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Research Article
In vitro Regeneration System of *Couroupita guianensis* Using Cotyledonary Nodes

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Abstract

Background and Objective: *Couroupita guianensis* (family: Lecythidaceae) is a medicinally important tree distributed in tropics and widely used in traditional medicine. Propagation of this plant species through seeds is not feasible under natural conditions, as the seeds are consumed along with fruit pulp by peccaries, pigs and monkeys when the fruits split open. However, the seedling emergence and growth is greatly affected by abiotic stress like heat and drought. Micropropagation system is required for clonal propagation, germplasm conservation and genetic improvement to satisfy the pharmaceutical demand of this medicinal plant. Therefore, the current investigation was focused to bring out an effective protocol for *in vitro* shoot multiplication of *C. guianensis* using cotyledonary nodes. **Materials and Methods:** An efficient micropropagation protocol has been established for *C. guianensis* using cotyledonary nodes from one week old *in vitro* germinated seedlings. Murashige and Skoog's (MS) medium and Woody plant (WP) medium amended with various combination of cytokinins [6-benzylaminopurine (BAP), kinetin (KIN), thidiazuron (TDZ)] and auxins [indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and α-naphthalene acetic acid (NAA)] were evaluated for their efficiency in inducing multiple shoots. **Results:** The WP medium was superior to MS medium in the multiple shoot induction (~2 folds) where a combination of 3.0 mg L^{-1} BAP+ 2.0 mg L^{-1} KIN induced highest number of shoots (11.04±0.17) per explant. The addition of 0.5 mg L^{-1} TDZ increased the number of shoots per explant (14.28±0.07) with shoot length of 6.18±0.11 cm. Micropropagated shoots rooted on MS medium supplemented with 3.0 mg L^{-1} IBA produced more number of roots (2.66±0.11) with root length of 2.96±0.06 cm. An increase in the number of roots (5.33±0.16) and root length (5.73±0.35 cm) was observed with the addition of activated charcoal (1.0 g L^{-1}) to the rooting medium. Rooted shoots transferred to quarter-strength liquid MS basal salts produced secondary roots. After 7 days of secondary roots initiation, healthy shoots were transferred to the soil mixture containing sand, red soil and organic manure (1:1:0.5, v/v/v). Monomorphic DNA fingerprinting pattern obtained using RAPD and ISSR markers confirmed the clonal fidelity of micropropagated plantlets. **Conclusion:** This study promotes the large-scale clonal propagation of *C. guianensis* for sustainable utilization in traditional medicine and reintroduction into its natural habitats.

Key words: *Couroupita guianensis*, micropropagation, cotyledonary node, plant growth regulators, activated charcoal

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Couroupita guianensis (Lecythidaceae) an important medicinal tree is widely used in folk medicine[1]. It is commonly called as cannonball tree because of large, ball-shaped fruits hanging down from the trunk[2]. In tamil, it is referred as nagalingam, as the flowers contain snake-shaped pollen at the centre[1]. In traditional medicine system, whole tree parts of *C. guianensis* are used to treat tumors, inflammation, malaria, skin infections and toothache[3,4]. The leaves possess anti-inflammatory[5], anti-bacterial, anti-microbial, anti-mycobacterial, anti-biofilm[6,7], anti-ulcer, anti-arthritic, anti-diarrheal[8-10], anti-cancer[11], anti-depressant[12], ovicidal[13,14] and anti-nociceptive properties[15]. The pharmaceutical industry uses the tree parts (leaf, flower and root) to prepare a syrup "Betalupe" for treating female infertility[16].

Couroupita guianensis is found mostly in sacred groves of temples. However, its distribution in natural habitats is threatened by human settlements[17]. Propagation of this plant species through seeds is not feasible under natural conditions, as the seeds are consumed along with fruit pulp by peccaries, pigs and monkeys when the fruits split open. Rarely, seeds that escape the degradation of their digestive enzymes germinate under suitable conditions. However, the seedling emergence and growth is greatly affected by abiotic stress like heat and drought. An efficient micropropagation system is required for clonal propagation, germplasm conservation and genetic improvement to satisfy the pharmaceutical demand of this medicinal plant. Micropropagation has been successfully employed for the large scale clonal propagation of medicinally important tree species like, *Stevia rebaudiana*[18] and *Morus alba*[19]. Previous report indicates the use of apical shoots and nodal segments from *in vitro* seedlings for micropropagation of *C. guianensis*[20].

Micropropagated plantlets, in general, are genetically identical as multiple shoots are formed from meristematic cells[21]. Genetic stability of *in vitro* regenerated plantlets is commonly analyzed using molecular markers such as randomly amplified polymorphic DNA [RAPD], inter simple sequence repeat [ISSR], restriction fragment length polymorphism [RFLP] and amplified fragment length polymorphism [AFLP][22]. Of these, RAPD and ISSR are rapid, cost effective and highly discriminating markers[23]. Both these markers were useful in analyzing the genetic integrity of micropropagated medicinal plants like *Lecythis pisonis* Cambess[24] and *Harpagophytum procumbens*[25].

Therefore, the current investigation was focused to bring out an effective protocol for *in vitro* shoot multiplication of *C. guianensis* using cotyledonary nodes and to authenticate the genetic homogeneity using RAPD and ISSR markers. This study adds further evidence to the potential of molecular markers in assessing the genetic integrity of *C. guianensis*.

MATERIALS AND METHODS

Collection of plant materials and surface sterilization: *Couroupita guianensis* fruits were collected from the Loyola College hostel garden, Chennai, India (13°03'41.7"N 80°13'57.5"E). Mature and healthy seeds were extracted from the ripened fruits after removing the outer shell mechanically. The pulp debris was removed from the seeds by washing under running tap water for 15 min and seeds were left for drying overnight at room temperature (28°C). The outer layer of the seed coat with exotestal hairs was removed manually and seeds were surface sterilized for 20 min in disinfecting solution containing 1 mL of Tween-20 per 100 mL in 1% (v/v) sodium hypochlorite (available Chlorine 4% w/v approx, Qualigens, Mumbai, India). The seeds were then subjected to 6-8 washes using autoclaved double distilled water. After surface sterilization, seed coat was removed with a sterile blade, embryos were blotted under sterile conditions on a filter paper and germinated on MS medium amended with 1.0 mg L^{-1} KIN with combination of 0.1 mg L^{-1} IBA. Culture tubes were maintained in dark for 3 days and then transferred to light [16/8 h (day/night) photoperiod] with a photosynthetic photon flux density (PPFD) of 50 µmol m^{-2} sec^{-1} supplied with cool-white fluorescent lamps (Philips, Chennai, India). Explants were derived from one week old *in vitro* seedlings. Cotyledonary nodes were separated from the hypocotyl and the cotyledonary leaves were trimmed to expose the shoot apex.

Effect of different media and PGRs on *in vitro* shoot regeneration: Cotyledonary nodes (7 mm) were cultured in MS[26] or Woody Plant (WP) medium containing 30 g L^{-1} sucrose, 8 g L^{-1} agar (HiMedia, Mumbai, India), 1% (w/v) polyvinylpyrrolidone (PVP) and supplemented with and without plant growth regulators (PGRs), such as 6-benzylaminopurine (BAP) or kinetin (KIN) (1.0-5.0 mg L^{-1}) alone or in combination with indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) and thidiazuron (TDZ) (0.5, 1.0 mg L^{-1}) (HiMedia, Mumbai, India) to determine the optimum conditions for multiple shoot induction (Table 1, 2 and Fig. 1). The pH of the medium was adjusted to 5.8 before gelling with agar (HiMedia, Mumbai, India). About 10 mL of culture medium was dispensed into

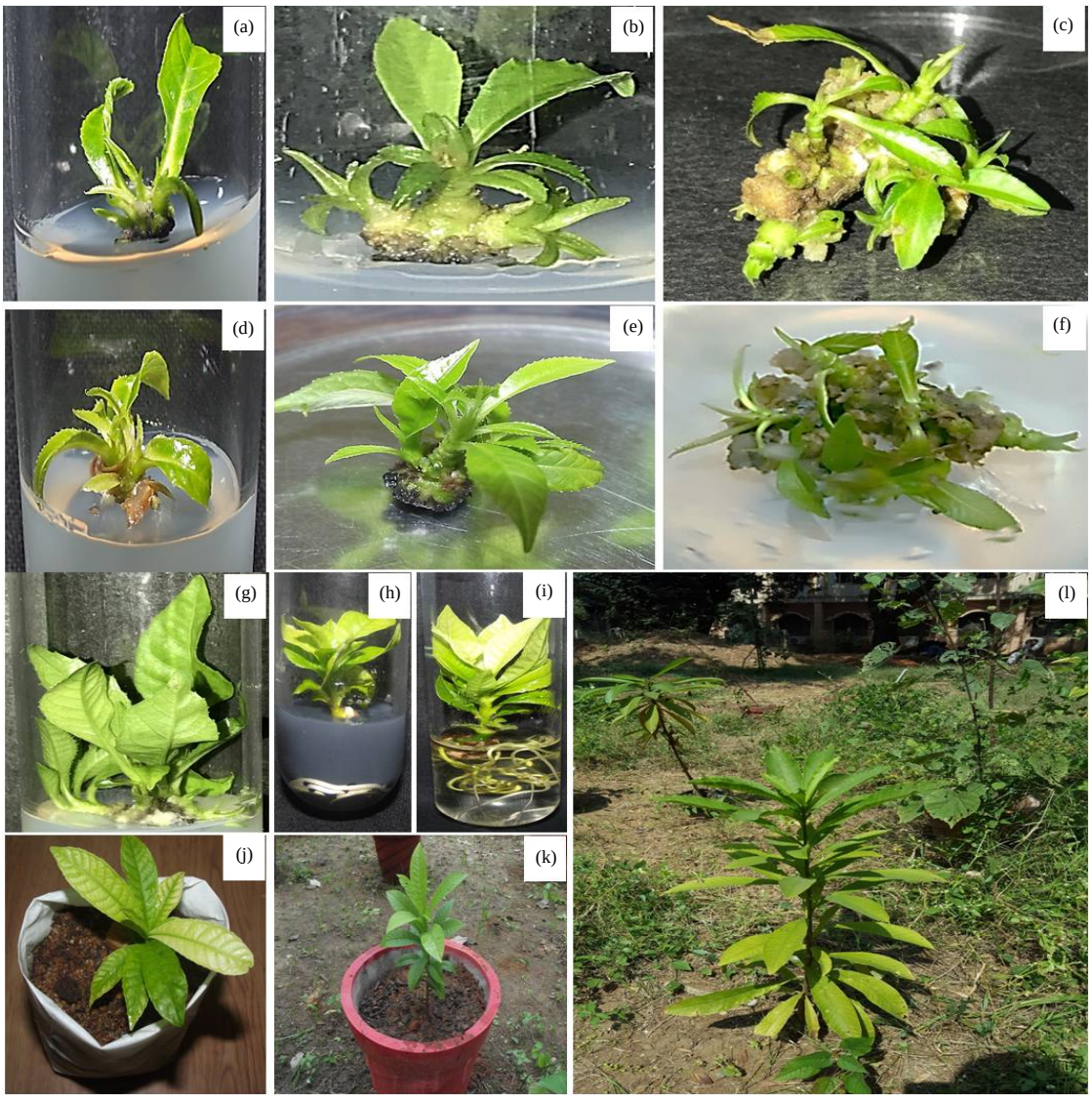

Fig. 1(a-k): Micropropagation and regeneration from cotyledonary node of *Couroupita guianensis*
(a) Shoot multiplication on MS medium supplemented with 3.0 mg L^{-1} KIN+1.0 mg L^{-1} NAA after 4 weeks, (b) After 6 weeks, (c) After 8 weeks, (d-f) Shoot multiplication on WP medium, (g) Highest no of shoots was produced in WP medium supplemented with BAP 3 mg L^{-1}+2.0 mg L^{-1} KIN+0.5 mg L^{-1} TDZ, (h) Rooting medium with 1 g L^{-1} activated charcoal, (i) Secondary roots initiation in quarter-strength MS liquid basal salts, (j) Secondary roots plantlets were transferred to HDPE grow bags to the soil mixture containing garden soil, red soil and sand (1:1:1, v/v/v) and (k) After one month of plants transferred to pot containing sand, soil and cow dung manure (1:1:0.5, v/v/v); (l) Hardening of plants

tubes (25 mm diameter×150 mm height) (Borosil, Chennai, India) and autoclaved at 121°C for 20 min. The cultures were maintained at 25±2°C in light [16/8 h (day/night) photoperiod] supplied by cool white fluorescent lamps (Phillips, Chennai, India) at 50 µmol m^{-2} sec^{-1} PPFD. Newly emerging shoots were sub-cultured at every 2 weeks intervals onto the same initial medium.

Rooting and acclimatization: The proliferated shoots (2-3 cm length) were individually separated and transferred to the rooting medium with (1.0 and 2.0 g L^{-1}) or without activated charcoal. Full-strength MS salts and vitamins supplemented with various concentrations of IAA or IBA (1.0-6.0 mg L^{-1}) was used for rooting (Fig. 2) with MS basal medium as control. Rooted plantlets (5 cm height) after 2 weeks of culture were removed from the culture tubes and washed with sterile water to remove the agar media, blot dried on filter paper and placed in quarter-strength liquid MS basal salts for the induction of secondary roots. After 2 weeks, plantlets (15-18 cm height) with well-established root system were

Table 1: Effect of different media and PGRs on multiple shoot induction of *Couroupita guianensis*

PGRs (mg L^{-1})					No. of shoots per explant		Shoot length (cm)	
BAP	KIN	IBA	IAA	NAA	MS	WP	MS	WP
-	-	-	-	-	1.0±0.00^{m}	2.72±0.13^{m}	1.60±0.05^{mn}	2.94±0.08^{j}
1.0	-	-	-	-	1.07±0.01^{l}	3.28±0.08^{lm}	1.62±0.06^{m}	3.14±0.13^{i}
1.5	-	-	-	-	1.04±0.01^{l}	3.76±0.10^{kl}	1.66±0.09^{m}	3.38±0.08^{hi}
2.0	-	-	-	-	1.20±0.05^{ij}	4.36±0.07^{jk}	1.90±0.10^{k}	3.48±0.09^{h}
2.5	-	-	-	-	1.72±0.08^{d}	5.20±0.07^{i}	2.64±0.13^{h}	3.76±0.06^{gh}
3.0	-	-	-	-	1.25±0.01^{i}	5.56±0.06^{h}	3.10±0.06^{c}	3.88±0.08^{g}
4.0	-	-	-	-	1.30±0.01^{gh}	6.44±0.08^{f}	1.96±0.03^{k}	4.62±0.10^{c}
5.0	-	-	-	-	1.22±0.01^{ij}	5.04±0.05^{ij}	2.28±0.10^{ij}	3.40±0.10^{h}
-	1.0	-	-	-	1.05±0.01^{l}	3.32±0.07^{l}	1.50±0.09^{o}	2.92±0.06^{j}
-	1.5	-	-	-	1.11±0.02^{k}	4.24±0.06^{k}	1.60±0.11^{mn}	3.14±0.10^{i}
-	2.0	-	-	-	1.06±0.01^{l}	5.08±0.11^{ij}	1.86±0.09^{kl}	3.48±0.09^{h}
-	2.5	-	-	-	1.32±0.06^{gh}	6.16±0.11^{g}	2.26±0.08^{ij}	4.26±0.08^{ef}
-	3.0	-	-	-	1.97±0.12^{c}	5.36±0.09^{hi}	3.28±0.14^{b}	3.42±0.18^{h}
-	4.0	-	-	-	1.36±0.06^{g}	4.52±0.09^{j}	2.82±0.06^{ef}	3.38±0.09^{hi}
-	5.0	-	-	-	1.12±0.06^{k}	4.24±0.06^{k}	2.54±0.09^{h}	3.14±0.12^{i}
2.5	-	0.5	-	-	1.18±0.04^{k}	7.64±0.05^{a}	1.90±0.05^{k}	4.92±0.15^{a}
2.5	-	1.0	-	-	1.16±0.03^{k}	6.84±0.05^{d}	2.46±0.08^{i}	4.38±0.25^{e}
2.5	-	-	0.5	-	1.31±0.04^{gh}	6.44±0.08^{f}	2.80±0.05^{ef}	4.24±0.25^{ef}
2.5	-	-	1.0	-	1.28±0.02^{i}	6.04±0.04^{gh}	2.94±0.05^{e}	4.02±0.14^{f}
2.5	-	-	-	0.5	1.61±0.05^{e}	7.24±0.17^{b}	2.86±0.07^{f}	4.42±0.24^{d}
2.5	-	-	-	1.0	2.11±0.02^{b}	6.32±0.07^{fg}	3.04±0.09^{d}	4.22±0.28^{ef}
-	3.0	0.5	-	-	1.19±0.08^{k}	7.16±0.08^{c}	2.70±0.15^{g}	4.86±0.23^{b}
-	3.0	1.0	-	-	1.23±0.02^{ij}	6.72±0.05^{de}	2.84±0.08^{ef}	4.74±0.13^{bc}
-	3.0	-	0.5	-	1.50±0.02^{f}	6.36±0.14^{fg}	3.12±0.06^{c}	4.68±0.13^{c}
-	3.0	-	1.0	-	1.50±0.04^{f}	6.08±0.12^{gh}	3.30±0.11^{b}	4.60±0.17^{c}
-	3.0	-	-	0.5	2.20±0.01^{b}	6.68±0.04^{e}	3.08±0.09^{d}	4.72±0.19^{bc}
-	3.0	-	-	1.0	5.66±0.08^{a}	6.52±0.06^{ef}	3.50±0.04^{a}	4.58±0.19^{cd}

Values represent Mean±SE. Different letters indicated significant difference among the treatment means analyzed using Duncan's multiple range test ($p<0.05$). This data recorded on 6 weeks

Table 2: Effect of TDZ supplementation in different media and PGRs on multiple shoot induction of *Couroupita guianensis*

PGRs (mg L^{-1})						No. of shoots per explant		Shoot length (cm)	
BAP	KIN	IBA	IAA	NAA	TDZ	MS	WP	MS	WP
2.5	-	0.5	-	-	0.5	5.12±0.04^{fg}	9.12±0.15^{d}	3.06±0.06^{e}	4.98±0.09^{c}
2.5	-	1.0	-	-	0.5	4.96±0.02^{h}	7.64±0.19^{fg}	2.86±0.06^{f}	4.90±0.13^{c}
2.5	-	0.5	-	-	1.0	4.92±0.06^{h}	7.52±0.11^{g}	2.42±0.09^{h}	4.80±0.16^{cd}
2.5	-	1.0	-	-	1.0	4.84±0.04^{h}	7.08±0.22^{hi}	2.26±0.05^{ij}	3.90±0.09^{e}
2.5	-	-	0.5	-	0.5	4.32±0.08^{hi}	6.72±0.24^{j}	2.60±0.09^{g}	4.92±0.16^{c}
2.5	-	-	1.0	-	0.5	3.92±0.05^{i}	6.40±0.26^{kl}	2.54±0.10^{gh}	4.82±0.17^{cd}
2.5	-	-	0.5	-	1.0	3.36±0.08^{g}	5.84±0.22^{l}	2.30±0.03i	3.98±0.07^{e}
2.5	-	-	1.0	-	1.0	3.32±0.09^{g}	5.60±0.13^{m}	2.18±0.05^{j}	3.86±0.06^{ef}
2.5	-	-	-	0.5	0.5	6.16±0.09^{e}	7.88±0.20^{f}	3.20±0.06^{de}	4.88±0.21^{cd}
2.5	-	-	-	1.0	0.5	7.24±0.19^{d}	7.48±0.08^{gh}	2.88±0.06^{f}	4.62±0.22^{d}
2.5	-	-	-	0.5	1.0	5.96±0.06^{f}	6.96±0.09^{i}	2.34±0.07^{i}	4.56±0.18^{de}
2.5	-	-	-	1.0	1.0	5.84±0.30^{f}	6.72±0.12^{j}	2.24±0.06^{ij}	3.84±0.06^{ef}
-	3.0	0.5	-	-	0.5	6.08±0.05^{e}	8.56±0.42^{e}	2.70±0.14^{fg}	4.92±0.24^{c}
-	3.0	1.0	-	-	0.5	5.76±0.05^{f}	7.68±0.18^{fg}	3.06±0.08^{e}	4.90±0.19^{c}
-	3.0	0.5	-	-	1.0	5.24±0.11^{f}	7.56±0.17^{g}	2.44±0.06^{h}	4.66±0.12^{d}
-	3.0	1.0	-	-	1.0	5.44±0.11^{fg}	7.32±0.18^{h}	2.56±0.08^{gh}	3.68±0.06^{f}
-	3.0	-	0.5	-	0.5	4.88±0.50^{h}	7.08±0.16^{hi}	2.94±0.14^{f}	4.88±0.22^{cd}
-	3.0	-	1.0	-	0.5	4.32±0.41^{hi}	6.80±0.11^{ij}	3.04±0.02^{e}	4.68±0.20^{d}
-	3.0	-	0.5	-	1.0	4.28±0.23^{hi}	6.72±0.08^{j}	2.66±0.10^{g}	3.94±0.09^{e}
-	3.0	-	1.0	-	1.0	3.92±0.17^{i}	6.56±0.11^{k}	2.62±0.09^{g}	3.88±0.10^{ef}
-	3.0	-	-	0.5	0.5	9.36±0.25^{b}	7.84±0.18^{f}	3.44±0.05^{d}	4.84±0.24^{cd}
-	3.0	-	-	1.0	0.5	10.04±0.18^{a}	6.88±0.16^{ij}	4.18±0.13^{a}	4.72±0.25^{d}
-	3.0	-	-	0.5	1.0	9.04±0.14^{bc}	6.60±0.17^{jk}	3.44±0.14^{d}	4.64±0.23^{d}
-	3.0	-	-	1.0	1.0	8.08±0.16^{c}	6.52±0.11^{k}	3.48±0.15^{d}	3.98±0.07^{e}
2.5	3.0	-	-	-	-	4.28±0.18^{hi}	11.04±0.17^{b}	3.98±0.07^{b}	4.64±0.16^{d}
2.5	3.0	-	-	-	0.5	5.06±0.17^{fg}	14.28±0.07^{a}	3.90±0.17^{b}	6.18±0.11^{a}
2.5	3.0	-	-	-	1.0	4.80±0.14^{h}	10.52±0.09^{c}	3.66±0.11^{c}	5.56±0.10^{b}

Values represent Mean±SE. Different letters indicated significant difference among the treatment means analyzed using Duncan's multiple range test ($p<0.05$)

transferred to greenhouse conditions in HDPE (High-density polyethylene) grow bags (10 cm diameter) filled with a soil mixture containing sterilized sand, red soil and organic manure (1:1:1, v/v/v). Hardened plants with 6-10 fresh leaves were transferred to clay pots (30 cm height×28 cm diameter) and plants grown to about 30 cm in height were transferred to soil.

Assessment of genetic fidelity: Genomic DNA was extracted from the leaves of three *ex vitro* micropropagated plants and the mother plant of *C. guianensis* as described[27]. A total of 20 randomly amplified polymorphic DNA [RAPD] and 10 inter simple sequence repeat [ISSR] primers were used for genetic fidelity analysis, out of which 10 RAPD and 6 ISSR primers were selected based on the amplification of distinct and scorable bands. The PCR reaction setup and cycling conditions were maintained as described[23], except for the primer annealing temperature (36°C for 30 sec-RAPD; 53-55°C for 30 sec-ISSR). The PCR products resolved on 1.5% (w/v) agarose were visualized under UV transilluminator and documented using the Gel documentation system (Gelstan 4X, Chennai, India). DNA fingerprinting profiles were compared using Labimage 1D software version 3.3.0 (Kapelan Bio-Imaging Solutions, Leipzig, Germany) to evaluate the clonal fidelity. The experiment was repeated three times to confirm the reproducibility of banding pattern by ISSR markers.

Statistical analysis: All the experiments were repeated three times with 30 explants per treatment. Data is presented as Mean±standard error and the analysis of variance (One-way ANOVA) was performed. For shoot proliferation, the regeneration frequency (%), mean number of shoots per explant and shoot length were recorded after 6 weeks of culture. For root induction, mean number of roots and root length were recorded after 2 weeks of culture on rooting medium. Duncan's multiple range test was used to compare the significant differences among the treatment means at 5% probability level using IBM SPSS statistics version 19.0.

RESULTS

Effect of different media and PGRs on *in vitro* shoot regeneration: Multiple shoots were successfully induced from cotyledonary node of one week old *in vitro* germinated seedlings of *C. guianensis*. The multiple shoot responses to different media (MS and WP) containing cytokinins alone (BAP and KIN) and in combination with auxins (NAA, IAA or IBA) and TDZ are summarized in Table 1, 2. The WP medium showed high frequency in shoot regeneration without any callus formation, while basal WP medium produced shoots per explant (Table 1). The synergistic application of auxins with the best possible concentration of cytokinins showed that the maximum number of shoots per explant (Table 1) occurred in MS medium containing 3.0 mg L^{-1} KIN+1.0 mg L^{-1} NAA (Fig. 1a-c) and in WP medium containing 2.5 mg L^{-1} BAP+0.5 mg L^{-1} IBA, respectively (Fig. 1d-f).

Effect of TDZ supplementation in different media and PGRs on *in vitro* shoot regeneration: The highest number of shoots (14.28±0.07) was produced in WP medium containing 2.5 mg L^{-1} BAP + 3.0 mg L^{-1} KIN + 0.5 mg L^{-1} TDZ (Table 2, Fig. 1g). This was 1.4 folds greater than that achieved in MS medium producing the maximum shoot number. Increase in shoot length was also observed in WP medium (Table 2). This implied that WP medium promoted elongation of shoots along with shoot proliferation.

Rooting and acclimatization: Rooting of *in vitro* shoots was carried out on MS medium supplied with various concentrations of IBA or IAA (Fig. 2). The IBA was significantly effective compared to IAA for root induction. The IBA (3.0 mg L^{-1}) induced the highest number of roots per shoot (2.66±0.11) followed by 6.0 mg L^{-1} IAA (2.40±0.13 roots per shoot) with an average root length of 2.96±0.06 and 2.90±0.06 cm. Regenerated shoots of *C. guianensis* showed two folds (5.33±0.16 roots per shoot) increase in the root induction when activated charcoal (1.0 g L^{-1}) was added to the rooting medium containing 3.0 mg L^{-1} IBA (Fig. 1h, Fig. 3). Acclimatization of rooted shoots in quarter-strength liquid MS basal salts resulted in prominent elongation of the shoots and induction of secondary roots after 2 weeks (Fig. 1i). Plantlets with secondary roots (70.21%) were transferred to HDPE grow bags (30 cm wide×35 cm tall, SK Organic Farms, Chennai, India) containing mixture of garden soil, red soil and sand (1:1:1, v/v/v) (Fig. 1j). After one month the plants were transferred to pot containing sand, soil and cow dung manure (1:1:0.5, v/v/v) (Fig. 1k). Healthy plantlets were morphologically similar and showed uniform growth characteristics (Fig. 1l).

Assessment of genetic fidelity: *Couroupita guianensis* plantlets propagated under *in vitro* conditions were subjected to clonal fidelity analysis using ISSR and RAPD markers. A total of 74 monomorphic bands was generated by ten RAPD primers. The number of bands ranged from 5 (OPA2 and OPA4) to 10 (OPA1 and OPA3) with 7.4 bands per primer on average (Table 3). The amplicons ranged in size from

Table 3: List of RAPD and ISSR primer sequences with the number and size of amplified fragments generated in *Couroupita guianensis* mother plant and *ex vitro* plants

Primer code	Primer sequence (5'–3')	Number of scorable bands	Range of band sizes (bp)
OPA 1	CAGGCCCTTC	10	390-2360
OPA 2	TGCCGAGCTG	5	430-1500
OPA 3	AGTCAGCCAC	10	470-1490
OPA 4	AATCGGGCTG	5	1070-1810
OPA 5	AGGGGTCTTG	7	400-1400
OPA 10	GTGATCGCAG	6	340-2170
OPA 11	CAATCGCCGT	7	330-2270
OPA 13	CAGCACCCAC	7	560-2150
OPA 15	TTCCGAACCC	8	300-2450
OPA 18	AGGTGACCGT	9	480-2100
	Total	74	
ISSR-1	$(AG)_8T$	7	530-1450
ISSR-2	$(AG)_8C$	6	360-1690
ISSR-3	$(GA)_8T$	7	330-1400
ISSR-4	$(GA)_8A$	7	500-1590
ISSR-5	$(AG)_8G$	7	740-1960
ISSR-6	$(GA)_8C$	7	610-1980
	Total	41	

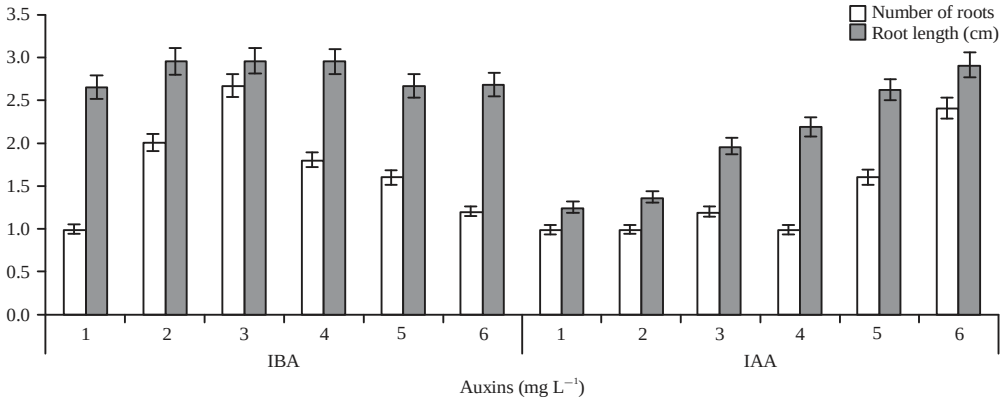

Fig. 2: Effect of auxins on root induction of micro-propagated plantlets of *Couroupita guianensis*

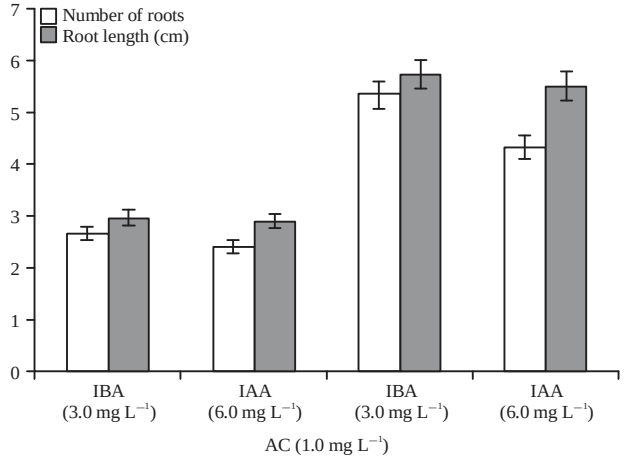

Fig. 3: Effect of activated charcoal on root induction of micropropagated plantlets of *Couroupita guianensis*

300-2400 bp. Likewise, $(AG)_8$ and $(GA)_8$ dinucleotide ISSRs anchored on the 3'-end produced 41 monomorphic bands. Six ISSR primers produced 6.8 bands per primer with a molecular size range of 330-1980 bp (Table 3). DNA fingerprinting pattern obtained using RAPD and ISSR primers confirmed the genetic homogeneity of micropropagated plants. (Fig.4).

DISCUSSION

Couroupita guianensis plantlets were propagated under *in vitro* conditions using cotyledonary nodes. The morphogenetic responses to different media (MS and WP) containing cytokinins alone (BAP and KIN) and in combination with auxins (NAA, IAA or IBA) and TDZ are summarized in Table 1 and 2. MS medium devoid of PGRs failed to induce multiple shoots, while basal WP medium produced 2.72±0.13 number of shoots per explant (Table 1), thereby indicating the

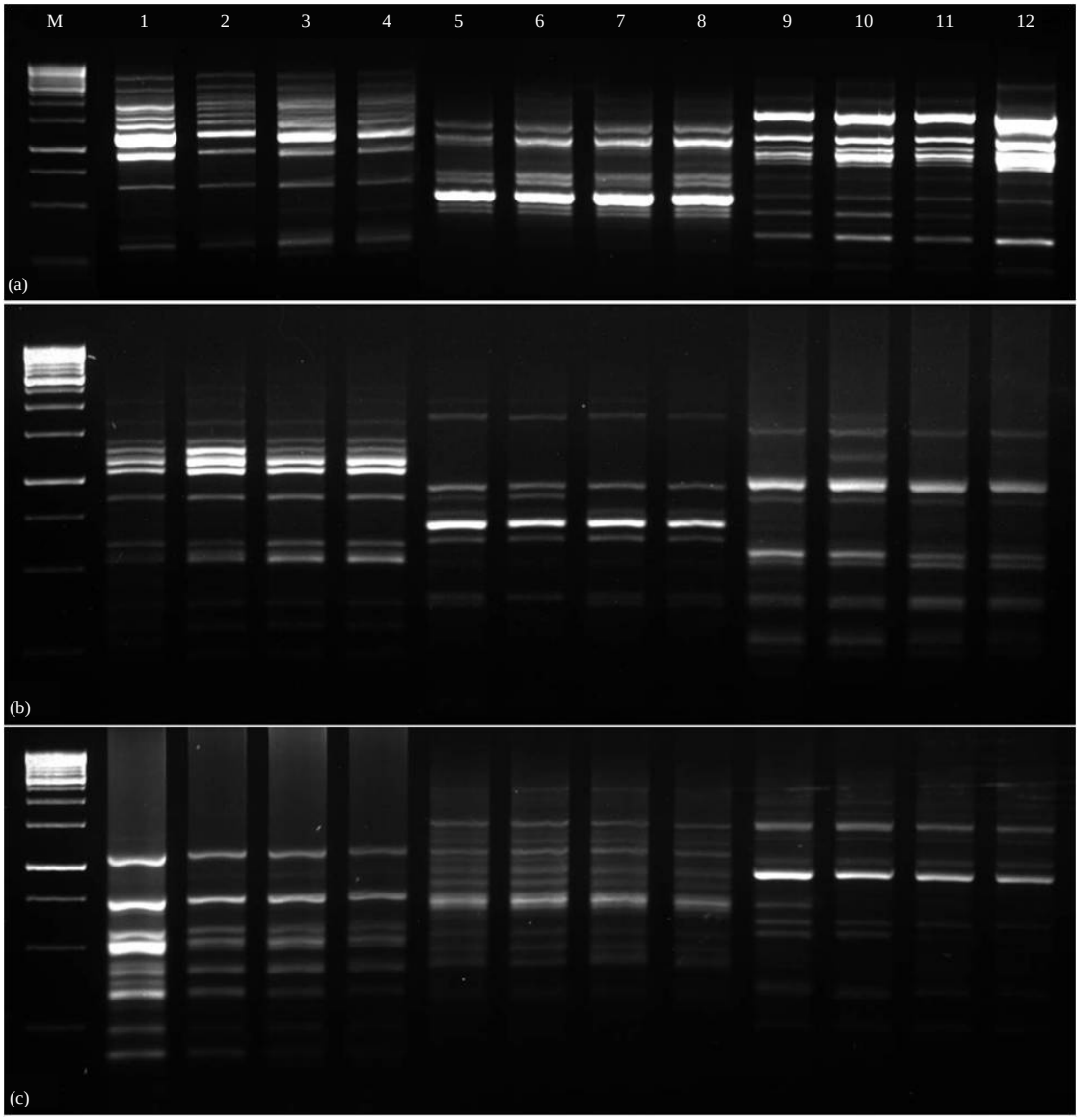

Fig. 4(a-c): Assessment of genetic fidelity of mother plants and *Couroupita guianensis* using RAPD and ISSR markers, (a) Lanes 1-4 (OPA 1), Lanes 5-8 (OPA 10), Lanes 9-12 (OPA 13), (b) Lanes 1-4 (ISSR-1), Lanes 5-8 (ISSR-2), Lanes 9-12 (ISSR-3) and (c) Lanes 1-4 (ISSR-4), Lanes 5-8 (ISSR-5), Lanes 9-12 (ISSR-6); Lane M-1 Kb DNA ladder, Lanes 1,5 and 9-mother plant, Lanes 2-4, 6-8,10-12-micropropagated plants

requirement of addition of certain salts in WPM such as $Ca(NO_3)2.4H_2O$ and K_2SO_4. $Ca(NO_3)2.4H_2O$ plays a role in nitrogen source may function as a signal molecule of plant growth via increased gene expression for enzyme responsible for the uptake and utilization of nitrate[28]. Growth on a poor nitrogen source is not sufficient to cause the induction of nitrate reductase and nitrite reductase enzymes essentially required for the consumption of nitrate[29]. Likewise K_2SO_4 in WP medium is enriched in potassium as compared to MS medium which has been reported to influence the flux of other minerals such as nitrogen, phosphorus and carbon and enhances the translocation of photosynthates which in turn enhance the quality of shoots[30]. The WP medium contains higher level of all the salt concentration compared to MS medium. Where as KI, KNO_3 and $CoCl_2.6H_2O$ were absent in WP medium. The superiority of KIN for the induction of multiple shoots was previously reported in medicinal plants such as *Eclipta alba*[31] *Matthiola incana* and *Eustoma grandiflorum*[32]. A further increase in the cytokinins higher than 3.0 mg L^{-1} resulted in decline of multiple shoot buds. Addition of

individual cytokinins is to WP medium resulted in significant increase in number of shoots by 3.7 folds (4.0 mg L^{-1} BAP) and 3.1 folds (2.5 mg L^{-1} KIN) in comparison with MS medium. Shaik *et al.*[33] reported that BAP produced more no of shoots compare to KIN in Lessertia frutescens. Similar observations were recorded in all the possible combinations of PGRs amended in WP medium. The superiority of WPM medium for the induction of multiple shoots was previously reported in *Ficus carica* L.[34], *Punica granatum* L.[35], *Shorea robusta*[36], *Quercus lusitanica* Lam[37].

The results clearly indicate the differential response of explants in different media×PGR interactions with greater number of shoots and shoot length in WP medium. The supplementation of different auxins had pronounced effect on shoot regeneration in WP medium than MS medium. High frequency shoot regeneration (8.2±0.17 shoots/explant) of *C. guianensis* in MS medium containing a combination of BAP and KIN 1.0 mg L^{-1}, NAA (0.5 mg L^{-1}) and organic additives (50 mg L^{-1} of ascorbic acid and 25 mg L^{-1} each of adenine sulphate, L-arginine and citric acid)[20]. Likewise, the positive influence of NAA supplementation in multiple shoot induction was reported in medicinal plants such as *Barringtonia racemosa*[38], *Romulea minutiflora*[39], *Khaya grandifoliola*[40], *Holarrhena antidysenterica*[41] and *Merwilla plumbea*[42].

Thidiazuron (TDZ) promotes *in vitro* shoot regeneration in majority of woody plants even at very low concentration either individually or in synergistic interaction with other PGRs[43]. TDZ alone at different concentrations produced only callus which failed to induce any shoots even upon transfer to medium containing other cytokinins. Low concentration of TDZ producing high no of shoots proliferation and high concentration of TDZ to producing callus in *Quercus robur* L[44]. In the present study, supplementation of TDZ to the regeneration medium (MS and WP) containing various combinations of PGRs showed a positive correlation to shoot proliferation such as *Bauhinia vahlii*[45], Cichorium intybus[46] Salix tetrasperma[47].

In vitro rooting of regenerated shoots was carried out in full strength MS medium with PGRs. Roots developed spontaneously after 14 days with IBA. However the inclusion of IBA in medium resulted in significant improvement of root number and root length. Similarly, IBA (2.0 mg L^{-1}) reported on *Maytenus senegalensis*[48], micro cutting of tree species like *Pappea capensis*[49], *Lessertia frutescens*[33], *Valeriana officinalis*[50] and *Morinda coreia*[51]. IBA profusely induced adventitious roots from *in vitro* leaf explants of *C. guianensis* Aubl.[52] and *ex vitro* mini cuttings of tree species like *Azadirachta indica*[53] and *Spondias pinnata*[54]. Regenerated shoots of *C. guianensis* showed two folds (5.33±0.16 roots per shoot) increase in the root induction when activated charcoal (1.0 g L^{-1}) was added to the rooting medium containing 3.0 mg L^{-1} IBA (Fig. 1h, Fig. 3). Similar results were reported in *Acacia leucophloea* and *Helicteres isora*[55] with the use of activated charcoal[56]. Acclimatization of rooted shoots in quarter-strength liquid MS basal salts resulted in prominent elongation of the shoots and induction of secondary roots after 2 weeks (Fig. 1i). The quarter-strength liquid MS basal salts facilitated secondary root initiation was reported in seed germination of *C. guianensis*[57]. Plantlets with secondary roots (70.21%) were transferred to HDPE grow bags (30 cm wide×35 cm tall; SK Organic Farms, Chennai, India) containing mixture of garden soil, red soil and sand (1:1:1, v/v/v) (Fig. 1j). After one month the plants were transferred to pot containing sand, soil and cow dung manure (1:1:0.5, v/v/v) (Fig. 1k). Healthy plantlets were morphologically similar and showed uniform growth characteristics (Fig. 1l).

Couroupita guianensis plantlets propagated under *in vitro* conditions were subjected to clonal fidelity analysis using ISSR and RAPD markers. A total of 74 monomorphic bands was generated by ten RAPD primers. The number of bands ranged from 5 (OPA2 and OPA4) to 10 (OPA1 and OPA3) with 7.4 bands per primer on average (Table 3). The amplicons ranged in size from 300-2400 bp. Likewise, $(AG)_8$ and $(GA)_8$ dinucleotide ISSRs anchored on the 3'-end produced 41 monomorphic bands. Six ISSR primers produced 6.8 bands per primer with a molecular size range of 330-1980 bp (Table 3). DNA fingerprinting pattern obtained using RAPD and ISSR primers confirmed the genetic homogeneity of micropropagated plants. (Fig. 4). In concurrence with this study, reports indicate the efficiency of PCR markers in determining the genetic fidelity of tissue cultured plants of *Gerbera jamesonii* Bolus[58], *Phoenix dactylifera* L.[59], *Rauvolfia serpentine*[60], *Psidium guajava*[61] and Ocimum basilicum Var[62]. Therefore, this protocol described here suitable for clonal propagation and helps in preserving rare tree species. *In vitro* shoot multiplication of *C. guianensis* using cotyledonary nodes and to authenticate the genetic homogeneity using RAPD and ISSR markers.

CONCLUSION

In this paper, a rapid, reproducible and large-scale micropropagation protocol was established from cotyledonary nodes of *C. guianensis.* This protocol facilitates sustainable utilization of *C. guianensis* in traditional medicine and reintroduction into its natural habitats.

SIGNIFICANCE STATEMENT

This study was increasing demand and importance to traditional medicinal species. In the present study conservation, sustainable utilization and reintroduction of *C. guianensis* using the cotyledonary nodes. Genetic stability of *in vitro* regenerated plantlets is commonly analyzed using molecular markers such as randomly amplified polymorphic DNA [RAPD], inter simple sequence repeat [ISSR]. These markers confirmed the clonal fidelity of micropropagated plantlets of *C. guianensis*.

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