IN VITRO PROPAGATION
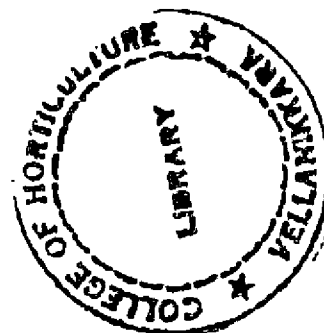# IN ASHOKA
(Saraca asoca (Roxb.*)* de Wilde*)*

By

BRINDHA DEVI I.

(2009 – 11 – 132)

THESIS

Submitted in partial fulfillment of the
requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT BREEDING AND GENETICS
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR-680 656
KERALA, INDIA

2011

DECLARATION

I hereby declare that this thesis entitled '*In vitro* propagation in *Saraca asoca* (Roxb) de Wilde' is a bonafide record of work 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.

Vellanikkara,

14.09.2011

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CERTIFICATE

Certified that this thesis entitled '*In vitro* propagation in *Saraca asoca* (Roxb.) de Wilde' is a record of work done independently by Mrs. Brindha devi, I. (2009-11-132) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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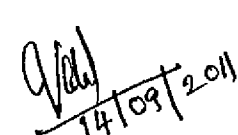
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LIST OF ABBREVIATIONS

Benzyl Amino Purine   BAP

Naphthalene Acetic Acid   NAA

2,4- Dichloro Phenoxy Acetic   - 2,4-D

Acid

Indole Acetic Acid   - IAA

Indole Butyric Acid   - IBA

Murashige and Skoog Medium   - MS

Schenk and Hildebrandt   - SH

Medium

Woody Plant Medium   - WPM

Introduction

1. INTRODUCTION

Asoka (*Saraca asoca*) is an important ayurvedic medicinal tree adapted to the agro climatic conditions prevailing in Kerala. Scientifically, asoka is known as *Saraca asoca* (Roxb.) de Wilde. It belongs to the family Leguminosae, sub family Caesalpiniaceae. Literally, the term asoka means 'remover of sorrow'. In Sanskrit it is known as asoka, in Hindi it is called asok. In Malayalam it is called asokam. In Tamil it is called asogam (Warrier *et al*,1996). It is believed that one who sits under the tree or who looks at the tree in full bloom, will forget their distress and grief and become happy and contended. Asoka is considered as a sacred tree of Hindus and Buddhists. Besides its medicinal properties, its decorative orange red flowers and evergreen beautiful foliage makes this tree favourable in gardens, national parks, roadsides, museums, temples and places for tourist attractions.

The tree is distributed throughout India up to an elevation of 750m except the northeastern part of the country. As a medicinal tree, the utility of asoka seems to have been mentioned first in 'Charaka Samhitha' as analgesic and antispasmodic. Other classical works, such as 'Susrutha Samhitha' and 'Ashtangahridaya' mention the therapeutical applications of asoka. During the Nighantu period, pharmacological properties as well as pharmacotherapeutics were indicated. In the medicinal and therapeutic texts, pharmaceutical forms made out of asoka and applications in single and poly herbal formulations have been mentioned (Karalam, 2007).

The bark of this tree is rich in tannins and phenols and is the primary medicinal part commercially used in ayurvedic preparations (Radhakrishnan *et al.*, 2007). Flowers, seeds, fruits and leaves are the other parts of tree used in medicinal preparations. Asoka is used not only to cure various diseases but also effectively utilized for its preventive, promotional and corrective properties (Govindapanicker, 1993).

The bark of asoka is useful in dyspepsia, fever, dypsia, burning sensation, colic ulcers and pimples. Its bark is refrigerant, astringent to bowel, anthelmintic, demulcent and emollient. Asoka bark is used in treatment of excessive menstruation as a uterine sedative. It has a stimulating effect on endometrium and ovarian tissue and is useful in menorrhagia. The well-known Ayurvedic preparations of bark are Asokarishtam and Asokaghrutham. Leaves are depurative and their juice mixed with cumin seeds is used for treating stomach ache. Ash of plant is good for external application in rheumatic arthritis. Flowers pounded and mixed with water are useful in haemorrhage dysentery. Dried flowers are used in diabetes. Flowers are used in the treatment of bleeding piles, scabies in children and other skin diseases. Seeds are reported to be used in the cure of excess urinary discharges. Seed extract is effective against dermatophytic fungi. The bark, flowers and fruits are prescribed in combination with other drugs for the treatment of snake bite and scorpion stings (Sivarajan and Balachandran, 1994).

Almost 70 tonnes of asoka bark is used annually by Ayurvedic industries in Kerala for preparing a variety of Ayurvedic medicines. The over exploitation of *Saraca asoca* has almost depleted its population from its natural habitats in India, particularly from the Western Ghats. International Union for conservation of Nature and Natural Resources (IUCN) has categorized this species as 'globally vulnerable' (Kurian, 2002). The requirements of Asoka bark is very high compared to other medicinal plants and also it is in short supply (Kala *et al.*,2006). The rarity has lead to substitution of the same with the bark of a few leguminous as well as non-leguminous trees. This affects the property and quality of the medicinal preparations. Due to its acute short supply compared to its demand, National Medicinal Plant Board has included *Saraca asoca* in the list of 32 medicinal plants identified and prioritized for development at the national level. Cultivation of asoka is a viable alternative to reduce the pressure on the resources in their natural habitats and also can ensure the quality and genuineness of the drugs.

The bioactivities of this plant are due to the large number of secondary metabolites present in the various parts of the plant. Conservation of this endangered species as well as taking up large scale new cultivation of elite genotypes rich in therapeutic content are of great importance. Conventional propagation is through seeds but seed viability is only for a short period and the seed propagated progeny need not reproduce plant with all therapeutic content as that of the mother plant.

Also the seeds get infested by insect attack, affecting its germination very seriously. Most of the *Saraca asoca* genotypes are naturally cross-pollinated through insects and are highly heterozygous. Hence seed propagation may not give true to type progeny. Clonal propagation is hence the best alternative to popularize identified elite genotypes as well as conservation of this endangered species. Hence, developing a simple and efficient method of propagation is the best alternative, for large scale multiplication and popularization of elite genotypes as well as ex-situ conservation of the species. With this view, the present study in asoka was undertaken for the standardization of suitable explants, surface sterilization procedures and culture establishment protocols along with induction of multiple shooting and rooting.

Plate 1 : Asoka tree with inflorescence
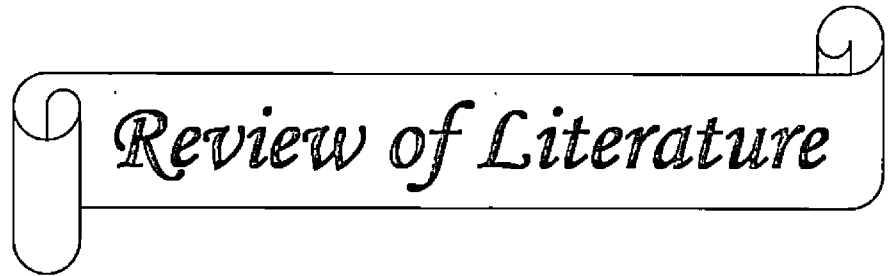
Plate 2 : Asoka bark pictures

Plate 3 : Inflorescence of Asoka

Review of Literature

2. REVIEW OF LITERATURE

Only limited literature is available on clonal propagation in *Saraca asoca*. Hence information available on other related tree species are also mentioned. The literature relevant for the conduct of the study on "*In vitro* propagation of *Saraca asoca* is reviewed here under the following titles.

2.1. BOTANY OF ASOKA

Saraca asoca is a small evergreen tree of Caesalpinaceae Family with several medicinal properties . It has numerous spreading and drooping glabrous branches. Its leaves are pinnate, 30-60cm long having 2-3 pairs of lanceolate leaflets. Flowers are orange or orange yellow in colour, arranged in dense corymbs and are very fragrant. Fruits are flat black pods, leathery and compressed with 4-8 seeds. Seeds are ellipsoid oblong and compressed. The bark is dark brown to grey or black with a warty surface. The thickness varies from 5mm to 10mm. The entire cut surface turns reddish on exposure to air. The taxonomic classification of *Saraca asoca* as suggested by Biswas and Debnath (1972) are given below.

Classification

Kingdom : Plantae
Divison : Magnoliophyta
Class   : Mgnoliopsida
Order   : Fabales
Family   : Caesalpinaceae
Genus   : Saraca
Species : Asoca

2.2. BIOCHEMICAL CONSTITUENTS IN ASOKA

Saraca asoca is reported to contain glycoside, flavanoids, tannins and saponins. The Phytochemical study shows that in the bark of plant presence of epicatechin, procyanidinp2,11'-deoxyprocyanidin B, (+) catechin, leucopelargonidin and leucocyanidin. The flower part of plant contain Oleic, linoleic palmitic and stearic acids, P-sitosterol, quercetin, kaempferol- 3-0-P-D-glucoside, quercetin-3-0-P-D-glucoside, apigenin-7-0-p-D-glucoside, pelargonidin-3, 5- diglucoside, cyanidin-3, 5-diglucoside, palmitic, stearic, linolenic, linoleic, p and y sitosterols, leucocyanidin and gallicacid. Seed and Pod contains oleic, linoleic, palmitic and stearic acids, catechol, (-) epicatechol and leucocyanidin . Five lignan glycosides, lyoniside, nudiposide, 5-methoxy-9-β-xylopyranosyl-(−)-isolariciresinol, icariside E3, and schizandriside, and three flavonoids, (−)-epicatechin, epiafzelechin-(4β→8)-epicatechin and procyanidin B2, together with β-sitosterol glucoside, were isolated from dried bark (Pradhan et al., 2009).

Flowers give □-sitosterol, flavonoids and flavone glycosides-quercetin, kaempferol-3-O-□-D-glucoside, quercetin-3-O-□-D-glucoside. The anthocyanins present are pelargonidin-3, 5-diglucoside and cyanadin-3, 5-diglucoside. Bark yields catechol and sterols-(24□)-24-cholest-5-en-3□-ol, a wax containing n-alkanes, esters and free primary alcohols. Alcoholic extract and glycoside P2 from stem bark is oxytoxic. Aerial part is Central Nerve System (CNS) active, hypothermic, CNS depressant and diuretic. Stem bark is anticancerous, has spasmodic action on rabbit intestine and cardiotonic action in frog and dog. Seed of asoka has antifungal properties. Stem bark is astringent, antileucorrhoeic, antibilious and uterine sedative. Flower is uterine tonic, antidiabetic and antisyphilitic. Stem bark and flower is antibilious (Husain et al, 1992).

2.3. THERAPEUTIC USES OF ASOKA

Saraca asoca possess wide ranging biological activities. Almost all parts of the tree possess medicinal properties. These multiple

bioactivities of the plant is due to the large number of secondary metabolites present in various parts of the plant. Hence it is a pre-requisite to have an investigative study of the secondary metabolites in *Saraca asoca*. (Sivarajan and Balachandran, 1994). Asoka is one of the most reputed medicinal plants applied not only to cure various diseases but also effectively utilized for its preventive, promotional and corrective properties. The bark rich in tannins and phenols is the primary medicinal part commercially used in ayurvedic preparations. Flowers, seeds, fruits, leaves are the other parts of tree used in medicinal preparations.

Asoka bark is used as an astringent in treatment of excessive menstruation as a uterine sedative. The plant is used to treat depression in women. It has a stimulating effect on endometrium and ovarian tissue and is useful in menorrhagia due to uterine fibroids, in leucorrhoea and internal bleeding hemorrhoids and hemorrhagic dysentery. It is diuretic, tonic, cooling, aphrodisiac. Ash of plant is good for external application in rheumatic arthritis. Extract of bark has anticancerous property. Bark is refrigerant, astringent to bowel, anthelmintic, demulcent, emollient. The bark of asoka is useful in dyspepsia, fever, dypsia, burning sensation, visceromegaly, colic ulcers, metropathy, leucorrhoea and pimples. Leaves are depurative and their juice mixed with cumin seeds is used for treating stomach ache. Flowers pounded and mixed with water are useful in haemorrhage dysentery. Dried flowers are used in diabetes. Flowers are used in the treatment of bleeding piles, scabies in children and other skin diseases (Sivarajan and Balachandran, 1994). Seeds are reported to be useful in reducing urinary discharges. Seed extract effective against dermatophytic fungi. Seeds are used for treating bone fractures, strangury and vesical calculi.

Dried fruits used in the cases of Spermatorrhoea, phosphaturia, diseases of genito-urinary tract such as dysuria, gonorrhoea, chronic cystitis, calculous affections, urinary disorders, incontinence of urine, gout and impotence. The bark, flowers and fruits are prescribed in combination with other drugs for the treatment

of snake bite and scorpion stings. Flowers of Asoka recorded higher tocopherol (vitamin A) content which characterizes its commercial utility.

2.4. PROPAGATION IN ASOKA

The plant is amenable to both sexual as well as asexual methods of propagation. It is vegetatively propagated by stem cuttings and air layering. It is conventionally propagated through seeds.

2.4.1. Seed propagation

Seeds are formed usually during February-April. Seeds are collected when they are ripen and are sown after soaking in water for 12 hours on the prepared beds. Seeds germinate within 20 days but Seed viability is only for a short period. Also the Seeds get infested by insect attack, and damping off disease hence affecting its germination very seriously. Most of the *Saraca asoca* genotypes are naturally cross-pollinated through insects and highly heterozygous. Hence seed propagation will not give true to type progeny. The seedlings shrink and die off within a few weeks of emergence. This makes the seed propagation of the species a difficult process (Pradhan *et al.*, 2009). Warrier *et al.* (2007) reported that the recalcitrant behavior of the seeds of the species results in loss of viability within short period.

Seeds of *thathiri* germinate within seven to twelve days of sowing but lose their viability within six months (Bhagat *et al.,* 1992). Bahuguna *et al.* (1988) reported that the seedlings of *thathiri* are highly susceptible to damping off and hence difficult to raise.

2.4.2. Vegetative propagation

The plant can be propagated by vegetative means by rooting of stem cuttings and air layering. Vegetative or Clonal multiplication through stem or root

cuttings is not easy in this species. Hard wood cuttings were tried from different selections in different IBA concentrations and potting mixtures. Zero percent rooting was observed (Pradhan *et al,* 2009). However, Sasidharan and Pathrose (2007) standardized the vegetative propagation through stem cuttings and air layering.

Bahuguna *et al.* (1988) attempted the raising of stem cuttings of *thathiri* dipped in different concentrations of growth regulators including IAA, IBA and NAA and reported the best response for the treatment of the cuttings with 200 ppm IBA for 24 hours. Rajesh *et al.* (1993) found that shoot cuttings of *Woodfordia fruticosa* failed to root effectively without added growth hormones and they reported that prolonged dipping in NAA 100ppm gave the best result. Raju *et al.* (1994) has reported that *Woodfordia fruticosa* is a hard to root species irrespective of seasons and hormonal treatments. Similarly in the case of *Lagerstroemia flos-reginae,* vegetative propagation is difficult (Paily and D'Souza, 1986).

2.4.3. *In vitro* propagation

Very few attempts have been made to propagate *saraca asoca* species through *in vitro* techniques. Ramasubbu *et al.* (2008) worked on the *in vitro* propagation in *Saraca asoca* (Roxb.) De Wilde using shoot tip , nodal and internodal explants cultured in MS medium supplemented with different concentrations (0.5-2mg/l) of Benzyl aminopurine (BAP), Kinetin (Kn), 2,4-D. The highest frequency of shoot organogenesis (82%) was found in nodal explant treated with 0.5mg/l of BAP and callus were formed more on 2,4-D. The microshoots rooted very well in MS medium supplemented with 4mg/l of IBA.

2.4.3.1. *In vitro propagation in other crops*

Devi *et al.* (2007) attempted an efficient tissue culture protocol for *in vitro* propagation, conservation of *Saraca asoca* and as an alternative source for medicinal compounds.

Tiwari *et al.* (2002) developed an improved micro propagation protocol for teak (*Tectona grandis*). Here the Nodal explants placed on MS medium supplemented with 22.2 µM benzylaminopurine and then were serially transferred to fresh medium after 12, 24, 48 and 72 h gave maximum culture establishment (76.8%). Establishment was reduced when explants were retained in the initial culture medium longer than 12 h. Explant on MS medium supplemented with 22.2 µM benzylaminopurine and 0.57 µM indole-3-acetic acid resulted in the maximum average number of shoots.

The morphogenetic potential of shoot tip explants of black pepper (*Piper nigrum*) was investigated and an effective multiple-shoot propagation method is described by Philip *et al.* (2002). The best establishment and proliferation of shoot tip explants was obtained on MS medium containing 1.5 mg l^{-1} BAP alone.

Tsai and Kinsella (2000) conducted an experiment involving callus culture from immature cotyledons of cacao in Gamborg B5 agar medium containing 1.0ppm of 2,4-D and 0.2 ppm of Kinetin and the suspension cultures of cacao bean were established from the calli and grown in a modified Murashige and Skoog medium containing 5ppm of 2,4-D and 0.1ppm Kinetin.

Krishnan and Seeni (1994) attempted *in vitro* shoot tip cultures of *Woodfordia fruticosa* (L.) Kurz in SH medium supplemented with BAP 0.2 mg per litre. They obtained regenerated plantlets with uniform morphological growth and flower characteristics.

In vitro clonal propagation of a neem tree through Somatic embryogenesis was attempted by (Shrikhande *et al.*,1993).

Several workers have attempted *in vitro* propagation in different species of *Lagerstroemia*. They include Ho and Lee (1985) in *L. speciosa*, Paily and D'Souza (1986) in *L. flos-reginae*, Yamamoto *et al.* (1994) and Quraishi *et al.* (1997) in *L. parviflora*, Eymar *et al.*, (2000) in *L. indica*, Sumana and Kaveriappa (2000) in *L. reginae*, Zobayed (2000) in *L. speciosa* and *L. thorellii* and

Grigoriadou *et al.* (2003) in *L. indica.* Nodal segments were used as explants in all the species and uniform plantlets were obtained when they were cultured on MS medium supplemented with hormones.

2.4.4. General Aspects of Tissue Culture

Gottlieb Haberlandt of Berlin (1902) reported that isolated cells under proper environment and nutrition are capable of regenerating into entire plants. Schleiden (1838) and Schwann (1839) postulated the cell theory, which revealed that plant cells are totipotent in nature. This forms the basis for plant cell, tissue and organ culture. Skoog and Miller (1957) made a landmark in the history of plant tissue culture with their discovery of auxins and cytokinins . They put forth the concept of hormonal control of organ formation and showed that root and shoot differentiation was a function of auxin-cytokinin ratio and that it could be regulated by altering the relative concentrations of these growth regulators in the medium. Many pioneer investigators like White (1934), Gautheret (1939), Nobecourt (1939), Miller *et al.* (1956), Reinert (1958), Steward *et al.* (1958), Bergmann (1960) and Vasil and Hildebrandt (1965) have contributed for the successful development of plant tissue culture concepts. Murashige and Skoog (1962) developed a completely defined nutrient medium for plant tissue culture .

Several aspects of plant tissue culture are being applied in agriculture, which include the production of haploid plants, secondary metabolite production, embryo rescue techniques etc. However, the best commercial application of tissue culture is the production of true to type plants at a very rapid rate compared to the conventional methods (Levy, 1981). Murashige (1974) found out the possibility of three routes of *in vitro* propagule production, which included enhanced release of axillary buds, production of adventitious shoots through organogenesis and somatic embryogenesis. Vasil and Vasil (1980) reported that the tissue culture derived plantlets grow faster and mature earlier than seed propagated plants.

2.4.4.1. *Factors influencing success of in vitro propagation*

Success of *in vitro* propagation depends on several factors directly and indirectly. These factors include age of the explant, size, genotype and type of explant, surface sterilisation, presence of systemic microbial contamination, presence or absence of other additives, nitrogen source and concentrations, physical conditions of the medium, pH, quality and intensity of light, temperature and relative humidity (Brown and Thorpe, 1986).

2.4.4.2. *Age of the explant* .

A tree is more amenable to vegetative propagation during its juvenile stage. Generally, the more juvenile the specimen, the easier it is to propagate by vegetative means. In most plants, there is no clearly defined transition from the juvenile to the mature phase. Often some parts of the tree may be mature or senescent, while other portions still display juvenile characters (Bonga, 1982). When the plant matures, the meristematic apices, the centres of growth and organization in the plants undergo changes. Hence the tissues from young and old parts of the plant show differences in their behaviour in culture conditions (Bonga, 1980). Bonga (1982) reported that in a recalcitrant *in vitro* culture from which true to type vegetative propagation is not possible, it is wise to develop materials from highly juvenile material.

Dormant vegetative buds present at the root-shoot junction can be induced to grow out and such tissues are juvenile in nature and the cells show low mitotic rate and low number of ribosomes (Bronchart and Nougarede, 1970). Peterson (1975) reported that the shoot buds were formed naturally near the apex of roots of some species and in root cultures of others. *In vitro* culture studies of the juvenile sprouts have resulted in cloned propagation of tree species like *Sequoia* (Ball, 1978). The low mitotic rate and low number of ribosomes may be significant in relation to the morphogenetic capacity of the tissue (Bonga, 1980).

Several workers have discussed the slow growth, low propagation rate and weak *in vitro* performance of mature explants as compared to juvenile shoots. Some of them are Paily and D'Souza (1986) in *Lagerstroemia flos-reginae;* Maarri *et al.* (1986) in pear; Messeguer and Mele (1987) and Rodriguez *et al.* (1988) in *Corylus avellana*; Rajmohan and Kumaran (1988) in jackfruit; Quraishi *et al.* (1997) in *Lagerstroemia parviflora*; Sumana and Kaveriappa (2000) in *Lagerstroemia reginae* .and Karale *et al.* (2005) in *Emblica officinalis.* In maple, the transfer of rooted plantlets into soil is more critical in the micro propagation process of mature trees than with plantlets regenerated from seedlings (Hanus and Rohr, 1987).

2.4.4..3 *Season for collecting explants*

The success of plant tissue culture is influenced to a large extent by the season of explant collection. Spring season (March-April) is the best to initiate tissue culture from mature trees. In *Corylus avellana,* Messeguer and Mele (1987) noted that at least 95 per cent aseptic shoot cultures were obtained and buds flushed within 10 to 12 days in spring season as compared to five to six weeks during other seasons. In chestnut, shoot explants taken during mid May gave rise to plantlets successfully (Chauvin and Salesses, 1988). Yu (1991) reported that in Litchi (*Litchi chinensis*), the explant collected after ten continuous rainy days was cent per cent contaminated and that taken after fifteen continuous sunny days had a contamination rate of only 20 per cent. Several workers like Yang (1992), Thakar and Bhargava ,1999 reported that in the medicinal tree *Gmelina arborea*, the axillary buds collected during summer responded better to *in vitro* culture than in winter. Mahale *et al.* (2005) reported that in the case of tamarind, bud break in *in vitro* culture was noticed when the explants were collected during April to June. In eagle wood, Nazeem *et al.* (2005) reported that nodal segments collected during March- April from current season shoots responded better to *in vitro* culture.

2.4.4.4. *Genotype*

Response to *in vitro* culture varies between plant species even within a single species, it may vary with varieties. Pierik and Steegman (1976) reported genotype specific influences for *Anthurium andreanum* and *Anthurium schezerianum*. Under *in vitro* culture, explants from three cultivars of *Begonia* x *Heimalis* differed in their survival rates (Welander, 1978). Influence of genotype on the callusogenesis of hypocotyl explants of *Cuphea wrightii* and *Cuphea procumbens* have been reported by Truta *et al.* (2002). However, Mallika *et al.* (1997) observed in nutmeg that there is no genotypic influence on the *in vitro* shoot initiation.

2.4.4.5. *Explant size and its position on the mother plant*

Skirvin (1980) pointed out that there is variation among the type of explant within each plant species and the most suitable explant for each plant species should be determined. Ho and Lee (1985) reported that in *Lagerstroemia speciosa*, nodal segments were superior to shoot tips when cultured artificially. Norton and Norton (1986) studied the effects of explant length (2.5 to 20.0 mm), axillary bud number (0 to 6), presence or absence of apical dome and explant retrieval (from top, middle or base of plant canopy) in the case of *Prunus* and *Spirea*. The number of shoots formed after four weeks increased with the explant length and decreased with the number of buds present. Explants taken from the top of the canopy produced more shoots, but the removal of the apex did not affect the shoot number. Rahman and Blake (1988) observed in jack that nodal explants gave more proliferation than shoot tips. Krishnan and Seeni (1994) observed that in *Woodfordia fruticosa,* shoot tips of size 0.8cm were the best explants for multiple shoot induction. In rose, complete organogenesis was observed when leaf callus was cultured whereas stem callus failed to differentiate under the same conditions (Arif and Khatamian, 1996). Mallika *et al.* (1997) reported in nutmeg that nodal explants produced more proliferation in the culture tubes. In cashew,

Keshavachandran (1998) reported that explants from *in vitro* grown seedlings responded better than explants from nursery grown seedlings. Zobayed (2000) recorded that single noded explants with two unfolded leaves of *Lagerstroemia speciosa* and *Lagerstroemia thorelli* responded to *in vitro* culture by shoot multiplication and plantlet regeneration. In *Citrus aurantifolia*, kamble *et al.* (2005) reported that cotyledonary segment was the best explants when compared to root tip, hypocotyls stem, leaf, epicotyl stem and shoot tip of *in vitro* grown seedlings. In peach, shoot tips are superior in shoot induction compared to nodal explants (Sharma *et al.*, 2005). Panimalar *et al.* (2005) observed that the best explant in *Centella asiatica* was nodal segments.

2.4.4.6. *Surface Sterilisation*

The explants especially those collected from the field grown conditions, may harbour a lot of microorganisms. When such explants are inoculated into a nutrient medium, it will contaminate the whole system. Sodium hypochlorite (0.5 to 2.0 per cent w/v), calcium hypochlorite (filtered 5.0 to 10.0 per cent w/v) and mercuric chloride (0.05 to 0.1 per cent w/v) are the commonly used surface sterilants. Ramasubbu *et al.* (2008) reported the surface sterilization of *Saraca asoca* explants with 1% Bavistin solution and 400ppm Chloramphenicol on a rotary shaker and thoroughly washed with sterile distilled water for 5 minutes. A final treatment was given with 0.1% $HgCl_2$ (w/v) for 5 minutes and again washed with sterile distilled water. In *Woodfordia fruticosa*, the explants were surface sterilised by treating with 0.1 per cent $HgCl_2$ for 5 minutes (Krishnan and Seeni, 1994). They also reported that period of surface sterilisation exceeding 5 minutes was lethal to the explants. Hu and Wang (1983) suggested magnetic stirring, ultra sonic vibration or keeping the soaked explants under vacuum to reduce the possibility of trapping air bubbles on the explant surface. Generally, a drop of detergent is added to the surface sterilant to reduce the surface tension and to increase the wetability. Surface sterilization is done to remove all the

microorganisms present on the explant with minimum damage to the plant parts. Bonga (1982) advocated the use of alcohol alone or in combination with other chemicals for disinfection.

2.4.4.7. *Presence of systemic contaminants*

Mathias and Anderson (1987) reported that bacteria, fungi or viruses on the surface of the bark, glandular hairs at the nodes and internal tissues could cause contamination in the explants. Woody plant tissue culture has a serious problem associated with it as it harbours a number of microorganisms within its tissues internally which causes latent contamination. Surface sterilisation removes only those microorganisms that are present on the outer surface. According to Mallika *et al.* (1992), growing stock plants under controlled conditions and regularly spraying the plants with systemic and contact fungicides can reduce or avoid the problem of contamination to some extent. Dodds and Roberts (1985) reported that the use of antibiotics for sterilisation should be avoided as they metabolise the plant tissues with unpredictable results

2.4.4.8. *Culture medium*

Selection of culture medium depends on the plant species and purpose of culturing. A wide variety of media have been reported by many researchers. The earliest and widely used basal media proposed were by White (1934) and Heller (1953). Since 1980, most researchers have been using MS (Murashige and Skoog, 1962) medium. Other derivatives of MS medium include the B5 medium developed by Gamborg *et al.* (1968), SH (Schenk and Hildebrandt, 1972) medium and the woody plant medium (WPM) developed by Lloyd and McCown (1980). The MS medium is characterised by high concentration of mineral salts. Skirvin (1980) and Griffins *et al.*, (1981) suggested that reducing the strength of MS medium by half was more beneficial for culturing.

Response of an explant to different media depends on the plant species. Das (1992) reported that rhizome explants of *Agave sisalana* responded better in SH medium when compared to MS medium. Krishnan and Seeni (1994) observed that shoot tip explants from *Woodfordia fruticosa* in the SH medium established well in *in vitro* condition. In the case of *Adhatoda beddomei*, Sudha and Seeni (1994) found that callus free proliferation of the stem node explants was obtained when they were cultured in SH medium. Ramasubbu and *et al* (2008) reported that *Saraca asoca* in the MS medium established well in *in vitro* condition.

2.4.4.9. *Exudations from the explant*

Lethal browning of the explant and exudations in the culture medium, hinders the establishment of *in vitro* cultures of several plant species, especially woody plants. During the course of *in vitro* growth and development, plant tissues not only deplete the nutrients that are furnished in the medium, but also release substances that can accumulate in the cultures. According to Zaid (1987), these substances called as phenols, have profound influence on the physiological effects of the cultured tissues. Polyphenols can be oxidised by peroxidases (Mayer and Harel, 1979) or polyphenol oxidases (Mayer and Harel, 1979 and Hu and Wang, 1983). Oxidation products of the polyphenols are known to be highly reactive and inhibit enzyme activity leading to the death of the explant (Hu and Wang, 1983).

Polyphenol interference in culture establishment of different woody plant species was reported by several workers. (Anderson, 1975; Lenartowicz and Millikan, 1977; Lloyd and McCown, 1980; Baleriola and Mullins, 1983; Zaid, 1987 and Amin and Jaiswal, 1988). Krishnan and Seeni (1994) observed the browning of the medium due to polyphenol exudation from different explants of *Woodfordia*. Mathew *et al.* (1987) and Mathew (1995) have reported polyphenol interference in clove. In *Lagerstroemia parviflora*, exudations from the culture continued upto the rooting stage (Quraishi *et al.*, 1997).

2.4.4.10 *Methods to overcome polyphenol interference under in vitro conditions*

Endogenous polyphenol concentration in the plant material is reduced by etiolation of branches (Ballester *et al.*, 1989) or growing the stock plants in dark (Marks and Simpson, 1990) or cold storage of the cuttings (Dalal *et al.*, 1992). The degree of wounding during explant preparation can greatly affect the amount of exudate produced during its establishment. Unwounded tissues like whole leaves or embryos do not exude phenol and they grow well in the culture medium (Reuveni and Kipinis, 1974). Lesser wounding or cutting with a sharp blade reduces the phenol exudation from the explants (Ripley and Preece, 1986).

Pretreatments like soaking explants in water (Gupta *et al.*, 1980) or in antioxidant solutions like ascorbic acid or citric acid (Gupta *et al.*, 1980; Zaid and Tisserat, 1983) or in adsorbants like 0.7 per cent polyvinyl pyrolidone (Gupta *et al.*, 1980) or in a solution of antioxidant and sucrose (Gupta *et al.*, 1981) reduce polyphenol exudation and consequent oxidation. In apple, Baleriola and Mullins (1983) treated the explants with Ca^{2+} ions to prevent the leakage of polyphenols into the medium. Keeping explants under running tap water or agitation of the explants with 0.5 per cent polyvinyl pyrolidone for 30 to 45 minutes reduced phenolic exudation in guava *in vitro* culture (Amin and Jaiswal, 1988). In *Dioscorea alata* L., the cut end of the explant was sealed with paraffin wax to prevent the exudation (Bhat and Chandel, 1991). Bhat and Chandel (1991) also reported in banana that the exudation could be prevented to an extent when the exposed ends were sealed. Suspending the explants in a solution of $25\mu M$ polyvinyl pyrolidone and 522.5 μM citric acid before culturing could check the polyphenol exudation in *Lagerstroemia parviflora* (Quraishi *et al.*, 1997).

Anderson (1975) and Chevre *et al.* (1983) reported that reducing the salt concentration of the basal medium was an effective method for reducing polyphenol exudation. But Hildebrandt and Harney (1988) observed that increasing the salt concentration of the medium had little effect on the amount of polyphenols released, until it reached five times that of normal MS medium.

Ichihashi and Kako (1977) reported that the browning of *Cattleya* shoot tip was most effectively controlled by the use of antioxidants into stationary liquid medium. However the same antioxidants were not effective when incorporated into semi solid medium. Many workers proved that the incorporation of antioxidants into the culture medium effectively controlled polyphenol interference in different crop plants (Ichihashi and Kako, 1977; Monaco *et al.,* 1977; Hildebrandt and Harney, 1988 and Quraishi *et al.,* 1997). Activated charcoal has the ability to adsorb toxic metabolites released into the culture medium (Fridborg and Erikson, 1975). Several workers like Bajaj (1978), Stevenson and Harris (1980) and Zaid and Tisserat (1983) have reported that addition of 1.0 to 2.0 per cent activated charcoal or 0.5 to 1.0 per cent polyvinyl pyrolidone in the culture medium prevented the accumulation of polyphenols in the culture medium and the subsequent browning of the explants. The adsorbants along with the phenols adsorb hormonal substances also. Hence the inclusion of charcoal reduces the availability of growth hormones and there is need for providing an abnormally high concentration of auxins in the culture medium (Tisserat, 1979 and Zaid and Tisserat, 1983).

Anderson (1975) observed that the green portion of the rhododendron shoot tips had to be transferred to a fresh medium every three weeks to be kept alive. Similar result was reported by Broome and Zimmerman (1978) in blackberry. In *Woodfordia fruticosa,* Krishnan and Seeni (1994) observed that the browning of the medium due to polyphenol oxidation was reduced to a minimum after three successive transfers into the same media each at three days interval.

Plant growth regulators play a major role in darkening the medium by oxidising the phenols. Bergmann (1964) and Asahira and Nitsch (1969) reported that the synthesis of polyphenols is stimulated by cytokinins. Increased secretion of polyphenols was also observed with the application of NAA (Zagoskina and Zaprometov, 1979) or abscissic acid (Bagratishvity *et al.,* 1984). In walnut, Rodriguez (1982) found that the callus induced with 2,4-D and kinetin

was incapable of organ formation because of the increased production of polyphenols.

Rabechault *et al.* (1976) suggested that addition of 1.0 per cent sucrose in the culture medium decreased the browning. Amorium *et al.* (1977) reported that the increased concentration of exogenous glucose level increased the phenol synthesis of rose cells.

Reduction of light intensity was found to reduce exudation of polyphenols effectively (Forrest, 1969; Hu and Wang, 1983 and Ziv and Halevy, 1983). Activity of enzymes concerned with both biosynthesis and oxidation of polyphenols was increased by light (Davis, 1972). Hildebrandt and Harney (1988) reported that the release of polyphenols was less at 7° C than at 27°C in *Pelargonium* x *Hortum*.

2.4.4.11. *Plant growth regulators*

For a successful plant tissue culture, Krikorian (1982) suggested that the selection and addition of growth regulators at the optimum level is one of the crucial factors. Commonly used growth regulators in tissue culture include four groups such as auxins, cytokinins, gibberellins and retardants like abscissic acid. Murashige (1974) utilised cytokinins to overcome the apical dominance of shoot to enhance the branching of lateral buds from leaf axils. Murashige (1974) reported that BAP is the most effective cytokinin for meristem, shoot tip and bud culture followed by kinetin. Lo *et al.* (1980) reported that a high content of cytokinin was deleterious to the initiation and elongation of roots of both monocotyledonous and dicotyledonous plants. In *Lagerstroemia* during micro propagation, the best growth regulator for multiple shoot induction was found to be BAP (Ho and Lee, 1985 and Paily and D'Souza, 1986). Krishnan and Seeni (1994) reported that the induction and proliferation of multiple shoots in the *in vitro* culture of *Woodfordia fruticosa* depended solely on the presence of BAP in the medium. However, in *Agave*

sisalana, Hazra *et al.* (2001) found that kinetin was the most effective plant growth regulator in inducing multiple shoots.

Exogenous auxin was not always needed for axillary bud proliferation. A high concentration of auxin induces callus formation (Hasegawa,1980) Lundergan and Janick (1980) advocated that one of the possible roles of auxin at elongation stage is to nullify the suppressive effect of high cytokinin concentration thereby restoring normal shoot growth. Although exogenous auxins do not promote axillary shoot proliferation, culture growth has been improved by its presence (Wang and Hu, 1980). Hu and Wang (1983) described the young shoot apex as an effective site of auxin biosynthesis. Auxins are used for the rooting of *in vitro* produced shoots.

For successful organogenesis in some cultures, a combination of auxins and cytokinins are used. Lee *et al.* (1987) found that a combination of BAP and NAA produced the maximum number of multiple shoots in the culture of *Lagerstroemia indica* f. *alba*. Plant regeneration in the stem and leaf derived callus of *Cuphea ericoides* was obtained in a media containing both auxins and cytokinins (Rita and Floh, 1995). In *Lagerstroemia reginae,* Sumana and Kaveriappa (2000) found that the best results in plant regeneration were obtained with a combination of auxins and cytokinins.

Different workers used auxins like IBA and IAA in different concentrations for successful *in vitro* rooting (Ho and Lee, 1985; Paily and D'Souza, 1986; Yamamoto *et al.*, 1994 in *Lagerstroemia;* Krishnan and Seeni, 1994 in *Woodfordia fruticosa;* Sudha and Seeni, 1994 in *Adhatoda beddomei;* Rita and Floh, 1995 in *Cuphea ericoides;* Quraishi *et al.*, 1997 in *Lagerstroemia;* Purohit and Singhvi, 1998 and Purohit *et al.*, 1999 in *Achras sapota* and Rout *et al.*, 2001 in *Lawsonia inermis*).

2.4.4.12. *Carbon and energy sources*

Sucrose is the most commonly used carbon energy source for plant tissue culture. Most of the workers have used 20 to 30g/l sucrose in the medium. Glucose and fructose may be substituted in some cases, but most often sugars are reported to be poor sources (George and Sherrington, 1984). In apricot, Marino *et al.* (1991) reported that shoot proliferation rate was increased with sorbitol as the carbon source than with sucrose. Nair and Gupta (2003) found that the sucrose concentration of the medium was crucial for the induction of somatic embryos in black pepper and 30g/l was the optimum quantity for somatic embryogenesis.

2.4.4.13. *Other organic compounds*

Vanoverbeek *et al.* (1941) reported that in *Datura* embryos, coconut water promoted the growth and differentiation. Adenine sulphate can enhance growth and shoot formation in many plants (Skoog and Tsui, 1948). In *Cinchona ledgeriana,* Hunter (1979) found that the addition of phloroglucinol to the medium promoted the culture growth. Conger (1981) reported the role of complex organic compounds like casein hydrolysate, coconut water, yeast, malt extract and fruit and vegetable juice for successful growth of tissues and organs. Role of these complex organic compounds is usually unpredictable and repeatability is also very poor, therefore it has been recommended to avoid their use as far as possible (Gamborg and Shyluk, 1981).

2.4.4.14. *Culture environment*

The physical form of the medium, whether liquid or semi solid, pH, other environmental factors like light, temperature, relative humidity and season of culture play an important role in the *in vitro* growth and differentiation.

Light requirement for differentiation involves a combination of several components namely intensity, quality and duration (Murashige, 1974). Broderick *et al.* (1974) reported that in onion and tomato, callus cultures were

formed and maintained in darkness at 23-27° C. According to Murashige (1977) the optimum day light period required is 16 hours for a wide range of plants. During *in vitro* culture, the environmental temperature of the species at the original habitat should be taken into consideration (Yeoman, 1986).

Relative humidity is rarely a problem except in arid climate where rapid drying occurs. Hu and Wang (1983) reported that air humidity is infrequently controlled and when it is controlled, 70 per cent has been found to be the most frequent setting. In *Lagerstroemia in vitro* cultures, the relative humidity in the vessels under forced ventilation was lesser compared to the sealed vessels (Zobayed, 2000).

2.4.4.15. *Rooting of in vitro produced shoots*

In vitro produced shoots can be rooted either through *in vitro* methods itself or through *ex vitro* methods. There are three phases involved in rhizogenesis; namely induction, initiation and elongation. All cytokinins inhibit rooting and auxins favour induction of rooting. Ancora *et al.* (1981) reported that among the different auxins, NAA is the most effective for root induction. The concentration of hormone required is often critical to provide sufficient stimulus to initiate roots while preventing the excessive formation of the callus (Yeoman, 1986). In *Woodfordia fruticosa, in vitro* rooting was done with IBA 0.2mg/l in SH medium (Krishnan and Seeni, 1994). A combination of auxins gave better response for *in vitro* rooting in teak and clove (Gupta *et al.*, 1980 and Mathew and Hariharan, 1990).

Some workers have reported successful *in vitro* rooting of some plant species in the media without any growth regulators. Bhattacharya and Sen (1980) noted that plantlets were produced when embryoids derived from callus tissue of *Brassica campestris* were transferred to SH medium devoid of hormones. Similarly in Crape myrtle, Zhang and Davies (1986) obtained successful *in vitro*

rooting of shoots in medium without auxins. Das (1992) also reported similar results in *Agave sisalana*.

Hu and Wang (1983) suggested that a medium with low salt concentration promotes rooting. Abundant rooting was observed when the salt concentration in the medium was reduced to half, one third or one fourth of the standard strength (Kartha *et al.*, 1974; Lane, 1979 and Skirvin and Chu, 1979). For rooting, the sugar content of the medium was also lowered (Roy *et al.*, 1990).

Activated charcoal has profound influence on the rooting of shoots *in vitro*. Activated charcoal adsorbs toxic substances in the medium thereby improving root regeneration and development of more roots (Jaiswal and Amin, 1987). It also adsorbs residual cytokinins from the shoot and shades the *in vitro* produced roots from high intensity of light, which inhibit its growth (Hu and Wang, 1983).

Concentration of agar used for rooting varies from zero in liquid medium to 0.7 per cent in solid medium. Liquid media facilitates the free diffusion of toxic plant wastes and when used with filter paper bridge system provides excellent aeration for root development (Hu and Wang, 1983). Rooting of shoots in medium filled with agar was reported by Ho and Lee (1985); Goh *et al.* (1988) and D'Silva and D'Souza (1992).

Hunter (1979) found that cinchona shoots raised in proliferation media rooted best by a non-aseptic implantation into peat blocks. These blocks were rehydrated with one-tenth concentration of MS salts following the application of an ethanolic IBA or NAA dip at 75mg/l or 100mg/l. In *Lagerstroemia speciosa*, 50 per cent rooting was observed in the *in vitro* produced shoots when raised in sand supplemented with 10mg IBA per litre (Ho and Lee, 1985). Successful *ex vitro* rooting in *Lagerstroemia indica* was reported by Zhang and Davies (1986). Yeoman (1986) advocated an *ex vitro* rooting approach which provides a simple, highly efficient and more economic methodology. *In vitro* produced shoots were transferred to pots containing a mixture of peat, vermiculite and sand in a ratio of 4:2:1. The shoots were maintained in a high humidity environment and watered

daily. During the first two weeks, a water solution containing 15 M NAA was administered four times at equally spaced intervals to promote rooting. Twenty per cent of shoots rooted after eight weeks. Das (1992) found that in Agave *sisalana*, the shoots rooted *ex vitro* in pots containing sand and covered with a polythene bag. In *Achras sapota*, Purohit and Singhvi (1998) and Purohit *et al.* (1999) obtained successful callus free rooting when the shoots, after treatment with pre autoclaved IBA at the rate of 200mg/l for 2 hours, were directly implanted on autoclaved soil rite and irrigated with one fourth SH solution. This allowed rooting and partial hardening simultaneously.
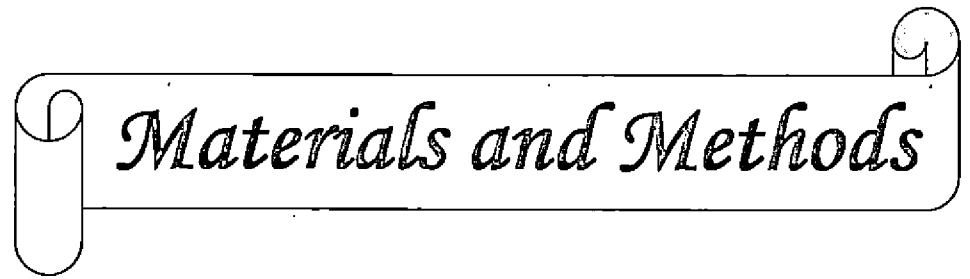
2.4.4.16. *Hardening and planting out*

Acclimatization is crucial to any micro propagation procedures since shoot and plantlets produced *in vitro* must be readapted to the environmental conditions outside the culture vessels. Plants produced by tissue culture techniques are generally more expensive than conventionally produced seedlings. The transfer of the plantlet from the culture vessels to the main field is time consuming, labour intensive and may vary with species or even with varieties. During the period of adaptation changes in both structures and physiology of shoots occur.

Leaves of *in vitro* cultured plantlets are characterised by the absence or reduced amount of epicuticular wax in comparison to the leaves of the green house or field grown plants (Grout, 1975). This affects the rate of water loss from the leaves. During acclimatization, as the humidity is gradually lowered, the density of wax on leaves increases (Wardle *et al.*, 1983).

Kyte and Briggs (1979) found that a porous potting mixture of peat: perlite: composted bark (1:1:1) was the best for rooting tissue cultured rhododendrons. Barnes (1979) suggested the method of covering the transplanted plantlet with polythene cover to maintain high humidity. Nutrition of the plantlets during acclimatization phase is dependent on the plant species. A period of humidity acclimatization was suggested for the newly transferred plantlets to make

them adapted to the external environment (Hu and Wang, 1983). Ziv (1986) reported that success in acclimatization depends not only on the post transfer conditions but also on the pretransfer conditions. Lee *et al.* (1987) used a mixture of perlite, vermiculite and peat in a ratio 1:1:1 by volume to harden the *in vitro* produced plantlets of *Lagerstroemia indica.* Rooting and partial hardening of the plantlets of *Achras sapota* were simultaneously done where the shoots were directly implanted on autoclaved soil rite in culture bottles and irrigated with one quarter strength SH solution directly after treatment with preautoclaved IBA (Purohit and Singhvi, 1998; Purohit *et al.*, 1999). Krishnan and Seeni (1994) reported that the rooted plantlets of *Woodfordia fruticosa* were directly transferred to the potting mixture to omit the step of hardening. Zobayed (2000) found that in *Lagerstroemia speciosa* the plantlets produced by forced ventilation treatment with lower relative humidity conditions survived better in *ex vitro* conditions compared to the plantlets produced in sealed culture vessels with higher relative humidity. In *Agave sisalana*, Das (1992) reported that rooted plantlets transfered to a mixture of soil and sand in a ratio of 1:1 survived better when placed in green house for 90-100 days. For increasing the survival rate of hardened plantlets in the main field, hardening of rooted plantlets of *Adhatoda beddomei* in humidity chamber was essential (Sudha and Seeni, 1994). Similar reports have been made in *Utleria salicifolia* (Gangaprasad *et al.*, 2003) and *Citrus reticulata* (Karwa, 2003)

Materials and Methods

3 MATERIALS AND METHODS

The present study was carried out at the Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara during the period 2009-2011. The details of the materials and methods followed for the conduct of the various experiments are detailed below. The study was undertaken through the conduct of the following three experiments.

Experiment 1: Standardization of suitable surface sterilization procedures explants, and culture establishment protocols.

Experiment 2: Induction of multiple shoots.

Experiment 3: Induction and elongation of rooting, hardening and planting out.

3.1 STANDARDIZATION OF SUITABLE SURFACE STERILIZATION PROCEDURES EXPLANTS AND CULTURE ESTABLISHMENT PROTOCOLS.

3.1.1 Source of Explants

The explants were collected from 6 month old seedlings . These seedlings were kept in the net house of All India Coordinated Research Project on Medicinal and Aromatic Plants ,COH, KAU. 0.1% Bavistin spray is given to the source plants for every three days and there were hence no symptoms of pest and diseases.

3.1.1.1 *Preparation of explants*

The tiny shoot cuttings were brought to the laboratory from 6 month old seedlings . From this shoot cuttings, Shoot tips, nodal and internodal segments were taken as explants for the study. Shoot tips of approximately 5 to 6mm in length were excised carefully from the plants. Nodal segment and internode segment of approximately 20-25mm in length were used as explants. The explants were immersed in 1.0 per cent Teepol solution for three to four minutes and were thoroughly washed in running tap water to remove all traces of the chemical

3.1.2 Culture Medium

3.1.2.1 *Composition of the medium*

The response of the explants in three different basal media was studied in the present investigation. The culture media used were Murashige and Skoog's (MS) medium , MS medium in half strength (½ MS) and Woody Plant medium (WPM) (Lloyd and McCown, 1980). Composition of these three media is given in Table 1. The basal media were supplemented with different levels of auxins like IAA, IBA, 2,4- D and cytokinins like BAP, and Kinetin singly or in combination to study the effect of different growth regulators on culture establishment.

3.1.2.2 *Preparation of the stock solutions*

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of the medium. Stock solutions of the major and minor nutrients were prepared first by dissolving the required quantity of chemicals in double distilled water and stored under refrigerated conditions in amber coloured bottles. Stock solutions for the major and minor nutrients were prepared afresh every three months. The vitamin stock solutions were prepared fresh every six to eight weeks and those of growth regulators were prepared fresh every four weeks.

3.1.2.3 *Preparation of the culture medium*

Specific quantities of the stock solution were pipetted out into a beaker. The required quantity of plant hormones was mixed. Sucrose was weighed, well dissolved and added freshly and volume made up to the required level using double distilled water. The pH of the solution was adjusted at 5.8 using 1.0N NaOH or 0.1N HCl. For obtaining a semisolid medium, agar was added at 0.75 per cent level and the medium was boiled till a clear solution was obtained. About 15ml of this molten medium was dispensed into the culture tubes (15x2.5cm or 20x2.5cm sizes).

3.1.2.4 *Sterilization of the culture medium*

The tubes were plugged with non-absorbent cotton and autoclaved at 121°C and 15psi (1.06 kg/cm^2) for 20 minutes (Dodds and Roberts, 1985). The medium was allowed to cool to room temperature and stored in a cool dry place.

Table 1 Composition of various basal media tried for *in vitro* culture of *Saraca asoca.*

Ingredients(mg/l)	MS	½ MS	WPM
Inorganic constituents			
$(NH_4)NO_3$	1650	825	400
KNO_3	1900	950	--
K_2SO_4	--	--	990
KH_2PO_4	170	85	170
$Ca(NO_3)_2.4H_2O$	--	--	386
$CaCl_2.2H_2O$	440	220	72.50
$MgSO_4.7H_2O$	370	185	180.70
$FeSO_4.7H_2O$	27.80	13.54	27.85
Na_2EDTA	37.30	18.56	37.30
$MnSO_4.4H_2O$	22.30	11.15	22.30
$ZnSO_4.7H_2O$	8.60	4.30	8.60
H_3BO_3	6.20	3.10	6.20
KI	0.83	0.415	
$Na_2MoO_4.2H_2O$	0.25	0.12	0.25
$CuSO_4.5H_2O$	0.03	0.012	0.25
$CoCl_2.6H_2O$	0.03	0.012	
Organic constituents			
Nicotinic Acid	0.50	0.50	0.50
Pyridoxine HCl	0.50	0.50	0.50
Thiamine HCl	0.10	0.10	1
Glycine	2	2	2
Sucrose	30000	15000	30000

3.1.3 Transfer Area and Asceptic Manipulation

All the aseptic manipulations such as surface sterilization of the explants, preparation and inoculation of the explants and subsequent sub culturing were carried out in a clean laminar airflow chamber. The working table of the laminar airflow chamber was initially surface sterilized by wiping with absolute alcohol and then by switching on the ultraviolet light for 30 minutes. The Petri dishes, forceps, knives and other inoculation aids were initially autoclaved and then flame sterilized before each inoculation. The hands were washed thoroughly with soap under running tap water. After drying, they were wiped with absolute alcohol before inoculation.

3.1.4 Surface Sterilisation

Surface sterilisation was carried out under perfect aseptic conditions in the laminar airflow chamber. Different Surface sterilisation treatments was tried to eliminate fungal and bacterial contamination from the explants. The explants were initially cleaned with teepol wash. Then they were surface sterilized by each treatments as given in table 2. During surface sterilization the explants were soaked in each solution for required period. Then they were continuously agitated manually to ensure thorough contact of the explants with the chemical. The explants after surface sterilization were rinsed thrice thoroughly with sterilized distilled water to remove traces of the chemical from the surface of the explants.

3.1.5. Inoculation

The explants that were surface sterilised were inoculated under perfect aseptic conditions into the different basal media supplemented with varying quantities of growth regulators and cultured. The cultures were incubated at $25\pm 2^{\circ}C$ in an air-conditioned culture room under a light intensity of 3000 lux supplied by cool fluorescent lamp. Relative humidity in the culture room varied between 60 to 80 per cent according to the climate prevailing.

As *S.asoca* contains a wide range of polyphenols, browning of the medium due to polyphenol exudation from the cut ends of the explants was noticed.

Within 48 hours of inoculation, upon retaining the explants in the brown media, the explants lost their viability. Hence, on the third day of the initial inoculation, the explants were transferred to a fresh medium with the same concentration of the growth regulators.

Table 2 Different surface sterilisation treatments carried out for the explants of *Saraca asoca* before inoculation.

Sl.no.	Treatment
T 1	0.1% Bavistin for 6 minutes + 400ppm chloramphenicol + 0.1% $HgCl_2$ for 5 minutes
T 2	0.1% Bavistin for 10 minutes + 400ppm chloramphenicol + 0.1% $HgCl_2$ for 5 minutes
T 3	0.1% Bavistin for 15 minutes + 400ppm chloramphenicol + 0.1% $HgCl_2$ for 5 minutes
T 4	0.1% $HgCl_2$ for 3 minutes
T 5	0.1% $HgCl_2$ for 4 minutes
T 6	0.1% $HgCl_2$ for 5 minutes
T 7	0.1% $HgCl_2$ for 6 minutes
T 8	0.1% $HgCl_2$ for 7 minutes
T 9	100% Ethyl Alcohol for 3 minutes
T 10	90% Ethyl Alcohol for 3 minutes
T 11	70% Ethyl Alcohol for 3 minutes
T 12	70% Ethyl Alcohol for 3 minutes + 0.1%$HgCl_2$ for 4 minutes
T 13	70% Ethyl Alcohol for 3 minutes + 0.1%$HgCl_2$ for 5 minutes
T 14	70% Ethyl Alcohol for 3 minutes + 0.1%$HgCl_2$ for 6 minutes

3.1.6. Types of Contamination

During the initial period of the project work there was contamination due to micro organisms like fungi and bacteria. The fungal contamination was identified by whitish or pinkish mycelial growth. The bacterial contamination was identified by slimy or milky layer formation in the media.

3.1.7. Polyphenolic Interference

Polyphenol from the cut end of the explants caused browning of the media. It inhibited the growth of the explants.

3.1.8. Standardisation of Basal Media and Explant

The best basal medium for *in vitro* culture of *S. asoca* was identified by inoculating the explants into the three basal media *viz.* MS medium, half MS and WPM which were supplemented with growth hormones to initiate culture in *S. asoca* (Table 3). Shoot tips, nodal and internodal segments were employed for establishing cultures. For each treatment about 20 replications were maintained. The response of each explants in each media was recorded. Also the effect of different media on culture establishment was recorded.

Table 3 Standardisation treatments of basal media for *in vitro* culture of *S. asoca* with different explants

Treatment	Medium
T 1	MS + BAP 0.5 mg/l
T 2	MS + BAP 1.0 mg/l
T 3	MS + BAP 1.5 mg/l
T 4	MS + BAP 2.0 mg /l
T 5	½ MS + BAP 0.5 mg/l
T 6	½ MS + BAP 1.0 mg/l
T 7	½ MS + BAP 1.5 mg/l
T 8	½ MS + BAP 2 mg/l
T 9	WPM+ BAP 0.5 mg/l
T 10	WPM + BAP 1.0 mg/l
T 11	WPM+ BAP 1.5 mg/l
T 12	WPM + BAP 2.0 mg /l
T 13	MS + 2,4-D 0.5 mg /l
T 14	MS + 2,4-D 1.0 mg /l
T 15	MS + 2,4-D 1.5mg /l
T 16	MS + 2,4-D 2.0 mg /l
T 17	½ MS + 2,4-D 0.5 mg /l
T 18	½ MS + 2,4-D 1.0 mg /l

T 19	½ MS + 2,4-D 1.5mg /l
T 20	½ MS + 2,4-D 2.0 mg /l
T 21	WPM+ 2,4-D 0.5 mg/l
T 22	WPM +2,4-D 1.0 mg/l
T 23	WPM+ 2,4-D 1.5 mg/l
T 24	WPM + 2,4-D 2.0 mg /l
T 25	MS + Kinetin 0.5 mg/l
T 26	MS + Kinetin 1.0mg/l
T 27	MS + Kinetin 1.5mg/l
T 28	MS + Kinetin 2.0mg/l
T 29	½ MS + Kinetin 0.5 mg/l
T 30	½ MS + Kinetin 1.0mg/l
T 31	½ MS + Kinetin 1.5mg/l
T 32	½ MS + Kinetin 2.0mg/l
T 33	WPM+ Kinetin 0.5 mg/l
T 34	WPM + Kinetin 1.0 mg/l
T 35	WPM+ Kinetin 1.5 mg/l
T 36	WPM + Kinetin 2.0 mg /l

3.2 EXPERIMENT 2. MULTIPLE SHOOT INDUCTION

Studies were conducted to determine the effect of various growth regulators on the induction of multiple shoots from nodal segments. surviving cultures were subcultured at an interval of 3-4 weeks to the same medium in which they were maintained, for a period of three months. The response of the cultures in each subculture was observed and recorded. The treatments that were tried for multiple shoot induction are presented in Table 4.

Table 4 Treatment combinations of growth regulators for multiple shoot induction in nodal segments of *S.asoca*

Treatment	Medium
T 1	½ MS + BAP 0.5 mg/l
T 2	½ MS + BAP 1.0 mg/l
T 3	½ MS + BAP 1.5 mg/l
T 4	½ MS + BAP 2 mg/l
T 5	½ MS + Kinetin 0.5 mg/l
T 6	½ MS + Kinetin 1.0mg/l
T 7	½ MS + Kinetin 1.5mg/l
T 8	½ MS + Kinetin 2.0mg/l
T 9	½ MS + BAP 0.5 mg/l+ IAA 0.5 mg/l
T 10	½ MS + BAP 0.5 mg/l+ IAA 1.0 mg/l
T 11	½ MS + Kn 0.5 mg/l+ IAA 0.5 mg/l
T 12	½ MS + Kn 0.5 mg/l+ IAA 1.0 mg/l

3.3 EXPERIMENT 3: INDUCTION AND ELONGATION OF ROOTING AND HARDENING OUT

Shoots obtained from the previous Experiment was subjected to *in vitro* rooting. The percentage of shooting was found to be very low . A maximum of 30 percentage shooting was observed in ½ MS medium supplemented with BAP 0.5mg/l . From a total of 20 observations only 6 responded for shooting. This was cultured using 0.5 mg/l IBA and 0.5 mg/l IAA in 1/2MS media. In each treatment 3 shoots were taken and observations were recorded.

Results

4 RESULTS

The results of the various experiments carried out for the 'Standardisation of *in vitro* propagation techniques in *Saraca asoca* (Roxb.) de Wilde. are presented in detail below.

4.1 CULTURE ESTABLISHMENT

4.1.1 Surface Sterilisation

All plant materials used for culture establishment were treated with an appropriate sterilization agent to inactivate the microbes present on their surface. The effects of the surface sterilization treatments on culture establishment in *Saraca asoca* are presented in Table 5. The most effective sterilization was achieved by the combination treatment of soaking the explants in $HgCl_2$ 0.1 per cent for five minutes followed by soaking them in 70 per cent alcohol for three minutes where the percentage of live cultures was 80 and there was no contamination. Sterilization with 0.1 per cent $HgCl_2$ for 4 minutes and 5 minutes also gave good results where 70 per cent cultures survived. However, there was a contamination rate of 30 per cent and 10 per cent respectively.

Increasing the time of soaking or the concentration of the sterilant adversely affected the survival of the explants. Soaking the explant in any chemical for more than five minutes resulted in the browning and death of the explants. Even though, higher concentration of the chemical sterilants fully controlled the contamination, it affected the survival of the explants as well. None of the explants survived in the treatment 0.1 percent $HgCl_2$ beyond 5 minutes and in 100 per cent or 90 per cent ethyl alcohol for 3 minutes.

Table 5: Effect of surface sterilants on culture establishment of nodal segments in *Saraca asoca* (one week after inoculation)

Treatment	Contaminati on (%)	Survival (%)
0.1% Bavistin for 6 minutes + 400ppm chloramphenicol + 0.1% HgCl$_2$ for 5 minutes	100	Nil
0.1% Bavistin for 10 minutes + 400ppm chloramphenicol + 0.1% HgCl$_2$ for 5 minutes	100	Nil
0.1% Bavistin for 15 minutes + 400ppm chloramphenicol + 0.1% HgCl$_2$ for 5 minutes	100	Nil
0.1% HgCl$_2$ for 3 minutes	40	20
0.1% HgCl$_2$ for 4 minutes	30	70
0.1% HgCl$_2$ for 5 minutes	10	70
0.1% HgCl$_2$ for 6 minutes	Nil	100
0.1% HgCl$_2$ for 7 minutes	Nil	100
100% Ethyl Alcohol for 3 minutes	Nil	100
90% Ethyl Alcohol for 3 minutes	Nil	100
70% Ethyl Alcohol for 3 minutes	70	10
70% Ethyl Alcohol for 3 minutes + 0.1%HgCl$_2$ for 4 minutes	20	60
70% Ethyl Alcohol for 3 minutes + 0.1%HgCl$_2$ for 5 minutes	Nil	80
70% Ethyl Alcohol for 3 minutes + 0.1%HgCl$_2$ for 6 minutes	Nil	20

Average of 20 observations

4.1.2. Effect of different explants on the culture establishment

Among the three explants tried, namely shoot tip, nodal segments and internodes the maximum response was shown by nodal segments. This was followed by the shoot tips. Nodal segments responded by callusing in all Media except WPM. Plate (4) This was followed by shoot tips. The Shoot tips responded by callusing in MS media. Plate (5) The internodal explants did not respond in any of the media. Hence for regeneration through callus mediated organogenesis, nodal segments was found better . The details are furnished in Table 6.

Plate 4: Callus formation in Nodal segment in ½ MS medium with BAP 0.5 mg/l

Plate 5: Callus formation in Shoot tip segment in MS medium with BAP 0.5 mg/l

Table 6 :Standardization of suitable explants

Medium	Response in percentage			Type of Response
	Shoot tip	Nodal segment	Internode	
MS + BAP 0.5 mg/l	10	10	Nil	Callusing
MS + BAP 1.0 mg/l	Nil	10	Nil	callusing
MS + 2,4-D 0.5 mg/l	Nil	Nil	Nil	
MS + 2,4-D 1.0 mg/l	Nil	Nil	Nil	
MS + Kinetin 0.5 mg/l	Nil	10	Nil	callusing
MS + Kinetin1.0 mg/l	Nil	Nil	Nil	
½ MS + BAP 0.5 mg/l	Nil	60	Nil	callusing
½MS + BAP 1.0 mg/l	Nil	10	Nil	callusing
½MS +2,4-D 0.5 mg/l	Nil	Nil	Nil	
½MS + 2,4-D 1.0 mg/l	Nil	Nil	Nil	
½MS + Kinetin0.5 mg/l	Nil	10	Nil	callusing
½MS + Kinetin1.0 mg/l	Nil	10	Nil	callusing
WPM + BAP 0.5 mg/l	Nil	Nil	Nil	
WPM + BAP 1.0 mg/l	Nil	Nil	Nil	
WPM + 2,4-D 0.5 mg/l	Nil	Nil	Nil	
WPM + 2,4-D 1.0 mg/l	Nil	Nil	Nil	
WPM + Kinetin0.5 mg/l	Nil	Nil	Nil	
WPM + Kinetin1.0 mg/l	Nil	Nil	Nil	

Average of 20 observations.

4.1.2.1 *Types of contamination*

During the initial period of the project work there was contamination due to micro organisms like fungi and bacteria. The maximum percentage of contamination was identified as fungal contamination . Plate(6) The presence of fungal contamination was identified by the whitish or pinkish mycelial growth. The bacterial contamination was also identified by slimy or milky layer formation in the media. The percentage of contamination was worked out and the details are furnished in Table 7

Table 7 : Types of and percentage of contamination

Total no of tubes innoculated	Total no of tubes contaminated		Percentage of contamination	
	fungus	bacteria	fungus	bacteria
25	11	4	44%	16%

Plate 6: Fungal contamination in culture

4.1.2.2 *Treatments to overcome Phenolic Interference*

When explants were inoculated, it was found that polyphenols exuded from the cut ends of the explant into the medium . When the explants were retained in the same medium it caused browning and death of the cultures (Plate 7). A decrease in the survival rate of explants was noticed when left as such in the medium that contained polyphenols. Hence, the explants were subcultured into the same basal media in which they were inoculated once in three days. When the explants were subcultured twice, the contamination rate increased to 44 per cent when compared to 10 per cent in one subculturing. Hence one subculturing on the third day of the initial inoculation was found to be the optimum for reducing polyphenol interference with a maximum survival rate of 86 per cent. The results are presented in Table 8.

Table 8: Effects of subculturing in polyphenol exudation.

Treatment	Culture conditions	Explant Discolouration (%)	Media Discolouration (%)	Live (%)	Contamination (%)
No subculture	Light	100	100	Nil	Nil
	Dark	100	100	Nil	Nil
One subculture	Light	4	Nil	86	10
	Dark	8	Nil	84	8
Two subculture	Light	6	Nil	44	50
	Dark	Nil	Nil	64	36
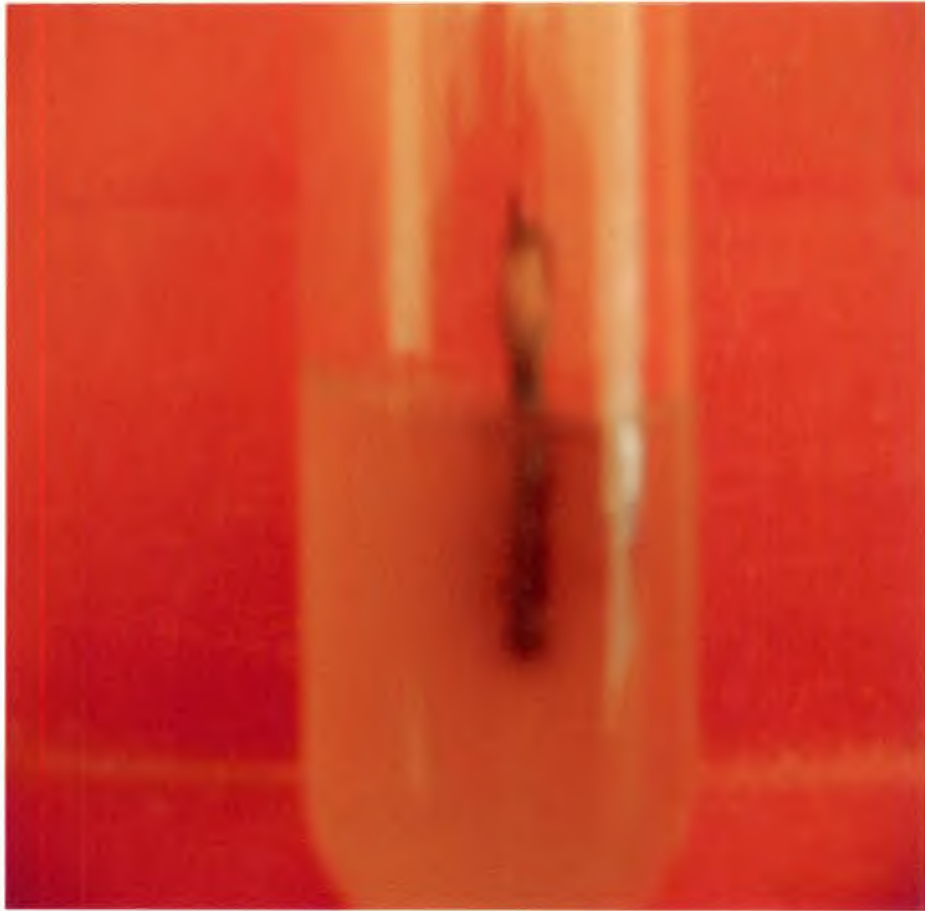
Plate 7: Phenolic exudation from explant

4.1.3 Effect of different basal media on the culture establishment

The effect of three different basal media on the culture establishment of the nodal segment in *Saraca asoca* is presented in Table 9. The results show that the explants responded maximum to ½ MS medium when compared to MS and WPM. In the MS medium, the response was shown in T 1, T 2, and T 25 with 10 per cent each response and in ½ MS, maximum response was shown in T 5 and T 7 where 60 per cent and 30 per cent of the culture responded correspondingly by callusing. In WPM no response were seen at all. Among all media ½ MS media was identified as the best media for *in vitro* culture of *Saraca asoca* and hence for further studies in Experiments 2 and 3, ½ MS media containing different concentrations of growth regulators were used.

Table 9 : Standardisation of Basal media for *in vitro* propagation in nodal segment of *Saraca asoca.*

Treatment	Medium	Nodal segment	Response in Percentage
T 1	MS + BAP 0.5 mg/l	10	Callusing
T 2	MS + BAP 1.0 mg/l	10	Callusing
T 3	MS + BAP 1.5 mg/l	Nil	
T 4	MS + BAP 2.0 mg/l	Nil	
T 5	½ MS + BAP 0.5 mg/l	60	Callusing
T 6	½MS + BAP 1.0 mg/l	10	Callusing
T 7	½ MS + BAP 1.5 mg/l	30	Callusing
T 8	½MS + BAP 2.0 mg/l	20	Callusing
T 9	WPM + BAP 0.5 mg/l	Nil	
T 10	WPM + BAP 1.0 mg/l	Nil	
T 11	WPM + BAP 1.5 mg/l	Nil	
T 12	WPM + BAP 2.0 mg/l	Nil	
T 13	MS + 2,4-D 0.5 mg/l	Nil	
T 14	MS + 2,4-D 1.0 mg/l	Nil	
T 15	MS + 2,4-D 1.5 mg/l	Nil	
T 16	MS + 2,4-D 2.0 mg/l	Nil	
T 17	½MS +2,4-D 0.5 mg/l	Nil	
T 18	½MS + 2,4-D 1.0 mg/l	Nil	
T 19	½MS +2,4-D 1.5 mg/l	Nil	
T 20	½MS + 2,4-D 2.0 mg/l	Nil	
T 21	WPM + 2,4-D 0.5 mg/l	Nil	
T 22	WPM + 2,4-D 1.0 mg/l	Nil	
T 23	WPM + 2,4-D 1.5 mg/l	Nil	
T 24	WPM + 2,4-D 2.0 mg/l	Nil	

T 25	MS + Kinetin 0.5 mg/l	10	Callusing
T 26	MS + Kinetin 1.0 mg/l	Nil	
T 27	MS + Kinetin 1.5 mg/l	Nil	
T 28	MS + Kinetin 2.0 mg/l	Nil	
T 29	½MS + Kinetin 0.5 mg/l	10	Callusing
T 30	½MS + Kinetin 1.0 mg/l	10	Callusing
T 31	½MS + Kinetin 1.5 mg/l	20	Callusing
T 32	½MS + Kinetin 2.0 mg/l	10	Callusing
T 33	WPM + Kinetin 0.5 mg/l	Nil	
T 34	WPM + Kinetin 1.0 mg/l	Nil	
T 35	WPM + Kinetin 1.5 mg/l	Nil	
T 36	WPM + Kinetin 2.0 mg/l	Nil	

Average of 20 observations

4.2 EXPERIMENT 2: MULTIPLE SHOOT INDUCTION

Those media combination which responded in experiment 1 was used here. There were no multiple shoot induction in any of the growth regulator combination. A maximum of 30 % culture showed response in T1 media Plate(8) . Single shoots were seen in T7, T9, T10 also. But percentage of cultures responded was found to be low. BAP induced better response in explants both when used alone and also in combination with IAA. Kinetin did not induce much response in shoot induction. The details are furnished below in Table 10, Table 11, and Table 12.

Table 10: Effect of BAP for Shoot Induction in Nodal segment of *Saraca asoca*

Treatment	% Culture Showing Shoot Induction	Days for Shoot Induction	Mean no of Shoots	Mean length of Shoot in cm
T 1	30	51	Single shoot	1.5
T 2	Nil·	------	------	------
T 3	10	69	Single shoot	1.3
T 4	5	54	Single shoot	1.3

Average of 20 observations
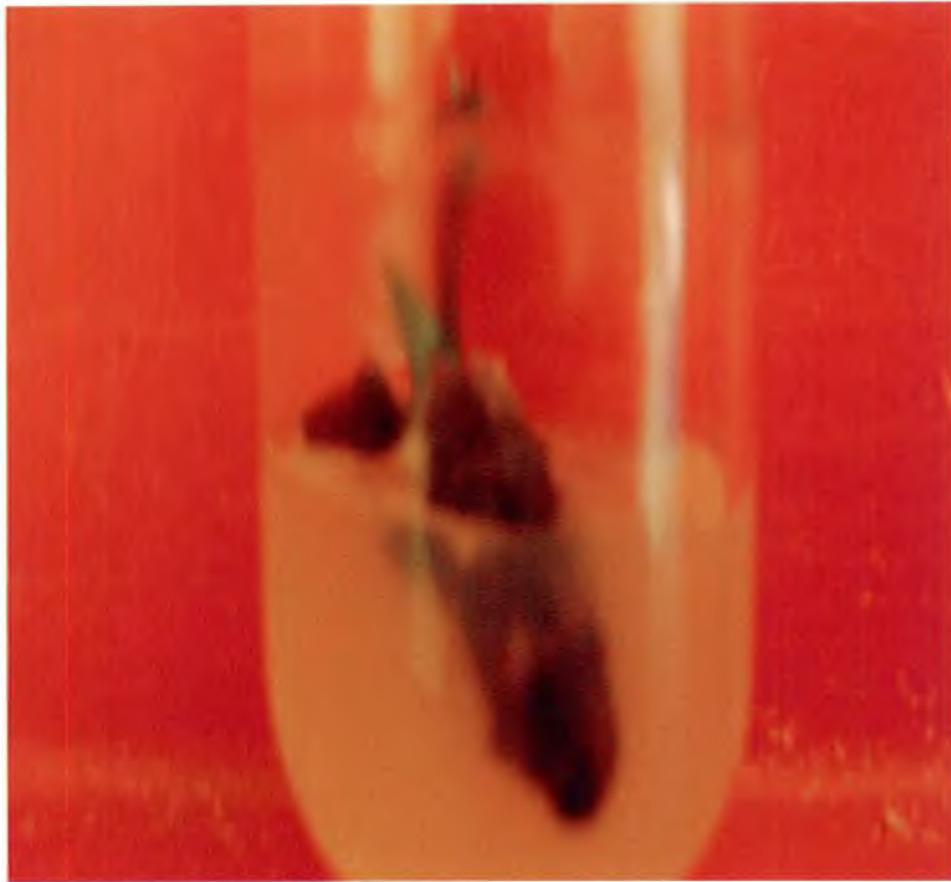
Plate 8: Shoot formation in Nodal Segment in ½ MS medium with BAP 0.5 mg/L

Table 11: Effect of Kinetin for Shoot Induction in Nodal segment of *Saraca asoca*

Treatment	% Culture Showing Shoot Induction	Days for Shoot Induction	Mean no of Shoots	Mean length of Shoot in cm
T 5	Nil	------	-------	-------
T 6	Nil	-------	-------	------
T 7	10	69	Single shoot	1.2
T 8	Nil	-------	--------	------

Average of 20 observations

Table 12: Effect of Auxin- Cytokinin Combination for Shoot Induction in Nodal segment of *Saraca asoca*

Treatment	% Culture Showing Shoot Induction	Days for Shoot Induction	Mean no of Shoots	Mean length of Shoot in cm
T 9	10	52	Single shoot	1.6
T 10	5	68	Single shoot	1.3
T 11	Nil	-----	-----	-----
T 12	Nil	-----	-----	-----

Average of 20 observations

4.3 ROOTING OF *IN VITRO* PRODUCED SHOOT

Since the no of shoots obtained was low , rooting was tried only in ½ MS medium supplemented with 0.5% IBA and 0.5% IAA. There was no rooting stage obtained in any of the cultures even after two months. The details are furnished in Table 13.

Table 13: Effect of IAA, IBA in Root Induction in Nodal segment of *Saraca asoca*

Medium	% Culture Showing root induction	Days for root induction	Mean length of root in cm
½ MS +0.5 mg/l IAA	Nil	------	------
½ MS +0.5 mg/l IBA	Nil	------	------

Average of 20 observations

Discussion

5. DISCUSSION

Saraca asoca is a plant with medicinal properties that is propagated both by sexual and asexual methods. Seed propagation is difficult in this species as the seedlings are highly susceptible to damping off (Bahuguna *et al.*, 1988). The seeds also lose their viability within six months (Bhagat *et al.*, 1992). Vegetative propagation in the plant is rather difficult as the plant is a hard to root species irrespective of the seasons and hormonal treatments (Bahuguna *et al.*, 1988; Rajesh *et al.*, 1993; Chauhan *et al.*, 1994; Raju *et al.*, 1994). In view of these facts, the standardization of *in vitro* propagation technique has great relevance in the production of true to type propagules at a cheaper rate within a reasonable time.

Attempts were made to standardize the *in vitro* propagation technique in *Saraca asoca* at the Plant Tissue Culture Laboratory, Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara during 2009-2011. The results obtained are discussed here.

5.1. CULTURE ESTABLISHMENT

5.1.1. Surface sterilization

The most effective surface sterilisation in *Saraca asoca* was achieved by soaking the explants in 70 percent alcohol for three minutes followed by soaking them in 0.1 per cent HgCl₂ for five minutes. Bonga *et al.* (1982), has advocated the use of alcohol alone or in combination with other chemicals for disinfection.

Exposure of the explants to chemicals beyond five minutes was found to be deleterious. This is in confirmation with the findings of Krishnan and Seeni (1994) in *Woodfordia fruticosa*.

After surface sterilisation, rinsing the explants thoroughly with sterile distilled water ensured the removal of the traces of the chemical present on

the explants. Hu and Wang (1983) reported that it is necessary to wash the tissues twice or thrice in sterile distilled water to ensure dilution of the chemical. Krishnan and Seeni (1994), also recommended the need for rinsing the explants five to six times in sterile distilled water before inoculation.

5.1.2. Explant

In the present investigation, nodal segments tended to be callusogenic and also it induced more no of single shoots. Among all explants taken nodal segment responded better when compared to others. However, Shoot tips of *Saraca asoca* were found to be the best in inducing multiple shoots in *in vitro* conditions by (Ramasubbu *et al.*,2008). Rahman and Blake (1988) observed in jack that nodal explants gave more proliferation than shoot tips In nutmeg, nodal segments were better in inducing multiple shoots than terminal buds (Mallika *et al.*, 1997). Similar reports have been done by Mathew (1995) in clove. The use of nodes in preference to shoot tips for proliferation of stem is also reported in other medicinal species like *Adhathoda beddomei* (Sudha and Seeni, 1994); *Aegle marmelos* (Ajithkumar and Seeni, 1998) and *Utleria salicifolia* (Gangaprasad *et al.*, 2003). Panimalar *et al.* (2005) observed that the best explant in *Centella asiatica* was nodal segments.

However, inferences in all these studies were based on relatively better morphogenic responses observed with nodal explant cultures compared to shoot tip cultures and not on exclusive regenerative capacity of the former.

5.1.3. Basal medium

In the present study, among all the media used the culture establishment was the best in 1/2 MS medium. Skirvin (1980) and Griffins *et al.* (1981) suggested that reducing the strength of MS medium by half was more beneficial for culturing.
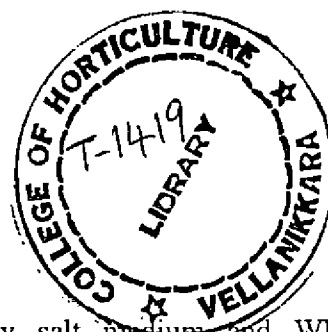
Woody plants usually require a low salt medium and WPM formulated by Lloyd and McCown (1980) was found to be the best basal medium for *in vitro* culture in other woody plants by several workers (Vieitez *et al.*,1983 in chestnut; Flynn *et al.*, 1990 in cocoa). However in *Saraca asoca* the response in WPM was negligible. Similar results have been reported in *thathiri* by Gayatri (2005).

5.1.4. Polyphenol interference

The polyphenol interference in *Saraca asoca* was very much pronounced and this lead to the browning of the medium. *Saraca asoca* is reported to contain polyphenols and tannins that ooze out into the medium during culture. Survival of the explants reduces if polyphenol interference is left unchecked. This has been reported by Mathew (1995) in clove. Explant establishment of *Saraca asoca* thus required special procedures to escape or avoid problems that are associated with polyphenol exudation. Similar problem has been reported in other woody plant species by Lenartowicz and Millikan (1977); Lloyd and McCown (1980); Mathew (1995). The explants were subcultured once, three days after the first inoculation, which checked the polyphenol interference to a great extent. Subculturing more than once was not found to be beneficial as it increased the contamination of the cultures. Similar observations have been made by Gayatri (2005) in *thathiri* .

No significant difference in polyphenol exudation was observed when the cultures were kept in the dark. Similar observation was made by Mathew (1995) in clove and Gayatri (2005) in *thathiri* , where providing dark culture conditions was not found effective in reducing polyphenol interference unlike in other crop plants as reported by Forrest (1969) and Hu and Wang (1983).

5.2. EXPERIMENT 2: MULTIPLE SHOOT INDUCTION

In *in vitro* culture of *Saraca asoca* nodal segments showed shoot induction in ½ MS medium containing 0.5 mg/l BAP. Only single shoots were obtained. The direct effect of cytokinin in tissue culture may vary according to the particular compound used, the type of cultures and the plant species from which it was derived (George and Sherrington, 1984). Generally, cytokinin has been utilized to overcome the apical dominance of shoots to enhance the branching of the lateral buds from leaf axils (Murashige, 1974). In the present investigation, for the shoot induction in *Saraca asoca* , cytokinin in the form of BAP was found to be highly essential. However kinetin did not invoke any response in the explants. 2,4-D, the commonly used auxin for callusogenesis, did not respond in *Saraca asoca*. This was in accordance with Gayatri (2005) in *thathiri*

The superiority of BAP over kinetin for the shoot induction, proliferation and subsequent growth was reported by Lundergan and Janick (1980) in apple; Yadav *et al.* (1990 a) in *Syzygium cumini*; Yadav *et al.* (1990 b) in mulberry; Mathew (1995) in clove and Gamboa and Abdelnour (1999) in *Gmelina arborea*. Unlike in the crops mentioned above, kinetin was found to be the most effective cytokinin in inducing multiple shoots in sisal (Hazra *et al.*, 2001)

5.3. ROOTING OF *IN VITRO* PRODUCED SHOOT

In the present study rooting was tried in the single shoots obtained using IAA and IBA. There was no response seen even after 2 months. Woody plants generally show a slower response to culture conditions than herbaceous angiosperms. This is in accordance with Mathew (1995) in clove.

In present study 6 month old seedlings was taken as explants source. It did not respond well to *in vitro* propagation. Compton and Preece (1988) reported that the age of the stock plant and the location on the stem from which the explants are removed greatly affect the establishment of tissues *in vitro*. In addition to this the recalcitrance of the species also affected its response to *in vitro* propagation.

Summary

6. SUMMARY

The present investigation was carried out during the period 2009-2011 in the Tissue Culture Laboratory of The Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara with the objective of standardizing *in vitro* propagation technique in *Saraca asoca* .Six month old rooted cuttings of *Saraca asoca* planted in earthen pots were used as source of explants. The salient findings of the investigation are presented below.

1. Among the different explants tried, nodal segments were found to be the best explant.
2. ½ MS medium was found to be the best for culture establishment. The response of explants to culture in MS medium and WPM was low.
3. Surface sterilization of the explants was carried out effectively by soaking the explant in 70 per cent alcohol for 3 minutes followed by soaking them in 0.1 per cent Hg Cl2 for 5 minutes.
4. Increasing the concentration of the surface sterilant or the time of soaking was deleterious to the explants.
5. Polyphenol exudation was checked by subculturing once in the same media three days after inoculation of the explant.
6. It was observed that among the cytokinins tried, BAP was superior to Kinetin and 2,4-D .

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IN VITRO PROPAGATION
IN ASHOKA
Saraca asoca (Roxb.) de Wilde

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Asoka (*Saraca asoca*) is an important medicinal and ornamental tropical tree currently facing the threat of extinction due to overexploitation of trees occurring in forests and other natural habitats. Unscientific and destructive extraction of bark from trees have lead to acute shortage of raw bark by ayurvedic industries. Hence, the International Union for Conservation of Nature and Natural Resources (IUCN) has listed this species under 'globally vulnerable' category. It is also enlisted among the 36 threatened and endangered medicinal plants of India.

It is considered as the sacred tree of buddhists and Hindus. Literally the term 'asoca' means ' sorrow-less' and the tree is believed to remove the grief and unhappiness. The tree has immense medicinal properties. Its bark is considered as the primary medicinal part. Due to its acute short supply compared to its demand, various development and research activities are being prioritized to conserve, utilize and improve this species. It is mainly propagated by seeds. Due to heterozygous and cross pollinated nature of the species, it never give a true to type progeny. Therefore the present study was undertaken to standardise the technique of *in vitro* propagation of *Saraca asoca*. Standardization of suitable explants, surface sterilization procedures and culture establishment protocol, Induction of multiple shoots and Elongation of root, hardening and planting out are the major objectives of the study.

Nodal segment, Internodal segment and shoot tip were the three explants tried. Various surface sterilisation procedures were tried using Chloramphenicol, ethyl alcohol , 0.1% mercuric chloride and combination of ethyl alcohol and mercuric chloride in various concentration and duration, using nodal segments as explant. Surface sterilisation using 70% ethyl alcohol for 3 minutes followed by 0.1% mercuric chloride for 5 minutes proved to be the best, which gave the maximum survival percentage of 80. The next part of the study was standardisation

of suitable explants for culture establishment. Among the three explants, Nodal segments gave maximum response of 60 per cent in ½ MS medium with BAP 0.5mg/l. This was followed by shoot tips in the same medium, which gave 10 per cent response. Internodal segments did not respond in any of the media used.

Standardisation of basal media for culture establishment was done using nodal segments as the explants. Three media supplemented with BA 0.5 mg.l^{-1} were tried viz. MS, Half strenght MS, Woody plant media. Among the three, ½ MS media was identified as the best basal medium followed by MS medium. No response was seen in WPM medium.

Culture establishment as well as Shoot bud initiation was attempted in ½ MS and MS media with various growth regulator combinations. Maximum response of 60 per cent was obtained in ½ MS medium containing BAP 0.5 mg/l followed by 30 percent in the same medium containing BAP 1.5 mg/l . There were no response with 2,4-D. The response obtained was callusing in all cases.

Induction of multiple shooting was tried in ½ MS medium supplemented with BAP, and Kn alone as well as combinations of BAP, IAA at various concentration. Here highest response of 30 per cent of single shoots was recorded in ½ MS media containing BAP 0.5 mg/l. Response was in the form of single shoot. The single shoots with a mean length of about 1.5mm after one week of growth was obtained. With BAP 2.0 mg/l, single shoots were produced in about 5% of cultures within 54 days. Effect of Kn in various concentration ranging from 0.5 to 2.0 mg/l was found to be low in shoot induction. The maximum length of shoot of about 1.6 cm was recorded in combination of BAP 0.5 mg/l and IAA0.5 mg/l. various combinations of IAA and IBA at different concentrations were tried for rooting of *in vitro* shoots. However there was no response in any of the combinations tried.