INDUCTION AND ESTABLISHMENT OF TRANSFORMED HAIRY ROOT CULTURES OF SARSAPARILLA (*Hemidesmus indicus* L.) R. Br.

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(2009-09-115)

M.Sc. INTEGRATED BIOTECHNOLOGY

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

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THESIS

Submitted in partial fulfillment of the

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KERALA, INDIA

2014

DECLARATION

I hereby declare that the thesis entitled **"Induction and establishment of transformed hairy root cultures of sarsaparilla (*Hemidesmus indicus* L.) R. Br."** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis, entitled **"Induction and establishment of transformed hairy root cultures of sarsaparilla (*Hemidesmus indicus* L.) R. Br."** is a record of research work done independently by **Mr. Sudheep Gopi (2009-09-115)** 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 him.

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We, the undersigned members of the advisory committee of Mr. Sudheep Gopi, a candidate for the degree of **Master of Science (Integrated) in Biotechnology**, agree that the thesis entitled **INDUCTION AND ESTABLISHMENT OF TRANSFORMED HAIRY ROOT CULTURES OF SARSAPARILLA (*Hemidesmus indicus* L.) R. Br.** may be submitted by Mr. Sudheep Gopi, in partial fulfillment of the requirement for the degree.

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Sudheep Gopi

Dedicated to My Parents

&

Beloved Sister

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µL	Microlitre
µ*M*	Micromolar
2,4- D	2.4- Dichlorophenoxy acetic acid
2iP	2-Isopentenyladenine
ABA	Abscisic acid
BAP	Benzylaminopurine
B.P.	Boiling point
cm	Centimetre
DW	Dry weight
FW	Fresh weight
g	gram
GA_3	Gibberellic acid
GC MASS	Gas chromatography mass spectrometry
GI	Growth index
$HgCl_2$	Mercuric chloride
HPLC	High performance liquid chromatography
HR	Hairy root
IAA	Indole 3- acetic acid
IBA	Indole 3- butyric acid
KIN	Kinetin
Lbs	pounds
mg/L	Milligram per litre

mM/L	Millimolar per litre
mM	Millimolar
Min	Minute
mm	Millimeter
mL	Milliliter
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
NMR	Nuclear magnetic resonance
PGR	Plant growth regulator
PSI	Pounds per square inch
RF	Relative flow
rpm	Rotations per minute
TLC	Thin Layer Chromatography
UV	Ultraviolet
WHO	World Health Organization
t	Tonne
v/v	Volume/volume
w/v	Weight/volume

INTRODUCTION

1.NTRODUCTION

Medicinal plants are those plants, in which one or more of its organ contains substances that can be used for therapeutic purposes or which, are precursors for the synthesis of useful drugs (WHO., 1977). The use of plants as medicine is as old as human civilization. People of all ages from developed and developing countries use plants as an attempt to cure various diseases and to get relief from physical sufferings. Natural products are sources for bioactive compounds and have potential for developing novel therapeutic agents (Avinash *et al*., 2011). India is full of biodiversity and has rich flora and fauna with many plants having medicinal values (Shrivastava, 2014).The use of traditional medicines and medicinal plants in most developing countries as therapeutic agents for the maintenance of good health has been widely observed (UNESCO., 1996).

The increasing demand of plant-based drugs is creating heavy pressure on some high-value medicinal plant populations in the wild due to over-harvesting. Several of these medicinal plant species have slow growth rates, low population densities, and narrow geographic ranges (Nautiyal *et al.,* 2002), therefore they are more prone to extinction (Jablonski, 2004). *In vitro* culture techniques in plant biotechnology provide new options for collection, multiplication and short to long term conservation of plant biodiversity. Significant progress has been made for conserving endangered, rare, crop ornamental, medicinal and forest species, especially for non-orthodox seed and vegetatively propagated plants of temperate and tropical origin. Cell and tissue culture techniques ensure the rapid multiplication and production of plant material under aseptic conditions (Cruz *et al*., 2013).

Hemidesmus indicus R. Br. commonly known as Indian Sarsaparilla, is a twining shrub, belonging to the family *Asclepiadaceae*. It is one of the most widely used medicinal plants in India, well known for its medicinal values. *H. indicus* is highly valued in the Indian systems of medicine; Charaka Samhita, the ancient Indian treatise which dates back to 1000 BC, mentions the medicinal uses of the

plant. The root extract of this plant has been widely used in the traditional systems of medicine in India such as Ayurveda, Siddha and Unani.

The roots of *H. indicus* are woody and aromatic. The essential oil containing 2-hydroxy-4- methoxy benzaldehyde is the main constituent of the root drug (Sreekumar *et al*; 2000). The aroma of the roots is due to the presence of 2-hydroxy-4-methoxy benzaldehyde and ledol. Other chemical components include *b*-sitosterol, α- and β-amyrins, lupeol, tetracyclic triterpene alcohols, resin acids, fatty acids, tannins, glycosides. The root extract serves as flavouring agent for the preparation of soft drinks (Sarasan *et al*., 1994) and bakery products (Patnaik and Debata, 1996). 2-hydroxy-4-methoxy benzaldehyde is often used as a substitute for vanilla in ice creams and as ingredient in herbal tea preparations (Khanna and Kannabiran, 2008).

The extract from the roots is used against the diseases of blood, inflammation, diarrhoea, respiratory disorders, skin diseases, syphilis, fever, bronchitis, asthma, eye diseases, epileptic fits in children, kidney and urinary disorders, loss of appetite, burning sensation and rheumatism (Austin, 2008). This plant is reported to be effective against viper venom and its haemorrhage (Cheruvathur *et al*., 2013).

The dried root of *H. indicus* costs about Rs. 120 per kilogram in Indian market (George *et al*., 2008).The economic importance of the plant has led to the indiscriminate collection from its habitats. Reports show that the plant is getting rare and endangered (Rahman, 2001).The commercial cultivation of these plants has not yet been attempted (Saha *et al*., 2003).

There are many constraints in utilizing natural population of the plant. The harvesting of the roots is labour intensive and uneconomic as it is deep rooted. The roots of the plant are usually harvested during autumn and dried and stored for further use (Saryam *et al*., 2012). The harvesting must be done at the right stage that is when the concentration of the compound is optimum. Application of plant tissue culture techniques is effective in solving these problems. Root culture of

H. indicus is found to be effective in the production of pharmaceutically important secondary metabolite like 2- hydroxy-4- methoxy benzaldehyde. Hairy The study aims to develop transformed hairy root cultures of *H. indicus* using wild strains of *A. rhizogenes* and study the growth pattern of hairy roots in different nutrient media for determining the production of 2-hydroxy-4-methoxy benzaldehyde. The scientific methods given in the topic are relevant to achieve the objectives and it will be useful for the sustainable harvest of roots as well as compound for product development without sacrificing natural population thereby conservation of the plants can be effectively realized.root culture (HRC), also called transformed root culture, is a type of plant tissue culture used to produce bioactive plant-derived compounds. The HRCs are generated from the plant of interest by infecting tissue with the bacterium *Agrobacterium rhizogenes*. Transformed roots display more rapid growth and are characterized by increased formation of root hairs. The secondary metabolites occur in higher concentrations in transformed root cultures than the normal *in vitro* cultures.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Historically plants have played an important role in medicine. Through observation and experimentation, human beings have learnt that plants promote health and well-being. The use of these herbal remedies is not only cost effective but also safe and almost free from serious side effects. The village elders, farmers and tribal have tremendous knowledge on medicinal plants for health reasons started thousands of years ago and is still part of medical practices by folks of various regions of Indian sub-continents as well as several other countries including China, Middle East, Africa, Egypt, South America and other developing countries of world (Mazid *et al*., 2012).

According to the WHO, over 80% of the world's population relies on traditional forms of medicine, largely plant based, to meet primary health care needs. In India, the collection and processing of medicinal plants and plant products contributes a major part each year to the national economy as a source of both full and part time employment (Holley *et al*., 1998).

Herbal medicine has a long history in the treatment of several kinds of disease (Holm *et al.,* 1998). Their use for the treatment of disease has been practiced by man for many years and is still being widely practiced even today (Kokwaro, 1993). For many years, people have developed a store of empirical information concerning the therapeutic values of local plants before orthodox medical practice appeared. Through periods of trial, error, and success, these herbalists and their apprentices have accumulated a large body of knowledge about medicinal plants. According to Iwu *et al.,* (1999) the first generation of plant drugs were usually simple botanicals employed more or less in their crude form. Several effective medicines used in their natural state were selected as therapeutic agents based on empirical study of their application by traditional societies from different parts of the world.

Infectious diseases account for approximately one-half of all deaths in tropical countries. Medicinal plants have been traditionally used for different kinds

of ailments including infectious diseases. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties. Historically, plants have provided a good source of anti-infective agents. The isoquinoline alkaloid, emetine, obtained from the underground part of *Cephaelis ipecuanha* and related species, have been used for many years as an amoebicidal drug for the treatment of abscesses due to the spread of *Escherichia histolytica* infections. Quinine, an alkaloid that occurs naturally in the bark of the *Cinchona* tree, is another important drug of plant origin with a long history of usage against malaria. The higher plants have made important contributions in areas beyond anti-infective, such as cancer therapies. Scientists from divergent fields are investigating plants with an intention to discover valuable phytochemicals. Laboratories all over the world have found literally thousands of phytochemicals which have inhibitory effects on all types of microorganisms *in vitro* (Cown, 1999).

Since the beginning of human civilization, medicinal plants have been used by mankind for its therapeutic value. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of the agents in traditional medicine. The plant-based, traditional medicine systems continue to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care (Owolabi *et al*., 2007).

India has several traditional medical systems, such as Ayurveda and Unani, which has survived through more than 3000 years, mainly using plant-based drugs. The *material medica* of these systems contains a rich heritage of indigenous herbal practices that have helped to sustain the health of rural people of India. The ancient texts like Rig Veda (4500-1600 BC) and Atharva Veda mention the use of several plants as medicine. The books on Ayurvedic medicines such as *Charaka Samhita* and *Susruta Samhita* refer to the use of more than 700 herbs (Jain, 1968).

Medicinal plants are plants containing inherent active ingredients used to cure disease or relieve pain (Okigbo *et al*., 2008). The use of traditional medicines and medicinal plants in most developing countries as therapeutic agents for the maintenance of good health has been widely observed (UNESCO., 1996). Modern pharmacopoeia still contains at least 25% drugs derived from plants and many others, which are synthetic analogues, built on prototype compounds isolated from plants. Interest in medicinal plants as a re-emerging health aid has been fuelled by the rising costs of prescription of drugs in the maintenance of personal health and well-being and the bioprospecting of new plant-derived drugs (Lucy and Edgar, 1999). The ongoing growing recognition of medicinal plants is due to several reasons, including escalating faith in herbal medicine (Kala, 2005). Furthermore, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of drugs and chemotherapeutics from these plants as well as from traditionally used herbal remedies (UNESCO., 1998). According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Nascimento *et al*., 2000).

Medicinal plants produce bioactive compounds used mainly for medicinal purposes. These compounds either act on different systems of animals including man, and/or act through interfering in the metabolism of microbes infecting them. The microbes may be pathogenic or symbiotic. In either way the bioactive compounds from medicinal plants play a determining role in regulating host-microbe interaction in favour of the host. So the identification of bioactive compound in plants, their isolation, purification and characterization of active ingredients in crude extracts by various analytical methods are important. The medicinal properties of plants could be based on the antioxidant, antimicrobial, antipyretic effects of the phytochemicals in them (Cowman, 1999; Adesokan *et al*., 2008).The instant rising demand of plant-based drugs is unfortunately creating heavy pressure on some selected high-value medicinal plant populations in the wild

due to over-harvesting. Several of these medicinal plant species have slow growth rates, low population densities, and narrow geographic ranges (Nautiyal *et al.,* 2002) and are therefore more prone to extinction (Jablonski, 2004). Conversely, because information on the use of plant species for therapeutic purpose has been passed from one generation to the next through oral tradition, this knowledge of therapeutic plants has started to decline and become obsolete through the lack of recognition by younger generations as a result of a shift in attitude and ongoing socio-economic changes (Kala, 2000). Furthermore, the indigenous knowledge on the use of lesser-known medicinal plants is also rapidly declining. The application of plant tissue culture techniques is effective in the sustainable use and conservation of plants.

2.1. TISSUE CULTURE

Plant tissue culture is the aseptic culture of cells, tissues, organs and their components under defined *in vitro* conditions. It is an important tool in both basic and applied studies as well as in commercial application. The ideas of the German scientist, Haberlandt, paved the way for the origin of plant tissue culture. He established the concept of totipotency that is, the ability of a plant cell to develop into a complete plant. He is considered as the father of tissue culture. The availability of the new techniques during the period 1940-1960 led to the application of tissue culture to five broad areas, namely, cell behaviour (including cytology, nutrition, metabolism, morphogenesis, embryogenesis, and pathology), plant modification and improvement, pathogen-free plants and germplasm storage, clonal propagation, and product (mainly secondary metabolite) formation. The application of the *in vitro* technologies to an increasing number of plant species was done in the 1990s. Cell cultures have remained an important tool in the study of basic areas of plant biology and biochemistry and have assumed major significance in studies in molecular biology and agricultural biotechnology (Thorpe, 2007).

The regeneration of plants under aseptic and controlled environmental conditions are referred to as micropropagation because very small pieces of plant

tissue organs are used as starting vegetative tissue (Davis and Becwar, 2007).Tissue culture has been successfully used for the commercial production of pathogen-free plants (Debergh and Maene, 1981), to conserve the germplasm of rare and endangered species (Fay, 1992). The application of biotechnology especially tissue culture provides an important tool to propagate the selected genotypes (Campbell *et al*., 2003).

The first true plant tissue cultures were obtained by Gautheret (1934, 1935) from cambial tissue of *Acer pseudoplatanus.* Success of plant tissue culture is governed by two main criteria – explant and tissue culture media (Gamborg *et al*., 1976). The earliest nutrient media used for growing plant tissues *in vitro* were based on the nutrient formulations for whole plants, but Knop's solution and that of Uspenski and Uspenskia were used the most, and provided less than 200 mg/L of total salts.

Based on an examination of the ash of tobacco callus, Murashige and Skoog (MS) developed a new medium. The concentration of some salts was 25 times that of Knop's solution. In particular, the levels of NO^{3-} and NH^{4+} were very high and the arrays of micronutrients were increased. MS formulation allowed for a further increase in the number of plant species that could be cultured, many of them using only a defined medium consisting of macro and micronutrients, a carbon source, reduced N, B vitamins, and growth regulators. Frequently used tissue culture basic media are Murashige and Skoog (MS) medium, Linsmaier and Skoog (LS) medium, Gamborg's (B5) medium and Nitsch and Nitsch (NN) medium.The MS salt formulation is now the most widely used nutrient medium in plant tissue culture.

The Murashige and Skoog salt formulation is now the most widely used nutrient medium in plant tissue culture. MS is a completely defined medium suitable for a culturing of large number of species. The media consists of mineral salts, a carbon source (generally sucrose), vitamins and growth regulators. MS media have inorganic nutrients at adequate proportion and concentration to satisfy

both physiological and nutritional requirements of the plants hence organic supplements like amino acids, casein hydrolysates, yeast extract or coconut milk are not added to the media. MS media have high nitrate, potassium and ammonium content compared to other nutrient media. Linsmaier - Skoog medium has similar salt composition to that of MS. Majority of the other commonly used media are combinations of the MS media.

Plants require certain elements in adequate concentration for proper growth and development. Epstein (1971) has devised some criteria to determine whether an element is essential or not. An element is considered to be essential if t is vital for completing the life cycle of the plant, its action cannot be replaced by any other element, it has direct influence on the organism and constituent of the molecule that is known to be essential (Gamborg *et al*., 1976).

Those elements required in concentrations greater than 0.5 mM/L are defined as macroelements and those required in concentrations less than 0.5 mM/L as microelements (Fossard, 1976).The major elements required by plants are ions of nitrogen(N), potassium(K), calcium (Ca), phosphorus (P) magnesium(Mg) and sulphur (S) and the minor plant nutrients or trace elements are iron(Fe), nickel (Ni), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper(Cu), and molybdenum (Mo). For each species the concentration of each nutrient for maximum growth rate varies (Saad and Elshahed, 2012). Along with these inorganic nutrients a carbohydrate source is required to provide carbon which the plant otherwise fixes by photosynthesis in the natural conditions. Sucrose, lactose, maltose, galactose, starch etc. can be used as carbohydrate sources, of these sucrose is the most effective carbon source (Saad and Elshahed, 2012). Organic compounds such as vitamins, amino acids and plant growth regulators are required for proper growth (Gamborg *et al*., 1976). These organic nutrients and undefined supplements are essential for morphogenesis and growth. The vitamins inevitable for cell and tissue culture media are thiamine (B1), nicotinic acid and pyridoxine (B6) of which thiamine is the most important (Saad and Elshahed, 2012). Myoinositol is

considered a vitamin source by some, while others consider it as a carbohydrate source (Gamborg *et al*., 1976).

2.1.1. Plant Growth Regulators

Plant growth regulators are endogenous chemicals produced by plants which have critical role in regulating the growth and development of plants. These compounds are required by plants in very low concentrations. Synthetic analogues of plant growth regulators are also available. The 5 main recognized classes of PGRs are Auxins, Cytokinins, Gibberelins, Abscissic acid and Ethylene. Of these auxins and cytokinins are important in regulating growth and morphogenesis of tissue culture plants. (Edwin *et al*., 2008). Two or more hormones can either act synergistically or antagonistically (Edwin *et al.*, 2008).

2.1.1. a. Auxins

Auxins are frequently used in plant tissue culture. The word auxin is derived from the Greek word Auxein meaning to grow. The primary auxin available in plants is Indole-3-acetic acid. Most abundant naturally occurring auxin is indole-3-acetic acid. It is either produced by *de novo* synthesis or by releasing from conjugates (Bartel, 1997). Auxin is involved in cell division and cell elongation. Auxin is used in combination with cytokinin for callus culture, cell suspension and organ culture. The weakest auxin phenyl acetic acid is also present endogenously in plants along with indole butyric acid and 4-chloro IAA (Edwin *et al*., 2008). NAA and 2, 4 - D are synthetic auxins. IAA, IBA and NAA are used for root induction. For callus induction 2, 4-D is mainly preferred. IAA becomes rapidly metabolized in plants so when used along with cytokinin the callus developed will give rise to shoots or embryo as its concentration has been diminished. Auxin will suppress morphogenesis at higher concentrations.

2.1.1. b.Cytokinins

Cytokinins promote shoot proliferation, its morphogenesis and cell division. Skoog and Miller discovered kinetin which thus became the first kinetin to be

discovered. Cytokinins are very important in shoot culture media where they break apical dominance and dormancy and release lateral buds (Edwin *et al*., 2008). Kinetin, zeatin, BA, 2iP can specifically promote cell division, proliferation and morphogenesis of shoots. N-phenyl N-1, 2, 3- thiadiazyol -5- yl urea known as thiadiazuron is found to have cytokinin activity and stimulate shoot initiation. Skoog and Miller in 1957 proposed that the ratio of auxin to cytokinin is crucial in determining the fate of undifferentiated callus tissues. High cytokinin to auxin ratio promotes shoot formation while a low ratio will induce root formation. A balanced ratio of cytokinin and auxin will retain the undifferentiated state (Haberer and Kieber, 2002).

The function of abscissic acid is involved in leaf, fruit abscission and dormancy. It is useful for *in vitro* propagation of embryo. There are about 90 gibberelins known of which GA3 is most common. GA is infrequently used in tissue culture, still it is useful in studying morphogenesis (Smith, 2012). It can induce corm and bulb formation, embryo maturation and stem elongation. It can negatively affect root induction and callus growth (Jha and Ghosha, 2005).

2.2. SECONDARY METABOLITE PRODUCTION

The evolving commercial importance of secondary metabolites has in recent years resulted in a great interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites by means of tissue culture technology. The principle advantage of this technology is that it may provide continuous, reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be extracted. In addition to its importance in the discovery of new medicines, plant cell culture technology plays an even more significant role in solving world hunger by developing agricultural crops that provide both higher yield and more resistance to pathogens and adverse environmental and climatic conditions. (Vanisree *et al*., 2003).

Secondary metabolites are compounds produced in plants that have role in attraction of pollinators, defense against predators and diseases. They are not essential to sustain plant life. They are produced in plants only during a particular developmental stage or under specific seasonal, stress or nutrient availability conditions (Verpoorte *et al*., 2002).

There is a series of distinct advantages to producing a valuable secondary product in plant cell culture, rather than *in vivo* in the whole crop plant. These include: a) Production can be more reliable, simpler, and more predictable, b) Isolation of the phytochemical can be rapid and efficient, as compared to extraction from complex whole plants, c) Compounds produced *in vitro* can directly parallel to the compounds in the whole plant, d) Interfering compounds that occur in the field-grown plant can be avoided in cell cultures, e) Tissue and cell cultures can yield a source of defined standard phytochemicals in large volumes, f) Tissue and cell cultures are a potential model to test elicitation, g) Cell cultures can be radio labelled, such that the accumulated secondary products, when provided as feed to laboratory animals, can be traced metabolically (Anand, 2010).

2.2.1. Hairy root cultures

Hairy roots originate from a plant disease caused by the Gram-negative soil bacterium *Agrobacterium rhizogenes*. Hairy root tumours are characterized by a proliferation of adventitious roots at the bacterial infection site. The development of genetically transformed plant tissue cultures and roots transformed by *Agrobacterium rhizogenes* (hairy roots), is a key step in the use of *in vitro* cultures for the production of secondary metabolites. Hairy roots are able to grow fast without phytohormones, and to produce the metabolites of the mother plant (Bensaddek *et al.,* 2008). Hairy roots (HRs) are differentiated cultures of transformed roots generated by the infection of wounded higher plants with *Agrobacterium rhizogenes.* This pathogen causes the HR disease leading to the neoplastic growth of roots that are characterized by high growth rate in hormone free media and genetic stability. HRs produce the same phytochemicals pattern of

the corresponding wild type organ. High stability and productivity features allows the exploitation of HRs as valuable biotechnological tool for the production of plant secondary metabolites (Pistelli *et al*., 2010).

Plant hairy roots offer a novel and sustainable tissue-based system that reserves the multiple specialized cell types which are important in maintaining better consistency in the synthesis of bioactive secondary molecules (Bhansali and Kumar, 2014). The transformed root cultures have been offered additional advantages such as rapid growth, uniformity, genetic stability and high biosynthetic capacity.

The genetic determinant of hairy root disease is a large plasmid called the root-inducing (Ri)-plasmid which is carried by virulent strains of *A. rhizogenes* (Chilton *et al*., 1982). The Ri plasmid is similar to the tumor-inducing (Ti) plasmid found in *Agrobacterium tumefaciens*, which is the causative agent of Crown Gall tumours in many dicotyledonous plants. Both the Ri and Ti plasmids transfer a segment of the plasmid called transfer DNA or T-DNA to the plant genome during infection. Transfer of the T-DNA to the plant genome is directed by a different region of the plasmid called the virulence (*vir*) region. The T-DNA contains genes that encode enzymes responsible for the biosynthesis of plant hormones, such as auxin and cytokinin. While *A. tumefaciens* produces transgenic tumours, *A. rhizogenes* produces transgenic roots. This provides a major advantage for root-related studies since it eliminates the lengthy and expensive process of regenerating whole transgenic plants.

Initially, hairy roots were thought to be a disease limited to dicotyledonous plants (De Cleene and De Ley, 1981). After further research, *A. rhizogenes* root induction was demonstrated on monocots (Porter, 1991) and in gymnosperms such as radiata pine (*Pinusradiate*) and larch (*Larix)* (Mcafee, *et al*., 1993; Li and Leung, 2003). The hairy root transformation system is adaptable to a very broad range of plant species, which is important for research purposes.

Hairy root cultures are potentially useful for the production of a large number of foreign proteins and secondary metabolites. The transformed roots are genetically stable and exhibit faster growth rates than normal roots (Hu and Du, 2006). Hairy root cultures of red beet (*Beta vulgaris*) have shown promise for the commercial production of peroxidase (Rudrappa *et al*., 2005). Brigham *et al.,* (1999) demonstrated that hairy root cultures of *Lithospermum erythrorhizon*, manufacture shikonins at an elevated level. Hakkinen *et al*., (2005) showed that hairy root cultures of *Nicotiana tabacum* expressing the *h6h* gene from *Hyoscyamus niger* had an enhanced secretion of the alkaloid scopolamine.

Hairy roots are not only able to anergise growth regulators but are also characterized by fast growth, which seems to make them appropriate for the production of valuable secondary metabolites (Shen *et al.*, 1988; Wysokinska and Chmiel, 1997; Giri and Narasu, 2000).

2.3. THE PLANT - *HEMIDESMUS INDICUS* (L.) R. BR.

Kingdom	:	Plantae
Phylum / Division	:	Magnoliophyta
Subphylum / Subdivision	:	Magnoliophytna
Class	:	Magnoliopsida
Subclass	:	Magnolidae
Order	:	Gentianales
Suborder	:	Gentianineae
Family	:	Asclepiadaceae
Subfamily	:	Asclepiadoideae
Genus	:	*Hemidesmus*

Species : *indicus*

Common names

English	:	Indian sarsaparilla
Hindi	:	Anantamul
Sanskrit	:	Anantamula, Sariva
Malayalam	:	Nannari, Naruneendi

Hemidesmus indicus is a perennial, fast-growing thin creeper vine that sends tendrils out at every node to cling to the surrounding vegetation for stability and support. The leaves are very slender, smooth and oval shaped, closely resembling blades of grass, and they maintain a uniform shiny dark green color throughout the year. The stems stiffens and become woody over time, and the bark varies in colour from dark red to rust to brown. In the right climate *H. indicus* will produce flowers almost all year round; the flowers are small, thin and elongated, and light green with a purple hue inside. The seeds are white and covered in tiny silvery white hairs. The root system is sparse, linear, and usually produces one main root with very few side branches. The roots are known to be very aromatic, emitting a sweet scent reminiscent of a combination of vanilla, cinnamon and almonds (Austin, 2008).

Traditional Ayurvedic medicine practitioners have used Sariva for hundreds and hundreds of years; it was used as a healing herb as well as a magical-spiritual dream herb. It is used to treat stomach problems, cure rashes, ease the mind, quell the symptoms of syphilis, induce trance states and deep meditation, and to clarify and prepare the mind for the dream world. The roots are also known to help relieve stress by inducing an overwhelming sensation of relaxation, euphoria, and tranquillity (Pole 2006).

Hemidesmus indicus is effective as an anti-inflammatory, diuretic and vulnerary. It prevents miscarriages, improves fertility, and treats syphilis (Arun *et al*., 2007). For hundreds of years Ayurvedic practitioners have used Sariva root to promote a calm and tranquil state of mind, to maintain mental clarity while falling asleep and to achieve lucidity while dreaming. This is definitely a powerful dream herb that is used by many people to aid in meditation, trance, and lucid dream induction. There is also significant scientific evidence that *H. indicus* can be used effectively as a treatment for arthritis, asthma, bronchitis, epileptic seizures, high blood pressure, immune disorders, and high stress (Arun *et al*., 2007). In traditional Hindi folk wisdom, healers or sages used the roots to cleanse the blood of toxins, soothe skin irritations and rashes, reduce the burning sensations caused by urinary tract infections, reduce fevers, and to heal moderate cases of acne. Women use Sariva root to promote a healthy pregnancy and to reduce the possibility of a miscarriage (Arun *et al*., 2007).

Hemidesmus indicus is known to naturally produce a wide variety of beneficial compounds known for their healing and calmative effects. The chemical investigations of the root drug showed the presence of an essential oil containing 80% 2-hydroxy-4-methoxy benzaldehyde. Other constituents present in the root are: β-sitosterol, α- and β- amyrins, lupeol, alcohols, resin acids, fatty acids, tannins, saponins, a glycoside and a ketone (Murti and Seshadri 1941 a, b., Chatterjee and Bhattacharyya, 1955), flavonoids and terpenoids (Gupta *et al.,* 1992), novel pregnane glycosides like desinine, indicine, hemidine, hemidesine and emidine (Oberai *et al.,*1985, Prakash *et al.,*1991, coumarinolignoids like hemidesminine (Mandal *et al.,*1991), hemidine-1 and hemidine-2 (Das *et al.* 1992) and an organic acid HI-RVIF (Alam *et al.* 1994) from stem and root extract of the plant have also been isolated.

Medidemine, hemisine and desmisine are pregnaneoligoglycosides isolated from *H. indicus* (Deepak *et al.,* 1997). Sigler *et al*. (2000) isolated denicunine and heminine which are novel pregnane C21 steroidal glycosides from dried stem. Triterpenoids are also present in *H. indicus*. Pentacyclic triterpenes including

oleanenes, ursenes and known compound beta amyrin were isolated in 2001 by Roy *et al*. A new triterpene lactone 3-keto-lup-12-ene-21, 28-olide was also characterized in the hexane soluble fraction of stem extract (Gupta *et al*., 1992). Phytochemical analysis revealed the presence of saponins, tannins, terpenoids, cardiac glycosides, phenolic compounds, phytosterols, sapogenins, flavonoids, cardenolides, volatile oils, carbohydrates, proteins (Khanna and Kanabiran, 2008).

There are over a hundred unique compounds that have been isolated from the roots, stems, leaves and flowers. Some of the many compounds found in this plant include: 2-hydroxy-4-methoxy benzaldehyde, 2-hyroxy-4-methoxy benzenoid, alpha-amyrins triterpene, benzoic acid, beta-amyrins, beta-sitosterol, coumarin, delta-dehydrolupeol acetate, delta-dehydrolupanyl-3-beta-acetate, desmine, glucosides, hemidesmin-1, hemidesmin-2, hemidescine, hemidesmic acid, hemidesmine, hemidesmol, hemidesterol, hemidine, hemisine, hexatriconate acid, hyperoside, indicine, indicusin, lactone, lupanone, lupeol acetate, lupeoloctacosonate, medidesmine, p-methoxy salicylic aldehyde, pregnane ester diglycosidedesinine, sarsapogenin, sarsaponin, sitosterol, smilacin, smilgenin, stigmasterol, tannin, triterpenoidsaponin, and vanillin, as well as many other potentially psychoactive compounds (Kainthla *et al*., 2006).

Hemidesmus indicus might prove to be a useful memory restorative agent for the treatment of dementia seen in the Alzheimer's disease. (Shete and Bodhankar, 2010). The chloroform and *n-butanol* fractions of ethanolic extract *H. indicus root* were screened for claimed potential in mice. The *n-butanol* fraction of *H. indicus* extract significantly improved learning and memory at all doses mice. Medicines containing *Hemidesmus* bark extracts were effective in treating several free radical mediated diseases because of its free radical scavenging property (Murali *et al*., 2011).Vijayakumari and Nishteswar (2012) studied the wound healing activity of *H. Indicus* and found out an increase in the rate of wound contraction and increase in the percentage of epithelialisation when root paste is applied on chronic wounds. Herbal formulation of *H. indicus* increases proliferation

of cells, promote granulation of tissue and thus improve wound healing (Ganesan *et al*., 2012).

Drug preparations from roots are also used to treat nutritional disorders, syphilis, fevers, foul odour from the body, bronchitis, piles, rat bite poisoning, epileptic fits in children, children's wasting diseases and 'tridosha' disease of the blood, leucorrhoea, 'kapha' and 'vata' (Kirtikar and Basu 1975). Prabakaran *et al.,* (*2*000) reported that the ethanol extract of *H. indicus* significantly prevented rifampicin and isoniazid induced hepatotoxicity in rats.

The chloroform and ethanol extracts were reported to possess antifungal activity against *Aspergillus niger* and weak antibacterial activity against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,Klebisella pneumonia, Salmonella aureus*, (Gayathri and Kannabiran, 2009), *Salmonella typhi, Staphylococcus aureus, Escherichia coli*, *Vibrio cholera* (Ratha *et al*., 2012) and *Propionibacterium* acnes (Kumar *et al*., 2007). The inhibitory effect varied based on the solvent in which extract was formed. Methanolic and ethanolic extracts showed significant results while chloroform has minimum and petroleum ether with no effect at all. The phytochemicals saponins and tannins render this antimicrobial activity (Joseph *et al*., 2011). *H. indicus* can inhibit the growth of *Propionibacterium* acnes (Kumar *et al*., 2007). The cytotoxic activity of *Salmonella typhimurium* in a macrophage cell line could also be blocked by chloroform fraction of *H. indicus* root extract (Das and Devaraj, 2008).

This plant is also used against various skin diseases and in the treatment of acne. Remarkable antimicrobial activity has been shown against *Staphylococcus aureus, Salmonella typhi, Klebisella pneumonia, Aspergiillus flavus, Aspergillus fumigates* and *Aspergillus niger* by pure saponin extract. Saponin fraction of antifungal activity was much stronger than antibacterial effect. Its antifungal activity was comparable to that of the standard fungicide amphotericin B. (Khanna and Kannabiran, 2008). *H. indicus* root extract has anti enterobacterial effect also

due to the presence of antimicrobial trace elements such as copper and zinc (Das and Devaraj, 2006).

Alam *et al*., (1994, 1996) discovered an organic acid isolated from root extract possesses viper venom inhibitory activity. Extracts significantly neutralized venom-induced lethality and hemorrhagic activity in rats and mice. The extract also antagonized venom-induced coagulant and anticoagulant activity. Later the compound was found to be 2- hydroxyl-4-methoxy benzoic acid which has antiviper venom action by inducing changes in serum phosphatase and transaminase activity in male albino rats thus neutralizing the venom (Alam and Gomes, 1998).

Sekhar *et al*., (2005) reported *H. indicus* as a potential phytoremediation agent. It has lead hyper accumulating property. Lead accumulation occurs maximum in shoots of the plant body. Mosquitoes cause major health menace and are vector in transmitting many diseases. Root extract has mosquitocidal and water sedimentation properties (Arjunan *et al*., 2012). *Culex quinquefasciatus,* endemic to Indian subcontinent is the vector of lymphatic filarisis. The root extract was found to have larvicidal effect on culex mosquitoes because of the presence of high concentrations of saponins and tannins (Joseph *et al*., 2011). Thus the plant can play a major role as environment friendly insecticides (Khanna and Kannabiran, 2007).

The root extract has potent anti-inflammatory, antipyretic and antioxidant properties (Dutta *et al.,* 1982; Rao *et al.,* 2005). Alam and Gomes, (1998) reported that the compound 2-hydroxy-4-methoxy benzoic acid has antioxidant properties. The root bark also possesses antioxidant activity (Ravishankara *et al.,* 2002). The altered levels of hemoglobin, blood glucose, plasma insulin in diabetic rats can be reverted back to basal levels by administration of ethanolic extract of roots (Subramanian *et al*., 2011). Its action is very rapid and can bring down glucose level within 5 hours. It stimulates the production of insulin by beta cells of islets of pancreas (Mahalingam and Krishnan, 2008). The glycogen content in muscles was also improved significantly by *H. indicus* (Subramanian *et al.,* 2011).

The root extract exhibited inhibitory activity against *Mycobacterium leprae* (Gupta, 1981) and keratinophilic fungi (Qureshi *et al.,* 1997). The ethanolic extract was reported to be effective chemoprotective agent and prevented oxidative stress and tumour in skin (Sultana *et al.,* 2003). Anoop and Jagadeesan (2003) found out the antiulcerogenic activity of the aqueous ethanolic root extracts of *Hemidesmus* in animal models. They also found out the effectiveness was more in roots collected during flowering seasons than during vegetative seasons in animal models.

Prasad *et al.* (1983) reported the antimicrobial studies on essential oils of *H. indicus.* Prabakan *et al.* (2000) reported the protective effect of *H.indicus* against rifampicin and isoniazid induced hepatotoxicity in rats. The effect of cell culture derived *H.indicus* in the prevention of hypercholesterolemia in normal and hyperlipidimic rats have been reported by Bopanna *et al.,* (1997). *H. indicus* can also protect liver microsomes (Shetty *et al*., 2005). Liver toxicity and damage caused by ethanol can be cured by *H. indicus* rendering hepatoprotective action (Saravanan and Nalini, 2008; Baheti *et al*., 2006).

The cytodifferentiating, cytotoxic and cytostatic activities of *Hemidesmus indicus* on a human promyelocytic leukemia cell line (HL-60) was estimated by Ferruzi *et al*., (2013). It can significantly increase calcium levels by mobilizing the intracellular calcium stores. It induces apoptosis of leukemic cells by caspase - 3 activity. Administration of *H. indicus* along with chemotherapeutic agents methotrexate, 6-thioguanine, cytarabine enhance antitumour activity (Fimognari *et al*., 2011). Thus *H. indicus* can be considered as a potent drug in anticancer pharmacology. It can render protection against hydroperoxide induced cutaneous oxidative stress and tumour promotion (Sultana, 2003). Cisplastin was used as positive mutagen in cultured human lymphocytes and it was found out that at lower concentrations *H. indicus* root extract has significant genoprotective effects (Ananthi, 2010). A decotion of *H. indicus* roots along with *Smilax glabra* rhizome and *Nigella sativa* seeds induce cytotoxicity to prevent chemically induced carcinogenesis in rats. This decotion is used in Sri Lanka to treat cancers by traditional medical practitioners. The cytotoxic effects of *H. indicus* ranged

between that of *N. Sativa* and *S. glabra* (Thabrew, 2005). Sultana *et al*., (2003) reported that the topical application will provide chemoprotection against skin carcinogenesis.

The cell culture extract of *H. indicus* along with atherogenic diet can lower serum, tissue and fecal lipid levels preventing hypercholesterolemia (Boppana et al., 1997). The elevated levels of lipid peroxides in the plasma and pancreatic tissues was brought back to normal by root extract, improving lipid profile (Subramanian *et al*., 2011). 217.5 µg/mL methanolic root extracts was capable of inhibiting 50% lipid peroxide formation and the same extract in 73.5 µg/mL could scavenge hydroxyl radicals. Chloroform ethanol extract of *H. indicus* has a new pregnane glycoside which has antioxidant and anti dyslipidemic activities (Sethi *et al*., 2010).

The use of *H. indicus* against leucorrhoea at Bargarh district in Orissa and Sattordem Village of Goa has been reported (Sen and Behera, 2008). Siddique *et al.,* (2004) have reported the use of *H. indicus* among the local people and herbal practitioners of Barind Tract of Bangladesh against diarrhoea, rheumatism, fever, headache, asthma, eye disease and wounds.

Alcoholic extract of *H. indicus* showed anti-nociceptive activity. Triterpenes, flavanoids and sterols present in root extract contributes to this activity (Verma *et al*., 2005). *H. indicus* can reduce the number of ectopic beats and duration of ventricular tachycardia. Thus it is a potent vasodilator, positive inotropic agent, and cardioprotectant (Khandewal *et al*., 2011). ADP induced platelet aggregation inhibition was possible with the extract whose activity was comparable to commercial heparin (Mary *et al*., 2003). Carragenan induced rat paw oedema and brewer's yeast induced pyrexia in rats can be reduced by root extract thus establishing its anti-pyretic and anti-inflammatory activity (Lakshman *et al*., 2006).

Mehta *et al.,* (2012) evaluated the anti-arthritic activity of roots and it was found out that the presence of terpenoid in hydroalcoholic extract as well as in ethyl acetate fraction attributes to this property. The altered levels of hemoglobin, blood glucose, plasma insulin in diabetic rats can be reverted back to basal levels by

administration of ethanolic extract of roots (Subramanian *et al*., 2011). Its action is very rapid and can bring down glucose level within 5 hours. It stimulates the production of insulin by beta cells of islets of pancreas (Mahalingam and Krishnan, 2008). The glycogen content in muscles was also improved significantly by *Hemidesmus* (Subramanian *et al.,* 2011).

Evans *et al.,* (2004) reported the enhancement in the absorption of water and electrolytes from rat intestine by water extract of roots of *H. indicus.* Administration of the extract along with oral rehydrating salt solution will enhance its efficiency. *H. indicus* can suppress the renal tubular reabsorption of water and electrolytes and thus increase the urine volume. It can maintain prolonged diuretic effect, curtailing the frequency of administration of drugs (Gadge and Jalalpure, 2011). Das *et al*., (2003) reported the anti-diarrhoeal activity of *H. indicus* in both *in vitro* and *in vivo* conditions. Gentamicin induced renal impairment in rats can also be treated with *Hemidesmus*, so can be used along with aminoglycosides as an adjunct therapy (Kotnis *et al*., 2004). It also has otoprotectant activities (Previati *et al*., 2007). Shetty *et al.,* (2005) reported the radiation protection of DNA and membrane *in vitro* by extract of *H.indicus.*

2.4. MICROPROPAGATION OF *HEMIDESMUS INDICUS*

The micropropagation of *H. indicus* has been attempted by many researchers for the purpose of conservation as well as the production of secondary metabolites like 2-hydroxy-4-methoxy benzaldehyde.

In vitro propagation of *H. indicus* was done by shoot tip culture, direct and indirect organogenesis and somatic embryogenesis (Sarasan and Nair, 1991). Sarasan *et al.,* (1994) tried both organogenesis and embryogenesis on MS and B5 medium from callus initiated from leaf or stem explants. Embryogenesis is dependent on the type of callus used, age of callus and plant growth regulator. MS medium containing NAA (2 mg/1) and KIN (0.5 mg/L) was used to induce organogenesis from callus. Plantlets were produced successfully by organogenesis.

Axillary bud culture was performed by Patnaik and Debata (1996). They observed that shoot multiplication occurred strongly in the presence of cytokinin and NAA. Reduction in shoot numbers with each subculture was also reported. They also successfully induced callus from leaf and stem segments in MS medium with NAA or NAA and kinetin. Shoots were then induced on the callus thus formed upon transfer to medium with kinetin and coconut milk as supplements.

Malathy and Pai, (1998) proposed a simple protocol for *in vitro* propagation of the plant. They achieved shoot initiation in MS media with 3.0 mg/L BAP. They also found out that decreasing ammonium nitrate concentration can increase internode length and shoot thickness. Decruse *et al.,* (1999) reported the effect of cryopreservation on seed germination of *H. indicus*.

Sreekumar *et al*., (2000) reported that an average micro propagated plant after cultivation in the field was observed to have 4-5 shoots, 5-8 branches per shoot and increased root biomass. With increasing maturity, the caulogenic response of the plant was decreasing. The concentration of 2- hydroxyl-4-methoxy benzaldehyde was also determined to be higher than in conventional plants.

A protocol for regeneration of plants from root segments excised from aseptic seedlings was developed by Ramulu *et al*., (2003). Root segments showed better regeneration of shoots in medium with cytokinins and alpha naphthalene acetic acid within 2 to 3 weeks. 85 % survival of *in vitro* plants was observed when transferred to mist chamber.

Saha *et al*., (2003) has done *in vitro* propagation via bud multiplication. 0.1 mg/L NAA and 2.0 mg/L BAP supplemented MS medium was best in bud multiplication. The bud break occurred 4 days after inoculation. Chromosome analysis was done using acetic orcein squash technique. Chromosome number and structure were stable in all the regenerates. 85 % survival rate was achieved in rooted plants when transferred to soil.

Neeta *et al*., (2005) established shoot cultures and callus cultures from roots and leaves of *H indicus* on Murashige and Skoog medium with various hormonal

combinations. The production of antioxidants (lupeol, vanillin, and rutin) in shoot cultures, callus cultures derived from leaf cells and root cells, was compared with root and aerial portions of the parent plant. Shoot cultures and leaf callus cultures produced more antioxidants than root callus cultures. BA- NAA was best suited for shoot cultures and NAA – KIN was best for leaf callus culture. Callus formation from roots occurred only in the presence of 2, 4 –D. Combinations of BA and NAA at all concentrations increased lupeol content. Leaf callus cultures could only produce rutin and lupeol but not vanillin. Rutin content was higher in leaf derived callus cultures nut lower in shoot cultures. The content and quantity of the compounds varied in different growth stages. The metabolic content of *in vitro* plants was similar to field plants.

Neeta *et al*., (2003) reported that premature leaf fall and callus formation are common problems that arise during shoot formation and rooting which can affect the plantlets survival during hardening. Work was done to overcome these problems. Addition of adenine sulphate, silver nitrate or calcium salts were significantly beneficial in reducing premature leaf fall. Addition of silver nitrate resulted in yellowing of explants and calcium salts will cause shoot tip necrosis, and so both of them were not preferred. Addition of phenyl alanine and shikimic acid will give broad leaves with good shoot proliferation but it will also cause increase callus formation and leaf abscission. 15 mg/L adenine sulphate in media produced broad and healthier leaves with a reddish tinge in plantlets and decreased the time required for shoot proliferation and elongation. It also increased the morphogenetic potential of the plants. Optimum concentration of sucrose was found to be 40g/L. Half strength MS medium was best suited for rhizogenesis. Activated charcoal 100 mg/L reduced caulogenesis and induced root formation within 10 days. The secondary metabolites content and genetic content remained stable for 3 years of culture, concentration of lupeol, vanillin and rutin was similar in regenerated plantlets and comparable to parent plant regenerated i*n vitro* plants by using nodal and leaf explants (Shanmugapriya and Sivakumar, 2011).They found out that exogenous supply of plant growth regulators is not always required, some

species have enough endogenous plant growth regulators. The protocol provided an efficient method for rapid regeneration and successful regeneration of plantlets.

Saryam *et al*., (2012) developed an efficient protocol for *in-vitro* micropropagation of *Hemidesmus indicus,* by using seeds, cotyledonary nodes, shoot tips, and nodal segment of explants. The explants used for the present experiment were seeds which were cultured on MS media with or without any growth hormone. The seedlings developed were further used for shoot initiation and multiplication. When cultured for 4 weeks on Murashige and Skoog's (MS) medium containing different concentrations and combinations of BAP and KIN either alone or with combination, highest percentage of shoot induction is observed (75%) in the presence of 1.0mg/L BAP, maximum number of 1-3 shoots/node were produced. Average shoot length is of 2cm. Multiple shoot percentage respond 80% in the presence of 2.0 mg/L BAP while maximum number of shoots/node were produced is 12-13 after 5 weeks of culture of shoot length 2-3cm. Rooting induction is observed in the medium containing 2.0 mg/L NAA and 200mg activated charcoal shows 85-90% rooting with root length 3-5cm.

Cheruvathur *et al*., (2013) developed a protocol for somatic embryogenesis. Rate of somatic embryogenesis was highest when callus subculturing was done in half strength MS medium with 2 µm IBA. Somatic embryos were formed from callus derived from nodal cuttings. Somatic embryos developed upto torpedo stage on induction medium. For synthetic seed formation, somatic embryos were suspended in MS medium in a matrix containing sodium alginate (3% W/V). These were then dropped into a medium containing 75 mM calcium chloride. After 120 days of storage at 4°C the seeds germinated. Plant regeneration occurs quickly through somatic embryogenesis. Therefore culture induced genetic changes is highly reduced. Traditional germplasm repositories are difficult to maintain because of lack of space and expenses required.

Synthetic seeds are the best alternatives to traditional seeds. Cost of production of synthetic seeds is very low and they are easy to handle and can be

stored for a long time. Synthetic seeds meet the quarantine of international standards. Easy delivery of plantlets, easy and mass production of plantlets is also possible by synthetic seeds. Pro-embryogenic light yellow calli was best suited for somatic embryogenesis among different calli (Cheruvathur *et al*., 2013).

Heble and Chadha (1978) done *in vitro* propagation by establishing different culture systems and the steroid content in each was estimated. Phytosterols were present in all cultures. Cholesterol, campesterol, sitosterol and 16-dehydropregnenolone were present in leaves, stem and roots of the plant, but 16-dehydropregnenolone was detected only in callus derived from stem. Maximum concentration of 16-dehydropregnenolone was in roots (0.04%) followed by stem (0.006%), the leaves contained trace amounts of this steroid.

Sreekumar *et al.,* (1998) reported that light has no particular effect on root biomass production. In case of liquid cultures the optimum speed for agitation in cultures was 70 rpm beyond which resulted in root damage. Sucrose was found out to be the best sugar source for *H. indicus*. The stability of root cultures were estimated over a period of 21 days with normal morphology, consistent biomass and product yields.

2- hydroxy-4- methoxybenzaldehyde is an unusual phenolic compound (Sircar *et al*., 2007), crystalline in nature (Nagarajan *et al*., 2003). This aldehyde compound is present in *Hemidesmus indicus* and *Decalpis hamiltonii*; both belonging to *Asclepediaceae* family. It is a potent tyrosinase inhibitor. It has structural similarity to vanillin and is used as a flavouring agent. It also possess insecticidal and anti-microbial property (Sircar *et al*., 2007). This compound is present in more than 90% in the volatile oils of these plants (Nagarajan *et al*., 2003). 2-hydroxy-4-methoxy benzaldehyde is produced in equal amounts in tissue culture plants and wild plants (Giridhar *et al*., 2004). Reverse phase HPLC was used for determining and validating 2- hydroxy 4- methoxybenzaldehyde and 2-hydroxy 4- methoxy benzoic acid in *H. indicus* roots. The method is very well suited for routine analysis and is fast and sensitive (Sircar *et al.,* 2007). *Decalpis hamiltoni* also

produces 2- hydroxy 4-methoxy in tubers and its production is increased *via* triacontanol treatment. (Giridhar *et al*., 2005). Gas chromatography is mainly used for assay of the compound (Sircar *et al*., 2007) in both fresh and dry roots (Nagarajan *et al*., 2003).

The enzymatic route of synthesis of 2- hydroxy 4- methoxybenzaldehyde is not yet known (Chakrabarthy *et al*., 2008) but is assumed to be by central phenylpropanoid pathway (Kundu *et al*., 2012). Benzoate pathways can originate by two means either by shikimate pathway or by phenyl propanoid pathway. Many experiments have been carried out to uplift the accumulation of the compound in roots. Substances like chitosan, methyl jasmonate and yeast extract were used as elicitors (Kundu *et al*., 2012). 200 mg/L chitosan on 24 hour treatment increased maximum phenolic content in both cortex and cork tissues. The phenolic accumulation decreased after 24 hours. Chitosan elicitation also improved phenyl alanine ammonia lyase activity with maximum activity after 12 hours of treatment (Chakrabarthy *et al*., 2008). Yeast extract treated roots showed a sharp increase in shikimate dehydrogenase and phenylalanine ammonia lyase activity. Among the three elicitors yeast extract was found to be the best elicitor (Kundu *et al*., 2012). Aminoxy acetic acid is an irreversible inhibitor of phenyl alanine ammonia lyase thus it can decrease the 2- hydroxy 4-methoxybenzaldehyde content (Chakrabarthy *et al*., 2008). Thus it can be concluded that shikimate pathway has a major role in modulating the biosynthesis of 2-hydroxy-4- methoxybenzaldehyde (Kundu *et al*., 2012).

Nagarajan and Rao (2003) developed a gas chromatographic procedure was developed forthe assay of 2-hydroxy-4-methoxybenzaldehyde in both fresh and dried roots of different origin. Benzyl butyrate was used as the internal standard. Among the methods tried, steam hydrodistillation was suitable for extraction of the volatile oils. The quantity of this aromatic compound varied from 0.03 to 0.54%.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Induction and establishment of transformed hairy root cultures of sarsaparilla (*Hemidesmus indicus* L.) R. Br." was conducted at Biotechnology and Bioinformatics division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute during 2013-2014. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1. PLANT SAMPLE COLLECTION

Plants were collected from the forest area of JNTBGRI during the months of October – November (Plate 1). Young tender shoot tips and nodes were used for initiating *in vitro* cultures.

3.2. PREPARATION OF GLASSWARE AND INSTRUMENTS (CLEANING AND STERILIZATION)

Glassware used in the study was cleaned with 1% teepol followed by thorough washing in tap water. After rinsing with distilled water the glassware was kept inverted on a clean draining tray and allowed to dry. The glassware was then sterilized by drying at 160^0C for 2 hours in a temperature controlled hot-air oven before use. Sterilization of Petri-dishes, forceps, scalpels, millipore filteration units etc. was done by autoclaving at 121°C, 15 lb psi for 20 min. These were separately wrapped with aluminum foil and then with brown paper, packed in autoclavable bag and autoclaved. Sterile filter-papers required for aseptic works were obtained by autoclaving desirably cut and folded filter-papers that were properly arranged in culture bottles. Boxes of micropipette-tips wrapped in brown paper and culture bottles containing distilled water were also autoclaved for aseptic use.

To maintain aseptic conditions inside the Laminar air flow chamber, the workbench was wiped using 70% ethanol before and after working. Tools such as forceps, scalpels, inoculation loop etc. used for inoculation were again sterilized by dipping in alcohol and flaming before and during use in the Laminar air flow.

Plate 1: *Hemidesmus indicus* plant

3.3. CHEMICALS AND OTHER CONSUMABLES

Chemicals used for surface sterilization procedures were Teepol (Reckitt Benckiser Ltd, India) and mercuric chloride (E Merck Ltd., Mumbai, India). Antibiotics Streptomycin (Ranbaxy Pvt. Ltd, India) were used at times, for controlling the growth of contaminating bacteria in plant tissue culture media. All culture media components and plant growth regulators used were plant tissue culture grade or equivalent and were procured from notable National or International companies. Standard 2-hydroxy 4 - methoxybenzaldehyde was procured from Biotechnology Laboratory of JNTBGRI and the extraction solvents for 2-hydroxy 4-methoxy benzaldehye analysis were all analytical and/or HPLC grade (E Merck, India). Glassware required for the work, with the exception of the culture bottles (Excel glassware Ltd., Kerala), were purchased from Borosil Glass Works Ltd., India and the plastic ware from Tarsons Pvt. Ltd., India.

3.4. EQUIPMENTS

The major equipments used in the present study include: Electronic Balance (Shimazu, Japan), pH meter (Eutech, Singapore), Autoclave (Nat Steel Equipment Private Limited, Bombay), Laminar airflow hood (Klenzaids, India), Gyrorotatory shaker G-10 (New Brunswick Scientific Co; USA), Vacuum rotavapour evaporator (Heidolf, Germany), UV-visible spectrophotometer (XP 3001 Xplorer, UK), HPLC (Gilson, France).

3.5. SOFTWARE

UV Win 5 software v 5.0.5 was used for Spectrophotometry analysis. Gilson Unipoint TM LC system software was used for analyzing HPLC samples.

3.6. PREPARATION OF CULTURE MEDIA

3.6.1. Tissue Culture Media

Murashige and Skoog (MS) (1962) media was used for the experiments. The compositions of these media are provided in Appendix.In all these cases, stock solutions of the media ingredients were prepared by dissolving weighed quantities of

salts in known volume of distilled water and were stored under refrigeration. Appropriate aliquots of these solutions were mixed to prepare the media of required salt strength in required quantities. The volume of stock solution required was calculated using the formula:

$$\text{Volume of stock solution} = \frac{\text{Concentration required x Volume of media}}{\text{Concentration of the stock solution}}$$

Sugars and chemicals not made into stock solutions (Calcium chloride, *myo*-inositol etc.) were added in required quantities. The PGRs were prepared as stock solutions of concentration 1 mg/mL. Weighed quantity of hormone powder were taken in clean reagent bottle/vial, dissolved in few drops of appropriate solvent and then made to the final volume with distilled water. The stock solutions of all PGRs were stored at 4 ºC. Following addition of desired concentrations of required PGRs, the medium was made-up to the final volume and the pH of the medium adjusted by addition of 1 N NaOH/ 1 N HCl. Subsequently, 0.6% (w/v) agar added in the media as gelling agent was melted by heating on a water bath. The molten media was thoroughly mixed and dispensed in aliquots of 15 mL in the case of culture tubes (20x150 mm), 50 ml for Erlenmeyer-flasks (250 mL) and 40 ml for culture bottles. In case of requirement of liquid media, the media could be dispensed in required quantities into the culture vessels, soon after correction of media pH. Tubes and flasks were then plugged with cotton plugs (non-absorbent cotton wrapped in surgical cloth) and culture bottles were capped with polypropylene closures before autoclaving at 121^0C/1.5 Kg/cm^{-2} for 18 min.

3.6.2 Bacteriological media

Yeast Extract Mannitol (YEM) medium (Appendix II) was used for maintaining cultures of *A. rhizogenes*. Media components were weighed and dissolved in distilled water. After making up the medium to the desired volume, the pH was adjusted to 7.0 using 1 N NaOH/1 N HCI. As per requirement, YEM broth was dispensed in 20 mL aliquots into 100 mL flasks or in 50 mL into 250 mL flasks. For preparation of YEM agar media, 1.5% (w/v) agar was added and the media melted.

Autoclaving of all the media was done at 121°C/1.5 Kg/cm^{-2} for 18 min. The autoclaved solid medium was cooled to bearable temperature and aseptically poured into disposable Petri-dishes for bacterial plating. The media was then left to cool and solidify under aseptic conditions. After solidification, the Petri-dishes were wrapped with parafilm and the media stored for impending use

3.7. PREPARATION FOR INOCULATION

All the experiments were carried out aseptically under conditions in a Laminar air flow hood fitted with a bactericidal UV tube (15 W). Before beginning work, the floor of the chamber was wiped clean with cotton dipped in 70% ethanol. Surface of all the vessels and other accessories (such as forceps, scalpels, inoculation loop, lighter etc. except the live plant material/culture) used were also cleaned with alcohol and arranged properly inside the Laminar air-flow chamber. The chamber was then sterilized with U.V. rays continuously for 20 min.

Before introduction into the Laminar air flow chamber, explants taken from the plants growing under *in vivo* conditions were systematically cleaned to remove dirt and adhering microbes. Further, to prepare the explants for inoculation into culture media, definite surface sterilization procedures were followed.

To maintain aseptic conditions inside the Laminar airflow chamber, hands and arms to be used inside the inoculation chamber were thoroughly scrubbed with alcohol before inoculation. The rims of the glass culture vessels were flamed before and after opening as well as after closing, to maintain sterile conditions. Instruments (like forceps, scalpels, inoculation loops, Petri-dishes etc.) were all sterilized by dipping in/spraying alcohol and flaming a few times, before and during inoculation. Adequate care was taken to cool these tools before putting into operation. All the dissections of explants for inoculation were carried out on autoclaved and sterilized glass Petri-dishes (90 mm diameter).

3.8. CULTURE CONDITIONS

After inoculation, all the cultures were incubated in a clean air-conditioned culture room at a temperature of 25±2ºC and relative humidity of 50-60%. Illumination, if provided, was using cool white fluorescent light (2.5 feet wide fluorescent tubes of 40 watt) and with photoperiod of 12 h light-dark cycles (30-35 $\mu Em^{-2}s^{-1}$). Cultures in suspension were kept on gyratory shakers at 80 rpm for roots.

3.9. SUBCULTURE, MAINTENANCE AND REVIVAL OF *A. RHIZOGENES*

Isolated colonies of bacteria from the mother plates of each strain of *A. rhizogenes* were carefully transferred to 30 mL YEM broth in the 100 mL flasks and kept for overnight incubation at 25±2º C on Gyratory shaker at 100 rpm. As and when required, optical density of the bacterial suspension was measured using UV-visible spectrophotometer at 600 nm. From the overnight grown culture of each bacterial strain, a loop full of broth was streaked on Petri-dishes containing YEM agar medium and again incubated overnight at 25°C. After sufficient growth and/or usage for hairy root induction, the bacterial cultures were stored under refrigeration at 4^0C. To assure proper maintenance, the bacterial cultures thus stored were gradually brought to room temperature and routinely subcultured at one week interval, following the procedure mentioned above.

3.10. *IN VITRO* REGENERATION OF PLANTS

3.10.1. Establishment of Aseptic Shoot Cultures

3.10.1.1. Culture initiation

Young shoots having 3-4 nodes were collected in conical flasks filled with distilled water and taken into the laboratory and followed that the shoots were defoliated and washed thoroughly with running tap water. The young tender shoot tips, first, second and third nodes were excised from the shoots, placed in a clean culture-bottle, the mouth of the bottle covered with surgical cloth and the explants kept under running tap water for 15 min to remove dust particles and microbes adhering to the surface. The explants were then thoroughly washed in 5% (v/v) Teepol and subsequently in running tap water for 5 min to remove any residual

detergent. The explants were rinsed with distilled water for a few times and then taken into the Laminar air-flow chamber for further surface sterilization.

The shoot tips and nodes were then transferred to a sterile culture-bottle, immersed in 0.1% (w/v) $HgCl_2$ and cleansed by shaking well for 1-5 minutes, and the optimum time for mercuric chloride treatment was standardized. The explants were then given 2-3 thorough rinses with sterilized distilled water to remove the traces of $HgCl_2$. After transfer to a sterile Petri-dish, fresh cuts were given to the sterilized nodes to remove undesirable or dead portions and to prepare uniform sized explants (of 1.0 – 1.5 cm length) with single node for inoculation. The explants were inoculated vertically into MS medium, such that the plane of nodal region just parallels that of the media. The media consists of full and half strength salts of MS media with 3% sucrose and agar (0.6% w/v). The explants were inoculated into media devoid of hormones and MS media supplemented with various concentrations (1.0 - 2.5 mg/L) of 6-benzylaminopurine (BAP) alone or in combination with auxin (NAA/ IAA). Periodic observations were made for 4 weeks. For each experiment 25 explants were used and repeated at least two times.

3.10.1.2. Multiplication of Shoots

After 4 weeks, individual shoot buds of 1.0- 1.5 cm long emerged from the axillary meristem of nodal explants, were separated and subcultured onto solid media supplemented with different concentrations (0.25 – 1.5mg/L) of BAP for inducing multiple shoots. The emergence of new shoot buds from the base of the subcultured shoot bud was taken into consideration for calculating shoot multiplication frequencies. The data on average number of shoots induced per explant and the average length of the shoots was recorded after 4 weeks of culture.

The multiple shoots so obtained after 4 weeks were separated individually and transferred to again to same media, for further multiplication of shoots. Similarly nodal segments of aseptic shoots were also recultured in half strength MS media with different concentrations of BAP (1.0-2.5 mg/L) for multiplication of shoots.

3.10.1.3. Elongation and Rooting

After 4 weeks, shoots of 1.0-1.5 cm long obtained from the multiplication media were transferred to half strength MS solid media supplemented with IBA and IAA at various concentrations (0.1 -1.5 mg/L) for both elongation and rooting.

3.10.1.4. Deflasking and Hardening

Healthy *in vitro* developed rooted shoots were taken out of the culture vessels and gently washed with running tap water to remove media remnants and agar adhering to the roots. The plantlets were then transplanted into plastic cups filled with sand and soil (2:1) and kept in mist chamber. The plantlets were irrigated daily and after 3 weeks healthy hardened plants were transferred to field.

3.11. BIOPRODUCTION STUDIES

3.11.1. Induction of Hairy Roots

3.11.1.1. Preparation of cultures for hairy root induction

Healthy shoots (5-7 cm) derived from the cultures in MS basal medium at the end of 5th subculture were transferred to pre-incubation media consisting of plain agar (1% w/v) and incubated for 2 days.

The different *Agrobacterium rhizogenes* strains used for hairy root induction were A4, R1022, LBA 9402, 15834, K599 and NCIM 5140. For hairy root induction using scalpel method, a loop full of broth from the overnight grown suspension culture of each bacterial strain was streaked on Petri-dishes containing YEM agar medium. After overnight incubation at 25±2^{0}C, isolated colonies grown on these YEM agar plates were taken for induction of hairy roots (Ooms *et al* 1985).

3.11.1.2. Scalpel method of inoculation

For induction of hairy roots using different strains of *A. rhizogenes*, infection of pre-incubated shoots was performed by wounding the internodal portion with a sterile scalpel blade containing bacteria scraped off from isolated colonies on YEM agar medium. The shoots so infected were planted vertically in

MS basal agar medium (1% w/v agar) with the infected portions 2-3 cm well above the medium to minimize the colonization of the medium by *A. rhizogenes*. The infected shoots were then incubated under complete darkness at 25±2^{0}C. For each strain of bacteria, there were 20 infected shoots with at least 5 wounds per shoot. An equal number of shoots subjected to wounding without bacterial culture served as control.

3.11.*1.3. Recording of response to hairy root induction*

Periodical observations were made on the response of the wounded sites towards infection.

3.11.1.4. Disinfection of hairy roots

After 3 weeks of root initiation, putatively transformed roots of approximately 2 cm length were carefully excised from the infected shoots and individually transferred to Petri-dishes containing MS basal agar medium (1% w/v agar). Of these roots, those showing bacterial infection were transferred to MS basal agar media supplemented with antibiotic (500 mg/L Streptomycin). Roots on the antibiotic media were observed regularly for infection and the infected roots were subjected to repeated transfer to the fresh antibiotic medium containing progressively decreasing concentrations of antibiotics, till the absence of bacterial growth was observed.

3.11.1.4. Confirmation of genetic transformation and establishment of hairy root cultures

Confirmation of genetic induction was done by recording the absence of hairy roots in the control. Decontaminated hairy roots were first established in full strength solid MS media devoid of hormones. Then the roots were transferred to hormone free liquid MS media and the cultures in liquid medium were subjected to continuous agitation on a gyratory shaker till end of the culture cycle.

3.11.2. Growth Curve Analysis of Hairy Root Cultures

The growth curve of hairy root cultures were studied over a period of 30 days with an interval of 5 days. Hormone free MS liquid media was prepared and 50 ml was poured into 20 Erlenmeyer flasks (250ml flasks). The media were then

sterilized by autoclaving. The roots established in liquid cultures were used for the experiment. Roots of 100mg FW was weighed under sterile conditions in a laminar air flow and transferred to the 50ml media in flasks and incubated in a gyratory shaker (New Brunswick Scientific, Germany) at 80rpm under culture room conditions. The cultures were harvested at 5 day intervals starting from the 5^{th} day onwards till 30^{th} day. Fresh Weight (FW) of the harvested hairy root mats was determined subsequent to washing of the roots with demineralised water to remove medium salts and blotting the excess water on filter paper. The mean weight of roots and growth index were determined after each harvest. Growth was then expressed as Fresh Growth Index calculated as per the formula given below (Kittipongpatana *et al*., 1998). The growth curve was plotted based on the data obtained.

$$\text{Growth index} = \frac{\text{Final Fresh Weight} - \text{Initial Fresh Weight}}{\text{Initial Fresh Weight}}$$

3.11.3. Phytochemical Analysis

3.11.3.1. Extraction of compounds from hairy roots

Phytochemical analysis for 2- hydroxy 4- methoxy benzaldehyde was performed. The hairy roots were harvested, washed with distilled water and blotted dry on a filter paper and dried at room temperature for 4 days. The dried hairy roots were powdered and 2g root powder was extracted with 90mL petroleum ether (BP 60-80° C) for 6hrs in a 500mL soxhlet apparatus. The extracts were then filtered through Whatman No. 1 filter paper and the residues were again extracted with methanol for 6 hrs. 5g root powder was also extracted at room temperature under constant stirring in 50ml petroleum ether. The extracts were then concentrated using a Rotavapor (Heidolf, Germany) under vacuum. The concentrated samples of the extracts were used for chromatographic analysis.

3.11.3.2. Identification of 2- Hydroxy 4- Methoxy Benzaldehyde by Thin Layer Chromatography

Thin layer chromatography was done in readymade plates (Merck). Standard sample 2mg of 2-hydroxy 4- methoxy benzaldehyde dissolved in 2 ml petroleum ether. Concentrated samples and standard sample was loaded on the plate about 2.0cm from the lower end using capillary tubes and allowed to dry the spots in air. The plate was then placed in the chromatographic chamber containing 20ml of different solvent systems (petroleum ether and ethyl acetate 4:1, petroleum ether and acetone 10: 1, benzene and hexane 1:1). The plates were then kept slanting on the wall of the chromatographic chamber and its mouth was sealed tightly with the lid. The chromatographic run was carried out at room temperature. The solvent migrated up and when reached three fourth of the total length of the plates, they were taken out and the solvent front was marked and allowed to dry in air. The different spots on the TLC plates were detected in a UV chamber (254-365 nm) chamber and the fluorescent spots were visualized. The plate was also kept in iodine chamber for few minutes and the spots were marked. The relative flow (R_f) was determined.

$$\text{Relative Flow} = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by solvent front}}$$

3.11.3.3. Confirmation of 2- Hydroxy 4- Methoxy Benzaldehyde by HPLC

The root samples were analyzed using high performance liquid chromatography (HPLC) attached with UV/VIS Detector (Gilson, UV/VIS 156 detector).The method consisted of a Gilson 321 series semi-preparative, LC system (Gilson, Inc. France) attached with following components: two pumps (Gilson 321 and 322), system interface (Gilson 506C) and a high pressure adjustable volume dynamic mixer (AVDM™, Gilson). Data acquisition and instrumental control were performed using Unipoint ™ LC system software version 5.1 (Gilson Inc. France). Separation of the compounds was performed on

a general purpose reverse phase Kromacil C-18 column (250 x 4.6mm, 5 µm particle size, VDS optilab Chromatographietechnik GmbH, Wiesenweg, Berlin) and the isocratic mobile phase consisted of methanol 80%, water 20%, Mobile phase was degassed with an Ultrasonicator (Dr. Hielscher, Germany) before use. The flow rate was 1.0 ml/min and the injection volume was 15µL. The analysis was performed at room temperature (25º C) and the compound was detected at 254 nm. The filtered (0.45µm nylon filter, Millipore Corporations, Massachusetts, USA) crude extract were injected into the column for identification of the compound.

3.11.3.4. Quantification of 2- Hydroxy 4- Methoxy Benzaldehyde by spectrophotometry

The percentage of 2-hydroxy-4-methoxy benzaldehyde in the hairy root extract was quantified using a UV-visible spectrophotometer (XP 3001Xplorer) by measuring the absorbance at 277 nm and comparison of the spectrum with that of authentic compound (Sigma, USA). The stock solution of standard was prepared in a concentration of 2mg/mL in petroleum ether. A calibration curve was set using different concentrations of standard ranging from 10-100µg/mL. Absorbance of extracts of hairy roots in petroleum ether was measured in quantified nm using petroleum ether as blank. The quantity was calculated by comparing the absorbance obtained with that of standard calibration curve.

3.12. STATISTICAL PROCEDURES

To analyse and compare the results through statistical methods, means were compared. The analysis of data was done using Microsoft Excel software.

RESULTS

4. RESULTS

The results of the study entitled "Induction and establishment of transformed hairy root cultures of sarsaparilla (*Hemidesmus indicus* L.) R. Br." done at Biotechnology and Bioinformatics division, Jawaharlal Nehru Tropical Botanic Garden and Research Institue during 2013-2014 are presented in this chapter.

4.1. IN VITRO REGENERATION OF PLANTS

First, second and third nodes and young tender shoot tips of *H. indicus* collected from JNTBGRI premises were surface sterilized with 0.1% $HgCl_2$ at different intervals like 1- 5 minutes. Maximum percentage (86 %) of shoot bud initiation was obtained in explants sterilized for 2 minutes with low percentage (7%) of contamination. Explants treated in $HgCl_2$ for 1 min showed positive response but the percentage of infection was 10 -12 %. Mercuric chloride treatment for more than 2 min had negative effect on explant. As the exposure time increased the response of explants decreased. Explants treated for 3 min in 0.1% $HgCl_2$ turned brown after 3-4 days of incubation indicating tissue death. More than 3 min exposure time resulted in tissue death and explants did not show any response in the medium. Young tender shoot tips were more easily damaged by increasing treatment time than the nodal explants. Surface sterilization using 0.1% $HgCl_2$ for 2 minutes was selected to be optimum for all types of explants used in this study (Table 1).

Shoot bud initiation was observed from axillary meristems of nodes after an incubation of 10 days. First, second, third nodes and young tender shoot tips of healthy plants varied in their bud initiation response (Table 2).

Young tender shoot tips showed maximum percentage of response (90%) with maximum number of shoot formation (2.58 ± 0.124) in 4 weeks of culture. From each axillary bud only single shoot bud was obtained (Plate 2(A)).

Table 1: Surface sterilization using 0.1 % mercuric chloride for different time intervals**.** 25 explants were used for each experiment.

Time (min)	Percentage of infection	Percentage of response
1	11	78
2	7	86
3	6	23
4	Nil	Nil
5	Nil	Nil

Table 2: Response of nodal explants of different position cultured in MS solid media containing different concentrations of BAP. Observations were made after 4 weeks.

Explant	Percentage of shoot initiation (%)	Number of shoots	Length of shoots (cm)
First node	70	1.83 ± 0.023	0.89 ± 0.012
Second node	65	1.12 ± 0.065	0.83 ± 0.063
Third node	62	1.04 ± 0.166	0.65 ± 0.054
Young tender nodes	90	2.58 ± 0.124	0.96 ± 0.022

Shoot bud break was observed in 10 days of culture in media containing 2 mg/L BAP whereas no response was obtained in basal media and media containing more than 2.5 mg/L of BAP or combination of BAP plus other auxins. However slight callusing was noticed on explants in media containing BAP and auxin. Response of explants in media supplemented with varied concentration of BAP was studied in detail. The results showed that media containing only 2.0 mg/L BAP favoured maximum shoot bud initiation 2.52 ± 0.873 in a period of 3 weeks. The average length of shoots was ranging from 0.71 to 0.9 cm (Table 3).

Shoot initiation was tried in both half strength and full strength MS media and recorded that more number of shoot formation in media with half strength salts (Table 4). Half strength media was found to be better for shoot induction than full strength. Average number of shoots emerged from half strength MS media was 2.25 ± 0.054 with length 0.83 ± 0.016. The shoots so produced were healthy.

4.1.1. Multiplication of Shoots

Shoot buds of 1.0 -1.5 cm long were subcultured individually in to half strength MS solid media supplemented with BAP at different concentrations showed multiple shoot formation in 3 weeks period (Plate 2(B)).

Media containing 1.0 mg/L BAP was found to be the best for shoot multiplication with average number of shoots of 3.571 ± 0.272 of length 3.29cm length (Table 5).Callusing on the nodal regions of the explants was noticed irrespective of the concentration of BAP used.

A) Shoot bud emerged on the axillary bud of nodal explants in MS solid media with 2mg/l BAP after 3 weeks.

B) Multiple shoots emerged in media containing 1.0mg/L BAP.

Observation made after 3 weeks.

Plate 2: *In vitro* regeneration of plants

Table 3: Effect of different concentration of BAP in Half strength Solid MS media on shoot initiation

BAP concentration (mg/L)	Mean number of shoots	Mean length of shoots (cm)
1.0	1.12 ± 0.356	0.80 ± 0.050
1.5	1.17 ± 0.389	0.71 ± 0.010
2.0	2.52± 0.873	0.82 ± 0.086
2.5	1.11± 0.333	0.90 ± 0.040

Table 4: Influence of salt strength of media on nodal segments or bud initiation. Observations were made after 4 weeks.

Media strength	Number of shoots	Length of shoots (cm)
Full strength media	1.14 ± 0.025	0.65 ± 0.011
Half strength media	2.25 ± 0.054	0.83 ± 0.016

Table 5: Influence of different concentration of BAP on shoot multiplication in half strength MS Solid media

BAP concentration (mg/L)	Number of shoots (cm)	Shoot length (cm)	Remarks *
0.25	2.54 ± 0.977	2.86 ±0.292	+
0.5	3.24 ± 0.130	3.09 ±0.888	+
1.0	3.57 ± 0.272	3.29±0.633	+
1.5	3.03 ± 0.115	2.63±0.563	+

'+'indicates degree of callusing

4.1.2. Elongation and Rooting

Shoots of 1.0 – 1.5 cm long were subcultured onto half strength MS solid media augmented with 0.1 – 1.5 mg/L IBA and IAA responded positively with the emergence of 2-3 roots in a period of 3 weeks (Plate 3).

The roots formed were so healthy and associated with slight callus formation. Maximum number of roots was obtained in media containing 0.1 mg/L IBA.

After 4 weeks of root formation on shoots, the plantlets were weaned away from the culture vessels and planted in plastic cups filled with sand and soil (2:1) got established and emergence of new leaves in 4 weeks under mist house condition. It was recorded that 90 % of the *in vitro* derived plants were established in plastic cups (Plate 4). Subsequently these hardened plants were successfully transplanted to the field and recorded 80 % survival.

4.2. BIOPRODUCTION STUDIES

4.2.1. Induction of hairy roots

Aseptic shoots were infected with overnight grown *A.rhizogenes* (Plate 5) by scalpel method. After two weeks of infection, small protuberances were appeared on the infected sites of the shoots and emerged small hairy roots on the 4^{th} week of the incubation (Plate 6). No such emergence was obtained in the control. Out of six strains (A4, R1022, LBA 9402, 15834, K599 and NCIM 5140) tested, only A4 strain was found to be effective for inducing hairy roots. Average number of roots emerged on the wounded sites of shoots was 1.0 ± 0. 07. Prolonged period of incubation of shoots in the same media helped elongation of roots and there was no additional root formation. The response of aseptic shoots to the infection of different strains of *A. rhizogenes* strains are given in Table 6.

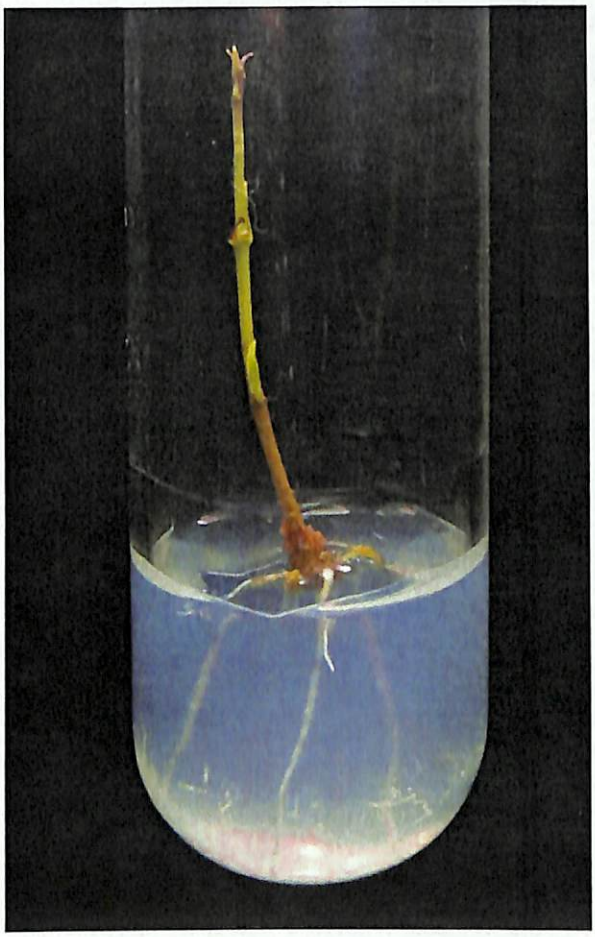


Plate 3: Roots emerged in media with IBA and combinations of IBA and IAA. Observation made after 3 weeks

Plate 4: *In vitro* plant planted in plastic cups filled with sand and soil

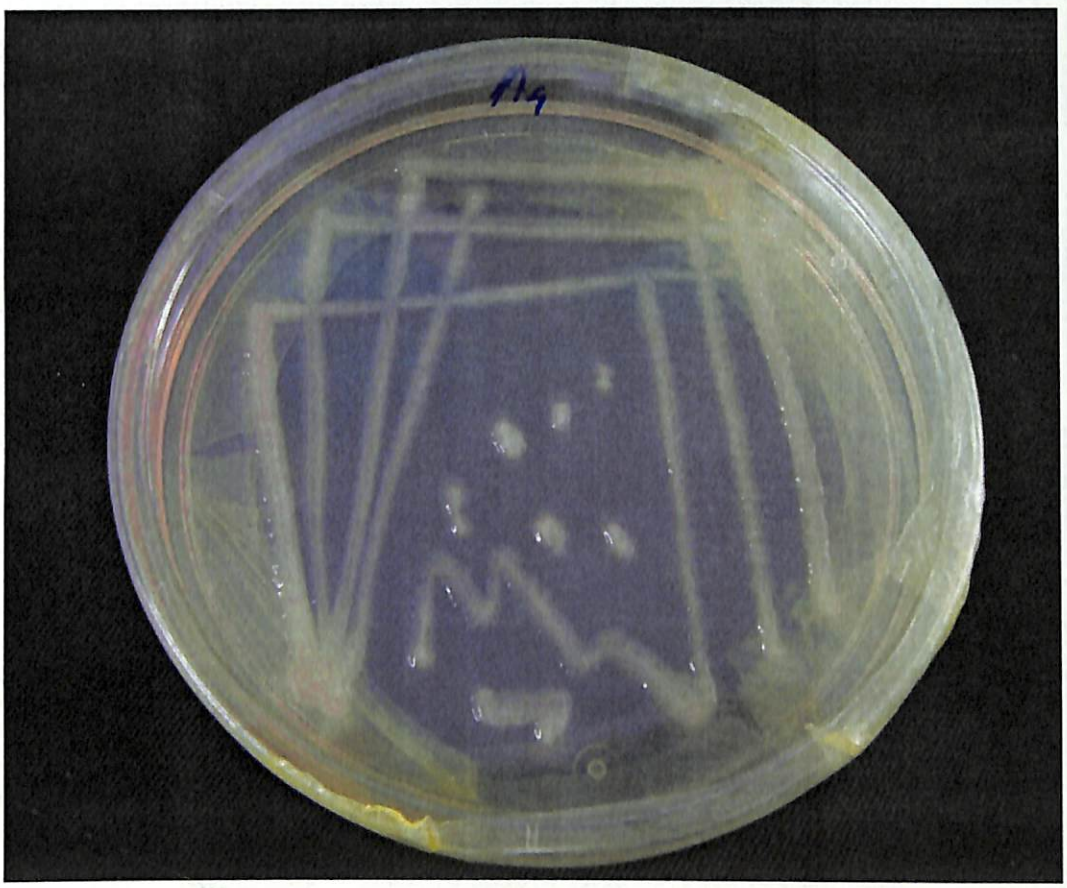


Plate 5: overnight grown *Agrobacterium rhizogenes*

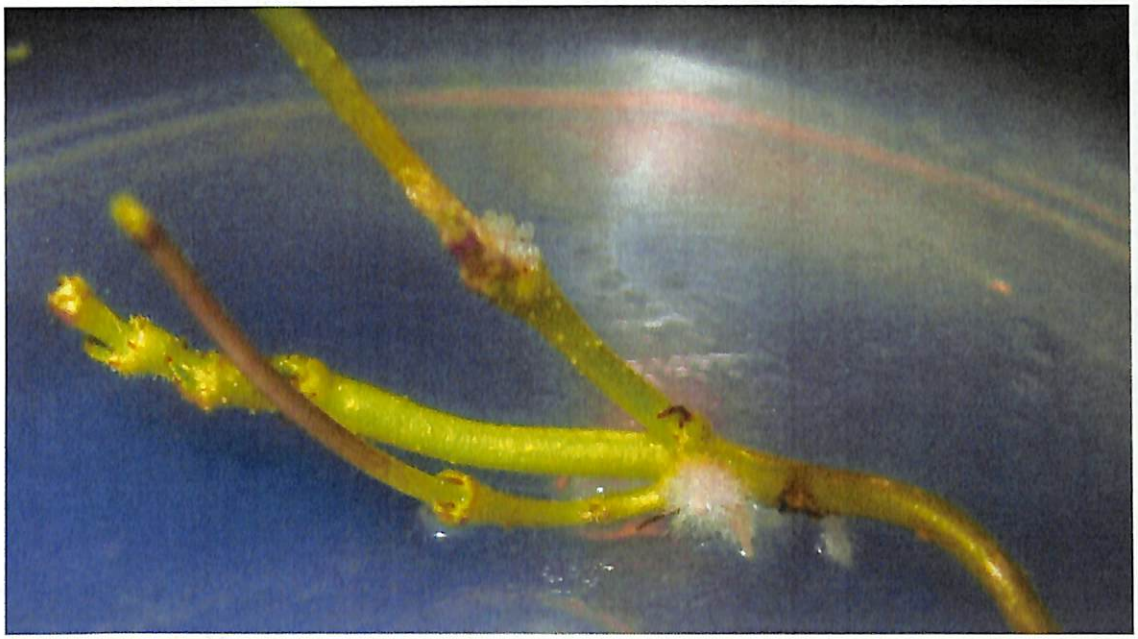


Plate 6: Small hairy roots on the 4th week of the incubation

Table 6: Response of aseptic shoots to the infection of different strains of *A. rhizogenes* strains

A. Rhizogenes strain	Percentage of rooting	Number of roots/wounded site after 4 weeks	Number of roots obtained after antibiotic treatment
LBA 9402	-	-	-
R1022	-	-	-
A4	28%	1.0 ± 0. 07	0.8 ± 0.447
K599	-	-	-
NCIM 5140	-	-	-
15834	-	-	-

The induced hairy roots attained 2.0-2.5 cm length in a period of 2 weeks. The hairy roots separated out from the shoot were decontaminated by frequent transfer to agar media containing antibiotics at two week intervals. After two transfers of hairy roots to antibiotic medium at two week intervals, the roots got bacteria free and those roots were used for further experiments.

The proliferation of roots by the formation of lateral branches was observed in 3 weeks in the MS solid media. After attaining adequate growth, the roots were transferred to liquid MS media and kept the cultures on a Gyratory shaker at 80 rpm. These fully established hairy roots (Plate 7) were used to study the growth pattern and compound production.

4.2.2. Growth curve analysis of hairy root cultures

The growth curve of hairy root cultures were studied over a period of 30 days with an interval of 5 days. The initial root inoculum fresh weight was 0.1g/L. The root biomass started increasing exponentially after a lag phase of five days. The root biomass reached maximum (14.10±0.045g) on 25th day. Fresh wt of the roots and Growth Index were determined (Table-7). After 25th day roots appeared to be brownish and there was no further increase in biomass which indicated that the root growth reached stationary phase. The initial growth index was 0.083 and it increased to 6.051 in a period of 25 days (Figure 1).

Plate 7: Bacteria free hairy roots established in MS basal liquid media after 4 weeks of incubation

Table 7: FW and Growth index of hairy roots grown in MS basal liquid media for a period of 30 days

Days	Fresh weight (g)	Growth Index
0	2.17±0.015	0.083
5	2.56±0.062	0.279
10	3.71±0.214	0.853
15	5.51±0.340	1.753
20	9.96±0.065	3.982
25	14.10±0.045	6.051
30	13.69±0.516	5.849

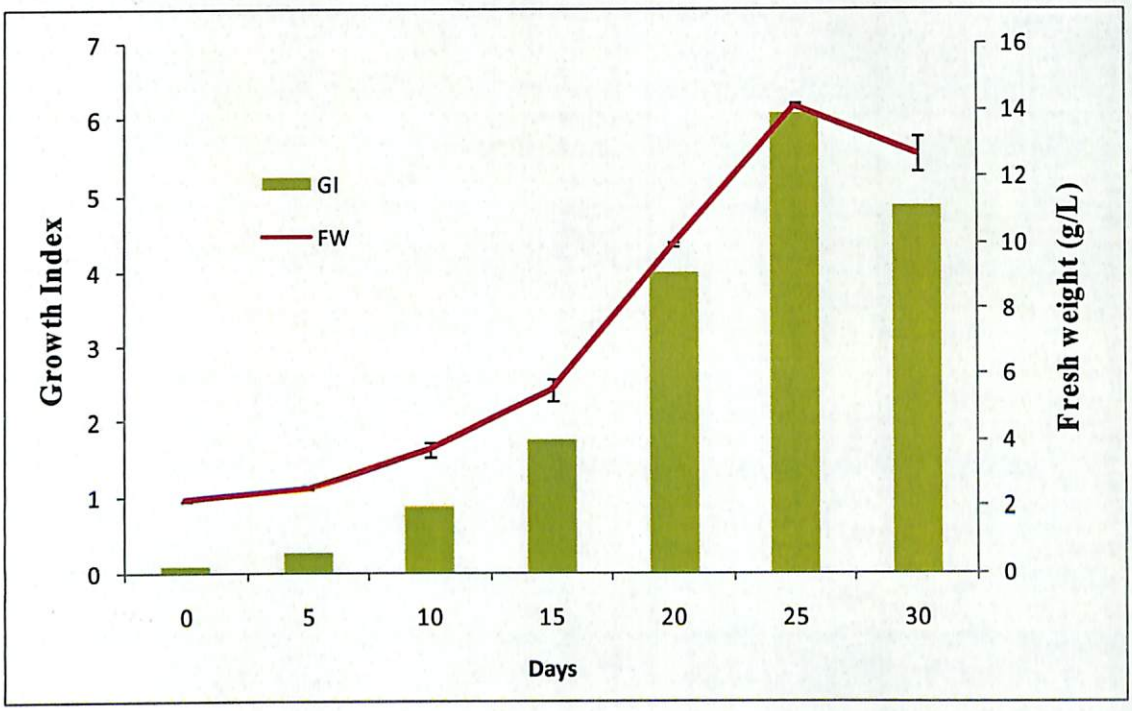


Fig. 1: Growth curve of hairy roots

The hairy roots established were harvested after 25 days and estimated the compound, 2-hydroxy 4-methoxy benzaldehyde.

4.2.3 Phytochemical analysis

4.2.3.1 *Extraction of 2-hydroxy 4-methoxy benzaldehyde*

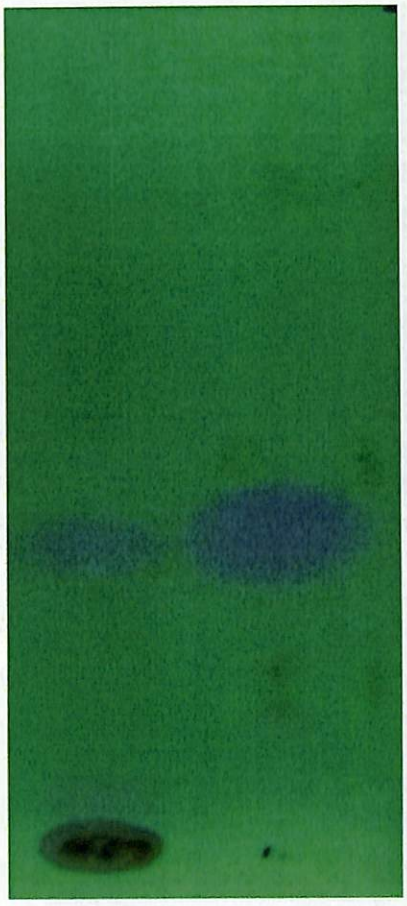
The compound was extracted as per the procedure described earlier. Three different solvent systems such as petroleum ether: ethyl acetate (4:1), petroleum ether: acetone (10: 1), and benzene: hexane (1:1). Among these solvent systems, well resolved bands were obtained in petroleum ether: acetone (10:1) mixture. The resolved root extracts showed bands (plate 8) corresponding to the standard 2- hydroxy-4-methoxy benzaldehyde at the Rf value of 0.4 cm.

4.2.3.2 *Confirmation of 2- hydroxy 4 – methoxy benzaldehyde using HPLC*

Authenticity of 2- hydroxy 4-methoxy benzaldehyde was further confirmed by HPLC (Fig.2 & 3). The retention time was found to be 4 .1 for the authentic compound and a peak was obtained in the chromatogram of the sample at same retention time.

4.2.3.3 *Quantification of 2-hydroxy- 4-methoxy benzaldehyde using Spectrophotometric Analysis*

The petroleum ether extract of hairy root samples were analyzed at 277nm in a UV-VIS spectrophotometer for the estimation of 2- hydroxy-4-methoxy benzaldehyde. It was found that the 30 day old hairy roots grown in liquid MS media supplemented with 0.1 mg/L IBA produced 0.081% g DW of 2- hydroxy-4-methoxy benzaldehyde. Though concentration of the molecule in *in vitro*-derived roots is slightly lower than the concentration of the compound in the roots of mature field grown plants, further experiments on root cultures can considerably reduce the difference of compound level.



A **B**

A: Root extract in methanol obtained by soxhlet extraction.

B: Authentic sample of 2- hydroxy 4-methoxy benzaldehyde

Plate 8: TLC plate loaded with different samples and standard. Bands obtained in samples identical to the band of authentic compound.

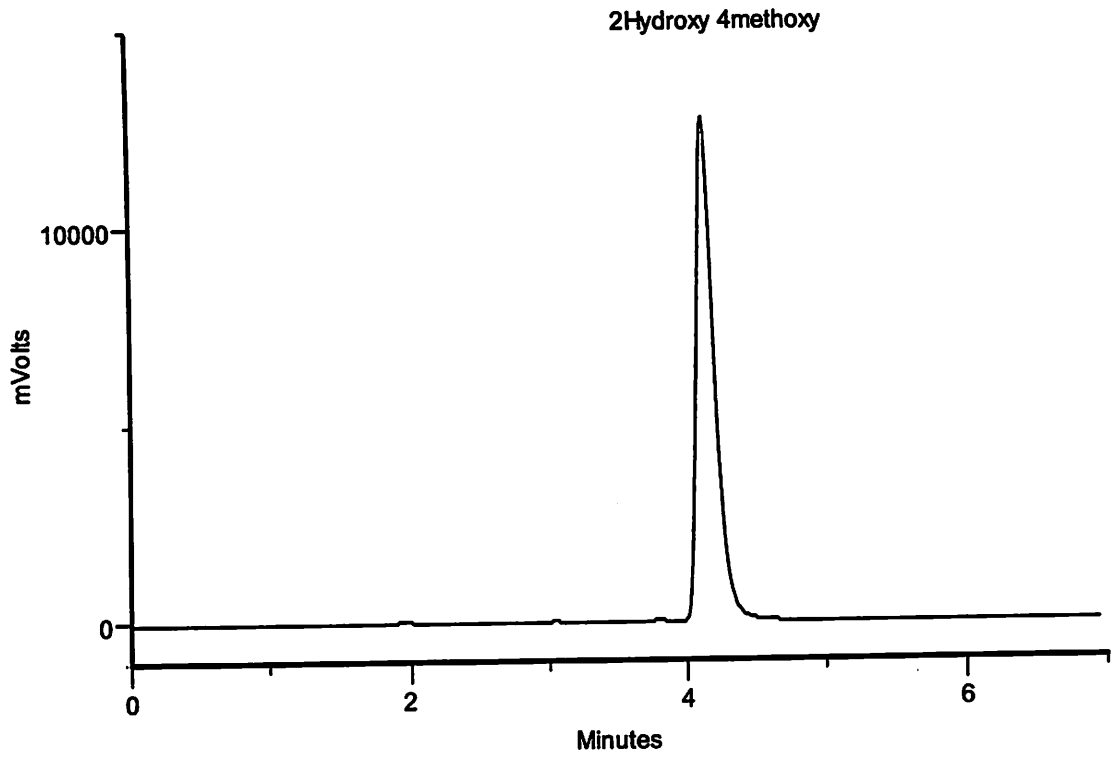


Fig 2: HPLC chromatogram of authentic compound

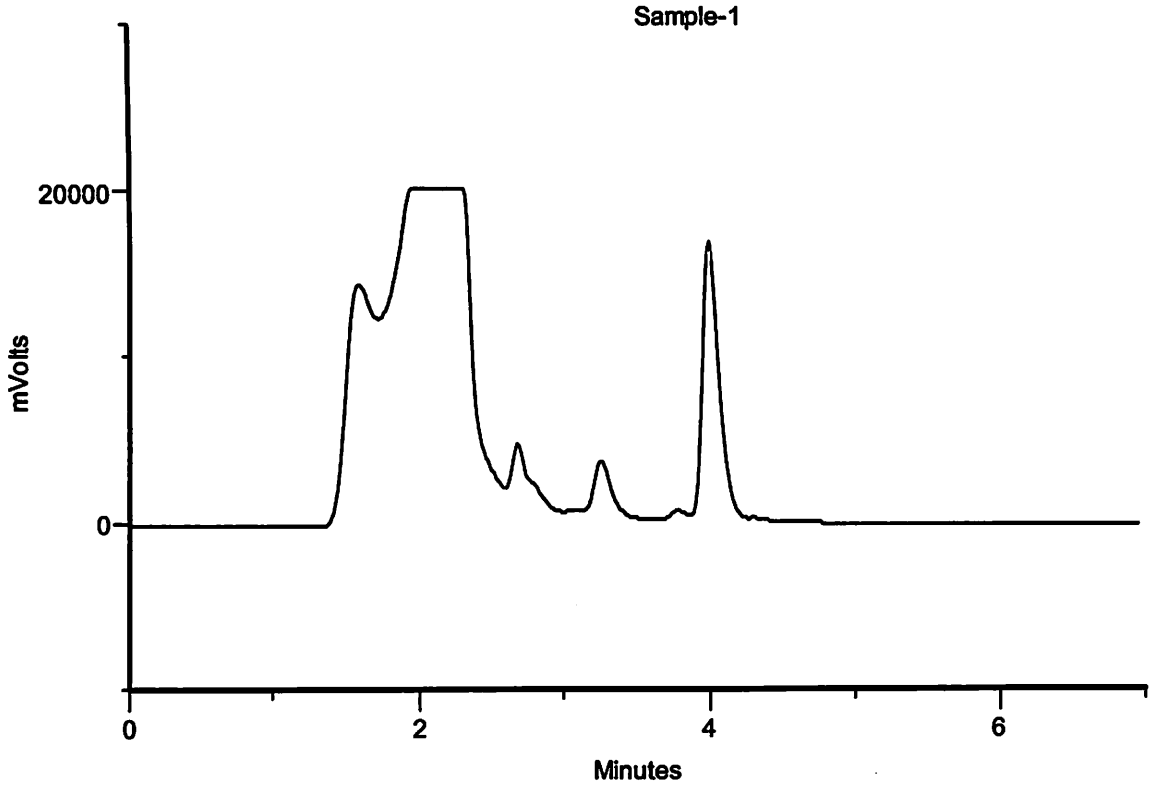


Fig 3: HPLC chromatogram of sample

DISCUSSION

5. DISCUSSION

Medicinal plants are the most exclusive source of life-saving drugs for majority of the world's population. *Hemidesmus indicus*, popularly known as Indian sarasaparila, a very important medicinal plant widely grown in the forest areas of the Western Ghats. Its benefits range from anti-pyretic property to anti-cancerous action. The major useful part in the plant is its tuberous roots and that contain an important aromatic compound 2, hydroxy 4-methoxy benzaldehyde (0.09% g DW). The plant is usually collected from wild and its availability has become a major problem. Conventional propagation and cultivation of the plant is seldom reported.

In vitro propagation of *Hemidesmus indicus* has been tried by many scientists considering their medicinal benefits. Protocol for micropropagation of a large number of plants has been reported and described at different stages of micropropagation (Murashige; 1974). The stages are (1) initiation of the aseptic culture from explant (2) multiplication (3) rooting. Later Deberge and Maene, (1981) and Torres, (1989) added two more stages to this: stock plant selection and preparation of explant that is stage 0 and acclimatization of plants in external environment stage 4. In order to reduce the cost of production, *ex vitro* rooting of explants is also done. In the present study the same procedure has been followed for micropropagation of *H. indicus*.

The objective of the present study was to develop tissue culture system in *H. indicus* and establishment of hairy root culture for the production of 2- hydroxy 4- methoxy benzaldehyde. The natural propagation of the plant is *via* seeds with 95.33 percent of regeneration. *In vitro* propagation of *H. indicus* was achieved by different methods that are *via* shoot nodes, axillary buds, cotyledonary nodes, leaf explants, root segments, organogenesis and embryogenesis.

In every tissue culture system contamination is a major problem that seriously affects the successful regeneration of plants under controlled environment. Bacteria, fungi, yeast and moulds are the common contaminants

which may be from air, water or soil in the surroundings of the plant or endophytic in nature (Bunn and Tan, 2002). The process of surface sterilization is nothing but the removal of contaminants that are grown on the surface of explants without incurring damage to the plant tissues. There are different methods being employed for surface sterilization of explants and many surface sterilants are used. The common agents used for surface sterilization are sodium hypochlorite, ethanol, calcium hypochlorite, mercuric chloride, hydrogen peroxide etc. In certain plants systemic growth of microbes in the internal tissues has been reported and in such occasions combination of different sterilants are used to remove the pathogens. No report of endophytic microorganisms in the tissues of *H. indicus* has been available. The contamination that occurs may be due to organisms in the environment and improper sterilization conditions. Contaminations can result reduced growth of plants, tissue necrosis, reduced shoot proliferation and reduced rooting (Odutaya *et al*., 2007). Contamination of explants also found to be related with seasonal variation.

Mercuric chloride (0.1% w/v) is a very efficient surface sterilant being widely used in plant tissue culture against all kinds of bacterial and fungal contamination .Time given for surface sterilization of explants is a very important factor that depends upon the maturity of the tissues used. In *H. indicus*, treatment of explants with 0.1% $HgCl_2$ for varied time tested and found 2 min incubation was best for effective removal of contaminants with maximum response. Long duration given for surface sterilization of explants together with the poor response and death of tissues noted in the present investigation clearly indicating the critical influence of $HgCl_2$. The result on surface sterilization of tender shoots at 2 min time showed the sensitivity of the explants to $HgCl_2$ and many such experiments have been reported in several plant taxa.

Nutrient media composition is an important factor deciding the response of explants. Murashige and Skoog (1962) media is extensively used for many plants irrespective of their habit including tree species. MS media is enriched with high salt content and favoured growth of many plants under *in vitro* (Seabrook, 1980).

Previous workers (Sarasan *et al.,* 1994, Patnaik and Debata 1996, Sreekumar *et al*., 1998) have reported that MS medium was suitable for tissue culture propagation of *H. indicus*. Response of explants in basal medium indicated the requirement of hormonal supplementation for inducing the resident axillary meristem to shoot buds. Plant growth regulators have a critical role in determining morphogenesis. Cytokinins can elicit axillary buds from apical dominance and promotes shoot development (Bhojwani and Razdan, 1983). A large number of plants require cytokinin for shoot induction Sen and Sharma, (1991), Purohit and Dave, (1995), Jain and Nessler, (1996), Patil and Jayanthi, (1997). BAP and KIN are found to be effective for shoot bud induction and multiple shoot formation (Lundergan and Janick 1980, Rahaman and Blake, (1988), Sen and Sharma, (1991), Bhat *et al.,* (*1995*), Benmoussa *et al.,* (1996), Misra, (1996), Saryam *et al*., (2012).

Another important factor determining the morphogenesis is the physiological age of the explants. Young shoot tips and first nodes induced shoot initiation better than others. Sreekumar *et al.,* (2000) has also reported similar results. Marginal callusing was evident in all the cultures. Sreekumar *et al*., (2000) reported the presence of callus formation on explants cultured in media supplemented with combination of BAP and NAA.

Shoot multiplication was also attained in half strength MS medium with BAP. The optimum concentration required for shoot multiplication was found to be 1.0 mg/L BAP. This indicates that cytokinin requirement for shoot multiplication was less than that for shoot initiation. Multiple shoot formation was pronounced in medium with half the concentration of ammonium nitrate signifying a low nitrogen concentration was enough for healthy shoots. The shoots so established were elongated and rooted in media containing low level of IBA is not uncommon as many reports are available with similar observations in several species. Sarasan *et al*., (1994) and Patnaik and Debata (1996) also reported that IBA was more beneficial for inducing rooting than IAA and NAA. This was documented in many other species also, Patnaik and Debata, 1996). Maximum rooting was achieved in half strength MS medium with 0.1 mg/L IBA. Combination of IBA and IAA gave thick and fleshy roots. Root initiation was very slow and took about 25-30 days.

Root initiation occurred equally in dark and light conditions. This was in contrast to findings of Sreekumar *et al*., (2000) that darkness has a stimulatory effect on root initiation. Success in micropropagation can only be achieved by establishing the *in vitro* plantlets in external environment. The shoots and plantlets developed *in vitro* will be very delicate and require a period of acclimatization to cope up with harsh environmental conditions. Cuticle formation occurs during hardening in *in vitro* plants. A mixture of sand and soil in the ratio 2:1 was used as the potting mixture. The plants were hardened in mist house for 2 weeks after which it was transferred to field and had 80 % survival. The high percent survival of plants during hardening under mist house conditions is presumed be due to the peculiar characteristic feature of *H. indicus* that also apparently demonstrating the amenability of the plant species under *in vitro* conditions.

Plants are the tremendous source for the discovery of new products with medicinal importance in drug development. The secondary metabolites are known to play a major role in the adaptation of plants to their environment and also represent an important source of pharmaceuticals. Today several distinct chemicals derived from plants are important drugs, which are currently used in many countries in the world. Secondary metabolites are economically important as drugs, flavor and fragrances, dye and pigments, pesticides, and food additives. Many of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances. The evolving commercial importance of secondary metabolites has in recent years resulted in a great interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites by means of tissue culture technology.

Plant cell and tissue culture technologies can be established routinely under sterile conditions from explants, such as plant leaves, stems, roots, and meristems for both the ways for multiplication and extraction of secondary metabolites. *In vitro* production of secondary metabolite in plant cell suspension cultures has been reported from various medicinal plants, and bioreactors are the key step for their commercial production. The utilization of plant cells for the production of natural

or recombinant compounds of commercial interest has gained increasing attention over past decades.

In India roots of nearly 50% of plants are used in traditional system of medicine. Plant roots have been used by man since ancient times, nowadays roots are mainly used for medicinal purposes. Secondary metabolites are produced in specific parts of the plant body. Roots are source of many industrial compounds like berberine, camptothecin etc. Therefore root cultures are potent source of chemicals as well as a means for conservation of economically important plants. In case of *H. indicus,* 2- hydroxyl- 4- methoxy benzaldehyde is produced in the roots which are also a root drug.

In medicinal plants, organ cultures for example root/ transformed hairy root cultures are found to be suitable as they are genetically more stable that other callus or cell suspension cultures with respect to compound yield. In the present study, hairy roots of *H. indicus* using a wild bacterial strain *A. rhizogenes* (*Rhizobiaceae*) was induced on the aseptic shoots and established the cultures in MS basal liquid media. The bacteria *A. rhizogenes* is capable of inducing hairy roots through genetic transformation.

Genetically transformed hairy roots are used in wide variety of research applications, and have received an increasing amount of attention over the past decade. Interest in hairy roots has expanded tremendously from initial work on the molecular biology of agrobacterial infection and investigations into the culture requirements and *in vitro* characteristics of excised hairy roots. Hairy roots are now applied in studies of plant secondary metabolism and its genetic manipulation, as hosts for production of foreign proteins, for plant propagation in agriculture, in environmental research, and for development of new engineering technology for large-scale production of plant chemicals.

Hairy roots possess the biosynthetic capabilities of differentiated tissue (Signs and Flores 1990; Hamill *et al.,* 1987; Doran 1989) and growth rates comparable to plant cell suspension cultures (Flores *et al.,* 1988). This unique combination offers limitless potential for production of chemicals in the controlled

environment in the bioreactor. However, low levels of production in plant tissue have prevented use of this technology on a commercial scale.

Hairy root cultures have been proven to be an efficient means of producing secondary metabolites that are normally biosynthesized in roots of differentiated plants (Hu & Du, 2006). Hairy roots are characterised by high growth rate and genetic stability. Successful initiation of hairy roots on shoots raised *in vitro* depended on several factors. The virulence of the soil-borne gram negative bacterium, *A. rhizogenes* is known to vary with strains and between the plant species used for infection (Rech, 1988, Pellegrineschi and Davolio-Mariani, 1996). The susceptibility of plant species to *Agrobacterium* strains varies greatly significant differences were observed between the transformation ability of different strains *of Agrobacterium.* Out of the six strains used for the study, only A4 was found to be successful in inducing hairy roots. The age and differentiation status of plant tissue can also affect the chances of successful transformation. The level of tissue differentiation also determines the ability to give rise to transformed roots after *A rhizogenes* inoculation (Trypsteen *et al*., 1991).

Several studies have established the suitability of MS medium for hairy root cultures of dicotyledonous herbs. Culture media found to be optimal hairy root growth through our study was full-strength MS media. However, many others have also utilized half strength MS media for establishment of hairy root cultures and such differences observed may be due to the difference in root clone chosen for the study.

The growth curve of hairy root cultures were studied over a period of 30 days with an interval of 5 days. The initial root inoculum fresh weight was 0.1g/L. The root biomass started increasing exponentially after a lag phase of five days. The root biomass reached maximum (14.10±0.045g) on 25^{th} day. After 25^{th} day there was no further biomass increase which indicated that the roots reached stationary phase of growth. Fast growth of roots in a short period of time indicates the characteristic feature of the root clone as well as the media tested for the study.

TLC is recognized as a reliable method for identifying compounds using suitable solvent systems for many molecules including 2- hydroxy -4-methoxy benzaldehyde isolated from the hairy roots. Qualitative detection of 2-hydroxy-4-methoxy benzaldehyde by TLC has already been reported by Sudha and Seeni, (2001). Out of different solvent systems used petroleum ether: acetone (10:1) was found to be the best solvent system for TLC. The relative flow of the sample was found to be same as that of reference standard. HPLC was used for determining and validating 2- hydroxy 4- methoxybenzaldehyde and 2- hydroxyl- 4- methoxy benzoic acid in *H. indicus* roots (Sircar *et al.,* 2007). HPLC analysis of the sample confirmed the presence of the compound. Further evidences for identification and purity of the compound can be obtained by FT-IR, GC – MS and NMR spectral analysis.

SUMMARY

6. SUMMARY

The study entitled "Induction and establishment of transformed hairy root cultures of sarsaparilla (*Hemidesmus indicus* L.)R. Br." was conducted at Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram from September 2013 to August 2014.

Hemidesmus indicus or Indian Sarasaparilla is a very important aromatic medicinal plant widely used for various traditional preparations. The tuberous roots are used as diuretic. In addition, the tuberous roots of the plant are also used as food flavours. The major compound obtained from the tuberous roots is 2-hydroxy 4- methoxy benzaldehyde, which has several properties including anti-microbial and antioxidant. In addition, the compound is used as a substitute of vanillin. Though synthetic substitutes of 2- hydroxy 4- methoxy benzaldehyde are available, importance of natural source has still immense potential.

The objective of the present study was to develop a tissue culture system and establishment of hairy root culture for the production of 2- hydroxy 4-methoxy benzaldehyde. In order to achieve the objectives, tissue culture protocol for the development of shoot cultures was standardized using shoot tips and nodal segments of the field grown plants. The explants were treated with 0.1 % $HgCl_2$ for surface sterilization at different periods of time and found 2 min treatment was optimal to obtain maximum contamination free explants (93%) with more response (73%). Shoot bud break was observed in 10 days of culture in media containing 2 mg/L BAP whereas no response was obtained in basal media and media containing more than 2.5 mg/L of BAP or combination of BAP plus other auxins. Among the explants tested, young tender shoot tips produced maximum number of shoot buds (2.58 ± 0.124) in a period of 4 weeks. Whereas in other explants only single shoot bud was emerged irrespective of the hormonal combinations tested. The results showed that media containing only BAP (2.0 mg/L) favoured maximum shoot bud initiation. The average length of shoots was ranging from 0.71 to 0.9 cm.

Shoot initiation was also tried in both half strength and full strength MS media and recorded that more number of shoot formation was in media with half strength salts. Shoot buds of 1.0 -1.5 cm long were subcultured individually on to half strength MS solid media supplemented with BAP at different concentrations showed multiple shoot formation in 3 weeks period. Media containing 1.0 mg/L BAP was found to be the best for shoot multiplication with average number of shoots of 3.571 ± 0.272 shoots with a mean length of 3.29cm. Shoots of 1.0 – 1.5 cm long were subcultured onto half strength MS solid media augmented with 0.1 – 1.5 mg/L IBA and IAA responded positively with the emergence of 2-3 roots in a period of 3 weeks. The roots formed were so healthy and associated with slight callus formation. Maximum number of roots was obtained in media containing 0.1 mg/L IBA. After 4 weeks of root formation, the plantlets were weaned away from the culture vessels and planted in plastic cups filled with sand and soil in the ratio 2:1 got established by the emergence of new leaves in 4 weeks under mist house condition. It was recorded that 90 % of the *in vitro* derived plants were established in plastic cups. Subsequently these hardened plants were successfully transplanted to the field and recorded 80 percent survival.

Bioproduction studies of *H. indicus* was carried out using the hairy roots induced from the shoots. For the induction of hairy roots, various wild strains of *A. rhizogenes* namely A4, R1022, LBA 9402, 15834, K599, NCIM 5140 were used. Only A4 strain was found to be successful in inducing hairy roots. The induced hairy roots were separated out and transferred on to agar medium with antibiotics for decontamination. The decontaminated hairy roots were established in solid as well as in liquid MS media devoid of hormones.

The measurement of growth parameters were done by growth curve study of hairy root cultures over a period of 30 days with an interval of 5 days. Fresh Weight (FW) of the harvested hairy root mats was determined subsequent to washing of the roots with demineralised water to remove medium salts and blotting the excess water on filter paper. The mean weight of roots and growth index were determined after each harvest. Growth was then expressed as Fresh Growth Index.

The root biomass started increasing exponentially after a lag phase of five days. The root biomass reached maximum (14.10 ± 0.045g) on 25th day. After 25th day there was no further biomass increase which indicated that the roots reached stationary phase of growth. The roots appeared to be brownish at the centre portion of roots. The initial growth index was 0.083 and it increased to 6.051 during the next 25 days, but after that the growth index decreased. The hairy roots so established were harvested and extracted for 2-hydroxy 4-methoxy benzaldehyde. The compound was quanitified using spectrophotometric analysis and recorded 0.081% DW in hairy root samples. A suitable solvent system was developed and the compound was separated through TLC. The Rf value was detected as 0.4 cm. The compound was further identified using HPLC co-chromatographed with authentic compound and recorded similar retention time.

The overall results obtained in the present investigation illustrate the suitability of culture conditions including media composition and other physical parameters for the large scale multiplication of plants and further establishment in the field. The result obtained with bioproduction studies, though preliminary, hairy root culture is a desirable option for the production of the bioactive molecule, 2-hydroxy 4-methoxy benzaldehyde. The experiments on hairy root cultures can enhance both biomass and compound production considerably for which further enhancement of the system using hairy root cultures leading to the production of the compound, 2 -hydroxy 4-methoxy benzaldehyde is imperative thereby a suitable system can be developed which would help avoiding harvest of plants from the field.

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7. REFERENCES

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APPENDICES

APPENDIX-I

Murashige and Skoog Medium (1962)

Components		**mg/L**
NH_4NO_3	-	1650
KNO_3	-	1900
$CaCl_2.2H_2O$	-	440
$MgSO_4.7H_2O$	-	370
$KH_2PO_4.3H_2O$	-	170
KI	-	0.83
H_3BO_3	-	6.2
$MnSO_4.4H_2O$	-	22.3
$ZnSO_4.7H_2O$	-	8.6
$Na_2MoO_4.2H_2O$	-	0.25
$CuSO_4.5H_2O$	-	0.025
$CoCl_2.6H_2O$	-	0.025
$FeSO_4.7H_2O$	-	27.8
Na_2EDTA	-	37.3
Nicotinic acid	-	0.5
Pyridoxine HCl	-	0.5
Thiamine HCl	-	0.1
Glycine	-	2
Inositol	-	100
Sucrose	-	3%
Agar		0.65%(w/v)
pH - 5.8		

APPENDIX-II

Specific medium for bacterial culture (YEM or Yeast Extract Mannitol medium)

Constituents	Concentration (g/l)
Yeast extract	0.4
Mannitol	10.0
K2HPO4	0.5
MgSO4.7H2O	0.2
NaCl	0.1
Agar	15
pH	7

ABSTRACT

INDUCTION AND ESTABLISHMENT OF TRANSFORMED HAIRY ROOT CULTURES OF SARSAPARILLA (*Hemidesmus indicus* L.) R. Br.

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ABSTRACT

The study entitled "Induction and establishment of transformed hairy root cultures of sarsaparilla (*Hemidesmus indicus* L.) R. Br." was conducted at the Biotechnology and Bioinformatics division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute during 2013-2014. The objective of the study was to induce and establish transformed hairy root cultures using wild strain(s) of *Agrobacterium rhizogenes* for determining the production of 2-hydroxy 4-methoxy benzaldehyde.

Murashige and Skoog media (1962) was used for the experiments. Young shoots from the collected plants served as the explants for the initiation of cultures. The explants were surface sterilised using 0.1% HgCl2 at different intervals like 1- 5 minutes and standardised the time as 2 minutes since maximum percentage (86 %) of shoot bud initiation was obtained in explants sterilized for 2 minutes.

The explants were inoculated into MS medium of full and half strengths with 3 %sucrose and agar (0.6% w/v) and augmented with various concentrations (1.0 - 2.5 mg/L) of 6-benzylaminopurine (BAP) alone or in combination with auxin (NAA/ IAA) and MS media devoid of hormones. The results showed that media containing only 2.0 mg/L BAP favoured maximum shoot bud initiation 2.52± 0.873 in a period of 3 weeks. The average length of shoots was ranging from 0.71 to 0.9 cm. Also young tender shoot tips showed maximum percentage of response (90%) with maximum number of shoot formation.

For inducing multiple shoots MS media supplemented with different concentrations (0.25 – 1.5 mg/L) of BAP were used. Media containing 1.0 mg/L BAP was found to be the best for shoot multiplication with average number of shoots of 3.571 ± 0.272 of length 3.29cm length.

The induction of hairy roots were done for the bioproduction studies. For the induction of hairy roots, various wild strains of *Agrobacterium rhizogenes* namely A4, R1022, LBA 9402, 15834, K599, NCIM 5140 maintained in Yeast extract mannitol (YEM) media were used. Infection of pre-incubated shoots was performed by

wounding the internodal portion with a sterile scalpel blade containing bacteria scraped off from isolated colonies on YEM agar medium. The hairy roots emerged at the site of infection in the 4th week. Only A4 strain was found to be successful in inducing hairy roots.

The initiated hairy roots were transferred to Petri-dishes containing MS basal agar medium (1% w/v agar). Of these roots, those showing bacterial infection were transferred to MS basal agar media supplemented with antibiotic (500 mg/L Streptomycin). After the decontamination of hairy roots, the roots were established in solid as well as liquid MS media devoid of hormones.

The measurement of growth parameters of hairy root cultures over a period of 30 days with an interval of 5 days were done. The mean weight of roots and growth index were determined after each harvest. Growth was then expressed as Fresh Growth Index. The root biomass reached maximum (14.10±0.045g) on 25th day. After 25th day there was no further biomass increase which indicated that the roots reached stationary phase of growth. The roots appeared to be brownish at the centre portion of roots. The initial growth index was 0.083and it increased to 6.051 during the next 25 days, but after that the growth index decreased.

The extraction of the commercially significant secondary metabolite, 2-hydroxy -4-methoxy benzaldehyde was done. The extracts were then concentrated using Rotavapor under vacuum. The concentrated samples of the extracts were used for chromatographic analysis.

Thin layer chromatography was done for the identification of the compound. Well resolved bands were obtained in petroleum ether: acetone 10:1 for samples identical to the band of authentic compound 2- hydroxyl- 4-methoxy benzaldehyde. The relative flow of the compound was obtained as 0.4. The presence of 2- hydroxyl- 4-methoxy benzaldehyde was confirmed by HPLC. The retention time was found to be 4 .1 for the authentic compound and a peak was obtained in the chromatogram of the sample at same retention time. Further scaling up studies needs to be done for the pharmaceutical purposes and commercial product development.