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Implications of Auxins in Induction of Adventitious Roots from Leaf Explants of Cannon Ball Tree (*Couroupita guianensis* Aubl.)

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ABSTRACT

An effective protocol has been developed for the adventitious root culture of Cannon Ball tree (*Couroupita guianensis* Aubl.) from the leaves of *in vitro* germinated 30 days' old seedlings. Seeds germinated on cytokinins and auxins supplemented with Murashige and Skoog (MS) medium. Half strength MS medium supplemented with 2.0 mg/L indole-3-butyric acid (IBA) was found the most suitable for the induction of adventitious roots (92%) from the midrib region of the leaves than the other auxins. The roots were proliferated on half strength MS liquid medium supplemented with IBA 0.5 mg/L. After eight weeks of inoculation the roots in the medium yielded 5.933 ±0.135 gm fresh and 2.731 ±0.010 gm dry biomass of adventitious roots. The growth rate was increased by 8-9 folds in this investigation.

***Keywords*:** *Couroupita guianensis*; Adventitious roots; Half strength MS medium

1. INTRODUCTION

Couroupita guianensis Aubl. is popularly known as Cannon ball tree belongs to the family Lecythidaceae (Brazil Nut Family). It is listed as threatened medicinal plant worldwide (Mitre 2012; Rai 2014). This species was over exploited during past century and today only few plants exist in protected areas. It is a large deciduous tree growing to a height of 20

meters and native to tropical South America. The leaves are alternate, inflorescence is racemose cauliflorus represents reddish and pink flowers with pleasant aroma. The tree bears large globose woody fruits, directly on the trunk and main branches. They look like big rusty cannon balls hanging in clusters (Fig. 1A-C) (Sai et al. 2011). This plant is also known as Naglingam, Ayahuma, Kailaspati, Calabasse Colin etc. in India (Lim 2012).

Fig. 1A. Morphology of the plant *Couroupita guianensis* Aubl.
Fig. 1B. Naglingam flower.
Fig. 1C & D. Cannon Ball fruit and Seeds.

It is a major ornamental plant in Caribbean and South-East Asian botanic gardens and listed as a rare tree and flower in India (Shah et al. 2012; Shete et al. 2013). The plant is under threat in the wild because the natural propagation of *C. guianensis* is very slow. In nature the plant propagated by seeds only and the seeds are recalcitrant with limited viability. The seeds are reported to have short life span and cannot withstand low temperatures (Gousia et al. 2013). Muniswamy and Sreenath (2000) were unable to germinate cannon ball seeds in the soil. Consequently, the long-term sustainability of remaining population of *C. guianensis* is in question. Recent accounts in the literature refer to the enormous secondary metabolites from *C. guianensis*.

Its flowers are reported to contain terpenoids, phenolic compounds, reducing sugars and triterpenoids, α-amirin, β-amirin, β-sitosterol, tannins, ketosteroids (Ramalakshmi et al., 2013). Eugenol, farnesol and triterpenoid esters of fatty acids as β-amirin palmitate were isolated from the leaves (Eknat and Shivchandraji, 2002). The seeds are reported to possess indigo, indirubin, stigmasterol and campesterol, linoleic acid, nerol and tryptanthrin (Tayade, 2013; Rastogi and Mehrotra, 1995; Bergman et al., 1985).

The varied chemical compositions of *C. guianensis* resulted in multi-specialty biological actions like antibacterial (Shah et al., 2012; Azimi et al., 2012), antioxidant (Bafna et al., 2011), antidepressant (Wankhede et al., 2009), antiulcer (Elumalai et al., 2012), analgesic, anti-inflammatory and antifertility (Geetha et al., 2004 and 2005), antitumor (Premanathan et al., 2012), antimicrobial, antibiofilm (Al-Dhabi et al., 2012; Ramalakshmi et al., 2013), immunomodulatory (Pradhan et al., 2009), antipyretic (Usman et al., 2012), neuropharmacological, anxiolytic (Vinod et al., 2012), wound healing (Umachigi et al., 2007), antiarthritic (Elumalai et al., 2012), antinociceptive (Pinheiro et al., 2010), antidiarrheal (Elumalai et al., 2013), ovicidal (Baskar and Ignacimuthu 2013), antifeedent and larvicidal (Lingathurai et al., 2011) activities. Traditionally, the leaves are employed to cure skin diseases, tumors, inflammations, odontalgia, cold, enteric gas formation and abdomen ache (Golatkar *et al.*, 2001; Elumalai *et al.*, 2012). The volatile oils from the flowers cure hemorrhage, piles, scabies, dysentery and scorpion poison (Shah *et al.*, 2012). The fruit pulp is rubbed to cure skin diseases (Sanz *et al.*, 2009).

The *in vitro* production of medicinal compounds can be possible through plant cell and organ culture under controlled conditions without any environmental fluctuations (Rao and Ravishankar, 2002). The *in vitro* root culture is highly advantageous method for the production of secondary metabolites of pharmaceutical interest, since it is relatively easy to maintain and manipulate (Sivanandhan et al., 2011). Due to the immense medicinal properties this plant was over exploited which resulted in dramatic reduction of its natural population. The Government of Puducherry (India) has declared *C. guianensis* flower (Nagalingam flower) as the Official State Flower to conserve this valuable tree under natural habitats (Deepa 2007). The development of fast growing root culture system offers unique opportunities to provide roots in the laboratory without disturbing the wild population. Moreover, development of root culture is highly advantageous, as it is an alternative method for clonal propagation and germplasm conservation (Chaturvedi and Sharma, 1981).

Realizing the threat of extinction of such threatened medicinal plant species, attention has already been focused towards developing production alternatives of root-derived phytomolecules in order to meet the growing demand of pharmaceutical industries. Plant cell and organ cultures are promising techniques for the production of valuable secondary metabolites, pigments and other chemical compounds (Mulabagal and Tsay, 2004). Plant roots serve as a source of bioactive molecules, flavors, dyes and fragrances (Bais et al., 2001; Fulzele et al., 2002). So far, adventitious root culture has been achieved in many medicinal plants due to its rapid growth and stable production of secondary metabolites of medicinal interest (Murthy et al., 2008). Recently adventitious root cultures have been conducted to increase biomass and secondary metabolite though adventitious root cultures in *Astragalus membranaceus* (Wu et al., 2011), *Stevia reboudiana* (Reis et al., 2011), *Withania somnifera* (Sivanandhan et al., 2012), Gynura procumbens (Saiman et al., 2012), *Echinacea angustifolia* (Cui et al., 2013), *Panax ginseng* (Wang et al., 2013), *Podophyllum hexandrum* (Rajesh et al., 2014), *Glycyrrhiza uralensis* (Yin et al., 2014) etc.

Development of adventitious roots is an intricate process involving various endogenous and exogenous factors (Sorin et al., 2005). The process of differentiation and induction pathways in rooting can be triggered by the supplementation of specific auxins exogenously (Praveen et al., 2009). In the present investigation, *in vitro* culture and proliferation of adventitious roots of *C. guianensis* have been established. It could provide an alternative way to produce the pharmaceutically important secondary metabolites from the roots of *C. guianensis*.

2. MATERIALS AND METHODS

2. 1. Seeds and their sterilization

The fruits were collected during the months of January-April; the seeds were separated using sterile forceps and rinsed several times with tap water. The seeds were immersed in 80% ethanol for 1 min, followed by 0.5% aqueous solution of sodium hypochlorite for 10 min, and rinsed three times in sterile double distilled water. The seeds were treated with 0.1% (w/v) Bavistin (systemic fungicide, BASF India Ltd.) for 5 min and $HgCl_2$ (disinfectant, HiMedia, India) for 4-5 min, and rinsed with autoclaved distilled water for 6-8 times under laminar air flow chamber. Surface sterilized seeds were inoculated on culture medium.

2. 2. Culture medium and incubation conditions

The seeds were cultured on MS basal medium (Murashige and Skoog 1962) containing 3% (w/v) sucrose, 0.8% agar, additives (50 mg/L ascorbic acid, 25 mg/L each of citric acid, L-arginin and adenine sulphate) and various plant growth regulators 6-benzylaminopurine (BAP), Kinetin (Kin), indole-3-acetic acid (IAA) and IBA. The pH of the medium was rendered 5.8 using 0.1 N NaOH or 0.1 N HCl before autoclaving and dispensed into culture tubes (25×150 mm). Culture experiments were maintained under light and dark field (40-45 µmol m^{-2} s^{-1} Spectral Flux Photon Density (SFPD) light intensity for 12 h/d photoperiod) at 2 5±2 °C temperature and 50-70% relative humidity.

2. 3. Induction of roots from *in vitro* leaf inoculum

The leaf explants (2 cm in length and 3.8 cm in width) from 30 days old *in vitro* germinated seedlings were inoculated on different strength of agar gelled MS medium (Full, half and one-fourth) fortified with different types of auxins (IAA, IBA, Naphthalene acetic acid and Naphthoxy acetic acid) at varied concentrations (1.0-4.0 mg/L). The cultures were incubated at 25^{o}C in the dark for adventitious root proliferation.

2. 4. Proliferation of adventitious roots in liquid medium

After four weeks, the adventitious roots were individually excised to a length of approximately 3-5 cm and sticking agar gel was removed, and inoculated to 250 ml flask containing 100 ml of different strength of MS liquid medium supplemented with various concentrations of IBA (0.5-4.0 mg/L) along with additives. The cultures were maintained at 25 ±2 °C in the dark on a rotary shaker at 100 rpm (Technico Pvt Ltd., Chennai, India). At the end of every four weeks, the roots were subcultured to fresh medium containing the same

optimal concentration of hormones. Finally, the adventitious roots were harvested after eight weeks.

2. 5. Determination of Biomass

The *in vitro* cultured adventitious roots were collected after eight weeks from the media and the fresh weight (FW) was measured after rinsing with sterile distilled water and the surface water was dried using blotting paper. The adventitious root dry weight (DW) was recorded after 2 days drying at 60 °C in hot air oven. The growth rate was calculated according to Sivakumar et al. (2005) and Ahmed et al. (2008).

$$\text{The Growth Rate} = \frac{\text{The harvested dry weight} - \text{The inoculated dry weight}}{\text{The inoculated dry weight}}$$

F/D ratio = Fresh weight/ Dry weight

2. 5. Statistical analysis

The experiments were repeated thrice with ten replicates. The percentage of response in root induction, fresh and dry weight of roots from leaf explants were monitored as growth parameters. Data collected in the experiments were analyzed using SPSS software (version 16.0). The mean values and the differences within the treatments were compared using one-way analysis of variance (ANOVA). Duncan's Multiple Range Test (DMRT) was performed at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3. 1. *In vitro* seed germination

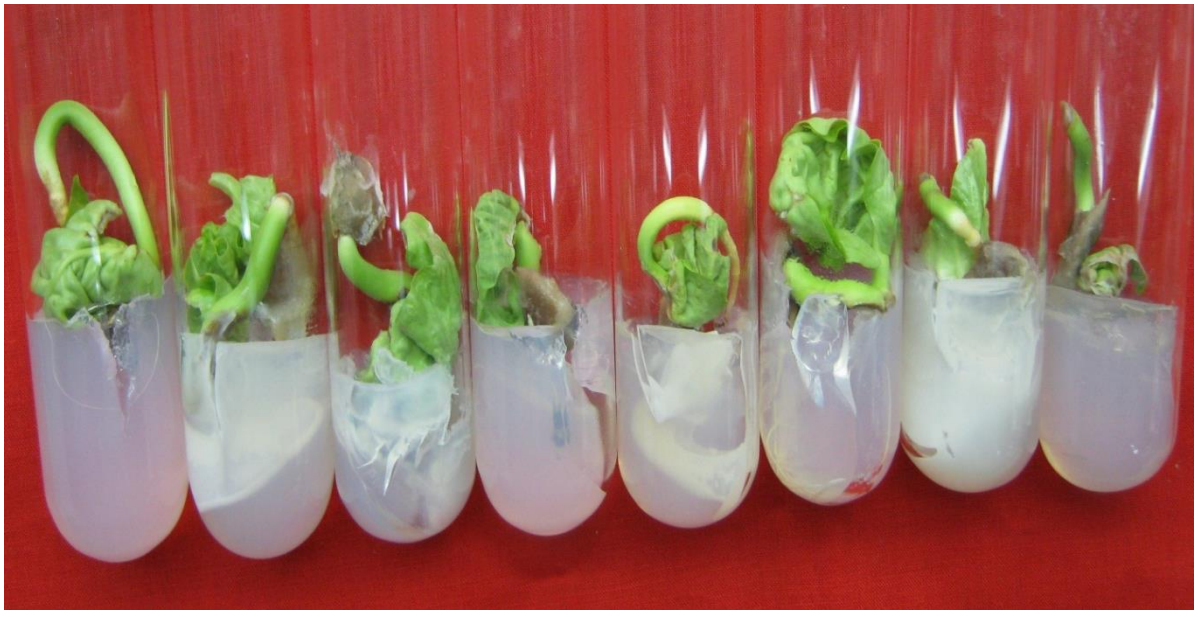

Fig. 2. *In vitro* seed germination of *C. guianensis*

The seeds inoculated on MS medium supplemented with all concentrations of plant growth regulators were responded with in a week. Most of the seeds were germinated *in vitro* due to sterilization procedure and absorption of nutrients from the medium. Maximum percentage of seed germination was recorded on MS supplemented with auxins (data not shown). The leaves from *in vitro* germinated seedlings were used as explants to induce adventitious roots. Initially the seed cultures were incubated under dark field and transferred to light field after one week (Fig. 2).

3. 2. Effect of auxins in adventitious root induction from *in vitro* derived leaves

Different types of auxins along with various strength of MS medium were tested for primary adventitious root induction from the leaves. Normal roots were produced from leaf explants with all the combinations after four weeks of incubation. Mature leaves from 30 days old seedlings exhibited higher response for root induction when cultured on MS semi-solid medium supplemented with different combinations and concentrations of IAA, IBA, NAA and NOA. Among the different concentrations of auxins tested individually, half strength MS medium supplemented with IBA was reported most effective (92%) than other auxins. IBA 2.0 mg/L was observed the best concentration to promote the growth of roots from leaves (Table 1). In the present study, roots induction occurred at the cut midrib part of the leaf explants. After four week of cultures, rooting frequency was comparatively less on IAA, NAA and NOA. Maximum 85% of leaves responded on half strength MS medium with 2.0 mg/L IAA, 77% and 54% on 2.0 mg/L NAA and NOA respectively.

Table 1. Effect of auxins in half strength MS medium on root induction from leaf.

Conc. of auxins (mg/L)				Percentage of Response (%)
IAA	IBA	NAA	NOA	
0.0	0.0	0.0	0.0	00[a]
1.0	-	-	-	63[f]
2.0	-	-	-	85[j]
3.0	-	-	-	72[g]
4.0	-	-	-	64[f]
-	1.0	-	-	78[i]
-	2.0	-	-	92[k]
-	3.0	-	-	80[i]
-	4.0	-	-	73[g]
-	-	1.0	-	56[e]
-	-	2.0	-	77[g]
-	-	3.0	-	70[f]

-	-	4.0	-	63[c]
-	-	-	1.0	49[d]
-	-	-	2.0	54[c]
-	-	-	3.0	50[b]
-	-	-	4.0	43[b]

Note: Mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and significance of variation between the concentrations was studied using DMRT at 0.05% level.

Fig. 3A. Adventitious root induction from in vitro leaves.
Fig. 3B. Adventitious roots induced within a week.
Fig. 3C. Adventitious roots induced within 4 weeks.

The cultures under complete dark environment induced callus, which inhibited the rooting frequency. Initially cultures were incubated in dark for a week and then transferred to light field for another week. This cycle repeated for four weeks. The root induction from the leaves was visible white and thick in appearance (Fig. 3A-C). Auxins supplemented with full strength MS medium always induced profuse callusing which subsequently turned brownish and prohibited initiation of roots from the leaves. Thin and fragile roots were reported from one-fourth MS medium augmented with various kinds of auxins. IBA was reported to have greater stability than other auxins (Pacurar et al., 2014).

The superiority of IBA in adventitious root cultures was discussed in many plants like *Psoralea corylifolia* (Baskaran and Jayabalan, 2009), *Nepeta cataria* (Yaang et al., 2010), *Ginkgo biloba* (Pandey et al., 2011), *Bixa orellana* (Mahendranath et al., 2011), (*Labisia pumila* (Ling et al., 2013) and *Luffa acutangula* (Umamaheswari et al., 2014). Root initiated within 2 weeks from the leaves incubated under total darkness, whereas, rooting was delayed (more than 6 weeks) under light condition (16 h photoperiod).

The results proved that half strength chemical environment (MS basal medium and sucrose) fortified with IBA (2.0 mg/L) under optimum dark and light field proved favorable for induction of adventitious roots from the leaves of *C. guianensis*. There were neither adventitious roots nor callus formation observed in the auxin free MS medium under either light or dark field.

3. 3 Effect of IBA on proliferation of adventitious roots in liquid medium

The *in vitro* regenerated adventitious roots obtained from the previous stage were excised and used as inoculums (0.5 gm) for the proliferation of adventitious roots. The roots (4-5 cm long) were inoculated on 250 ml culture flask containing half strength MS liquid medium fortified with different concentrations IBA. The cultures were agitated at 100 rpm on a gyratory shaker in dark field at 25 ±2 °C for 4 weeks. The maximum fresh and dry biomass was reported on half strength MS medium supplemented with 1.0 mg/L IBA (Fig. 4).

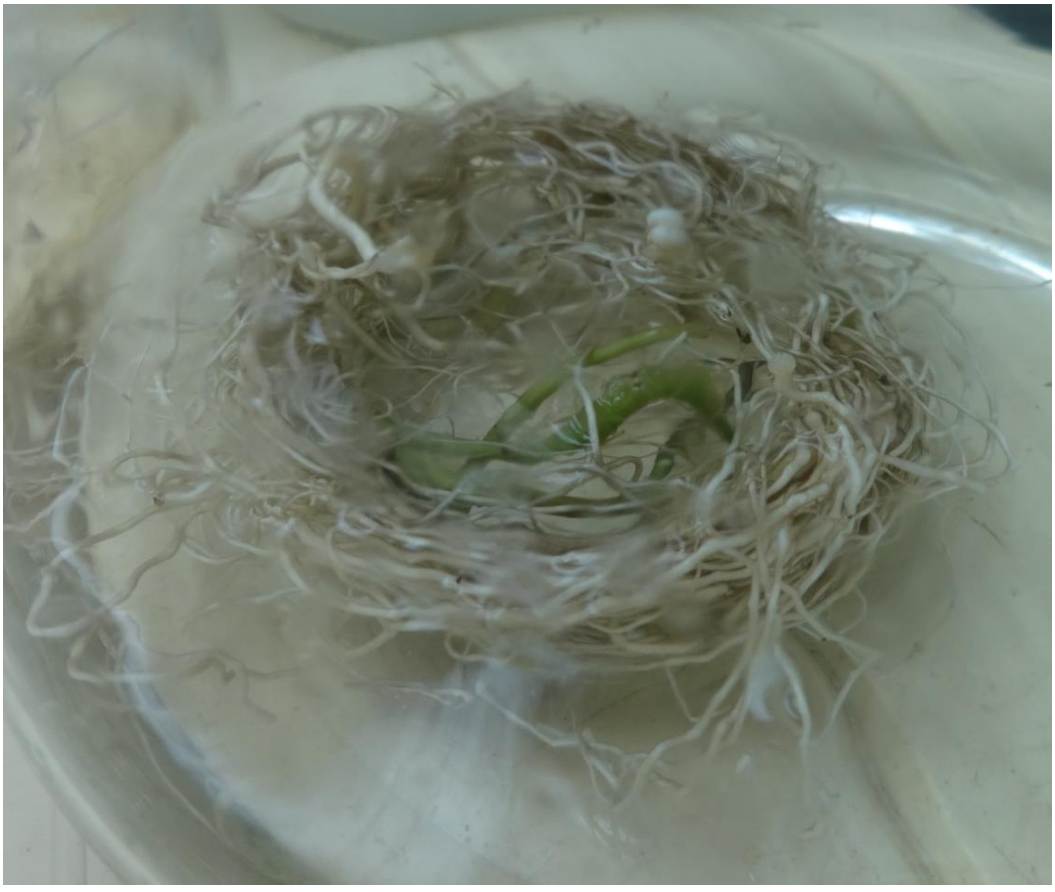

Fig. 4. Proliferation of Adventitious roots on MS liquid medium (shake flask culture).

This concentration yielded 5.933 ±0.135 gm fresh and 2.731 ±0.010 gm dry biomass. This value was higher than other concentrations of IBA tested. There was a correlation observed between the increase in concentration and biomass production up to the optimal level. It was observed that the fresh and dry weights were gradually decreased beyond the optimum concentration. Higher concentrations of IBA (4.0 mg/L) produced 0.802 ±0.286 and 0.498 ±0.009 gm biomass with high intensity of callus (Table 2). The effect of increased auxins concentrations on the hindrance of root induction and biomass production also reported in *Luffa acutangula* (Umamaheswari et al., 2014). The growth ratio was increased up to 8-9 folds using half strength liquid MS medium with 2.0 mg/L IBA, and the F/D ratio was 3.202 after 8 weeks of culture (Fig. 5).

Fig. 5A. Harvested fresh adventitious roots
Fig. 5A. Harvested adventitious roots dried

Table 2. Effect of IBA on fresh and dry weight of adventitious roots induced on liquid MS medium under dark field.

Conc. of IBA (mg/L)	Fresh weight of roots (gm) (Mean ± SD)	Dry weight of roots (gm) (Mean ± SD)	Growth Ratio (GR)	F/D ratio
0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0	0.0
0.5	3.290 ± 0.088^{d}	1.264 ± 0.011^{c}	1.528	2.026
1.0	5.933 ± 0.135^{f}	2.731 ± 0.010^{e}	4.462	3.202
2.0	4.720 ± 0.205^{e}	2.053 ± 0.014^{d}	3.106	2.667

3.0	1.525 ±0.209[c]	0.847 ±0.018[b]	0.694	0.678
4.0	1.502 ±0.286[b]	0.800 ±0.009[b]	0.600	0.702

Note: Mean separation was analyzed by ANOVA using SPSS software (var. 16.0) and significance of variation between the concentrations was studied using DMRT at 0.05% level.

Incubating adventitious root cultures under dark field induced roots due to the slow metabolism of endogenously applied auxins than light. It is agreed with the results of adventitious roots in *Cichorium intybus* (Nandagopal and Kumari, 2007), *Morinda citrifolia* (Baque et al., 2012), *Psammosilene tunicoides* (Zhang et al., 2013) and *Luffa acutangula* (Umamaheswari et al., 2014).

4. CONCLUSION

To conclude the present study, *in vitro* culture system for adventitious root cultures of threatened plant *C. guianensis* have been established, which provides an alternative way to produce roots, so as to avoid the exploitation of roots for pharmaceutical industries. Maximum percentage of adventitious roots was induced from mature *in vitro* leaves of 30 days old seedlings. Half strength MS semisolid medium supplemented with 2.0 mg/L IBA was found most suitable for the induction of primary adventitious roots. Liquid half strength MS medium with 0.5 mg/L IBA was found optimum for the production of maximum biomass of adventitious roots. To the best of our knowledge; this is the first report on influence of exogenous auxins in adventitious root cultures of *Couroupita guianensis* Aubl.

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