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IN VITRO MICROPROPAGATION OF SIAMESE ROSEWOOD *DALBERGIA COCHINCHINENSIS* PIERRE, A TIMBER YIELDING TREE

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Abstract

Dalbergia cochinchinensis, a woody legume, is a high-value timber-yielding tree that is important for both ecological and commercial purposes. To allow efficient *In vitro* micropropagation, we evaluated the efficiency of plant growth regulators (including kinetin, gibberellic acid, and thidiazuron), different basal media (¼ Murashige and Skoog medium (MS), ½ MS, 1 MS, 2 MS, ¼ woody plant medium (WPM), ½ WPM, 1 WPM, and 2 WPM), gelling agents (phytagel, crystal agar gel G180, food grade agar powder, and bacteriological grade agar powder), and carbon sources (sucrose and glucose) for shoot induction in *D. cochinchinensis*. Nodal segments, derived from the shoots of 1-year old trees, were surface sterilized and used. Shoots induced by gibberellic acid showed the highest shoot length (17.28 ± 0.20 mm) but were thin, pale green, and displayed shoot tip necrosis. However, shoots induced by kinetin were shorter (15.20 ± 0.41 mm) but were greener and more vigorous. The best conditions for improving shoot induction and elongation were 30 g/L sucrose and 2.6 g/L phytagel. *In vitro* microshoots were rooted on half-strength WPM augmented with 0.05 mg/L indoleacetic acid. After 1 month of culture, the highest number of roots per shootlet was 6.8 ± 0.58, with an average root length of 54.76 ± 5.22 mm. The plantlets were acclimatized in coconut husks with an 80% survival rate.

Key words: Woody legume, Plant growth regulators, Basal medium, Gelling agent, Carbon source.

Introduction

Dalbergia cochinchinensis, or Siamese rosewood, a legume species in the family Fabaceae is indigenous to Thailand, Cambodia, Vietnam, and Laos. *D. cochinchinensis* is typically 25–30 m tall and 0.6–1.2 m in diameter. This tree has many potential uses, and its red-colored durable heartwood or rosewood has made it valuable worldwide (Moestrup *et al*., 2006). The timber is mainly exported to China to produce luxury furniture, boats, and musical instruments (Bhagwat *et al*., 2015).

The visual appeal, durability, and cost of heartwood contribute to its great demand, resulting in illegal harvesting for domestic use and export to neighboring countries. In addition, illegal logging at borders has caused territorial conflicts (Siriwat & Nijman, 2018). Many important tropical forest trees, including *D. cochinchinensis*, have a low germination capacity because of seed coat impermeability (Werker, 1980; Bell & van Staden, 1993; Cervantes *et al*., 1996; Seng & Cheong, 2020). Additionally, forests, which are *D. cochinchinensis* habitats, have been invaded for use in construction and agriculture. Although Thailand and Cambodia have begun commercial cultivation and conservation, there are no mature *D. cochinchinensis* plants because they are very slow-growing; therefore, all harvesting occurs in natural forests. *D. cochinchinensis* also has many natural enemies: their leaves are affected by a severe rust disease caused by *Maravalia pterocarpi*, and three black spot diseases, associated with two unidentified ascomycetes and one coelomycete (Pongpanich, 1998). As a result, *D. cochinchinensis* has been classified as an endangered plant species and listed in CITES Appendix II, since 2013.

Many organizations such as the Greater Mekong Subregion have worked to find solutions to these problems by preserving and rehabilitating, by improving capacity for seed storage, germplasm supply, promoting reproduction (Luoma-aho *et al*., 2003), and incorporating valuable and endangered species such as *Dalbergia* in forest restoration programs (Kaewkrom *et al*., 2005; Sakai *et al*., 2009). Current research surrounding *D. cochinchinensis* is mainly related to identification of species (Hartvig *et al*., 2015; Zhang *et al*., 2016; Moritsuka *et al*., 2017; Wang *et al*., 2019; Hartvig *et al*., 2020) and extraction of biological substances (Chuankhayan *et al*., 2007; Ratananikom *et al*., 2013; Liu *et al*., 2016; Xiang *et al*., 2018; Zhu *et al*., 2018). Research concerning the conservation and propagation of *D. cochinchinensis* is limited, especially that involving tissue culture.

Here, we focused on four factors to develop a micropropagation protocol for shoot induction and elongation in *D. cochinchinensis*: (1) suitable plant growth regulators (PGRs), (2) basal medium, (3) gelling agent, and (4) carbon source. We aimed to increase the quantity of *D. cochinchinensis* and allow for its greater use. Moreover, extinction can thereby be avoided by preserving and sustaining the species.

Materials and Methods

Plant material and surface sterilization: Explants were taken from 1-year old *D. cochinchinensis* shoots. Approximately 20 mm-long shoots were cut with one node. The shoots were washed thoroughly with tap water for 15 min, and surface sterilized by applying three drops of Tween-20 + 1% (v/v) carbendazim and rinsed after 30 min with sterile water once. Subsequently, three drops of Tween-20 + 0.1% (w/v) mercuric chloride ($HgCl_2$) were added, and the shoot was rinsed after 5 min with sterile water once. Finally, three drops of Tween-20 + 0.1% (w/v) $HgCl_2$ + 0.1% (v/v) plant preservative mixture (PPM) were added to the shoot, and the shoot was rinsed after 5 min with sterile water four times.

Table 1. Effect of cytokinins (Kn and TDZ) and gibberellin (GA_3) on bud-breaking and shoot growth in *D. cochinchinensis* nodal explants.

Plant growth regulator (mg/L)			Shoot regeneration frequency (%)	Shoot length (mm)
Kn	GA_3	TDZ		
0.0	0.0	0.0	40	$1.27^{p} \pm 0.23$
3.0			80	$4.80^{l} \pm 0.43$
3.5			70	$6.50^{k} \pm 0.25$
4.0			80	$8.42^{i} \pm 0.17$
4.5			70	$10.38^{g} \pm 0.28$
5.0			70	$15.20^{b} \pm 0.41$
5.5			70	$13.74^{c} \pm 0.58$
6.0			70	$13.30^{cd} \pm 0.75$
6.5			70	$12.39^{ef} \pm 0.43$
7.0			70	$10.02^{gh} \pm 0.39$
	1.0		70	$6.50^{k} \pm 0.67$
	1.5		80	$7.66^{j} \pm 0.44$
	2.0		80	$9.74^{h} \pm 0.28$
	2.5		80	$12.93^{de} \pm 0.68$
	3.0		80	$17.28^{a} \pm 0.20$
	3.5		80	$14.79^{b} \pm 0.59$
	4.0		80	$12.77^{de} \pm 0.65$
	4.5		80	$11.94^{f} \pm 0.47$
	5.0		80	$8.51^{i} \pm 0.61$
		0.5	80	$11.87^{f} \pm 0.49$
		1.0	90	$9.71^{h} \pm 0.55$
		1.5	80	$4.97^{l} \pm 0.73$
		2.0	80	$3.91^{m} \pm 0.17$
		2.5	70	$2.66^{n} \pm 0.29$
		3.0	70	$2.53^{no} \pm 0.21$
		3.5	70	$2.31^{no} \pm 0.37$
		4.0	60	$1.97^{o} \pm 0.13$
		4.5	60	$1.32^{p} \pm 0.72$
		5.0	60	$1.16^{p} \pm 0.13$

Values are means ± SE. Differences in letters within a column are statistically significant at $p<0.05$

Shoot induction: For shoot induction, explants were transferred to shoot induction media, woody plant medium (WPM) + sucrose (30 g/L), supplemented with varying concentrations of kinetin (Kn), gibberellic acid (GA_3), and thidiazuron (TDZ). Initially, we tested concentrations from 1 to 9 mg/L in 2 mg/L steps and then increased to 0.5 mg/L steps over the optimal concentration range. After we identified the best concentration of each PGR, we combined the best concentration. All cultures were incubated at 25 ± 2 degrees celsius under light provided by white fluorescent tube lights for 16 h and in the dark for 8 h. All cultures were transferred to fresh medium every 2 weeks. Percent responses and shoot lengths were recorded after 1 month.

Effects of basal medium, gelling agent, and carbon source: Two different basal media, WPM (Lloyd & McCown, 1981), and MS medium (MS) (Murashige & Skoog, 1962) at different concentrations (¼, ½, 1, and 2) were tested. The gelling agents tested were 2.6 g/L phytagel (*Phyto*Technology Laboratories), 7 g/L crystal agar gel G180 (Central Gel co., Ltd.), 10 g/L food grade agar powder (Pattanasin Enterprise) and 15 g/L bacteriological grade agar powder (Himedia). Two sugars, sucrose and glucose, were tested as carbon sources for plants in tissue culture, as at concentrations from 10 to 50 g/L, in steps of 10 g/L, to identify the optimal combination with the best concentration of PGRs from shoot induction.

***In vitro* rooting and acclimatization:** *In vitro* rooting was performed using the two-step root induction method established by Anis *et al.*, (2005). Healthy micro-shoots from 4-month old plantlets were cultured in high concentrations (20 mg/L) of indoleacetic acid (IAA) and indolebutyric acid (IBA) (Ahmad & Anis, 2019) in half-strength WPM liquid medium. Microshoots were then transferred to half-strength WPM semi-solid medium supplemented with IAA and IBA at various concentrations (0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 mg/L). The percentage of plants rooting, number of roots, and mean root length after 1 month of culture transfer were recorded.

After 1 month, shootlets with well-developed roots were transferred to 100 mm-diameter cups containing coconut husks covered with clear plastic bags to protect plantlets from photo-oxidation. The transplanted shootlets were washed to remove the adhering gelling agent using tap water. All transplants were cultured under a 16 h light + 8 h dark photoperiod; artificial light was provided using white fluorescent tubes. After planting, the explants were watered daily for 2 weeks. After 1 month, well-developed plantlets were moved to a greenhouse under natural daylight.

Experimental design and data analysis: All experiments had 10 replicates per treatment. Data were analyzed using SPSS v.26 (SPSS, Chicago, IL, USA). The significance of differences between means was tested using Duncan's multiple range test at $p<0.05$. All results are expressed as means ± standard error.

Results and Discussion

Effect of various cytokinin and gibberellin concentrations on shoot induction and elongation from nodal segments: The results showed that explants exhibited no response in media cultured without PGRs. In contrast, explants cultured in media containing PGRs tended to exhibit shoot induction and shoot elongation. (Table 1) summarizes our results. We found that 5 mg/L kinetin (Kn) induced shorter shoots (15.20 ± 0.41 mm) than those induced by gibberellic acid (GA_3) (17.28 ± 0.20 mm). Similarly, Fráguas *et al.*, (2004) showed that increased Kn levels in the growth medium resulted in reduced shoot lengths, but Kn was essential for shoot induction (Leshem *et al.*, 1988). In this experiment, 5 mg/L Kn was optimal for shoots induction because shoots from Kn were greener and more vigorous than those induced by GA_3 (Fig. 1C).

The longest shoots (17.28 ± 0.20 mm) were induced by 3 mg/L GA_3. However, shoots exposed to GA_3 were less green, thin, and showed tip necrosis (Fig. 1A), compared with shoots with other PGRs. These morphological variations in plantlet survival had previously been reported and were mainly related to photosynthesis and transpiration (Ziv, 1991). Our observations were similar to those of previous research on lentil plants, where GA_3 increased plant growth by 43%, whereas seed mass was decreased by 26% (Giannakoula *et al*., 2012) and GA_3 increased shootlet lengths compared to culture medium without GA_3 (Alansi *et al*., 2020).

In addition, we tested thidiazuron (TDZ) for shoot induction. It resulted in the shortest shoot lengths compared with other PGRs that we tested: the optimal TDZ concentration was 0.5 mg/L, with shoot length of 11.87 ± 0.49 mm. Although TDZ led to the shortest shoot lengths, but their color was greener than that of shoots induced by other PGRs. TDZ was shown to promote shoot induction, particularly in woody plant species, because it exerted high cytokinin-like activity when used at low dosages and, on the other hand, higher concentrations caused no shoot elongation. (Pai & Desai, 2018).

We found an optimal concentration for each PGR, as shown in Table 1. Best concentration to the combined is shown in Table 2. High shoot length values were obtained under 3 mg/L GA_3 (17.77 ± 0.52 mm) and 5 mg/L Kn + 3 mg/L GA_3 (16.61 ± 0.42 mm). However, we found that the results of these experiments were the same as the previous results in Table 1. For shoots formed in GA_3, shoot length was extensive, but the shoots were unhealthy. According to Fráguas *et al*., (2004), GA_3 can increase the length of shoots but, in turn, can negatively affect shoots, for example, by causing hyperhydricity. When GA_3 was combined with Kn, some leaves formed and emerged in greater numbers than when GA_3 was used alone. However, the leaves were small, yellowish, and most fell before the end of 1 month (Fig. 1B). TDZ treatment resulted in shorter and slower shoot growth than in the other treatments (Table 2). Therefore, the PGR that had the greatest effect on growth was 5 mg/L Kn, resulting in extensive shoot length (15.85 ± 0.39 mm) and, most importantly, the characteristics of the shoots indicated that they were green, healthy, and strong enough to continue growth and induce roots in the next step.

Effect of basal medium: In previous section experiments, 5 mg/L Kn was the optimal concentration. In this section, we brought 5 mg/L Kn to combine with various types and concentrations of media for culturing plants. The best response and elongation characteristics for shoot induction were found using half strength WPM, yielding 22.36 ± 0.31 mm shoot length and 95% response (Table 3 & Fig. 1D). Shoots were also more vigorous and healthier than other media (¼ MS, ½ MS, 1 MS, 2 MS, ¼ WPM, 1 WPM, and 2 WPM). We observed hyperhydration and defoliation symptoms in explants grown in full-strength MS medium similar to (Rathore *et al.*, 2004). Moreover, MS led to worse results than WPM at every medium strength. WPM contained potassium sulfate, whereas MS contained potassium nitrate. Because of this difference, WPM can increase the uptake of certain media elements (Khamushi *et al.*, 2019).

Table 2. Effect of cytokinins (Kn and TDZ) and gibberellin (GA_3) combinations on bud-breaking and shoot growth in *D. cochinchinensis* nodal explants.

Plant growth regulator (mg/L)			Shoot regeneration frequency (%)	Shoot length (mm)
Kn	GA_3	TDZ		
0.0	0.0	0.0	50	$2.15^{g} \pm 0.37$
5.0			70	$15.85^{c} \pm 0.39$
	3.0		80	$17.77^{a} \pm 0.52$
		0.5	80	$11.27^{f} \pm 0.53$
5.0	3.0		70	$16.61^{b} \pm 0.42$
5.0		0.5	70	$12.33^{e} \pm 0.61$
5.0	3.0	0.5	80	$13.70^{d} \pm 0.63$

Values are means ± SE. Differences in letters within a column are statistically significant at $p<0.05$

Table 3. Effects of culture media on shoot induction and elongation in *D. cochinchinensis.*

Media type	Shoot regeneration frequency (%)	Shoot length (mm)
1/4 MS	50	$5.40^{e} \pm 0.37$
1/2 MS	50	$4.75^{f} \pm 0.59$
1 MS	30	$3.34^{g} \pm 0.39$
2 MS	25	$2.24^{h} \pm 0.17$
1/4 WPM	90	$18.67^{b} \pm 0.40$
1/2 WPM	95	$22.36^{a} \pm 0.31$
1 WPM	85	$16.96^{c} \pm 0.21$
2 WPM	80	$12.64^{d} \pm 0.20$

Values are means ± SE. Differences in letters within a column are statistically significant at $p<0.05$

Table 4. Effect of gelling agent on shoot induction and elongation in *D. cochinchinensis.*

Gelling agent	Shoot regeneration frequency (%)	Shoot length (mm)
Phytagel	90	$19.01^{a} \pm 0.80$
Crystal agar gel G180	70	$16.54^{b} \pm 0.96$
Agar powder, food grade	70	$16.67^{b} \pm 1.11$
Agar powder, bacteriological grade	50	$8.52^{c} \pm 1.06$

Values are means ± SE. Differences in letters within a column are statistically significant at $p<0.05$

Table 5. Effect of carbon source on shoot induction and elongation in *D. cochinchinensis.*

Carbon source (g/L)		Shoot regeneration frequency (%)	Shoot length (mm)
Glucose	Sucrose		
10		80	$4.92^{g} \pm 0.79$
20		90	$17.74^{b} \pm 0.63$
30		80	$16.57^{c} \pm 0.98$
40		90	$7.58^{f} \pm 0.72$
50		90	$3.89^{h} \pm 0.29$
	10	90	$9.11^{e} \pm 0.59$
	20	70	$11.27^{d} \pm 0.54$
	30	80	$18.82^{a} \pm 0.75$
	40	80	$18.24^{ab} \pm 1.46$
	50	90	$5.69^{g} \pm 0.48$

Values are means ± SE. Differences in letters within a column are statistically significant at $p<0.05$

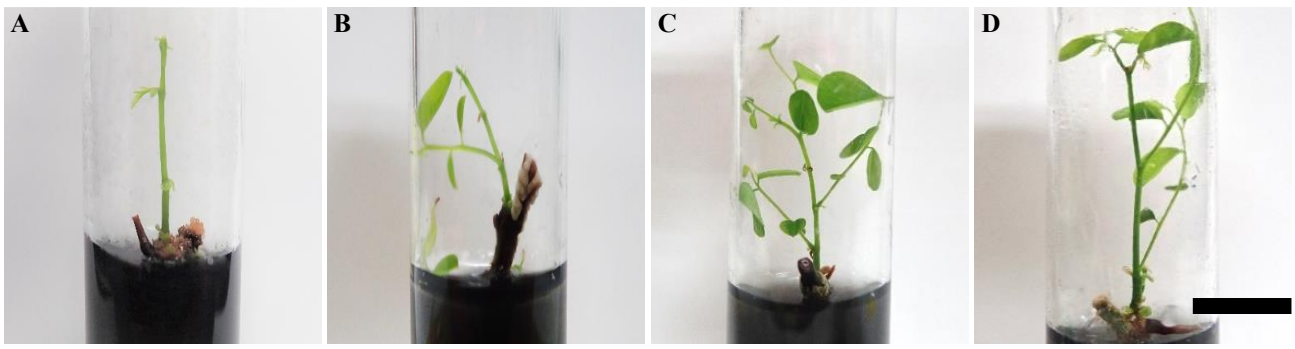

Fig. 1. Shoot bud induction and elongation in nodal explants of *D. cochinchinensis* after 1 month. A) Shoot bud induction in nodal explants on WPM + 3 mg/L GA_3. B) Shoot bud induction in nodal explants on WPM + 3 mg/L GA_3 + 5 mg/L Kn. C) Shoot bud induction in nodal explants on WPM + 5 mg/L Kn. D) Shoot elongation in ½ WPM with 5 mg/L Kn. (scale bar 1 cm).

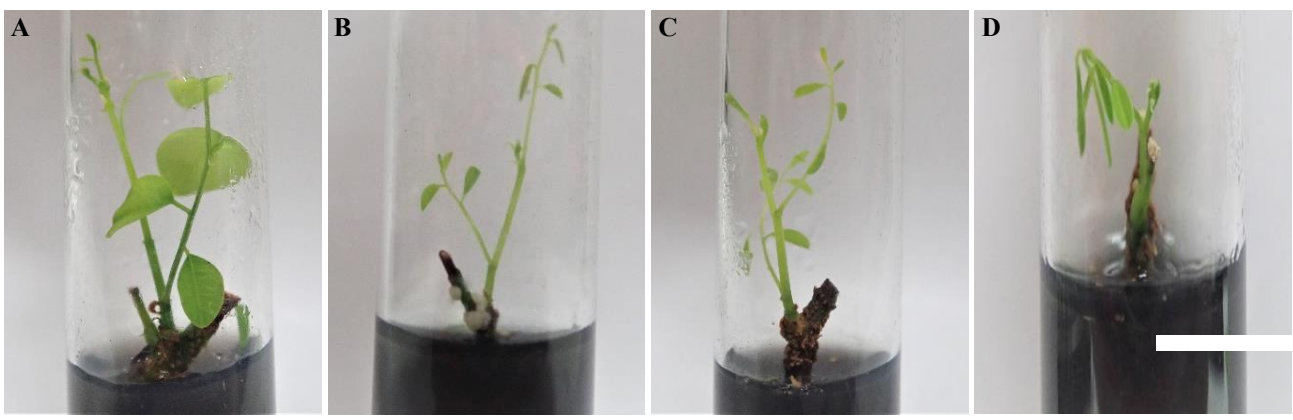

Fig. 2. Different gelling agents for shoot induction and elongation in nodal explants of *D. cochinchinensis* on WPM supplemented with 5 mg/L Kn after 1 month of culture. A) Shoot induction and elongation on phytagel. B) Shoot induction and elongation in crystal agar gel G180. C) Shoot induction and elongation in food grade agar powder. D) Shoot induction and elongation in bacteriological grade agar powder. (scale bar 1 cm).

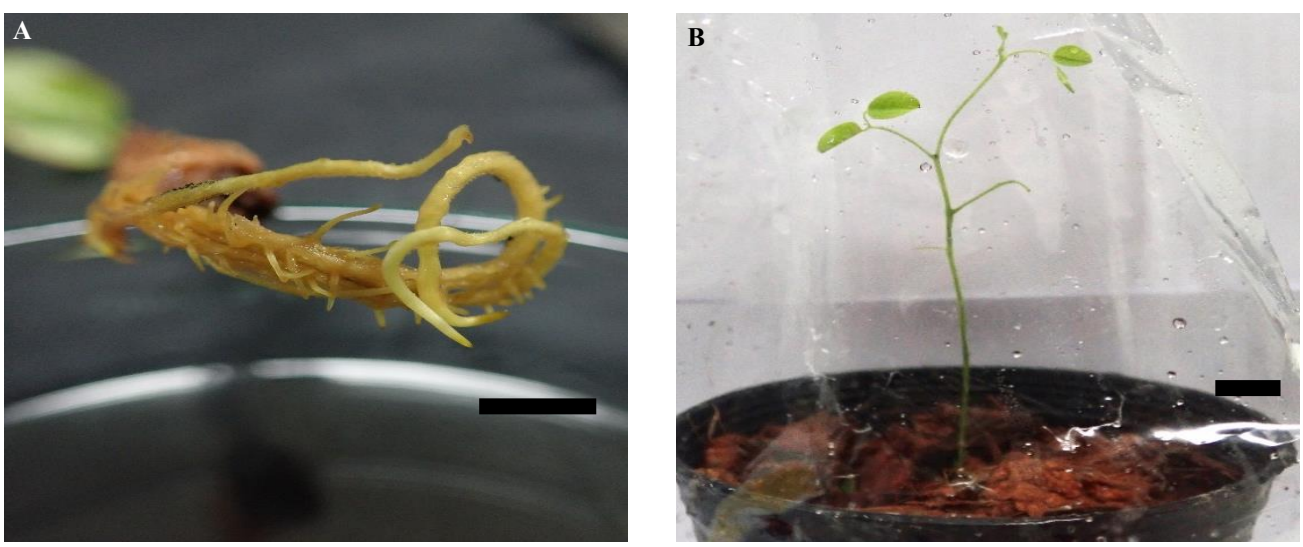

Fig. 3. Root induction and acclimatization of *D. cochinchinensis.* A) *In vitro* rooted shoots treated with 20 mg/L IAA for 5 days, then transferred to 0.05 mg/L IAA for 1 month. B) Acclimatized plant in the greenhouse after 1 month. (scale bar 1 cm).

Effect of gelling agent: We used the gelling agents listed in Table 4 and found the optimum response with phytagel. The shoot length was 19.01 ± 0.80 mm with 90% response (Table 4). Phytagel is a high quality, clear and low impurities, but the disadvantage of phytagels is that they are the most expensive gelling agent. The result showed gelling agents, crystal agar gel G180 and food grade agar powder; produced green and vigorous shoots almost similar to that of phytagel (Fig. 2). Therefore, industrial applications, the crystal agar gel G180 or food grade agar powder, may be preferred for use to reduce costs. According to Schmauder (1985), gelling agents can promote good growth of plant cultures and are more profitable for commercial practices if the low costs are involved.

Effect of carbon source: Two carbon sources were tested: glucose and sucrose. Sucrose was more effective than glucose. We found sucrose at 30 g/L gave the highest shoot lengths (18.82 ± 0.75 mm) as shown in (Table 5). This was consistent with a study by Romano *et al.*, (1995) who found 30 g/L sucrose showed the highest number of shoots and high rate of shoot proliferation and elongations in *Quercus robur* (English Oak). Good quality *D. cochinchinensis* shoots were observed with 30 and 40 g/L sucrose. Glucose and sucrose at concentrations as low as 10 g/L revealed signs of hyperhydricity (vitrification), whereas at higher concentrations (50 g/L), shoots were small and exhibited browning and defoliation of shoot tips and leaves, respectively. This was attributed to high sucrose concentrations, which reduced the water potential of the culture medium and depressed plant transpiration, with consequent reductions in nutrient absorption (Santana *et al*., 2011).

Effect of various auxin concentrations on *In vitro* rooting and acclimatization by plantlets: Roots were observed under IAA longer than IBA. The mean root length under IAA was 54.76 ± 5.22 mm, with 60% of shoots producing roots, whereas the mean root length under IBA was only 21.34 ± 2.06 mm, with 40% of shoots producing roots (Table 6 & Fig. 3A). IAA has been found to be superior to IBA and other auxins *In vitro* rooting in leguminous plants (Polanco & Ruiz, 1997; Monteuuis & Bon, 2000; *Barik et al*., 2004; Barik *et al*., 2005; Patil *et al.*, 2009). According to Borthakur *et al.*, (2012), the addition of IAA at higher concentrations reduces the percentage of rooting and root length. Similarly, we observed that for IAA concentrations of 0.2 mg/L or more, root formation was not detected in *D. cochinchinensis*. Numbers of roots from IAA and IBA were not significant. The well-developed plantlets were acclimatized in coconut husks (Fig. 3B) and exhibited an 80% survival rate.

Table 6. Effect of low auxin concentrations on *In vitro* root induction in shoots of *D. cochinchinensis.*

Auxins (mg/L)		Root regeneration	No. of roots per	Root length (mm) per
IAA	IBA	Frequency (%)	microshoot	microshoot
0.00	0.00	0	0.00 ± 0.00	0.00 ± 0.00
0.05		60	6.80^{a} ± 0.58	54.76^{a} ± 5.22
0.10		40	4.20^{b} ± 1.15	28.57^{b} ± 4.39
0.20		0	0.00 ± 0.00	0.00 ± 0.00
0.40		0	0.00 ± 0.00	0.00 ± 0.00
0.80		0	0.00 ± 0.00	0.00 ± 0.00
	0.05	40	6.20^{a} ± 1.64	21.34^{c} ± 2.06
	0.10	20	3.20^{bc} ± 0.84	20.32^{cd} ± 0.75
	0.20	20	2.80^{c} ± 0.45	15.84^{de} ± 4.60
	0.40	20	2.60^{c} ± 0.55	12.03^{e} ± 0.76
	0.80	20	2.00^{c} ± 0.71	14.73^{e} ± 0.50

Values are means ± SE. Differences in letters within a column are statistically significant at $p<0.05$

Conclusions

It was possible to propagate *D. cochinchinensis* using tissue culture methods to increase plant numbers in large quantities to meet industrial demand, reduce the effects of illegal harvesting, and maintain an endangered species. We assessed various combinations of PGRs, medium types, gelling agents, and carbon sources. We showed that the most successful combination was half-strength WPM supplemented with Kn, sucrose, and phytagel, leading to vigorous shoots. However, phytagel was the most expensive gelling agent, and inexpensive alternatives, such as food grade agar powder, were almost as effective. For successful *In vitro* root induction using the two-step method, explants were successfully acclimatized in coconut husk material.

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