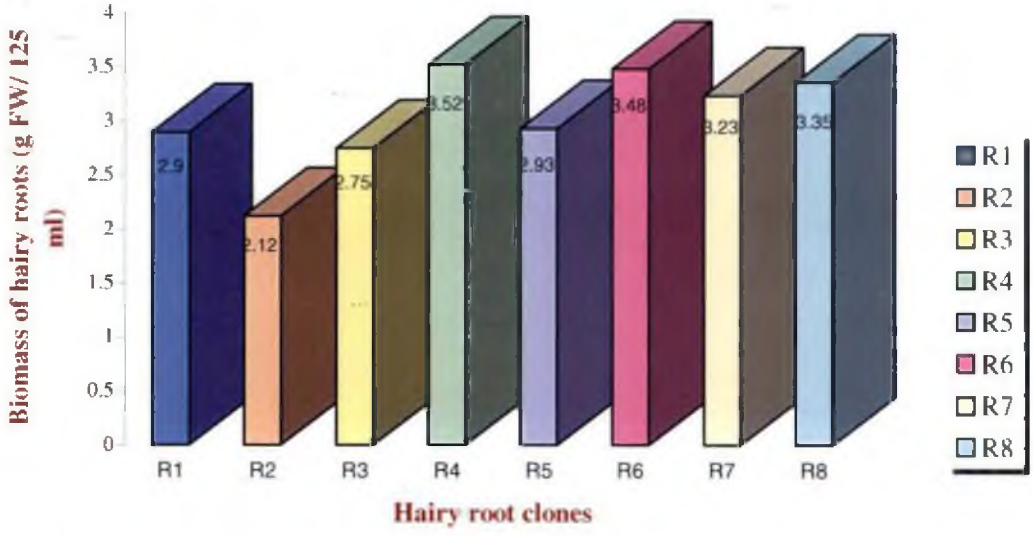


Fig. 7. Variation in biomass production within A4 derived hairy root clones

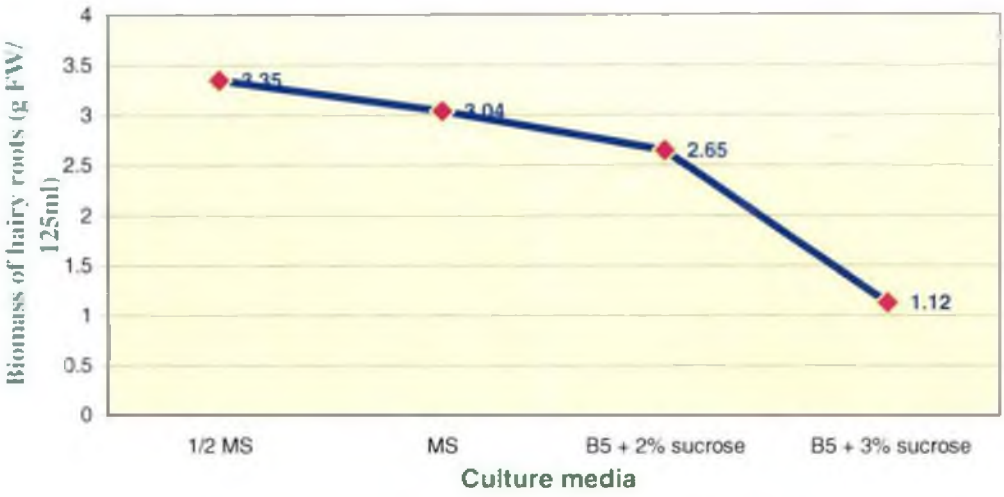


Fig. 8. Effect of culture media on the growth of hairy roots

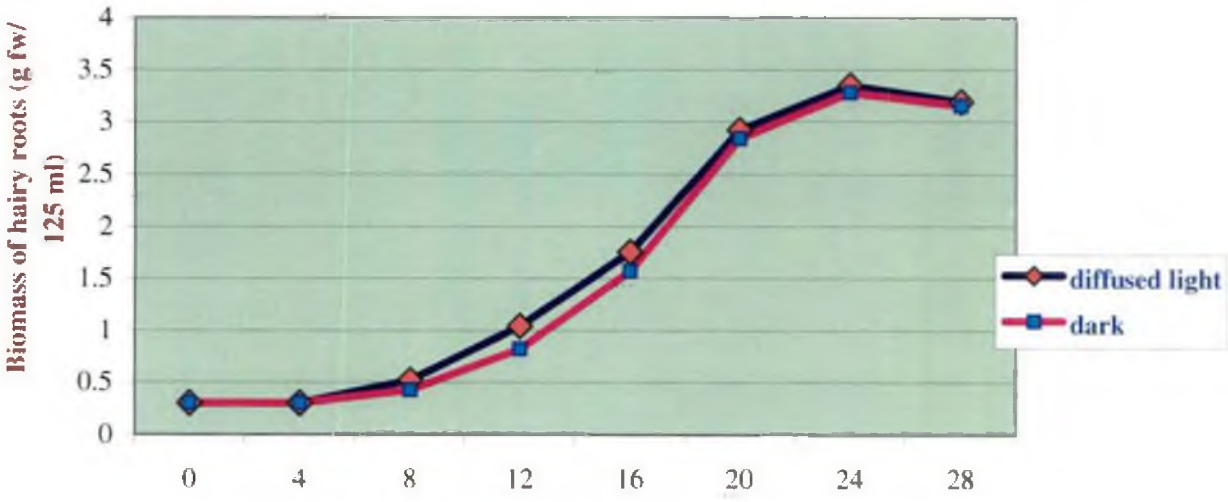


Fig. 9. Growth curve of *W. somnifera* hairy roots under diffused light and dark

gradually changed the colour from creamish white to brown and the biomass began to decrease thereafter.

The growth of the roots was initially slow under dark photoperiod. However by the end of 25 days, roots produced almost equal (3.28 g FW/125 ml) compared to roots incubated under diffused light (3.35 g FW /125 ml). The hairy roots exhibited a sigmoid growth pattern (S- shaped curve) (Fig. 9).

4.3.8 Growth rate of hairy roots

The growth of seven hairy root tips from a single root clone inoculated at the fifth subculture cycle in half MS + 3.0 per cent sucrose and B5 + 2.0 per cent sucrose are shown in the Fig. 10

The fresh weight of hairy roots 25 DAI (1.24g) was 15.5 times more than its weight on 15 DAI in half MS medium, where as the fresh weight was 19 times more in B5 medium (0.95 g). The dry weight was found to be 4.38 times less than the fresh weight. The growth of the hairy roots was at the rate of 0.116 g/ day (between 15-25 days) in half MS and each root tip grew at the rate of 0.016 g / day. In B5 the root grew at the rate of 0.09 g/ day and each root tip at the rate of 0.012 g/ day.

4.4 CONFIRMATION OF TRANSFORMATION

4.4.1 Morphology of hairy roots

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. The hairy roots showed a faster growth rate compared to control roots or non-transformed roots. The hairy roots produced high lateral branching with secondary and tertiary branches that are normally hairy with plagiotropic growth habit and some showed reduced geotropism (Plate 13). Normal roots or control roots were positively geotropic with less branches and root hairs.

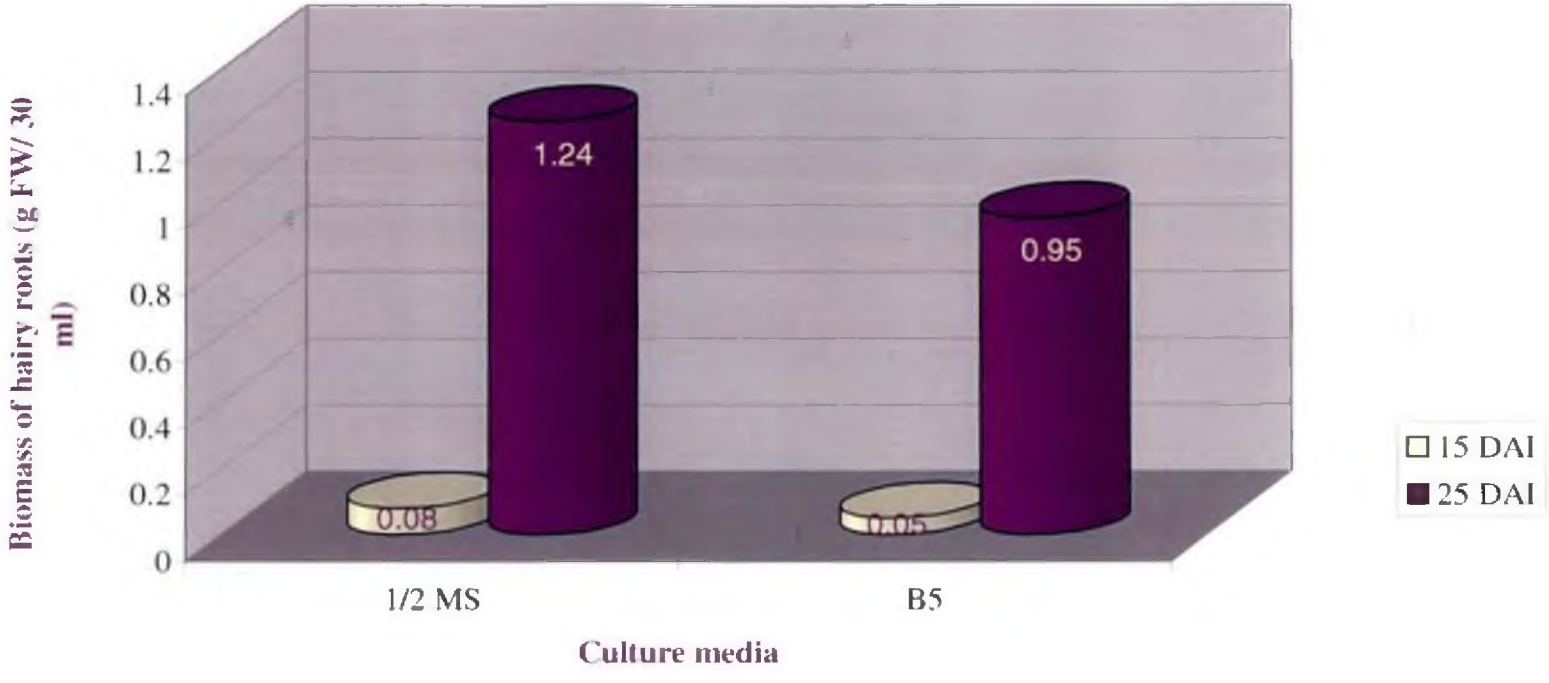


Fig. 10. Growth rate of seven hairy root tips in half MS and B5 media

4.4.2 Opine analysis

Transformation of plant tissues by *A. rhizogenes* could be confirmed by biochemical detection of the production of opines in root tissues. Agropinic acid, mannopinic acid and mannopine were used as standards of opines. In the first method, opines were extracted using water alone and in the second method 0.1M HCl was used to extract the opines.

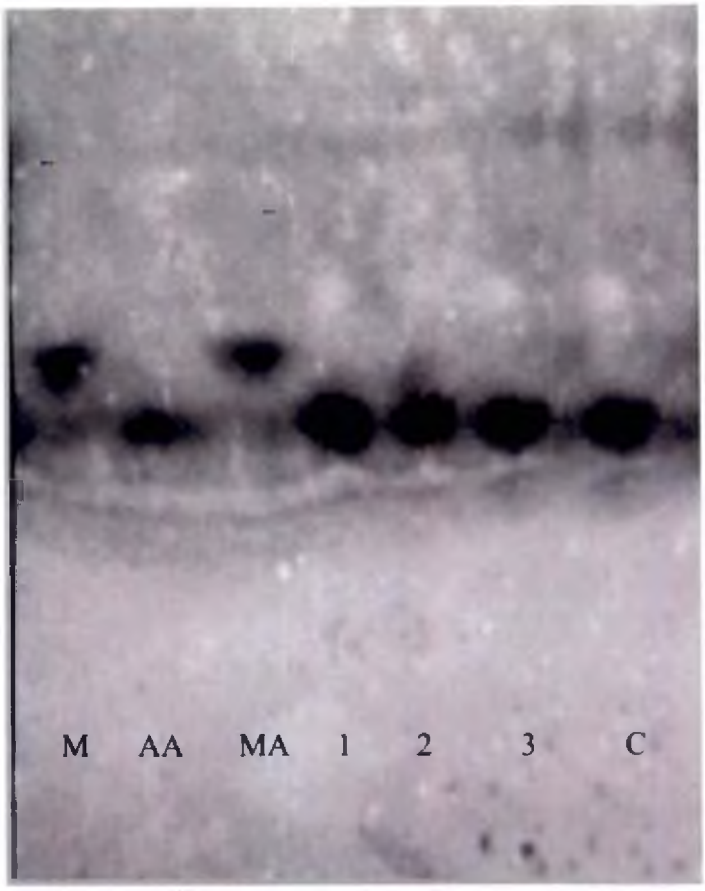
Following the first procedure, the opines were extracted from A4 and ATCC 15834 induced hairy roots and also the roots produced by control explants and MTCC 2364 infected explants.

The response of transformed and normal roots to the presence of opines is given in the Plate 16.

It was noticed that both the transformed and control roots produced spots at positions corresponding to agropinic acid after silver staining. No spot was produced at positions corresponding to mannopine and mannopinic acid. To verify, the opines were also extracted from *in vitro* control roots and shoots and the from the roots and shoots of field grown plants and it was found that all the extracts produced spots in positions corresponding to agropinic acid.

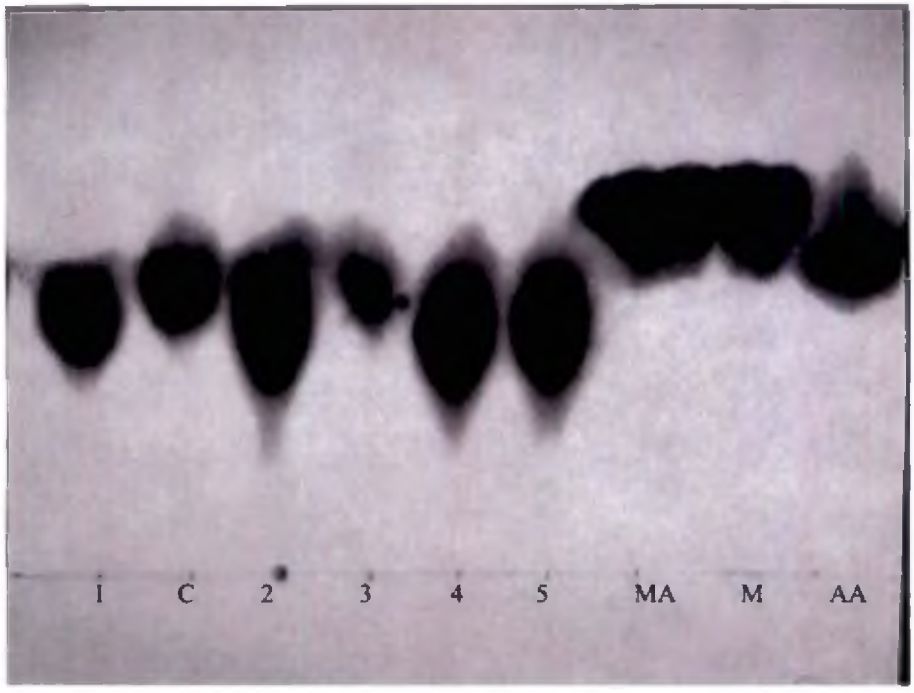
The opines extracted following the second procedure using 0.1M HCl also showed similar results. Here, opines were also extracted from the callus formed at the wound site of leaf segment after infection with ATCC 15834.

All the extracts produced spots near the positions corresponding to agropinic acid and no spots were observed at positions corresponding to mannopinic acid and mannopine. But the intensity of the spots showed variation. The opines extracted from roots produced by control explants and MTCC 2364 infected explants and the leaf callus showed only less intense spots compared to transformed roots.



M - Mannopine
AA - Agropinic acid
MA- Mannopinic acid
1 - A4 derived root clone
2 - ATCC 15834 derived root clone
3 - Root from MTCC 2364 infected explant
C - Control roots (normal)

(Opines extracted using water)



(Opines extracted using 0.1 M HCl)

1- Root from MTCC 2364 infected explant, C- Control roots (normal), 2 - ATCC 15834 derived root clone, 3- ATCC 15834 derived leaf callus, 4, 5- A4 derived root clone, MA- Mannopinic acid, M- Mannopine, AA- Agropinic acid

Plate 16. Detection of opines by High Voltage Paper Electrophoresis

Confirmation of the transformation by detection of opines using high-voltage paper electrophoresis was found to be unsuccessful because of the existence of interfering substances, which produced spots near the positions of agropinic acid after silver staining. Therefore the DNA was extracted from roots and subjected to PCR and Southern hybridization.

4.4.3 Confirmation by PCR analysis

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

4.4.3.1 Isolation of genomic DNA

Upon electropohoresis on one per cent agarose gel, intact DNA was observed in all the wells (Plate 17).

4.4.3.1 Isolation of cosmid from E.coli

The cosmids pLJ1 and pLJ85 were isolated from *E.coli* and cosmids had high molecular size (Plate 18).

4.4.3.3 PCR analysis of rol B and rol C genes

PCR analysis was carried out using three sets of primers, Rol BF1R1, Rol BF2R2 and Rol CF1R1. The output of primer 3 soft ware using which Rol BF2R2 primer was designed is shown in the Fig. 11.

The alignment of forward and reverse primers of primer sets, Rol BF1R1 and Rol CF1R1 with T_L- DNA for *rol* B and *rol* C genes of pRiA4 plasmid is given in Appendix VI. The amplified DNA samples electrophoresed on 0.7 per cent agarose gel is given in Plate 19.

Amplification was obtained with Rol BF2R2 and Rol CF1R1 primer sets which confirmed the presence of *rol* B and *rol* C genes in the transformed roots as well as positive control. Amplification with Rol BF2R2 primer set corresponding

Fig. 11. Output of Primer 3 software

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Primer3 Output
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PRIMER PICKING RESULTS FOR A4

No mispriming library specified
Using 1-based sequence positions
WARNING: Unrecognized base in input sequence

OLIGO            start  len      tm      gc%    any    3'  seq
LEFT PRIMER       2604   20    60.02    55.00   7.00  2.00
GAAGCCTGCTGCAGTAAACC
RIGHT PRIMER      2812   20    59.99    50.00   4.00  0.00
TTCAGCAGCAGGATCAACAC
SEQUENCE SIZE: 2951
INCLUDED REGION SIZE: 2951

PRODUCT SIZE: 209, PAIR ANY COMPL: 8.00, PAIR 3' COMPL: 0.00

1    TTCAAAAAAAGCGCGATGAAATCAAGTATCCAGTTATTAAACCAGATCTTGATTTTTATT
61   ACATTAATACTTCTTAAAGGGAAACAAGTGACACACTCAGCTTCTCCTGACATCAAACTC
121  GTCAGCCCATCGACTAACCATTAGCCGATTGCAAACTTGCACTCGCCATGCCTCACCAAC
181  TCACCAGGTTCGAACCTAAGCTGGGTGCTGGCATAGAGGTCGAATGTCAGACACTTTCCC
241  TTTGTCGAAGTTAGCTCCATCTGCTCATTCAGCTTGATGACACGCCCAGGGAAAGAAAAT
301  GCAGCGAAGTAGGCGCTCCGCATAGGATCCGCGTAACCGAAAAAACCTGATGCTTGCCTG
361  AGCCCTCTATTGACCTCATCGAGAGTCACATCATGCTGGTACGGGGGAAGGTTGGTGAGG
421  AGTGCGCCTTGCATCCAATTGTAAGGCAAGGACCCTCCATAGAAGCAGAGCATCATCGTC
481  GGGCAGTCGACGTAGAGGTACAGCAGCCCGTGGCCCACCGACCCTCCTTCTTCGTCAATA
541  TCCATCGATTGATTCTCACCCGGCTCGATCAAGGGTTTACGCTCATTTGAGGCGTTCTTC
601  ATGTGTCTAGCTAGCTCATCGCTGCTTGTCACATCCTCCACTTTGAGCTTGAAAAAGAGA
661  GAACACAGGTCGTCTTCAGCCATGTTAACAAAGTAGGAAACAGGTTGCTTGTACCCATAA
721  CTCGAAGCATCCAAACTATTTATAATCCAGAGCTCTTAGCGTATTGGAGCAAATAATAAA
781  AATATTTGAGTTAGGTATAGGTCCACAAATTTATTAATTAATGGATGCGGCTGTCGATGC
841  TCCACGTCGCCTCGTTGTGCGAATATAGAAGGGACACCTGCCTGCTTTTAATTGTTTTTA
901  CTTATTAGGTTTAAAGCTCAGCGACCCACGTGAGGGAATAGGTTCTGTCCTCCAATAATG
961  GTGGTTTGTGGTCTATGGAATCTCAGCTTTTGCTTTTTCCTTTAATAATGGGACGCACCC
1021 GCCTACCTTTATTTATGCTGCGGAAAGGCGCTACTTTGTCAAGCTAAACCCCACGTTAGG
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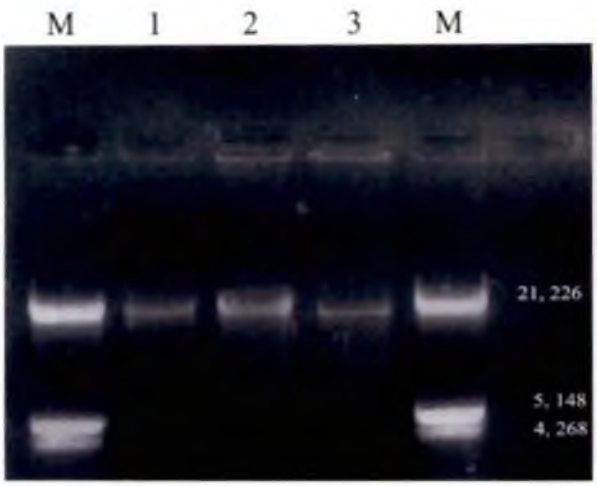
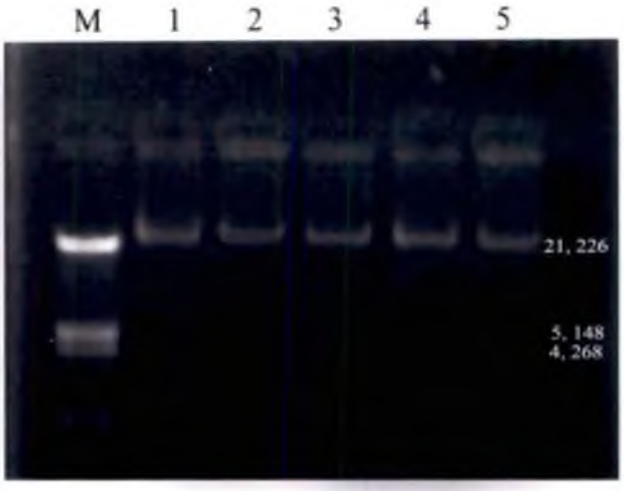
1081 CCTGTCGGCACCAAAGCAACACAGCTGGCAATTTATTATAGGGTCTCTTAAAAGCACGTA
1141 CATGGCCACCATTCCTTCTTAGGCTAACTGCTATTCGCCCTGCTTATTTATTTTTTATTA
1201 AACTCAGAAACAGAGATAATAGTTTTGCACCCACTAAACTAGTGTAAATTTGGCATATAT
1261 CTATAGCAGGTTCTCCAAAACCATGTATTTCCTTTTGGAATAATTTATTCAACACGATAA
1321 TCGCTTAAACGTATTCCCGATTGAAAAAGATTAACACATATTATTATTGCTATTATTAAT
1381 ACATCATTTTGGCATAGATAATTTCCGCACTATATTACATATACAATGTTAGTAATTAAT
1441 ATGATTGTGGCAATACTTTACATCTTAACCAGAAAATAGAAAAAGTATCTTGAATGCAGC
1501 AATCTTCACGTTTGCCAATGCGCTTTCGCGAAATCCAATAGCGGGCCAACTTTAAGCTTT
1561 ACCCCACTCCTCCCAATTATGGGCATCCTTTTTTGACATCCTTTCGCTAAGGCCAAACAA
1621 CACGACAAGAAAAAGCAAACCCAGTACTATGAACCCATGAACGATCACGCATGCAGTATT
1681 TTCGCTTGATCCAGATTTTAAGGCCTCCCGTGTCTTGCGCATCGATCTTGATGTGATTGT
1741 TGCCAAACTACCTCTGGCTTTCCCGACCAGAGACTGTGTCTGATTGGCGCCAGTTTCCGA
1801 CGCCGTATTTTAAATGGCCCTGGCACTTGCCTTTTTCGTAACTATCCAACTCACATCACA
1861 ATGGATCCCAAATTGCTATTCCTTCCACGATTTCAACCAGTAGATCTCACTCCAGCATGG
1921 AGCCAGATAAACCTATTCGAGGGGATCCGATTTGCTTTTGCAATCTATAGCCGTGACTAT
1981 AGCAAACCCCTCCTGCATTTCCAGAAACGATGGGCTCTTGCAGTGCTAGATTTGAAGGAA
2041 AACTCTCCACCGATATATATACTTAAACAACTAGCTGAGCTCTTGAAGAACAAAGTCTGC
2101 TATCATCCTCCTATGTTTGTTAGTCAGCCGGATCTGGCTCGAGAAAACGACCAACATGTA
2161 TTTGTCTATCTTTCTCGCGAGAAGATGCAGAAAGTGCTGAAGGAACAATCCATTACATTT
2221 GGAATGGAGGCCGTGCTGGCGACAACGATTCAACCATATCGGAGCGAGCTCGCCCTCCAG
2281 GAGATGCTCCGTGTTCACAACCTTGCTTGGCCGCACAGCCGCACGGAGGAACCTGATTTA
2341 GAATGCTTCATCGCCATTTTCGCAAGTTCCTTGTTCATTCACTTGCTGGAGTTAAAAGTG
2401 ACCAACGTTTACGGGAGAGAGGTAGCTTGCACCTTCTTTCTGCGGCGAGGGACTGAAAAC
2461 CGCCCCTATGATGTTGTAGCTTGCGGCACCACACAATTCACCAAAAATGCCCTCGGGATA
2521 TCACGTCCGGCCGCCTCCTCACCGGAGCCAGACCTAACCCTGCGACTCTCGGGGCCTGAT
2581 CAGGAAGGCGAGGAGGGCGTCATGAAGCCTGCTGCAGTAAACCTGAAGAAAGAAGCCTAA
                          >>>>>>>>>>>>>>>>>>>>
2641 AGCCGACTTGAACTCCCCCTGCAGGCAACCTTTATCTATGAGTTTGTCATAAGTTCTATG
2701 TACCCTCCCGCAGTCTGTGACACAGAACCTTGGGAGTTGTAGCGTACGTTGTAATGTGTT
2761 GACCTATTTTCTTGTACTAAATATTTTCTTCTGTGTTGATCCTGCTGCTGAATTTTGCCA
                                   <<<<<<<<<<<<<<<<<<<<
2821 AAAACAGCACATGCTCATATGACTATCTAATCTACTACACATATATTGCAGTATCAACAA
2881 CAACGACACACCTGGACTTATAATATTATAGTTCAACAGTACATTTGACATAAAACATTT
2941 TCACGACAT//
KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer
```



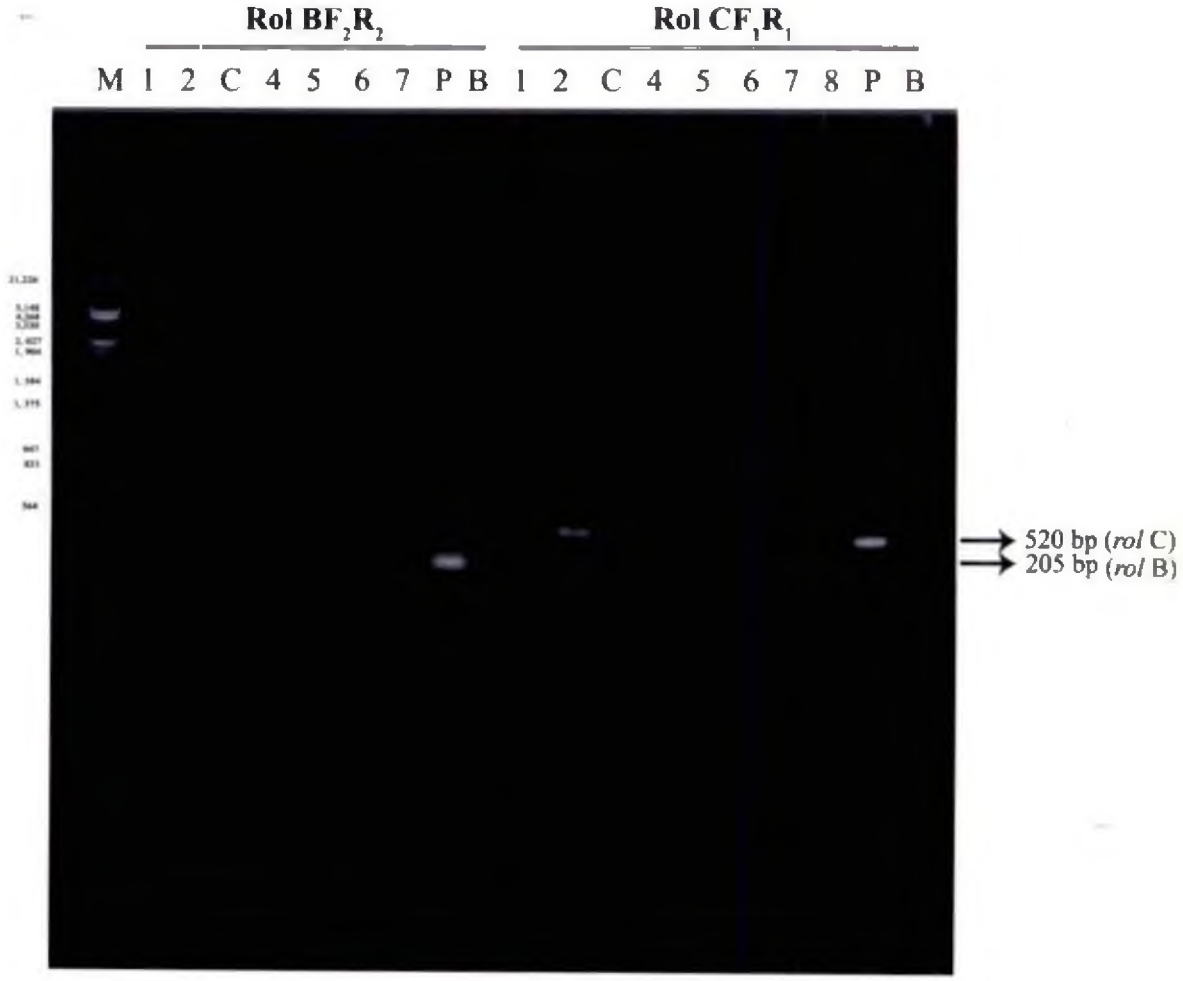
M- Molecular weight marker lambda DNA/ *Eco*RI+ *Hin*d III digest, 1,2,3- hairy roots

Plate 17. Isolation of genomic DNA from hairy roots



1, 2, 3- pLJ1 cosmid, 4, 5- pLJ85 cosmid

Plate 18. Isolation of cosmids



M- Molecular weight marker lambda DNA/ *Eco*RI+ *Hin*d III digest, 1,2- A4 derived root clones C- Control roots (normal), 4- Root from MTCC 2364 infected explant, 5, 6, 7, 8 - ATCC 15834 derived root clone P- Positive control (pLJ1), B- Blank (without DNA)

Plate 19. PCR analysis of hairy roots for *rol* B and *rol* C genes

to *rol* B gene produced a 205 bp band for the transformed roots (A4 & ATCC 15834) and for the cosmid pLJ1. When Rol CF1R1 primer set (*rol* C) was used for amplification, a 520 bp band was visualized for transformed root clones (A4 & ATCC 15834) and cosmid pLJ1.

No amplification occurred in negative control as well as roots obtained from MTCC 2364 infected explants. These results indicated the non-transformed nature of roots produced from the MTCC 2364 infected explants.

The transgenic nature of the hairy roots was thus confirmed by PCR analysis.

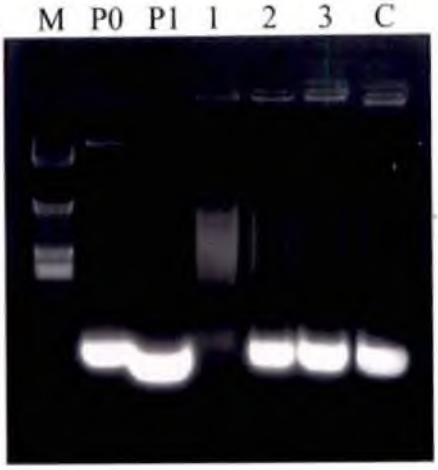
4.4.4 Southern hybridization

On viewing the gel using UV transilluminator, it was observed that the entire DNA after restriction digestion with *Bam* H1, appeared as a smear and no separate intact bands are visible (Plate 20).

Southern hybridization was carried out further for confirming the presence of *rol* B and *rol* C genes of TL-DNA in the transformed roots. The regions of hybridization were detected by autoradiograpy. On developing the film after the required exposure time, it was observed that clear intact band was produced only in the region corresponding to positive control and no other sample DNAs produced band. When PCR amplified product obtained using Rol BF2R2 primer set was used as the probe, a single intact band was produced where as the PCR amplified product of Rol CF1R1 produced two separate bands (Plate 21).

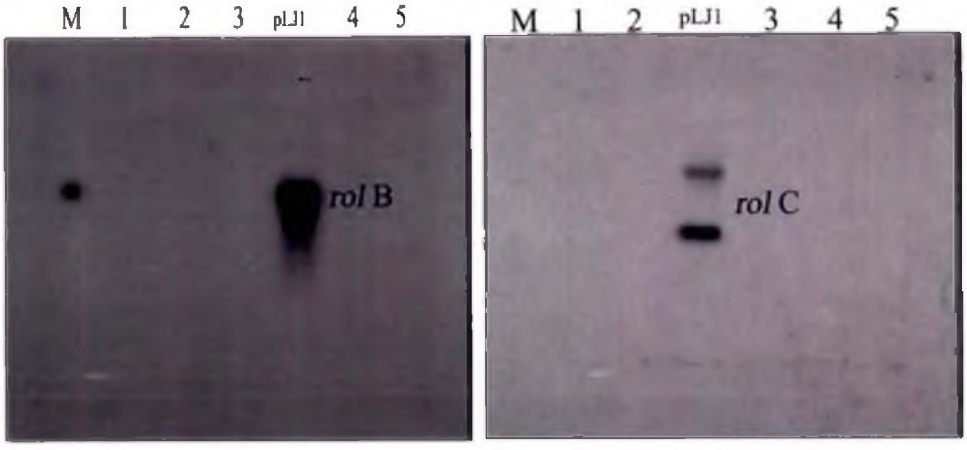
4.4.5 Dot blot analysis

In dot blot analysis DNA was directly loaded on to nitrocellulose membrane. On using Rol BF2R2 and Rol CF1R1 primer sets derived probe of pLJ1 cosmid, positive signal (dot) of radioactivity was obtained from A4 and 15834 induced hairy roots, whereas roots produced by MTCC 2364 infected explants and control roots showed no signal. Positive control produced darker spot



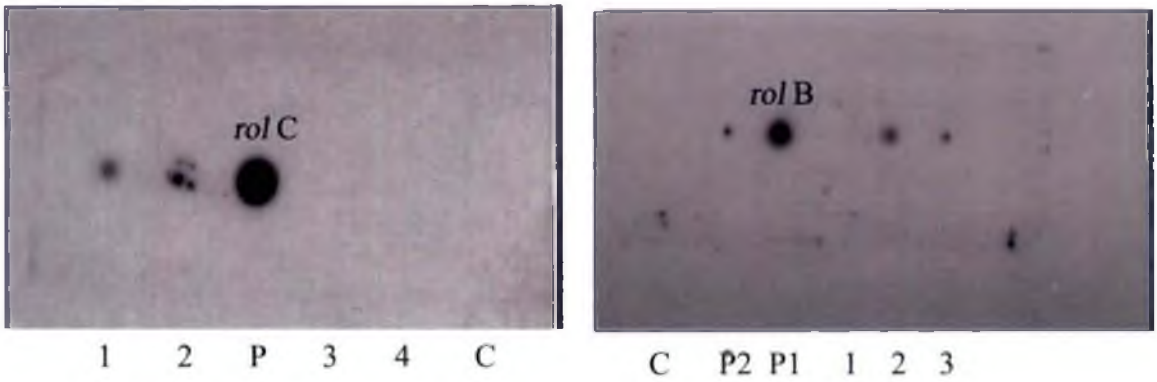
M- Molecular weight marker lambda DNA/ *Eco*RI+ *Hind* III digest, P0- unrestricted pLJ1, P1- restricted pLJ1, 1- A4 derived root clone, 2 - ATCC 15834 derived root clone, 3- Root from MTCC 2364 infected explant, C- Control roots (normal)

Plate 20. Restriction digestion of genomic DNA and pLJ1 cosmid



M- Molecular weight marker lambda DNA/ *Eco*RI+ *Hind* III digest, 1,2- A4 derived root clone, 3- ATCC 15834 derived root clone, pLJ1- positive control, 4- Root from MTCC 2364 infected explant, 5- Control roots (normal).

Plate 21. Autoradiagram of *Bam* H1 restricted DNA of hairy root clones



1- A4 derived root clone, 2- ATCC 15834 derived root clone, P- Positive control (pLJ1), 3,4- Root from MTCC 2364 infected explant, C-Control roots (normal)

C-Control roots (normal), P2- pLJ85 cosmid, P1 Positive control (pLJ1), 1- Root from MTCC 2364 infected explant, 2- A4 derived root clone, 3- ATCC 15834 derived root clone

Plate 22. Dot blot analysis of hairy roots for *rol* B and *rol* C genes

compared to others. This confirmed the presence of TL-DNA with *rol* B and *rol* C genes in A4 and ATCC 15834 induced hairy roots (Plate 22).

4.5 STANDARDIZATION OF WITHANOLIDE ESTIMATION

4.5.1 Standardization of withanolide extraction

Thin Layer Chromatography was employed for the estimation of withanolides (Plate 23).

The *W. somnifera* root powder on refluxing with hexane, the fats, waxes and oils present in the root material get solubilized. Hexane vaporizes at 65-70 °C and so, attaching a condenser to the resfluxing flask helped to keep the volume intact. On analyzing the extract obtained after refluxing with hexane by TLC, it was observed that a spot corresponding to withaferin A was produced by the extract.

When the ethanol extract powder was treated with HCl followed by ethyl acetate, alkaloids formed hydrochlorides and withanolides come to the ethyl acetate. This produced a clear fraction containing withanolides and a cloudy fraction containing alkaloids. Each fraction was collected separately over anhydrous sodium sulphate. Anhydrous sodium sulphate absorbed the residual moisture and makes the fractions clear. The solvent from the withanolide fraction was removed over a water bath.

The pH of the alkaloid fraction was made alkaline (pH 10) by the addition of ammonium solution. At this alkaline condition, alkaloids came out of the hydrochlorides and became free alkaloids. The addition of chloroform in the separating funnel facilitated the solubilization of the alkaloid. Addition of sodium chloride removed the cloudiness and makes the lower fraction (alkaloid) clear by absorbing the moisture. The supernatant was vaporized in a water bath so as to remove chloroform.

The withanolides and alkaloids in ethanol were spotted on the TLC plate separately. The standard corresponding to withaferin A was also spotted. On developing the plate, it was found that alkaloids and withanolides developed separate spots.

In another TLC plate, the ethanol extract of the root powder (without separating withanolides and alkaloids) along with withanolides in ethanol, alkaloids in ethanol and withaferin A standard were spotted separately. It was found that the ethanol extract of root powder produced separate spots corresponding to withaferin A and alkaloids. However, the presence of alkaloid is in no way interfering with the development of spot corresponding to withferin A. Hence it can be concluded that the separation of alkaloid is not necessary for the estimation of withanolides.

4.5.2 Selection of TLC plates

In RP_{18} F_{254} plate the spot corresponding to withaferin A was observed as brown spot in an yellow background whereas in Silica gel_{60} F_{254} plate, under UV, corresponding to withaferin A, a black spot was observed in a green background. Both types of plates were found to be good in withnolide analysis. Since RP_{18} F_{254} plate with methanol: water solvent system was costly, Silica gel_{60} F_{254} plate was used in further analysis.

4.5.3 Standardization of spray reagent

Two types of spray reagents were tried separately for increasing the sensitivity. On using $FeCl_3$ + Water + Acetic acid + Conc. H_2SO_4 spray reagent, spots after charring appeared brown. The spray reagent vanillin + boric acid + Conc. H2SO4 + MeOH was found to be more sensitive. By using this reagent the spots corresponding to different metabolites appeared in different colours like black, magenta, violet, brown etc. and the spots were more clear and visible to eyes. Hence this reagent was used in the further studies.

4.5.4 Estimation of withaferin A from fresh root samples

Estimation of the withaferin A content of fresh root samples including roots from field grown plants, non transformed *in vitro* roots and transformed hairy roots was carried out. On analyzing the developed plate it was found that five spots of different colours developed in all the three samples, however roots from field grown plants produced an additional maroon spot of Rf value 0.68. The colour of each spot and the corresponding Rf value is given in the Table (27).

Table 27. Colour of each spot and the corresponding Rf value

Sl. No. of spot	Description of spots			
	Standard withaferin A		Ethanol extract of root biomass	
	Rf	colour	Rf	colour
1			0.78	Magenta
2			0.65	Black
3	0.56	Magenta to bluish violet	0.56	Magenta to bluish violet
4			0.23	Blue to Black
5			0.17	Brown

The spot corresponding to withaferin A standard was initially magenta and on further charring the colour turned to bluish violet (Rf: 0.56). The other spots developed might be other metabolites present in the root including the alkaloids. However, due to the lack of standards corresponding to each spot, what each spot represents is still unclear.

The SPOT DENSO tool present in the Tool box 3 of the Alfa Imager was used in withaferin A quantification. The amount of withaferin A content in different fresh root samples obtained by using TLC densitometry analysis is given in the Table (28).

Table 28. Amount of withaferin A in different samples – TLC densitometry analysis

Sl. No.	Sample	Values in mg g^{-1}
1	Roots of field grown plants	0.258 (0.025)
2	Normal in vitro roots	0.174 (0.017)
3	Hairy roots	0.189 (0.019)

% value is given in paranthesis

4.6 ENHANCEMENT OF SECONDARY METABOLITE PRODUCTION

4.6.1 Addition of osmoregulants

The addition of osmoregulant PEG (6000g) Silica gel_{60} F_{254} was found in no way affecting the hairy root growth. The root cultures remained white or creamy white and were healthy in this stress media. The root culture in 5.0 per cent PEG developed slight creamy brown colour.

However, the osmoregulant PEG at 2.0 and 5.0 per cent failed to elicit a positive response in the biosynthesis of withaferin A in root cultures. The stress medium in which the roots were cultured, was also subjected to TLC analysis. But the extract obtained from the media was highly viscous; hence normal running was not obtained in a TLC plate. So withanolide analysis could not be performed in this medium.

4.6.2 Addition of precursors

The hairy root culture grown in half MS supplemented with 1.0 m*M* methionine showed good growth. The lateral branching was prominent here and the biomass of the roots increased progressively in this culture medium compared to half MS medium with 2.0 m*M* methionine. However the withaferin A content was almost similar to control hairy roots in all the combinations. No positive influence in the biosynthesis of withaferin A was observed here.

On TLC analysis of the culture medium, (after separating the hairy roots) corresponding to the position of withaferin A standard (magenta colour), yellow coloured spots developed in the control media as well as in methionine containing media.

4.6.2 Addition of elicitors

4.6.2.1 Elicitation by Aspergillus homogenate

Two types of biotic elicitors, *Aspergillus* homogenate and yeast extract was used in the elicitation studies. Elicitation by *Aspergillus* homogenate at the rate of 250 µl/ 125ml as well as 500µl/ 125 ml elicited a positive response on biosynthesis

of withaferin A in the hairy roots of *W. somnifera*. The accumulation of withaferin A increased progressively in the hairy roots. The metabolite was found to be released into the media as well. The *Aspergillus* homogenate at the rate of 250 µl/ 125ml was found to be the optimum concentration compared to 500µl/ 125 ml in the enhancement of secondary metabolites.

Aspergillus homogenate at the rate of 250 µl/ 125ml produced 2.25 times increase in withaferin A content (0.436 mg/ g) over control hairy roots. The homogenate at the rate of 500µl/ 125 ml produced 1.77 times increase (0.334 mg/ g) in withaferin A content.

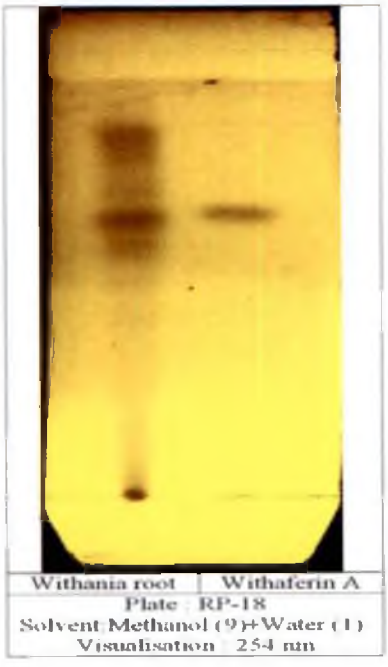
The culture media was also subjected to TLC analysis after separating the roots. Magenta coloured spot corresponding to withaferin A was produced by 250 and 500 µl *Aspergillus* homogenate media samples. The control media produced yellow coloured spot instead of magenta spot in position corresponding to withaferin A. Withaferin A content in 250 and 500 µl *Aspergillus* homogenate media samples was 0.088 µg ml^{-1} and 0.078 µg ml^{-1} respectively.

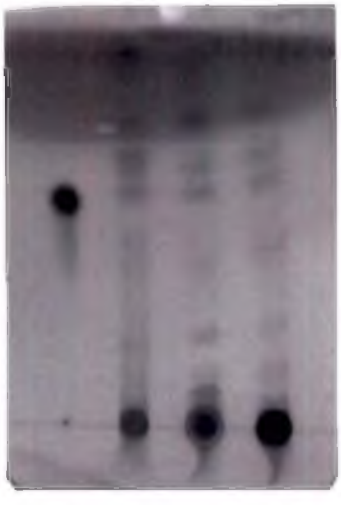
4.6.2.2 Elicitation by yeast extract

The hairy root cultures showed reduction in the accumulation of withaferin A content on adding yeast extract at the rate of 2.5 and 5.0 g l^{-1}. The root cultures supplemented with 2.5 and 5.0 g l^{-1} produced 0.008 per cent and 0.004 per cent withaferin A respectively. The withaferin A content (0.017) of 21 days grown control hairy roots was found to be slightly lower than 28 days grown control hairy roots.

The culture media, when subjected to TLC analysis, it was found that, in all the samples including control only a light yellow spot was present instead of magenta spot in position corresponding to withaferin A.

Amount of withaferin A in hairy root samples after elicitation by *Aspergillus* homogenate and yeast extract is shown in the Table 29.





Std FR HR IR

Std- Withaferin A standard,
FR- Field root , HR- Hairy root,
IR- *In vitro* root

Std HR AH_{250} AH_{500} M

Std- Withaferin A standard,
HR- Hairy root, Hairy roots -
(AH_{250} - *Aspergillus* homogenate 250 mg l^{-1}, AH_{500} - *Aspergillus* homogenate 500 mg l^{-1},
M- 1mM methionine)

Plate 23. Thin Layer Chromatography of *W. somnifera* root extracts for withaferin A estimation

Table 29. Quantitative estimation of withaferin A content in elicitor treated hairy root samples

Culture	Sample name	Withaferin A content ($mg\ g^{-1}$)
Root biomass	S_{AH}-250	0.436 (0.043)
	S_{AH}-500	0.334 (0.033)
	$S_{Control}$ (28days)	0.189 (0.019)
	S_{YE}- 2.5	0.086 (0.008)
	S_{YE}- 5.0	0.043 (0.004)
	$S_{control}$ (20 days)	0.174 (0.017)
Media	M_{AH}- 250	0.088 $\mu g\ ml^{-1}$
	M_{AH}-500	0.078 $\mu g\ ml^{-1}$
	Control	0.000 $\mu g\ ml^{-1}$

(AH-Aspergillus homogenate, YE-Yeast extract, % value is given in paranthesis)

Through out the enhancement studies, when the root culturing media was subjected to TLC analysis, five different coloured spots were produced corresponding to different metabolites. The colour of each spot and the corresponding Rf value is shown in the Table 30. In all the samples except *Aspesrgillus* homogenate supplemented media, yellow spot was produced instead of magenta spot in position corresponding to withaferin A.

Table 30. Colour of each spot and the corresponding Rf value- culture media

Sl. No.	Colour of spot	Rf value
1	Black	0.64
2	Yellow or magenta	0.56
3	Magenta	0.32
4	Violet	0.24
5	Brown	0.17

Discussion

5. DISCUSSION

Withania somnifera (L.) Dunal commonly known as *Aswagandha* or Indian ginseng is a high valued medicinal plant of Indian system of medicine, classically known for its rejuvenative benefits. The medicinal properties of *Aswagandha* have been attributed to its chemical constituents, mainly alkaloids and steroidal lactones (primarily of the withanolide class).

It is important to note that in approximately 60 per cent of the medicinal plants used in the traditional systems of medicine (Ayurveda, Siddha, Unani), roots are the principal materials for drug preparation. It is estimated that more than 90 per cent of the plant species used by the industry is collected from the wild and more than 70 per cent of the plant drugs involved destructive harvesting and very few are in cultivation. Development of biotechnological methods such as micropropagation, cell, root and hairy root cultures is one of the major solutions to circumvent these problems (Sudha and Seeni, 2001).

In this context, development of fast growing root culture system offers unique opportunities for providing root drugs in the laboratory, without resorting to field cultivation. Hairy root culture systems are more efficient than normal root cultures, because of their genetic and biochemical stability over long periods and are ideal for introducing genes to elevate growth and secondary metabolites. Moreover, the fast growth rate of hairy roots over normal roots give biotechnologists an interesting option to exploit the genetic transformation techniques for the enhancement of secondary metabolite production.

The results obtained in the study on " Genetic transformation for hairy root induction and enhancement of secondary metabolites in *Aswagandha* (*Withania somnifera* L. Dunal)" are described in this chapter.

5.1 STANDARDIZATION OF *IN VITRO* REGENERATION

5.1.1 *In vitro* seed germination

The seed germination was favoured in half MS nutrient medium with 2.5 per cent sucrose. Eighty four percent of the seeds germinated within 6-15 days under dark, whereas only 67 per cent seeds germinated within 10-20 days under 16 hr (1000 lux) photoperiod. Dark photoperiod was found to have a positive influence on *in vitro* seed germination of *W. somnifera* by increasing the germination percentage and lowering the time for germination. This result was in conformity with the findings of Sen and Sharma (1991) who reported eighty percent germination of seeds within 20 days under dark and 60 per cent germination within 20-25 days under 16 h photoperiod on half MS with one per cent sucrose.

The high rate of fungal and bacterial contamination of inoculated seeds from fresh berries, especially on the month of June and July may be due to the high humidity and rainfall prevailing in the season, which influences the growth of fungi and bacteria in the stock mother plants maintained in the shade house.

The explants for micro propagation studies were excised from *in vitro* germinated seedlings to avoid contamination.

5.1.2 Hypocotyls as explants

5.1.2.1 Regeneration

The hypocotyl segments whether placed directly or upside down, maximum regeneration response and shoot bud differentiation was obtained on MS with BAP 1.0 and 1.5 mg l^{-1} in combination with IAA (0.5 mg l^{-1}). On increasing and decreasing the concentration of BAP (from the above value) a large difference in the regeneration response was observed. The results suggest that there exists an optimum concentration of growth regulators, which produced maximum differentiation of shoots.

Ohki *et al.* (1978) reported that with regard to the differentiation of shoots from hypocotyls there exists one optimum concentration of growth regulators for each line and hybrid of *Lycopersicon esculentum*. When stem segments were cultured on medium with optimum concentration of growth regulators for the

hypocotyls (a juvenile, homologous organ of the stem) no or little shoot formation was noticed. It seems that each organ has a specific concentration of growth regulators favourable for shoot formation.

Kulkarni *et al.* (2000) obtained good regeneration from hypocotyl segments of *W. somnifera* on MS with BAP 0.5 mg l^{-1} alone.

It was observed that more number of shoot buds differentiated from the hypocotyl segments placed upside down compared to segments placed directly. Here the upper portion of the hypocotyls is in contact with the nutrient media. Once the differentiation of shoot buds as well as callus proliferation initiated the surface area got increased facilitating faster absorption of nutrients directly from the media. This reason may be attributed to the high shoot bud differentiation from the hypocotyl segments placed upside down.

Girija *et al.* (2004) reported that in *Capsicum annuum* L.cv. California Wonder, high regeneration was obtained from hypocotyl segments when placed upside down in MS medium supplemented with IAA 0.3 mg l^{-1} and BAP 5.0 mgl^{-1} or IAA 0.2 mg l^{-1} with BAP 3.5 mg l^{-1}.

In all the cases, that is when the hypocotyls segments whether placed directly or upside down, shoot buds were differentiated only from the upper portion of the hypocotyl segments. An increased gradient of growth substances from the base of the hypocotyls could be responsible for this. Similar differential morphogenic responses were observed by Fari and Czako (1981), in the cultivar "T. Hatvani". They related this phenomenon to a hypothetical gradient of endogenous growth substances along the axis between the cotyledonary node and root neck. The growth substances administered exogenously through the culture medium complemented the variable endogenous levels in step-wise shifts.

In the present study 70 per cent regeneration response was also obtained on MS with BAP 1.0 mg l^{-1} and IAA 0.5 mg l^{-1} along with 10 per cent coconut milk.

Here the multiple shoot induction was observed even in the absence of coconut milk indicating that it is not an essential factor of multiplication. However coconut milk supported the production of multiple shoots. The natural cytokinins in coconut milk promote shoot multiplication. Anilkumar and Nair (2004) reported that the stimulatory effect of coconut milk is due to the presence of cytokinins and zeatin ribosides.

5.1.2.2 Multiplication

The combination that contains BAP (0.4 mg l^{-1}) and IAA (0.5 mgl^{-1}) in almost equal proportion was found to be the best for multiplication and leaf differentiation of hypocotyl derived shoot buds. Auxin causes cell division and cell elongation that in combination with cytokinin produced good elongation of multiple shoot buds.

In the combination MS with IAA 0.5 mg l^{-1} without BAP, rooting initiated and also the leaf size increased, but multiplication was found to be nil or very less. Auxin favours the formation of adventitious roots in addition to cell division and elongation, but reduces the shoot regeneration frequency.

The combination MS with IAA 0.5 mg l^{-1} with BAP 1.0 or 2.0 mg l^{-1} produced good multiplication and no rooting was observed here so also the elongation of differentiated shoots was found to be very less. Cytokinins at higher concentrations induce adventitious shoot formation but generally inhibit root formation and it promotes axillary shoot formation by decreasing the apical dominance.

This is in agreement with the findings of Lo *et al.* (1980) who reported that high cytokinin content was deleterious to the initiation and elongation of roots. According to Muthuvel *et al.* (2005) the increase and decrease in regeneration frequency is attributed to the cytokinin concentration.

5.1.2.3 Elongation and rooting

The shoots produced maximum elongation on MS with GA_3 0.5 mg l^{-1}. Here the elongation of the leaf petiole was also found to be high, whereas the rooting percentage was low in the presence of GA_3. Compared to other combinations the leaf size was also found to be more here. Gibberellins can increase the cell division, cell elongation so also the size of leaf, flower and fruit.

The relative concentration of GA_3 plays a vital role in organogenesis (Thorpe, 1980). The stimulatory effect of GA_3 on the growth of stem and its promotory effect on shoot elongation were reported by Seetharam *et al.* (2003). They observed maximum shoot elongation with BAP and GA_3 at 1.0 mg l^{-1} in *Solanum surattense.*

In the present study, the elongation of the internode was higher in MS with GA_3 1.0 mg l^{-1}. In general gibberellins can induce the elongation of internodes, but it usually inhibits adventitious root as well as shoot formation. This is in accordance with the results of Gupta and Chandra (1981) in *Solanum surattense*, who reported that the shoots formed at lower levels of GA_3 (0.5 –1.0 mg l^{-1}) had longer internodes.

The culture medium half MS with 2.5 per cent sucrose produced good rooting at a fast pace. The plantlets showed maximum health and vigour with dark green leaves and the number of internodes, leaves and roots were highest in this culture medium among the combinations tested. The lowering of nitrogen and sugar content was found to be favourable at later stages of the culture cycle for producing healthy *in vitro* rooted plantlets. Usually a medium with low salt concentration promotes rooting (Hu and Wang, 1983). Lane (1979); Skirvin and Chu (1979) reported that *in vitro* rooting can successfully be achieved when the salt concentration in the media was reduced to half, one third or one forth of the standard strength. Kulkarni *et al.* (2000) reported the use of half MS medium without growth regulators for rooting of elongated shoots in *W. somnifera.*

During rooting, sugar content of the medium is also lowered in most of the cases (Roy *et al.*, 1990). Watanabe *et al.* (1991) reported that root formation in *Asparagus* cultures was reduced with increasing sugar levels.

Inoculation on half MS medium after pulse treatment with IBA 1000 mgl^{-1} also efficiently rooted the elongated shoots. Pulse treatment for five seconds was found to be ideal for rooting. The shoots inoculated after elongation in GA_3 showed efficient rooting with very less callusing tendency, where as the shoots obtained after multiplication in BAP and IAA showed more callusing tendency at the shoot base along with efficient rooting. The presence of residual cytokinin and auxin in the shoots from the multiplication media might have resulted in callus proliferation.

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

5.1.3 Cotyledonary segments as explants

5.1.3.1 Regeneration

Regeneration was obtained from cotyledonary segments only on MS with IAA 0.5 mg l^{-1} and BAP 1.0 and 1.5 mg l^{-1}. All other combinations of BAP tested did not supported regeneration. Each organ has a specific concentration of growth regulators favourable for shoot formation and in the case of cotyledonary segments1.0-1.5 mg l^{-1} BAP was found to be the optimum value.

Mathew (2002) reported that cotyledon explants of *Capsicum annuum* L.cvs Byadagai Dabbi and Arka Lohit showed best shoot morphogenesis in MS medium supplemented with BAP 2.0 mg l^{-1} and IAA 1.0 mg l^{-1}.

The proximal and distal parts of the cotyledon also showed differential regenerative behaviour. More number of shoot buds was produced from the

petiolar end. An increased gradient of growth substances from the base of the hypocotyls could be responsible for this differential regenerative behaviour as suggested by Fari and Czako, (1981).

Cotyledonary segments also produced friable creamish green callus in the same culture medium. Muthuvel *et al.* (2005) reported that cotyledonary segments of *Lycopersicon esculentum* Mill. produced greenish compact nodular calli in medium containing auxin and cytokinin.

Cotyledon explants of *Capsicum annuum* cvs. Pico and Piquillo differentiated shoot buds and only sporadically roots often with callus formation on auxin and cytokinin containing combination (Arroyo and Revilla, 1991). Gaikwad and Prasad (2003) found that media containing NAA 1.0 mg l^{-1}, BAP 2.0 mg l^{-1} and KN 2.0 mg l^{-1} was the best medium for callus induction and shoot regeneration from cotyledonary explants of *W. somnifera.*

5.1.3.2 Regeneration from cotyledonary calli

A maximum of 20 per cent regeneration was obtained on MS + BAP 2.0 mg l^{-1}. For axillary bud proliferation, exogenous auxin was not always needed. The increase and decrease in regeneration frequency is attiributed to the cytokinin concentration.

This result is in conformity with the results obtained by Rani and Grover (1999). They obtained multiple shoots from callus of *W. somnifera* on MS + BAP 2.0 mg l^{-1}. Cytokinin, whether alone or in combination with an auxin is the critical growth regulator for adventitious shoot induction (Steinitz *et al.*, 1999). Manickam *et al.* (2000) obtained regeneration of multiple shoots from nodal callus cultured on MS medium with BAP (4.44 μM) and IAA (0.57 μM).

In the present study, regeneration of cotyledonary callus was not favoured in BAP and KN combination. In *Lycopersicon esculentum* Mill, MS medium

containing 2.0 mg l^{-1} IAA and 1.0 mg l^{-1} KN produced high regeneration from cotyledon calli (Muthuvel *et al.*, 2005).

Four percent regeneration was obtained on MS with 3.0 per cent sucrose. It may be due to the presence of small-programmed buds already present in callus before inoculation. Multiple shoot formation without the supplementary stimulation of growth hormones may be attributed to the increase in the endogenous growth hormone level (Norstog, 1970).

5.1.3.3 In vitro flowering

In vitro flowering was obtained on MS with GA_3 0.5 mg l^{-1} in 10 per cent of the cotyledonary segment derived shoots. Gibberellins in general have the ability to induce flowering.

5.1.4 Shoot tips and nodal segments as explants

5.1.4.1 Multiplication

From both shoot tips and nodal segments, the combination MS + BAP 1.0 mg l^{-1} and IAA 0.5 mg l^{-1} produced maximum number of shoots and the number of shoot buds were more on MS + BAP 2.5 mg l^{-1} and IAA 0.5 mg l^{-1}. On taking shoots and shoot buds together, maximum multiplication (up to 25) occurred in the latter combination. The number of elongated shoots was more at low concentrations of BAP with respect to IAA. Increasing the concentration of BAP resulted in increased multiplication of shoot buds inhibiting the apical dominance.

This is in conformity with the findings of Govindaraju *et al.* (2003). They obtained maximum number (up to 24) from nodal segments on MS medium fortified with BAP 2.5 mg l^{-1} + IAA 0.5 mg l^{-1} and 10 per cent coconut milk.

In the present study, callusing was observed from the cut surface of explants (nodal segment and shoot tips), when they were cultured on MS with BAP and IAA. The initiation of callus from the cut surfaces may be due to higher accumulation of the endogenous auxin level favouring an active cell division

producing a mass of cells. This can also be attributed to the exposure of cells at the surface to an excessive supply of nutritive substances as compared with the cells adjoining intact region of the plant (Gayathri, 2005).

Callus formation at the basal cut ends of node explants of *W. somnifera* on axillary bud multiplication medium is in agreement with results obtained by Rani and Grover (1999). They observed callus initiation at the base of *in vitro* derived shoots on MS medium with 3.32 M BAP, 1.16 M KN and 0.98 M IBA. According to Marks and Simpson (1994) callus formation may be due to the action of accumulated auxin at basal cut ends, which stimulate cell proliferation especially in the presence of cytokinin.

Regeneration of shoot buds from the basal callus was also obtained in the same culture cycle and it was highest in MS+ BAP 2.5 mg l^{-1} + IAA 0.5 mg l^{-1}. At high concentrations of BAP (2.0-2.5 mg l^{-1}) multiplication of shoot buds from the basal callus was also high resulting in increased number of shoot buds.

Govindaraju *et al.* (2003) reported that on using BAP alone at 1.0-3.0 mg l^{-1}, differentiation of large number of shoots from callus as well as directly from the explants was noticed in *W. somnifera*. Supplementing IAA at 0.5 mg l^{-1} led to substantial increase in the height of the shoots. Similar reports of shoot elongation on supplementing with low concentration of IAA were reported in *Crotalaria lutescens* (Naomita and Rai, 2000) and *Cajanus cajan* (Geetha *et al.* 1998).

Nodal segments produced more multiple shoots compared to shoot tip. The results are in agreement with those of Rani and Grover (1999); Kulkarni *et al.* (2000) and Govindaraju *et al.* (2003) who also reported maximum shoot formation from nodal segments of *W. somnifera*.

5.1.4.2 Subculturing

The subculturing was found to be effective for multiplication of shoot buds from nodal segments and shoot tips obtained from the first culture cycle.

The shoot multiplication at an enhanced pace was achieved by subsequent culturing. This is in accordance with the results of Vadawale *et al.* (2004). They also obtained high shoot multiplication from *in vitro* derived shoot of *W. somnifera* by subsequent cultures up to 4-5 cycles.

5.1.4.3 Elongation and Rooting

Giberrellic acid alone at low concentrations (0.5 mg l^{-1}) had a promoting effect on shoot elongation. Such a shoot elongation response was reported in *Vigna sublobata* (Bhadra *et al.*, 1994) and *Phaseolus vulgaris* (Franklin *et al.*, 1991) where, NAA and BAP were used besides GA_3. Govindaraju *et al.* (2003) used GA_3 alone (0.1mg l^{-1}) for elongating the dwarf statured multiple shoots of *W. somnifera.*

Rooting on half MS with 0.25 per cent activated charcoal after pulse treatment with IBA 1000 mg l^{-1} was found to be highly effective for inducing roots *in vitro*, followed by half MS with 0.5 mg l^{-1} IBA.

Manickam *et al.* (2000) obtained maximum rooting (5.1± 0.49) of *in vitro* derived shoots of *W. somnifera* on MS with IBA 9.8 μ*M*. Govindaraju *et al.* (2003) reported that the shoots were rooted successfully on half strength MS medium (both liquid and solid) supplemented with IBA (0.5- 1.0 mg l^{-1}) or IAA (0.5 mg l^{-1}).

According to Rani and Grover (1999) IBA 2.0 mg l^{-1} alone or in combination with IAA 2.0 mg l^{-1} was effective for root induction. The effectiveness of IBA in rooting has been reported in many species. According to Ludwig-Muller (2000) transport velocity of IBA was markedly slower compared to that of IAA and NAA. The slow movement and slow degradation of IBA facilitates its localization near the site of application and thus its better function in inducing roots (Nickell, 1982).

Inoculation of shoots on half MS without activated charcoal after pulse treatment produced numerous short and stout roots with prominent callus in the shoot base. Activated charcoal has a profound influence on rooting of shoots *in vitro*. Activated charcoal adsorbed the residual auxin and cytokinin from the shoots and hence no callusing occurred on shoots inoculated after pulse treatment in charcoal containing media. Auxin (IBA) at high concentration stimulate callus proliferation. This was also overcome by the absorbing capacity of 0.25 per cent activated charcoal.

This is in agreement with the findings of Hu and Wang (1983). They reported that activated charcoal can absorb residual cytokinin from shoot and it also shades *in vitro* roots from light, which in high intensity may inhibit root growth.

On adding IAA along with IBA, roots became thicker and shorter with less laterals and proliferation of callus also occurred at the cut ends. According to Govindaraju *et al.* (2003), addition of IAA 0.5 mg l^{-1} did not show much effect on root length. They also reported that roots produced in solidified media were thick and long with callus at base. Compared to IAA, IBA was found to be more effective for rhizogenesis.

5.1.5 Leaf as explant

A maximum of 30 per cent regeneration response was obtained on MS with BAP 1.0 mg l^{-1} and IAA 0.5 mg l^{-1} and the mean number of shoot buds was highest in MS with BAP 2.0 mg l^{-1} and IAA 0.5 mg l^{-1}. It is thus quite evident that different explants vary in their regenerative behaviour. The leaf explants showed lower regenerative ability compared to other explants (except root). This may be due to the difference in the physiological maturity of the tissues.

Ohki *et al.* (1978) stated that the stage of maturation of the plant related to the change of genome expression causes the different sensitivity to these exogenous regulators. Govindaraju *et al.* (2003) obtained a maximum of 29 per

cent regeneration from leaf explants of *W. somnifera* on MS with BAP 2.0 mg l^{-1} and IAA 0.5 mg l^{-1}. Abhyankar and Chinchanikar (1996) found that shoot buds developed on leaf margins of *W. somnifera* after subculturing on MS medium containing IAA 0.4 mg l^{-1} + KN 0.2 mg l^{-1} + BAP 1.0 mg l^{-1}.

Leaf explants showed more tendency for callusing. There are differences in intrinsic hormonal balances in different explants of the same plant and the environment inside the cells plays a crucial role in organogenesis. Gupta and Chandra (1981) reported that incorporation of IAA (0.5 –5.0 mg l^{-1}) into the medium containing KN (1.0 mg l^{-1}) resulted in suppression of direct differentiation of shoot buds from leaf explants of *Solanum surattense*. But due to the intervening callus formation, the number of differentiated shoot buds increased at lower IAA levels (0.5- 1.0 mg l^{-1}). Thus higher number of shoot buds was observed on MS with KN (1.0 mg l^{-1}) + IAA (0.5- 1.0 mg l^{-1}) than with KN (1.0 mg l^{-1}) alone.

5.1.6 Roots as explants

The roots of *in vitro* seedlings and *in vitro* rooted plantlets produced good callusing but failed to regenerate shoots by direct organogenesis in all growth regulator combinations tested. Each explant differs in their regenerative behaviour. Root showed least tendency for regeneration.

Similar results have been reported by Rani and Grover (1999) and Govindaraju *et al.* (2003) in *W. somnifera* roots. Govindaraju *et al.* (2003) reported that the roots of *W.somnifera* showed good callusing response both under dark and light conditions, however failed to regenerate shoots at all the concentrations and combinations of growth regulators tested.

But the root explants of *Solanum surattense* produced hard green callus at higher levels of KN (1.0-3.0 mg l^{-1}), which subsequently differentiated a few shoot buds. Direct differentiation of a large number of shoot buds without any callus formation occurred at higher levels of KN (5.0 mg l^{-1}) or BAP (3.0-5.0 mg l^{-1}) (Gupta and Chandra, 1981).

In the present study somatic embryo like structures were produced on MS + BAP 1.5 mg l^{-1} and IAA 0.5 mg l^{-1} from a single seedling root. The auxin cytokinin balance in this combination might have favoured the formation of somatic embryo. The explant cells differ in their DNA synthesis and cell division ability due to the difference in the physiological maturity of the cells.

In general, auxins are known to stimulate the induction of embryogenically competent tissue and initiation of early stages of embryogenisis (Fujimura and Komamine, 1980). Cytokinins on the other hand are required for growth and maturation of differentiated embryoids (Krikorian and Kaun, 1981). A combination of auxin and cytokinin proved effective in differentiation of somatic embryos.

Combinations and levels of exogenous hormones were found to control the mode of embryogenesis in *Fresia refracta* (Wang *et al.*, 1989).

5.2 CULTURING AND SENSITIVITY SCREENING OF *AGROBACTERIUM* AND EXPLANTS

Complete elimination of the bacteria from the explants after co-cultivation is very essential; otherwise it will interfere with the growth and hairy root production of explants. Overgrowth of the bacteria causes death of the explants and disrupts the experiment. Elimination of the bacteria from the explants is done using antibiotics. The antibiotic chosen should be such that it efficiently kills the bacteria; at the same time it does not affect the growth and morphogenesis of the explants. The most commonly used antibiotics for this purpose were ampicillin, carbenicillin and cefotaxime. However the sensitivity of each *A. rhizogenes* strains and explants towards these antibiotics has to be studied carefully so as to find out the most appropriate one to be used in the further experiments.

All the three strains of *A.rhizogenes* (A4, MTCC 2364 & ATCC 15834) were found to be sensitive to cefotaxime whereas, the strains A4 and MTCC 2364 showed resistance to ampicillin. The strain MTCC 2364 also showed resistance to

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

5.2.1 Preculturing of explants

The explants were cultured for two days on MS medium without antibiotics prior to transformation. This was done to make the explants acquainted to the new culture condition, since each explant was now in direct contact with the media. The cell division will be initiated and the endogenous hormones will be used up during this phase, thereby preparing the explants for transformation. Yu *et al.* (2001) reported that hairy roots emerged 3-4 days earlier than with usual treatment, if leaf explants of *Pueraria lobata* were pre cultivated for 2-3 days before transformation with *A. rhizogenes* strain, R 1601.

5.2.2 Wounding of explants

A plant cell becomes susceptible to *Agrobacterium* when it is wounded. The wounded cells release phenolic compounds, such as acetosyringone, that activate the *vir* region of the bacterial plasmid (Binns and Thomashow, 1988).

In the present study, the pricked region turned slightly brown within one week. The hairy roots were induced in and around the wounded regions, which are the site of *Agrobacterium* infection. The exudation of phenolic compounds as well as the damage of plant cells by pricking produced browning of the tissues. This is in accordance with the observations of Chaudhuri *et al.* (2005) who reported that

the wounded areas turned brown within one week in all the explants of *Tylophora indica,* which includes leaf, stem and shoots.

Sevon and Oksman-Caldentey (2002) reported that the transferred T-DNA derived from the Ri plasmid causes the plant cells to proliferate adventitious roots at or near the site of infection. Zdravkovic-Korac *et al.*, 2004 found that hairy roots appeared from the deep wounds of androgenic embryos of *Aesculus hippocastanum* usually at a frequency of one thin root per wounded point. They also found that deep wounding (1-2 mm) was more efficient than mere scratching for *Agrobacterium* infection.

Moore *et al.* (1979) showed that most hairy roots emerged from the pericycle tissue of the carrot vascular cylinder. According to Hildebrand (1934), *A. rhizogenes* has to enter a wound deep enough to reach the phloem region in order to induce hairy roots on apple trees. Nilsson and Olsson (1997) hypothesized that only cells containing high levels of auxin and sucrose (which regulate *rol* B and *rol* C promoters, respectively) are able to act as root meristem initials and are also ideal targets for *A. rhizogenes* infection. Since ray cells and phloem cells are positioned in the region with the highest amount of sucrose and considerable amount of IAA, they could be convenient targets for *A. rhizogenes* infection. This explanation may clarify why wounding of explants of *W. somnifera* is favoured for inducing transformation.

5.3 STANDARDISATION OF TRANSFORMATION TECHNIQUES

5.3.1 Influence of bacterial inoculum

The bacterial inoculum used affects the transformation frequencies. In the present study, when the bacterial colonies were used as the inoculum transformation was produced only by A4 strain whereas no transformation was shown by ATCC 15834 and MTCC 2364. But when bacterial suspension was used as the inoculum, both A4 strain and ATCC 15834 showed successful transformation.

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

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

In accordance to the present results, Subroto and Doran (1994) obtained hairy roots by wounding *Solanum aviculare* plantlets with a syringe needle containing *A. rhizogenes* (A4) single cell colonies. Similarly long stem explants inoculated with a loop full of bacterial culture (A4TIII and A4T) produced hairy roots. The reports of successful transformation by using bacterial suspension were given by Yoshimatsu *et al.*, 2003, Koike *et al.*, 2003 and Lee *et al.*, 2004.

Here the superior performance of ATCC 15834 in suspension form than single cell colonies may be attributed to two reasons, firstly ATCC 15834, being motile, the strain can take advantage of the liquid medium to facilitate better attachment to the wounded cells than they get when applied as colonies. Secondly the optimum concentration of bacteria (ATCC 15834) for producing successful transformation might be present in bacterial suspension compared to colonies. But the A4 strain could exhibit virulence both when inoculated as suspension or as single cell colonies. The greater concentration of bacteria in the colonies of A4 might have favoured the transformation. The strain MTCC 2364 was found to be avirulent irrespective of the nature of the inoculum and hence failed to produce any transformation.

5.3.2 Influence of co-culture period on hairy root induction

Transformation by co-culture is an effective and stable method for introducing desired gene into plant DNA (Malabadi and Nataraja, 2003). Co-

cultivation plays an important role in the success of tranformation. It is during this period that the *vir* genes are activated and the T-DNA is transferred into the plant cell. However, increasing the co-cultivation period might led to necrosis and death of the explant due to the hypersensitive response of the tissue (Sarmento *et al.*, 1992). Hence the length of co-cultivation period should always be the shortest interval necessary to obtain the maximum frequency of transformation.

In the present study, transformation was obtained only from leaf segments and shoot tips under different co-culture period. Taking together both leaf and shoot tip into consideration, co-cultivation period of one or two days gave higher transformation rates than three-day co-cultivation period (1 day for leaf segment and 2 day for shoot tip). These differences could be explained by the fact that the concentration of *A. rhizogenes* was optimum after one and two days of co-cultivation than after three days, and that could increase considerably the probability of gene transfer. The decreases in transformation rate after three days of co-cultivation may not reflect a decrease in the virulence of *A. rhizogenes* strains, but an overgrowth of bacteria, which killed the explant tissues. Furthermore, after three days of co-cultivation it was difficult to eliminate the *Agrobacterium* strains completely. Similar results were reported by Pawlicki *et al.* (1992) in carrot.

Bacterial cells multiplied in the co-culture medium and after 24 h, the optimum quantity of bacteria were available for transformation and hence a higher transformation was achieved. After 36 hrs, the level of bacterial cells reached supra optimum level and competitive inhibition of competent bacterial cells resulted in inhibition of transformation (Karmarkar *et al.*, 2001b).

The strain MTCC 2364 was fast growing and it overgrow on the explant, hence it was difficult to eliminate the bacteria completely after the co-cultivation period. Moreover it was noticed that this strain could survive up to 400 mg l^{-1} cefotaxime.

Necrosis of the hypocotyl explants was observed on increasing the co-cultivation period (2-3 days). The probable reasons for this study may be the delicate nature, age of the explant and the virulence of the bacterium. The bacterial strains dominated over the explant, resulting in the death of tissues during co-cultivation. Similar results were reported by Pawar and Maheshwari (2004) about hypocotyls explants in *W. somnifera*.

Callusing and rooting of the explants may be attributed to the endogenous auxin as well as by the auxin synthesis directed by T-DNA. Schmulling *et al.* (1988) reported that the T-DNA from the Ri plasmid has a direct effect on auxin synthesis and sensitivity to auxin in plants. Alteration of auxin metabolism in transformed cells has been supposed to play an important role in expression of the hairy root phenotype (Gelvin, 1990).

In the present study, callusing and rooting tendency vary with the bacterial strain, explant and wound site. This is in conformity with the findings of Chaudhuri *et al.* (2005). They reported that scanty yellow-green wound callus appeared in some of the explants, those infected with either of the bacterial strains (A4 and LBA 9402) as well as in some of the uninfected controls. Proliferation of wound callus into friable white callus and the apperance of roots occurred within 42 days after infection with A4 strain. The induction of wound callus and transformed roots were significantly correlated with bacterial strain, explant type and wound site.

One day or 24 h co-culture period for *A. rhizogenes* infection in leaf segment was earlier reported in *Solanaceous* plants (Shimomura *et al.*, 1991) and *Lawsonia inermis* (Bakkali *et al.*, 1997). Success reports of two-day co-culture period were there for *Pueraria phaseoloides* (Shi and Kintzios, 2003), *Cephaelis ipecacuanha* (Yoshimatsu *et al.* (2003) and *Withania somnifera* (Pawar and Maheshwari, 2004).

5.3.3 Influence of acetosyringone in hairy root induction

The accumulation of phenolic compounds, such as acetosyringone or those released by cultured cells activate the *vir* genes of Ti plasmid and stimulate transformation (Binns and Thomashow, 1988).

In the present study acetosyringone dissolved in DMSO was supplemented at a concentration of 100 µM prior to (bacterial suspension) or/ and during co-cultivation in MS media in order to increase the transformation efficiency. Tepfer (1984) reported that successful infection of some species could be achieved by the addition of acetosyringone.

In the present study, acetosyringone enhanced the transformation percentages with A4 strain. This is in conformity with the findings of Zdravkovic - Korac *et al.* (2004) who reported that the presence of 50 µM acetosyringone in the co-cultivation media significantly increased the number of putative transformants using A4GUS in *Aesculus hippocastanum*. The addition of acetosyringone in co-cultivation media might have resulted in the activation of *vir* genes and thereby increased the virulence of A4 strain.

However, no such positive influence was noticed with ATCC 15834 by the addition of acetosyringone. Actually the transformation percentage showed reduction with the addition of acetosyringone. It appears that the phenolic compounds released by the plant cells were sufficient to induce the virulence genes and stimulate the genetic transformation in the case of ATCC 15834. This is in agreement with the results of Pawlicki (1992) with carrot, Sangwan *et al.* (1991) on *Datura* and Kumar *et al.* (2002) on *Lucerene* by using *A. tumefaciens* strains.

According to our observations, the activation of *A. rhizogenes* strain by the addition of acetosyringone in the bacterial suspension was found to have a negative effect on transformation response. This observation was contradictory to the findings of Chaudhuri *et al.* (2005) and Tsuro *et al.* (2005) who reported the

addition of acetosyringone in the bacterial suspension A4 and A13 respectively for improving the transformation efficiency.

The direct addition of acetosyringone in the bacterial suspension and further incubation in the static condition for two hours might have affected the growth and virulence of bacterial strains. However, MTCC 2364 failed to produce any transformation both in the presence and absence of acetosyringone. Pawar and Maheshwari (2004) obtained transformation using MTCC 2364 in the absence of acetosyringone from the same plant. But none of the tests for the confirmation of transformation was presented in this report.

5.3.4 Standardization of explants for efficient transformation

It has been reported that the virulence of *Agrobacterium* strains varies among the plant hosts (Hobbs *et al.*, 1989) and that the transformation efficiency of host species can vary between different bacterial strains (Godwin *et al.*, 1991).

In the present study, different explants such as hypocotyl segements, cotyledonary segments, leaf segments, shoot tips and nodal segments of *W. somnifera* showed differential responses to transformaion. The leaf segments of *W. somnifera* were found to be the best explant for efficient transformation under each variable studied followed by shoot tip.

The age and differentiation status of plant tissue can affect the chances of successful transformation (Trypsteen, 1991). Citovsky *et al.* (1991) have reported that specificity of *Agrobacterium* transformation is closely connected with the physiology and growth of the plant. Nin *et al.* (1997) have reported that the age and hormonal balance of the host tissue affect the transformation frequencies.

Stachel *et al.* (1986) reported that the induction of *vir* genes was not merely a response of *Agrobacterium* to necrotic plant cells, but it required an active plant celluluar metabolism. Citovsky *et al.* (1991) stated that cell division and DNA synthesis are involved in the incorporation of T- DNA into the plant genome.

Therefore absence of cell division may prevent successful T- DNA transfer. The explant cells differ in their DNA synthesis and cell division ability due to the difference in the physiological maturity of the cells (Karmarkar, 2001b).

Nilsson and Olsson (1997) hypothesized that only cells containing high levels of auxins and sucrose (which regulate *rol* B and *rol* C promoters respectively) are able to act as root meristem initials and are also ideal targets for *A. rhizogenes* infection.

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

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

Trasformation varies with plant species, plant organs, infected sites etc. The leaf explants followed by shoot tips have enough competent cells for successful transformation. Compared to shoot tips, leaf segments especially the petiolar region possess more competent cells for successful transformation. The age, hormonal balance and differentiation status of these tissues (leaf and shoot tips)

were found to be favourable for effective transformation. Since cell division in the host target tissue is required for successful *Agrobacterium* transformation (Binns and Thomashow, 1988), it can therefore be attributed that wound sites associated with actively dividing cells of leaves and shoot tips show higher transformation rates. The TL-DNA (*rol* B in particular) confers the competence to respond to auxin (Cardarelli *et al.*, 1987). Therefore explants capable of auxin synthesis (leaves and shoot tips with young leaves) showed higher transformation rates since these cells contains high levels of auxins and sucrose that can act as root meristem initials which are the ideal targets of *Agrobacterium* infection.

Here the hairy roots were produced directly from explants as well as *via* callus. Proliferation of hairy roots without intervening callus was reported in *Camptotheca acuminata* with *A. rhizogenes* strains ATCC 15834 and R-1000 from wounded hypocotyls and true leaves (Lorence *et al.*, 2004). Similar reports were there in *Isatis indigotica* with A4, R 1601 and ATCC 15834 strains from cotyledon and hypocotyls explants (Xu *et al.*, 2004).

In the present study, from leaf segments, hairy roots were induced more from the petiolar region and in the absence of petiole hairy roots were produced from the proximal cut edges.

Shi and Kintzios (2003) reported that when leaf explants with petioles of *Pueraria phaseoloides* were inoculated with *A. rhizogenes* suspension (ATCC 15834), hairy roots were induced from micro-calli of leaf petioles or directly from the cut edges of leaf petiole, but the frequency of root induction (mean number of roots per explant) was higher from the microcalli than directly from the cut edges of leaf petioles (70%). When the leaf explants without petioles were infected, hairy roots were only induced via callus from the leaf vein of cut edges of leaf explants 6-8 weeks after infection (40%). This increased hairy root induction from leaf explants with petioles as compared with cotyledons and leaf explants without petioles, may be due to the fact that petioles are more competent for transformation than cotyledon or leaf explants without petioles.

The inability of hypocotyls, cotyledonary segments and nodal segments to induce hairy roots may be due to the lack of wound adjascent competent cells or differences in the physiological maturity of the tissues.

Normal rooting and callusing of the control explants in growth regulator free MS medium indicate the presence of endogenous auxins in the cells even after preculturing. In the present study, the transformed explants also produced normal roots also along with hairy roots. The cells with high levels of auxins and sucrose that act as root meristem initials, if not transformed by *Agrobacterium* might produce normal roots. The occurrence of normal rooting from infected explants was also reported by Karmarkar (2001) in *Holostemma ada-kodien*, whereas Chaudhuri *et al.* (2005) reported that roots never appeared on the control explants or similar explants inoculated with LBA 9402 in *Tylophora indica*.

Success reports of hairy root induction from leaf segments were reported in *Atropa belladonna* (Jaziri *et al.*, 1994), *Dubosia mycoporoides* x *D. leichhardti* (Celma *et al.*, 2001), *Withania somnifera* (Pawar and Maheshwari, 2004; Kumar *et al.*, 2005) and *Solanacea* spp. (Knopp *et al.*, 1988).

Similarly reports of transformation from shoots were reported in *Plumbago rosea* L. (Komaraiah *et al.*, 2002), *Rauvolfia serperntina* (Benjamin *et al.*, 1993) *Withania somnifera* (Ray *et al.*, 1996) and *Gentiana* spp. (Momcilovic *et al.*, 1997).

5.3.5 Efficiency of strains in inducing hairy roots

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

In the present study ATCC 15834 showed the highest efficiency in transforming plant tissues, followed by A4. The strain MTCC 2364 however failed to produce any successful transformation. But Pawar and Maheshwari, 2004 reported transformation in *W. somnifera* using MTCC 2364 and MTCC 532 or ATCC 2364.

Agrobacterium rhizogenes strain A4 was significantly better than strains R1601 and ATCC 15834 for inducing roots in *Isatis indigotica* (Xu *et al.*, 2004). Benjamin *et al.* (1993) reported that *A. rhizogenes* 15834 was an effective vector for specific gene transformations in *Rauvolfia serpentina.*

Knopp *et al.* (1988) reported that *A. rhizogenes* strain A4 obviously has the ability to induce well growing hairy roots in a wide range of *Solanaceous* host plant compared to 8196 strain. *Agrobacterium rhizogenes* strains, 15834, A4, 1855 and 2659 were tested for their ability to induce hairy roots on rhizome of *Scopolia japonica* and strain 15834 was found to be most active in inducing hairy roots (Mano *et al.*, 1986).

Petit *et al.* (1983) studied the pathogenicity of different strains of *A. rhizogenes*. They found that the strains A4, 15834 and HRI were the most virulent on all hosts tested. The strains 8196, TR7, and TR 101 were found to be less virulent and transformed only a limited number of hosts. They suggested that the difference in host range and pathogenicity of *A. rhizogenes* strains is due to the difference in the plasmids they harbour. Similarly Cardarelli *et al.* (1987) attributed the difference in transformation ability of different strains of *Agrobacterium* to the plasmids harboured by them.

Rhodes *et al.* (1989) reported that the agropine strains (15834, A4, TR-7 etc.) have wide host range that is attributed to the presence of TR-DNA fragment of the T-DNA harbouring genes for auxin synthesis (tms1 and tms 2). These genes trigger cellular division by auxin synthesis due to which these strains are able to transform a wide range of species. On contrary, the mannopine strains and

cucumopine strains are deficit in the TR-DNA fragment; hence they cannot trigger auxin synthesis and so can infect only a limited number of hosts. In the present study, it was found that only the agropine strains such as 15834 and A4 induced hairy roots while the strain MTCC 2364 strain did not induce any hairy roots.

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

Spencer and Towers (1989) reported that different *A. rhizogenes* strains respond differently to the wound induced compounds. The wide host range (WHR) strains like 15834 and A4 are more sensitive to the wound induced compounds than the limited host range (LHR) strains like 8196 and 2659. So the lack or low levels of inducer molecules from wounded plant cells inhibit the virulence of these LHR strains resulting in inhibition of tranformation. So also in addition to the *vir*-inducing compounds certain compounds induced from wounds are inhibitory to the *vir* induction. Not all the *Agrobacterium* strains are capable of degrading the inhibitory wound induced compounds. So they differ in the transformation ability. In the present study, WHR strains A4 and ATCC 15834 produced transformation. This may be due to their ability to respond to the wound inducer molecules as well as to degrade the inhibitory wound induced compounds. On the contrary, the strain MTCC 2364 was either unable to respond to the levels of inducer molecules from wound or to degrade the inhibitory compounds. More over the plasmid harboured by the strain might not be efficient in inducing hairy roots.

Leroux *et* al. (1987) reported that the host range of WHR and LHR strains might be affected by the differences in structure of *vir* A protein. Though the *vir* A proteins in both types are structurally related, the proteins in their amino acid termini are quite divergent. So they differ in signal recognition and hence the

transformation ability. In the present study, the strains; A4 and ATCC 15834 are form the WHR type so they possess similar type of *vir* A proteins. The strain MTCC 2364 may possess different type of *vir* A proteins due to which their *vir* gene system is not sufficiently activated resulting in their failure to induce transformation.

5.3.6 Number of days for root induction

Hairy root induction was achieved in a time period of 1-4 weeks in majority of the plant species. In the present study almost 9-20 days (1-3 weeks) was taken for hairy root induction from leaf and shoot tip explants irrespective of the method of inoculation.

The hairy roots developed 2-4 weeks after infection in *Angelonia salicarifolia* (Koike *et al.*, 2003), 2-3 weeks after infection in *Tylophora indica* (Chaudhuri *et al.*, 2005), 3-4 weeks after infection in *W. somnifera* (Ray *et al.*, 1996), and 4-10 weeks after infection in *Camptotheca acuminata* (Lorence *et al.*, 2004).

5.3.7 Establishment of hairy root cultures

The hairy roots showed fast growth with high lateral branching in hormone free basal media. Most of the hairy roots exhibited plagiotropic growth habit and some showed reduced geotropism. The results are in agreement with the observations of Sevon and Oksman-Caldentey (2002) who reported that hairy roots are fast growing and laterally highly branched and are able to grow in hormone free medium. Moreover, these organs are not susceptible to geotropism any more. Banerjee *et al.* (1995) have reported that hairy roots generally showed negative geotropism or reduced gravitropism, while normal roots show strong gravitropism.

In the present study, the growth of the root clones differed in the same culture medium. Among the hairy root clones, some showed faster growth, some showed slow growth whereas some root clones failed to grow at all. Variation was also observed in the amount of root hairs. In *Scopolia japonica* (Mano *et al.*,

1986), *Dubosia leichhardtii* (Mano *et al.*, 1989) and *Catharanthus roseus* (Batra *et al.*, 2004) the variation between transformed root clones has been attributed to the nature of T-DNA integration into the host genome.

Guivarc'h *et al.* (1999) reported that only seven carrot hairy root lines (out of 160) retained growth capacity for two years. They concluded that hairy roots harbouring TR-DNA alone grew poorly and stopped growing during initial subcultures. Vilaine and Casse- Delbart (1987) reported that the transfer of TL-DNA alone, as well as the transfer of TR-DNA alone, leads to root induction in plant tissues transformed by agropine-type Ri plasmids such as pRi A4 and pRi HR1.

Celma *et al.* (2001) reported that the variation in the severity of hairy root syndrome symptoms is known to occur due to the integration of the TL/ TR – DNAs in different parts of the plant genome resulting in varying levels of expression of the introduced genomes. The variation within clone can be due to deletion of TR-DNA, that are known to enhance the action of the *rol* genes contained on the TL-DNA.

It has also been shown that deletion of one or more of the *rol* genes of the TL-DNA may be responsible for the frequently observed variation of the severity of the hairy root symptoms (Sevon *et al.*, 1997).

Jouanin *et al.* (1987) have shown that in pRi A4 plasmid-transformed plants, the size of the TR-DNA (including the region *tms*) varies from about 5-28 kb although the size of the TL-DNA (19-20 kb) seems to be relatively constant. They also showed that the copy number varies from 1-4 per plant genome and that TL and TR- DNAs are not always present in the same number of copies; in some cases the regions are linked together.

On the basis of this evidence, the considerable variation in the characteristics of *W. somnifera* hairy roots can be attributed to variation in the copy

number, size and chromosomal location of the Ri T-DNA fragments integrated into the plant genome resulting in variable levels of gene expression. The variation can also be attributed to presence of TR- DNA alone or due to the deletion of TR-DNA that is known to enhance *rol* genes of TL-DNA. It may also be attributed to the deletion of one or more of the *rol* genes of the TL-DNA.

In the present study, control roots showed only less lateral branching with slow growth and the roots were least hairy and perished after 2-3 subcultures on growth regulator free MS medium. This is in agreement with the findings of Chaudhuri *et al.* (2005) who reported that non-transformed roots showed necrosis and poor growth with the absence of lateral branching. Shi and Kintzios (2003) also reported that control roots inoculated in growth regulator free MS grew very slowly, did not branch and perished after 2-3 subcultures.

5.3.8 Rapid culturing of hairy roots

Variation was noticed within clones (A4 derived) in the biomass production. The growth of the root clones differed in the same culture medium. The integration of the TL/ TR-DNAs at different locations cannot be responsible for this within clone-variation unless the original root culture was chimaeric, possibly due to somaclonal variation. It can be due to the deletion of *rol* genes. Also, deletion of the TR-DNA may be possible, as we did not detect the presence or absence of the TR-DNA genes that are known to enhance the action of the *rol*-gene contained on the TL-DNA.

Compared to ATCC 15834 induced hairy roots, the hairy roots induced by A4 strain showed faster growth producing more biomass. The growth of hairy roots was influenced by the strain used in hairy root induction. This observation was supported by the findings of Sevon and Oksman-Caldentey (2002) who reported that the *A. rhizogenes* strain could also have effect on biomass and alkaloid productivity of hairy roots.

In comparison with the roots in solid culture, hairy roots cultured in liquid growth regulator free MS medium grew rapidly and had high lateral branching. This is in consistent with the findings of Shi and Kintzios (2003) and Chaudhuri *et al.* (2005) who also reported that the roots cultured in liquid medium showed more rapid growth and higher branching than those cultured in solid medium.

When the hairy roots are cultured in the liquid medium, with increasing incubation time, older parts of such roots became dark brown eventually darkening the culture medium. This can be related to the effects of some metabolites released into the medium as suggested by Shi and Kintzios (2003).

It is important to note that in the culturing of hairy roots, the elongation of root tips as well as the production of lateral roots is responsible for higher biomass. Bapat and Ganapathi (2005) reported that hairy roots have more number of apical zones showing a high degree of cell division.

Sevon and Oksman-Caldentey (2002) also reported that the greatest advantage of the hairy roots compared to conventional roots is their ability to form several new growing points and consequently lateral branches. The growth rate of hairy roots may vary greatly between species, but differences are also observed between different root clones of the same species.

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

In the present study, the culture medium was found to have a significant effect on *W. somnifera* hairy root growth. Among the four liquid media tested, half MS was found to be superior for promoting hairy root growth followed by MS, B_5 + 2.0 per cent sucrose and B_5 + 3.0 per cent sucrose respectively.

The results are in accordance with the reports of Xu *et al.* (2004) who found that the culture medium have 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.

Factors such as the carbon source and its concentration, ionic concentration of the medium, pH of the medium, light, phytohormones, temperature and inoculum are known to influence the growth and alkaloid production of hairy roots (Sevon and Oksman-Caldentey, 2002).

Hilton and Wilson (1995) reported that Gamborg's B5 medium is the most widely used medium for the hairy roots of many species. Christen *et al.* (1992) reported that modifying the culture conditions can increase the growth rates and biomass yields of the hairy roots of *Datura stramomium*.

Toivonen *et al.* (1991) studied the effect of varying concentrations of sucrose, phosphate, nitrate and ammonium on the growth of hairy root cultures of *Catharanthus roseus*. They found that biomass yields were maximal in media containing high concentrations of sucrose. These results have some contradictions with our results. The presence of 3.0 per cent sucrose in half MS favoured the growth whereas, B5 media with 3.0 per cent sucrose was found to have a negative influence on growth. At the same time B5 with 2.0 per cent sucrose supported growth. Low concentration of ammonium (half MS) was found to be more favourable for the growth of hairy roots than high ammonium concentration (MS).

Hairy root cultures of *Plumbago rosea* L. showed an enhancement in biomass with an increase in the sucrose concentration up to 3.0 per cent (Komaraiah *et al.*, 2002). Higher concentration of sucrose was also reported to produce optimum biomass in *Catharanthus roseus* hairy root cultures (Toivonen *et al.*, 1991).

The hairy roots of different species behave differently in the same culture conditions. Individual hairy root clones can also have different optimum concentrations of sucrose or mineral ions (Oksman- Caldentey *et al.*, 1994). It can

be concluded that hairy roots of each species or specifically each root clone has a particular optimum concentrations of sucrose and mineral ions for producing maximum biomass.

5.3.10 Growth pattern of hairy roots

The growth pattern of hairy roots of *W. somnifera* obtained in the present study was almost in complete agreement with the findings of Xu *et al.*, 2004 on *Isatis indigotica* hairy roots.

In the present study, the hairy roots exhibited a sigmoid growth pattern reaching its apex on the 25th day of culturing with a 12-fold increase in the fresh weight as compared with the starting inoculums. The hairy roots of *Isatis indigotica* grew fast and showed as S-shaped growth curve that reached its apex on the day 24 with the starting inoculums (Xu *et al.*, 2004).

Babaoglu *et al.* (2004) reported that the hairy roots induced in *Lupinus mutabilis* exhibited a sigmoid growth pattern with lag (0-5 days), exponential (5-15 days) and stationary phases.

The hairy roots incubated under dark accumulated almost equal biomass as compared to those incubated under diffused light. This is in consitent with the observations of Babaoglu *et al.* (2004). They reported that the growth of hairy roots of *Lupinus mutabilis* was not significantly different in the light or the dark. However, Yoshimatsu *et al.* (2003) found that the growth of the transformed roots of *Cephaelis ipecacuanha* was superior under dark than under light.

The hairy roots originally white, gradually changed colour to brown and the biomass began to decrease, owing to depletion of nutrients in the liquid medium and lysis of cells. A similar phenomenon was also observed in the hairy root cultures of *Isatis indigotica* (Xu *et al.*, 2004).

5.3.11 Growth rate of hairy roots

During early stages of the root growth in *W.somnifera* and *Solanum surattense*, the increase in the number of hairy root branches was almost logarithmic. The extensive branching was due to the presence of many meristems, which accounted for high growth rates of hairy roots in culture. This particular characteristic was observed to be the most common for members of *Solanaceae* (Pawar and Maheshwari, 2004).

The average growth rate of the hairy roots varies from 0.1 to 2.0 g dry weight/ litre /day. This growth rate exceeds that of virtually all conventional roots and is comparable with that of suspension cultures (Sevon and Oksman-Caldentey, 2002).

In the present study the growth rate of hairy roots was found to 0.116 g/ day. In *Plumbago rosea,* the specific growth rate of the hairy root cultures was calculated as 0.15 g/ day with a doubling time of 4.5 days. (Komaraiah *et al.*, 2002).

5.4 CONFIRMATION OF TRANSFORMATION

5.4.1 Morphology of hairy roots

In the present study, the hairy roots induced by ATCC 15834 was initially creamy white in colour, relatively thick with high root hairs compared to that of A4 strain which produced relatively thin white hairy roots with comparatively less root hairs.

Hairy roots show morphological variations depending upon the interaction nature of plant cell phenotype and strain of the bacterium and show differences in root thickness, degree of branching and amount of hairy root production (Bapat and Ganapathi, 2005).

Banerjee *et al.* (1995) reported that hairy roots are whitish in colour with numerous root hairs and show a high degree of lateral branching. Among the

several root clones of *Gentiana lutea*, a range of morphological features was observed, from those that produced calluses, to those with thin, elongated roots, all with few root hairs (Momcilovic *et al.*, 1997).

Here the TR and / or TL-DNA may be present in a different copy number(s) as suggested as Merlo *et al.* (1980) resulting in varied level of expression of hairy roots symptoms. There may be differences in the length of the T-DNA of Ri plasmid that get stably integrated into the plant genome bringing out differences in the morphological features.

5.4.2 Opine analysis

Transformation of plant tissues by *A. rhizogenes* could be confirmed by biochemical detection of the production of opine in plant tissues. The opine synthesizing enzyme genes in T-DNA could be transferred and integrated into the genome of the host plant, when expressed in the host plant, the genes can produce opine synthesizing enzymes and catalyse the production of opine, which does not exist in the host plant in nature. Therefore the presence of opine in hairy roots can be used as a proof and index of successful transformation and integration of T-DNA in to plant tissues (Xu *et al.*, 2004).

In the present study, spots were produced in positions corresponding to agropinic acid by both transformed and non-transformed roots. However, variation in the amount of opines (agropinic acid) was evident from the relative intensity of staining. Even then, confirmation of transformation by HVPE was unsuccessful here because of the presence of interfering substances that show positive reaction to silver nitrate staining.

The results were in agreement with the findings Davey *et al.* (1987) who reported that silver nitrate positive compounds in both transformed and non-transformed root material of *Solanum* and *Nicotiana* species mask the detection of agropinic acid in transformed tissues.

Yoshimatsu *et al.* (2003) reported that confirmation of the transformation by detection of opines (agropine and mannopine) using a high-voltage paper electrophoresis was unsuccessful in *Cephaelis ipecacuanha*, because of the existence of interfering substances, which produced spots near the positions of agropine and mannopine after silver staining.

In the present study, compounds migrating to similar positions as mannopine and mannopinic acid were absent in all the tissues examined. Karmarkar (2001) reported that though agropine was detected in the hairy roots of *Holostemma adakodien* induced by *A. rhizogenes* strains ATCC 15834, PcA4 and A4 but none of the transformed roots showed the presence of mannopine.

Moreover, opine production can be unstable in hairy roots and may disappear after a few passages (Flores *et al.*, 1987). For this reason, detection of T-DNA by PCR or Southern hybridization is often necessary to confirm genetic transformation.

5.4.3 Confirmation by PCR analysis

PCR analysis of hairy roots was carried out for the confirmation of transformation. Polymerase Chain Reaction is a powerful technique for confirming DNA insertion in transformed tissues. Primers are designed such that they can simultaneously amplify specific regions of T-DNA that are expected to integrate into the plant genome. Advantages include the rapid manner in which large collections of transformed tissues can be analysed and the very small amount of plant tissues required (Lassner *et al.*, 1989).

For PCR analysis, the DNA was isolated from hairy roots subcultured in liquid medium (25 days) in order to make sure that the root material is free of bacterial (A4 and ATCC 15834) contamination.

By using DNAs from the hairy roots as template and the non-transformed roots as control, PCR products amplified with *rol* B primers and *rol* C primers,

respectively could be detected. It was demonstrated that two fragments, with length 205 and 520 bp corresponding to *rol* B and *rol* C, respectively were amplified only from the hairy root cultures (A4 and ATCC 15834) and cosmid pLJ1 but not from untransformed roots (control, blank and MTCC 2364). These results indicated that the *rol* B and *rol* C genes from the Ri plasmid of *A. rhizogenes* A4 and ATCC 15834 were successfully integrated into the genome of *W. somnifera* hairy roots. The roots produced by MTCC 2364 infected explants were non-transformed.

The *rol* B gene plays a major role in root induction and the *rol* C gene product confers optimal growth capacity to newly transformed roots (Lee *et al.*, 2004).

The confirmation of transformation using PCR analysis for detecting the presence of *rol* genes was reported in many species like *Pueraria phaseoloides* (Shi and Kintzios *et al.*, 2003), *Papaver somniferum* (Flem-Bonhomme *et al.*, 2004) and *Tylophora indica* (Chaudhuri *et al.*, 2005).

5.4.4 Confirmation by Southern hybridization

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

The DNA from hairy roots A4, ATCC 15834, roots produced by MTCC 2364 infected explants and control was subjected to restriction digestion using *Bam* H1 restriction endonuclease prior to blotting. The amplified products corresponding to *rol* B and *rol* C genes obtained by PCR reaction using Rol BF2R2 and Rol CF1R1 primers were used as probes separately.

It was found that the probe for *rol* B genes gave a single band corresponding to positive control. No bands were detected in the positions corresponding to hairy roots and control. The probe for *rol* C genes produced two bands for positive control. Here also no bands were detected in the positions corresponding to hairy roots and negative control.

The DNA sequence corresponding to the probe (*rol* C fragment) may be remaining as two separate fragments (pLJ1) following restriction with *Bam* H1. The restriction endonuclease *Bam* H1, restrict the sequence within the recognition sequence, which would be present inside the corresponding probe sequence of DNA and this resulted in the separation of DNA corresponding to probe sequence into two separate fragments.

Since PCR gives amplification corresponding to hairy roots of *rol* B and *rol* C genes in hairy roots, the absence of bands in hairy root samples would not be due to the non-transformed nature. It can be due to low concentration of the DNA samples or may be due to low DNA binding capacity of nitrocellulose filter.

However the presence of band in the positive control rule out the possibility of low DNA binding capacity of nitrocellulose filter to some extend and also the technical faults in carrying out the hybridization procedure.

Southern hybridization by detecting the presence of T-DNA for confirming the transformation was carried out in *Aesculus hippocastanum* (Zdravkovic- Korac *et al.*, 2004) *Gentiana species* (Momcilovic *et al.*, 1997) *Taraxacum platycarpum* (Lee *et al.*, 2004).

5.4.5 Dot blot analysis

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

The double stranded DNA does not bind efficiently to the filters hence; the denaturation treatment facilitated the efficient binding of DNA to the membrane in single stranded form. Dark spot was produced in the photographic plate

corresponding to the positions of the membrane loaded with DNA of A4, ATCC 15834 and cosmid pLJ1 and pLJ85 on using *rol* B probe (PCR amplified product of pLJ1 cosmind using Rol BF2R2 primers). No spot was present in positions spotted with DNA from control roots and roots produced by MTCC 2364 infected explants. It can be attributed that the samples (A4 and ATCC 15834) contained the sequences that hybridized with pLJ1-derived probe (*rol* B), showing the incorporation of TL-DNA.

This positive hybridization confirms the presence of *rol* B genes (TL-DNA) in hairy roots transformed by A4 and ATCC 15834. The absence of positive signal or spot indicated the non-transformed nature of control roots as well as the roots derived from MTCC 2364 infected explants. The presence of small dot in pLJ85-loaded position indicated the occurance of sequence with similarity to *rol* B genes in pLJ85, that is in TR-DNA. Bouchez and Camilleri, 1990 reported that a *rol* B gene sequence homologue, *rol* B_{TR}, has been identified on the TR-DNA 3' of the aux1 gene of *A. rhizogenes* A4 Ri plasmid. The restriction map of the TR region of pRiA4 showing the inserts of pLJ85 cosmids contains *rol* B gene (Jouanin, 1984).

The pLJ1 probe corresponding to *rol* C gene fragment (amplified by Rol CF1R1 primers) also hybridizes with DNA of A4 and ATCC 15834 derived roots, indicating the presence of TL-DNA in particular the *rol* C gene. Non-transformed roots (control and MTCC 2364 derived) did not hybridize with the probe confirming their non-transformed nature.

The spot corresponding to the positive control pLJ1 was much darker and larger compared to others, whereas the spots corresponding to others (A4 and ATCC 15834) were lighter indicating the high DNA concentration of the former sample over later samples.

Davey *et al.* (1987) carried out dot blot analysis for the confirmation of transformation in *Solanum nigrum*.

5.5 STANDARDISATION OF WITHANOLIDE ESTIMATION

Withanolides are predominantly associated with the members of Solanacea, and in particular for the genus *Withania* (Ganzera *et al.*, 2003).

Withaferin A was selected for the study due to its marked bioactivity. In the present study thin layer chromatographic method was employed for the estimation of withaferin A present in the roots due to its simplicity, accuracy and low cost over HPLC.

Gupta *et al.* (1996) also used TLC densitometry technique for the quantitative determination of withaferin A in different plant parts like leaves, roots, stems, seeds and persistent calyx, since this method was simple, rapid, accurate and less expensive.

The hexane extract of root powder on TLC anlaysis produced a spot corresponding to withaferin A. It means that on refluxing with hexane, some amount of withanolides also came into the extract. So it is better to avoid the use of hexane so as to minimize the loss of metabolites. However, Gupta *et al.* (1996) reported the use of hexane for defatting the methanol extract of *W. somnifera* root powder.

The withanolide portion of the ethanol extract of root powder was extracted with ethyl acetate and the alkaloid portion with chloroform and finally each fraction was dried by passing over anhydrous sodium sulphate.

Gupta *et al.* (1996) used diethyl ether for extracting withanolides and chloroform for extracting alkaloids from methanol extract of plant material and both were dried over anhydrous sodium sulphate.

In the present study, two types of precoated plates were found to be good for TLC analysis of withanolides that includes RP_{18} F_{254} plates with methanol-

water (9: 1) solvent system and Silica gel_{60} F_{254} plate where chloroform-methanol (9.8: 0.2) was used as the solvent system.

Dinan *et al.* (2001) reported the use of RP_{18} F_{254} plates with methanol-water (7: 8) solvent system and Si O_2 F_{254} plates with isobutyl methyl ketone-hexanol-hexane-acetic acid (30: 30: 40: 1) solvent system for TLC analysis of withanolides from *Iochroma gesnerioides*. Gupta *et al.* (1996) used precoated Silica gel_{60} F_{245} with Chloroform-methanol (9.5: 0.5) developing solvent.

In the present study, the spray reagent vanillin + boric acid + H_2 SO_4 + MeOH was found to be more sensitive for TLC analysis. Gupta *et al.* (1996) also reported the use of same spray reagent for visualizing the plate loaded with withaferin A.

The spot corresponding to withaferin A was initially magenta and on further charring, the colour turned to bluish violet. Gupta *et al.* (1996) reported the formation of a blue coloured spot for withaferin A.

The presence of withaferin A was confirmed in roots of field grown plants, normal *in vitro* roots as well as hairy roots by TLC analysis. Ganzera *et al.* (2003) reported that the analysis of *W. somnifera* root, stem and leaf confirmed the presence of withaferin A and withanolide D in all the parts of the plant but with significant differences in the ratio.

Banerjee *et al.* (1994) studied the production of withanolides with special reference to withaferin A, in the A4 induced hairy root lines of different growth phases (4, 10 and 24 weeks) using HPLC and found that maximum levels of withaferin A was observed in the media and hairy roots of 10 weeks old.

The roots of field grown plants were found to possess the highest level of withaferin A which was followed by hairy roots and then by the *in vitro* normal roots. According to Ray and Jha (2002) the withanolide content of six month old

seed derived plants showed a higher accumulation of withaferin A than tissue cultured plants of the same age. Kumar *et al.*, (2005) reported that in *W. somnifera* there is enhancement of secondary metabolites in hairy roots, which is indicated through significant enhancement of the antioxidant activity.

Contrary to the present results, Vitali *et al.* (1996) reported that though limited production of withanolides was observed in shoot and callus cultures, no withanolides were detected in hairy roots. Gupta *et al.* (1996) reported that leaf was the only source of withaferin A among the samples analysed which includes leaves, root, stem, seed and persistent calyx.

5.6 ENHANCEMENT OF SECONDARY METABOLITE PRODUCTION

5.6.1 Addition of osmoregulants

The addition of osmoregulant PEG (2.0 % and 5.0 %) failed to elicit a positive response on the biosynthesis of withaferin A in root cultures. This finding is in agreement with the results obtained by Sindhu (1999). She reported that none of the osmoregulants added to the basal growth medium such as poly ethylene glycol, mannitol and sorbitol (1.5 –3.0 %) could sustain callus growth and berberine synthesis.

Contrary to the present results Sankar (1998) reported that the osmoregulant PEG at 2.0 per cent elicited a positive response in leaf calli of *Sida cordifolia* on biosynthesis of ephedrine.

In the present study the hairy root cultures were found to be in more health and vigour in 2.0 per cent PEG (6000g) compared to 5.0 per cent PEG. This variability can be due to the difference in the osmotic pressures offered by the media.

Akcam-oluk (2003) reported that the application of osmotic stress using PEG 3350 (90 g l^{-1}) to the *Catharanthus roseus* cell suspension cultures increases ajmalicine production capacity, although it does not trigger the production of other

alkaloids involved. However, the cells exposed to PEG 600 (100g l^{-1}) did not survive at all. Polyethylene glycol with different molecular weight was found to have different influence on secondary metabolite production.

5.6.2 Addition of precursors

In the present study, the addition of precursor, methionine at 1.0 mM and 2.0 mM was found to be ineffective in increasing the withanolide production of hairy root cultures. However, the biomass of the roots increased progressively in this culture medium.

This result is in conformity with the findings of Veleiro *et al* (1985). They administered (^{14}C – methyl) methionine to the excised leaves and 15 days old seedlings of *Acnistus breviflorus* to study the accumulation of withaferin A and jabrosalactone and found that (^{14}C – methyl) methionine was a much poorer precursor of withanolides in terms of absolute incorporation. The lower absolute incorporation obtained with (^{14}C – methyl) methionine may be explained considering that this precursor is involved in a large number of biosynthetic pathways many of which are probably more active than those leading to withanolides.

Morgan and Shanks (2000) observed that feeding higher levels of auxin or tryptophan resulted in increased branching and thickening of the *Catharanthus roseus* hairy root cultures, which also resulted in dramatic reduction in flux to indole alkaloids.

5.6.3 Addition of Elicitors

5.6.3.1 Addition of Aspergillus homogenate

Elicitation is one of the methods that have been used to enhance secondary metabolites of cell cultures (Eilert, 1987).

In the present study *Aspergillus* homogenate at the rate of 250 µl/ 125ml as well as 500 µl/ 125 ml elicited a positive response on biosynthesis of withaferine A in the hairy roots of *Withania somnifera.*

The result is in conformity with the findings of Vazquez-Flota *et al.* (1994) who reported that the use of *Aspergillus* homogenate produced an increase in both the accumulation and yield of ajmalicine in *Catharanthus roseus* hairy root cultures.

The communication between elicitor and plant cell can be effected by various means. For example, fungal glycoprotein and carbohydrate elicitors may bind to certain receptor sites and elicit a phytochemical response from the cell (DiCosmo and Misawa, 1985).

The response of plant cells and tissues to elicitation with fungal homogenates is in direct relation with the composition of the cell wall of the fungi, where their different components are the true elicitors. Some types of cellulose-glucans are better elicitors than chitin-glucan (Kombrink and Hahlbrock, 1986).

The elicitors may act as hormones (in that they trigger a metabolic cascade) regulating secondary metabolism in those plant cells which are able to receive, decode and further modulate the molecular signal. The treatment of cultured plant cells with fungal elicitors can alter gene expression for secondary metabolite synthesis (DiCosmo and Misawa, 1985).

Sevon and Oksman-Caldentey (2002) reported that the elicitors increase the cell permeability. Enhancement of cell permeability may increase the formation of secondary products, because feed back inhibition and intracellular degradation of the products decrease.

In the present study, the oligosaccharide liberated from the cell wall of *Aspergillus* homogenate were the best elicitors, even though their identity is still

unknown. It would have increased the cell permeability of hairy root cultures facilitating enhancement in the secondary metabolite production. It was also noticed that the metabolite withaferin A was released into the *Aspergillus* homogenate containing culture medium.

Biotic elicitors have been reported to release the products from hairy roots into the medium without any loss of viability and production capacity of the hairy roots (Sevon and Oksman-Caldentey, 2002). Nef *et al.* (1991) reported that low concentration of the fungal homogenates induced the liberation of 90 per cent of the total ajmalicine into the medium.

The hairy root cultures were exposed to *Aspergillus* homogenate for 72 hrs to study the influence on secondary metabolite production. DiCosmo and Misawa (1985) reported that in elicitor: plant cell culture systems, the elicitation (induction) and accumulation of antibiotic secondary metabolites shows rapid kinetics, with maximum levels often elicited within 48-72 hrs after exposure to elicitor.

In the present study, the *Aspergillus* homogenate at the rate of 250 µl/ 125ml was found to be optimum concentration compared to 500 µl/ 125ml in the enhancement of secondary metabolites. Liu *et al.* (1997) reported that, homogenate of *Aspergillus oryzae* (5 ml per flask) elicited artemisinin accumulation after 48 hrs.

Tyler *et al.* (1988) reported that 500 µl of fungal homogenate (*Botrytis*) to be the maximum "dosage" of elicitor preparation at which cell viability could be consistently maintained.

5.6.3.2 Addition of Yeast extract

The hairy root cultures showed reduction in the accumulation of withaferin A content on adding yeast extract at the rate of 2.5 and 5.0 g l^{-1}. The yeast extract might have inhibited the metabolite synthesis and its accumulation in hairy root

cultures. Also, the addition of yeast extract did not favour the secretion of metabolites from hairy root cultures into the media. Hence the media also failed to respond in TLC analysis.

Linsafors *et al.* (1989) observed that treatment of *Panax ginseng* tissue cultures with yeast glucans and chitins did not stimulate accumulation of ginsenoides. The use of yeast (*Rhodotorula marina*) failed to elicit the accumulation and yield of alkaloids in *Catharanthus roseus* hairy root cultures (Vazquez- Flota *et al.*, 1994)

DiCosmo and Misawa (1985) reported that low-molecular weight products from certain fungi could inhibit elicitation activity by a process, which may involve competition for membrane receptor sites. Specific fungal molecules can therefore suppress plant cell metabolism to synthesize antibiotic secondary metabolite.

Contrary to the present results, Wibberley *et al.* (1994) reported that with the use of yeast extract elicited the accumulation of sesquiterpene phytoalexins (capsidol and debneyol) in *Nicotiana tabacum*.

The withaferin A content of 20 day grown hairy root cultures (control) were found to be lower than 28 day grown hairy roots. It is reasonable to assume that decreasing growth rate leads to accumulation of primary metabolites, amino acids, nucleic acids and other precursors. Accumulation of these products is perhaps directly involved in stimulating secondary metabolism. This may help to explain why increase in secondary metabolite synthesis is observed late in the growth cycle (DiCosmo and Misawa, 1985).

Summary

SUMMARY

The present study entitled "Genetic transformation for hairy root induction and enhancement of secondary metabolites in *Aswagandha* (*Withania somnifera* (L.) Dunal)" was carried out at the Centre for Plant Biotechnology and Molecular Biology and Biochemistry Laboratory of the College of Horticulture, Vellanikkara and the Biochemistry Laboratory of Aromatic and Medicinal Plants Research Station, Odakkali. The salient findings of the study are stated below:

1. The seeds of *Withania somnifera* showed early and high germination percentage under dark compared to 16hr (1000 lux) photoperiod.
2. Maximum regeneration of hypocotyl segments placed upside down (70%) was obtained on MS medium supplemented with BAP 1.5 mg l^{-1} and IAA 0.5 mg l^{-1} or BAP 1.0 mg l^{-1}, IAA 0.5 mg l^{-1} and coconut milk 10 per cent whereas the hypocotyl segments placed directly showed maximum regeneration response on MS medium supplemented with BAP 1.0 mg l^{-1} and IAA 0.5 mg l^{-1}.
3. The number of shoot buds regenerated was more from hypocotyl segments placed upside down compared to segments that were placed directly and the shoot buds were produced only from the upper portion of hypocotyl segments, whether placed directly or upside down.
4. Among the different combinations tested, the multiplication of hypocotyl derived shoot buds were more on MS medium supplemented with BAP 0.4 mg l^{-1} and IAA 0.5 mg l^{-1} and the number of elongated shoot buds decreased with the increase in concentration of BAP with respect to IAA.
5. Maximum elongation of hypocotyl and cotyledonary segment derived shoots occurred on MS medium supplemented with GA_3 0.5 mg l^{-1} and this combination produced elongation of the leaf petiole whereas, GA_3 1.0 mgl^{-1} produced elongation of the internode. The leaf size was also more in the former combination.

6. The plantlets from hypocotyls derived shoots showed maximum health and vigour in half MS with 2.5 per cent sucrose and the number of internodes and leaves were also high in this combination.
7. Pulse treatment with IBA 1000 mg l^{-1} for five seconds was found to be the best for efficient rooting of hypocotyl derived shoots.
8. Shoots elongated on MS with GA_3 0.5 mg l^{-1} showed efficient rooting with very less callusing tendency whereas the shoots obtained after multiplication on BAP and IAA showed high callusing tendency at the shoot base on rooting media after pulse treatment.
9. Both direct as well as indirect organogenesis was obtained from cotyledonary segments in the same culture cycle.
10. Cotyledonary segments showed maximum regeneration response (44%) on MS medium supplemented with BAP 1.5 mg l^{-1} and IAA 0.5 mg l^{-1} and cotyledonary segments derived calli showed good regeneration in BAP alone without KN.
11. The shoots produced from hypocotyls showed faster elongation compared to shoot produced from cotyledonary segments.
12. *In vitro* flowering was obtained from shoot produced by cotyledonary segment in MS with GA_3 0.5 mg l^{-1} and leaf in half MS with 0.25 per cent activated charcoal.
13. Maximum multiplication was obtained from shoot tips and nodal segments on MS with BAP 2.5 mg l^{-1} and IAA 0.5 mg l^{-1}. Regeneration from basal callus was also high in this combination and compared to shoot tips, nodal segments produced more multiple shoots.
14. The number of differentiated shoot buds from basal callus of shoots tips and nodal segments increased with the increase in concentration of BAP (up to 2.5 mg l^{-1}) with respect to IAA.
15. Subculturing was found to be highly promising for efficient multiplication of shoot tip derived shoots.
16. Pulse treatment with IBA 1000 mg l^{-1} for five seconds and further inoculation on half MS with 0.25 per cent activated charcoal was found to be the best for efficient rooting.

17. Pulse treatment with IBA 1000 mg l^{-1} for five seconds and further inoculation on half MS without activated charcoal produced numerous short and stout roots along with prominent callus on the shoot base.
18. Production of lateral roots was more on half MS with IBA (0.5/1.0 mg l^{-1}) and on adding IAA along with IBA, roots became thicker and shorter with less lateral root production.
19. Proliferation of callus was more at the cut ends of shoot on rooting medium supplemented with IAA.
20. Increasing the concentration of IBA from 0.5 to 1.0 mg l^{-1} in the presence of IAA increased the number as well as the length of the roots.
21. Maximum regeneration response was obtained from leaf segments (30%) on MS with BAP 1.0 mg l^{-1} and IAA 0.5 mg l^{-1}.
22. Somatic embryo like structures were produced from *in vitro* seedling root on MS supplemented with BAP 1.5 mg l^{-1} and IAA 0.5 mg l^{-1}.
23. Roots showed good callusing tendency but very less regeneration tendency.
24. 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.
25. Among the three *Agrobacterium rhizogenes* strains tested, the strains A4 and ATCC 15834 were able to induce hairy roots in *Withania somnifera* whereas, the strain MTCC 2364 failed to induce hairy roots by any of the transformation methods.
26. Of the two strains A4 and ATCC 15834, the strain ATCC 15834 showed a greater potential for transformation compared to A4.
27. Among the various explants tested, the leaf segments and shoot tips showed efficient transformation and the leaf segments showed a greater percentage of hairy root formation (70%) than the shoot tips (33.33%).
28. The bacterial concentration or the type of inoculum affected the transformation frequencies. The bacterial cells from single cell colonies (DIM) of A4 produced transformation, whereas single cell colonies of ATCC 15834 and MTCC 2364 did not produce any transformation. Suspension culture of A4 and ATCC 15834 (SM) produced transformation.

29. The transformation frequency was influenced by the co-culture period. Leaf segments showed maximum response to induce hairy roots in one-day co-culture period with A4 and ATCC 15834 whereas; shoot tips produced maximum response by two-day co-culture. In general 9-20 days was taken for hairy root induction.
30. Necrosis of hypocotyl explants increased with the increasing co-culture period and they showed least tendency for callusing among the various explants tested.
31. Callusing and normal rooting also occurred from transformed explants along with hairy root induction.
32. Callusing of explants was more with strain ATCC 15834 and the leaf segments produced more callus compared to other explants, both from the petiolar end as well as from the wound sites present in the inner leaf lamina.
33. Hairy roots initiated directly as well as from the proliferated callus of leaf explants and the size of the callus grew in the subsequent subcultures.
34. Non-transformed (control) leaf segments produced callus only from the petiolar end.
35. The presence of 100 μM acetosyringone in the co-culturing media increased the number of putative transformants with A4 strain following DICA and SCA method. With the strain 15834, the presence of acetosyringone was not found to positively influence the transformation percentage.
36. The inner laminar region of leaf segment produced only one hairy root per wound site, whereas 2-3 hairy roots were produced around the petiolar end on infection with A4.
37. A maximum of ten hairy roots were produced per single leaf segment by ATCC 15834 infections (proximal end) with a mean of 3.33 roots and the shoot tips produced a maximum of three hairy roots per single explant (mean: 2.66).
38. The hairy roots were induced more from the petiolar region of leaf segments and in the absence of petiole hairy roots were produced from the

proximal end. From the shoot tips, hairy roots were produced from and around the basal portion only and no roots developed from the leaves attached to shoots.

39. On transformation of leaf segments with ATCC 15834, it was clearly visible that once the explant got transformed, the tendency for producing normal roots got decrease and more number of hairy roots was produced in place of normal roots.
40. Among the root cultures, some root lines showed faster growth with high lateral branching whereas some hairy roots showed only slower growth and at the same time some failed to grow and later the colour turned brown. The hairy roots normally showed a plagiotropic growth habit and some showed reduced geotropism. The colour of the hairy roots gradually changed from white to cream and later to brown almost 25-28 days after incubation.
41. Control roots grew slowly compared to transformed roots and is positively geotropic with comparatively less lateral branching and hairiness and got killed after 2-3 subcultures in growth regulator free media.
42. Compared to ATCC 15834 induced hairy roots, roots induced by A4 strain showed faster growth producing more biomass.
43. In comparison with roots in solid cultures, hairy roots cultured in liquid growth regulator free half MS medium grew rapidly with high lateral branching.
44. Among the four liquid media tested, half MS was found to be superior for promoting hairy roots in *W. somnifera* followed by MS, B_5 with 2.0 per cent sucrose and B5 with 3.0 per cent sucrose respectively. Bulging or callusing of hairy roots occurred in B_5 with 3.0 per cent sucrose. In all the culturing media the newly growing regions of hairy roots were creamy white in colour, whereas the initial roots incubated turned brown.
45. The hairy roots exhibited a sigmoid growth pattern and no significant differences were observed in the growth of hairy roots under dark and diffused light.

46. 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.
47. Confirmation of transformation by opine detection using high-voltage electrophoresis was found to be unsuccessful in *W. somnifera* because of the existence of interfering substances which produced spots near the positions of agropinic acid after silver staining.
48. Genomic DNA could be isolated from *W. somnifera* using modified Doyle and Doyle method.
49. The Polymerase Chain Reaction confirmed the presence of *rol* B and *rol* C genes of TL-DNA in the hairy roots induced by A4 and ATCC 15834 strain.
50. In Southern hybridization, high concentration of DNA might be required to obtain positive signal from transformed roots.
51. Dot blot analysis also confirmed the presence of *rol* B and *rol* C genes of TL-DNA in the hairy roots induced by A4 and ATCC 15834 strains based on the positive hybridization signal obtained.
52. In dot blot analysis, the PCR amplified product of *rol* B gene (probe) showed positive signal from pLJ1 and pLJ85 cosmid, indicating the presence of *rol* B gene in TL and TR- DNA of A4 Ri plasmid.
53. Thin Layer Chromatography method can be employed for the estimation of withanolides.
54. It is better to avoid the use of hexane for removing fats, waxes and oil from root material so as to avoid the risk of metabolite losage and separation of alkaloid was not found to be necessary for withanolide estimation.
55. RP_{18} F_{254} plate with methanol-water (9:1) solvent system and Silica gel_{60} F_{254} plates with chloroform-methanol (9.8:0.2) solvent system can be used for TLC analysis of withanolides.
56. The spot corresponding to withaferin A can be viewed under UV at 254 nm. The spot was initially magenta and on further charring the colour turned to bluish violet. The spray reagent vanillin + boric acid + H2SO4 + Methanol was found to be more sensitive for TLC analysis.

57. Withaferin A content was more in *W. somnifera* field root, followed by hairy root, normal *in vitro* root respectively.
58. The addition of osmoregulant PEG (2.0 and 5.0 %) failed to elicit a positive influence in the biosynthesis of withaferin A in root cultures.
59. The addition of precursor methionine at 1.0 mM and 2.0 mM were found to be ineffective in increasing the withanolide production of hairy root cultures, though the root growth was favoured in this media.
60. Elicitation by *Aspergillus* homogenate at the rate of 250 µl/ 125ml as well as 500µl/ 125 ml elicited a positive response on biosynthesis of withaferin A in the hairy roots of *W. somnifera*. The accumulation of withaferin A increased progressively in the hairy roots on addition of *Aspergillus* homogenate, so also the metabolite was found to be released into the media.
61. The *Aspergillus* homogenate at the rate of 250 µl/ 125ml was found to be the optimum concentration compared to 500µl/ 125 ml in the enhancement of secondary metabolite.
62. The hairy root cultures showed reduction in the accumulation of withaferin A content on adding yeast extract at the rate of 2.5 and 5.0 g l^{-1}.
63. The withaferin A content of 20 day grown hairy root cultures (control) were found to be lower than 28 day grown hairy roots i.e. the increase in secondary metabolite synthesis occurred late in the growth cycle.

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* originals not seen

Appendices

APPENDIX I

Composition of plant tissue culture media

Constituent	Murashige and Skoog (1962) – MS (mg l^{-1})	Gamborg's (1968) – B_5 (mg l^{-1})
KCl	-	-
$MgSO_4.7H_2O$	370	250
$NaH_2PO_4.H_2O$	-	150
$CaCl_2.2H_2O$	440	150
KNO_3	1900	2500
$CaCl_2$	-	-
Na_2SO_4	-	-
NH_4NO_3	1650	-
KH_2PO_4	170	-
$Ca(NO_3)_2.4H_2O$	-	-
$(NH_4)_2SO_4$	-	134
$FeSO_4.7H_2O$	27.8	-
$MnSO_4.4H_2O$	22.3	-
$MnSO_4.H_2O$	-	10
KI	0.83	0.75
$CoCl_2.6H_2O$	0.025	0.025
$Ti(SO_4)_3$	-	-
$ZnSO_4.7H_2O$	8.6	2
$CuSO_4.5H_2O$	0.025	0.025
H_3BO_3	6.2	3
$Na_2MoO_4.2H_2O$	0.25	0.25
$Fe_2(SO4)_3$	-	-
EDTA disodium salt	37.3	-
EDTA-Na ferric salt	-	43
m-inositol	100	100
Thiamine	0.1	1.0
Pyridoxine	0.5	1.0
Nicotinic acid	0.5	1.0
Glycine	2	-
Cysteine	-	10
Sucrose	30,000	20,000

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	1.0	-	5.0	-
K_2HPO_4	-	0.5	-	-
Yeast extract	2.0	1.0	1.0	5.0
$MgSO_4.7H_2O$	-	0.2	0.5	-
Peptone/Trypton	5.0	-	5.0	10.0
Mannitol	-	10.0	-	-
NaCl	5.0	0.1	-	10.0
Sucrose		-	5.0	-
Agar	15.0	20	20	20

Adjust pH to 7.0

APPENDIX III

Reagents used for DNA isolation

1) Extraction buffer (4X)

Sorbitol	-	2.5 g
Tris- HCl	-	4.8 g
EDTA	-	0.74 g

The chemicals were dissolved in 60 ml sterile distilled water. The pH was adjusted to 7.5 and the final volume was made up to 100 ml with distilled water and then autoclaved.

2) Lysis buffer

1M Tris- HCl (pH- 8.0)	-	20 ml
0.25 M EDTA	-	20 ml
5M NaCl	-	40 ml
CTAB	-	2 g
Distilled water	-	20 ml

Cetyl Trimethyl Ammonium Bromide (CTAB) was dissolved in 20 ml sterile distilled water. To this solution the required volumes of other stock solutions are added.

3) Tris- HCl 1M (pH- 8.0)

Tris-m HCl (15.76 g) was dissolved in 60 ml sterile distilled water. The pH was adjusted to 8.0 and final volume was made up to 100 ml with distilled water and then autoclaved.

4) EDTA 0.25 M

Ethylene Diamine Tetra Acetic acid (EDTA) 9.305 g was dissolved in 100 ml sterile distilled water and autoclaved.

5) NaCl 5M

Sodium chloride 29.22 g was dissolved in 100 ml sterile distilled water and autoclaved.

6) Sarcosine 5%

Sarcosine 5 g was dissolved in 100 ml sterile distilled water and autoclaved

7) TE buffer

(Tris HCl –10.0 mM; EDTA- 1.0 mM)

Tris HCl 1.0 M (pH 8.0)	-	1.0 ml
EDTA 0.25 M (pH 8.0)	-	0.4 ml
Distilled water	-	98.6 ml

Autoclaved and stored at room temperature.

8) Ice-cold Isopropanol

9) Chloroform- isoamyl alcohol (24:1 v/v)

To 24 parts of chloroform, one part of isoamyl alcohol was added and mixed properly. The mixture was stored in refrigerator before use.

10) Ethanol 70 per cent

To 70 parts of absolute ethanol, 30 parts of double distilled water was added.

APPENDIX IV

Buffer and dyes used in gel electrophoresis

1) 6X Loading / Tracking dye

Bromophenol blue	-	0.25 %
Xylene xyamol	-	0.25 %
Glycerol	-	30 %

The dye was prepared and kept in fridge at 4 °C.

2) Ethidium bromide (intercalating dye)

The dye was prepared as a stock solution of 10 mg ml^{-1} in water and was stored at room temperature in a dark bottle.

3) 50X TAE buffer (pH8.0)

Tris base	-	242.0 g
Glacial acetic acid	-	57.1 ml
0.5 M EDTA (pH 8.0)	-	100 ml
Distilled water	-	1000 ml

The solution was prepared and stored at room temperature.

APPENDIX V

Reagents used for cosmid isolation

1) Solution I (Resuspension buffer)

Glucose	-	50 mM
Tris	-	25 mM
EDTA	-	10 mM
pH	-	8.0

2) Solution II (Lysis buffer)

NaOH	-	0.2 M
SDS	-	0.1 %

3) Solution III (Neutralization buffer)

CH_3COOK	-	5M
pH	-	5.5

Appendix VI

Sequence alignment of designed primers with *A. rhizogenes* pRiA4 plasmid TL-DNA *rol* B-C genes

```
TTCAAAAAAAGCGCGATGAAATCAAGTATCCAGTTATTAAACCAGATCTTGATTTTTATT 60

ACATTAATACTTCTTAAAGGGAAACAAGTGACACACTCAGCTTCTCCTGACATCAAACTC 120
            ROL CF1- TTAGCCGATTGCAAACTTGCTC
GTCAGCCCATCGACTAACCATTAGCCGATTGCAAACTTGCTCTCGCCATGCCTCACCAAC 180
                     **********************
TCACCAGGTTCGAACCTAAGCTGGGTGCTGGCATAGAGGTCGAATGTCAGACACTTTCCC 240

TTTGTCGAAGTTAGCTCCATCTGCTCATTCAGCTTGATGACACGCCCAGGGAAAGAAAAT 300

GCAGCGAAGTAGGCGCTCCGCATAGGATCCGCGTAACCGAAAAAACCTGATGCTTGCCTG 360

AGCCCTCTATTGACCTCATCGAGAGTCACATCATGCTGGTACGGGGGAAGGTTGGTGAGG 420

AGTGCGCCTTGCATCCAATTGTAAGGCAAGGACCCTCCATAGAAGCAGAGCATCATCGTC 480

GGGCAGTCGACGTAGAGGTACAGCAGCCCGTGGCCCACCGACCCTCCTTCTTCGTCAATA 540

TCCATCGATTGATTCTCACCCGGCTCGATCAAGGGTTTACGCTCATTTGAGGCGTTCTTC 600

ATGTGTCTAGCTAGCTCATCGCTGCTTGTCACATCCTCCACTTTGAGCTTGAAAAAGAGA 660
 AACACAGGTCGTCTTCAGCCAT- ROL CR1
GAACACAGGTCGTCTTCAGCCATGTTAACAAAGTAGGAAACAGGTTGCTTGTACCCATAA 720
 **********************
CTCGAAGCATCCAAACTATTTATAATCCAGAGCTCTTAGCGTATTGGAGCAAATAATAAA 780

AATATTTGAGTTAGGTATAGGTCCACAAATTTATTAATTAATGGATGCGGCTGTCGATGC 840

TCCACGTCGCCTCGTTGTGCGAATATAGAAGGGACACCTGCCTGCTTTTAATTGTTTTTA 900

CTTATTAGGTTTAAAGCTCAGCGACCCACGTGAGGGAATAGGTTCTGTCCTCCAATAATG 960

GTGGTTTGTGGTCTATGGAATCTCAGCTTTTGCTTTTTCCTTTAATAATGGGACGCACCC 1020

GCCTACCTTTATTTATGCTGCGGAAAGGCGCTACTTTGTCAAGCTAAACCCCACGTTAGG 1080

CCTGTCGGCACCAAAGCAACACAGCTGGCAATTTATTATAGGGTCTCTTAAAAGCACGTA 1140

CATGGCCACCATTCCTTCTTAGGCTAACTGCTATTCGCCCTGCTTATTTATTTTTTATTA 1200

AACTCAGAAACAGAGATAATAGTTTTGCACCCACTAAACTAGTGTAAATTTGGCATATAT 1260

CTATAGCAGGTTCTCCAAAACCATGTATTTCCTTTTGGAATAATTTATTCAACACGATAA 1320

TCGCTTAAACGTATTCCCGATTGAAAAAGATTAACACATATTATTATTGCTATTATTAAT 1380

ACATCATTTTGGCATAGATAATTTCCGCACTATATTACATATACAATGTTAGTAATTAAT 1440

ATGATTGTGGCAATACTTTACATCTTAACCAGAAAATAGAAAAAGTATCTTGAATGCAGC 1500
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```
AATCTTCACGTTTGCCAATGCGCTTTCGCGAAATCCAATAGCGGGCCAACTTTAAGCTTT 1560

ACCCCACTCCTCCCAATTATGGGCATCCTTTTTTGACATCCTTTCGCTAAGGCCAAACAA 1620

CACGACAAGAAAAAGCAAACCCAGTACTATGAACCCATGAACGATCACGCATGCAGTATT 1680

TTCGCTTGATCCAGATTTTAAGGCCTCCCGTGTCTTGCGCATCGATCTTGATGTGATTGT 1740

TGCCAAACTACCTCTGGCTTTCCCGACCAGAGACTGTGTCTGATTGGCGCCAGTTTCCGA 1800

CGCCGTATTTTAAATGGCCCTGGCACTTGCCTTTTTCGTAACTATCCAACTCACATCACA 1860

              ROL BF1-    TCCACGATTTCAACCAGTAG
ATGGATCCCAAATTGCTATTCCTTCCACGATTTCAACCAGTAGATCTCACTCCAGCATGG 1920
                          ********************
AGCCAGATAAACCTATTCGAGGGGATCCGATTTGCTTTTGCAATCTATAGCCGTGACTAT 1980

AGCAAACCCCTCCTGCATTTCCAGAAACGATGGGCTCTTGCAGTGCTAGATTTGAAGGAA 2040

AACTCTCCACCGATATATATACTTAAACAACTAGCTGAGCTCTTGAAGAACAAAGTCTGC 2100

TATCATCCTCCTATGTTTGTTAGTCAGCCGGATCTGGCTCGAGAAAACGACCAACATGTA 2160

TTTGTCTATCTTTCTCGCGAGAAGATGCAGAAAGTGCTGAAGGAACAATCCATTACATTT 2220

GGAATGGAGGCCGTGCTGGCGACAACGATTCAACCATATCGGAGCGAGCTCGCCCTCCAG 2280

GAGATGCTCCGTGTTCACAACCTTGCTTGGCCGCACAGCCGCACGGAGGAACCTGATTTA 2340

GAATGCTTCATCGCCATTTTCGCAAGTTCCTTGTTCATTCACTTGCTGGAGTTAAAAGTG 2400

ACCAACGTTTACGGGAGAGAGGTAGCTTGCACCTTCTTTCTGCGGCGAGGGACTGAAAAC 2460

CGCCCCTATGATGTTGTAGCTTGCGGCACCACACAATTCACCAAAAATGCCCTCGGGATA 2520

TCACGTCCGGCCGCCTCCTCACCGGAGCCAGACCTAACCCTGCGACTCTCGGGGCCTGAT 2580

                                                    ROL BR1 - GCCTAA
CAGGAAGGCGAGGAGGGCGTCATGAAGCCTGCTGCAGTAAACCTGAAGAAAGAAGCCTAA 2640
                                                              ******
AGCCGACTTGAACT
AGCCGACTTGAACTCCCCCTGCAGGCAACCTTTATCTATGAGTTTGTCATAAGTTCTATG 2700
**************
TACCCTCCCGCAGTCTGTGACACAGAACCTTGGGAGTTGTAGCGTACGTTGTAATGTGTT 2760

GACCTATTTTCTTGTACTAAATATTTTCTTCTGTGTTGATCCTGCTGCTGAATTTTGCCA 2820

AAAACAGCACATGCTCATATGACTATCTAATCTACTACACATATATTGCAGTATCAACAA 2880

CAACGACACACCTGGACTTATAATATTATAGTTCAACAGTACATTTGACATAAAACATTT 2940

TCACGACAT 2949
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Appendix VII

Reagents for Southern hybridization

1) Pre hybridization solution
   6X SSC
   5X Denhardts reagent
   0.5 % SDS
   Denatured salmon sperm DNA- 10 mg ml^{-1}

2) Hybridisation solution
   6X SSC
   5X Denhardts reagent
   0.5 % SDS
   Radiolabelled probe

   a) 20X SSC
      Sodium chloride - 175.3 g
      Sodium citrate (trisodium citrate 2-hydrate) - 88.2 g
      The pH was adjusted to 7.0 and the final volume was made up to 1000 ml with distilled water.

   b) Denhardts reagent (50X)
      Ficoll (type 400) - 5.0 g
      Poly vinyl pyrolidone - 5.0 g
      Bovine sperm albumin (fraction V) - 5.0 g
      Finally made up to 500 ml with distilled water, filter sterilized and stored at –20 °C.

   c) Salmon sperm DNA
      Stock solution 10 mg ml^{-1} was boiled for 10 minutes and stored at –20°C as aliquots. Just before use it was heated in water bath for 5 minutes and quickly chilled on ice.

3) Developer solution (For developing X- ray film)
   Metol - 4 g
   Sodium sulfite (anhydrous) - 300 g
   Hydroquinone - 16 g
   Potassium bromide - 10 g
   Sodium carbonate (anhydrous) - 200 g
   The chemicals were dissolved in distilled water and the final volume was made up to 2.0 litre.

4) Fixer

   Hypo (Sodium thiosulfate) - 200 g/ 2 litre

GENETIC TRANSFORMATION FOR HAIRY ROOT INDUCTION AND ENHANCEMENT OF SECONDARY METABOLITES IN *ASWAGANDHA* (*Withania somnifera* (L.) Dunal)

By

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ABSTRACT OF THE THESIS

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ABSTRACT

The present study entitled 'Genetic transformation for hairy root induction and enhancement of secondary metabolites in *Aswagandha* (*Withania somnifera* (L.) Dunal) was carried out at the Centre for Plant Biotechnology and Molecular Biology and Biochemistry Laboratory of the College of Horticulture, Vellanikkara and the Biochemistry Laboratory of Aromatic and Medicinal Plants Research Station, Odakkali. The study was undertaken to standardize the *in vitro* regeneration protocol in *W. somnifera* from different explants, to standardize the genetic transformation using *Agrobacterium rhizogenes*, to standardize the biochemical techniques for the estimation of secondary metabolites in roots and also to enhance the secondary metabolite production in the hairy root cultures of *W. somnifera*.

An efficient method for *in vitro* plant regeneration was developed in *W. somnifera*. Different explants such as hypocotyls, cotyledonary segments, leaf segments, shoot tips, nodal segments and roots from *in vitro* germinated seedlings were used for the study. The seeds showed early and high germination under dark compared to 16 h photoperiod.

Multiple shoot formation was observed from hypocotyl segments placed directly and upside down. Maximum regeneration response was obtained from hypocotyls, cotyledonary segments and leaf segments in MS + BAP 1.0/ 1.5 mg l^{-1} + IAA 0.5 mg l^{-1}. Shoot buds produced from hypocotyls and cotyledonary segments showed good multiplication in MS + BAP 0.4 mg l^{-1} + IAA 0.5 mg l^{-1}. Cotyledonary segments derived callus regenerated in MS + BAP 2.0 mg l^{-1}. Shoot tips and nodal segments taken from axenic seedlings showed highest frequency of multiple shoot formation in MS + BAP 2.5 mg l^{-1} and IAA 0.5 mg l^{-1}. Regeneration from basal callus, produced by shoot tips and nodal segments was also obtained in the same culture cycle. The roots taken from *in vitro* seedlings and *in vitro* rooted plantlets showed high callusing but failed to regenerate by direct

organogenisis. However, somatic embryo like structures were produced from seedling roots in MS + BAP 1.5 mg l^{-1} and IAA 0.5 mg l^{-1}.

The shoot/ shoot buds produced good elongation in MS + GA3 0.5 mg l^{-1}. *In vitro* flowering of cotyledonary segment derived shoots was also obtained in this combination. The shoots were successfully rooted in half MS + 0.25 per cent activated charcoal by pulse treatment with IBA 1000 mg l^{-1} for five seconds. The plantlets were successfully hardened and transferred to large pots in the green house.

Genetic transformation was carried out in *W. somnifera* using three different *A. rhizogenes* strains like A4, ATCC 15834 and MTCC 2364 for inducing hairy roots. The explants such as hypocotyls, cotyldonary segments, leaf segments, shoot tips and nodal segments were used for genetic transformation. Here the influence of different parameters such as type of explants, type of bacterial inoculum, co-cultivation periods and acetosyringone effects on transformation frequencies were studied. Among the three *A. rhizogenes* strains, the strains A4 and ATCC 15834 produced successful transformation. Of this two successful strains ATCC 15834 showed a greater potential for transformation. Among the various explants used, only the leaf segments and shoot tips produced hairy roots. Leaf segments showed a greater percentage of transformation than the shoot tips. Though A4 strain produced successful transformation in *W. somnifera* by direct inoculation of bacteria from single cell colonies as well as in the suspension form, the strain ATCC 15834 produced transformation only in the suspension form. A co-cultivation period of one day was found to be the best for leaf segments, whereas shoot tips responded more under two day co-culture period. The acetosyringone (100 μM) enhanced the transformation percentages with A4 strain, whereas no such influence was observed with ATCC 15834 strain.

The hairy root cultures established on MS + 250 mg l^{-1} cefotaxime showed phenotypic variations in growth habit. The hairy roots normally produced high lateral branching with plagiotropic growth habit and showed sigmoid growth

pattern. Among the four liquid media tested, half MS was found to be superior in promoting hairy root growth followed by MS, B5 + 2.0 per cent sucrose, B5 + 3.0 per cent sucrose respectively

The confirmation of transformation by opine detection was found to by unsuccessful in *W. somnifera* because of the presence of interfering substances which produced spots near the positions of agropinic acid including control. Because of the low concentration of DNA, Southern hybridization technique failed to produce band corresponding to hairy root samples. However, the transformation was confirmed in A4 and ATCC 15834 induced hairy roots by PCR and dot blot analysis.

A Thin Layer Chromatographic method was employed for withanolide estimation. Withaferin A was used as the standard in estimation studies. Silica gel_{60} F_{254} plate chloroform- methanol (9.8: 0.2) was used as the solvent system. The spot was observed under UV at 254 nm and also the sensitivity was improved by using vanillin (0.05 g) + boric acid (1.0 g) + H2SO4 (2.0 ml) + Methanol (100 ml) spray reagent. Withaferin A produced magenta spot, which changed to bluish violet on further charring. Field root possess more withaferin A followed by hairy roots and in vitro roots contained the least.

Enhancement of secondary metabolite production was studied using techniques such as addition of osmoregulants, precursor feeding and elicitation. The withaferin A content in the hairy root biomass and the culture medium were estimated. The osmoregulant PEG (2.0 % and 5.0 %) and methionine precursor (1mM and 2mM) failed to enhance the withaferin A content. With the addition of yeast extract (2.5 and 5.0 g l^{-1}) a reduction in withaferin A content was observed in the root biomass. However, the biotic elicitor *Aspergillus* homogenate (250 and 500 µl /125 ml) elicited a positive influence on the biosynthesis of withafein A in the hairy root cultures.