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Micropropagation and conservation strategies of the potentially medicinal and economically-important tropical deciduous tree - *Drypetes roxburghii* (Wall.) Hurursawa

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The present study was designed to understand seed germination barriers and to develop an efficient micropropagation system in *Drypetes roxburghii*. Fresh seeds with coats removed, cultured on MS medium supplemented with + 1.4 µM gibberellic acid (GA$_3$) + 0.01% activated charcoal (AC) resulted in maximum (83.30 ± 0.63%) seed germination with healthy seedling development. Cotyledonary nodal explants showed best shoot proliferation on Murashige and Skoog (MS) medium supplemented with 1 µM 6-benzylaminopurine (BAP) with maximum number of shoots (1.80 ± 0.24) and shoot length (4.10 ± 0.34 cm). The combination of hormones did not show any significant effect compared with the individual cytokinins tested. The maximum number of shoots (1.70 ± 0.26) with shoot length 3.44 ± 0.22 cm was observed on MS medium supplemented with 1 µM BAP + 2 µM kinetin (Kn). In encapsulation experiments, the nodal explants encapsulated with liquid MS medium + 2 µM BAP + 3% sucrose + 3% sodium alginate showed maximum shoot sprouting (1.70 ± 0.33) on MS medium supplemented with 1 µM BAP + 3% sucrose. The *in vitro* derived shoots were best rooted on MS medium supplemented with 2 µM IBA with maximum number of roots (2.10 ± 0.34) and root length (6.21 ± 0.24 cm) per shoot. The plantlets developed *in vitro* were successfully established under field conditions with 90% survival rate.

Key words: Seed germination, micropropagation, phytohormones, synthetic seeds.

INTRODUCTION

Drypetes roxburghii (Wall.) Hurusawa (Euphorbiaceae), which was previously well known as *Putranjiva roxburghii*

Wall., is an important medicinal plant and native tree of India and popularly known as Kudrajuvi, Patravanti,

Jivputrak and Nageia. Roxburgh (1832) explains the name of the tree "Pootranjeeva, the Sanskrit name Pootra signifies a son and Jeeva means life. It is a moderately-sized, deciduous, evergreen tree, up to 25 m height. This plant is widely used in the traditional system of Indian medicine for treating azoospermia, diuretic, catarrh, ophthalmopathy and for constipation (Varma et al., 2010). *D. roxburghii* is one of the constituents of "Y-spur", an Ayurvedic formulation prepared by Vilco laboratories, which is found to be very effective in male infertility (Badole et al., 2011).

A number of phytochemical compounds were isolated in pure form from various parts of *D. roxburghii*. Triterpenoids, namely putranjivanonol, putranjic acid, friedelin, putranjivadione, friedelanol, roxburgholone (Sengupta and Mukherjee, 1968), putric acid (Chopra et al., 1970), putranjivandione (Sengupta et al., 1968) were isolated from stem bark, and other compounds namely methyl putranjivate, putranjivic acid, putrone I a and putrol I b (Chopra et al., 1968) were isolated from leaf extracts. Chopra and Et (1970) evaluated the chemical composition of leaves and root bark of *D. roxburghii* and found that the leaf extract contains polyphenolic compounds in addition to triterpenoids, and this extract yielded ellagic acid, gallic acid, gallocatechin, ellagi and gallo-tannins and saponins. The main components of the root bark were triterpenoids, friedelin, putranjivadione, roxburgholone, methyl putrate and saponins derived from oleanolic acid.

Due to the presence of a number of phytochemicals, the plant has a large number of pharmacological proper-ties, namely antibacterial, antifungal, antinociceptive, antipyretic, anti-inflammatory (Reanmongkol et al., 2009), antioxidant, analgesic, anthelmintic, cytotoxic, hypoglycemic, insecticidal and larvicidal activities. Along with the medicinal properties, this plant has great commercial importance because of its valued wood and seed oil. However, recently, the plant in its natural population has become rare due to large scale destruction of trees for its wood and due to short viability of seeds and low seed germination. It is therefore important to conserve these trees because of their biological and economic importance. Conventional propagation methods cannot replace the depleting population because the seeds show a low percentage germination and vegetative propagation methods are unsuccessful. Development of standardized micropropagation technique for the improvement of native species, mainly forest tree species, is an important issue for preserving biodiversity (Ishii and Kambou, 2007). Reinforcement of wild plant populations using individuals raised *ex situ* is considered a valid means of reducing the risk of threatened species (Bowes, 1999). *In vitro* culture can be a valuable technique for clonal mass propagation and conservation of this tree within a short

period of time.

Recently, there has been increased interest in using *in vitro* techniques for propagation of trees using different explants because this is a valuable technique for faster *ex situ* conservation of plants by using minimum amount of explant material. Rescue of an endangered forest tree, *Givotia rottleriformis,* by using *in vitro* techniques was well documented by Rambabu et al. (2005). The present communication demonstrates the *in vitro* seed germination studies and an effective high frequency shoot regeneration method for producing a large number of plants from Cotyledonary node explants of *D. roxburghii*.

MATERIALS AND METHODS

Collection of plant material

The freshly-ripened fruits of *D. roxburghii* were collected from dry deciduous forests of Rangareddy district, Andhra Pradesh and Madurai Kamaraj University, Madurai, Tamil Nadu in the month of January, 2011 and 2012. The collected fruits were dried in the shade at room temperature in the laboratory and the hard seed coat removed from seeds. The seeds with coats removed were used for the present study.

Surface sterilization and *in vitro* seed germination

The seeds were surface sterilized by directly dipping in 0.1% (w/v) $HgCl_2$ for 5 min and then rinsed thoroughly (4 to 5 washes) with sterilized double distilled water under aseptic conditions. The seeds were then tested for germination under both sterile and non-sterile conditions. In non-sterile germination tests, seeds were placed on wet blotting papers in Petri dishes. Seeds were incubated in a culture room under the following conditions: 16/8 h light/dark cycle at 25 ± 2°C. Petri dishes were randomized every two days (Yang et al., 1999). Seeds showing radicle emergence were recorded as germinated. In every replicate, germination percentage after 45 days was recorded.

In sterile germination tests, the surface sterilized seeds were transferred aseptically on to an agarified MS medium (Murashige and Skoog, 1962) with 3% sucrose and 4.41 g/L MS salts (HiMedia laboratories, India). Media was adjusted to pH 5.7 prior to autoclaving at 121°C for 15 min. For every replicate, the germination percentage was recorded after 45 days of culture.

To study the effect of strength of the medium and sucrose concentration on *in vitro* seed germination of *D. roxburghii,* the surface sterilized seeds were inoculated on full strength MS medium with 3% sucrose, full strength MS medium with 1.5% sucrose, half strength MS medium with 3% sucrose and ½ strength MS with 1.5% sucrose. In all the media, 1.4 μM GA_3 and 0.01% activated charcoal were added. In every replicate, percentage germination was recorded at different intervals. The effect of auxins, cytokinins and gibberellins on *in vitro* seed germination and seedling morphology of *D. roxburghii* was studied. Length of the radicle and hypocotyl length were recorded.

The effect of different storage time intervals on *in vitro* seed germination was studied. The seeds collected were stored at room temperature for one week to one year and the stored seeds were inoculated periodically on to the MS medium supplemented with 3%

sucrose, 1.4 µM GA$_3$ and 0.01% activated charcoal. The percentage seed germination and fresh weight of the seedlings, root and shoot individually, were recorded to determine seed viability and seedling vigour. In all the experiments, 100 seeds per replicate were inoculated and the experiments were conducted in three replicates.

Culture initiation and shoot proliferation

In the primary experiment, one month old aseptic seedlings raised *in vitro* were used for culture initiation. Different seedling-derived explants were inoculated on MS medium supplemented with various concentrations of plant growth regulators (BAP and 2,4-D) to observe the morphogenetic response. In the second experiment, the best explant for shoot proliferation was selected and cultured on medium containing individual cytokinins or containing combinations of cytokinins and auxins. In all the experiments the medium was supplemented with 3% (w/v) analytical grade sucrose and solidified with 0.8% (w/v) agar agar (HiMedia laboratories, India). The cultures were maintained under controlled conditions with 25 ± 2°C temperatures and with 16 h light and 8.0 h dark with the help of cool white fluorescent lights (Philips, India – 40 wattage).

Synthetic seeds

The shoot tips, nodal and cotyledonary nodes excised from the *in vitro* grown cultures were used for encapsulation to determine the best explant for production of seed analogues. Sodium alginate solution of different concentrations (1 to 5% w/v) were prepared by mixing sodium alginate with calcium-free liquid MS medium containing 3% sucrose and at the same time different concentrations of calcium chloride (25 to 100 mM) solution were also prepared. Then, the explants were suspended in sodium alginate solution in the laminar air flow cabinet for 1 to 2 min and dropped one by one through a modified 1000 µl micropipette into a sterile aqueous solution of calcium chloride. Due to the exchange of ions between sodium alginate and calcium chloride, calcium alginate beads were formed within 20 to 30 min. The beads were then collected by discarding the calcium chloride solution, washed with sterilized double distilled water and surface dried by placing them in sterilized Petri dishes containing blotting papers. Finally, prepared beads were directly cultured on MS medium supplemented with 2 µM BAP.

Rooting and acclimatization

Individual shoots of 3 to 4 cm obtained either from direct explants or from synthetic seeds were separated and sub cultured on fresh MS medium supplemented with different concentrations of individual auxins (IAA, IBA, and NAA). After 3 to 4 weeks of root initiation the complete plantlets developed *in vitro* were removed and washed with sterile double distilled water to remove the traces of agar. The plantlets were then potted in paper cups containing sterilized soil, sand and vermicompost in 1:1:2. These plantlets were covered initially with polythene bags and maintained under culture room conditions to maintain relative humidity. The plantlets were irrigated with half strength MS medium devoid of sucrose and plant growth regulators. After 4 to 6 weeks the covers were removed and the primary hardened plants were transferred to earthen pots and irrigated with tap water and observed for further growth.

Experimental design and scoring of data

Each experiment was repeated at least three times and for each experiment, twenty replicates were used. Data was recorded periodically for shoot multiplication and rooting, respectively. Data was subjected to analysis of variance (ANOVA) carried out by the SPSS 20 (SPSS Inc. Chicago, IL, USA). Values with P ≤ 0.05 were considered to be statistically significant.

RESULTS

In vitro seed germination

In vitro seed germination was influenced by several factors, of these, the first and most important step is the standardization of the surface sterilizing agent concentration and duration of exposure. Seed germination at different concentrations of HgCl$_2$ (0.01 to 1.0%) were evaluated to determine the best concentration for reducing contamination. All the treatments were effective for disinfecting the seeds. However treatment with 0.1% HgCl$_2$ for 4 min was effective for both disinfecting the seeds and to initiate the healthy seedlings *in vitro*. In the non-sterile germination test, the seed germination of *D. roxburghii* was initiated within 7 to 8 days. The seeds that showed radicle emergence were recorded as germinated. The percentage germination was recorded as 73.3% in Petri dishes containing wet blotting papers (Figure 1A). However in sterile germination experiments, the initiation of seed germination was observed 10 to 14 days after culture. 80.0% of seeds sprouted within 45 days of culture on ½ strength MS medium supplemented with 3% sucrose (Figure 1B). After sprouting initially, radicle elongation was observed followed by hypocotyl elongation. After nearly one month of seed germination the cotyledonary leaves opened and 10 to 15 days after that, the first primordial leaves appeared on some seedlings. It was observed that there was significant variation in seed germination percentage with manipulating the strength of medium and sucrose concentration. The maximum seed germination percentage (83.3 ± 0.63) was noticed in full strength MS medium supplemented with 3% sucrose. Whereas the minimum seed germination (66.6 ± 0.46%) was recorded on ½ strength MS medium supplemented with 1.5% sucrose (Table 1).

In the present study, depending upon the hormones, the seedlings showed different morphological response as follows (Table 2). In control (MS basal medium with 3% sucrose) the seeds sprouted and showed normal seedlings with elongated hypocotyls (66.6 ± 0.43 cm). Medium with 1.4 µM GA$_3$ induced healthy seedlings with (7.70 ± 0.53 cm) radicles and (8.40 ± 0.56 cm) hypocotyls. Medium with 1.4 µM GA$_3$ and 0.01% AC

Table 1. Effect of strength of the medium and sucrose concentration on percentage of seed germination in *D. roxburghii* on MS medium with 1.4 µM GA3 and 0.01% AC.

Strength of the MS medium + % of sucrose	Mean percentage of germinated seeds ± SE	Mean percentage of abnormal germination ± SE	Mean percentage of non germinated seeds ± SE
Full + 3	83.3 ± 0.63[c]	6.6 ± 0.33[a]	10.0 ± 0.30[a]
Full + 1.5	76.6 ± 0.53[b]	10.0 ± 0.37[b]	13.3 ± 0.28[b]
Half + 3	80.0 ± 0.73[c]	6.6 ± 0.53[a]	13.3 ± 0.14[b]
Half + 1.5	66.6 ± 0.43[a]	13.3 ± 0.47[c]	20.0 ± 0.17[c]

Data indicate mean ± standard error of the 20 replicates per treatment in three repeated experiments. Mean followed by the same letter was not statistically significant at 0.05% probability.

Table 2. Influence of different hormones on *in vitro* seed germination and seedlings morphology of *D. roxburghii* on MS medium.

Hormones (µM)	Mean Length of the radicle (cm) ±SE	Mean Length of hypocotyls (cm) ± SE	Nature of radicle	Nature of hypocotyls	Cotyledonory leaf nature (size, shape and colour)
Control	7.00 ± 0.42[cd]	9.30 ± 0.36[fg]	Normal	Normal	Normal
1.4 GA$_3$	7.70 ± 0.53[cd]	8.40 ± 0.56[f]	Normal	Normal	Normal
1.4 GA$_3$ + 0.01% AC	8.80 ± 0.61[de]	7.50 ± 0.63[df]	Normal	Normal	Normal
4.44 BAP	6.40 ± 0.30[bc]	2.20 ± 0.38[a]	Normal	Short	Un opened
4.56 KN	1.80 ± 0.38[a]	3.20 ± 0.64[ab]	Inhibited	Short	Small, wrinkled
5.77 IAA	7.20 ± 0.59[cd]	10.00 ± 0.39[h]	Normal	Thin elongated	Small, pale yellow
4.92 IBA	8.20 ± 0.53[f]	8.00 ± 0.49[f]	Normal	Normal	Un opened
5.37 NAA	5.60 ± 0.73[bc]	4.10 ± 0.65[bc]	Bulged	Short	Large, pale yellow
4.52 2,4-D	4.60 ± 0.83[ab]	5.20 ± 1.02[cd]	Thin	Short	Wrinkled

Data indicate mean ± standard error of the 20 replicates per treatment in three repeated experiments. Mean followed by the same letter was not statistically significant at 0.05 % probability.

showed healthy seedlings with well developed (8.80 ± 0.61 cm) radicle and (7.50 ± 0.63 cm) hypocotyls. The formed seedlings were similar to *in vivo* germinated seedlings and initiation of first primordial leaves was observed. In medium with 4.44 µM BAP the hypocotyls got inhibited (2.20 ± 0.38 cm) and cotyledonory leaves failed to open. In medium with 4.56 µM Kn the radicle (1.80 ± 0.38 cm) and hypocotyls (3.20 ± 0.64 cm) got inhibited and cotyledonory leaves were small and wrinkled.

In medium with 5.77 µM IAA the radicle was normal whereas the hypocotyls were thin and elongated (10.00 ± 0.39 cm) and the cotyledonory leaves were small, pale yellow in colour. Medium with 4.92 µM IBA showed normal radicle and hypocotyls, whereas cotyledonory leaves failed to open. Medium supplemented with 5.37 µM NAA induced bulged radicles (5.60 ± 0.73 cm) and hypocotyls were short (4.10 ± 0.65 cm) whereas

cotyledonory leaves were large and pale yellow in colour. Medium with 4.52 µM 2,4-D induced short, thin radicles (4.60 ± 0.83 cm), short hypocotyls (5.20 ± 1.02 cm) and wrinkled cotyledonory leaves. Seed storage time influence the percentage of seed germination and fresh weight of the seedlings strongly. The percentage of germination increased significantly (83.3 ± 0.63%) with decrease in storage period. The fresh weight of the seedlings was also more (0.94 ± 0.01 mg)

Table 3. Effect of different storage periods (one week to one year) on viability of *D. roxburghii* seeds.

Storage duration in days	Mean percentage of germination ± SE	Mean Fresh weight of seed lings ± SE	Mean fresh weight of shoots ± SE	Mean fresh weight of root ± SE
`7	83.3 ± 0.63[e]	0.94 ± 0.01[e]	0.42 ± 0.00[d]	0.51 ± 0.00[c]
30	56.6 ± 0.23[d]	0.93 ± 0.01[d]	0.41 ± 0.01[c]	0.51 ± 0.01[c]
60	46.6 ± 0.49[c]	0.79 ± 0.01[bc]	0.32 ± 0.01[ab]	0.45 ± 0.01[ab]
90	30.0 ± 0.56[b]	0.78 ± 0.01[ab]	0.33 ± 0.00[ab]	0.44 ± 0.01[ab]
180	16.6 ± 0.65[a]	0.66 ± 0.01[a]	0.29 ± 0.00[a]	0.37 ± 0.01[a]
365	-	-	-	-

Data indicate mean ± standard error of the 20 replicates per treatment in three repeated experiments. Mean followed by the same letter was not statistically significant at 0.05 % probability.

when the storage period was minimum (7 days) (Table 3), whereas at maximum storage period (1 year) the seeds lost their viability and failed to germinate. Freshly sown seeds showed maximum percentage of germination.

Shoot induction and proliferation

The *in vitro* morphogenetic response of every plant species depends on the type and physiological status of the explant. Normally in trees, rejuvenation is the major problem, so seedling derived explants were used to develop successful micropropagation. In the present experiment among all the explants (shoot tips, nodes and cotyledonory nodes) tested for active proliferation of shoot buds in *D. roxburghii*, cotyledonory nodes showed the best response followed by shoot tips and nodes (Figure 1C and D).

The cotyledonory nodes of *D. roxburghii* cultured on MS medium, with or without hormonal supplements, exhibited varied responses. The cotyledonory nodes failed to induce morphogenetic response in the medium devoid of plant growth regulators. Addition of plant growth regulators specifically cytokinins (BAP and Kn) to the medium showed positive effect on multiple shoots induction. In the present study, both BAP and Kn favored shoot bud initiation and proliferation. However, among both the hormones tested, BAP was more effective than the Kn in initiation and subsequent proliferation of multiple shoots (Table 4). The first visible appearance of shoot buds was noticed within 10 to 14 days after inoculation. Initially, small protuberances were induced in the cotyledonory nodes which later developed into shoot buds and elongated into healthy shoots. The maximum number of shoots (1.80 ± 0.24) with maximum shoot length (4.10 ± 0.34 cm) was noticed at lower concentration (1.0 µM) of BAP (Figure 1E). Later, gradual decrease in shoot number with increasing concentration

of BAP was observed. MS medium augmented with Kn showed comparatively less response. The maximum number of shoots (1.70 ± 0.21) with maximum shoot length (4.05 ± 0.35 cm) was noticed with 2.0 µM Kn. Increase or decrease in the concentration of Kn beyond or below 2 µM resulted in decrease of shoot number and shoot length.

The combined effect of cytokinins was evaluated for multiple shoot induction in *D. roxburghii*. The cotyledonory nodal segments of *D. roxburghii* were cultured on MS medium supplemented with various concentrations and combinations of cytokinins. But the combination of cytokinins did not show any significant effect than the individual cytokinins tested. The maximum number of shoots (1.70 ± 0.26) with shoot length (3.44 ± 0.22 cm) was noticed on MS medium supplemented with 1.0 µM BAP + 2.0 µM Kn. However the shoots formed are healthy with dark green leaves and are similar to the *in vivo* growing shoots.

BAP (1.0 µM) along with different auxins (IAA, IBA and NAA) were used to investigate their effect on induction of multiple shoots. In IAA of the three concentrations tested, BAP 1.0 µM + IAA 1.0 µM showed the best response with high frequency of shoots (1.60 ± 0.22) and shoot length (2.71 ± 0.25 cm). Out of three concentrations of IBA tested, the maximum number of shoots (1.70 ± 0.21) with shoot length (3.11 ± 0.19 cm) was recorded on MS medium supplemented with BAP 1.0 µM + IBA 2.0 µM. Among three concentrations of NAA tested, BAP 1.0 µM + NAA 1.0 µM showed the superior response with mean number of shoots (1.70 ± 0.26) and shoot length (2.98 ± 0.26 cm). The cultures that grow on the medium supplemented with cytokinins and auxins showed slight narrow leaves with light green in colour.

Synthetic seeds

In *D. roxburghii,* of all the concentrations of sodium alginate and calcium chloride tested, 3.0% (w/v) sodium

Table 4. Influence of different concentrations and combinations of phytohormones such as cytokinins (BAP and Kn) and auxins (IAA, IBA and NAA) on multiple shoot induction and proliferation in *D. roxburghii* on MS medium.

BAP (µM)	KN (µM)	IAA (µM)	IBA (µM)	NAA (µM)	Mean Shoot number ± SE	Mean shoot length (cm) ± SE
0.0	0.0	-	-	-	0.0	0.0
1	-	-	-	-	1.80 ± 0.24^h	4.10 ± 0.34^l
2	-	-	-	-	1.60 ± 0.22^{ef}	3.80 ± 0.41^{ij}
3	-	-	-	-	1.50 ± 0.22^{cde}	3.10 ± 0.45^{gh}
4	-	-	-	-	1.30 ± 0.30^{bc}	2.72 ± 0.35^{cd}
5	-	-	-	-	1.09 ± 0.21^a	2.61 ± 0.30^{bc}
	1	-	-	-	1.60 ± 0.22^{ef}	3.79 ± 0.32^{ij}
-	2	-	-	-	1.70 ± 0.21^{fg}	4.05 ± 0.35^{jk}
-	3	-	-	-	1.50 ± 0.22^{cde}	3.69 ± 0.25^{ij}
-	4	-	-	-	1.40 ± 0.22^{bcd}	3.17 ± 0.25^{gh}
-	5	-	-	-	1.20 ± 0.20^{ab}	2.92 ± 0.29^{ef}
1	1	-	-	-	1.60 ± 0.22^{ef}	3.17 ± 0.22^{gh}
1	2	-	-	-	1.70 ± 0.26^{fg}	3.44 ± 0.22^{hi}
1	3	-	-	-	1.40 ± 0.22^{bcd}	3.23 ± 0.2^{gh}
2	2	-	-	-	1.60 ± 0.26^{ef}	3.03 ± 0.29^{gh}
3	2	-	-	-	1.50 ± 0.22^{cde}	2.97 ± 0.21^{ef}
1	-	1	-	-	1.60 ± 0.22^{ef}	2.71 ± 0.25^{cd}
1	-	2	-	-	1.50 ± 0.22^{cde}	2.45 ± 0.26^{ab}
1	-	3	-	-	1.40 ± 0.22^{bcd}	2.15 ± 0.20^a
1	-	-	1	-	1.50 ± 0.22^{cde}	2.89 ± 0.29^{de}
1	-	-	2	-	1.70 ± 0.21^{fg}	3.11 ± 0.19^j
1	-	-	3	-	1.40 ± 0.22^{bcd}	2.78 ± 0.25^{cd}
1	-	-	-	1	1.70 ± 0.26^{fg}	2.98 ± 0.26^{fg}
1	-	-	-	2	1.60 ± 0.22^{ef}	2.96 ± 0.26^{ef}
1	-	-	-	3	1.40 ± 0.16^{bcd}	2.79 ± 0.30^{cd}

Data indicate mean ± standard error of the 20 replicates per treatment in three repeated experiments. Mean followed by the same letter was not statistically significant at 0.05 % probability.

alginate with 100 mM calcium chloride solution showed best results by the formation of identical beads with both the explants tested (Figure 1F). The lower concentrations of sodium alginate (1.0 to 2.5% w/v) and calcium chloride (25 to 75 mM) were not suitable for encapsulation because the resulting beads were irregular in shape and too soft to handle, whereas at higher concentrations of sodium alginate (4 to 5% w/v), the beads were too hard and caused considerable delay in sprouting. In the present experiment, shoot tips and nodes were used as explants for encapsulation. Both the explants responded well and sprouted within two weeks under *in vitro* conditions, however among both the explants tested nodal explants showed maximum shoot sprouting from synthetic seeds than the shoot tips on culture medium that is, MS + 1.0 µM BAP + 3% sucrose. Among all the encapsulation mixtures tested, maximum shoot sprouting (1.70 ± 0.33) was observed from the nodes encapsulated with liquid MS medium fortified with 2.0 µM BAP + 3% sucrose and 3% sodium alginate (Table 5 and Figure 1G).

Rooting and acclimatization

The shoots developed from direct inoculated explants and encapsulated explants were taken out from the test tubes and were inoculated on root induction medium to develop complete plantlets. In the present study, MS medium supplemented with different concentrations of auxins (IAA, IBA and NAA) were used as root induction medium. Among different concentrations of hormones tested, IBA at lower concentrations favored root induction in *D. roxburghii*. The maximum root induction (2.10 ± 0.34) with root length (6.21 ± 0.24 cm) was noticed on MS medium supplemented with 2.0 µM IBA (Table 6 and Figure 1H). Rooted plantlets were transferred to the

Table 5. Influence of synthetic endosperm on shoot sprouting percentage, germination time (days) and on shoot number formation from encapsulated explants (shoot tips and nodes) of *D. roxburghii.*

Explants	BAP (µM)	KN (µM)	Germination period in days	Mean percentage of sprouting ± SE	Mean Shoot number ± SE
Shoot tips	1.0	-	10-15	90 ± 0.57[c]	1.20 ± 0.20[bc]
Shoot tips	2.0	-	08-12	100[e]	1.50 ± 0.22[f]
Shoot tips	3.0	-	12-16	85 ± 0.85[bc]	1.00 ± 0.21[a]
Shoot tips	-	1.0	11-15	90 ± 0.53[c]	1.00 ± 0.21[a]
Shoot tips	-	2.0	13-16	80 ± 0.58[b]	1.40 ± 0.22[e]
Shoot tips	-	3.0	15-18	75 ± 0.67[a]	1.30 ± 0.26[d]
Nodes	1.0	-	08-14	95 ± 0.54[cd]	1.30 ± 0.26[d]
Nodes	2.0	-	08-12	100[e]	1.70 ± 0.33[h]
Nodes	3.0	-	12-16	75 ± 0.39[a]	1.30 ± 0.26[d]
Nodes	-	1.0	10-14	95 ± 0.43[cd]	1.10 ± 0.27[ab]
Nodes	-	2.0	12-16	80 ± 0.44[b]	1.60 ± 0.26[g]
Nodes	-	3.0	14-18	80 ± 0.57[b]	1.50 ± 0.22[f]

Data indicate mean ± standard error of the 20 replicates per treatment in three repeated experiments. Mean followed by the same letter was not statistically significant at 0.05 % probability.

Table 6. Effect of different concentrations of individual auxins such as IBA, NAA and IAA on *in vitro* root regeneration from shoots of *D. roxburghii* on MS medium under controlled conditions

IBA (µM)	NAA (µM)	IAA (µM)	Mean root number ± SE	Mean root length (cm) ± SE
1.0	-	-	1.60 ± 0.22[de]	6.08 ± 0.19[g]
2.0	-	-	2.10 ± 0.34[h]	6.21 ± 0.24[ef]
3.0	-	-	1.90 ± 0.34[fg]	5.39 ± 0.20[ce]
4.0	-	-	1.40 ± 0.32[cd]	4.97 ± 0.19[bc]
5.0	-	-	1.10 ± 0.17[ab]	3.83 ± 0.50[ab]
-	1.0	-	1.40 ± 0.26[cd]	4.28 ± 0.25[bc]
-	2.0	-	1.60 ± 0.30[de]	4.82 ± 0.57[bc]
-	3.0	-	2.00 ± 0.33[g]	6.07 ± 0.20[ef]
-	4.0	-	1.00 ± 0.21[ab]	3.71 ± 0.67[ab]
-	5.0	-	0.90 ± 0.23[a]	3.63 ± 0.84[a]
-	-	1.0	1.90 ± 0.34[fg]	5.82 ± 0.26[ce]
-	-	2.0	1.70 ± 0.33[ef]	5.31 ± 0.29[ce]
-	-	3.0	1.60 ± 0.22[de]	4.98 ± 0.25[bc]
-	-	4.0	1.20 ± 0.24[bc]	4.05 ± 0.52[bc]
-	-	5.0	1.10 ± 0.27[ab]	3.90 ± 0.68[ab]

Data indicate mean ± standard error of the 20 replicates per treatment in three repeated experiments. Mean followed by the same letter was not statistically significant at 0.05 % probability.

MS medium supplemented with 2.0 µM IBA (Table 6 and Figure 1H). Rooted plantlets were transferred to the paper cups containing soil, sand and vermicompost in 1:1:2 ratios and irrigated with liquid ½ strength MS medium devoid of sucrose and plant growth regulators (Figure 1I). It was noticed that the plantlets growing in a plastic caps got hardened by the formation of new leaves. After 4 weeks, the plantlets were transferred to the earthen pots, irrigated with normal tap water and maintained under shade conditions. Later the hardened plants were directly transferred to the field conditions with 90% survival.
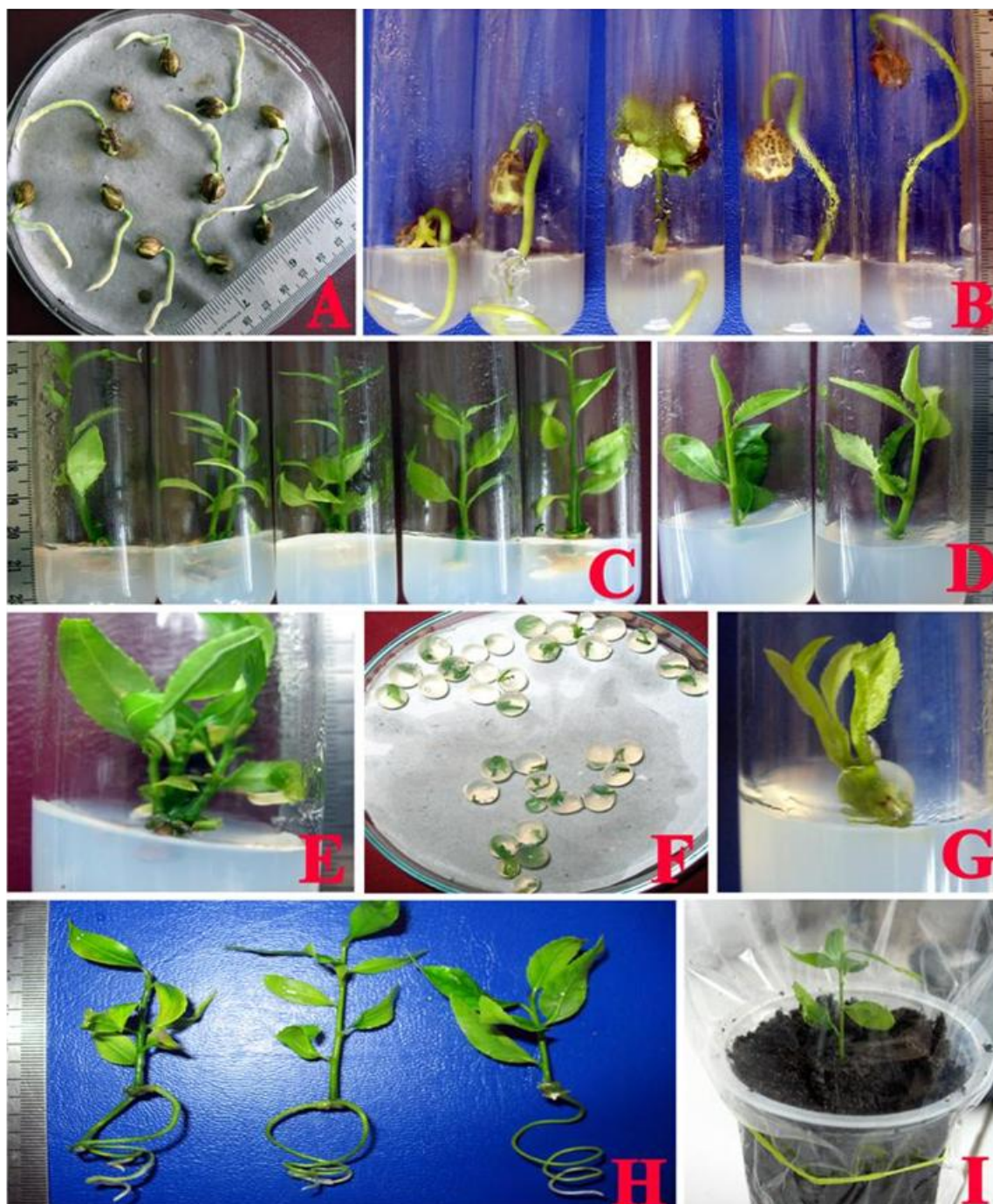
Figure 1. *in vitro* seed germination and micropropagation of *Drypetes roxburghii*. (A) *In vitro* non sterile seed germination of *D. roxburghii* seeds in petri dish containing wet blotting papers; (B) *In vitro* sterile germination of *D. roxburghii* seeds on ½ strength Murashige and Skoog (MS) medium supplemented with 3% sucrose and 0.8 % agar; (C) Shoot initiation from cotyledonory nodes of *D. roxburghii* on Murashige and Skoog (MS) medium supplemented with 1.0 µM BAP; (D) Initiation of shoots from shoot tip explants of *D. roxburghii* on Murashige and Skoog (MS) medium supplemented with 1.0 µM BAP; (E) Multiple shoot induction in *D. roxburghii;* (F) Synthetic seeds of *D. roxburghii* formed by the encapsulation of explants with 3% sodium alginate and 100 mM calcium chloride; (G) Shoot sprouting from nodes encapsulated with calcium free MS liquid medium supplemented with 2.0 µM BAP + 3% sucrose + 3% sodium alginate; (H) *In vitro* rooting of *D. roxburghii* shoots when cultured on Murashige and Skoog (MS) medium supplemented with 2.0 µM IBA and (I) Primary hardened plantlets of *D. roxburghii* ready to transfer into field conditions.

DISCUSSION

In the Euphorbiaceae, seed germination is more difficult in most of the tree species. It is usually due to the presence of hard seed coat (Reddy et al., 2001). So, it is important to study the *in vitro* seed germination by using various methods. Under natural conditions, the transit of seeds through intestines of wild animals and birds breaks the tegument dormancy and favors the imbibitions phenomenon, resulting in the rapid and homogeneous germination of seeds (Ishii and Kambou, 2007). However, *in vitro* seed germination can be favored by various treatments like acid treatment (Samuel et al., 2009; Sambe et al., 2010), cold and hot water treatment (Thokozani et al., 2011), hormonal treatment (Maridass and Thangavel, 2008), breaking the hard seed coat etc.

In the present study, the hard seed coat was removed to get the maximum percentage of seed germination. Non sterile and sterile germination tests were conducted to compare the germination percentage in *D. roxburghii*. Sterile and non sterile seed germination tests in *Physoplexis comosa* and *Primula glaucescens* was conducted to conserve these endangered species (Cerabolini et al., 2004). They found that conservation of *P. comosa* required more stringent requirements like sterile environment and phytohormones supply. Whereas for *P. glaucescens* non sterile conditions without phytohormones allowed wide spread propagation. However in *D. roxburghii* the germination percentage was more in sterile environment when compared to non sterile conditions.

The present study confirms that the medium strength and sucrose concentration played a significant role on *in vitro* seed germination of *D. roxburghii*. In an earlier work, Rambabu et al. (2006) described that the zygotic embryos of *Givotia rottleriformis* germinated with 100% on full strength MS medium. However, conversely to the present findings, Pickens et al. (2003) found that there was no effect of sucrose on seed germination or seedling growth in *Tillandsia eizii*.

Supplying of phytohormones to the medium favors *in vitro* seed germination (Miller et al., 1992; Maridass and Thangavel, 2008; Samuel et al., 2009). Addition of GA$_3$ and activated charcoal to the medium enhanced the seed germination and favored the production of healthy seedlings in *D. roxburghii*. Addition of GA$_3$ to the medium favored seed germination and seedling survival was reported in some other species like *P. comosa* (Cerabolini et al., 2004), Strawberry (Miller et al., 1992) etc. In contradiction to the present findings, Ishii and Kambou (2007) found that BAP favours the *in vitro* seed germination in *A. digitata*. Whereas, Pickens et al. (2003) found that addition of NAA to the medium inhibited the seedling growth but not the germination in *Tillandsia cizii*.

In *Thamnocalamus spathiflorus,* improvement of seed germination with quite often callus formation from embryogenic axis with incorporation of BAP 2,4-D, and GA$_3$ to the medium was reported by Bag et al. (2000). Addition of activated charcoal to the medium favored further enhancement of *in vitro* seed germination in *Pterocarpus santalinus* reported by Chaturani et al. (2006).

This study confirms that the increasing of storage period of the seeds of *D. roxburghii* results in gradual decrease of the percentage of germination. The similar observations was also reported in few other species like *A. malaccensis* (Shankar, 2012) and *Pterocarpus santalinus* (Chaturani et al., 2006). It is proved that storing seeds in cool conditions such as in a refrigerator may prolong viability (Ahmed and Gogoi, 2000).

The present study provides an efficient method for shoot regeneration from cotyledonory nodes of *D. roxburghii*. Frequency of shoot bud formation and further development were greatly influenced by the type of cytokinin and its concentration present in the medium. Among different concentrations of cytokinins present in the medium, 1.0 µM BAP was found to be more effective to induce multiple shoots in *D. roxburghii*. Similar type of results that is, BAP induced axillary shoot proliferation from cotyledonory nodes reported in *Aegle marmelos* (Arumugam and Rao, 1996), *Sterculia urens* (Purohit and Dave, 1996), *Dalbergia sissoo* (Pradhan et al., 1998) and *Quercus floribunda* (Purohit et al., 2002). Contradictory to the present findings, Husain et al. (2007) reported the efficient shoot regeneration from cotyledonory nodes of *Pterocarpus marsupium* on MS medium supplemented with TDZ.

The significant role of the combinations of cytokinins, cytokinins and auxins on *in vitro* shoot proliferation was reported previously in *Dlabergia sissoo* (Thirunavoukkarasu et al., 2010). The combinations of cytokinins did not play much role in *D. roxburghii* when compared with individual cytokinins. However the shoots formed in combinations are healthy with dark green leaves. Vengadesan et al. (2002) reported that in *Acacia sinuate,* maximum number of shoots are induced from cotyledonary node explants on MS medium supplemented with 6.66 µM BAP and 4.65 µM Kn. Ajithkumar and Seeni (1998) obtained enhanced shoot production from nodal segments of *Aegle marmelos* when they were cultured in the medium augmented with BA + IAA, rather than on medium supplemented with BAP or Kn alone. Danthu et al. (2003) reported that addition of 0.26 µM IBA as a supplement with BAP improved the production of shoots in *Khaya senegalensis*. Maximum response from *Acacia senegal* nodal cultures were noticed when BAP and NAA were added to the culture medium (Kaur et al., 1998). Wasel (2000) reported that multiple shoot formation was better in *Acacia seyal* when the culture medium was

supplemented with NAA.

In *D. roxburghii* among different concentrations of sodium alginate and calcium chloride tested for production of artificial seeds, 3.0% sodium alginate and 100 mM calcium chloride formed identical beads. The present findings was similar with the Castillo et al. (1998) who reported that 2.5% sodium alginate solution was optimum for maximum synthetic seed germination (77.5 %) in *Carica papaya*. Among both the explants tested for synthetic seed formation, nodal explants encapsulated with liquid MS medium fortified with 2.0 µM BAP + 3% sucrose and 3% sodium alginate showed good shoot sprouting. Efficient plantlet regeneration from different encapsulated explants was reported in a number of forest trees (Reddy et al., 2012). *In vitro* conservation of *Cederla fissilis* via encapsulation of shoot tips; cotyledonory and epicotyl nodal segments were reported by Nunes et al. (2003).

Hung and Trueman (2011) studied the alginate encapsulation of shoot tips and nodes for short term storage of *Corymbia torelliana* × *Corymbia citriodora*. They found 100% survival in nursery when pre-converted shoot tip derived synthetic seeds were transferred on to an organic compost substrate. Asmah et al. (2011) developed a protocol for encapsulation of *Acacia* hybrid *in vitro* derived shoots and axillary buds and found that the germination rate was within 73.3 to 100% in the duration of six to 20 days.

Induction of rooting is effected by several intrinsic and extrinsic factors. The cloned shoots rooted *in vitro* on MS medium containing root inducing auxins. It has been found that in *D. roxburghii* maximum lateral root initiation and primordial growth is promoted when micro shoots cultured on MS medium supplemented with 2.0 µM IBA. The positive roles of IBA on *in vitro* rooting of trees were reported by many others.

Similar to the present findings, Rao et al. (1998) reported that MS medium supplemented with 0.23 µM IBA induced maximum rooting in *Excoecaria agallocha.* In *Garcinia indica* maximum rooting (91.66%) occurred in shoots cultured on half-strength MS medium supplemented with 10 µM IBA (Malik et al., 2005). Whereas in *Pterocarpus marsupium,* two-step culture system was developed that is, pulse treatment and subsequent transfer of treated shoots to a low concentration of IBA along with phloroglucinol by Husain et al. (2007).

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ABBREVIATIONS

2,4,D, 2,4, dichlorophenoxyacetic acid; **AC,** activated charcoal; **BAP,** 6-benzylaminopurine; **GA$_3$,** gibberellic acid; **HgCl$_2$,** mercuric chloride; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **Kn,** kinetin; **MS,** Murashige and Skoog; **NAA,** *α*-naphthaleneacetic acid.

Conflict of interest

The authors report no declarations of interest.

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