ESTABLISHMENT OF *IN VITRO* ROOT CULTURES OF SARSAPARILLA (*Hemidesmus indicus* L.) R. Br.

By

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(2009 - 09 - 103)

THESIS

Submitted in partial fulfilment of the

requirements for the degree of

MASTER OF SCIENCE (INTEGRATED) IN BIOTECHNOLOGY

Faculty of Agriculture

Kerala Agricultural University

M.Sc. (Integrated) BIOTECHNOLOGY COURSE

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM – 695 522

KERALA, INDIA

2014

DECLARATION

I hereby declare that the thesis entitled **"Establishment of *in vitro* 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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ACKNOWLEDGEMENT

Writing of this dissertation has been a great experience. Without the support, patience and guidance of the following people, this study would not have been completed. It is to them that I owe my deepest gratitude.

First and foremost I want to thank my major advisor Dr. K. Satheeshkumar. It has been an honour to be his student. I appreciate all his contributions of time and ideas to make my project experience productive and stimulating. The joy and enthusiasm he has for research was motivational for me. I am also thankful for the excellent example he has provided as a successful scientist.

Besides my major advisor, I would like to thank the rest of my advisory committee members Dr. P.N.Krishnan, Dr. Padmesh P. Pillai, Dr. K.K.Sabu for their encouragement and insightful comments. I am thankful for their aspiring guidance, invaluably constructive criticism and friendly advice during the project work. I am sincerely grateful to them for sharing their truthful and illuminating views on a number of issues related to the project. I am also thanking Dr. B.R. Reghunath who was always ready to help. I express my warm thanks to him for his support and guidance.

My sincere thanks also go to Dr. Lekha Sreekantan, Dr. Swapna Alex, Dr. K.B. Soni and Dr. Deepa S. Nair for their love and care.

I would also thank Silja P. K., Sibi, Krishna kumar, Deepthi and Binoy who have wholeheartedly helped me despite their enormous work pressures. Their expertise and camaraderie was appreciable. I don't think I can ever repay the individual kindnesses that they showed me; I can only promise to pay it forward to others. I would also like to specially thank Midhu Krishnan who was a constant source of love and energy.

My time at JNTBGRI was made enjoyable in large part due to my dear friends Soumya S. Dharan, Dhanya.R., Dhanya C.S., Pamita N.S., Sudheep Gopi, Pareeth C.M. and Achuth J. Sankar. I am completely indebted to them for their support.

I would like to thank my dad, mom and my beloved brother who have always supported, encouraged and believed in me, in all my endeavours and who so lovingly and unselfishly cared for me. Last but not the least I thank Lord Almighty.

Dedicated to My Parents and

Brother

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µM	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
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. INTRODUCTION

Man has been using plants for medicinal purposes since time immemorial. Traditional and other forms of medicine like Ayurveda, Unani and Siddha utilizes the therapeutic potential of plants. About 80 percent of the third world population still depends entirely on traditional medicine for primary healthcare (Gayathri and Kannabiran, 2008). World Health Organization (WHO) has proposed guidelines for standardizing the herbal medicines (Kotnis *et al.*, 2004). India is rich in biodiversity and has about 45000 plant species, among which eight thousand are found to have medicinal properties (Grover *et al.*, 2002). The medicinal uses of most plants are not yet fully known and it is assumed that there are many plants with potential therapeutic activity against chronic diseases like Alzhemeirs, AIDS, cancer and so on which are yet to be explored (Ganesan *et al.*, 2012). The enduring commercial exploitation of most of the medicinal plants from wild has resulted in receding of the population of many species in their natural habitat. If timely steps are not taken for their conservation, cultivation and mass propagation, they may be lost from the natural vegetation forever. *In situ* conservation of these resources alone cannot meet the ever increasing demand of pharmaceutical industry. Cultivation of these plants is urgently needed to ensure their availability to the industry as well as to people associated with traditional system of medicine. Finally, the trend in the domestication, biotechnological intervention and genetic improvement of medicinal plants, instead of the use of plants harvested in the wild, will offer great advantages since it will be possible to obtain uniform and high quality raw materials which are fundamental to the efficacy and safety of herbal drugs (Calixto, 2000).

Hemidesmus indicus (L.) R. Br., commonly known as Indian sarsaparilla, is a perennial shrub belonging to the dicotyledonous family Asclepiadaceae. The shrub is twining or prostrate in nature with woody rootstock. It is widely distributed in the upper Gangetic plain eastwards to Bengal and Sundribuna and from the central provinces to South India and Ceylon. The plant prefers mesophytic to semi dry conditions and are commonly found in open scrub

jungles, hedges and uncultivated soil (George *et al.*, 2008). *H. indicus* is an official drug in both Indian pharmacopoeia and British pharmacopoeia. This is one the most widely used medicinal plants in the Indian system of medicine and is one among the *rasayana* plants of ayurveda. It is also recognized as the "medicinal plant as a highly traded one from tropical forests" by the National Medicinal Plant Board of India. The plant has wide ranging medicinal benefits. The main medicinal properties of the plant are attributed to its roots even though leaf and stem extracts also have medicinal values (Saha *et al.*, 2003).

The plant is rich in phyto-constituents like tannins, sterols, flavanoids, glycosides and volatile oils (Austin, 2008). The essential oil containing 2-hydroxy 4- methoxy benzaldehyde (0.9% g DW) is the main constituent of the root drug (Sreekumar *et al*; 2008). The extracts from the roots of the plant is said to have diuretic, diaphoretic, demulcent, alterative, anti-rheumatic, anti-diarrhoeal, anti- bacterial, anti- inflammatory, anti-pyretic and anti-oxidant properties.

The root is used externally as a poultice over swellings, boils and painful parts. The drug from root extracts can be used as a tonic and blood purifier. The plant is used in the treatment of a large number of diseases like syphilis, kidney and urinary disorders, chronic rheumatism, ulcer, piles, bronchitis, skin diseases, fever, epileptic fits in children, leprosy, leucoderma, leucorrhoea, eye diseases, burning sensation, loss of appetite. The root extract can act against lethality of viper venom and its induced hemorrhage (Cheruvathur *et al.*, 2013).The milky latex from the plant is useful for relieving inflammation of the eyes (Saha *et al.*, 2003). The leaves are also used against vomiting, colds, wounds and leucoderma. The root drug is usually formulated in the form of syrup. Even the whole plant can be pounded and used or infusion can be prepared with dried leaves and served with milk and sugar as a tonic for diarrhoea and chronic cough.

The roots of the plant are usually harvested during autumn and dried and stored for further use (Saryam *et al.*, 2012). 2-3 year old plant will yield 14-16 kg

of roots. The dried root of the plant costs about Rs. 120 per kilogram (George *et al.*, 2008). Root extracts are used in soft drinks (Sarasan *et al.*, 1994) and bakery products (Patnaik and Debata, 1996) and as perfumery in cosmetic products. 2-hydroxy-4-methoxy benzaledhyde is a high value flavor product and is expensive. It is often used as a substitute for vanilla in ice creams. It is used as an ingredient in herbal tea preparations (Khanna and Kannabiran, 2008). The plant is often adulterated with *Ichnocarpus frutescens* or *Decalepis hamiltonii* (Giridhar *et al.*, 2004).

Hemidesmus indicus is of economic interest for its wide ranging pharmacological activity. However, there are many constraints in utilizing natural population of the plant. The major problem is the scarcity of the plant in the wild. The plant is usually uprooted for its single long tap root (Neetha *et al.*, 2003). The plant is rare and getting endangered (Misra *et al.*, 2005). The chemical constituents among the plants need to be qualitatively and quantitatively uniform for preparation of standardized formulations. The wild plants showed plant to plant chemo variability (Chaturvedi *et al.*, 2007). Harvesting of the roots from the wild is very difficult since it is very deeply rooted (Sreekumar *et al.*, 1998). Moreover the harvesting must be done at the right stage that is when the concentration of the compound is optimum. In *H. indicus*, the major bioactive compound is 2- hydroxy 4 – methoxy benzaldehyde, which is accumulated in the tuberous roots.

The commercial cultivation of these plants has not yet been attempted and only wild population is available (Saha *et al.*, 2003). These problems could be overcome by tissue culture techniques which help large scale multiplication leading to conservation of the plant. However, isolation of the bioactive compound, 2- hydroxy 4 – methoxy benzaldehyde from the tuberous roots also requires field cultivation and harvest of roots, which is again labour intensive. Methods of *in vitro* production of such pharmaceutically important compounds are many and extensive reports are available with several taxa including *H. indicus* (Sreekumar *et al.*, 1998). The main advantage of *in vitro* production of

important compounds using cell or organ cultures over conventional cultivation is that the later one is free from vagaries of weather and will reduce the collection pressure on plants (Misra *et al.*, 2005).

Secondary metabolites can be produced by cell suspension cultures and organ cultures. Plant cell cultures are very much susceptible to variations and are not suitable for continuous synthesis of secondary metabolites over extended periods of time and hence root cultures are the best possible technique for production of 2- hydroxy 4- methoxy benzaldehyde. Therefore the present study describes development of tissue culture system and establishment of root culture for the production of 2- hydroxy 4-methoxy benzaldehyde in *H. indicus*.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Man has been using plants as medicine since ancient times. There may be hardly any plant without medicinal or pharmaceutical value. WHO has estimated that 80 percent of the inhabitants of the world rely on traditional medicine which is based on plant extracts and its active components. The beneficial effects of plants on health have been found out by experimentation and the use of these herbal remedies are cheap and free from side effects.

The use of medicinal plants is prevalent in both developing and developed countries (Patil *et al.*, 2012). Their use can be classified into two distinct areas of medicine: traditional system of medicine and modern system of medicine. Traditional system of medicine is again divided into (1) local or folk or tribal stream and (2) codified and organized Indian systems of medicine like Ayurveda, Siddha, Unani etc (Mazid *et al.*, 2012). Traditional medicine is widely in practice in China, India, Japan, Pakistan, Sri Lanka and Thailand. In case of pharmaceutical industry 40% of the compounds are derived from plants directly or indirectly because chemical synthesis is either not possible or is very expensive.

Majority of the plant species are collected from natural sources except Opium, Senna, Psyllium, Periwrinkle and Cinchona (Inamadar *et al.*, 2008). Medicinal plants have industrial applications also, ranging from traditional medicines, herbal tea, herbal foods, phytopharmaceuticals and industrially produced pharmaceuticals. Moreover medicinal plants provide valuable foreign exchange for developing countries as they are sources of drugs such as reserpine and quinine, tinctures, intermediates in production of semi synthetic drugs. The world market for these plant based products exceeds billions of dollars every year. Examples for these products are vinblastine, taxol, colchicines, vincristine, forskolin and artemisinin. Annually the value of a medicinal plant varies from $ 0.2 to $ 340 million (Kumar, 2004). Surveys are conducted to discover new plant sources of herbal medicines to keep up with the growing market demand. In the

research and developments in the pharmaceutical sector also medicinal plants constitute an integral part. Bulgaria, Germany and Poland are the major exporters of plant based medicinal products.

Information and facilities for various processes like upstream processing, downstream bioprocessing, extraction, purification, and marketing of the industrial potential of medicinal plants are required for proper development and commercialization of medicinal plant based bio industries. Absence of infrastructure, financial support and governmental interest hinders the development of authenticated market products from traditional herb extracts. Rural people rely directly on traditional medicinal herbs and plants rather than pharmaceutical market products (Horeau and Dasilva, 1999).

According to reports of Food and Agricultural Organization (FAO) medicinal plant exports increased from 375,000 in 1991 to a staggering 600,000 in 2002.The major importers of medicinal plant and plant products are China, Hong Kong, United States and Japan. Many worlds' leading pharmaceutical companies are based in Germany and so it is the largest importer of medicinal plants in the European Union.

The contribution of medicinal plants to biodiversity is crucial. India is rich in biodiversity. Approximately 3000 plant species in India are known to have medicinal value. About 1000 single drugs and 3000 compound preparations have been registered in India. The earliest references to use of medicinal plants are recorded in Rigveda (3700 BC). Attempts have been made to make use of indigenous knowledge of medicinal plants for the treatment of various ailments (Mazid *et al.*, 2012).

The increasing consumption of medicinal plants has led to loss of genetic diversity and habitat destruction (Canter *et al.*, 2005), resulting in extinction of medicinal plants (Horeau and Dasilva, 1999). Environmental pollution has also resulted in loss of medicinal plants, 19 species are already extinct and 1236 species are facing various degrees of threat across different biogeography regions

in India according to the Red list of threatened plants (Naik *et al.*, 2012). The extinction of medicinal plants will lead to considerable loss to biodiversity in the current situation as well as it deprives the future availability of their benefits in the society. To overcome these conditions plant biotechnology has come up with micropropagation as a solution.

2.1 TISSUE CULTURE

Starting from 1960s the application of plant biotechnology was directed to five broad areas - cell behavior (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. Plant tissue culture is the aseptic culture of cells, tissues, organs and their components under defined *in vitro* conditions. It is a useful tool for basic and applied sciences as well as in commercial application. German scientist Haberlandt is considered as the father of plant tissue culture. It was his ideas that paved way for the development of tissue culture. By 1990 *in vitro* propagation was extended to a large number of species. Today the plant tissue culture has advanced to a stage where virtually any plant can be propagated successfully under *in vitro* conditions (Gamborg *et al.*, 1976). Plant tissue cultures have evolved as an important tool in the areas of agricultural biotechnology and molecular biology (Thorpe, 2007). This is also a desirable alternative to produce useful medicinal compounds from plants (Rao and Ravishankar, 2002).

Success of plant tissue culture is governed by two main criteria – explant and tissue culture media (Gamborg *et al*; 1976). The usage of suitable nutrient media will result in successful *in vitro* culture of plants. White's root culture medium and Gautheret's callus culture medium were the initially developed medium. Uspenski and Uspenska's medium for algae was the basis of White's medium, while Knop's salt solution was the basis of Gautheret's medium. 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 .

Murashige and Skoog medium 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

1) It is vital for completing the life cycle of the plant.
2) Its action cannot be replaced by any other element.
3) It has direct influence on the organism
4) 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. 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. 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. 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 . 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. Two or more hormones can either act synergistically or antagonistically.

2.1.1. 1. 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-3-butyric acid and 4-chloro IAA. NAA and 2,4-D are synthetic auxins. IAA, IBA and NAA are used for root induction (Smith, 2012). 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 (Smith, 2012).

2.1.1.2. Cytokinins

Cytokinins promote shoot proliferation, its morphogenesis and cell division (Smith, 2008). 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 (Smith, 2012). 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.

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 (GA) known of which GA_3 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.

2.2. SECONDARY METABOLITE PRODUCTION VIA DIFFERENT CULTURE SYSTEMS

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). Distributions of these compounds vary widely in plant species and sometimes occur only in a single plant species and related plants. Alkaloids, phenyl propanoids and terpenoids are the major groups of secondary metabolites (Rhodes, 1994).

Secondary products have attained high commercial interest as plant products. They are mainly classified into three categories based on their commercial purpose – essential oils, glycosides, alkaloids. The essential oils are mixtures of terpenoids. They are used as flavouring agents, solvents and perfumes. Glycosides include tannins, phenolics, flavanoids, saponins, cyanogenic glycosides and mustard oils. They are used as medicinal, food colours and dyes. Alkaloids are much more diverse group with new structures being reported continuously. Morphine, nicotine and cocaine are some examples of alkaloids. Alkaloids are very active physiologically in humans (Shuler, 1981).

The synthesis of secondary metabolites in plants is tightly regulated. The main problem that we face is the lack of knowledge of the biosynthetic pathway of secondary metabolite. Biotransformation, feedback inhibition and elicitors can enhance the production. Biotransformation involves the use of exogenous supply of biosynthetic precursors that increase the accumulation of compounds. Feedback inhibition and inhibition of membrane transport can enhance production. Elicitors are compounds that increase the synthesis of secondary metabolites and can be abiotic or biotic. Pectin, chitosan are examples of biotic elicitors (Dornenburg and Knorr, 1995).

Secondary metabolites are usually obtained by extraction of whole plants. Production of these compounds by plants is not satisfactory; they are restricted to a particular species, or to particular developmental stages of the plant. Certain plants like cinchona trees are very slow to grow and require about 10 years for harvesting. Marketing of these compounds also has some difficulties. Supply of

these materials is erratic due to natural calamities. At times seasonal variations can cause undesirable changes in plants. Even when sufficient plant material is obtained for extraction, they must be maintained at a peak physiological pH to obtain high yields (Shuler, 1981). Hence different tissue culture methods are beneficial in overcoming these problems and to obtain high amounts of secondary metabolites.

2.2.1. Plant Cell Cultures

Plant cell cultures are potential source of many metabolites. Plant cell cultures can be developed from any plant but, some plants can render cells faster than others (Verpoorte *et al.*, 2002). Cell suspensions are derived by transferring friable portion of callus to liquid cultures. They are maintained under suitable conditions of light, temperature, agitation, aeration etc. They have slow growth rate. The oxygen requirement of these cultures is very low (Dicosmo and Misawa, 1985) but their shear sensitivity is a constraint (Verpoorte *et al.*, 2002). Cell suspensions are very much susceptible to somaclonal variations. Elicitation by methyl jasmonate, immobilization and *in situ* extraction are techniques used to improve secondary metabolite content in cell cultures (Roberts and Shuler, 1997). The disadvantage of plant cell cultures is that it does not continue synthesis of secondary metabolites over extended periods like whole plant (Dicosmo and Misawa, 1985).

2.2.2. Callus Cultures

The term callus is derived from the latin word callum meaning hard. Callus are disorganized cell masses which are totipotent in nature. Callus cultures are widely used in research and industrial development (Ikeuchi *et al.*, 2013). Callus tissues are derived by dedifferentiation of different explants induced by exogenous plant growth regulators. Plants can be regenerated from callus. The

auxin to cytokinin ratio is very critical in determining the fate of the plant, a high ratio favours rooting and low ratio promotes shoot regeneration. A ratio intermediate between the two is suited for callus. Other hormones like abscissic acid and brassinosteriods can also induce callusing to an extent. Wound induced calli can also be formed from vascular cells, cortical cells, and pith cells and are highly pluripotent in nature. The calli derived by different means vary in their physiological and morphological properties (Ikeuchi *et al.*, 2013). To maximize production from callus cultures, callus is developed from that part of the plant which is the major producer of the compound (Chattopadhyay *et al.*, 2002). Callus cultures provide an easy way for long term maintenance of cells with regular subculturing at 2- 4 week intervals.

2.2.3. Root Cultures

The main functions of plant roots are nutrient and water uptake and in symbiotic associations. Besides these functions roots are a major source of plant secondary metabolites (Signes and Flores, 1990). *In vitro* adventitious roots are similar to normal roots. These roots have high rate of proliferation in auxin supplemented culture media. Roots have tremendous potential in accumulating secondary metabolites. Secondary metabolites from *in vitro* roots are produced in many stages: induction of roots in *in vitro* shoots, culturing of these roots in solid medium and liquid medium in flask scale and bioreactor scale according to the requirement, establishing growth kinetics, strategies for higher metabolite production. The parameters that influence root cultures are aeration, inoculation density and culture period. Root cultures are genetically stable (Chadopadhyay *et al.*, 2002). Hairy root cultures are also useful in large scale culture of secondary metabolites.

2.3. MEDICINAL PROPERTIES OF *Hemidesmus indicus*

The medicinal benefits of *H. indicus* are many and much new potential are yet to be discovered. Scientists all over the world had worked on the plant to identify its pharmacological properties. Numerous reports on the medicinal

properties of the plant are found in the literature. *H. indicus* can act against immunotoxicity and other physiological and pharmacological disorders (Sultana, 2003).

The presence of many compounds was discovered in *H. indicus* which renders them medicinal properties. Medidemine, hemisine and desmisine are pregnane oligoglycosides 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* (Padhy *et al.*, 1973). 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, flavanoids, cardenolides, volatile oils, carbohydrates, proteins (Khanna and Kanabiran, 2008).

We are often exposed to free radicals in the environment or that arise as a result of the metabolic reactions in the body. Antioxidants scavenge these free radicals and render protection to cells. Medications containing Hemidesmus bark extracts were effective in treating several free radical mediated diseases because of its free radical scavenging property. The antioxidant property of the methanolic bark extracts were estimated in several *in vitro* and *ex vivo* models (Ravishankar *et al.*, 2002). The compound 2- hydroxy-4- methoxy benzoic acid is the active component that gives antioxidant property (Jayaram and Dharmesh, 2011). The leaf extract also possess significant antioxidant activity in par with standard antioxidant silymarin. Administration of aqueous extracts of *H. indicus* to rat liver mitochondria prior to bromobenzene induced oxidative stress promoted protection and its effectiveness was more than that of vitamin E (Gopi and Shetty, 2010).

Cancers occur due to mutations which result in aberrant signaling pathways. Ferruzi *et al.* (2013) estimated the cytodifferentiating, cytotoxic and cytostatic activities of *Hemidesmus indicus* on a human promyelocytic leukemia cell line (HL-60). 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* root extract 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 decoction 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). Topical application will provide chemoprotection against skin carcinogenesis (Sultana *et al.*, 2003).

Antimicrobials are substances that can either inhibit or kill pathogens in the host cells. Plant based antimicrobials have less side effects and so they are preferred to chemical antimicrobials. Extract can inhibit *Pseudomonas aeruginosia, Klebisella pneumonia, Salmonella aureus* (Gayathri and Kannabiran, 2009), *Salmonella typhi, Staphylococcus aureus, Escherichia coli* and *Vibrio cholera* (Ratha *et al.*, 2012). 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 chlorofoam fraction of *H. indicus*

root extract (Das and Devaraj, 2008). 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 antifungal activity was much stronger than antibacterial effect. Its antifungal activity was comparable to that of the standard fungicide amphiotericin 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).

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).

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). Gentamicin induced renal impairment in rats can also be treated with *H.indicus*, so can be used along with aminoglycosides as an adjunct therapy (Kotnis *et al.*, 2004).

H. indicus can reduce the number of ectopic beats and duration of ventricular tachycardia. Thus it is a potent vasodilatior, positive inotropic agent, and cardioprotectant (Khandewal *et al.*, 2011).

Herbal formulation of *H. indicus* increases proliferation of cells, promote granulation of tissue and thus improve wound healing (Ganesan *et al.*, 2012). Being good emulsifier, anti inflammatory and free radical scavenging property of phytosterols present can penetrate deep into the wound, prevents prolongation of initial phase, reduce redness and swelling thus induces wound healing (Vijayakumari and tarwar, 2012).

Alcoholic extract of *H. indicus* showed anti nociceptive activity. The optimum dosage preferred is 25-100mg/kg. Triterpenes, flavanoids and sterols present in root extract contributes to this activity (Verma *et al.*, 2005).

Anti diarrhoeal activity of *H. indicus* in both *in vitro* and *in vivo* conditions were reported (Das *et al.*, 2003). Water and electrolyte absorption in the intestine can be enhanced by root powder from rat intestine. Administration of the extract along with oral rehydrating salt solution will enhance its efficiency (Evans *et al.*, 2004).

Anoop and jagadeesan found out the anti ulcerogenicity of the aqueous ethanolic root extracts of *H. indicus* in animal models. They also found out the effectiveness was more in roots collected during flowering seasons than during vegetative seasons in animal models (Anoop and Jagadeesan, 2003).

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). 217.5µg/ml methanolic root extracts was capable of inhibiting 50% lipid peroxide formation and the same extract in 73.5µg/ml could scavenge hydroxyl radicals (Mary, 2003). Chloroform ethanol extract of *H. indicus* has a new pregnane glycoside hindiscusine which has antioxidant and anti dyslipidemic activities (Sethi *et al.*, 2010).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).

Liver toxicity and damage caused by ethanol can be cured by *H. indicus* rendering hepatoprotective action (Saravanan and Nalini, 2008; Baheti *et al.*, 2006). *H. indicus* can also protect liver microsomes (Shetty *et al.*, 2005). Prabakaran *et al* estimated the protective effect against rifampicin and isoniazid induced hepatotoxiciity of the ethanolic extracts. It also has otoprotectant activities (Previati *et al.*, 2007).

Anti arthritic activity of roots was evaluated by Mehta *et al.* (2012) and it was found out that the presence of terpenoid in hydroalcoholic extract as well as in ethyl acetate fraction attributes to this property.

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 brewers yeast induced pyrexia in rats can be reduced by root extract thus establishing its anti pyretic and anti inflammatory activity (Lakshman *et al.*, 2006).

Very few works have been done to find out the effect of *H. indicus* on nervous system. In mice butanol fraction has been shown to improve memory. Thus the extract has potential in treating dementia seen in alzheimers (Shete and Bodhankar, 2010).

Alam *et al.* (1994) discovered a compound which antagonized the viper venom induced hemorrhagic activity and lethality. Later the compound was found to be 2- hydroxyl-4-methoxy benzoic acid which has antisnake viper venom action by inducing changes in serum phosphatase and transaminase activity in male albino rats thus neutralizing the venom. (Alam and Gomes, 1998).

H. indiucs has many environment friendly characteristics apart from medicinal values. It has lead hyper accumulating property. Lead accumulation occur maximum in shoots of the plant body. Thus the plant proved its potential as a good phytoremediation agent (Sekhar *et al.*, 2005). Mosquitoes cause major health menace and are vector in transmitting many diseases. Root extract has mosquitocidal and water sedimentation properties (Arjunan *et al.*, 2011). *Culex quinquefasciatus,* endemic to Indian subcontinent is the vector of lymphatic filariasis. 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).

2.4. MICROPROPAGATION OF *Hemidesmus indicus* L.

Many researchers have tried micropropagation of *H. indicus* for large scale planting, improvement and conservation (Sharma and Yelne (1995). Propagation *via* many methods that is by using different explants like shoot tips, seeds, cotyledonary nodes were done. *In vitro* seed germination was successfully achieved on plain half MS media. This helped in obtaining juvenile plantlets which are contamination free and have better regeneration capacity in tissue culture (Saryam *et al.*, 2012).

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.

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- hydroxy-4-methoxy benzaldehyde was also determined to be higher than in conventional plants (Sreekumar *et al.*, 2000).

Ramulu *et al.*, (2003) developed a protocol for regeneration of plants from root segments excised from aseptic seedlings. 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.

Shoot and callus cultures using root and leaves as explants were established on Murashige and Skoog medium using different combinations of enzymes. The production of anti oxidants was tested in different types of culture of *H. indicus*. 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 (Neetha *el al.*, 2005).

Patnaik and Debata (1996) performed axillary bud culture. They observed that shoot multiplication occurred strongly in the presence of cytokinin and NAA. Reduction in shoot numbers with each subculture was also reported.

In vitro propagation via bud multiplication was also done by Saha *et al.* (2003) 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.

In vitro plants were regenerated by nodal and leaf explants by Shanmugapriya and Sivakumar (2011). From their studies they found out that exogenous supply of plant growth regulators is not always required, some species have enough endogenous plant growth regulators. The protocol provides an efficient method for rapid regeneration and successful regeneration of plantlets.

Protocol for somatic embryogenesis has been developed. 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 (Cheruvathur *et al.*, 2013). 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. Proembryogenic light yellow calli was best suited for somatic embryogenesis among different calli (Cheruvathur *et al.*, 2013).

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 mgl^{-1}) and Kn (0.5 mgl^{-1}) was used to induce organogenesis from callus. Plantlets were produced successfully by organogenesis. *In vitro* propagation of *Hemidesmus* was done by shoot tip culture, direct and indirect organogenesis and somatic embryogenesis (Sarasan and Nair, 1991).

In vitro propagation was done 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.

Callus was successfully induced 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.

Sreekumar *et al.*, (1998) reported that roots of *H. indicus* are slow growing and subculturing was done with long time intervals. Thick and fleshy roots were obtained in woody plant medium with very slow growth rate compared to MS

medium. Combinations of auxins in MS medium gave poor calloid root growth and also report 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- methoxy benzaldehyde 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 *Decalepis 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- methoxy benzaldehyde and 2- hydroxy 4- methoxy benzoic acid in *Hemidesmus* roots. The method is very well suited for routine analysis and is fast and sensitive (Sircar *et al.*, 2007). *Decalepis hamiltonii* also produces 2- hydroxy 4-methoxy benzaldehyde in tubers and its production is increased by 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- methoxy benzaldehyde is not yet known (Chakrabarthy *et al.*, 2008) but is assumed to be by central phenyl propanoid 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 h 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- methoxy benzaldehyde 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- methoxy benzaldehyde (Kundu *et al.*, 2012).

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 three years of culture. Concentration of lupeol, vanillin and rutin was similar in regenerated plantlets and comparable to parent plant (Neeta *el al.*, 2003). Decruse *et al.* (1999) reported the effect of cryopreservation on seed germination of *Hemidesmus*.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Establishment of *in vitro* 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)

Glasswares 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 filtration 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 airflow 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 indiucs* L. R. Br. 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). All culture media components, 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 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 final volume with distilled water. The stock solutions of all PGRs were stored at 4 °C. Following addition of desired concentrations of required PGRs, the medium was made-up to 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 adjusting 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.

3.7. 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.

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±2°C 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. *IN VITRO* REGENERATION OF PLANTS

3.9.1. Establishment of Aseptic Shoot Cultures

3.9.2. Culture initiation

Young shoots having 3-4 nodes were collected in conical flasks filled with distilled water and taken into the laboratory and then 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 sucrose (3% w/v) and agar (0.6% w/v). The explants were inoculated into media devoid of hormones and MS media supplemeted 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.

3.9.3. 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 – 1.5 mg/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.

3.9.4. 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.

3.9.5. 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.

3.10. BIOPRODUCTION STUDIES

3.10.1. Initiation and Establishment of Adventitious Root Cultures

3.10.1.1. Initiation of Callus Cultures

Nodal cuttings and shoot tips collected as mentioned previously, surface sterilized as described earlier and inoculated for initiating callus cultures. Explants were inoculated horizontally onto MS half strength solid media supplemented with different concentrations and combinations of BAP (1.5 - 3.5 mg/L) and NAA (0.2-2.0) and incubated in culture room at dark and light (12 h photoperiod; 30-35 $\mu Em^{-2}s^{-1}$) conditions.

The callus initiated from the explants was subcultured onto solid media with same growth regulator for further multiplication. For each experiment at least twenty replicates were used and repeated at least twice.

3.10.1.2. Initiation of Adventitious Roots from Callus

6 week old callus tissues were used for inducing adventitious roots. Callus pieces of around 100 mg FW was transferred to MS half strength solid medium containing different concentrations and combinations of IBA, NAA and GA_3 for root induction media and incubated under conditions of light (30-35 $\mu Em^{-2}s^{-1}$) and complete dark in a culture room 25±2°C. Observations were made regularly

and recorded the data. The roots differentiated from callus tissues were taken out after four weeks and used for establishing isolated root cultures.

3.10.1.3. In vitro Derived Roots from Aseptic Shoots

Adventitious roots initiated from *in vitro* multiplied individual shoots were dissected out and transferred onto half strength MS solid media with 0.6% agar and 3% sucrose containing different concentrations (0.1 – 0.4 mg/L) and combination of IBA and IAA. The cultures were maintained in culture room at a temperature of 25±2°C with photoperiod of 12 h lightdark cycles(30-35 $\mu Em^{-2}s^{-1}$).

3.10.2. Establishment of Isolated Root Cultures

Approximately 250mg (fresh weight) of adventitious roots were transferred onto half strength MS solid and liquid media containing different concentrations of IBA (0.1- 1.5mg/L) and combinations with IAA (0.1 and 1.0 mg/L). The liquid cultures were incubated on a Gyrotory shaker at 80 rpm in culture room conditions. The difference in growth of the cultures in solid and liquid media containing different hormonal regimes was recorded.

3.10.3. Growth Curve Analysis of Root Cultures

The growth pattern of adventitious root cultures was studied over a period of 40 days at an interval of 10 days. Roots of 300mg FW was weighed under sterile conditions in a laminar air flow and transferred to 250 ml conical flasks filled with 50ml MS liquid media supplemented with 0.1mg/l IBA and incubated on a gyrotary shaker at 80rpm under culture room conditions. The cultures were harvested at 10 day intervals starting from the 10^{th} day onwards till 40^{th} day. Fresh weight and dry weights of the cultures were recorded. The mean weight of roots and growth index were determined after each harvest. The growth curve was plotted based on the data obtained. Growth index (GI) was determined according to the formula given below. For each experiment six replicates were used and repeated twice.

$$\text{Growth index} = \frac{\text{Final fresh weight} - \text{Initial fresh weight}}{\text{Initial fresh weight}}$$

3.10.4. Fresh Weight and Dry Weight Determination

The harvested roots were washed in distilled water to remove the media remnants and the roots were blotted dry on a filter paper and recorded its fresh weight (FW). The roots were air dried for two days and dry weight (DW) was determined. The percentage of water content in roots was determined.

$$\text{Water content} = \text{fresh weight of roots} - \text{dry weight of roots}$$

$$\text{Percentage of water in roots} = \frac{\text{Water content}}{\text{Fresh weight}} \times 100$$

3.10.5. Phytochemical Analysis

3.10.5.1. *Extraction of 2- hydroxy 4- methoxy benzaldehyde*

Phtytochemical analysis for 2- hydroxy 4- methoxy benzaldehyde was performed. The roots were harvested, washed with distilled water and blotted dry on a filter paper and dried at room temperature for 4 days. The dried roots were powdered and 2g root powder was extracted with 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.

3.10.5.2. *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 (Rf) was determined.

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

3.10.5.3. 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 15 µl. The analysis

was performed at room temperature (25° 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.

3.10.5.4. Quantification of 2- hydroxy 4- methoxy benzaldehyde by spectrophotometry

The percentage of 2-hydroxy-4-methoxy benzaldehyde in the *in vitro* grown 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 *in vitro* grown roots in petroleum ether was measured using petroleum ether as blank. The quantity was calculated by comparing the absorbance obtained with that of standard calibration curve.

3.11. Statistical Procedures

To analyze 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 "Establishment of *in vitro* 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).

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

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).

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

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).

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).

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

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.

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

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 3).

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

Plate 3: Multiple shoots emerged in media containing 1.0mg/L BAP. Observation made after 3 weeks.

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.

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	3.01 ± 0.130	2.90 ±0.888	+
1.0	3.57 ± 0.272	3.29±0.633	+
1.5	3.03 ± 0.115	2.63±0.563	+

'+' indcates 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 4).

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 (Table 6).

Table 6: Response of shoots in half MS solid media with IBA and IAA for rooting. Observation made after 5 weeks.

Hormone concentration (mg/l)	Number of roots	Length of roots (cm)
0.1 mg/l IBA	3.02 + 0.823	1.89 ± 0.162
0.2 mg/l IBA	2.81 + 0.136	1.69 ± 0.324
0.3 mg/l IBA	2.08 + 0.901	1.65 ± 0.122
0.1 mg/l IBA + 0.1 mg/l IAA	2.60 + 0.141	1.24 ± 0.148

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). The plantlets got established and new leaves emerged in 4 weeks under mist house condition. It was recorded that 90 % of the *in vitro* derived plants were established in plastic cups (Plate 5). Subsequently these hardened plants were successfully transplanted to the field and recorded 80 % survival.

4.2. BIOPRODUCTION STUDIES

4.2.1. Initiation and Establishment of Adventitious Root Cultures

4.2.1.1. Initiation of Callus Cultures

Callus initiation was noticed in media containing all the combination of BAP and NAA in a period of three weeks with the emergence of mild cellular growth on throughout the surface of the explants including both cut ends.

Maximum percentage of explants responded (90.02%) was in half strength MS media with 2.5 mg/l BAP and 1.0mg/l NAA (Plate 6) and obtained 0.732 g FW callus tissues. There was sign of callus formation on explants inoculated on media devoid of hormones. The callus tissues so induced on the explants were semi friable type. Callus initiation on explants maintained under complete dark was between 8 and 12 days and in light it was delayed to 14-16 days. Proliferation of callus tissues was independent of light and dark and no marked difference was noticed between the two conditions tested. Colour of the calli varied from light yellow to light green. The callus tissue raised on explants kept under light was green in color (Table 7).

Table 7: Effect of different combinations of BAP and NAA in half strength MS solid media on callus induction. Observations were made 30 days.

Hormone concentration (mg/l)		Percentage of response (%)	Fresh weight of calli (g)
BAP	NAA		
1.5	0.2	68.93	0.538 ± 0.021
2.0	0.5	75.52	0.588 ± 0.065
2.5	1.0	90.02	0.732 ± 0.038
3.0	1.5	86.05	0.541 ± 0.026
3.5	2.0	70.25	0.438 ±0.014

4.2.1.2. Initiation of Adventitious Roots from Callus Tissues

Callus tissues cultured in half strength MS solid media supplemented with IBA, NAA and GA_3 induced adventitious roots in 2 weeks (Plate 7). Among the hormonal combinations tested, the best combination for root induction was MS media with 1.5mg/l IBA, 1.0 mg/l NAA and 0.5 mg/l GA_3 (61 %) with length

Plate 4: Roots emerged in media with IBA and combinations of IBA and IAA. Observation made after 3 weeks.

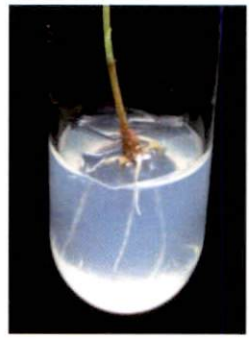


Plate 5: *In vitro* plant planted in plastic cups filled with sand and soil

Plate 6 : Callus initiation on nodal explants cultured in MS half strength media with 2.5 mg/L BAP and 1.0 mg/L NAA after 4 weeks

1.25cm (Table 8). Root length was also limited (1.25 ± 0.026). The roots so induced on callus tissues were thick and fleshy with many root hairs.

Table 8: Adventitious root formation on callus tissues cultured in MS solid media with different concentration of IBA, NAA and GA_3. Observation made after 4 weeks.

Hormone combination (mg/l)			Percentage of root induction (%)	Number of roots	Length of roots Cm
IBA	NAA	GA_3			
1.5	1.0	0.5	61 %	9.1± 0.026	1.25 ±0.026
2.5	1.0	0.5	42 %	6.2 ± 0.068	1.09 ± 0.013
3.0	1.0	0.5	38 %	5.6 ± 0.031	1.03 ± 0.035

4.2.1.3. Roots from aseptic shoots

Individual roots obtained in aseptic shoots cultured in MS solid media containing 0.1 mg/l IBA+ 0.1 mg/l IAA were also used for establishing isolated root cultures.

4.2.2. Establishment of Isolated Root Cultures

In order to establish root cultures, roots from different sources such as callus tissues and *in vitro*- derived aseptic shoots were inoculated onto half strength MS solid and liquid media with different concentration and combinations of auxins (IBA and IAA) and recorded the growth. Roots from callus tissues were fleshy, not lengthy as roots of aseptic shoot derived. Roots exhibited different responses in both solid and liquid media. Roots of callus–derived in solid media were profusely branched than roots of aseptic shoots and in media containing 0.3mg/l IBA showed maximum elongation. Elongation of roots in 0.1 mg/l IBA was low but had profuse lateral branching. Roots cultured in media containing 0.1 mg/l IBA and 0.1 mg/l IAA produced callus and the roots appeared were thick and fleshy. Maximum fresh weight of roots was obtained in media containing 0.1 mg/l

IBA after a period of 25 days (Plate 8) (Table 9). The fresh weight of roots was found decreasing in media containing high concentrations of IBA (> 0.1 mg/l).

Table 9: Isolated root cultures established in solid half strength MS media with different concentrations of IBA and combinations of IBA and IAA. Data taken after 25 days.

IBA (mg/l)	IAA (mg/l)	Fresh weight	Growth index
0.1	-	1.21 ± 0.026	3.84
0.2	-	0.98 ± 0.031	2.92
0.3	-	0.87 ± 0.009	2.48
0.1	0.1	0.76 ± 0.029	2.04
0.1	0.2	0.72 ± 0.052	1.88

Isolated root cultures transferred to half strength MS liquid media supplemented with different concentrations of IBA and combinations of IBA and IAA (Table 10). In liquid cultures the isolated roots started aggregating due to gyration and after a period of 5 days elongation of roots was noticed in media with 0.3 mg/l IBA. Profuse lateral branching of roots was found in media containing 0.1mg/l IBA and the roots entangled to become a puff like structure (Plate 10). It was also noticed that isolated roots reared in media containing IAA and IBA (0.1 mg/l IBA + 0.1 mg/l IAA) were produced callus tissues. Colour of the roots turned brown after a period of 20 days. Results of roots in both solid and liquid media showed that liquid media supplemented with 0.1 mg/l IBA found better and used for further studies (Table 10).

Plate 7: Roots emerged on callus tissues cultured in MS media with IBA, NAA and GA_3. Observations were made after 4 weeks.

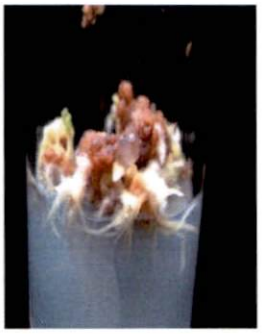


Plate 8: Isolated root cultures established in half strength MS solid medium supplemented with 0.1 mg/L IBA after 25 days

Plate 10: Isolated root culture established in 0.1 mg/L IBA supplemented solid half strength MS medium. Observations made after 25 days.

Table 10: Growth of roots in half strength MS liquid media with different concentrations of IBA and combinations of IBA and IAA. Observations were made after 25 days

IBA (mg/l)	IAA (mg/l)	Fresh weight (g)	Growth index
0.1	-	2.30 ± 0.021	2.05
0.2	-	2.01 ± 0.013	1.76
0.3	-	1.62 ±0.056	1.37
0.1	0.1	1.36 ± 0.016	1.11
0.1	0.2	1.19 ±0.020	0.94

4.2.3. Growth Curve Analysis of Root Cultures

Growth curve of the roots was studied in detail up to 40 days at an interval of 10 days. The root biomass started increasing exponentially after a lag phase of five days. The root biomass was maximum (2.009 ± 0.4904 g) on 20th day and recorded high growth index (Table 11). After a period of 20 days, the root growth found ceased by turning the colour into brown and reached stationary phase. After 20 days there was an abrupt decline of root growth. The established isolated roots were harvested and estimated the compound, 2-hydroxy 4-methoxy benzaldehyde.

Table 11: Growth curve of roots for a period of 40 days

Period (Days)	FW of roots (g)	Growth index
10	1.661± 0.1369	4.537
20	2.009 ± 0.4904	5.697
30	1.787 ± 0.1383	4.9566
40	1.732 ± 0.3620	4.7733

4.2.4. Fresh Weight and Dry Weight Determination

The average fresh weight of *in vitro* root was found to be 2.116 ± 0.112 and average dry weight was 0.536 ± 0.002g. Percentage of water content in *in vitro* roots was 74.669.

The established isolated roots were harvested and estimated the compound, 2-hydroxy 4-methoxy benzaldehyde..

4.2.5. Phytochemical analysis

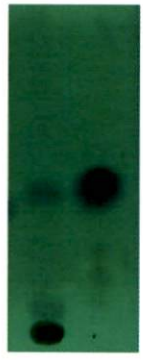
4.2.5.1.Extraction of 2-hydroxy 4-methoxy benzaldehyde

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 corresponding to the standard 2-hydroxy-4-methoxy benzaldehyde at the Rf value of 0.4 cm (Plate 11).

4.2.5.2. Confirmation of 2- hydroxy 4 – methoxy benzaldehyde using HPLC

Authenticity of 2- hydroxy 4-methoxy benzaldehyde was further confirmed by HPLC (Fig.1 & 2). The retention time was found to be 4 .1 for the

Plate 11: TLC plate loaded with different samples and standard. Bands obtained in samples identical to the band of authentic compound.



A B

A: Root extract in methanol obtained by soxhlet extraction.

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

authentic compound and a peak was obtained in the chromatogram of the sample at same retention time.

4.2.5.3. Quantification of 2- hydroxy 4 – methoxy benzaldehyde using Spectrophotometric Analysis

The petroleum ether extract of *in vitro* grown 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 20 day old adventitious roots grown in liquid MS media supplemented with 0.1 mg/l IBA produced 0.072% 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.

Fig 1. HPLC chromatogram of authentic compound

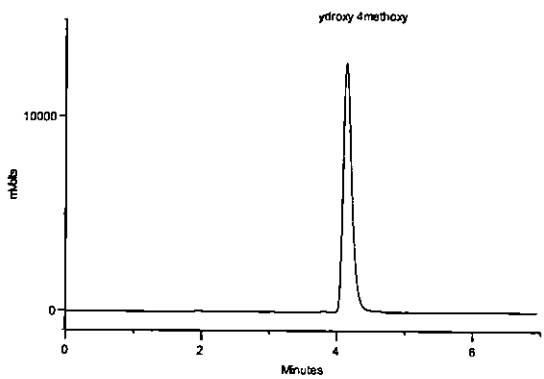
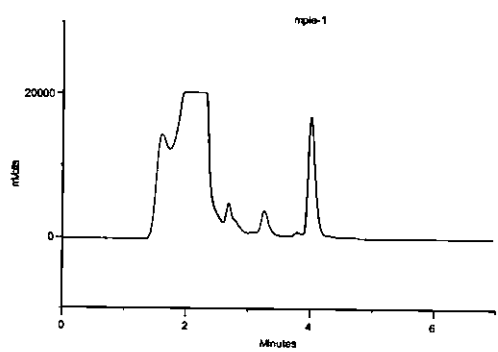


Fig 2: HPLC chromatogram of sample



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 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 *Hemidesmus indicus* and establishment of 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 *Hemidesmus* 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. 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 in 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 (Zryd, 1988). 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 surface sterilization of explants resulted in poor response and death of tissues 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 (Sreekumar *et al.*, 2000).

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 Kinetin are found to be effective for shoot bud induction and multiple shoot formation (Lundergan andJanick 1980, Rahaman and Blake 1988, Sen and Sharma 1991, Bhat *et at. 1995,* Benmoussa *et at.* 1996, Misra 1996) (Saryam et al., 2012).

Another important factor determining the morphogenesis is the physiological age of the explants (Ammirato, 1986). 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 (Rout and Das 1993, Raghuvanshi and

Srivastava 1995, Pattnaik and Chand 1996, 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 (George, 1996). 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 (Agarwal and Ghosh, 1985). 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 *Hemidesmus,* 2- hydroxy 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 than other callus or cell suspension cultures with respect to compound yield. In the present study induction of callus was possible in MS media supplemented with combination of BAP and NAA. Influence of light and dark conditions also played significant role in callus formation besides the influence of salt strength. Physical stature of the callus tissues was also subjective by the concentration of BAP. The results obtained in *H. indicus* are agreeing with many other reports already published by Chandramu *et al.*, (2003) and Siddique *et al.* (2002). Rao and Lee (1986) reported that intermediate level of auxin and cytokinin in the medium promoted callusing. According to Sreekumar *et al.*, (2000) auxins were more desirable than cytokinins for callus initiation and proliferation. NAA supplemented with BAP induced significant level of callusing is corroborated by the published works of others (Delfel and Rothfus 1977, Nikogosyan 1981, Han *et al.* 1993, Yan-Xiu 1993, Maljokeskitalo *et al.* 1995). NAA is preferred over 2, 4–D for obtaining pigmented and photosynthetically active callus in *Hemidesmus*

(Seeni and Gnanam 1981). 2.5 mg/L BAP and 1.00 mg/L NAA induced 90 percent of callus induction.

Many sources of adventitious roots are used for establishing isolated root cultures. Root induction on callus cultures was possible by transferring them to half strength medium supplemented with auxins and gibberellins. Root differentiation on callus tissues in presence of auxins and gibberellins was also reported in many other medicinal plants.

Rhizogenesis is a function of auxin activity (Kartha, 1975). Rhizogenic response was maximum by individual concentrations followed by substantive rooting in IBA and IAA and least in IAA and NAA this indicates the desirability of using IBA alone for root cultures (Sreekumar *et al.*, 2000). IBA induced long roots. Liquid media was more suitable for establishing root cultures than solid media. Basal medium promotes elongation of roots. There was a marked difference in root growth between solid and liquid cultures. Roots in liquid cultures fostered maximum growth though the roots became puff like structures due to continuous agitation. Shaking has profound influence on proliferation of roots in liquid media (Islam *et al.*, 2005). Agitation (80 rpm) promotes biomass production as the system gets increased rate of respiration and provides equal exposure to all components of the medium.

A growth curve of roots was sigmoid pattern that the root biomass was found to be increasing after an initial period of 5 days. After a lag phase, roots attained the exponential phase with maximum weight 2.009 ± 0.4904 on 20^{th} day. There after the root biomass was found to be decreasing constantly indicating that they might have attained the stationary phase.

TLC is used in identifying the presence of compounds in suitable solvent systems for many molecules including 2- hydroxy 4-methoxy benzaldehyde isiolated from the *in vitro*-derived roots. Petroleum ether: acetone (10:1) was the best solvent system for TLC. HPLC analysis also confirmed the presence of the compound. Further evidences for identification and purity of the compound can be obtained by FT-IR, GC – MASS and NMR spectral analysis.

Overall results obtained with the present investigation demonstrate the suitability of the species for mass propagation leading to field establishment. Besides, the data presented in the bioproduction studies using adventitious root cultures for the production of the bioactive compound, 2- hydroxy 4- methoxy benzaldehyde also necessitates further improvement of the system.

SUMMARY

6. SUMMARY

The study entitled "Establishment of *in vitro* 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 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 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 (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.

As part of bioproduction studies using adventitious roots, roots from different sources such as callus and roots from *in vitro* –derived aseptic shoots were used. Callus (0.732 g FW) was established using nodal explants in half strength MS media with 2.5 mg/L BAP and 1.0mg/L NAA. There was no sign of callus formation on explants inoculated on media devoid of hormones. The callus tissues so induced on the explants were semi friable type. Callus initiation on explants maintained under complete dark was between 8 and 12 days and in light it was delayed to 14-16 days.

Callus tissues cultured in half strength MS solid media supplemented with IBA, NAA and GA_3 induced adventitious roots in 2 weeks. Among the hormonal combinations tested, the best combination for root induction was MS media with 1.5mg/L IBA , 1.0 mg/L NAA and 0.5 mg/L GA_3 (61 %) with 1.25cm length. The roots so induced on callus tissues were thick and fleshy with many root hairs.

Similarly individual roots obtained from aseptic shoots cultured in MS solid media containing 0.1 mg/L IBA + 0.1 mg/L IAA were also used for establishing isolated root cultures.

In order to establish root cultures, roots from different sources were inoculated onto half strength MS solid and liquid media with different concentration and combinations of auxins (IBA and IAA) and recorded the growth. Roots exhibited different responses in both solid and liquid media. Roots of callus –derived in solid media were profusely branched than roots of aseptic shoots and in media containing 0.3mg/l IBA showed maximum elongation. Elongation of roots in 0.1 mg/L IBA was low but had profuse lateral branching. Roots cultured in media containing 0.1 mg/L IBA and 0.1 mg/L IAA produced callus and the roots appeared were thick and fleshy. Maximum fresh weight of roots was obtained in media containing 0.1 mg/L IBA after a period of 25 days. The roots established after 20 days under *in vitro* condition were harvested and extracted for 2-hydroxy 4-methoxy benzaldehyde and quantified. 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, adventitious root culture is a desirable option for the production of the bioactive molecule, 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, experiments on root cultures can enhance both biomass and compound production considerably for which further enhancement of the system using adventitious 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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APPENDIX 1

Composition of Murashige and Skoog Medium (1962) 1000 ml

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		

ESTABLISHMENT OF *IN VITRO* ROOT CULTURES OF SARSAPARILLA (*Hemidesmus indicus* L.) R. Br.

By

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(2009 - 09 - 103)

ABSTRACT

Submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE (INTEGRATED) IN BIOTECHNOLOGY

Faculty of Agriculture

Kerala Agricultural University

M.Sc. (INTEGRATED) BIOTECHNOLOGY COURSE

DEPARTMENT OF PLANT BIOTECHNOLOGY

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2014

8. ABSTRACT

The study entitled "Establishment of *in vitro* 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 L. R. Br., commonly known as Indian sarsaparilla, belonging to Asclepiadaceae family is one among the most widely used plants in the Indian system of medicine. Roots of the plant is rich in many medicinal compounds especially 2- hydroxy 4- methoxy benzaldehyde which is the main constituent of the root drug. The objective of the present study was to develop a tissue culture system and establishment of root culture for the production of 2-hydroxy 4- methoxy benzaldehyde.

In order to achieve the objectives, 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. 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 in 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 of length 3.29cm length.

As part of bioproduction studies using adventitious roots, roots from different sources such as callus and roots from *in vitro*–derived aseptic shoots were used. Callus (0.732 g FW) was established using nodal explants in half strength MS media with 2.5 mg/L BAP and 1.0mg/L NAA. Callus tissues cultured in half strength MS solid media supplemented with IBA, NAA and GA_3 induced adventitious roots in 2 weeks. Among the hormonal combinations tested, the best combination for root induction was MS media with 1.5mg/L IBA , 1.0 mg/L NAA and 0.5 mg/L GA_3 (61 %) with 1.25cm length. The roots so induced on callus tissues were thick and fleshy with many root hairs. Similarly individual roots obtained from aseptic shoots cultured in MS solid media containing 0.1 mg/L IBA + 0.1 mg/L IAA were also used for establishing isolated root cultures.

In order to establish root cultures, roots from different sources were inoculated onto half strength MS solid and liquid media with different concentration and combinations of auxins (IBA and IAA) and recorded the growth. Maximum fresh weight of roots was obtained in media containing 0.1 mg/L IBA after a period of 25 days. The roots so established after 20 days under *in vitro* condition were harvested and extracted for 2-hydroxy 4-methoxy benzaldehyde and quantified. 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, adventitious root culture is a desirable option for the production of the bioactive

molecule, 2-hydroxy 4-methoxy benzaldehyde. For further enhancement of the system using adventitious root cultures leading to the production of the compound, 2 -hydroxy 4-methoxy benzaldehyde requires more experiments, thereby a suitable system can be developed which would prevent harvesting of plants from the field.