INVESTIGATIONS ON *IN VITRO* REGENERATION OF *COUROUPITA GUIANENSIS* AUBL. (NAGALINGAM TREE) –A THREATENED BUT MEDICINALLY IMPORTANT PLANT

A PROJECT REPORT

(Research Project - Grant-in-Aid)

Submitted to

DEPARTMENT OF SCIENCE, TECHNOLOGY & ENVIRONMENT

GOVT. OF PUDUCHERRY

III FLOOR, PHB BUILDING, ANNA NAGAR

PUDUCHERRY

BY

DR. MAHIPAL SINGH SHEKHAWAT

Principal Investigator

Kanchi Mamunivar Centre for Post Graduate Studies

(Centre with Potential for Excellence by UGC)

Puducherry-605 008

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KANCHI MAMUNIVAR CENTRE FOR POST GRADUATE STUDIES
LAWSPET, PUDUCHERRY-605 008

FOREWORD

It is gratifying to note that the Plant Biology and Biotechnology Department of Kanchi Mamunivar Centre for Post Graduate Studies, Puducherry has undertaken a project on "Investigations on *In vitro* Regeneration of *Couroupita guianensis* Aubl. (Nagalingam Tree) – A Threatened but Medicinally Important Plant", under the sponsorship of the Department of Science, Technology and Environment, Govt. of Puducherry.

The natural population of Nagalingam Trees has been decreased day by day especially after the Thane cyclone in Puducherry region. The Principal Investigator Dr. Mahipal Singh Shekhawat and his team of scholars have developed an efficient micropropagation protocol for the regeneration of this threatened medicinal plant of South India.

The biotechnological method developed in this project will be useful to the authorities of Puducherry administration, environmentalist, researchers, forest department, policy makers and others who are directly or indirectly involve in the conservation of plant biodiversity.

I congratulate the Principal Investigator and his team on the successful completion of the project and thanks to the DSTE and DHTE, Govt. of Puducherry for their financial support and encouragement respectively.

Director

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The Principal Investigator is grateful to the Department of Science, Technology and Environment, Government of Puducherry, for the financial support.

The Principal Investigator also wishes to place on record his sincere gratitude to:

The Director, Technical and Higher Education, Govt. of Puducherry.

Director, Kanchi Mamunivar Centre for Post Graduate Studies, Puducherry.

Former Head of the Department Dr. V. Ramassamy and Present HOD of Plant Biology and Plant Biotechnology, Kanchi Mamunivar Centre for Post Graduate Studies, Puducherry.

Colleagues, Department of Plant Biology and Plant Biotechnology, Kanchi Mamunivar Centre for Post Graduate Studies, Puducherry.

Dr. Mohandas, former Controller of Examination, Kanchi Mamunivar Centre for Post Graduate Studies, Puducherry.

Thanks are also due to the following persons who helped the Principal Investigator in various capacities:

Mr. N. Kannan, Ms. M. Manokari, Ms. J. Revathi and I. Ilavarsi Research Scholars in the Biotechnology Unit, Department of Plant Biology and Biotechnology, Kanchi Mamunivar Centre for Post Graduate Studies, Puducherry.

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2.	Title of the project	Investigations on *In vitro* Regeneration of *Couroupita guianensis* Aubl. (Nagalingam Tree) – A Threatened but Medicinally Important Plant
3.	Funding agency	Department of Science, Technology & Environment, Govt. of Puducherry.
4.	Number and date	G.O. No. 10/DSTE/GIA/RP/JSA-I/2013/213 Dt.08.04.2013
5.	Name of the Principal Investigator	Dr. Mahipal Singh Shekhawat
6.	Abstract: *In vitro* regeneration protocol for *Couroupita guianensis* Aubl. (Nagalingam tree) has been developed during the present investigation. Fresh nodal shoot segments were used as explants. 3 to 5 shoots were differentiated within 10 to 15 days from the each node on MS (Murashige and Skoog) medium supplemented with 4.0 mgl^{-1} 6-benzylaminopurine (BAP). The cultures were multiplied on MS medium + 0.5 mgl^{-1} Indole-3 acetic acid (IAA) + 1.0 mgl^{-1} each BAP and Kinetin at 25±2° C under 30 µmol m^{-2} s^{-1} Spectral Flux Photons (SFP) light. The shoots were rooted on half strength MS medium + 2.5 mgl^{-1} Indole-3 butyric acid (IBA). The rooted plantlets were hardened in green house and finally transferred to the field.	
7.	Distribution statement	For limited circulation only
8.	Institute of Origin	Department of Plant Biology and Biotechnology, Kanchi Mamunivar Centre for Post Graduate Studies, Puducherry-605 008

EXECUTIVE SUMMARY

The present study deals with the development of *in vitro* regeneration protocol for *Couroupita guianensis* Aubl. (Nagalingam tree/Cannon ball tree) using somatic tissues. It is a highly medicinal plant. Each and every part of this plant is used in all types of systems of medicine. *C. guianensis* is mentioned under threatened plants category in IUCN Red Data Book, because the habitat of this species has declined widely. The natural propagation of *C. guianensis* is very slow and seeds shown directly in the soil could not germinate. Hence, there is urgent need to develop a plant regeneration protocol to conserve this plant using biotechnological interventions. Fresh nodal shoot segments were found to be most suitable type of explants. These were sterilized with help of 0.1% solution of Bavistin and $HgCl_2$. 3 to 5 shoots were differentiated within 10 to 15 days from the each node. Maximum number of explants responded on MS medium (Murashige and Skoog) supplemented with 4.0 mgl^{-1} 6-benzylaminopurine (BAP) where 57% explants regenerated shoots from the nodes. The effect of concentrations of Kinetin (Kn) on response of explants and shoot induction from nodal explants was not so impressive. The cultures were multiplied on MS medium + 0.5 mgl^{-1} Indole-3 acetic acid (IAA) + 1.0 mgl^{-1} each BAP and Kn at 25±2° C under 30 μmol m^{-2} s^{-1} Spectral Flux Photons (SFP). The most suitable medium for root induction from the *in vitro* raised shoots was found to be half strength MS medium + 2.5 mgl^{-1} Indole-3 butyric acid (IBA). The rooted plantlets were hardened in green house in bottles containing soilrite which were moistened with one-fourth strength of MS salts. Finally the hardened plantlets were transplanted in the field.

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ABBREVIATIONS

BAP	6-benzylaminopurine
Kn	Kinetin
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog medium
WP	Woody Plant's medium
SFP	Spectral Flux Photons

INTRODUCTION

Couroupita guianensis Aubl. (family Lecythidaceae), commonly known as cannon ball tree grown in Indian gardens as an ornamental tree for its beautiful flowers. It is also known as Nagalingam in Tamil and Kailaspati in Hindi (Satyavati et al., 1976). The trees are grown extensively in Shiva temples in South India. Hindus revere it as a sacred tree because the staminal sheath resembles the hood of the Naga, a sacred snake, protecting a Shiva Lingam, represented by reduced stigma. Hence, the name 'Naga Linga' tree. The "Cannonball Tree" is so called because of its brown cannon-ball-like fruits. The majority of these trees outside their natural environment have been planted as a botanical curiosity, as they grow very large with distinctive flowers. The genus *Couroupita* represents more than 30 recognized species throughout the world. In French it is known as Calabasse Colin. It is native to South India and Malaysia. The Puducherry Government has announced cannon ball flower (Nagalingam flower) as the State Flower (Deepa, 2007).

This magnificent tree can be seen in good condition at the Fairchild Tropical Botanical Gardens in Coral Gables, Florida. The cannonball tree was given its species name *Couroupita guianensis* in 1775 by the French botanist J. F. Aublet. The cannon ball tree is planted in gardens because the flowers are large, beautiful, pleasantly aromatic, and unlike any other flower a newcomer to the tropics has ever seen. Even the fruits are a botanical curiosity because they are in the shape and size of cannon balls and

the flowers arise from the trunk of the tree. In contrast to the flowers, fruits release a foetid aroma when they break open.

The fruit contains small seeds in a white, unpleasant smelling jelly, which are exposed when the upper half of the fruit goes off like a cover. The hard shells are used to make containers and utensils. Cannon ball flowers are considered of special significance in Buddhist culture in Sri Lanka. The long dangling fruity branches give the tree an unkempt appearance.

Couroupita guianensis is a large deciduous evergreen tree growing to a height of 20 meters. Leaves are alternate, oblong-obovate, up to 20 cm long, entire to slightly serrate and hairy on the veins beneath. Inflorescence is racemose, arising from the trunk and other large branches. It flowers in racemes which cauliflorus. The amazingly complex, yellow, reddish and pink flower of the cannonball tree are heavenly scented - a cross between a fine expensive perfume and a wonderful flower scent. These are 3" to 5" waxy, pink and dark-red flowers growing directly on the bark of the trunk. The tree bears large globose woody fruits, directly on the trunk and main branches. They look like big rusty cannonballs hanging in clusters, like balls on a string (Plate 1).

Fruits are edible and are occasionally eaten, but the smell of the white flesh discourages most people from trying them. This plant has been used widely in traditional medicine and grown in Indian gardens as an ornamental tree. It is a gigantic tree with deeper roots holding out delicate flowers for the world to see. Behold this

Plate 1.

A. *Couroupita guianensis* tree in natural habitat, B. Cannon ball flowers and fruits, C. Cauliflorus Inflorescence D. Mature fruit.

beauty shining pink within a pond of fragrance filling the air (Naga Linga flower: Poem from India).

Herbal and natural products of folk medicines have been used for centuries in every culture throughout the world. The infusion of *C. guianensis* flowers had been used to treat cold, intestinal gas formation, stomachache (Anonymous, 1950), barks used to treat hypertension, tumours, pain and inflammatory process (Sanz et al., 2009; Naif et al., 2012). The fresh fruit pulp is used in preparation of cooling medicinal drink and various parts are useful in skin disease. Methanolic extract of the plant parts exhibited antimicrobial, antifungal, antiseptic (Khan et al., 2003; Kavitha et al., 2011)), antidepressant activities (Sivakumar et al., 2012) whereas petroleum ether and chloroform extract showed antimalarial, antihelmintic (Aruna and Laddha, 2011; Desal et al., 2003), anticancer activity (Velliangiri and Subban, 2012) and immune modulatory activity (Pradhan et al., 2011). Leaves are widely used as an analgesic medicine by the rural population worldwide (Geetha et al., 2005; Mariana et al., 2010; Chandolu et al., 2011).

Religious significance of *Couroupita guianensis* in Asia

The trees are grown extensively in Shiva temples in India. In Hindi it is called Shiv Kamal and also known as "Kailaspati". It is called the Nagalingam tree in Tamil. The flowers are called Shivalinga flowers in Hindi; Nagalinga Pushpa in Kannada; Nagamalli flowers or Mallikarjuna flowers in Telugu. Hindus consider it a sacred tree as

the petals of the flowers resemble the sacred snake, Naga, a cobra protecting a Shiva lingam with its hood. In some parts of India, the tree is worshipped by childless couples.

In Sri Lanka, Thailand and other Buddhist countries the tree is often planted at Buddhist temples. It is here mistaken as the Sala tree, *Shorea robusta*, the tree under which the Buddha died and under which the previous Buddha Vessabhu attained enlightenment.

Traditional uses of *Couroupita guianensis*

The trees are used to cure colds and stomach aches. Juice made from the leaves is used to cure skin diseases, and shamans of South America have even used tree parts for treating malaria. Traditionally, the leaves of this plant have been used in the treatment of skin diseases (Satyavati et al., 1976), while the flowers are used to cure cold, intestinal gas formation and stomachache. The fruit pulp can disinfect wounds and young leaves ease toothache (Umachigi et al., 2007).

Fruit edibility: Fruits are edible, but only occasionally eaten because of the unpleasant odor of the white flesh.

Folkloric: Used to treat colds and stomachaches. Juice from leaves used for skin diseases. **Fragrance**: Fragrant flowers used to scent perfumes and cosmetics.

Wood: (1) Hard shells of the fruit sometimes used as containers and utensils.

(2) Wood used for making incense.

Phytochemical profile of *Couroupita guianensis*

The phytochemical profile of *C. guianensis* is relatively complete; there is some variability in the content of specific chemicals. Its Flowers yield an alipathic hydrocarbon and stigmasterol, alkaloids, phenolics and flavonoids, and has the active principles isatin and indirubin (vital to its antimicrobial activity) (Wong and Tie, 1995). Phytochemical screening yielded flavonoids, like 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone,7-hydroxy-5-methoxy-6,8-dimethylflavanone and the phenolic acid 4-hydroxybenzoic acid (Rane et al., 2001). In flowers, mainly eugenol, linalool and stigma sterols were identified and aliphatic hydrocarbon have been isolated by Jayashree et al., (2001).

The plant also contains several chemical constituents with novel structures and possesses bio-active moieties. These include eugenol, linalool, fernesol, nerol, tryptanthrine, indigo, indirubin, isatin, linoleic acid, α, β- amirins, carotenoids, sterols (Bergman et al., 1985 and Sen et al., 1974) and some acidic and phenolic compounds (Rajamanickam et al., 2009). Desal et al., (2003) have also been mentioned that *C. guianensis* leaves are rich in Phytochemicals and reported to contain quercertin, saponins and tryptanthrin.

The tree is also rich in providing anthocyanin, flavanoids, volatile constituents like eugenol and farsenol. The stem extracts of this plant is known to contain flavonoids, tannins, steroids, saponins, glycosides, amino acids, phenols, anthraquinones and triterpenoids (Manimegalai and Rakkimuthu, 2012).

Cellular Totipotency of Plant Cells

The concept of cellular totipotency was an integral part of the 'cell theory' as first proposed by Schleiden and Schwann. In 1849 Schleiden commented "the possibility of each cell, in any situation, on occasion, going through all the phases of cell life, and becoming developed in any way that the circumstances under which it is placed render necessary". This idea was based on his observation of the considerable regenerative powers of plants. It was implicitly postulated that the cell is capable of autonomy and even that it is totipotent (Gautheret, 1983). This is obvious in the cases of eggs and spores, which are able to transform/develop into complete organisms. Theoretical concept of somatic cell culture was finally imagined in 1902 by the German botanist Haberlandt (Gautheret, 1985).

In 1958, Reinert described for the first time somatic embryogenesis in carrot tissues. In these two experiments, there was no evidence that a single cell could be transformed into plantlet. Demonstration of this would require a start from isolated cells. Muir et al., (1954) undertook an exercise and set a cell of tobacco or *Tagetes erecta* on a small fragment of filter paper and placed this on top of a colony. Eight percent of these isolated cells multiplied. Several methods enabled the observation of isolated cell multiplying and offered the possibility to demonstrate cellular totipotency (Lutz, 1963; Bergmann, 1959). But they did not prove that the whole plant resulted from somatic embryogenesis affecting a single cell of the colony. Proof for this last

stage was achieved by Backs-Husemann and Rienert (1970) when they proved that a single cell of carrot transformed into an embryo.

Vasil and Hildebrandt (1965) observed that an isolated tobacco cell could divide and form a colony able to produce whole plants. In 1971, Takebe et al., regenerated whole plant from protoplasts and Carlson et al., (1973) were able to regenerate plants from fusion between protoplasts of *Nicotiana glauca* and *N. langsdorffii*. Melchers et al., (1978) obtained a hybrid between potato and tomato through fusion of protoplasts of the two species. The products of two types of protoplasts gave a plant not attainable from usual cross-binding.

The history of subject of evolution of plant tissue culture, and somatic cell genetics was extensively reviewed and discussed by Durzan and Campbell (1974), Krikorian (1996). Gautheret (1983, 1985) has described the history of plant tissue culture as part of applied biology and biotechnology. Role of somatic cell genetics in the genetic manipulation and plant breeding has been described in many articles/reviews (Griesback, 1984; Thorpe, 2000).

In vitro Propagation

Plant tissue culture, also known as the *in vitro* culture or microculture of plants, concerns the growth and developments of plants, plant cells, plant organs, or plant tissues in an aseptic, strictly controlled chemical and physical micro-environment. Regeneration of plants from cultured cell/tissues/organs/protoplasts is a unique property

of higher plants, and it has become a critical component of plant biotechnology (Vasil, 1991). Large-scale propagation of plants from cultured shoot meristems, commonly described as micropropagation, has been the most widely used aspect of plant biotechnology for more than three decades.

Kurtz et al., (1991) and Debergh and Read (1991) have described current methods of micropropagation and the various stages that lead to large-scale plant production. Micropropagation laboratories utilize the following basic methods to multiply plants *in vitro*: (1) enhanced apical meristem axillary branching, (2) adventitious shoots and (3) somatic embryogenesis. The other plant production methods are based on differentiation of callus/suspension cultures through organogenesis and somatic embryogenesis.

Individual nodal selections with their associated axillary vegetative buds are excised upon subculture. By manipulation of the culture multiplication techniques and the culture environment, particularly the growth regulators in the culture medium, apical dominance is suppressed an axillary branching are induced. Cultures may be divided as either single shoots or shoot clumps and further multiplied to obtain the desired number of shoots. Although the initial multiplication rate is slow, it increases rapidly and reaches a steady plateau for several subcultures. The excised shoots may then be rooted *in vitro* on direct by in soil/soilrite/vermiculite *ex vitro*. The rates of culture multiplication and amplification are affected by several factors (Debergh and Read, 1991; Shekhawat et al., 2014).

Micropropagation is in fact a complex multi step process involving numerous different starting tissues and cell types, ranging from cotyledons and vegetative meristem to reproductive tissues which can be induced to differentiate into plantlets, via either the organogenesis pathway or the embryogenesis pathway. The starting tissues, or explants, may be taken from either juvenile or mature/tested plant/tree of any age in between (Aitken-Christie and Connett, 1992). The micropropagation of mature plants is carried out to provide plants for clonal forestry or horticulture if the micropropagules are rejuvenated. The subject of rejuvenation and micropropagation has been reviewed (Pierik, 1990).

Maturation, a complex phenomenon, is the major problem preventing a wider application of tissue culture technology to woody plants. Despite the problems mentioned, the possibility of multiplying mature trees by cloning and establishing trials with micropropagated materials has been demonstrated for several tree species including *Eucalyptus* spp. (Sederoff, 1999; Shekhawat and Dixit, 2008), *Sequoia sempervirens* (Bonga and Von-Aderkas, 1992) and other tree species like *Maytenus emarginata* (Rathore et. al., 1992) and *Capparis decidua* (Deora and Shekahwat, 1995), *Achras sapota* (Purohit and Singhvi, 1998).

Several factors contribute to the production of plantlets, their physiology and biochemistry *in vitro*. These are:

(i) The chemical microenvironment: medium composition, medium pH and

composition of vessel headspace.

(ii) The physical microenvironment: Headspace temperature, incident light, vessel/ closure/design, gel strength and other factors.

(iii) Facilities and aseptic methods; and

(iv) Techniques used.

A thorough understanding of production costs serves as a meaningful competitive advantage in the micropropagation industry (Zimmerman, 1996). It is important that cost-effective procedures should be defined for plant species under micropropagation. Several general strategies to reduce current production costs include: (1) *in vitro* biological optimization, (2) elimination of production stages or repeated subcultures, and (3) automation and mechanization.

In vitro propagation of *C. guianensis*

Since, this plant is mentioned under threatened plants category in IUCN Red Data Book, because the habitat of this species has declined widely because of clearing for settlement, timber and agriculture, resulting in populations becoming threatened at a national level (Mitre, 2012). Again, due to Thane cyclone in Puducherry more than 50% population of this plant have been either uprooted or severely damaged (Uthayakumari, 2012; Jayalakshmi, 2012). The natural propagation of this plant is very slow and seeds directly shown in the soil could not germinate (Muniswamy and Sreenath, 2000).

This plant has strong religious and social relevance because it is part of South Indian Shiva temples and the followers of Shiva use this flower in their daily worship.

- This plant's flowers are declared as State Flower of Puducherry. It is the bound duty of Pondicherrians to propagate and conserve this plant in and around Puducherry.
- Each and every part of this plant is used to treat several human ailments from centuries but now the plant is facing survival problems due to natural and anthropogenic reasons.

Most of the work done on *C. guianensis* is related to phytochemical analysis for its medicinal values. Work related to cloning, tissue culture and micropropagation from mature tissues is largely deficit in literature. Muniswamy and Sreenath (2000) germinated *C. guianensis* seed embryo *in vitro* because seeds directly shown in the soil could not germinate. They were successful in *in vitro* germination of seeds and development of plantlets using excised embryos from seeds, cultured on half strength MS medium supplemented with a low level of the cytokinin BA (benzyladenine) 0.1 mgl^{-1}).

To the best of our knowledge any types of biotechnological work to conserve or clone this plant species using tissue culture and micropropagation is not available at national as well as international level. With help of Biotechnological methods (i.e. micropropagation, tissue culture etc.) we can multiply this plant species within short period of time which can also fulfill the demand of this plant to the pharmaceutical industries.

Objectives of the Present Study

Hence, there is urgent need to develop a plant regeneration protocol to conserve this threatened, religiously, socio-economically and ethno-botanically important plant species using biotechnological interventions. And this plant has been declared as Puducherry State Flower Plant, this is the bound duty of the Puducherry people to propagate, save and conserve this plant.

The objectives of the present investigation is to establish *in vitro* cultures to develop a rapid and high frequency plantlet regeneration protocol from mature tissues of *C. guianensis*, for providing continuous supply of a better source of elite plant to be used as standard material for replantation of this plant in the forest and in the field of drug research as well as in manufacturing of drugs (laboratory to field approach).

Due to higher demand and unavailability of farming practices, the plant population is decreasing day by day. It is urgent need to develop a cost effective and rapid micropropagation protocol to restore the plant genome. Furthermore, an efficient tissue culture protocol is must to get transgenic plants in future.

MATERIALS AND METHODS

The research project deals with the investigations/methods developed for establishment of *in vitro* cultures of *Couroupita guianensis* Aubl. (Cannonball Tree or Nagalingam Tree) selected in East Coast region of South India in general and Pondicherry in particular. Leaves, stems, and roots are used in various medicinal preparations, healing protocols, and treatment methods (including Sidha as well as Ayurveda) throughout the South India to treat various ailments from ancient times.

The plants were identified with the help of "Flora of Presidency of Madras" (Gamble, 1957) and the Professors of Department of Plant Biology and Plant Biotechnology, K.M. Centre for Post Graduate Studies, Puducherry. Field surveys were conducted for selection of mature and superior plants of *C. guianensis*. These include various sites of Tamil Nadu and Pondicherry. Plants were selected and marked at K.M.C.P.G.S. Fresh shoots of *C. guianensis* were collected from KMCPGS campus, Puducherry during the months of March to December, 2013. The fruits produced from plants, were carried to the Plant Biotechnology Laboratory.

Different types of explanting materials namely, axillary/terminal shoots, shoot apices/nodal shoot segments, seeds were harvested from selected trees. The explants were cleansed, dressed and treated with 0.1% solution of Bavistin, (a systemic fungicide) (BASF, India Ltd. Bombay). These were surface sterilized with ethanol and $HgCl_2$ (0.1%) solution under Laminar Air Flow Cabinet.

1. Chemicals

Pure and analytical grade chemicals of E. Merck (India) Ltd., E. Merck, Germany; British Drug House (BDH), Mumbai; Sigma Chemical Company, USA, Ranbaxy Laboratories Ltd., SAS Nagar, Lucerne, Switzerland; Koch Light Laboratories, England; Hi-Media, Mumbai; Qualigenes Fine Chemicals, Mumbai; Loba Chemical Company; S.d. Fine Chem. Pvt. Boisar; India and Boehringer Mannheim, Germany were used throughout the period of study.

2. Glassware/Plasticware

The glassware used for all the experiments were of either Corning or Borosil or Vensil make. Erlenmeyer flask (100, 150, 250 ml capacity), culture tubes (25x150 mm and 25x200 mm size) and culture bottles (400 ml) were used as culture vessels for raising tissue cultures. Flat bottom flasks (1000, 3000 and 5000 ml. Capacity), petridishes and beakers, volumetric flasks, measuring cylinders and pipettes (Gilson Pipettmen) were used for media preparation and inoculation.

The glassware was first rinsed with chromic acid followed by thorough washing with tap water and then soaked in liquid detergent for 2 hours and finally washed with tap water until the detergent was completely removed. These were rinsed with distilled water and allowed to oven dry at 60-80°C for 24 hours.

3. Culture media

The plant tissue culture media generally comprise of inorganic salts, organic compounds, vitamins, gelling agents i.e. agar-agar etc. All the components were dissolved in distilled water except growth regulators. Auxins were dissolved in 0.5N

NaOH or ethanol and cytokinins were dissolved in dilute 0.1N HCl or NaOH. For the present study two types of nutrient media were used. These include MS basal medium (Murashige and Skoog, 1962) and Woody Plant (WP) medium (Lloyd and McCown, 1980).

Both the media were used either as such or with certain modification in their composition. Sucrose and sugar cubes (Daurala Sugar Works, Daurala, U.P.) were added as a source of carbohydrate. The pH of the media was adjusted to 5.8±2 with 0.5N NaOH or 0.1N HCl before autoclaving. The medium was poured in the culture vessels. Finally the medium was steam sterilized by autoclaving at 15 psi pressure at 121°C for 15 minutes (Technico Systems, Chennai).

4. Culture Methods

Inoculation: The plant materials were inoculated under Laminar Air Flow Cabinet under sterile conditions. Explants were surface sterilized with $HgCl_2$ and were washed thoroughly with sterilized distilled water for 7-8 times and inoculated to various nutrient media. The cultures were kept in growth room under controlled conditions at the temperature 26±2°C with 14h/d illumination of 30-40 µmol $m^{-2}s^{-1}$ Spectral Flux Photons (SFP) and 60-70% relative humidity (RH). The light was provided by fluorescent tubes and incandescent bulbs. Temperature was maintained by air conditioning system. Positive air pressure was maintained in the culture rooms, in order to regulate temperature and maintain aseptic conditions.

Explants collected (Plate 2) from various sites were thoroughly washed with water containing 0.1% Bavistin for 4-5 minutes. These were surface sterilized with 0.1% $HgCl_2$ for 4-5 minutes and washed 6-8 times with autoclaved water under Laminar Air Flow Cabinet (Technico Systems, Chennai) and inoculated aseptically onto culture media. The final wash was given with aqueous sterilized solution of (0.1%) ascorbic acid. The surface sterilized explants were dipped in 90% ethanol for a short period (40 seconds).

The explants were inoculated horizontally and vertically on the medium for culture initiation. Different concentration and combination of cytokinins (BAP and Kinetin ranging from 0.5 to 6.0 mgl^{-1}) and auxins (IAA and IBA ranging from 0.1 to 1.0 mgl^{-1}) were incorporated in the medium to induce bud breaking. These cultures were incubated at 28±2°C in the dark for 2-3 days. Subsequently these were kept under diffused light (22 µmol m^{-2} s^{-1} SFP) for 8-10days.

Experiments were also conducted to find out the suitability of liquid medium for the present study. Cultures were first induced on agar gelled medium and then these were transferred liquid medium to get good numbers of shoots in minimum time period.

Multiplication of the cultures

The *in vitro* regenerated shoots were multiplied by repeated transfer of mother explants and subculturing of *in vitro* produced shoots on fresh medium. The shoot-clumps were subcultured in the culture flasks. For multiplication of cultures MS medium supplemented with various concentration and combination of cytokinins

Plate 2.

Explants used for culture initiation

Seeds

Shoot tips

Nodal shoot segments

(BAP and Kin; 0.1 to 3.0 mgl^{-1} and IAA/IBA ranging from 0.1 to 2.0 mgl^{-1}) were used. The cultures were incubated at 28±2°C temperature, 60-70% RH and 30-35 µmol $m^{-2}s^{-1}$ SFP for 12 h/d.

Rooting of *in vitro* produced shoots

For the rooting of *in vitro* produced shoots, the shoots were isolated of appropriate size and these were rooted by following methods

***(a) In vitro* rooting:** The shoots generated *in vitro* were cultured on half, one- fourth strengths of MS medium or WP medium containing auxins (IBA and IAA).

(b) *Ex vitro* rooting: The shoots produced in cultures were pulse treated with IBA for short time periods. The pulsed shoots were then transferred in the sterilized bottles containing moistened soilrite (Kel Perlite, Bangalore) covered with polycarbonate caps and allowed to remain under green house conditions.

Hardening of plantlets and field transfer: The *ex vitro* and *in vitro* produced plantlets were hardened in the green house. The *in vitro* rooted plantlets were washed with autoclaved distilled water to remove adhered nutrient agar and then transferred to sterilized soilrite mixture filled in glass bottles moistened with one-fourth strength of MS basal salts. Initially the hardened plantlets were kept covered but after 15-20 days the caps were loosened and finally removed. Plants hardened in bottles were transferred to polybags containing sand, soilrite, organic manure and black soil in 1:1:1:1 ratio. The hardened plantlets were finally transferred to the pots, polybags and were transferred to the field.

1. Observation and Data Analysis

The cultures were regularly subcultured on fresh medium after 4-5 weeks interval. The observations were taken after every five days of inoculation. The experiments were repeated thrice with ten replicates per treatment. The rate of multiplication represents number of shoots produce per explant on a specific medium after number of days of its inoculation as mentioned in the results. The data were subjected to statistical analysis.

RESULTS AND DISCUSSION

1. Establishment of the cultures of *Couroupita guianensis*

Herbal drugs in recent years have gained sufficient importance because of their efficacy and cost effectiveness and their ability to provide an effective alternative, especially for psychiatric patients with lingering conditions and intolerance to adverse effects of synthetic molecules. Numerous medicinal plants and their formulations are used by mankind in ethno-medical practice as well as traditional system of medicine in India (Manna et al., 2006). Plant products play a beneficial role in the management of various disorders (Zhao et al., 2009; Lee et al., 2009). The World Health Organization (WHO) has estimated that about three quarters of the world's population still relies on plant-derived medicines usually obtained from traditional healers, for their basic health-care needs (Kuruvilla, 2002; Farnsworth et al., 1985).

Explant is the material used as initial source of tissue culture procedure. Plant tissue culture success mainly depends on the age, types and position of explants (Gamborg et al., 1976) because not all plant cells have the same ability to express totipotency. In nature the trees/plants experience various kinds of stresses induced by very severe winters and high temperatures and high wind velocities. It seems that the tissues of adult plants accumulate metabolites during seasonal cycle of the year. These prevent bud breaking in culture as such the response of the explants taken from nonpruned/ unlopped trees do not respond in culture.

Explants harvested during April and May months found most suitable as compared to remaining period of the year for shoot induction and regeneration in *Couroupita guianensis* in present study. Growth regulators regulate various physiological and morphological processes in plants (Srivastava, 2002). Plant Growth Regulators are synthesized by plants; therefore many plant species can grow successfully without external medium supplements (Bhavisha and Jasrai, 2003; Baksha et al., 2005).

The different types of explants of *C. guianensis* exhibited variable responses on the culture media. Since, seeds could not be germinated naturally if directly sown in the soil. Embryos/seeds were tried to germinate during present investigation. The mature and immature fruits were collected from the trees and dissected in the laboratory to get seeds of different stages. The seeds were sterilized with fungicide (Bavistin) and $HgCl_2$ solutions. The embryos were collected under laminar air flow bench and inoculated on MS and Woody plant's media. Most of the seeds (80%) were germinated *in vitro* due to sterilization procedure and absorption of nutrients from the medium (Plate. 3). These seedlings were further multiplied and elongated on fresh MS medium which contains BAP 2.0 mgl^{-1}. Plumule was separated out from the seedling in the next stage and inoculated on fresh medium to get multiple shoots.

Hormones can also be added into cultures to improve plant growth and to enhance metabolite synthesis. As observed in woody trees, *in vitro* growth and shoot formation was not achieved without adequate concentrations of exogenous hormones.

Plate 3.

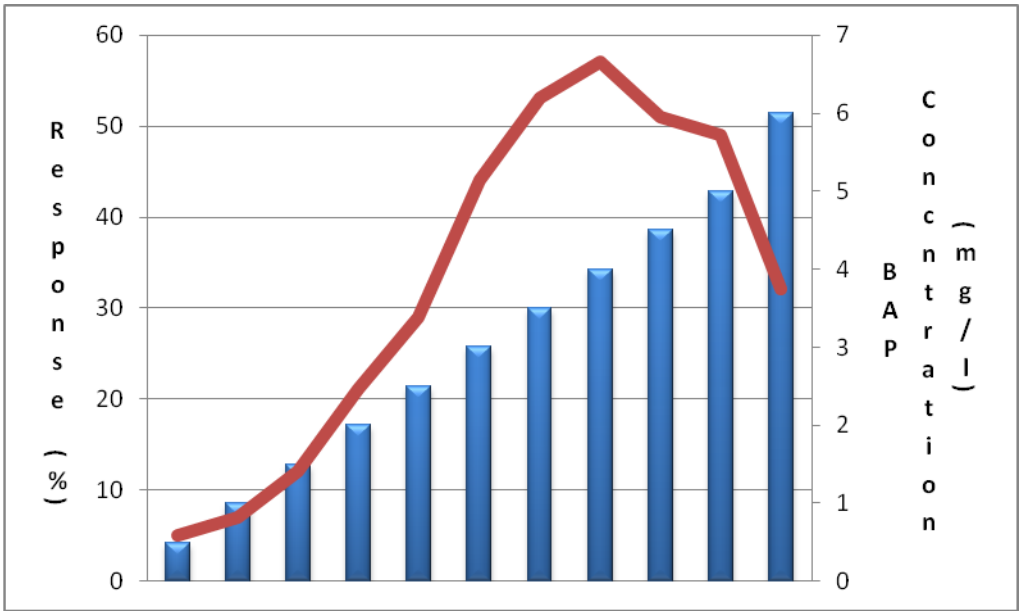
A-C. *In vitro* germination of seeds on MS medium, D-F. Elongation of shoots from seeds *in vitro*, G & H. Multiple shoot induction from seedlings of *C. guianensis*.

However, inadequate or excessive amount of growth hormones can cause morphological and physiological abnormalities (Bouza et al., 1994). As callus formation was observed in *C. guianensis* when higher concentration of IAA added in the culture medium. Several re-juvenilization methods for rejuvenation of mature woody trees have been suggested and discussed by Rathore et al., (1992) and, Deora and Shekhawat (1995). Sanchez et al., (1997) suggested many invigoration treatments for the micropropagation of mature chestnut trees. John et al., (1997) suggested cold treatment at 15°C for 72 hours for bud breaking to occur after 30-35 days on MS medium + BAP + Kn + biotin + calcium pantothenate in woody trees. No such treatment was required during our study in this system.

The axillary shoot apices responded quickly in cultures. From each node 3 to 5 shoots were differentiated within 10 to 15 days on different concentrations of BAP and Kn on MS medium. It took longer time (more than 20 days after inoculation) to induce axillary buds on WP medium. Maximum number of explants responded on BAP 4.0 mgl^{-1} where 57% explants regenerated shoots from the nodes (Fig. 1A). The effect of concentrations of Kn on response of explants and shoot induction from nodal explants was not so impressive. Only 43% shoots responded on this media combination (Fig. 1B). Four to five and 3-4 shoots regenerated from each node on MS medium supplemented with 4.0 mgl^{-1} BAP (Plate 4) and Kn respectively (Fig. 2A and 2B).

Fig. 1.

A.



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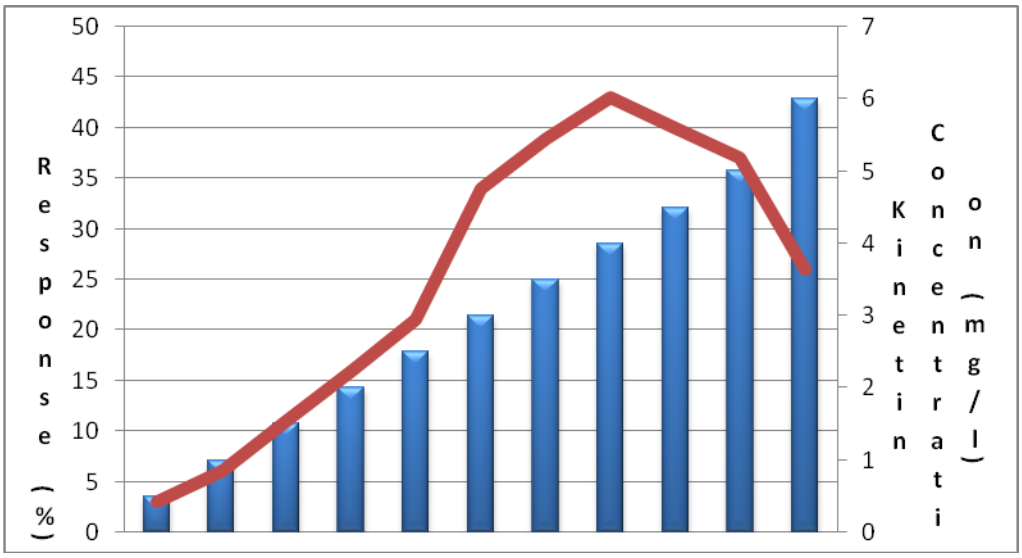
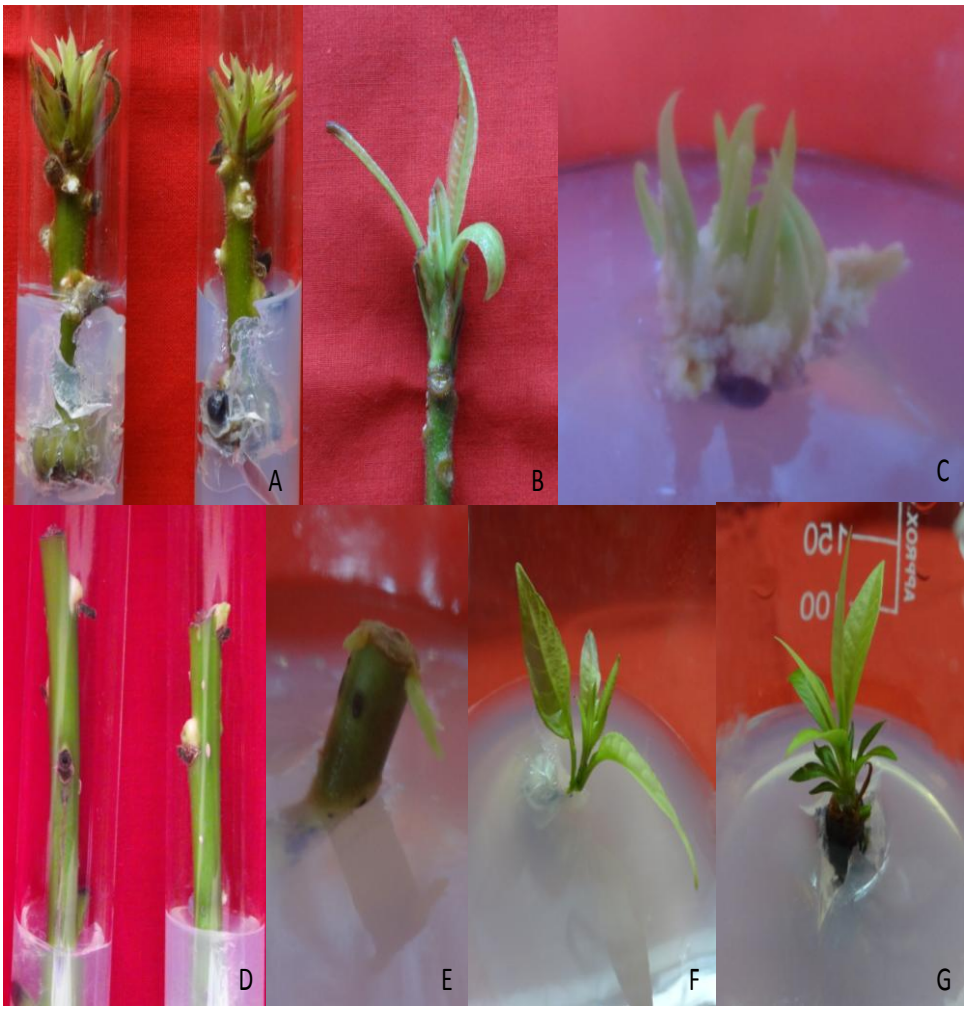


A. Effects of BAP concentration on the response of explants of *Couroupita guianensis* on MS medium.

B. Effects of Kinetin concentration on the response of explants of *Couroupita guianensis* on MS medium.

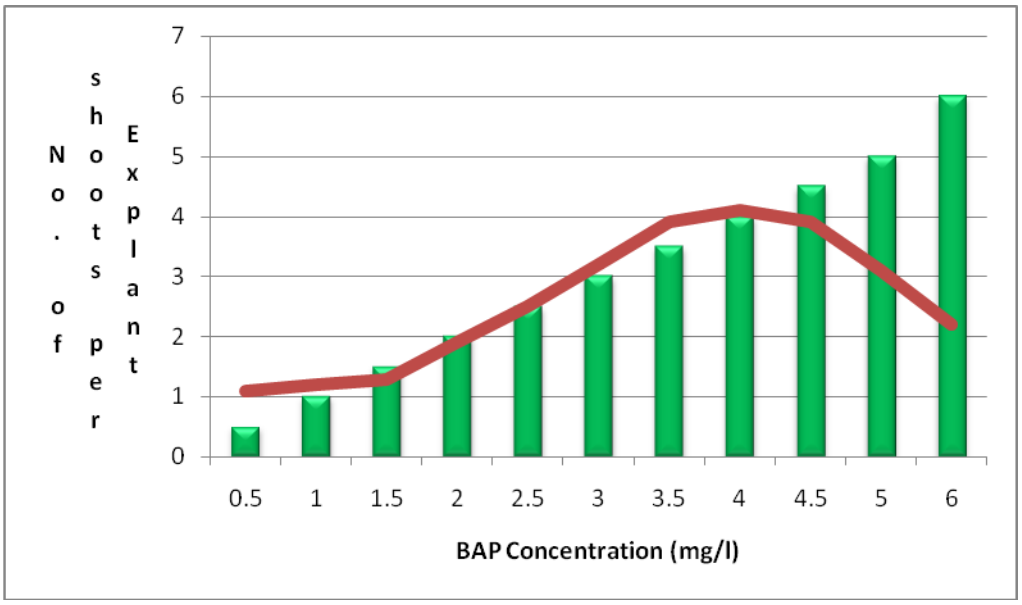
Plate 4.



A&B. Induction of shoots from the shoot tips, C. Formation of multiple shoots through apical shoot tips, D&E. Induction of shoots from nodal shoot segment cultured *in vitro* on MS medium, F&G. Elongation and multiple shoot formation from nodal segments *in vitro*.

Fig. 2.

A.



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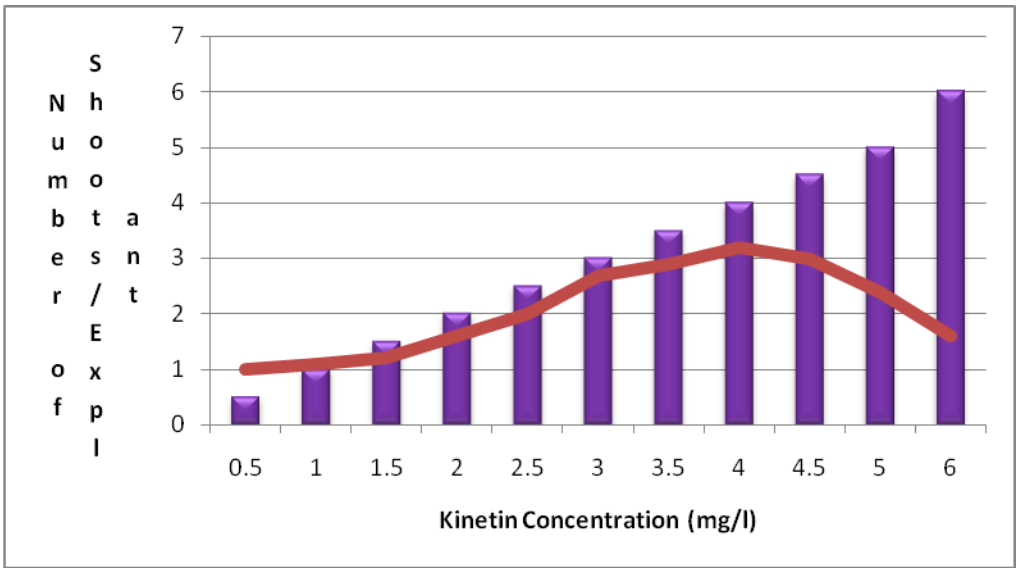


A. Effects of BAP concentration on initiation of number of shoots per explants of *Couroupita guianensis* on MS medium.

B. Effects of Kinetin concentrations on induction of number of shoots per explant on MS medium.

The MS medium supplemented with BAP + Kn was also not impressive as far as the number of shoots regeneration is concern (3-4 shoots per explant) and 47% explants responded on these concentrations (Fig. 3A). The explants harvested during April-May responded earlier in the culture (within two weeks) while the explants collected in November-December (responded after 3 to 4 weeks). Again incorporation of auxin (IAA and IBA) in the medium was not found very impressive for the induction of shoots. Less number of shoots regenerated from the nodes with these auxins. Basal medium containing 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} of IAA, increased the number and growth rate of multiple shoots in *Aloe barbadensis* (Ujjwala, 2007; Baksha et al., 2005). While higher rates of shoot proliferation were also reported in *Momordica dioica* on MS medium containing 0.8 mgl^{-1} of IAA and 2.0 mgl^{-1} of BAP (Shekhawat et al., 2011).

2. Multiplication of cultures *in vitro*

Shoots with explants taken from the inoculation medium were multiplied by following methods:

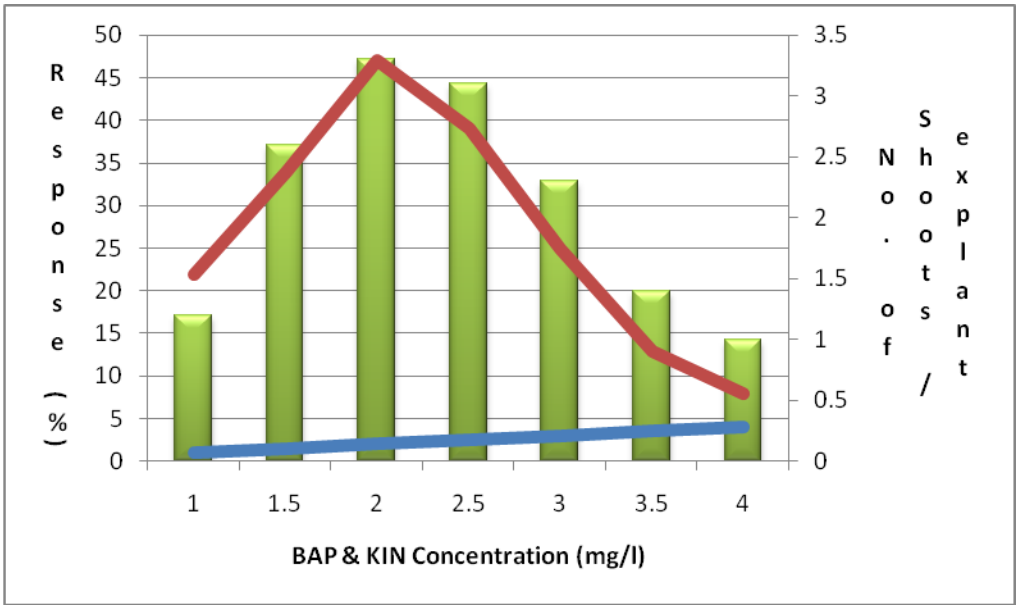
(i) Subculturing of the *in vitro* produced shoots, and

(ii) Repeated transfer of shoot clumps.

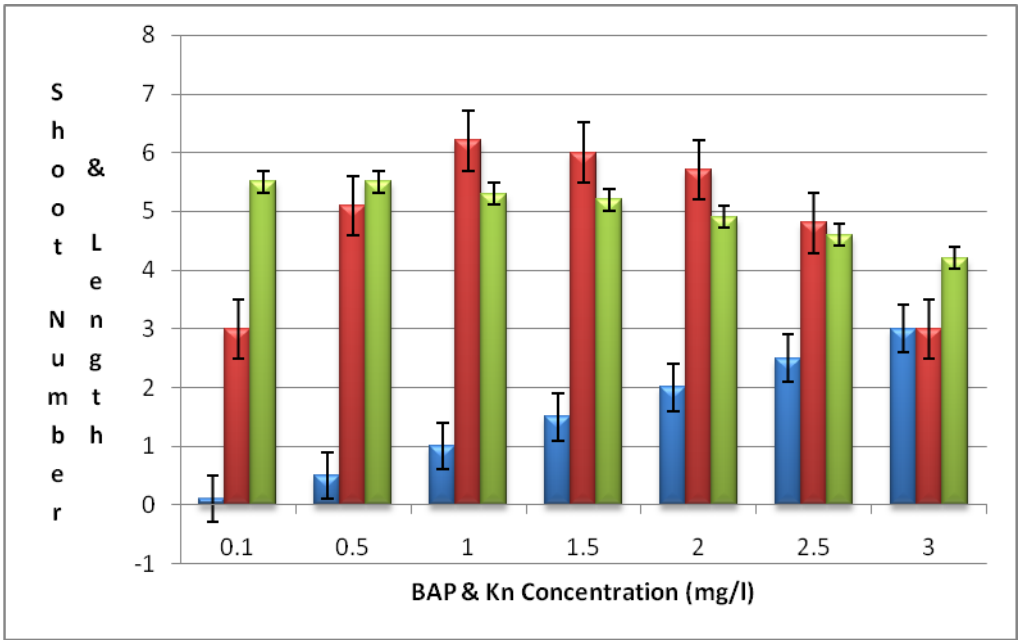
The *in vitro* regenerated shoots were sub-cultured for further multiplication on fresh medium. It was observed that cytokinin requirement for sub culturing of shoots was low. If the cultures were multiplied in shoot initiation medium they exhibited 'hyperhydricity' and low numbers of shoots were formed.

Fig. 3

A.



B.



A. The combined effect of BAP + Kinetin concentrations on % of response and initiation of shoots from the nodes of explants of *Couroupita guianensis* on MS medium.

B. Combined effects of Cytokinins (BAP + Kinetin) concentrations on multiplication of shoots from *in vitro* regenerated shoots on MS Medium containing 0.5 mgl^{-1} IAA.

Comparatively low concentration of BAP and Kn (1.0 mgl^{-1} each) promoted the shoots multiplication and also minimized the hyperhydricity. Sub culturing was essential within 30 to 35 days. The rate of multiplication of shoots through subculturing of *in vitro* produced shoots was relatively low and 6 to 7 shoots were produced in each culture flask (Plate 5).

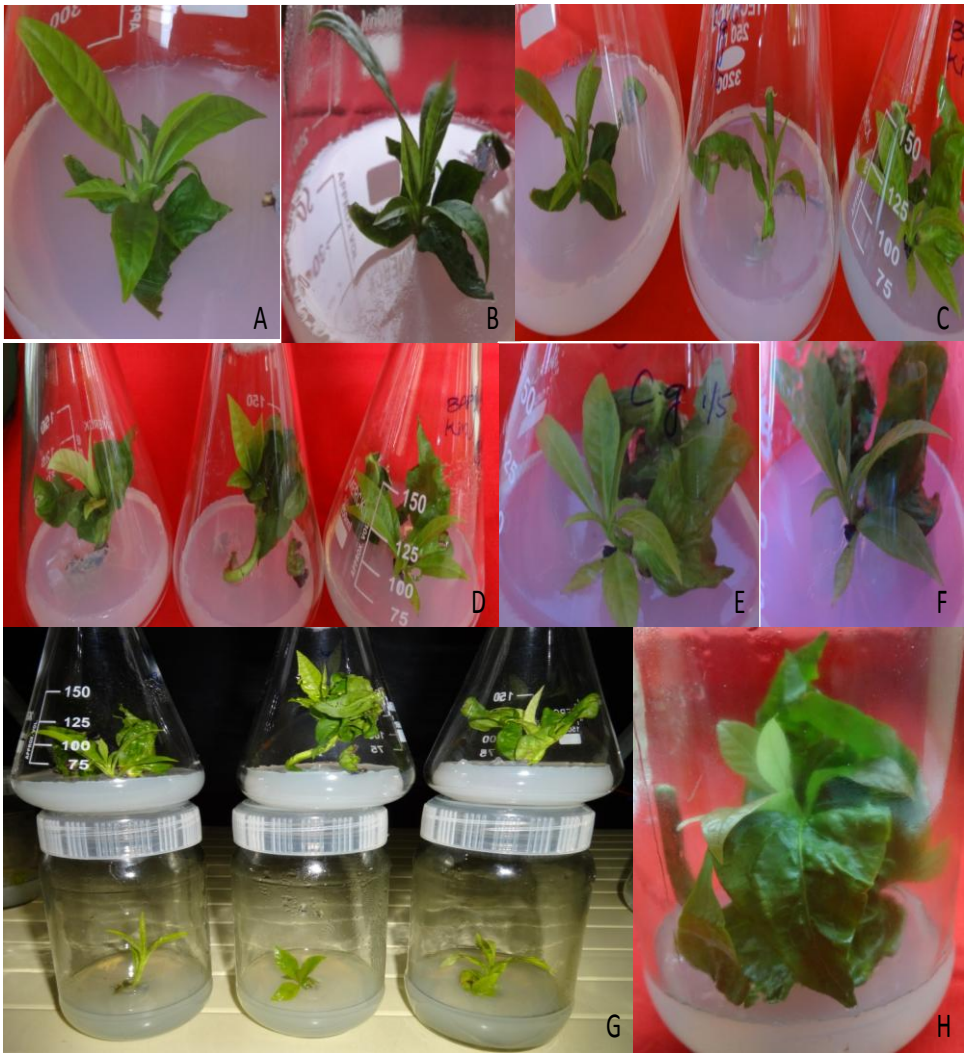
In order to enhance the rate of multiplication shoot clumps were repeatedly transferred to the fresh medium. This proved to be the most effective method of production and amplification of healthy shoots in cultures. The cultures were maintained on MS medium + 0.5 mgl^{-1} IAA + 1.0 mgl^{-1} each BAP and Kn at 25±2° C under 30 μmol m^{-2} s^{-1} SFP (Fig. 5D-J).

The effect of concentrations of BAP and Kn on shoot multiplication is shown in Fig. 3B. If sub culturing was delayed beyond 4 to 5 weeks the shoots of clumps turned yellow and subsequently became brown. This proved to be detrimental for the cultures. It was therefore, necessary to subculture/transfer the shoot clumps on the fresh medium after 30 days in order to maintain the sustained growth of the shoots and rate of multiplication (Plate 6 A to D).

Experiments were designed to study the response of explants with *in vitro* induced shoots in liquid MS medium. But it was not so impressive as far as shoot multiplication is concern because it took longer time (more than 6 weeks) to multiply the shoots *in vitro* as compared to semi solid medium (Plate 6 E to G).

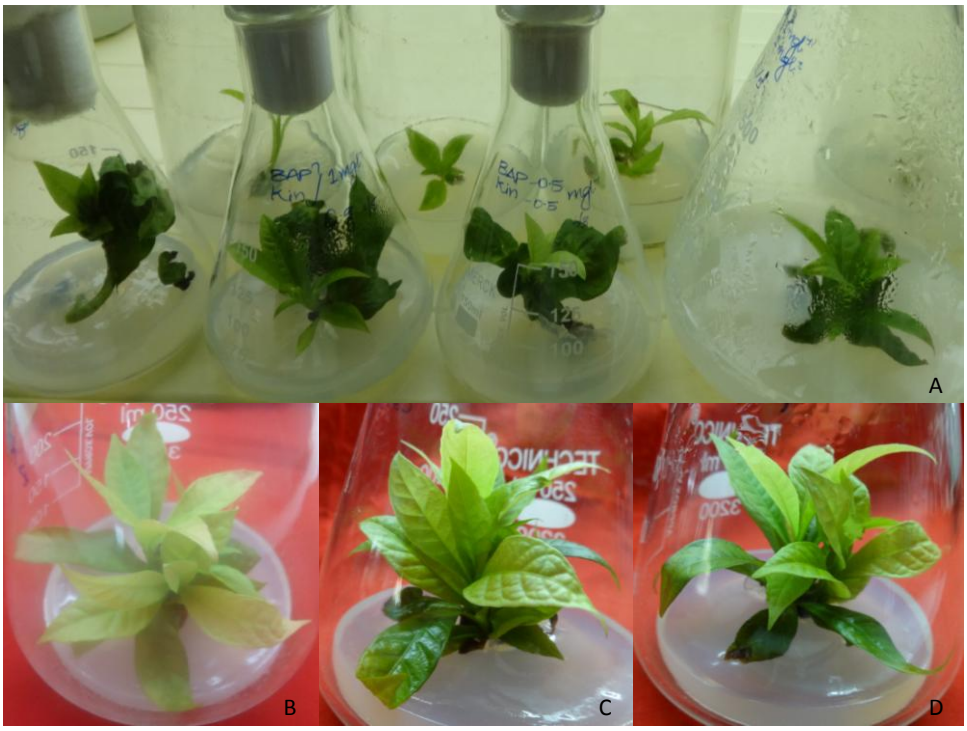
In case of *Salvadora persica,* MS medium containing 0.5 mgl^{-1} BAP, 0.5 mgl^{-1} Kn with 0.2 mgl^{-1} NAA showed rapid shoot multiplication (Phulwaria et al., 2011). MS

Plate 5.



A-H. Different stages of shoot multiplication in *C. guianensis*

Plate 6.



A-D. Different stages of multiple shoots formation in *C. guianensis*

E-G. Multiplication of shoots in liquid medium *in vitro*.

medium containing 1.0 mgl^{-1} BAP with 0.2 mgl^{-1} IAA reported the best for growth and number of shoots in *Leptadenia reticulata* (Rathore et al., 2010). After producing healthy shoots, plantlets transferred to different media combinations for root induction.

3. Rooting of *in vitro* regenerated shoots

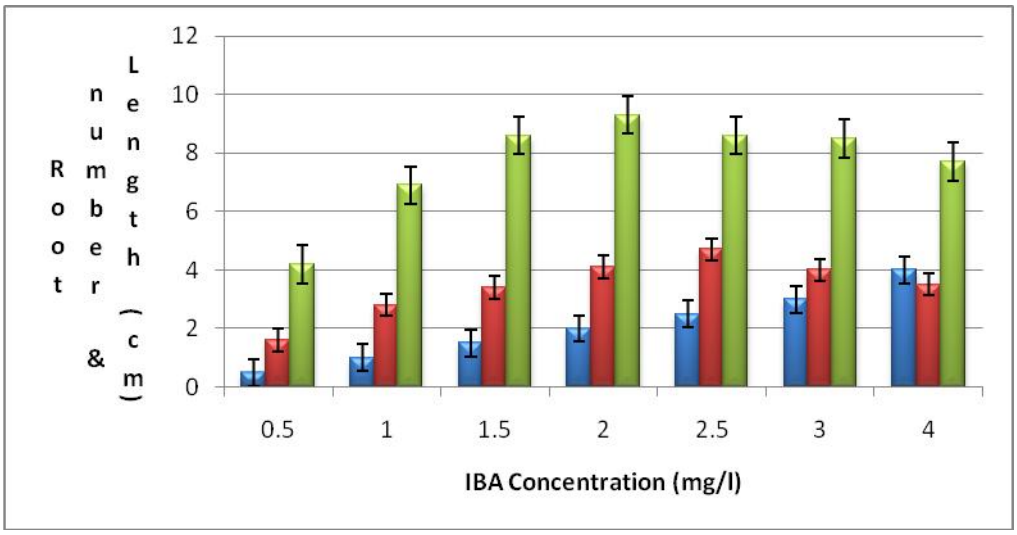
Well developed, healthy and sturdy micro-shoots (4-5 cm long) were excised and transferred to different strength of MS medium for rooting. Among these, 1/2 strength MS medium was found to be superior as compared to other media. Strength of MS medium appeared to be an important factor in influencing the rooting efficiency. However, at times a very low concentration of exogenous auxin is required for better rhizogenesis. Auxins are mainly used in root induction and their effect varies with type and concentration used in different plant species (Swamy et al., 2002).

Rooting of shoots was achieved by *in vitro* methods only. The *in vitro* generated shoots of *C. guianensis* rooted on MS Medium. However, the most suitable medium for root induction was found to be half strength MS medium + 2.5 mgl^{-1} IBA. On this medium about 78% of the shoots rooted. The root initiation was visible on 15th day of inoculation and 4-5 roots were initiated from cut ends of each shoot (Fig. 4A). Whereas 46% of the shoots were rooted on IAA containing medium and 3-4 roots were induced on this media combination (Fig. 4B) (Plate 7 A, B, E and F). The shoots rooted the best at 26±2°C temperature under diffused light conditions.

McClelland et al., (1990) investigated the effect of *in vitro* and *ex vitro* root initiation on subsequent microcutting root quality in woody plants *Acer rubrum* and *Betula nigra*. These were distinguished by several important anatomical and morphological traits that continued to regulate both root system and whole plant quality in later stages of production. The plantlets were hardened in green house for one to two months. During hardening about 85-90% of the plants survived (Plate 7 C, D, G to I). These could be pot transferred and finally transferred to the field.

Fig. 4

A.



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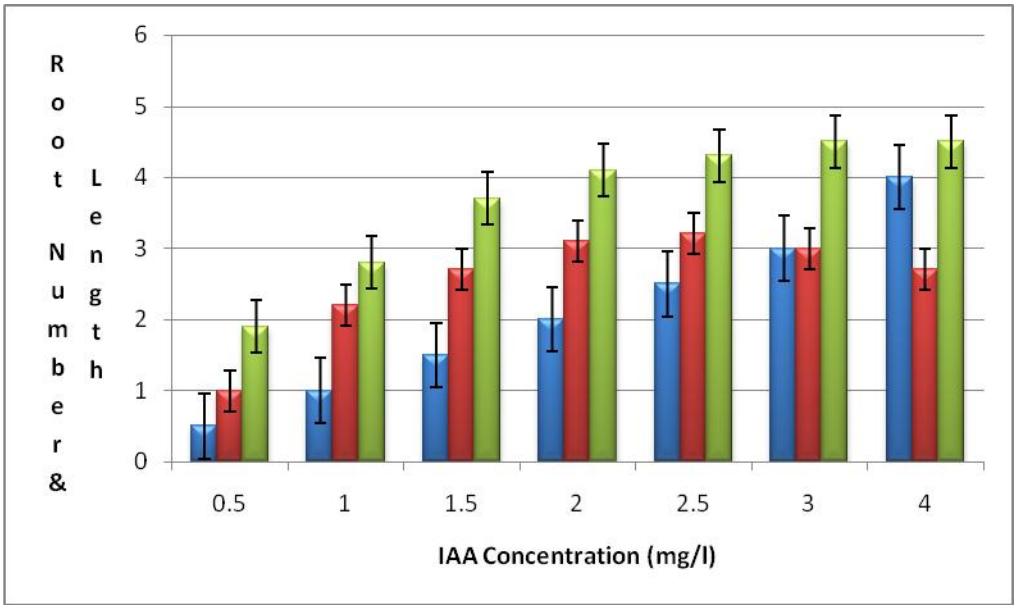

A. Effects of IBA concentrations on *in vitro* root induction from the shoots on half strength MS medium.

B. Effects of IAA concentrations on initiation of roots (number and length of roots) from *in vitro* regenerated shoots of *Couroupita guianensis* on half strength MS medium.

Plate 7.

A-B & E-F. *In vitro* root initiation and multiplication in *C. guianensis,*

C-D & G-I. Hardening stages of *in vitro* raised plantlets in green house.

CONCLUSION

The present study describes some advanced insight to develop direct regeneration protocol for *Couroupita guianensis* employing stem segment and seeds as explants. The micropropagation system has assured effective establishment, multiplication, rhizogenesis and acclimatization of *Couroupita guianensis* and could be exploited to multiply elite genotypes and develop *in vitro* strategies for the conservation of this valuable but threatened medicinal tree.

The tree systems have long life and they accumulate phenolic compounds and other secondary metabolites during the adverse conditions for survival. Researchers have to wait for the favorable time period when the accumulated metabolites leached out from the apical tissues. The woody trees require more time and experimentations to develop a complete tissue culture protocol.

It was first attempt to develop an efficient tissue culture protocol for regeneration of *C. guianensis* in cultures from mature tissues. Now, it has been possible to generate clones of *C. guianensis* through tissue culture. The process defined is highly reproducible, efficient and can be followed for cloning of selected and mature genotypes of *C. guianensis* selected for coastal area of South India.

These regenerative steps which are described in this research project report can be used by the authorities of Puducherry administration, environmentalist, researchers, forest department, policy makers and others who are directly or indirectly involve in the conservation of plant biodiversity.

REFERENCES

Aitken-Christie J, Connett M. Micropropagation of forest trees. In: Kurata K, Kozai T. (eds) Transplant Production System, Kluwer Academic Publishers, The Netherlands, 1992; 163-194.

Anonymous. Wealth of India. Council for scientific and Industrial research, New Delhi. 1950; 2: 362.

Aruna EA, Laddha KS. Scientific Abstracts: 53rd Indian Pharmaceutical Congress, New Delhi, CP, 2011; 29: 212.

Backs-Husemann D, Reinert J. Embryobildung durch isolierte Einzelzellen aus Gewebekulturen von *Daucus carota*. Protoplasma, 1970; 70: 49-60.

Baksha R, Alam R, Karim MZ, Mannan SA, Podder BP, Rahman ABMM. Effect of auxin, sucrose and pH level on *in vitro* rooting of callus induced micro shoots of sugarcane (*Saccharum officinarum*) J Biol Sci, 2005; 3: 915–920.

Bergman J, Lindstrom JO, Tilstam U. The structure and properties of some indolic constituents in *Couroupita guianensis* Aubl. Tetrahedron, 1985; 41: 2879-2881.

Bergmann D. A new technique for isolating and cloning of cells of higher plants. Nature, 1959; 184: 648-649.

Bhavisha BW, Jasrai YT. Micropropagation of an Endangered Medicinal Plant: *Curculigo orchioides* Gaertn. Plant tissue Culture, 2003;13: 13-19.

Bonga JM, Von Aderkas P. Rejuvenation of tissues from mature conifers and its implications for propagation *in vitro*. In: Ahuja MR, Libby WJ. (eds) Clonal Forestry I. Genetics and Biotechnology, Springer-Verlag, Tokyo, 1992; 182-199.

Bouza L, Jacques M, Miginiac E. *In vitro* propagation of *Paeonia suffruticosa* Andr. cv. 'Mme de Vatry': developmental effects of exogenous hormones during the multiplication phase. Scientia Hort, 1994; 57: 241–251.

Carlson PS, Smith HH, Dearing RD. Parasexual interspecific plant hybridization. Proc Nat Acad Sci, 1973; 69: 2292-2294.

Chandolu SK, Gaddala N, Vanamala S, Naresh VA. Elumalai. A short review on therapeutic uses of *Couroupita guianensis* aubl. Int Res J Pharm App Sci, 2011; 1(1): 105-108.

Debergh PC, Read PE. Micropropagation. In: Debergh PC, Zimmerman RH. (eds) Micropropagation: Technology and Application. Kluwer Academic Publishers, The Netherlands, 1991; 1-13.

Deepa HR. Puducherry comes out with list of State symbols. The Hindu. Online edition. Saturday, 21. Chennnai, TN, 2007.

Deora NS, Shekhawat NS. Micropropagation of *Capparis decidua* (Forsk.) Edgew - a tree of arid horticulture. Plant Cell Reports, 1995; 15: 278-281.

Desal T, Golatakar SG, Rane JB, Ambaye RY, Kamath VR. Larvicidal property of *Couroupita guianensis* Aubl. Indian drugs, 2003; 40: 484-486.

Durzan DJ, Campbell RA. Prospects of the mass production of improved stock of forest trees by cell and tissue culture. Can J Forest Research, 1974; 4: 151-174.

Farnsworth NR, Akerele O, Bingel AS, Soejarto DD, Guo ZG. Medicinal plants in therapy. Bull WHO, 1985; 63: 83-97.

Gamble JS. Flora of Presidency of Madras. Botanical Survey of India, Calcutta, India, 1957.

Gamborg OL, Shyluck JP, Brar DS, Constabel F. Morphogenesis and plant regeneration from callus of immature embryos of sorghum. Plant Sci Lett, 1976; 10: 67–74.

Gautheret RJ. History of plant tissue and cell culture: A personal account. In: Vasil IK. (ed) Cell Culture and Somatic Cell Genetics of Plants. Vol. 2. Cell Growth, Nutrition, Cytodifferentiation and Cryopreservation. Academic Press, London, 1985; 1-59.

Gautheret RJ. Plant Tissue Culture : A History Bot Mag, Tokyo, 1983; 96: 393-410.

Geetha M, Shankar MB, Mehta RS, Saluja AK. Antifertility Activity of *Artabotrys odoratissimus* Roxb. and *Couroupita guianensis* Aubl, Journal of Natural Remedies, 2005; 5(2): 121 – 125.

Griesback RJ. Introduction to somatic cell genetics. Hort Science, 1984; 19: 367-371.

Jayalakshmi J. Impact of Thane cyclone on Botanical Garden, Puducherry. M. Sc. Dissertation submitted to the K.M. Centre for PG Studies, Pondicherry University, 2012.

Jayashree BR, Severina JV, Suprabha GG, Ambaye RY, Khadse BG. Chemical examination of the Flowers of *Couroupita Guianensis* Aubl. Indian J Pharm Sci, 2001; 63(1): 72-73.

John M, Sanda SI, Amasino AM. Analysis of flowering time in ecotypes of *Arabidopsis thaliana*. Journal of Heredity, 1997; 88: 69–72.

Kavitha R, Kamalakannan P, Deepa T, Elamathi R, Sridhar S, Suresh Kumar J. *In vitro* Antimicrobial Activity and Phytochemical Analysis of Indian Medicinal Plant *Couroupita guianensis* Aubl. J Chem Pharm Res, 2011; 3(6): 115-121.

Khan MR, Kihara M, Omoloso AD. Antibiotic Activity of *Couroupita guianensis*. Journal of Herbs, Spices and Medicinal Plants, 2003; 10(3): 95-108.

Krikorian AD. Strategies for "minimal growth maintenance" of cell cultures: A perspective on management for extended duration experimentation in the microgravity environment of a space station. The Botanical Review, 1996; 62: 42-108.

Kurtz SL, Hartman RD, Irwin IYE. Current methods of commercial micropropagation. In: Vasil IK. (ed) Scale-up and Automation in Plant Propagation: Cell Culture and Somatic Cell Genetics of Plants, 1991; 8: 7-34.

Kuruvilla A. Herbal formulations as pharmacotherapeutic agents. Indian J Exp Biol, 2002; 40: 7-11.

Lee JR, Park SJ, Lee HS. Hepatoprotective activity of licorice water extract against Cadmium-induced toxicity in rats. Evidence-Based Complementary and Alternative Medicine, 2009; 6(2): 195–201.

Llyod, G, McCown BG. Commercially feasible micropropagation of mountain laurel *Kalmia latifolia* by use of shoot -tip culture. Int Plant Propagator's Soc, Combin Proc, 1980; 30: 421-427.

Lutz A. Description d' une techniqued' isolement cellulaire en vue de l' obtention de culture de

tissus vegetaux provenant d' une cellule unique. C R Ac Sc, 1963; 256: 2676-2678.

Manimegalai S, Rakkimuthu G. Phytochemical Screening of stem of *Couroupita guianensis* IJPSR, 2012; 3(11): 4434-4437.

Manna P, Sinha M, Sil PC. Aqueous extract of *Terminalia arjuna* prevents carbon tetrachloride induced hepatic and renal disorders. BMC Complementary Alternative Med, 2006; 6: 33-33.

Mariana MGP, Sidnei OB, Catharina EF, Ricardo MK, Maria EM, Fabio SM, Patricia DF. Antinociceptive activity of fractions from *Couroupita guianensis* Aubl. leaves. J Ethnopharmacol, 2010; 127: 407–413.

McClelland MT, Smith MAL. Vessel type, closure and explant orientation influence *in vitro* performance of five woody species. Hort Sci,1990; 25: 797–800.

Melchers G, Sacristan MD, Holder A. Somatic hybrid plants of potato and tomato regenerated from fused protoplasts. Carlsberg Res Comm, 1978; 43: 203-218.

Mitre M. *Couroupita guianensis*. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. www.iucnredlist.org.

Muir WH, Hildebrandt AC, Riker AJ. Plant tissue cultures produced from single isolated cells. Science, 1954; 119: 877-878.

Muniswamy B, Sreenath HL. *In-vitro* development of plants from cultured embryos of cannon ball tree (*Couroupita guianensis* Aubl.) Indian J Forest, 2000; 23(2): 202-204.

Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco cultures. Physiol Plant, 1962; 15: 473-497.

Naif Abdullah Al-Dhabi, Chandrasekar Balachandran, Michael Karunai Raj, Veeramuthu Duraipandiyan, Chinnasamy Muthukumar, Savarimuthu, Ignacimuthu, Inshad Ali Khan, Vikrant Singh Rajput. Antimicrobial, antimycobacterial and antibiofilm properties of *Couroupita guianensis* Aubl. Fruit extract. BMC Complementary and Alternative Medicine, 2012; 12: 242.

Phulwaria M, Ram K, Gahlot P, Shekhawat NS. Micropropagation of *Salvadora persica* - a tree of arid horticulture and forestry. New Forests, 2011; 10: 1007.

Pierik RLM. Rejuvenation and micropropagation In: NijKamp HJJ, Plas Vander LHW, Van J Aartrijk. (eds) Progress in Plant Cellular and Molecular Biology, Kluwer Academic Publishers, The Netherlands, 1990; 91-101.

Pradhan D, Panda PK, Tripathy G. Evaluation of the Immune Modulatory activity of the methanolic extract of *Couroupita guianensis* aubl. flowers in rats. Natural Product Radiance, 2009; 8(1).

Purohit SD, Singhvi A. Micropropagation of *Achras sapota* through enhanced axillary branching. Scientia Horticulturae, 1998; 76: 219-229.

Rajamanickam V, Rajasekaran A, Darlin quine S, Jesupillai M, Sabitha R. Anthelmintic activity of the flower extract of *Couroupita guianensis*. The Internet Journal of alternative, Medicine, 2009; 8.

Rane JB, Vahanwala SJ, Golatkar SG, Ambaye RY, Khadse BG. Chemical examination of the flowers of *Couroupita guianensis* Aubl. Indian J Pharm Sci, 2001; 63:72-73.

Rathore MS, Rathore MS, Shekhawat NS. *Ex vivo* implications of phytohormones on various *in vitro* responses in *Leptadenia reticulata* (Retz.) Wight & Arn. an endangered plant. J Environ Exp Bot, 2010; 10:1016.

Rathore TS, Deora NS, Shekhawat NS. Cloning of *Maytenus emarginata* (Willd.) Ding Hou- a tree of the Indian Desert, through tissue culture. Plant Cell Reports, 1992; 11: 449-451.

Reinert J. Uber die kontrolle der Morphogenese und die Induktion von Adventiv-embryogen an Gewebekultwren aus Karotten. Planta, 1958; 53: 318-333.

Sanchez PA, Sherpherd KD, Soule MJ, Place FM and Izac RJ. Soil Fertility Replenishment in Africa: An Investment in Natural Resource Capital. In: Replenishing Soil Fertility in Africa, Buresh, RJ, Sanchez PA, Calhoun F. (eds) SSSA and ICRAF, Madison, USA., 1997; 1-46.

Sanz BJ, Campos-de-la CJ, Epiquien RMA, Canigueral S. A first survey on the medicinal plants of the Chazuta valley (Peruvian Amazon). J Ethnopharmacolo, 2009; 122: 333-362.

Satyavati GV, Raina MK, Sharma M. Medicinal Plants of India. New Delhi: Cambridge printing Works, 1976: 286.

Sederoff R. Building better trees with antisense. Nature Biotechnology, 1999; 17: 750-751.

Sen AK, Mahato SB, Dutta NL. Couroupitine A, A new alkaloid from *Couroupita guianensis*. Tetrahedron Letters, 1974; 7: 609-610.

Shekhawat MS, Dixit AK. Micropropagation of *Eucalyptus camaldulensis* Dehn. clones selected for tsunami affected areas. Int J Plant Sci, 2008; 3(2): 531-535.

Shekhawat MS, Kannan N, Manokari M, Ramanunam MP. An Efficient Micropropagation protocol for High-Frequency Plantlet Regeneration from Liquid Culture of Nodal Tissues in a Medicinal Plant, *Turnera ulmifolia* L. J Sustainable Forestry, 2014; 33:327–336.

Shekhawat MS, Shekhawat NS, Harish, Kheta Ram, Phulwaria M, Gupta AK. High frequency plantlet regeneration from nodal segment culture of female *Momordica dioica* (Roxb.) J Crop Sci Bioteh, 2011; 14(2): 133-137.

Sivakumar T, Shankar T, Vijayabaskar P, Geetha G. Efficacy of *Couroupita guianensis* Against Selected Human Pathogens. Advances in Biological Research, 2012; 6(2): 59-63.

Srivastava LM. Plant growth and development: hormones and environment. Academic Press. 2002; 140.

Swamy RBV, Himabindu K, Lakshmi Sita G. *In vitro* micropropagatipon of elite rosewood (*Dalbergia latifolia* Roxb.) Plant Cell Rep, 2002; 11: 126–131.

Takebe I, Labib C, Melchers G. Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwissen, 1971; 58: 318-320.

Thorpe TA. History of plant tissue culture. In: Smith RH. (ed) Plant tissue culture: Techniques and Experiments. Academic Press, Tokyo, 2000; 1-32.

Ujjwala J Supe. *In vitro* Regeneration of *Aloe barbadensis*. Biotechnology, 2007; 6(4): 601-603.

Umachigi SP, Jayaveera KN, Ashok kumar CK, Kumar GS. Antimicrobial, wound healing, and antioxidant potential of *Couroupita guianensis* in rats. Pharmacology online, 2007; 3: 269-281.

Uthayakumari U. Impact of Thane cyclone on Tagore Arts College campus, Puducherry. M. Sc. Dissertation submitted to the K.M. Centre for PG Studies, 2012.

Vasil IK. (ed) Scale-up and Automation in Plant Propagation: Cell Culture and Somatic Cell Genetics of Plants, 1991; 8: 7-34.

Vasil V, Hildebrandt AC. Differentiation of tobacco plants from single isolated cells in micro cultures. Science, 1965; 150: 889-890.

Velliangiri P, Subban R. Quantification of quercetin and stigmasterol of *Couroupita guianensis* aubl by HPTLC method and *in-vitro* cytototoxic activity by mtt assay of the methanol extract against hela, nih 3t3 and hepg2 cancer cell lines. Int J Pharm Pharmac Sci, 2012; 4(4): 126-130.

Wong KC, Tie DY. Volatile Constituents of *Couroupita guianensis* Aubl. Flowers, J Essential Oil Research, 1995; 7:225-227.

Zhao J, Liu T, Ma L. Antioxidant and preventive effects of extract from *Nymphaea candida* flower on *in vitro* immunological liver injury of rat primary hepatocyte cultures. Evidence-Based Complementary and Alternative Medicine, 2009.

Zimmerman RH. Commercial application of tissue culture to horticultural crops in the United States. J Korean Soc Hortic Science, 1996; 37: 486-490.

APPENDIX

Table 1: Chemical composition of MS medium (Murashige and Skoog, 1962) used in present study.

Constituents	Quantity in mgl^{-1}
Macronutrients	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Na.EDTA	37.23
$FeSO_4.7H_2O$	27.95
Micronutrients	
KI	0.83
H_3BO_3	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Meso-Inositol	100
Glycine	2.0
Thiamine.HCl	0.1
Nicotinic acid	0.5
Pyridoxine.HCl	0.5
Sucrose (%w/v)	3%
pH	5.8

Table 2: Chemical composition of Woody Plants medium (Lloyd and McCown, 1980) used in present study.

Constituents	Quantity in (mgl^{-1})
Macronutrients	
NH_4NO_3	400
K_2SO_4	990
$Ca(NO_3)_2.4H_2O$	556
$CaCl_2.2H_2O$	96
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Na.EDTA	37.30
$FeSO_4.7H_2O$	27.80
Micronutrients	
H_3BO_3	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Meso-Inositol	100
Glycine	2.0
Thiamine.HCl	0.1
Nicotinic acid	0.5
Sucrose(%w/v)	3%
pH	5.8