IN VITRO REGENERATION OF STRIPE EBONY (DIOSPYROS FRUTESCENS BLUME)

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Abstract

The Stripe *ebony tree* (*Diospyros frutescens* Blume) is considered a species in danger of extinction due to overexploitation. Therefore, there is an urgent need to regenerate and conserve the species for future generation. In this study micropropagation protocol of *Diospyros frutescens* Blume was developed using nature zygotic embryos. Zygotic embryos isolated from germinated on MS medium containing 0.4mg L^{-1} Kinetin + 1.0mg L^{-1} BA. There were (83.11±0.79%) of explants induced shoots after 30 days of culture. The highest number of shoot on 1/2 MS medium supplemented with 2.5mg L^{-1} BA + 0.6mg L^{-1} IBA was 5.84±0.035 shoots per explant after 45 days of culture. The percentage of shoot produced roots the highest 85.52±0.48% after 45 days culture on 1/2 MS medium contain 0.8mg L^{-1} IBA.

Keywords: stripe ebony, shoot multiplication, rooting, in vitro propagation

1. Introduction

Diospyros frutescens belongs to (Ebenaceae) family, the tree grows about 12 to 20m tall and 30-80cm in diameter. Cylindrical stem, early branching, dark brown husk, many cracks. Single leaves separated from each other and 5.5-15cm length, 2.5-6cm width when dry it is black. Small flowers, solitary or gather each cluster from 1-3 flowers on a branch (Ho, 1993; Chan and Hien, 2000). Trees scattered or small groups in dry forests or Rocky Mountains, feralit soil, grow very slowly, the stripe ebony adapt to drought and high light conditions, distributed at an altitude not exceeding 70m. In the world, *Diospyros* genus has 450-750 species. Most of them are origins the tropics, and can be found in Sri Lanka, Laos, Cambodia, South China and Vietnam, West Africa and only a few in temperate areas. Some species in *Diospyros* genus are ranked-in group very endangered and have in the red list of IUCN 2016 such as *Diospyros mun* (IUCN, 2016). This genus includes valuable and commercial species, all of them are species such as *Diospyros kaki* and *D. virginiana*) There are two ebony groups for commercial value, that is black ebony: *D. ebenum*, *D. mun*, *D. crassiflora* and stripe ebony: *Diospyros celebica* and *Diospyros frutescens* (Ho, 1993). In Vietnam, there are two species *D. mun and D. frutescens*. The species *D. frutescens* is reduced by exploiting to take wood. It could be risk of extinction in nature. Because of the sssabitlity regeneration in the natural condition very low and the mother trees are exploited exhaust. At present, research on propagation and conservation ebony specieses of genus *Diospyros* have not cared much yet. Compared to other woody plants species, stripe ebony is very hardwood, the trunk, leafs, roots, flowers contain a lot of phenolic compounds, so the *in vitro* propagation from these parts is very difficult inside the epidermis cell contains many endogenous fungi, so the treatment and antiseptic of samples in the *in vitro* condition is very difficult. On the other hand, the phenolic compound is secretion very much. As a result, the rate of infection sample and death was high. But the research of stripe ebony *in vitro* propagation from zygotic embryos of seeds was suitable and necessary to conserve rare precious genetic resources in condition this species was reduced in nature.

2. Materials and methods

2.1 Collected stripe ebony and prepare the culture medium

Stripe ebony fruits were collected in nature and clean processing. The MS medium include macronutrients and micronutrients and supplement thiamine, pyridoxine, nicotinic acid at 1mg L^{-1}, myo-inositol (100mg L^{-1}), coconut water (100ml L^{-1}), agar (5g L^{-1}), sucrose (20g L^{-1}), activated carbon (1g L^{-1}) and Fe-EDTA 100mg L^{-1} (Evans *et al*., 1983, He *et al*., 2000).

2.2 Explant processing

The stripe ebony fruits are washed by soaps and continue re-rinse 5 times in distilled water, continue dip fruit in javel water (15%) for 10 minutes re-rinse with distilled water 3 times, after processed in alcohol at 70^0 for 1 minute and re-rinse distilled water 5 times and separating the seeds.

2.3 Effects of Kinetin and BA on shoot regeneration from explants of seed stripe ebony

The stripe ebony seeds were separated from fruit and cultured in MS medium + 0.4mg L^{-1} kinetin and BA at concentration from 0.0-2.0mg L^{-1} to inductive shoots regeneration. The experiment was performed with five treatments (Table 1). Each treatment was repeated 3 times. The ratio of explants created the shoots was evaluated after 30 days of culture (Jenik and Barton, 2005; Nguyen et al., 2016).

Effect of BA and IBA concentrations on shoot regeneration of stripe ebony

The shoots were hight from 2-2.5cm and had 1-2 pairs of leaves were moved to the rapid multiplicative stage on 1/2 MS medium, supplement BA and IBA at different concentrations (Manoj *et al*., 2009, Hoang Thi The *et al*., 2013). The shoots were cultured on 1/2 MS medium after 25 days to subculture new jar once to stimulates the ability to be rapid multiplication of the shoots and shoots cluster on the 1/2 MS medium.

Effects of growth regulators NAA and IBA to induce the roots of the stripe ebony shoot

The shoots were 2-3cm in height normal growth and development are cultured in medium 1/2 MS supplemental auxin (NAA, IBA) or activated carbon to stimulate root formation (Sharma *et al*., 1990, Farooq *et al*., 2008). The culture medium was adjusted pH = 5.8 before taking boil at 121°C for 20 minutes. *In vitro* culture conditions: Lighting time is 24 hours, light intensity: 2000 lux, temperature: 24^0C ± 2°C.

2.4 Experimental design, data collection and statistical analysis

The experiments are setuped at BA concentration from 0.0-2.0mg L^{-1} and designed by different treatments with 3 replicates, explants were seeds of stripe ebony fruit. Indicators were observed and evaluated after 30 days for the experiment into the explant 45 days for the rapid shoot multiplication experiment and 45 days for rooting experiment.

The collected data were subjected to statistical analysis of Statgraphics Centurion XV software.

3. Results

3.1 Effects of Kinetin and BA on shoot regeneration from explants of seed stripe ebony

At the control formula was only 0.4mg L^{-1} kinetic. There was 51.28±0.026% of the explants induced shots. When incorporating 0.4mg L^{-1} kinetin with BA concentration from 0.5-1.0mg L^{-1}, the ratio of shoot forming was higher than that of control formula 67.53±0.54% and 83.11±0.79%, the quality of the shoots was very good (Brown *et al*., 1995). The concentration of BA was increased from 1.5-2.0mg L^{-1}, the ratio of shoots formation and shoots per explant was lower and the quality of the shoots was also reduced Table 1. But the most suitable medium for shoot regeneration of stripe ebony was MS + 0.4mg L^{-1} kinetin + 1.0mg L^{-1} BA. (Goh *et al*., 1994, Ma *et al*., 2010) (Fig. 1a-e).

TABLE 1. Effects of Kinetin and BA on shoot regeneration of ebony after 30 days of culture.

Kinetin (mg L^{-1})	BA (mg L^{-1})	Percent shoot induction (%)	Quality of the shoots
0.4	0.0	51.28 ± 0.026^{a}	+
0.4	0.5	67.53 ± 0.54^{c}	++
0.4	1.0	83.11 ± 0.79^{d}	+++
0.4	1.5	68.58 ± 0.57^{c}	++
0.4	2.0	54.51 ± 0.76^{b}	+

Note: + : *Growth shoots are weak*; ++ : *Average shoots growth*; +++ : *Shoots grow well*
Data are given as means ± standard error of means with significant ($p \leq 0.05$)

BA concentration influenced shoot formation of striped ebony seeds in MS medium. In the medium without BA with only 0.4mg L^{-1} Kinetin, the shoot formation was very low (51.28±0.026%), some of explants showed black expression and did not germinate, when adding 0.5mg L^{-1} BA into the culture medium the number of shoot forming explants increased 67.53±0.54% the BA concentration increased 1.0mg L^{-1} the maximum ratio of shoot forming explants was 83.11±0.79%, the shoots grew well. Increase the concentration of BA from 1.5-2.0mg L^{-1} the ratio of shoot formation reduced, and the quality of shoots were least Table 1.

The results in Table 2 showed that in the presence of BA in the culture medium the shoots multiplication coefficient was 2.46±0.04–4.45±0.07 times higher than control treatment 0.85±0.06 times without BA. The stripe ebony shoots multiplication factor increases to 4.80±0.02 times when concentration of BA increases from 2.0-2.5mg L^{-1}. However, at the high concentration–3.0mg L^{-1} BA inhibited shoot multiplication factor, and reduced shoot height and shoot quality was least (George, 1993, Manoj *et al*., 2009, Josh *et al*., 2003). However, BA concentration at 2.5mg L^{-1} was the best for shoot multiplication of the stripe ebony with height and quality of shoots were very good (Fig. 1f).

Table 2. The effect of BA on the ability to multiply shoots of stripe ebony after 45 days of culture.

BA (mg L^{-1})	Shoot multiplication factor (times)	Average height of the shoots (cm)	Quality of the shoots
0.0	0.85 ± 0.06^{a}	1.42 ± 0.043^{a}	+
0.5	2.46 ± 0.04^{b}	2.52 ± 0.087^{c}	+
1.0	3.29 ± 0.05^{c}	2.95 ± 0.036^{d}	+
1.5	4.45 ± 0.07^{e}	3.54 ± 0.049^{e}	++
2.0	4.67 ± 0.03^{f}	3.82 ± 0.011^{f}	++
2.5	4.80 ± 0.02^{g}	4.17 ± 0.026^{g}	+++
3.0	4.29 ± 0.01^{d}	1.77 ± 0.031^{b}	++

Note: + : *Growth shoots are weak*; ++ : *Average shoots growth*; +++ : *Shoots grow well*.
Data are given as means ± standard error of means with significant ($p \leq 0.05$)

During the shoot rapid multiplication phase shoots at the shoot regeneration stages were transferred to culture in MS½ medium without using Kinetin only BA at different concentrations. BA at concentration 0.0mg L^{-1} with shoot multiplication factor was very low with 0.85±0.06 times. When BA concentration increases 0.5mg L^{-1} gave multiplication 2.46±0.04 times and 1.0mg L^{-1} was 3.29±0.05 times. The highest shoot multiplication factor at the concentration of BA 2.5mg L^{-1} was 4.80±0.02 times after 45 days of culture, the shoot quality was very good Table 2, at the concentration 3.0mg L^{-1} BA the multiplication factor started to reduce and there was glass formation on the surface of the agar medium. Thus, BA affects not only the shoot regeneration process but also the role in the shoot rapid multiplication and cluster of striped ebony. The results Table 3 showed that in 1/2 MS medium with only BA 2.5mg L^{-1}, the shoot multiplication factor was only 3.28±0.025 times and after increased when the concentration of IBA increased from 0.3-0.5mg L^{-1}, the ratio of shoot multiplication increased from 4.27±0.015-4.65±0.041 times. Shoot multiplication factor was the highest at IBA concentration 0.6mg L^{-1} was 5.84±0.035 times, shoot height was 3.51cm and shoot quality was good, IBA concentration increased from 0.7-1.0mg L^{-1} ratio of shoot multiplication reduced 3.47 ± 0.026 times, shoot height and quality least. Thus, when the culture medium combined of BA 2.5mg L^{-1} and IBA 0.6mg L^{-1}, the shoot multiplication coefficient was the most effective and shoot quality is very good after 45 days of culture (Manoj *et al*., 2009) (Fig. 1f).

3.2 Effect of BA and IBA concentrations on shoot multiplication of stripe ebony

TABLE 3. Effect of BA and IBA concentrations on shoot multiplication of stripe ebony.

BA (mg L^{-1})	IBA (mg L^{-1})	No. of shoots per explant	Average height of the shoots (cm)	Quality of the shoots
2.5	0.0	3.28 ± 0.025^{a}	2.58 ± 0.068^{b}	+
2.5	0.3	4.27 ± 0.015^{c}	2.78 ± 0.076^{c}	++
2.5	0.5	4.65 ± 0.041^{d}	2.81 ± 0.065^{c}	++
2.5	0.6	5.84 ± 0.035^{e}	3.51 ± 0.03^{d}	+++
2.5	0.7	4.26 ± 0.030^{c}	3.82 ± 0.029^{e}	++
2.5	1.0	3.47 ± 0.026^{b}	2.21 ± 0.026^{a}	+

Note: +: *Growth shoots are weak*; ++: *Average shoots growth*; +++: *Growth shoots well.*
Data are given as means ± standard error of means with significant ($p \leq 0.05$)

The shoot multiplication efficiency was high when there was combination of BA and IBA at different concentrations in 1/2 MS medium, with BA concentration 2.5mg L^{-1} and IBA concentration in the order: 0.0mg L^{-1}: 0.3mg L^{-1}: 0.5mg L^{-1}: 0. 6mg L^{-1}: 0.7mg L^{-1}: 1.0mg L^{-1} will gave the number of shoots per explant was 3.28±0.025: 4.27±0.015: 4.65±0.041: 5.84±0.035: 4.26±0.03 and 3.47±0.026 in which the IBA concentration 0.6mg L^{-1} gave number of shoots per explant was the highest 5.84±0.035 (Table 3). When the concentration of IBA increased from 0.7-1.0mg L^{-1}a, the number of shoots per explant reduced 4.26±0.03 and 3.47±0.026. Thus, shoot multiplication efficiency has been cultured on 1/2 MS medium with combination of BA 2.5mg L^{-1} and IBA 0.6mg L^{-1} was 5.84±0.035 shoots per explant higher than the 1/2 MS medium only BA 2.5mg L^{-1} was 4.80±0.02 shoots per explant after 45 days of culture. On 1/2 MS medium without NAA-IBA the shoots were not root. When adding NAA, IBA and other nutrients into the culture medium, the ability to stimulate rooting was expressed Table 4, the ratio of root-forming shoots were the highest on the medium contain 1.0mg L^{-1} NAA only 78.08±0.14% with 3.53±0.04 roots per shoot after 45 days of culture and reduced gradually 70.21 ±0.41% when the concentration of NAA increased up 1.5mg L^{-1}. The number of roots per shoot as well as the quality of the roots reduced. While IBA has a more positive effect on the ability to root stripe ebony shoots compared with NAA. When the IBA with concentration from 0.5-0.8mg L^{-1}, ratio of shoots induced root was the highest 85.52 ±0.48%, with 3.59–4.21 roots per shoot and 4.26-5.21cm roots length (Edwin, 1996, Gamborg, 2002, Azadi *et al*., 2007).

3.3 Effects of growth regulators NAA and IBA to induce the roots of the stripe ebony shoot

TABLE 4. Effects of growth regulators (NAA and IBA) on *in vitro* root induction in regenerated shoots of stripe ebony.

Concentration NAA/IBA (mg L^{-1})	Ratio of the shoots were rooted (%)		Average roots per shoot		Average length of the roots (cm)	
	NAA	IBA	NAA	IBA	NAA	IBA
0	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
0.1	31.44 ± 0.60^{b}	38.45 ± 0.61^{b}	2.41 ± 0.17^{b}	2.72 ± 0.015^{b}	3.20 ± 0.014^{b}	3.63 ± 0.056^{b}
0.3	67.31 ± 1.34^{c}	71.66 ± 0.84^{c}	2.56 ± 0.03^{c}	3.57 ± 0.031^{d}	3.37 ± 0.025^{c}	3.80 ± 0.09^{d}
0.5	70.43 ± 0.78^{d}	80.90 ± 0.17^{e}	2.74 ± 0.05^{d}	3.59 ± 0.011^{d}	3.51 ± 0.035^{d}	4.26 ± 0.07^{e}
0.8	77.05 ± 0.34^{e}	85.52 ± 0.48^{f}	3.33 ± 0.03^{e}	4.23 ± 0.022^{e}	4.18 ± 0.08^{f}	5.21 ± 0.05^{f}
1.0	78.08 ± 0.14^{e}	81.00 ± 0.58^{e}	3.53 ± 0.04^{f}	2.82 ± 0.026^{c}	3.71 ± 0.026^{e}	3.78 ± 0.011^{d}
1.5	70.21 ± 0.41^{d}	76.10 ± 0.39^{d}	2.55 ± 0.02^{c}	2.11 ± 0.01^{a}	3.21 ± 0.04^{b}	3.31 ± 0.023^{b}

Data are given as means ± standard error of means with significant ($p \leq 0.05$)

During the rooting stage of stripe ebony shoots were separated from the shoots at the shoot multiplication stage and cultured on MS½ medium containing NAA and IBA at different concentrations. The shoot did not produce roots in the medium without NAA, IBA, when the culture medium had NAA/IBA 0.1mg L^{-1} the shoots started to root the was 31.44 ±0.62% with NAA and 38.45±0.61% with IBA and this ratio increased gradually to the increasing of NAA/IBA concentration in the culture medium. In the same concentration, the rooting ratio and number of roots per shot of NAA and IBA was different. At concentration 0.5mg L^{-1}, the rooting ratio and number of roots per

shoot of NAA were 70.43±0.78% and 2.74 roots per shoot, root length was 3.51cm while IBA was 80.90±0.17% and 3.59 roots per shoot, root length was 4.26cm. This ratio was the highest at the concentration 0.8mg L^{-1}, NAA was 77.05±0.34% and 3.33 roots per shoot, root length was 4.18cm while IBA reached 85.52 ±0.48% and number of roots per shoot was 4.23 and 5.21cm root length Table 4. At concentrations 1.0mg L^{-1} and 1.5mg L^{-1}, these ratio and indicators reduced. Thus, compared with NAA, IBA had better effication on root formation rate, number of roots per shoot, root length and quality of striped ebony after 45 days of culture was very good (Fig.1g).

In vitro plants had grown in net houses on substrates: 80% sand + 20% coir + organic fertilizers; temperature: 28-30^{0}C; light: 20-25%; humidity: 75-80%; watering 3 times per day; survival rate was 89% after 60 days of planting (Fig. 1h-i).

Fig. 1. The stages of in *vitro* propagation of the stripe ebony (*Diospyros frutescens* Blume)
MS + 0.4mg L^{-1} Kinetin + 1.0mg L^{-1} BA after 30 days of culture
(a; b; c): Stripe ebony fruit separated to take the seeds and cultured on MS medium
(d; e): The seeds germinated and grown into the shoots after 30 days of culture
1/2 MS + 0.6mg L^{-1} IBA + 2.5mg L^{-1} BA, after 45 days of culture
(f): Shoot multiplication of stripe ebony
1/2 MS + 0.8mg L^{-1} IBA, sucrose 20g L^{-1}, activated carbon 1g L^{-1}, pH: 5.8 after 45 days of culture
(g): Rooting stage of stripe ebony
In vitro plants had grown in net houses on substrates:
(h): *In vitro* plants after 60 days of planting outside the nursery, survival rate was 89%
(i): *In vitro* plants after 75 days of growing in plastic bags

4. Conclusion

The ratio of explants had induced shoots was 83.11±0.79% on MS medium combined with growth regulators at different concentrations include: 0.4mg L^{-1} kinetin + 0.8mg L^{-1} BA, shoots were formed from startup cultured. After it would be moved into 1/2 MS medium include 0.6mg L^{-1} IBA + 2.5mg L^{-1} BA; sucrose 20g L^{-1}, activated carbon 1g L^{-1}, agar 5g L^{-1} and addition other nutrients to shoots rapid multiplication. After 45 days of culture the highest shoot multiplication factor was 5.84±0.035 times, 3.51±0.03cm shoot height and quality of the shoots were good. The stripe ebony shoots were moved to rooting 1/2 MS medium with growth regulators at concentrations NAA/IBA 0.8mg L^{-1}, sucrose 20g L^{-1}, activated carbon 1g L^{-1}, agar 5g L^{-1} and other nutrients after 45 days of culture. Ratio of shoots induced root the highest was 85.52±0.48% with 4.23±0.022 roots per shoot and 5.21cm root length. The results of this study are important data for further studies on other woody plants containing many phenolic compounds that are difficult to *in vitro* propagation by stem and shoots.

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