STANDARDISATION OF *IN VITRO* PROPAGATION TECHNIQUES IN *THATHIRI* (*Woodfordia fruticosa* (L.) Kurz.)

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that this thesis entitled **'Standardisation of *in vitro* propagation techniques in *thathiri* (*Woodfordia fruticosa* (L.) Kurz.)'** 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 to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara,
31.08.05

Gayathri, G.

CERTIFICATE

Certified that this thesis entitled **'Standardisation of *in vitro* propagation techniques in *thathiri* (*Woodfordia fruticosa* (L.) Kurz.)'** is a record of work done independently by Mrs. **Gayathri, G.** 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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EXTERNAL EXAMINER

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LIST OF ABBREVIATIONS

Benzyl Amino Purine	**- BAP**
Naphthalene Acetic Acid	**- NAA**
2,4- Dichloro Phenoxy Acetic Acid	**- 2,4-D**
Indole Acetic Acid	**- IAA**
Indole Butyric Acid	**- IBA**
Murashige and Skoog Medium	**- MS**
Schenk and Hildebrandt Medium	**- SH**
Woody plant Medium	**- WPM**

INTRODUCTION

1. INTRODUCTION

Plants have been used as therapeutic agents from the earliest days of man's existence. The ancient Indian system of medicine is predominantly a plant based materia medica, making use of our native plants. It caters to almost the entire rural population of our country. A perusal of literature showed that Indian medicinal plants attracted the attention of various scholars both from within the country and abroad.

Indigenous medicinal flora has always provided a base for upgradation and synthesis of biologically active drugs. For example, the discovery of reserpine from *Rauwolfia serpentina* and its role as an effective antihypertensive and tranquilizing agent was a major breakthrough in the twentieth century. Later isolation of vinblastine and vincristine from *Cantharanthus roseus* as anticancer drugs and discovery of natural drugs like diosgenine from *Dioscorea deltoidea* and solasodine from *Solanum laciniatum* used in the synthesis of steroid hormones denoted the importance of herbal medicines. Similarly, other uses of natural drugs for treatment of leucoderma, cardiac diseases, liver cirrhosis, etc attracted world attention for herbal medicine as effective remedies. This brought about an ever increasing demand for the medicinal plants and necessitated their cultivation on a commercial scale.

Woodfordia fruticosa (L.) Kurz commonly called as *thathiri* in Malayalam, is a spreading arborescent shrub of the hillsides distributed in the tropical regions of Asia and Africa (Biswas and Chopra, 1982). It is a large and deciduous shrub commonly distributed in the Western Himalayas. The plant has various medicinal uses. It is used for the treatment of bowel complaints, as an antihaemorrhagic and antipyretic (Badola,1987). It grows abundantly on the rocky and dry sites, landslips and other abandoned areas, which makes it an important crop for reclamation of degraded soils (Bahuguna *et al.,* 1988).

Flowers of *thathiri* are the most effective fermentation agents in ayurvedic medicines (Kroes *et al*., 1990). In Kerala, the dried flowers along with the buds and broken pieces of the inflorescence is an essential ingredient in many ayurvedic preparations like arishtams and asavams which aids in alcoholic fermentation (Anon 2003). This poses a threat to the seed set of the plant as the flowers are harvested in bulk. Raju *et al*. (1994) reported that the cuttings of *thathiri* are difficult to root irrespective of the season of rooting and the hormonal treatments given to the cuttings. Hence there remains the possibility of propagating the plant through *in vitro* culture.

Micropropagation owns a unique distinction as a quick and easy method of deriving plants with identical genetic constitution. This technique has been used by researchers to a large extent for the commercial exploitation of many taxa which are difficult to propagate, rare or endangered (Wochok, 1981). This promises the possibility of extensive multiplication of elite plants through exploitation of tissue culture techniques. There has been only a single report on the micropropagation of *Woodfordia fruticosa* (L.) Kurz by Krishnan and Seeni (1994).

Keeping all this in view, the present study was undertaken with the following objectives.

1. To identify the best explant for *in vitro* propagation of *thathiri*
2. To identify a suitable media for *in vitro* culture
3. To work out the best combinations of growth regulators for *in vitro* culture establishment, proliferation and rooting
4. To standardise the protocol for direct and indirect regeneration of *thathiri*
5. To assess the variability present, if any, in the callus mediated plantlets produced

Review of Literature

2. REVIEW OF LITERATURE

Plants have always been used as an important source of medicine for treating diseases in many societies. Transition from synthetic drugs to green medicines is gaining importance. The recent resurgence of interest in herbal foods, medicines and cosmetics has resulted in an increased demand for the medicinal plants leading to over exploitation, unsustainable harvesting and a virtual extinction of several plant species in the wild. The increased demand from the developed countries for the therapeutically active alkaloids has necessitated the cultivation of important medicinal plants on commercial scale.

Woodfordia fruticosa (L.) Kurz is a much-branched beautiful shrub belonging to the family Lythraceae with fluted stems and long spreading branches. It has a height of about 1-3m on an average. It commonly occurs in North India but is scarce in South India. *Woodfordia* is a small genus of arborescent shrubs distributed in the tropical parts of Asia and Africa. *Lagerstroemia, Duabanga, Lawsonia, Sonneratia, Crypteronia* and *Cuphea* are the other genera reported in the family Lythraceae (Brandis, 1906). Only one species of *Woodfordia* is found in India, which is named *Woodfordia fruticosa* (L.) Kurz (CSIR, 1969).

The plant is known by several names. In English, it is called Fire flamed bush. Known by the name *Dhataki* in Sanskrit and *Dhai* in Hindi, the plant is called *Velakkai* in Tamil (Chatterjee and Pakrashi, 1994). The systematic position of *thathiri* as given by Bentham and Hooker (1884) is as follows.

Systematic position of *thathiri*

Kingdom: Plantae.

Division: Phanerogams.

Subdivision: Angiosperms.

Class: Dicotyledonae.

Sub Class: Polypetalae.
Series: Calyciflorae.
Order: Myrtales.
Family: Lythraceae.
Genus: Woodfordia.
Species: fruticosa

Woodfordia fruticosa (L.) Kurz is synonymous to *Woodfordia tomentosa* Bedd.man., *Woodfordia floribunda* Salisb., *Grislea tomentosa* Roxb. and *Lythrum fruticosum.*

Albeit a lot of research having been done on the biochemical constituents and their properties in the plant, basic studies on the morphology and crop improvement are meagre. The available literature on *Woodfordia fruticosa* is limited. Hence the literature citations for the present work were broadened to cover the related genera of *Woodfordia*, woody plants with medicinal properties and vegetatively propagated species.

2.1 Botany of *thathiri*

Thathiri (*Woodfordia fruticosa* (L.) Kurz) is a bushy arborescent perennial shrub with medicinal properties. The bark is reddish brown in colour that peels off in thin fibrous strips. The branches are long, spreading and pubescent with young branchlets and numerous small black glands. The leaves are ovate or linear lanceolate with grey pubescence on the underside. They are arranged in an opposite or sub opposite manner. High temperature and long photoperiod leads to extensive flowering in the plant during February and March (Awasthi *et al.,* 2002). Flowers are numerous, brilliant red in colour produced in dense axillary paniculate cymose clusters. The showy scarlet flowers are tubular and borne on slender pedicels dilated above. Fruits, which are produced in April to June, are capsules that are ellipsoid and membranous. Capsules are included in

the calyx. Seeds within the capsule are minute, brown, smooth and cuneate-obovate.

2.2 Biochemical constituents in *thathiri*

Different workers have attempted biochemical investigations of *Woodfordia fruticosa* (L.) Kurz and have identified several polyphenols and tannins in the leaves, flowers and fruits of the plant. Polyphenols isolated from the leaves and fruits include polystachoside, myricetin-3-galactoside, pelargonodin-3,5- diglucoside, octacosanol, β-sitosterol and chrysophanol-8-O- beta- D-glycopyroside (Nair *et al.*,1976; Chauhan *et al.,*1979). Tannins identified include Woodfordin A to I, Oenothin A and Isoschimawalin A (Kadota *et al.,* 1990a; Kadota *et al.*, 1990b; Yoshida *et al.*, 1990 and Yoshida *et al.*, 1992). Woodfruticosin (Woodfordin C) has an inhibitory activity towards DNA topoisomerase II (Kadota *et al.*,1990b). Kroes *et al.* (1993b) reported that gallic acid released by the flowers of *thathiri* acted as an antioxidant in Ayurvedic medicines.

2.3 Therapeutic uses of *thathiri*

Almost all parts of the plant are having medicinal properties. Flowers are reported to have immunomodulatory activities (Labadie *et al. ,* 1989). Leaves of the plant are used in the traditional medicines of India and Nepal as they have antibiotic and sedative properties (Kadota *et al.,* 1990a). The flowers of *thathiri* known as *Sedowaya* or *Sidowaya* are used in the preparation of medicines against diarrhoea, rheumatism and sprue (Yoshida *et al.,* 1992). The flowers are also reported to be the most effective fermentation agents in Ayurvedic medicines (Kroes, *et al*., 1990; Kroes, *et al*., 1993a). The root-bark decoction is used against leucoderma, stem-bark decoction for septic wound wash, leaf decoction against indigestion and flower decoction against seminal weakness (Pal and Jain, 1998). In folk medicine, it is used for the treatment of burns (Bhattacharya, 1991) and for

teething trouble in children (Jain, 1994). Mukherjee *et al.* (1996) used the flowers of *Woodfordia fruticosa* with other ingredients for the preparation of a herbal uterine tonic, which had similar action of oxytocin without any toxic effects. In the Unani system of medicine, it is used in the preparation of vaginal tablets as haemostatic, astringent, anti-inflammatory and analgesic (Fatima *et al.,* 2000).

In veterinary medicine, the extract of the flowers of *Woodfordia fruticosa* is used as a wound wash and also to cure diarrhoea (Pal and Jain, 1998). Charu *et al.* (2003) reported that the methanolic extracts of the plant have fungicidal activity against the fungal pathogens *Fusarium oxysporum* and *Macrophomina phaseolina.*

2.4 Propagation in *thathiri*

The plant is amenable to both sexual as well as asexual methods of propagation. Seeds collected from the fruits during March- April are used to raise plants of the next generation. The plant can also be propagated by vegetative means by rooting of cuttings.

2.4.1 Seed propagation

The seedlings shrink and die off within a few weeks of emergence. This makes the seed propagation of the species a difficult process. Bahuguna *et al.* (1988) reported that the seedlings of *thathiri* are highly susceptible to damping off and hence difficult to raise. Seeds germinate within seven to twelve days of sowing but lose their viability within six months (Bhagat *et al.,* 1992). According to Paily and D'Souza (1986), seed germination in *Lagerstroemia flos-reginae,* another member of the Lythraceae family, is also slow and difficult.

2.4.2 Vegetative propagation

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. The effect of growth regulators like NAA and IBA in the rooting of stem cuttings of *thathiri* was studied by Chauhan *et al.* (1994) and they reported that prolonged dipping in NAA 100ppm gave the best result. Raju *et al.* (1994) has reported that *thathiri* 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 *Woodfordia* species through *in vitro* techniques. 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.

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.

In *Cuphea ericoides*, another member of Lythraceae, Rita and Floh (1995) obtained plant regeneration on stem and leaf derived callus when cultured on MS medium supplemented with hormones. Similar results were obtained in other species of *Cuphea viz., C wrighti* and *C. procumbens* by Truta *et al*. (2002).

Micropropagation of *Lawsonia inermis* in the Lythraceae family was attempted by Rout *et al.* (2001). They developed a successful protocol for mass propagation of the species by culturing the apical and axillary meristems on MS medium supplemented with different growth regulators.

2.4.3.1 General Aspects of Plant Tissue Culture

Schleiden (1838) and Schwann (1839) postulated the cell theory, which revealed the totipotent nature of plant cells. This forms the basis for plant cell, tissue and organ culture. Haberlandt (1902) reported that isolated cells are capable of resuming uninterrupted growth. Skoog and Miller (1957) with their discovery of auxins and cytokinins made a landmark in the history of plant tissue culture. 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. A completely defined nutrient medium for plant tissue culture was developed by Murashige and Skoog (1962).

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) advocated the possibility of three routes of *in vitro* propagule production, which included enhanced release of axillary buds, production of adventitious shoots through organogenesis and somatic embryogenesis. Vasil and Vasil (1980) reported that the tissue culture derived plantlets grow faster and mature earlier than seed propagated plants.

2.4.3.1.1 Factors influencing success of *in vitro* propagation

Success of *in vitro* propagation depends on several factors directly and indirectly. These factors include genotype of the source plant, size, age 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).

2.4.3.1.1.1 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.

2.4.3.1.1.2 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. 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.

2.4.3.1.1.3 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 and Hackett, 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.* (1987) 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).

2.4.3.1.1.4 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 explant when compared to root tip, hypocotyl stem, leaf, epicotyl stem and shoot tip of *in vitro* grown seedling. 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.

2.4.3.1.1.5 Surface Sterilisation

The explants, especially those collected from the field grown conditions, may harbour a lot of microorganisms. When such explants are inoculated onto a nutrient medium, it will contaminate the whole system. Surface sterilisation is done to remove all the microorganisms present on the explant with minimum damage to the plant parts. 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. As

the surface sterilants are toxic to the plant cells, it is necessary to wash the tissues twice or thrice in sterile distilled water to ensure dilution of the chemical (Hu and Wang, 1983).

Bonga (1982) advocated the use of alcohol alone or in combination with other chemicals for disinfection. 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. 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.

2.4.3.1.1.6 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

2.4.3.1.1.7 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 (1943) 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. Callusogenesis in *Rosa* species was different in MS and SH media.The calluses that developed in the MS medium were hard and dark green while those in the SH medium were soft, pale greenish and nodular (Datta *et al*., 2002). Similarly in *Rosmarinus officinalis,* Misra (2002) noticed that in MS medium, the calluses were compact, fresh and green upto 30 days but growth was slow whereas in SH medium, the growth was rapid but the callus turned brown within 15 days.

2.4.3.1.1.8 Exudations from the explant

Lethal browning of the explant and exudations in the culture medium, hampers 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).

2.4.3.1.1.8.1 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 at 100 rpm 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 25μM 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. In *Lagerstroemia parviflora*, culturing the explants on a medium that was

supplemented with 100µM polyvinyl pyrolidone and 522.5µM citric acid controlled polyphenol exudation (Quraishi *et al.,* 1997).

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^{o} C than at 27^{o}C in *Pelargonium* x *Hortum*.

2.4.3.1.1.9 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. 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*).

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.

2.4.3.1.1.10 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.

2.4.3.1.1.11 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).

2.4.3.1.1.12 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^{o} 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).

2.4.3.1.1.13 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 0.9 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 preautoclaved 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.

2.4.3.1.1.14 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; Sutter and Langhans, 1982). 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 soilrite 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 investigations reported herein on the 'Standardisation of *in vitro* propagation techniques in *thathiri* (*Woodfordia fruticosa* (L.) Kurz.)' were carried out at the Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara during the period 2003-2005. The study was undertaken through the conduct of the following two experiments.

Experiment 1: Direct regeneration

Experiment 2: Regeneration through callus mediated organogenesis

3.1 Source of explants

Three month old rooted cuttings were brought from the Nagarjuna Nursery at Thodupuzha and were planted in earthen pots of diameter 45cm and depth 35cm which were filled with a mixture of sand, soil and dry cow dung in the ratio 1:1:1. The plants were kept in a net house covered with polythene sheet and were watered on alternate days. The plants were checked for the occurrence of pests and diseases. Hand weeding was done every two months to control the weeds in the pots.

3.2 Culture medium

3.2.1 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 (Murashige and Skoog, 1962), Schenk and Hildebrandt (SH) medium (Schenk and Hilderbrandt, 1972) 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, NAA and 2,4-D and cytokinins that included BAP and Kinetin singly or in combination in different experiments to study the effect of different growth regulators on culture establishment.

3.2.2 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.

3.2.3 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 and inositol were added fresh and well dissolved and volume made upto 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).

3.2.4 Sterilization of the culture medium

The tubes were plugged with non-absorbent cotton and autoclaved at 121^{o}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 *thathiri.*

Ingredients (mg/l)	MS	WPM	SH
Inorganic constituents			
$(NH_4)NO_3$	1650	400	
$(NH_4)H_2PO_4$			300
KNO_3	1900		2500
K_2SO_4		990	
KH_2PO_4	170	170	
$Ca(NO_3)_2.4H_2O$		556	
$CaCl_2.2H_2O$	440	96	200
$MgSO_4.7H_2O$	370	370	400
$FeSO_4.7H_2O$	27.8	27.8	15
Na_2EDTA	37.3	37.3	20
$MnSO_4.4H_2O$	22.3	22.3	
$MnSO_4.H_2O$			10
$ZnSO_4.7H_2O$	8.6	8.6	1
H_3B0_3	6.2	6.2	5
KI	0.83		1
$Na_2MoO_4.2H_2O$	0.25	0.25	0.1
$CuSO_4.5H_2O$	0.025	0.25	0.2
$CoCl_2.6H_2O$	0.025		0.1
Organic constituents			
Myoinositol	100	100	1000
Nicotinic Acid	0.5	0.5	5
Pyridoxine HCl	0.5	0.5	0.5
Thiamine HCl	0.1	1	5
Glycine	2	2	
Sucrose	30000	30000	30000

3.3 Preparation of the explants

Shoot tips, nodal segments and leaf bits were taken as explants for the study. Shoot tips of approximately 0.5 to 1.0cm in length were excised carefully from the plants and tender unfolded leaves were clipped off and were used as explants. Both mature and tender stem segments upto 3cm size were taken and cut into bits with one node each and used as explants. For the leaf explants, the mature and young leaves were collected and cut into 0.5cm long bits.

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. Fungicidal and insecticidal treatments were not given to the explants, as there were no symptoms of pest attack on the source plants.

3.4 Transfer area and aseptic manipulations

All the aseptic manipulations such as surface sterilisation of the explants, preparation and inoculation of the explants and subsequent subculturing were carried out in a clean laminar airflow chamber. The working table of the laminar airflow chamber was initially surface sterilised 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 sterilised before each inoculation. The hands were washed thoroughly with soap under running tap water. After drying, they were wiped with absolute alcohol before inoculation.

3.5 Culture establishment

3.5.1 Surface Sterilisation

Surface sterilisation was carried out under perfect aseptic conditions in the laminar airflow chamber. The washed explants were put into the sterilant and kept immersed for the required period. They were continuously agitated manually to ensure thorough contact of the explants with the chemical. The different sterilisation treatments tried for the explants are listed in Table 2. The explants after surface sterilisation were rinsed five times thoroughly with sterilised distilled water to remove traces of the sterilant from the surface of the explant.

3.5.2 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. As *thathiri* 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. A second sub culture was also attempted in some cases and its effect studied. The effect of light on polyphenol exudation was studied by incubating the inoculated culture tubes in the dark as well.

3.5.3 Culture conditions

The cultures were incubated at 25± 2^{o}C in an air-conditioned culture room with 10 hours photoperiod (1000 lux) supplied by cool white

Table 2 Different surface sterilisation treatments carried out for the explants of *thathiri* before inoculation

Sterilant	Concentration (%)	Duration (minutes)
$HgCl_2$	0.1	3
	0.1	4
	0.1	5
	0.1	6
	0.1	7
$HgCl_2$	0.5	1
	0.5	2
	0.5	3
	0.5	4
	0.5	5
Ethyl Alcohol	100	2
	90	2
	70	2
Ethyl Alcohol and $HgCl_2$	70, 0.1	2,4
	70, 0.1	2,5
	70, 0.1	2,6
	70,0.5	2,1
	70,0.5	2,2
	70,0.5	2,3

fluorescent light. Relative humidity in the culture room varied between 60 to 80 per cent according to the climate prevailing.

3.5.4 Standardisation of basal medium and explant

The best basal medium for *in vitro* culture of *thathiri* was identified by inoculating the explants into the three basal media *viz.* MS medium, SH medium and WPM which were supplemented with growth hormones to initiate culture in *thathiri* (Table 3). Shoot tips, nodal segments and leaf bits were employed for establishing cultures. The response of each explant in each media was recorded. The best media and explant were identified and carried over for Experiment 1 and Experiment 2.

3.6 Experiment 1: Direct regeneration

The best basal medium for multiple shoot induction was identified as SH medium and shoot tips were identified as the best explant for regeneration. Hence culture of shoot tips in different concentrations of growth regulators was attempted to identify the best growth regulator for multiple shoot induction and proliferation.

3.6.1 Shoot induction

3.6.1.2 Effects of medium supplements

Studies were conducted to determine the effect of various growth regulators on the induction of multiple shoots from shoot tips. Details of the treatments conducted are presented in Table 4. Surviving cultures were subcultured at an interval of 3-4 weeks to the same medium in which they were inoculated, for a period of three months. The response of the cultures in each

Table 3 Standardisation treatments of basal media for *in vitro* culture in *thathiri*

Treatment	Medium
T 1	MS + BAP 0.5 mg/l
T 2	MS + BAP 1.0 mg/l
T 3	MS + Kinetin 0.5 mg/l
T 4	MS + Kinetin 1.0 mg/l
T 5	MS + BAP 0.5 mg/l + NAA 0.5 mg/l
T 6	MS + BAP 1.0 mg/l + NAA 0.5 mg/l
T 7	SH + BAP 0.5 mg/l
T 8	SH + BAP 1.0 mg/l
T 9	SH + Kinetin 0.5 mg/l
T 10	SH + Kinetin 1.0 mg/l
T 11	SH + BAP 0.5 mg/l + NAA 0.5 mg/l
T 12	SH + BAP 1.0 mg/l + NAA 0.5 mg/l
T 13	WPM + BAP 0.5 mg/l
T 14	WPM + BAP 1.0 mg/l
T 15	WPM + Kinetin 0.5 mg/l
T 16	WPM + Kinetin 1.0 mg/l
T 17	WPM + BAP 0.5 mg/l + NAA 0.5 mg/l
T 18	WPM + BAP 1.0 mg/l + NAA 0.5 mg/l

subculture was observed and recorded. Survival rate in each sub culture, number of leaves, number of shoots and shoot length were recorded.

3.6.2 Shoot elongation

Multiple shoots in the proliferation medium were subcultured to different combinations of media for shoot elongation. Growth regulator concentrations were altered for obtaining shoot elongation. The treatments that were tried for multiple shoot elongation are presented in Table 4.

3.7 Experiment 2: Regeneration through callus mediated organogenesis

Shoot tips, nodal segments and leaf bits were cultured in SH media for callusing.

3.7.1 Effect of growth regulators in callus induction

SH medium was supplemented with different levels of auxins to induce callusing in the cultures. NAA, 2,4-D and NAA in combination with BAP were used to induce the calli and their effect studied. Details are given in Table 5.

Cultures were incubated in 16-hour photoperiod light at 25±2° C at a relative humidity of about 60 to 80 per cent depending on the external climatic conditions. The relative performance of different explants for callus induction and proliferation was observed. Observations were recorded for callus induction, growth rate and morphology. Callus Index (CI) was worked out as below:

$$\mathbf{CI = P \times G}$$

where P = percentage of callus initiation and

G = growth score.

Table 4 Treatment combinations of growth regulators for multiple shoot induction and elongation from shoot tips of *thathiri*

Treatment	Medium
For shoot induction	
T 19	SH + BAP 0.1 mg/l
T 20	SH + BAP 0.2 mg/l
T 21	SH + BAP 0.3 mg/l
T 22	SH + BAP 0.4 mg/l
T 23	SH + BAP 0.5 mg/l
T 24	SH + BAP 1.0 mg/l
T 25	SH + Kinetin 0.5 mg/l
T 26	SH + Kinetin 1.0 mg/l
T 27	SH + Kinetin 1.5 mg/l
T 28	SH + BAP 0.1 mg/l + NAA 0.5 mg/l
T 29	SH + BAP 0.2 mg/l + NAA 0.5 mg/l
T 30	SH + BAP 0.3 mg/l + NAA 0.5 mg/l
T 31	SH + BAP 0.4 mg/l + NAA 0.5 mg/l
T 32	SH + BAP 0.5 mg/l + NAA 0.5 mg/l
T 33	SH + BAP 1.0 mg/l + NAA 0.5 mg/l
For shoot elongation	
T 34	SH + BAP 0.2 mg/l
T 35	SH + BAP 0.4 mg/l
T 36	SH + BAP 0.5 mg/l
T 37	SH + BAP 1.0 mg/l
T 38	SH + BAP 0.2 mg/l + NAA 0.5 mg/l
T 39	SH + BAP 0.4 mg/l + NAA 0.5 mg/l
T 40	SH + BAP 0.5 mg/l + NAA 0.5 mg/l
T 41	SH + BAP 1.0 mg/l + NAA 0.5 mg/l

Scoring was done based on the spread of callus and a maximum score of four was given to those calli that have occupied the whole surface of the media, three months after incubation.

Score 1 = callus occupies ¼ of the media surface

Score 2 = callus occupies ½ of the media surface

Score 3 = callus occupies ¾ of the media surface

Score 4 = callus occupies complete media surface

3.7.3 Effect of media supplements in organogenesis / embryogenesis from the callus

Calli obtained from the nodal segments were subcultured in media containing different combinations of cytokinins and auxins for shoot induction. The various treatments tried to induce multiple shoots from the calli are presented in Table 5. The response of the calli was observed and recorded at fortnightly intervals.

3.8 Root induction

Shoots of length of more than 2.5cm from both Experiment 1 and Experiment 2 were excised from the elongated shoot cultures and were subjected to *in vitro* rooting. The shoots were cultured in different concentrations of auxins, which included both IBA and IAA. Treatments tried for root induction are given in Table 6. Root induction was also attempted in media devoid of growth hormones. Observations on root induction were recorded at weekly interval.

3.9. Hardening, acclimatisation and establishment

Rooted plants were removed from the culture vessels after sufficient number of roots was formed. The plants that were taken out of the culture tubes were washed in running tap water to remove all the remnants of

Table 5 Treatment combinations of growth regulators for callus induction and callus regeneration in *thathiri*

Treatment	Medium
For callus induction	
T 42	SH + NAA 0.1 mg/l
T 43	SH + NAA 0.5 mg/l
T 44	SH + NAA 1.0 mg/l
T 45	SH + 2,4-D 0.5mg/l
T 46	SH + 2,4-D 1.0 mg/l
T 47	SH + 2,4-D 1.5 mg/l
T 48	SH + BAP 0.2 mg/l + NAA 0.5 mg/l
T 49	SH + BAP 0.4 mg/l + NAA 0.5 mg/l
T 50	SH + BAP 0.5 mg/l + NAA 0.5 mg/l
For regeneration	
T 51	SH + BAP 0.1 mg/l + NAA 0.5 mg/l
T 52	SH + BAP 0.2 mg/l + NAA 0.5 mg/l
T 53	SH + BAP 0.4 mg/l + NAA 0.5 mg/l
T 54	SH + BAP 0.5 mg/l + NAA 0.5 mg/l
T 55	SH + BAP 1.0 mg/l + NAA 0.5 mg/l

Table 6 Treatment combinations of growth regulators for rooting of multiple shoots in *thathiri*

Treatment	Medium
T 56	SH + IBA 0.1 mg/l
T 57	SH + IBA 0.2 mg/l
T 58	SH + IBA 0.4 mg/l
T 59	SH + IBA 0.5 mg/l
T 60	SH + IBA 1.0 mg/l
T 61	SH + IAA 0.1 mg/l
T 62	SH + IAA 0.2 mg/l
T 63	SH + IAA 0.4 mg/l
T 64	SH + IAA 0.5 mg/l
T 65	SH + IAA 1.0 mg/l
T 66	SH without growth regulators (Control)

agar. They were transferred to clay pots of size 8cm diameter and 6cm depth containing autoclaved fine river sand and watered regularly. The pots were covered with polythene sheet to ensure sufficient relative humidity within the system. They were maintained in controlled nursery conditions for four weeks and after that the established plants were transferred to larger clay pots of diameter 45cm and depth 35cm containing fresh potting mixture (sand, soil and dry cow dung in the ratio 1:1:1 and reared in a shade net house under irrigation. Data on the percentage survival were recorded after four weeks.

3.7.4 Presence of somaclonal variants

The hardened plants which were planted in the main field were observed for the presence of any morphological abnormalities and chlorophyll mutants which might have arisen due to somaclonal variation. After four months of establishment, plant height, number of branches and presence of chlorophyll abnormalities were recorded.

RESULTS

4 RESULTS

The results of the various experiments carried out for the 'Standardisation of *in vitro* propagation techniques in *thathiri* (*Woodfordia fruticosa* (L.) Kurz.)' are presented in detail below.

4.1 Culture establishment

4.1.1 Surface Sterilisation

All plant materials used for culture are treated with an appropriate sterilisation agent to inactivate the microbes present on their surface. The effects of the surface sterilisation treatments on culture establishment in *thathiri* are presented in Table 7. The most effective sterilization was achieved by the combination treatment of soaking the explants in 70 per cent alcohol for two minutes followed by soaking them in $HgCl_2$ 0.1 per cent for five minutes where the percentage of live cultures was 80 and no cultures were contaminated. Sterilisation 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 20 per cent and 10 per cent respectively with the above treatments.

Increasing the time of soaking or the concentration of the sterilant adversely affected the survival of the explants. The explants did not survive in higher concentration of the sterilant at a longer period of sterilisation. Soaking the explant in any chemical for more than five minutes was not desirable. It 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 0.5 percent $HgCl_2$ beyond 2 minutes and in 100 per cent or 90 per cent ethyl alcohol for 2 minutes.

Table 7 Effect of surface sterilants on culture establishment of different explants in *thathiri* (one week after inoculation)

Sterilant	Concentration (%)	Duration (minutes)	Contamination (%)	Uncontaminated cultures	
				Dead (%)	Live (%)
$HgCl_2$	0.1	3	40	40	20
	0.1	4	20	10	70
	0.1	5	10	20	70
	0.1	6	Nil	100	Nil
	0.1	7	Nil	100	Nil
$HgCl_2$	0.5	1	10	40	50
	0.5	2	Nil	70	30
	0.5	3	Nil	100	Nil
	0.5	4	Nil	100	Nil
	0.5	5	Nil	100	Nil
Ethyl Alcohol	100	2	Nil	100	Nil
	90	2	Nil	100	Nil
	70	2	70	20	10
Ethyl Alcohol and $HgCl_2$	70, 0.1	2,4	20	20	60
	70, 0.1	2,5	Nil	20	80
	70, 0.1	2,6	Nil	80	20
	70, 0.5	2,1	30	20	50
	70, 0.5	2,2	10	60	30
	70, 0.5	2,3	Nil	100	Nil

*Average of 25 observations

4.1.1.2 Effect of different basal media on the culture establishment

The effect of three different basal media on the culture establishment of the explants of *thathiri* is presented in Table 8. The results show that the explants responded maximum to Schenk and Hildebrandt (SH) medium. In the MS medium, the response was shown only in T 5 where 30 per cent of the shoot tips cultured responded by multiple shoot induction. In WPM, response was limited to T 13 where only 10 percent of shoot tips cultures responded. The SH media was identified as the best media for *in vitro* culture of *thathiri* and hence for further studies in Experiments 1 and 2, SH media containing different concentrations of growth regulators were used.

4.1.1.3 Effect of different explants cultured on the culture establishment.

Among the three explants tried, namely shoot tip, nodal segments and leaf bits, the maximum response for multiple shoot induction was shown by the shoot tip explants and for callusing by the nodal segments. Shoot tips responded by multiple shoot induction in all the three media tried but the response was maximum in SH medium (Plate 1). Nodal segments responded by callusing only in T 11 and T 12. The leaf bit explants did not respond in any of the media. Hence for direct regeneration shoot tip was identified as the best explant and for regeneration through callus mediated organogenesis, nodal segments were the best. The details of the same are furnished in Table 8.

4.1.1.4 Standardisation of treatments to overcome polyphenol interference

When explants were inoculated, it was found that polyphenols exuded from the cut ends of the explant into the medium within 48 hours of culturing (Table 9). When the explants were retained in the same medium it caused browning and death of the cultures (Plate 2). The treatments undertaken to check the polyphenol interference in cultures are discussed further.

Table 8 Response of explants in different basal media in culture establishment of *thathiri* within 8 weeks

Treatment	Medium	Explant	Cultures responding (%)	Response
T 1	MS+BAP0.5mg/l	Shoot tip	Nil	
		Nodal segment	Nil	
		Leaf bit	Nil	
T 2	MS+BAP1.0mg/l	Shoot tip	Nil	
		Nodal segment	Nil	
		Leaf bit	Nil	
T 3	MS+Kinetin0.5mg/l	Shoot tip	Nil	
		Nodal segment	Nil	
		Leaf bit	Nil	
T 4	MS+Kinetin1.0mg/l	Shoot tip	Nil	
		Nodal segment	Nil	
		Leaf bit	Nil	
T 5	MS+BAP0.5mg/l+NAA 0.5 mg/l	Shoot tip	30	Multiple shoot
		Nodal segment	Nil	
		Leaf bit	Nil	
T 6	MS+BAP1.0mg/l+NAA 0.5mg/l	Shoot tip	Nil	
		Nodal segment	Nil	
		Leaf bit	Nil	
T 7	SH + BAP 0.5mg/l	Shoot tip	80	Multiple shoot
		Nodal segment	Nil	
		Leaf bit	Nil	
T 8	SH + BAP 1.0 mg/l	Shoot tip	50	Multiple shoot
		Nodal segment	Nil	
		Leaf bit	Nil	
T 9	SH + Kinetin 0.5 mg/l	Shoot tip	Nil	
		Nodal segment	Nil	
		Leaf bit	Nil	

Table 8 continued

T 10	SH + Kinetin 1.0 mg/l	Shoot tip	Nil	
		Nodal segment	Nil	
		Leaf bit	Nil	
T 11	SH+BAP0.5mg/l+NAA 0.5 mg/l	Shoot tip	90	Multiple shoot
		Nodal segment	50	Callusing
		Leaf bit	Nil	
T 12	SH+BAP1.0mg/l+NAA 0.5 mg/l	Shoot tip	80	Multiple shoot
		Nodal segment	20	Callusing
		Leaf bit	Nil	
T 13	WPM + BAP 0.5 mg/l	Shoot tip	10	Multiple shoot
		Nodal segment	Nil	
		Leaf bit	Nil	
T 14	WPM + BAP 1.0 mg/l	Shoot tip	Nil	
		Nodal segment	Nil	
		Leaf bit	Nil	
T 15	WPM+Kinetin 0.5mg/l	Shoot tip	Nil	
		Nodal segment	Nil	
		Leaf bit	Nil	
T 16	WPM+Kinetin 1.0 mg/l	Shoot tip	Nil	
		Nodal segment	Nil	
		Leaf bit	Nil	
T 17	WPM+BAP0.5mg/l+NAA 0.5 mg/l	Shoot tip	Nil	
		Nodal segment	Nil	
		Leaf bit	Nil	
T 18	WPM+BAP1.0mg/l+NAA 0.5 mg/l	Shoot tip	Nil	
		Nodal segment	Nil	
		Leaf bit	Nil	

*No. cultured in each treatment = 10

Plate 1: Shoot tip inoculated in SH medium

Plate 2: Polyphenol exudation from the explant

4.1.1.4.1 Effect of physiological age of the explant on polyphenol exudation

Nodal explants and leaf bits from the mature and tender parts of the plants along with shoot tips were evaluated for the exudation of phenols and it was found that mature nodal segments and bits of older leaves produce more polyphenols than tender nodal segments and bits of younger leaves. However, the intensity of polyphenol exudation was maximum in shoot tips. The results are given in Table 10.

One week after inoculation, all the older leaf bits inoculated were surviving in the culture medium. Only 60 per cent of the young leaf bits and 50 per cent of the tender nodal segments survived. Shoot tip cultures were the maximum affected where only 30 per cent of the explants inoculated survived after a week.

Upon retaining the explants in the same medium that contained the polyphenols, the survival rate of the explants decreased further. Maximum live cultures at two weeks were observed in older leaf bits (60 per cent) followed by tender nodal segments, mature nodal segments and young leaf bits (20 per cent each) and minimum in shoot tips (10 per cent). At three weeks after inoculation none except 10 per cent mature leaf bits were alive in the medium. None of the explants survived beyond three weeks in the medium containing polyphenols.

4.1.1.4.2 Effect of subculturing on the polyphenol exudation

The survival rate of explants when left as such in the medium that contained polyphenols decreased. Hence the explants were subcultured into the same basal media in which they were inoculated once in three days. This increased their survival rate to 86 per cent. When the explants were subcultured twice, the contamination rate increased to 48 per cent when compared to 10 per cent in one subculturing. Hence one subculturing on the third

Table 9 Effect of time on the polyphenol exudation from different explants of *thathiri*

Time / Explant	6 Hours	12 Hours	24 Hours	36 Hours	48 Hours
Shoot tip	Nil	Nil	Slight	Medium	High
Tender nodal segment	Nil	Nil	Nil	Slight	Medium
Mature nodal segment	Nil	Nil	Nil	Slight	Medium
Young leaf bit	Nil	Nil	Nil	Slight	Slight
Older leaf bit	Nil	Nil	Nil	Nil	Slight

Table 10 Effect of physiological age of the explant on the polyphenol exudation in *in vitro* culture of *thathiri*

Explant Type	Explant discoloura-tion	Medium discoloura-tion	Live cultures (%)			
			1 week	2 weeks	3 weeks	4 weeks
Shoot tip	High	High	30	10	Nil	
Tender nodal segments	Medium	Medium	50	20	Nil	
Mature nodal segments	Medium	High	40	20	Nil	
Young leaf bit	Low	Medium	60	20	Nil	
Older leaf bit	Low	Medium	100	60	10	Nil

***** No. of cultures for each type of explant = 10

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day of the initial inoculation was found to be the optimum for reducing polyphenol interference. The results are presented in Table 11.

4.1.1.4.3 Effect of light on the polyphenol exudation

Light did not have much influence on the behaviour of the culture establishment. The cultures which were kept in the dark did not show any significant difference in survival rate compared to those kept in the light. The results are given in Table 11.

4.2 Experiment 1- Direct regeneration

4.2.1 Enhanced release of multiple shoots

Detailed experiments were conducted to identify the most ideal growth regulator combination for the multiple shoot induction and proliferation in *thathiri*. Different levels of cytokinins like BAP and Kinetin and auxins like of NAA were incorporated in the different basal media.

4.2.1.1 Effect of cytokinins

BAP at levels of 0.1 to 1.0 mg/l were tried for multiple shoot induction using SH as the basal medium. The results are presented in Table 12.

In treatment T 23, 92 per cent of the cultures showed shoot proliferation. The effect of T 20, T 21 and T 22 were on par with each other having 88 per cent of the cultures with shoot proliferation. When the BAP concentration was increased to 1.0mg/l in T 24, only 72 per cent of the cultures responded by shoot induction. Kinetin at any level failed to induce multiple shoots in the cultures.

Table 11 Standardization of treatments to overcome polyphenol interference (after 3 weeks)

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	4	Nil	44	48
	Dark	Nil	Nil	64	36

*No. of tubes for each treatment = 20

The number of days taken for shoot proliferation ranged from 45 days for T 20 to 70 days for T 24. The treatments T 19 and T 22 were on par with 52 days for shoot induction. The cultures took 59 days for shoot initiation in T 23.

The mean number of multiple shoots induced per explant four weeks after proliferation was maximum in T 20 (3.7 shoots) followed by T 21 (3.4 shoots) and T 22 (2.8 shoots). When the BAP concentration was increased beyond 0.2 mg/l, the average number of multiple shoots decreased with a mean of only 1.5 shoots in T 24.

The mean length of the shoots induced ranged from 1.0cm in T 24 to 2.3 cm in T 20. When the concentration of BAP was increased from 0.2 mg/l to 1.0 mg/l, there was a proportionate decrease in the mean length of the shoots induced.

The average number of leaves induced per shoot also varied considerably. The highest number of leaves on an average was recorded in T 20 (5.6) followed by T 21 (4.2) and T 22 (3.8). The minimum number of leaves induced was for T 24 (2.5).

When BAP concentration was increased it reduced the average number of multiple shoots produced. It also resulted in shorter shoots with less number of leaves and lesser vigour. The days taken for shoot proliferation also increased considerably when the BAP concentration was either greater or lesser than 0.2 mg/l. Hence BAP at 0.2mg/l (T 20) was found to be the optimum for inducing multiple shoots in *thathiri* (Plate 3).

Table 12 Effect of cytokinins on multiple shoot induction from shoot tip explants of *thathiri* in SH medium

Treatment	Cytokinin	Conce-ntration in mg/l	Culture (%) showing shoot prolifer-ation	Days for shoot prolifer-ation	Average no.of multiple shoots induced per explant	Average length of shoots (cm)	Average no.of leaves induced per shoot.
T 19	**BAP**	0.1	80	52	2.5	1.6	3.1
T 20		0.2	88	45	3.7	2.3	5.6
T 21		0.3	88	48	3.4	1.9	4.2
T 22		0.4	88	52	2.8	1.5	3.8
T 23		0.5	92	59	1.8	1.3	3.5
T 24		1.0	72	70	1.5	1.0	2.5
T 25	**Kinetin**	0.5	Nil				
T 26		1.0	Nil				
T 27		1.5	Nil				

* No. of cultures in each treatment =25

4.2.1.2 Effect of combination of BAP and NAA

The response of shoot tip explants in the multiple shoot induction in different concentrations of BAP (0 to 1.0mg/l) in combination with NAA (0.5mg/l) is given in Table 13.

In T 32, 96 per cent of the cultures showed shoot proliferation. Ninety two per cent of the cultures responded by shoot induction in T 33. The effects of T 30 and T 31 were on par with each other with 88 per cent of the cultures showing multiple shoot induction. Only 84 percent of the cultures showed shoot proliferation in T 29. The treatments T 28 failed to induce multiple shoots in culture.

The number of days taken for shoot proliferation was maximum for the treatment T 33 (71 days) and it was minimum for T 32 (46 days). The number of days taken for shoot proliferation increased as the concentration of BAP increased.

The mean number of multiple shoots per explant ranged from 2.2 in T 29 to 4.0 in T 32. Increasing the BAP concentration beyond 0.5 mg/l in T 33 resulted in lowering the number of multiple shoots induced per explant to 3.2.

The mean length of the shoots induced was maximum in T 31 (2.8 cm). The minimum mean length of 1.2 cm of the shoots was observed in T 33. in T 32, the average length of the shoots induced was 2.7 cm.

The maximum number of leaves were induced in T 31 with a mean of 4.4 leaves. Only 2.4 leaves on an average were induced per shoot in T 33. The average number of leaves induced per shoot ranged from 2.4 to 3.9 in T 33 and T 32.

Table 13 Effect of BAP and NAA combination on multiple shoot induction and proliferation from shoot tip explants in SH medium

Treatment	BAP concen-tration (mg/l)	NAA concen-tration (mg/l)	Cultures showing shoot proliferation (%)	Days for shoot proliferation	Average number of multiple shoots per explant	Average length of shoots (cm)	Average no. of leaves induced per shoot
T 28	**0.1**	**0.5**	Nil				
T 29	**0.2**	**0.5**	84	67	2.2	1.4	2.8
T 30	**0.3**	**0.5**	88	62	2.5	1.8	3.5
T 31	**0.4**	**0.5**	88	58	3.1	2.8	4.4
T 32	**0.5**	**0.5**	96	46	4.0	2.7	3.9
T 33	**1.0**	**0.5**	92	71	3.2	1.2	2.4

* No. of cultures per treatment = 25

The multiple shoots produced in media rated as best (SH + BAP 0.5 mg/l + NAA 0.5 mg/l) attained a maximum length of 4.0cm with an average length of 2.7cm (Plate 4).

4.2.2 Elongation of *in vitro* produced shoots

Shoot elongation was attempted in the SH medium which was supplemented with BAP singly or in combination with NAA. The results obtained after three weeks of culturing are presented in Table 14.

Maximum shoot elongation was obtained in T 34 (9.8 mm) followed by T 40 (6.2 mm) and T 35 (5.9 mm) with 4 to 6 well developed leaves in all these treatments (Plate 5). The least elongation was observed in T 36 (3.5 mm) with 3 to 4 narrow leaves.

4.3 Experiment 2: Regeneration through callus mediated organogenesis

4.3.1 Callus induction and proliferation

The best medium for culture of *thathiri* was observed to be SH medium and the best explant for callus induction was found to be nodal segment (Table 8). The effect of different media combinations on callus induction and proliferation from the nodal segments of *thathiri* is given in Table 15.

Among the different treatments tried for callus induction, T 43 induced callus in 80 per cent of the cultures (Plate 6). In T 42 and T 44, 48 per cent and 32 per cent of the cultures induced callus respectively. When a combination of BAP and NAA was used for callusing, the callus percentage ranged from 32 per cent in T 49 to 44 per cent in T 50.

Table 14 Effect of different media combinations on elongation of *in vitro* multiple shoots in the SH medium

Treatment	Growth regulator	Concentration (mg/l)	Average increase in length (mm)	Change in leaf morphology.
T 34	**BAP**	0.2	9.8	4 to 6 well developed leaves
T 35		0.4	5.9	4 to 6 well developed leaves
T 36		0.5	3.5	3 to 4 narrow leaves
T 37		1.0	Nil	Nil
T 38	**BAP, NAA**	0.2, 0.5	Nil	Nil
T 39		0.4, 0.5	4.4	4 to 6 narrow leaves.
T 40		0.5, 0.5	6.2	4 to 6 well developed leaves
T 41		1.0, 0.5	Nil	Nil

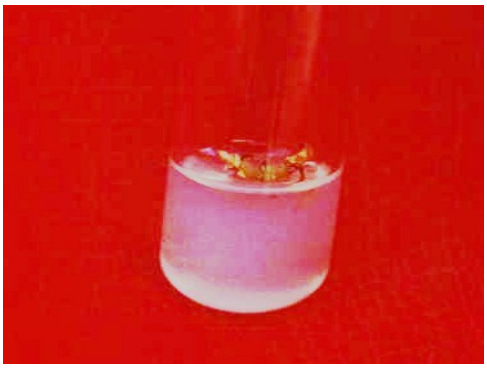

Plate 3: Multiple shoot induction in SH medium with BAP 0.2 mg/l

Plate 4: Multiple shoots induced in SH medium with BAP 0.5 mg/l and NAA 0.5 mg/l

Plate 5: Shoot in elongation medium with BAP 0.2 mg/l

Table 15 Effect of different media combinations on callus induction and proliferation from nodal segments of *thathiri*

Treatment	Growth regulator	Concentration (mg/l)	Days for callusing	Callus percentage	Growth score	Callus index	Nature of callus one month after induction
T 42	**NAA**	0.1	24	48	2	96	Cream, friable
T 43		0.5	24	80	4	320	Cream, friable
T 44		1.0	24	32	2	64	Cream, friable
T 45	**2,4-D**	0.5	Nil				
T 46		1.0	Nil				
T 47		1.5	Nil				
T 48	**BAP, NAA**	0.2, 0.5	32	36	2	72	Cream, friable
T 49		0.4, 0.5	35	32	1	32	Cream, friable
T 50		0.5, 0.5	35	44	1	44	Cream, friable

* No.of cultures per treatment= 25

The spread of the callus in the culture tubes ten weeks after incubation was maximum for T 43. The treatments T 42, T 44 and T 48 gave the same spread of callus. The lowest spread of the callus was noted for T 49 and T 50.

Callus Index (C.I) was highest (320) in medium containing 0.5mg/l of NAA (T 43). Callus index in other media combinations varied between 32 in T 49 to 96 in T 42. Any level of 2,4-D failed to induce callus in the cultures. In all cases, the calli produced were creamish yellow and friable one month after induction.

4.3.2 Regeneration from the callus

The calli were cultured in media that contained different concentrations of BAP and 0.5 mg/l NAA. The details are furnished in Table 16.

Maximum callus regeneration percentage was observed when the callus was cultured in T 54 (84 per cent). In T 53, the callus regeneration percentage was 76 while in T 55, it was reduced to 64 per cent.

The days for the proliferation of the callus were least in T 54 (12 days). It took 14 days for callus proliferation in T 53 and T 55. The creamish callus turned green during proliferation (Plate 7). The average number of multiple shoots induced six weeks after proliferation was maximum in T 54 (5.2). In T 55 the average number of shoots was reduced to 2.8.

The treatment T 54 was found to be the best treatment for callus regeneration with an average of 5.2 shoots per culture with a mean length of 2.5cm and 4.1 leaves on an average (Plate 8). An average of 3.7 shoots of length 2.3cm and 2.8 numbers of leaves were induced per culture in T 53. In T 55, the

Table 16 Effect of growth regulators on callus regeneration in *thathiri*

Treatm-ent	BAP concentr-ation (mg/l)	NAA concentr-ation (mg/l)	Callus showing prolifera-tion (%)	Days for prolifera-tion	Average No. of mutiple shoots per culture	Average length of the shoots (cm)	Average No. of leaves
T 51	0.1	0.5	Nil				
T 52	0.2	0.5	Nil				
T 53	0.4	0.5	76	14	3.7	2.3	2.8
T 54	0.5	0.5	84	12	5.2	2.5	4.1
T 55	1.0	0.5	64	14	2.8	2.0	3.1

***** No. of cultures per treatment = 25

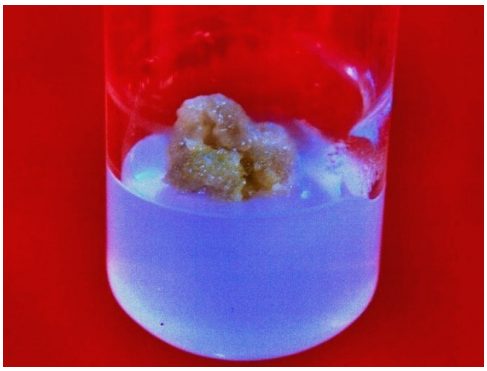

Plate 6: Six week old callus from nodal segment in SH medium with NAA 0.5 mg/l

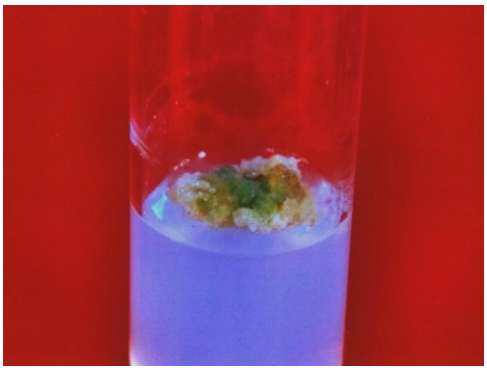

Plate 7: Callus turning green in regeneration medium with BAP and NAA @ 0.5 mg/l

Plate 8: Multiple shoots arising from callus in SH medium

mean number of multiple shoots (2.8) as well as the mean length of induced shoots (2.0) was low. No regeneration from the callus was observed in the treatments T 51 and T 52.

4.4 Rooting of *in vitro* produced shoots

The elongated multiple shoots from Experiment 1 and Experiment 2 having a length of more than 2.5cm were subjected to *in vitro* rooting. Results obtained are presented in Table 17.

The days for root induction were the lowest in T 57 (13 days) followed by T 58 (15 days), T 56 (18 days) and T 59 (18 days). The highest number of days for root induction was in T 66 (25 days) where auxins were not supplemented to the basal medium.

An average of 8.8 roots were induced in T 57. In the treatments, T 56 and T 58, the mean number of roots induced was 8.1 and 7.9 respectively. The lowest number of roots induced was for the treatment T 61 (4.3 roots).

The average length of the roots induced also varied considerably in the different treatments. It was the lowest for T 61 (3.6cm) followed by T 62 (3.9cm). The highest mean length of roots was recorded for T 58 (5.3cm) followed by T 60 (5.0cm).

In all the treatments tried, the roots produced were well developed and white in colour (Plate 9). The rooting media containing IAA was found to be inferior to the rooting media with IBA, with respect to number and length of roots induced.

Table17 Effect of different concentrations of growth regulators tried in the *in vitro* rooting of multiple shoots in SH medium

Treatment	Growth regulator	Concentr-ation (mg/l)	Days for root induction	Mean number of roots induced	Average length of roots (cm)
T 56	**IBA**	0.1	18	8.1	4.8
T 57		0.2	13	8.8	4.9
T 58		0.4	15	7.9	5.3
T 59		0.5	18	6.1	4.8
T 60		1.0	20	5.9	5.0
T 61	**IAA**	0.1	22	4.3	3.6
T 62		0.2	19	5.8	3.9
T 63		0.4	19	4.9	4.5
T 64		0.5	21	3.8	4.0
T 65		1.0	21	4.4	4.1
T 66	**No growth regulators**		25	6.5	4.4

4.5 Hardening and acclimatization

The rooted plants were planted in earthen pots filled with fine river sand which had been autoclaved previously and kept covered with polythene sheets. This maintained the relative humidity within the units. They were watered regularly. In experiment 1, 66.7 per cent of the plantlets and in experiment 2, 58.8 per cent of the plantlets survived the hardening treatment (Table 18). After four weeks in the net house conditions, the hardened plants were transferred to larger earthen pots containing potting mixture with sand, soil and dry cow dung in the ratio 1:1:1 (Plate10).

4.6 Survival in the main field and presence of somaclonal variants

The plants that had survived the hardening treatment were planted in large earthen pots containing potting mixture. Two months after transplantation, 56.3 per cent of the hardened plants from Experiment 1 and 60 per cent from Experiment 2 established well in the main field (Plate11). The established plants were free from any morphological abnormalities and chlorophyll mutations when observed during four months after transplantation (Table 19).

Table 18 Effect of hardening and establishment of *in vitro* cultured plantlets of *thathiri*

Experiment	Number planted	Number survived	Survival rate (%)	No. established in main field	Establishment rate (%)
1	24	16	66.7	9	56.3
2	17	10	58.8	6	60.0

Table 19 Morphological characters of the plants of *thathiri* in the field (four months after planting)

Plant No.	Height (cm)	No. of branches	Chlorophyll abnormality
Expt. 1- 1	48.2	6	Absent
2	45.7	6	Absent
3	38.9	4	Absent
4	47.0	5	Absent
5	35.5	4	Absent
6	38.6	4	Absent
7	42.6	6	Absent
8	44.9	6	Absent
9	47.3	6	Absent
Expt. 2 -1	41.4	5	Absent
2	39.7	5	Absent
3	38.1	4	Absent
4	46.5	6	Absent
5	43.7	6	Absent
6	44.3	6	Absent

Plate 9: Rooting of the shoots in media with IBA 0.2mg/l

Plate 10: Hardened plant in the field

Plate 11: Plants in the main field two months after planting

Discussion

5. DISCUSSION

Thathiri, *Woodfordia fruticosa* (L.) Kurz, 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 *Woodfordia fruticosa* (L.) Kurz at the Plant Tissue Culture Laboratory, Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara during 2003-2005. The results obtained are discussed hereunder.

5.1. Culture establishment

5.1.1. Surface sterilization

The most effective surface sterilisation was achieved by soaking the explants in 70 percent alcohol for two minutes followed by soaking them in 0.1 per cent $HgCl_2$ 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 the chemical beyond five minutes in low concentration (0.1) and beyond two minutes in higher concentration (0.5) 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) opined 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. Basal medium

In the present study, the culture establishment was the best in Schenk and Hildebrandt (SH) medium. Similar results were obtained by Krishnan and Seeni (1994) in *Woodfordia fruticose*; Das (1992) in *Agave sisalana*; Mallika *et al*. (1997) in nutmeg; Keshavachandran (1998) in cashew; Purohit and Singhvi (1998) and Purohit *et al*. (1999) in *Achras sapota*; Hazra *et al*. (2001) in sisal and Datta *et al*. (2002) in rose.

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 *thathiri* the response in WPM was negligible.

5.1.3. Explant

The best explant for direct regeneration in *thathiri* was found to be shoot tips. Shoot tips of *Woodfordia fruticosa* were found to be the best in inducing multiple shoots in *in vitro* conditions by Krishnan and Seeni (1994). The response of shoot tip in inducing multiple shoots may be due to their inherent regenerative capacity and the balance between auxin present in the explant and the cytokinin provided in the medium leading to the optimum auxin- cytokinin ratio.

In the present investigation, nodal segments tended to be callusogenic in the culture conditions and hence were used for indirect or regeneration through callus mediated organogenesis. However, 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). 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.

5.1.4. Polyphenol interference

The polyphenol interference in *thathiri* in the study was much pronounced and after 48 hours of culturing, the medium turned brown. *Thathiri* 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 *thathiri* 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) and 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. This is however in contrast with the reports of Krishnan and Seeni (1994) in *Woodfordia fruticosa* where they found that the browning of the medium was reduced to a minimum after three successive transfers each at three day intervals.

In the present study, apical shoots released more polyphenols than nodal segments. Similar results have been reported in clove by Mathew (1995). 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*.

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 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 1: Direct regeneration

In *in vitro* culture of *thathiri* shoot tips showed multiple shoot induction in SH medium containing 0.2 mg/l BAP. 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). 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). In the present investigation, for the induction of multiple shoots in *thathiri*, cytokinin in the form of BAP was found to be highly essential. Similar observations have been made in guava by Amin and Jaiswal (1988) and in cumin by Yadav *et al*. (1990a). However kinetin did not invoke any response in the explants. The superiority of BAP over kinetin for the induction, proliferation and subsequent growth of multiple shoot was also 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)

In *thathiri*, when BAP concentration was increased from 0.2 to 1.0 mg/l, it reduced the average number of multiple shoots produced. It also resulted in shorter shoots with less number of leaves and lesser vigour. Similar inhibitory effects of BAP at higher concentrations has been reported in guava (Jaiswal and Amin, 1987); mangosteen (Goh *et al*., 1988); pepper (Philip *et al*., 1992) and clove (Mathew,1995). Production of stunted shoots at higher levels of BAP has been reported by Goh *et al*. (1988) in mangosteen; Yadav *et al*.(1990b) in mulberry and Mathew (1995) in clove.

Shoot multiplication was a function of cytokinin activity but sustained growth of shoots depended on a synergistic balance between BAP and NAA. When a combination of BAP and NAA was supplemented in the culture medium, there was a better response in multiple shoot induction as the average number and length of the multiple shoots increased than in the medium where BAP alone was added. Hence the best combination of growth regulators in the present study for the induction of multiple shoots in the SH medium was found to be 0.5mg/l BAP and 0.5mg/l NAA. The combination of BAP and NAA for shoot initiation and multiplication corresponds well with the findings of Razdan (1990) where NAA was considered more effective than IAA due to its better stability. The combination of BAP and NAA is reported to be the best for multiple shoot induction in several crops like melina (Yang *et al*., 1992); nutmeg (Mallika *et al*.,1997); cashew (Keshavachandran, 1998); *Lagerstroemia reginae* (Sumana and Kaveriappa, 2000); *Utleria salicifolia* (Gangaprasad *et al*.,2003) and jute (Nandy *et al*., 2005).

When the concentration of BAP was increased from 0.5mg/l, it reduced the number and vigour of the induced multiple shoots. This has also been reported in *Gmelina arborea* by Thirunavoukkarasu and Debata (1998) where higher concentrations of BAP (1.0 to 1.5mg/l) in combination with NAA (0.5mg/l) resulted in very few or no shootlets.

For the elongation of the multiple shoots, BAP at 0.2mg/l or a combination of BAP and NAA each at 0.5mg/l was found to give good results in the study conducted herein. Maximum elongation of 5.0 to 15.0mm with four to six well-developed leaves in four weeks was observed in SH medium supplemented with 0.2mg/l BAP. Low BAP concentration has been used for elongation of the induced multiple shoots in other crops like guava (Amin and Jaiswal, 1988); chestnut (Chauvin and Salessses, 1988); walnut (Pennela, 1988); mulberry (Yadav *et al*., 1990b) and clove (Mathew, 1995).

5.3. Experiment 2: Regeneration through callus mediated organogenesis

Plants regenerated from the excised shoot apices and axillary buds *in vitro* appear to preserve the integrity of the parental genotype, whereas those regenerated from callus often demonstrate considerable instability (Amato, 1978). But as far as the rate of multiplication and induction of genetic variation are concerned, callus mediated somatic organogenesis or embryogenesis are reported to have greater potentialities than axillary bud release method.

Evans *et al*. (1981) suggested that within a given plant, success of callus initiation is dependent on the explant source and that the variation in different explants may reflect the difference in the phenotypic physiological expression of cells in the original explant.

In the present study, callusing was observed from the cut surface of explant above the medium when nodal segments were cultured in SH medium. The initiation of callus from the cut surfaces may be due to higher accumulation of the endogenous auxin level favouring an active cell division producing a mass of cells. This can also be attributed to the exposure of cells at the cut surfaces to an excessive supply of nutritive substances as compared with the cells adjoining intact region of the explant (Mitra *et al*., 1965). Callusing from cut ends was also observed in clove (Mathew, 1995).

Among the different growth regulators tried for inducing callus in the explants, NAA was found to be the best. Maximum growth of the callus was obtained in the SH medium containing 0.5mg/l of NAA. Hasegawa (1980) reported that a high concentration of auxin induces callus formation. Similar observations have been made by Datta *et al*. (2002) in rose. 2,4-D, the commonly used auxin for callusogenesis, did not induce callus in *thathiri*.

The combination of BAP along with NAA did not substantially increase the percentage of callus formation in the explants. This is in contrast with the observations of Datta *et al*. (2002) in rose and Nandy *et al*. (2005) in jute that a combination of BAP and NAA produced better calli than when cultured with NAA alone.

When NAA alone was used in the medium, callusing was induced within 24 days. However when a combination of NAA and BAP were added to the medium, it took more than 30 days for callusing. This is in contrast with the observations of Sondahl and Sharp (1977) in coffee and Mathew (1995) in clove that callus formation was a very slow process and it took two to three months of culture for substantial callus formation. Woody plants generally show a slower response to culture conditions than herbaceous angiosperms.

Callus obtained was initially creamy yellow in colour and friable in all cases one month after induction. This is in compliance with the reports of Mathew (1995) in clove and Nandy *et al*. (2005) in jute.

The calli when cultured in SH medium that contained 0.5mg/l BAP and 0.5mg/l NAA turned green within a period of two weeks. Multiple shoots were produced from this callus in a period of six weeks that ranged from two to five in number. Similar observations have been made in *Rosmarinus officinalis* by Misra (2002) and jute by Nandy *et al*. (2005).

5.4. *In vitro* rooting of the multiple shoots

The best response to *in vitro* rooting was observed when the multiple shoots were cultured in SH medium with 0.2mg/l IBA. IAA was less effective in inducing rooting when compared to IBA. This is in concurrence with the observations of other workers in different crop plants including *Woodfordia fruticosa* (Krishnan and Seeni, 1994); fig (Kumar *et al*., 1988); chestnut (Sanchez *et al*., 1997) and *Utleria salicifolia* (Gangaprasad *et al*., 2003).

Well-developed white roots were obtained when the multiple shoots were cultured on SH medium devoid of any growth hormones but the days taken for root induction was more. Rooting in media devoid of growth regulators has been observed in different crop species like *Brassica campestris* (Bhattacharya and Sen, 1980); crape myrtle (Zhang and Davies, 1986); *Agave sisalana* (Das, 1992) and jute (Nandy *et al*., 2005).

5.5. Hardening and Planting Out

The rooted plants were transferred to autoclaved fine river sand filled in small earthen pots and watered regularly. The pots were kept covered with polythene sheet to maintain humidity. Hu and Wang (1983) have suggested a period of humidity acclimatization for the newly transferred plantlets to make them adapted to the external environment. This method has been used for hardening of the rooted plants by Das (1992) in *Agave sisalana* and Karwa (2003) in *Citrus reticulata*. Maintenance of humidity was reported to be essential in the hardening phase of *Adhatoda beddomei* (Sudha and Seeni, 1994) and *Utleria salicifolia* (Gangaprasad *et al*., 2003). For experiment 1, 66.7 per cent of the plants and in Experiment 2, 58.8 per cent plants survived this hardening treatment. After four weeks in the net house the hardened plants were transferred to earthen pots filled with potting mixture. Krishnan and Seeni (1994) reported that the

rooted plants of *Woodfordia fruticosa* were directly transferred to the potting mixture to omit the step of hardening usually followed so as to make the system more economical and less cumbersome.

The reproductive biology of the tissue culture derived plantlets could not be studied within the time frame of this investigation as the plants will come to flowering only after one year of establishment in the field. Hence as a further work, the plants should be monitored and studied. Since the protocol of producing tissue culture derived plantlets has been standardised, this could be further expanded on commercial and profitable basis to meet the demands of ayurvedic drug industry after working out the economics of the cost of production.

SUMMARY

6. SUMMARY

The present investigation was carried out during the period 2003-2005 in the Tissue Culture Laboratory of The Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara with the objective of standardizing i*n vitro* propagation technique in *thathiri* (*Woodfordia fruticosa* (L.) Kurz. Six month old rooted cuttings of *thathiri* 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, shoot tips were found to be the best explant for direct regeneration and multiple shoot induction and nodal segments were suited for regeneration through callus mediated organogenesis.
2. Among the different basal media tried, SH 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 2 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 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 Kinetin did not evoke any response in multiple shoot induction.
7. BAP at a concentration of 0.2mg/l and in a combination with NAA (each at 0.5mg/l) produced the best response in multiple shoot induction.
8. Higher concentration of BAP showed inhibitory action on multiple shoot induction and proliferation.

9. BAP at 0.2mg/l gave elongated shoots with four to six well developed leaves.
10. Nodal segments did not favour multiple shoot formation and were callusogenic in nature.
11. Among the auxins tried for callusing, NAA was found to be superior to 2,4-D.
12. The best callus was obtained in media with NAA 0.5 mg/l.
13. Callus regeneration was the highest in media containing BAP 0.5mg/l and NAA 0.5mg/l.
14. IBA was found to be superior to IAA in inducing rooting in the shoots.
15. IBA at 0.2mg/l gave the best response in rooting with highest number of roots and lowest days for root induction.
16. Rooting was also observed in media devoid of growth regulators.
17. Rooted plants were planted in small earthen pots filled with autoclaved river sand for hardening and kept covered with polythene sheet.
18. After four weeks of hardening, the hardened plants were transferred to the main field.
19. After four months of planting in the pots, the height of the plants ranged from 35.5cm to 48.2cm with four to six branches in experiment 1.
20. In experiment 2, the height of the plants were in a range of 38.1 to 46.5 cm with four to six branches and chlorophyll abnormality was absent in both experiments.

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STANDARDISATION OF *IN VITRO* PROPAGATION TECHNIQUES IN *THATHIRI* (*Woodfordia fruticosa* (L.) Kurz.)

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ABSTRACT OF THE THESIS

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ABSTRACT

The present study 'Standardisation of *in vitro* propagation techniques in *thathiri* [*Woodfordia fruticosa* (L.) Kurz.]' was undertaken in the Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara during 2003-2005 through the conduct of two experiments *viz*. (i) direct regeneration and (ii) regeneration through callus mediated organogenesis.

SH medium was found to be the best basal medium for in vitro culture of *thathiri*. Shoot tips were the best explants for direct organogenesis and nodal segments were used as explants for indirect organogenesis. Surface sterilization of the explants was done by soaking them in 70 per cent alcohol for two minutes followed by soaking them in 0.1 per cent $HgCl_2$ for five minutes. One subculturing three days after inoculation checked the polyphenol interference. Multiple shoot induction was obtained when shoot tips were cultured in medium supplemented with BAP 0.5 mg/l and NAA 0.5 mg/l. The shoot elongation was best in media with BAP 0.2mg/l. Callus formation in the nodal explants of *thathiri* was best in media with NAA 0.5 mg/l while callus regeneration was superior in media containing BAP 0.5 mg/l and NAA 0.5 mg/l. The best response in rooting was observed in media with IBA 0.2 mg/l. Rooted plants were hardened in earthen pots containing sterile sand and covered with polythene cover. After four weeks they were transferred to larger pots in the main field.