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Adventitious Shoots Regeneration from Root and Stem Explants of *Eurycoma longifolia* Jack - An Important Tropical Medicinal Plants

[1]Sobri Hussein, [1]Rusli Ibrahim and [2]Anna Ling Pick Kiong
[1]Agrotechnology and Bioscience Division,
Malaysian Institute For Nuclear Technology Research,
Bangi, 43000 Kajang, Selangor, Malaysia
[2]Department of Bioscience, Faculty of Engineering and Science,
Universiti Tunku Abdul Rahman, 53300 Setapak, Kuala Lumpur, Malaysia

Abstract : This study reported the first successful adventitious shoots regeneration from the root and stem explants of *E. longifolia*. Generally, stem explants appeared to have a lower regeneration capability when treated with the cytokinins alone compared to the root explants. In the root explant, the best medium formulation determined was basal Juglans medium (DKW) supplemented with 1.0 mg L^{-1} of kinetin + 1.0 mg L^{-1} of zeatin whereas in the stem explants, basal woody plant medium (WPM) enriched with 2.0 mg L^{-1} of BAP + 2.0 mg L^{-1} of zeatin was found to be the best medium formulation in increasing the regeneration rate. Stem and root explants that were 2.0 cm distant from one another has been identified as the most suitable position for attaining the maximum percentage of adventitious shoots formation. Rootlets induction of adventitious shoots formed was also achieved and the highest induction rate was attained in basal MS medium supplemented with 0.5 mg L^{-1} of Indole-3-butyric acid. Acclimatization of the *in vitro* plantlets produced survived well with no morphological differences from the parent plants after two months of transplantation to the soil.

Key words: Adventitious shoots, direct regeneration, *Eurycoma longifolia*, micropropagation, tissue culture

Introduction

Eurycoma longifolia Jack, from the family of Simaroubaceae is commonly distributed in South East Asia. It owes its popularity to its aphrodisiac claim and has been sought after as an essential component for the treatments of fevers, aches and also as health supplements (Ang and Cheang, 1999). Over exploitation of natural stand associated with poor seed set and germination has caused depletion of this plant in nature. In Malaysia for example, *E. longifolia* shrub has now been declared a protected plant, as over-collection in the wild has almost eradicated it from the forests of that country.

Corresponding Author: Dr. Sobri Bin Hussein, Agrotechnology and Bioscience Division,
Malaysian Institute For Nuclear Technology Research,
Bangi, 43000 Kajang, Selangor, Malaysia
Tel: 603-89250510 Fax: 603-89258262

Although *in vitro* propagation is a potential alternative for the production of *E. longifolia* for commercial and conservation purposes, not many tissue culture studies have been conducted on this species. Many researchers focused on the determination of the bioactive components that lead to its medicinal values especially its aphrodisiac properties (Ang *et al*., 2001), phytochemical analysis of its quassinoid and canthinone alkaloids content (Ang *et al*., 2002; Choo and Chan, 2002; Tan *et al*., 2002; Jiwajinda *et al*., 2001; Morita *et al*., 1993; Darise *et al*., 1982) as well as the determination of its cytotoxic, antimalarial (Kuo *et al*., 2003), anti-tumor promoting and anti parasitic properties (Jiwajinda *et al*., 2002). Successful somatic embryogenesis was reported in *E. longifolia* (Sobri *et al*., 2005). However, this tissue culture method is difficult and susceptible to the low regeneration rate as well as increases the possibility of somaclonal variations (Lakshmanan and Taji, 2000). Besides, some treatments in somatic embryogenesis that coinciding with increased yield of somatic embryos caused adverse effects on the embryo quality, thereby impairing germination and *ex vitro* growth of somatic embryo plants (von Arnold *et al*., 2002). In view of this, a study was carried out to establish the effective tissue culture protocols for the production of high quality *E. longifolia* plantlets or seedlings from root and stem explants through direct plant regeneration techniques.

Materials and Methods

Plant Material

The source of *E. longifolia* plant used in this study was obtained from Selangor, Malaysia between June 2003 to April 2004.

Regeneration of Adventitious Shoots

Root and stem explants used in this studies were obtained from the two months old *in vitro* germinated plantlets. In the preliminary experiment, root and stem explants (approximately 2.0 cm in length) were cut at the distance of 2.0 cm from the *in vitro* stem and root, respectively. The explants were implanted either in the basal MS, DKW, WPM or NM supplemented with different cytokinins (BAP, kinetin and zeatin) alone or in combination at the concentration of 1.0 to 10.0 mg L^{-1}. In this study, the treatments were carried out in the transparent glass tubes (8.4x2.4 cm). Sucrose at 3% (w/v) and 2.5 g L^{-1} of gelrite agar were also added into the media. The pH of the media was adjusted to 5.7±0.02 prior to autoclaving. A 10 mL of the medium was placed in the tubes and covered with a layer of heavy-duty aluminum foil. The autoclaved media were left to stand (45°) until the media is fully solidified.

The cultures were maintained at 25±2°C and a 16 h photoperiod with cool white fluorescent light of 150 μmol m^{-2} sec^{-1} (supplied by Philips TLD fluorescent light tubes). The experiments were conducted in ten replicates and repeated thrice. The day of shoot formation from the explants were recorded. Percentage of shoot formation, number of leaves and length of the stem were evaluated after two months of culture.

Effects of the Explant Position on Adventitious Shoots Regeneration

In order to obtain the best regeneration capacity, the root and stem explants with 2.0 cm long each were trimmed to about 2.0, 4.0, 6.0 and 8.0 cm distant from the *in vitro* stem or root, respectively. The explants were transferred into the best medium and hormone concentration as determined in the preliminary studies. The experiments were conducted in ten replicates with three times of repetition.

Rooting

Healthy shoots (2.0±0.2 cm) induced from root and stem explants were transferred into the basal MS medium containing IBA at the range of 0.1 to 1.0 mg L^{-1} for rooting. The effects of these auxins on rootlets induction as well as the length of the rootlets were examined after two months of culture.

Hardening and Acclimatization

The plantlets with well-developed rootlets and shoots were thoroughly washed under tap water for 2 to 3 min to remove traces of agar-gelled medium sticking to them. *In vitro* hardening was done in culture bottles containing water and covered with polypropylene caps. After two weeks, the caps were opened and plantlets were transferred to plastic pots containing mixture of soil, that have the composition of nitrogen, phosphate, potassium at the ratio of 1:3:2. Plants were maintained at the envirounent with 25 to 28°C. The plants were initially covered with glass to maintain high humidity. After three weeks, the glasses were removed. The percentage of plant survival was calculated after two months.

Statistical Analysis

Variance analysis of the data was carried out and means were statistically compared using Tukey's test ($\alpha = 0.05$). Data were analyzed using the SPSS (version 11.5) analysis.

Results and Discussion

Regeneration of Adventitious Shoots from Root Explants

Studies on effects of six basal media (the basal DKW, WPM, NM and MS medium) supplemented with various cytokinins showed that adventitious shoots were only formed from root explants cultured in the basal DKW, WPM and NM supplemented with kinetin (Table 1). The data obtained revealed that, shoot could be induced at the concentrations of 1.0 to 6.0 mg L^{-1} of kinetin with the highest rate (70%) was observed in 1.0 mg L^{-1} of kinetin. The data obtained also revealed that increased concentrations of kinetin brought about a decrease in the regeneration rate. No significant difference in terms of adventitious shoots formation was observed in 2.0, 3.0 and 4.0 mg L^{-1} of kinetin. Apart from that, an earlier shoot formation was shown in 1.0 mg L^{-1} of kinetin (28 days) followed by 2.0 mg L^{-1} of kinetin (29 days) and 3.0 mg L^{-1} of kinetin (29 days). By taking the four parameters; leaflets, rachis, stem and leaflets per rachis into consideration, it was found that among the concentrations tested, 1.0 mg L^{-1} of kinetin managed to produce healthy and balance shoots in terms of number of leaflets (18.2±1.5), rachis (2.5±0.3), number of leaflets per rachis (7.2±0.5) and stem length (2.7±0.5 cm). Similarly, *Prunus avium* L. plantlet derived from media that contained kinetin had better leaf expansion and were more vigorous than those derived from media that contain either BAP or zeatin (Muna *et al*., 1999).

In order to shorten the five weeks culture period required for the induction of adventitious shoots, the root explants were also cultured in the basal DKW medium containing kinetin in combination with either zeatin or BAP at the concentration of 1.0 to 3.0 mg L^{-1}. Results obtained showed that the combination of 1.0 mg L^{-1} kinetin and 1.0 mg L^{-1} zeatin treated explants produced the highest regeneration rate (90%)(Table 2). Root explants treated with the combination of 2.0 mg L^{-1} of kinetin + 2.0 mg L^{-1} of zeatin also managed to regenerate, but at a lower percentage (70%). Apart from that, the results obtained in this study also showed that by using the combinations of kinetin and zeatin in the basal DKW medium, the regeneration period was shorten from five weeks to two weeks. As demonstrated in Table 2, it was found that shoot was formed as early as 15 days of culture in DKW medium supplemented with 1.0 mg L^{-1} of kinetin + 1.0 mg L^{-1} of zeatin (Fig. 1A). However,

Table 1: Effects of different basal media, Juglan Medium (DKW), McCown Woody Plant Medium (WPM) and Nitsch medium (NM) supplied with kinetin at the concentrations of 0.0 to 10.0 mg L^{-1} on the adventitious shoots regeneration from root explants of *E. longifolia*

Treatments							
Media	Concentration of Kinetin (mg L^{-1})	Days shoot start to form*	No of leaf lets*	No. of rachis*	No. of leaflets per rachis*	Stem length (cm)*	Regeneration (%)*
DKW	0.0	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
	1.0	28[b]	18.2[c]	2.5[c]	7.2[cde]	2.7[b]	70[f]
	2.0	29[b]	17.4[c]	2.0[bc]	8.7[e]	2.0[b]	60[f]
	3.0	29[b]	12.2[b]	2.5[c]	4.9[bc]	2.3[b]	60[f]
	4.0	30[bc]	12.5[b]	2.4[c]	5.2[bcd]	2.2[b]	60[f]
	5.0	35[c]	10.2[b]	1.3[b]	7.8[de]	0.6[a]	40[b]
	6.0	43[d]	10.5[b]	3.8[d]	2.8[ab]	0.9[a]	30[b]
WPM	0.0	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
	1.0	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
	2.0	38[b]	30.2[d]	3.1[c]	9.7[bc]	2.4[d]	60[d]
	3.0	46[c]	20.9[e]	1.7[b]	12.3[bc]	2.1[cd]	50[cd]
	4.0	51[c]	19.5[c]	1.5[b]	13.4[c]	1.5[bc]	40[bc]
	5.0	58[d]	10.5[b]	1.2[b]	8.8[b]	0.8[b]	30[b]
	6.0	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
NM	0.0	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
	1.0	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
	2.0	51[b]	8.3[c]	1.1[b]	7.5[b]	0.8[b]	30[b]
	3.0	56[c]	5.4[b]	1.0[b]	5.4[b]	1.3[b]	30[b]
	4.0	60[c]	0.0[a]	0.0[a]	0.0[a]	0.9[b]	30[b]
	5.0	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
	6.0	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]

a: NR indicates that no shoot was observed from the explants after two months of culture

b: No adventitious shoots regeneration was observed in the basal DKW, WPM and NM supplemented with the BAP and zeatin at the concentrations of 1.0 to 10.0 mg L^{-1} as well as concentrations of kinetin higher than 6.0 mg L^{-1}.

c: No direct plant regeneration was observed in the basal MS supplemented with the cytokinins (BAP, zeatin and kinetin) at the concentrations of 1.0 to 10.0 mg L^{-1}

d:* Means followed by the same letter (s) are not significantly different ($p<0.05$) using Tukey's test (N = 3)

Table 2: Effects of the basal DKW medium supplemented with the combination of kinetin and zeatin at the concentrations of 1.0 to 3.0 mg L^{-1} on the adventitious shoots regeneration from root explants of *E. longifolia*

Concentration (mg L^{-1})							
Kinetin	Zeatin	Days shoot start to form	No. of leaflets*	No. of rachis*	No. of leaflets per rachis*	Stem length (cm)*	Regeneration(%)*
0	0	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
1		27[b]	19.2[d]	2.2[b]	8.7[c]	2.0[b]	70[b]
2		27[b]	14.5[c]	2.0[b]	7.3[c]	2.0[b]	60[b]
3		30[c]	9.2[b]	2.0[b]	4.6[b]	2.3[b]	60[b]
0	1	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
1		15[b]	25.1[d]	1.9[b]	13.2[d]	3.1[c]	90[c]
2		17[b]	15.3[c]	1.8[b]	8.5[c]	2.5[c]	40[b]
3		36[c]	7.5[b]	2.0[b]	3.7[b]	1.2[b]	30[b]
0	2	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[b]
1		17[b]	17.3[b]	2.6[c]	6.7[b]	2.5[b]	60[c]
2		21[c]	15.3[b]	1.5[b]	10.2[c]	2.2[b]	70[c]
3		27[d]	16.4[b]	1.3[b]	12.6[d]	0.8[a]	40[b]
0	3	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
1		20[b]	7.2[b]	1.1[b]	6.5[c]	2.7[b]	40[b]
2		29[c]	0.0[a]	2.5[c]	0.0[a]	0.5[a]	30[b]
3		30[c]	12.7[c]	3.2[d]	4.0[b]	0.9[a]	30[b]

a: NR indicates that no shoot was observed from the explants after two months of culture

b: * Means followed by the same letter (s) are not significantly different ($p<0.05$) using Tukey's test (N = 3)

Table 3: Effects of the basal DKW medium supplemented with the combination of kinetin and BAP at the concentrations of 1.0 to 3.0 mg L^{-1} on the adventitious shoots regeneration from root explants of *E. longifolia*

Concentration (mg L^{-1})							
Kinetin	BAP	Days shoot start to form	No. of leaflets*	No. of rachis*	No. of leaflets per rachis*	Stem length (cm)*	Regeneration(%)*
0	0	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
1		28[b]	19.2[c]	2.0[b]	9.6[b]	2.0[b]	70[b]
2		29[b]	13.0[b]	2.0[b]	6.5[b]	2.0[b]	60[b]
3		29[b]	14.0[b]	2.0[b]	7.0[b]	2.3[b]	60[b]
0	1	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
1		29[b]	25.4[c]	2.8[c]	9.1[b]	1.7[c]	60[c]
2		37[c]	8.1[b]	1.1[b]	7.4[b]	0.9[b]	30[b]
3		41[c]	7.5[b]	1.0[b]	7.5[b]	0.4[ab]	20[b]
0	2	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
1		46[d]	7.1[c]	1.3[c]	5.5[b]	1.8[b]	30[ab]
2		35[c]	5.1[b]	1.1[b]	4.6[b]	0.3[a]	20[b]
3		30[b]	7.3[c]	1.0[b]	7.3[b]	1.2[b]	40[c]
0	3	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
1		31[b]	28.3[b]	2.7[b]	12.9[c]	2.9[c]	50[c]
2		39[c]	27.9[b]	3.3[b]	8.5[b]	1.3[b]	40[bc]
3		40[c]	0.0[a]	0.0[a]	0.0[a]	0.7[ab]	30[b]

a: NR indicates that no shoot was observed from the explants after two months of culture

b: * Means followed by the same letter (s) are not significantly different ($p<0.05$) using Tukey's test (N = 3)

Table 4: Adventitious shoots regeneration from different positions of root explants of *E. longifolia*

Root position-distant from stem (cm)	Days shoot start to form*	No. of leaflets*	No. of rachis*	No. of leaflets per rachis*	Stem length (cm)*	Regeneration* (%)
2.0	15±2[b]	24±2[a]	3.0±0.5[a]	8.0±1.0[a]	3.0±1.0[a]	90[a]
4.0	18±3[b]	15±2[b]	2.5±0.5[a]	6.0±0.5[b]	2.0±0.1[b]	40[b]
6.0	25±2[a]	6±1[c]	1.5±0.5[b]	4.0±0.8[c]	1.5±0.2[c]	10[c]
8.0	27±4[a]	2±1[d]	1.4±0.3[b]	1.4±0.5[d]	0.7±0.1[d]	10[c]

a: * Means followed by the same letter (s) are not significantly different ($p<0.05$) using Tukey's test (N =3)

at the higher concentrations of cytokinins such as in 3.0 mg L^{-1} of kinetin + 3.0 mg L^{-1} of zeatin, the root explant required 30 days to form shoot.

As for the combination of kinetin and BAP, the data obtained revealed that the highest regeneration percentage (70%) was obtained in 1.0 mg L^{-1} of kinetin + 3.0 mg L^{-1} of BAP (Table 3). Analysis on the leaflets formation indicated that the maximum value was observed in the combination of 1.0 mg L^{-1} of kinetin + 3.0 mg L^{-1} of BAP with the number of leaflets produced was 28.3±2.2, followed by 27.9±1.6 leaflets in 2.0 mg L^{-1} of kinetin + 3.0 mg L^{-1} BAP, 25.4±1.2 leaflets in 1.0 mg L^{-1} of kinetin + 1.0 mg L^{-1} of BAP and 8.1±1.4 leaflets in 2.0 mg L^{-1} of kinetin + 1.0 mg L^{-1} of BAP. The results presented also showed that the highest number of leaflets per rachis could be obtained in 1.0 mg L^{-1} of kinetin + 3.0 mg L^{-1} of BAP with the production of 12.9±0.4, followed by 9.1±0.5 for 1.0 mg L^{-1} of kinetin + 1.0 mg L^{-1} of BAP and 7.4±0.5 in 2.0 mg L^{-1} of kinetin + 1.0 mg L^{-1} of BAP. The combination of 1.0 mg L^{-1} of kinetin + 3.0 mg L^{-1} of BAP was also found to produce the longest stem (2.9±0.3 cm) within the treatment tested.

Effects of Root Explant Position on Adventitious Shoots Regeneration

The effects of the position of the root explants used on adventitious shoots induction were also investigated in order to resolve the location of regeneration and the minimum explant required for regeneration. Regeneration studies using root explants revealed that root that was 2.0 cm distant from the stem is the most suitable root explants in obtaining adventitious shoots. The data obtained revealed that the maximum frequency in terms shoot formation was significantly observed in 2.0 cm explants with the rate of 90% after two months of culture (Table 4). The regeneration capability was reduced

Fig. 1: *In vitro* propagation of *Eurycoma longifolia* Jack. (A) Adventitious shoots regenerated from root explants after one month of culture in the basal DKW medium supplemented with the combination of 1.0 mg L^{-1} kinetin and 1.0 mg L^{-1} of zeatin. (B) Adventitious shoots regenerated from stem explants after one month of culture in the basal WPM medium supplemented with the combination 2.0 mg L^{-1} BAP and 2.0 mg L^{-1} zeatin. (C) Root induction from adventitious shoots formed. (D) *In vitro* rooted plantlets after two months of transplantation into the soil with the composition of nitrogen, phosphate, potassium at the ratio of 1:3:2

Table 5: Effects of the basal Woody Plant Medium (WPM) supplied with zeatin at the concentrations of 0.0 to 10.0 mg L^{-1} on the adventitious shoots regeneration from stem explants of *E. longifolia*

Concentrations (mg L^{-1})	Days shoot start to form*	No. of leaflets*	No. of rachis*	No. of leaflets per rachis*	Stem length (cm)*	Regeneration* (%)
0.0	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
1.0	34[b]	14.3[c]	2.0[b]	7.2[c]	1.8[b]	30[b]
2.0	31[b]	20.5[d]	3.0[c]	6.8[bc]	1.7[b]	50[c]
3.0	39[c]	10.3[b]	2.0[b]	5.2[b]	1.5[b]	40[bc]

a: NR indicates that no shoot was observed from the explants after two months of culture

b: No direct plant regeneration was observed in the basal WPM supplemented with the BAP and kinetin at the concentrations of 1.0 to 10.0 mg L^{-1} as well as concentrations of zeatin higher than 3.0 mg L^{-1}

c: No direct plant regeneration was observed in the basal DKW, NM and MS medium supplemented with the cytokinins (BAP, zeatin and kinetin) at the concentrations of 1.0 to 10.0 mg L^{-1}

d: * Means followed by the same letter (s) are not significantly different ($p<0.05$) using Tukey's test (N = 3)

to 10% when the root explants were taken from 6.0 and 8.0 cm away from stem. In the meantime, 40% of the roots explant that was 4.0 cm distant from the stem managed to regenerate. These results contradicted with the pervious studies on *Alstroemeria* in which the younger explant, which originally located closer to the stem apex, showed a higher percentage of shoot regeneration than the older explants (Lin *et al*., 1998). The higher percentage of shoot formation in older root explant compared to the younger explant could be a result of the higher endogenous cytokinin accumulation in the older explant. Apart from the successful high regeneration percentage, an earlier shoot formation (15 days) was also shown in root explant that was 2.0 cm away from the stem. This was then followed by root explant that was 4.0, 6.0 and 8.0 cm distant from the stem, which demonstrated sign of shoot formation after 18, 25 and 27 days, respectively.

Regeneration of Adventitious Shoots from Stem Explants

Studies on stem explants revealed that the basal WPM with 1.0 to 3.0 mg L^{-1} of zeatin were the only treatments that managed to produce adventitious shoots from stem explant after two months of culture. Among the different concentrations of cytokinins examined, the maximum regeneration percentage (50%) was observed in culture medium supplemented with 2.0 mg L^{-1} of zeatin followed by 3.0 mg L^{-1} of zeatin (40%) and 1.0 mg L^{-1} of zeatin (30%). Meanwhile, analysis on the leaflets formation disclosed that maximum number of leaflets was noticed in 2.0 mg L^{-1} of zeatin with the number of leaflets produced was 20.5±2.1. Similarly, 2.0 mg L^{-1} of kinetin also produced the shoots with the longest stem, 1.7±0.1 cm (Table 5).

In order to increase the percentage of shoot formation, zeatin was combined with BAP or kinetin at the concentrations of 1.0 to 3.0 mg L^{-1}. The study revealed that for the direct regeneration of *E. longifolia* from stem explants, combination of two cytokinins is more ideal compared to single cytokinin test that produced lower regeneration percentage. The percentage of adventitious shoots formed in the combination of 2.0 mg L^{-1} of zeatin + 2.0 mg L^{-1} of BAP and 1.0 mg L^{-1} of zeatin + 1.0 mg L^{-1} of BAP was 70% while only 30% was achieved in the treatments using 3.0 mg L^{-1} of zeatin + 3.0 mg L^{-1} of BAP and 1.0 mg L^{-1} of zeatin + 3.0 mg L^{-1} of BAP (Table 6). Stem explant treated with the combination of 1.0 mg L^{-1} of zeatin + 1.0 mg L^{-1} of BAP produced shoot within 14 days of culture. Meanwhile, Table 7 represents the influences of kinetin and zeatin on shoot induction from stem explants cultured in the basal WPM. Even though the regeneration capability in this combination was not as high as that achieved in the previous treatment, one of the combinations, 2.0 mg L^{-1} of zeatin + 2.0 mg L^{-1} of kinetin managed to produce shoot within 20 days. This combination also produced maximum number of leaflets (28.7±1.5) rachis (3.1±0.2) (Fig. 1B).

Table 6: Effects of the basal WPM supplemented with the combination of zeatin and BAP at the concentrations of 1.0 to 3.0 mg L^{-1} on the adventitious shoots regeneration from stem explants of *E. longifolia*

Concentration (mg L^{-1})		Days shoot start to form*	No. of leaflets*	No. of rachis*	No. of leaflets per rachis*	Stem length (cm)*	Regeneration (%)*
BAP	Zeatin						
0	0	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
1		NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
2		NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
3		NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
0	1	32[b]	15.5[b]	2.0[a]	7.8[b]	1.7[ab]	50[ab]
1		14[a]	30.3[c]	2.6[ab]	11.7[c]	2.6[b]	70[b]
2		17[a]	13.6[b]	2.3[ab]	5.9[b]	2.5[b]	60[ab]
3		41[c]	0.0[a]	3.2[b]	0.0[a]	0.8[a]	30[a]
0	2	31[c]	19.3[b]	2.0[a]	9.7[b]	1.7[a]	30[a]
1		17[ab]	20.4[b]	2.5[ab]	11.4[c]	1.6[a]	50[ab]
2		16[a]	38.9[c]	3.5[b]	11.1[c]	4.3[b]	70[b]
3		20[b]	12.2[a]	1.9[a]	6.4[a]	0.9[a]	50[ab]
0	3	38[c]	11.2[c]	2.0[b]	5.6[b]	1.6[b]	30[a]
1		24[a]	11.3[c]	1.8[b]	6.3[b]	1.3[ab]	40[a]
2		31[b]	7.3[b]	1.1[a]	6.6[b]	0.5[a]	40[a]
3		40[c]	0.0[a]	2.5[c]	0.0[a]	0.7[ab]	30[a]

a: NR indicates that no shoot was observed from the explants after two months of culture
b: * Means followed by the same letter (s) are not significantly different ($p<0.05$) using Tukey's test (N = 3)

Table 7: Effects of the basal WPM supplemented with the combination of zeatin and kinetin at the concentrations of 1.0 to 3.0 mg L^{-1} on the adventitious shoots regeneration from stem explants of *E. longifolia*.

Concentration (mg L^{-1})		Days shoot start to form*	No. of leaflets*	No. of rachis*	No. of leaflets per rachis*	Stem length (cm)*	Regeneration (%)*
Kinetin	Zeatin						
0	0	NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
1		NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
2		NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
3		NR[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0[a]
0	1	33[b]	13.8[b]	2.0[ab]	6.9[a]	1.5[a]	40[a]
1		35[b]	9.4[a]	1.2[a]	7.8[a]	1.9[ab]	60[a]
2		28[a]	17.6[c]	1.5[ab]	11.7[b]	3.2[c]	50[a]
3		36[b]	15.7[bc]	2.2[b]	7.1[a]	2.8[bc]	40[a]
0	2	29[b]	18.7[c]	2.5[ab]	7.5[ab]	7.5[c]	50[a]
1		25[b]	15.2[b]	1.9[a]	8.0[bc]	0.9[a]	50[a]
2		20[a]	28.7[d]	3.1[b]	9.3[c]	3.8[b]	70[b]
3		38[c]	10.5[a]	1.7[a]	6.2[a]	2.7[b]	40[a]
0	3	38[a]	9.5[b]	2.0[c]	4.8[b]	1.4[b]	30[a]
1		51[b]	0.0[a]	1.1[b]	0.0[a]	0.5[a]	20[a]
2		37[a]	8.2[b]	1.1[b]	7.5[c]	2.2[c]	40[a]
3		36[a]	0.0[a]	0.0[a]	0.0[a]	1.8[bc]	30[a]

b: NR indicates that no shoot was observed from the explants after two months of culture
c: * Means followed by the same letter (s) are not significantly different ($p<0.05$) using Tukey's test (N = 3)

Effects of the Position of the Stem Explants on Adventitious Shoots Regeneration

The results obtained in this study from Table 8 showed that the position of the explants has a great influence on the regeneration ability from the stem explant of *E. longifolia*. It was found that 90% of the regenerated plantlet was derived from the stem explant which was 2.0 cm distant from root. The stem explant that was 4.0, 6.0 and 8.0 cm away from the root produced only 70, 30 and 10% of regeneration, respectively. This could be resulted by a kind of age-related response because the position was related to the physiological age of the explants (Lin *et al*., 1998). The regeneration potential of the explant had been reported varied with the developmental stage of the stem explant

Table 8: Adventitious shoots regeneration from different positions of stem explants of *E. longifolia*

Stem position -distant from root (cm)	Days shoot start to form*	No. of leaflets*	No. of rachis*	No. of leaflets per rachis*	Stem length (cm)*	Regeneration* (%)
2.0	16±2^{c}	35±3^{a}	3.0±0.4^{a}	11.6±2.0^{a}	4.0±0.5^{a}	90^{d}
4.0	20±2^{c}	20±3^{b}	3.0±0.5^{a}	6.6±1.5^{b}	2.5±0.4^{b}	70^{c}
6.0	28±2^{b}	6±1^{c}	1.5±0.2^{b}	4.0±0.7^{c}	1.5±0.3^{c}	30^{b}
8.0	35±3^{a}	0±0^{d}	0.0±0.0^{c}	0.0±0.0^{d}	1.2±0.2^{c}	10^{a}

a: * Means followed by the same letter (s) are not significantly different ($p<0.05$) using Tukey's test (N = 3)

Table 9: Effects of the basal MS medium supplemented with different concentrations of IBA on the root induction in the adventitious shoots regenerated from root and stem explants of *E. longifolia*

	Adventitious shoots regenerated from root explants			Adventitious shoots regenerated from stem explants		
Concentration of IBA (mg L^{-1})	Days of rootlets formation*	Percentage of rootlets formation(%)*	Rootlets length (cm)*	Days of rootlets formation*	Rootlets formation(%)*	Rootlets length (cm)*
0.0	NR^{a}	0^{a}	0.0^{a}	NR^{a}	0^{a}	0.0^{a}
0.1	NR^{a}	0^{a}	0.0^{a}	NR^{a}	0^{a}	0.0^{a}
0.2	NR^{a}	0^{a}	0.0^{a}	NR^{a}	0^{a}	0.0^{a}
0.3	NR^{a}	0^{a}	0.0^{a}	NR^{a}	0^{a}	0.0^{a}
0.4	24±3^{c}	40^{b}	5.0^{b}	NR^{a}	0^{a}	0.0^{a}
0.5	19±1^{b}	70^{c}	7.0^{c}	18^{b}	60^{b}	6.0^{b}
0.6	NR^{a}	0^{a}	0.0^{a}	NR^{a}	0^{a}	0.0^{a}

a: NR indicates that no root was induced from the shoots after two months of culture

b: * Means followed by the same letter (s) are not significantly different ($p<0.05$) using Tukey's test (N = 3)

(Chen *et al*., 2001). Nagori and Purohit (2004) also stated that these different responses may be due to variation in the endogenous auxin concentration in different regions of stem. Two months of observation also discovered that the highest number of leaflets (35.0±4.0) was yielded in stem explant that was 2.0 cm away from the root followed by 20.0±3.0 and 6.0±1.0 leaflets that were detected in the stem explant 4.0 and 6.0 cm away from the root.

There was no sign of leaflets formation in treatment using stem explant that was 8.0 cm distant from the root. Meanwhile, analysis on the rachis formation showed that the maximum number of rachis formed was also observed in the treatment using stem explant 2.0 cm away from the root. The results obtained also further suggested that the stem explant that was 2.0 cm away from the root possessed higher regenerative potential than the other stem regions tested in *E. longifolia*. Thus, direct plant regeneration from stem explant could be another alternative in obtaining *in vitro* plantlets of *E. longifolia* as plant regenerated from stem explants of woody species were morphologically normal (Palacious *et al*., 2002).

Rooting of Adventitious Shoots

For the adventitious shoots induced from root explant, the highest percentage of rootlets (70%) was observed in the treatment using 0.5 mg L^{-1} of IBA (Table 9). Apart from this, medium supplemented with 0.4 mg L^{-1} of IBA gave the second best response by exhibiting 40% of rootlets formation. Application of 0.6 mg L^{-1} of IBA depressed rootlets in these plantlets. Meanwhile, the data obtained also revealed that the formation of rootlets start to occurred after 19.0 days in 0.5 mg L^{-1} of IBA and 24 days in 0.4 mg L^{-1} of IBA. After two months, the rootlets could be elongated until 7.0±1.0 cm in length in the treatments using 0.5 mg L^{-1} of IBA (Fig. 1C). In the root meristem, auxin is implicated in regulating the pattern of cell division and differentiation (Friml, 2003). According to Puente and Martin (1997), if the shoots are competent to root, rooting rate could be increased easily. It has been reported that shoot characteristics such as size and shoot culture origins, lack of attaiument of a stabilized growth phase or apparent rejuvenation can also lead to a variable rooting response

(Marks and Simpson, 2000). Meanwhile, the analyses on the rootlets formation from stem explants derived shoots showed that only one treatment (0.5 mg L^{-1} IBA) managed to produce rootlets. A 60% of rootlets formation was observed in 0.5 mg L^{-1} of IBA and the length of rootlets produced was 6.0±1.5 cm after two months of culture.

Hardening and Acclimatization

The *in vitro* rooted plantlets were successfully acclimatized with 70% survival rate. Acclimatized plantlets were healthy and well developed when transferred to the soil. The plants grew as high as 9.0±1.5 cm and no morphological difference from the parent plants was shown after two months of transplantation (Fig. 1D).

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