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IN VITRO PROPAGATION OF *EUCALYPTUS CITRIODORA* HOOK. UNDER CONTROL OF SOME PHYTOHORMONES AND SUCROSE

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Abstract

In vitro propagation of *Eucalyptus citriodora* Hook. was carried out in the tissue culture laboratory of Vegetable and Fruit Research and Development Centre (VFRDC), Yemon, Hlegu Township, Yangon Region. In multiplication, the shoot apex and nodal segment of *Eucalyptus citriodora* Hook. were cultured in modified (Murashige and Skoog, 1962) MS medium containing various combinations of naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP). Among them, EuS_6 (NAA 0.75 + BAP 3.0 mg L^{-1}) showed the longest shoot length (2.25 cm) but maximum number of shoot (8.97) and maximum number of leaves (16.74) were obtained from EuS_5 (NAA 0.15 + BAP 3.0 mg L^{-1}). It is therefore EuS_5 (NAA 0.15 + BAP 3.0 mg L^{-1}) was regarded as the suitable treatment for shoot multiplication. *In vitro* shoot elongation, micro shoots (1 - 1.5 cm) were sub-cultured on MS medium added on BAP (0.1 mg L^{-1}) and the combination of BAP (0.1 mg L^{-1}) and (Gibberellic acid) GA_3 (0.1, 0.5 and 1.0 mg L^{-1}). Among them, BAP (0.1 mg L^{-1}) gave the longer shoot length than the combination of BAP and GA_3. Therefore, individual BAP treatment was assumed as a suitable treatment for *Eucalyptus* shoot elongation. In rooting experiment, half-strength MS medium supplemented with various concentrations of (indole-3-butyric acid) IBA (0, 0.5, 1.0 and 1.5 mg L^{-1}) and also supplemented with sucrose (0, 2, 4, 6 and 8 %) on elongated shoots (1.5 - 2.5 cm). The maximum number of root (1.7) was obtained from 2% sucrose.

Key words; *Eucalyptus citriodora* , Multiplication, NAA, BAP, GA_3

Introduction

Genus *Eucalyptus* belongs to the family Myrtaceae, which are mostly found in tropical regions. The native of this genus is the Australia (Logeswari

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and Kanagavalli, 2014). *Eucalyptus citriodora* Hook. is assumed as essential oil producing cultivar and also called Hnet-chauk in Myanmar (Krass et al., 2003). The oil of *Eucalyptus citriodora* Hook. extracted from leaves, has various medicinal benefits, as a stimulant, aphrodisiac, antispasmodic and antiseptic, used in the treatment of septic fevers, asthma, ulcers and spongy and bleeding gums. It is also reported as good for digestion as a nerve sedative and anti-malarian (Santos, 1997).

Eucalyptus is traditionally propagated through the seedling route. In such genetically diverse stocks, trees with the better qualities, such as a straight clear bole, disease and pest resistance, drought tolerance, fast growth, etc., occur at very low frequencies. Due to extensive cross pollination, seed progeny of superior trees do not maintain their superior characteristics. Various methods of vegetative propagation have been attempted, but most have resulted in failure, specifically when applied to explants from adult eucalypt tissues (Durand-Cresswell *et al*., 1982 ***In:*** Sharma and Ramamurthy, 2000). Although clonal propagation of *Eucalyptus* hasbeen achieved using many *in vitro* techniques, the establishment of cultures directly from mature trees or tissues still poses many problems, like difficulty in disinfestation, production of phenolics, rooting rates, etc. (Jones and Staden, 1997 ***In:*** Sharma and Ramamurthy, 2000). Eucalypt plantations raised using micropropagation techniques have been reported to yield higher biomass than seedling-derived plantations (Khuspe *et al.,* 1987 ***In:*** Sharma and Ramamurthy, 2000) if preceded by a selection of superior trees (Sharma and Ramamurthy, 2000).

Micropropagation through axillary proliferation and adventitious shoot proliferation on nodal explants has been successful (Cid *et al.,* 1999; Glocke *et al.,* 2006 ***In***: Cintra, 2007). The most common culture medium is MS medium (Murashige and Skoog, 1962) with a low auxin and cytokinin ratio is most commonly used for shoot multiplication (Watt *et al.,* 2003 ***In***: Cintra, 2007). To stimulate shoot elongation, gibberellic acid was added to some media (Cid *et al.,* 1999; Glocke *et al.,* 2006 ***In***: Cintra, 2007).

Several reports used different cytokinin and gibberellins combination to induce shoot elongation, mainly for genetic materials of difficult *in vitro* propagation (Brondani *et al.,* 2011).

The induction of adventitious roots is difficult in some *Eucalyptus* species
(*E. marginata* and *E. nitens*) but relatively easy in others (*E. camaldulensis*). *Eucalyptus globulus* is moderately easy to multiply, but difficult to root *in vitro* even when explants are taken from seedlings (Bennett *et al.,* 1994). Blomstedt *et al.* (1991) found that the frequency of rooting *E. regnans* F. Muell. *In vitro* was higher and callusing was prevented when a high IBA pulse (98 mM for 7 days in the dark) was applied instead of continual maintenance on 5 mM IBA. Le Roux and van Staden (1991) employed 9.8 mM IBA throughout rooting of a clone of *E. grandis* x *E. macarthurii*, plants and roots developed from callus at basal ends of shoots but these roots did not survive the hardening-off period. Muralidharan and Mascarenhas (1987) reported somatic embryogenesis in *Eucalyptus citriodora* on semisolid B5 medium supplemented with increased sucrose concentration (5%).

Regarding to the above facts, the germinated shoots are cultured on modified MS medium, the aim and objectives of NAA and BAP on formation of callus, to investigate the growth of nodal segments of *Eucalyptus citriodora* Hook. on different concentrations and combination of NAA and BAP, to study the shoot multiplication using by the plant growth regulators, and to study the effect of BAP and GA_3 on shoot elongation and to induced roots in the elongated shoots.

Materials and Methods

Study 1. Shoot multiplication of *Eucalyptus citriodora* Hook. using the combination of

NAA + BAP

The shoot multiplications of the germinated seedlings were conducted in the tissue culture laboratory of Vegetable and Fruit Research and Development Centre (VFRDC), Yemon, Hlegu Township, Yangon Region. The experiment was conducted in 2015.

Inoculation of shoot apex and nodal segment for shoot multiplication

Thirty days old shoot apex about 1 cm size and nodal segment of *in vitro* raised seedlings from germination treatments were excised in the culture chamber. These segments were cultured in modified MS medium containing various combinations of NAA and BAP, sucrose 30 g L^{-1} and agar 5 g L^{-1} at pH 5.8. Thirty mililiter medium was poured in a culture bottle. These cultures were maintained under 1000 - 1200 lux white fluorescent light for 16 hours light period and 8 hours dark period, temperature at 25 ± 2˚C and relative humidity 30 - 50%. A regular sub-culturing was carried out every 30 days to MS fresh medium. The treatments were MS basal medium supplemented with (NAA 0.15 + BAP 2.25) mg L^{-1}, (NAA 0.75 + BAP 2.25) mg L^{-1}, (NAA 1.50 + BAP 2.25) mg L^{-1}, (NAA 2.25 + BAP 2.25) mg L^{-1}, (NAA 0.15 + BAP 3.0) mg L^{-1}, (NAA 0.75 + BAP 3.0) mg L^{-1} and Control. Each treatment had 10 replications.

Data collection and statistical analysis

The number of shoot per cultured bottle, average shoots length, number of leaves were recorded. The collected data were analyzed using IRRISTAT software version 4.0 developed by International Rice Research Institute (IRRI), Los Baños, the Philippines.

Study 2. Shoots elongation of *Eucalyptus citriodora* Hook. in MS medium supplemented with combination of BAP + GA_3

The experiment was conducted in the laboratory of Vegetable and Fruit Research and Development Centre (VFRDC), Yemon, Hlegu Township, Yangon Region.

Inoculation of shoots for elongation

Shoot explants were obtained from the multiplicated shoots of the previous experiment. These shoots (1 - 1.5 cm size) were cultured on MS basal medium supplemented with 0.1 mg L^{-1} each of BAP and GA_3 for shoot elongation. In this experiment, 0.1 mg L^{-1} of BAP was combined with three concentrations of GA_3 (0.1, 0.5 and 1.0 mg L^{-1}), each had ten replicates were set up in completely randomized design (CRD).

Data collection and statistical analysis

The following characters were measured: number of shoots per cultured bottle, average shoots length, number of leaves, leaf width and leaf length. The collected data were analyzed using IRRISTAT software version 4.0 developed by International Rice Research Institute (IRRI), Los Baños, the Philippines.

Study 3. Rooting of multiplicated shoots of *Eucalyptus citriodora* Hook. using IBA and sucrose

The experiment was conducted in the laboratory of Vegetable and Fruit Research and Development Centre (VFRDC), Yemon, Hlegu Township, Yangon Region in 2015.

Rooting of multiplicated shoots by IBA treatments

In rooting, the explants were obtained from the elongated shoots of the previous experiment. The size of shoots 1.5 - 2.5 cm were cultured on half strength MS medium supplemented with different concentrations of IBA for rooting. In IBA treatment, four concentrations of IBA and each concentration with ten replications were set up in completely randomized design (CRD). However, Thirty seven (37) days after cultured, the contamination was observed. Therefore the next cultured of rooting had to establish in half strength MS medium supplemented with the same concentrations of IBA (0, 0.5, 1.0 and 1.5 mg L^{-1}). Each treatment had 10 replications.

Rooting of multiplicated shoots by sucrose treatments

In this experiment, 1.5 - 2.5 cm length shoots from the previous experiment were transferred to half strength MS medium supplemented with various concentration of sucrose (0, 2, 4, 6 and 8%) for rooting (followed by Muralidharan and Mascarenhas, 1987). Five concentrations of sucrose with ten replications each were set up in completely randomized design (CRD).

Culture medium preparation

The elongated shoots were cultured on half-strength MS medium supplemented with various concentrations and combination of IBA as well as different concentrations of sucrose. The pH was adjusted 5.6 ± 0.2. Thirty milliliter medium was poured in a culture bottle. These cultures were maintained for 16 hours light period and 8 hours dark period using 1000 - 1200 lux from 4 feet white fluorescent tubes. The cultures were also maintained at the temperature of 25 ± 2ºC and relative humidity of 30 - 50%.

Data collection and statistical analysis

The following parameters were measured: number of shoot per cultured bottle, average shoots length, number of roots and average roots length. The collected data were analyzed using IRRISTAT software version 4.0 developed by International Rice Research Institute (IRRI), Los Baños, the Philippines.

Results

Study 1. Shoot multiplication of *Eucalyptus citriodora* Hook. using the combination of NAA + BAP

Shoot length

The developed shoot length was observed 14 days after culture the segments. However, the initial measurement of shoots did not carry out because of the minute size of developed segments. It is therefore the data collection was started 30 days after culture and it was done in every 3 weeks

intervals. The longest shoot length (2.25 cm) was obtained from EuS_6 (NAA 0.75 + BAP 3.0 mg L^{-1}) followed by 2.00 cm from EuS_3 (NAA 1.50 + BAP 2.25 mg L^{-1}). The shortest shoot length (1.38 cm) was observed from EuS_1 (NAA 0.15 + BAP 2.25 mg L^{-1}). EuS_7 (Control) had 2.01 cm which was lower than EuS_6 but higher than the other treatments. By statistics, the treatment means were not significantly different from each other (Table 1).

Table 1. Effect of NAA and BAP on shoot length of *Eucalyptus citriodora* Hook.

Treatment	Shoots length (cm)					
	30 DAI	51 DAI	72 DAI	93 DAI	114 DAI	Mean
EuS_1 (NAA 0.15 + BAP 2.25 mg L^{-1})	0.98	1.33	1.35	1.58	1.68	1.38
EuS_2 (NAA 0.75 + BAP 2.25 mg L^{-1})	0.98	1.42	1.67	2.07	2.32	1.69
EuS_3 (NAA 1.50 + BAP 2.25 mg L^{-1})	1.01	1.35	1.78	2.49	3.35	2.00
EuS_4 (NAA 2.25 + BAP 2.25 mg L^{-1})	0.98	1.29	1.17	1.73	2.03	1.44
EuS_5 (NAA 0.15 + BAP 3.0 mg L^{-1})	0.87	1.09	1.72	2.52	2.77	1.79
EuS_6 (NAA 0.75 + BAP 3.0 mg L^{-1})	1.33	1.42	1.84	2.44	4.2	2.25
EuS_7 (Control)	1.07	1.44	1.59	2.29	3.64	2.01
F-test	ns	ns	ns	ns	*	-
5 % LSD	0.40	0.49	0.64	1.19	1.58	-
cv %	22.10	20.70	22.50	31.20	31.10	-

DAI=Days after inoculation EuS=Shoot of eucalypt ns=non significan *=significant

Number of shoots

The maximum number of shoots (8.97) was observed from EuS_5 (NAA 0.15 + BAP 3.0 mg L^{-1}) followed by 8.03 from EuS_6 (NAA 0.75 + BAP 3.0 mg L^{-1}). The minimum number of shoot (3.19) was obtained from EuS_7

(Control). By statistics, the treatment means were highly significant from each other (Table 2).

Table 2. Effect of NAA and BAP on number of shoot of *Eucalyptus citriodora* Hook.

Treatment	Number of shoots					
	30 DAI	51 DAI	72 DAI	93 DAI	114 DAI	Mean
EuS_1 (NAA 0.15 + BAP 2.25 mg L^{-1})	4.00	5.33	6.33	7.67	8.83	6.43
EuS_2 (NAA 0.75 + BAP 2.25 mg L^{-1})	5.00	6.17	7.67	8.17	10.03	7.41
EuS_3 (NAA 1.50 + BAP 2.25 mg L^{-1})	3.83	5.50	7.50	9.00	11.90	7.55
EuS_4 (NAA 2.25 + BAP 2.25 mg L^{-1})	3.00	4.50	6.67	8.50	10.50	6.63
EuS_5 (NAA 0.15 + BAP 3.0 mg L^{-1})	6.50	6.77	8.43	10.30	12.87	8.97
EuS_6 (NAA 0.75 + BAP 3.0 mg L^{-1})	4.00	5.83	8.30	9.90	12.13	8.03
EuS_7 (Control)	1.00	2.13	2.93	3.87	6.00	3.19
F-test	**	**	**	**	**	-
5 % LSD	1.34	1.85	2.15	2.33	2.88	-
cv %	44.00	30.50	30.80	29.00	26.50	-

DAI = Days after inoculation EuS = Shoot of Eucalypt ** = highly significant

Number of leaves

The highest number of leaves (16.74) was obtained from EuS_5 (NAA 0.15 + BAP 3.0 mg L^{-1}) followed by 15.89 from EuS_6 (NAA 0.75 + BAP 3.0 mg L^{-1}). The lowest number of leaves (8.97) was observed from EuS_7 (Control). By statistics, the treatment means were not significantly different from each other (Table 3).

Table 3. Effect of NAA and BAP on number of leaves of *Eucalyptus citriodora* Hook.

Treatment	Number of leaves					
	30 DAI	51 DAI	72 DAI	93 DAI	114 DAI	Mean
EuS_1 (NAA 0.15 + BAP 2.25 mg L^{-1})	4.67	6.37	9.33	12.90	14.53	9.56
EuS_2 (NAA 0.75 + BAP 2.25 mg L^{-1})	7.67	11.33	13.17	16.33	20.00	13.70
EuS_3 (NAA 1.50 + BAP 2.25 mg L^{-1})	8.00	12.67	15.10	18.23	19.30	14.66
EuS_4 (NAA 2.25 + BAP 2.25 mg L^{-1})	7.00	9.00	12.83	16.73	20.00	13.11
EuS_5 (NAA 0.15 + BAP 3.0 mg L^{-1})	8.00	11.83	15.33	19.83	28.70	16.74
EuS_6 (NAA 0.75 + BAP 3.0 mg L^{-1})	7.00	11.30	15.53	19.77	25.87	15.89
EuS_7 (Control)	6.00	6.83	8.67	10.33	13.00	8.97
F-test	ns	ns	*	ns	**	-
5 % LSD	4.29	4.82	4.51	6.85	7.15	-
cv %	35.00	27.30	19.70	23.60	19.90	-

DAI = Days after inoculation EuS = Shoot of Eucalypt ns = non significant * = significant
** = highly significant

Study 2. Shoots elongation of *Eucalyptus citriodora* Hook. in MS medium supplemented with combination of BAP + GA_3

Shoot length

Shoot development was observed 14 days after culture. It is therefore the data collection was started 14 days after culture. Fifty-six days after inoculation, the longest shoot length (3.01 cm) was observed from EuE_1 (BAP 0.1 mg L^{-1}) followed by 2.90 cm was obtained from EuE_3 (BAP 0.1 + GA_3 0.5 mg L^{-1}). The shortest shoot length (1.38 cm) was produced from EuE_5 (Control). By statistics, the treatment means were highly significant from each other (Table 4).

Table 4. Effect of BAP and GA_3 on shoot length of *Eucalyptus citriodora* Hook.

Treatment	Shoot length (cm)				
	14 DAI	28 DAI	42 DAI	56 DAI	Mean
EuE_1 (BAP 0.1 mg L^{-1})	1.86	2.34	3.74	4.11	3.01
EuE_2 (BAP 0.1 + GA_3 0.1 mg L^{-1})	1.81	1.99	2.75	3.23	2.45
EuE_3 (BAP 0.1 + GA_3 0.5 mg L^{-1})	1.76	2.24	3.53	4.06	2.90
EuE_4 (BAP 0.1 + GA_3 1.0 mg L^{-1})	1.34	1.96	2.56	2.81	2.17
EuE_5 (Control)	1.11	1.26	1.41	1.74	1.38
F-test	**	**	**	**	-
5%LSD	0.37	0.44	0.42	0.53	-
cv %	21	20.6	13.6	15.1	-

DAI = Days after inoculation, EuE = Elongation of Eucalypt, ** = highly significant

Number of shoots

The maximum number of shoots (26.81) was produced from EuE_1 (BAP 0.1 mg L^{-1}) followed by 24.61 from EuE_2 (BAP 0.1 + GA_3 0.1 mg L^{-1}). The minimum number of shoots (7.32) was obtained from EuE_5 (Control). By statistics, the treatment means were highly significant from each other (Table 5).

Table 5. Effect of BAP and GA_3 on number of shoot of *Eucalyptus citriodora* Hook.

Treatment	Number of shoots				
	14 DAI	28 DAI	42 DAI	56 DAI	Mean
EuE_1 (BAP 0.1 mg L^{-1})	9.51	19.86	33.29	44.57	26.81
EuE_2 (BAP 0.1 + GA_3 0.1 mg L^{-1})	11.29	19.14	29.29	38.71	24.61
EuE_3 (BAP 0.1 + GA_3 0.5 mg L^{-1})	8.14	16.43	27.29	37.43	22.32
EuE_4 (BAP 0.1 + GA_3 1.0 mg L^{-1})	9.14	15.14	24.43	32.29	20.25
EuE_5 (Control)	4.71	6.29	8.00	10.29	7.32
F-test	*	**	**	**	-
5%LSD	4.02	3.63	4.42	4.21	-
cv %	42.60	21.40	16.40	11.70	-

DAI = Days after inoculation, EuE = Elongation of Eucalypt, * = significant, ** = highly significant

Number of leaves

The maximum number of leaves (40.43) was observed from EuE_1 (BAP 0.1 mg L^{-1}) followed by 37.36 from EuE_3 (BAP 0.1 + GA_3 0.5 mg L^{-1}). The minimum number of leaves (15.22) was obtained from EuE_5 (Control). By statistics, the treatment means were highly significant from each other (Table 6).

Table 6. Effect of BAP and GA_3 on number of leaves of *Eucalyptus citriodora* Hook.

Treatment	Number of leaves				
	14 DAI	28 DAI	42 DAI	56 DAI	Mean
EuE_1 (BAP 0.1 mg L^{-1})	15.57	28.14	48.29	69.71	40.43
EuE_2 (BAP 0.1 + GA_3 0.1 mg L^{-1})	11.86	23.00	44.00	67.71	36.64
EuE_3 (BAP 0.1 + GA_3 0.5 mg L^{-1})	11.71	23.29	44.43	70.00	37.36
EuE_4 (BAP 0.1 + GA_3 1.0 mg L^{-1})	12.00	21.00	37.29	55.71	31.50
EuE_5 (Control)	9.29	13.00	17.29	21.29	15.22
F-test	ns	**	**	**	-
5%LSD	4.86	5.82	8.68	8.04	-
cv %	36.4	24.3	20.6	12.8	-

DAI = Days after inoculation, EuE = Elongation of Eucalypt, ns = non-significant,

** = highly significant

Leaf width

The best results of leaf width (0.33 cm) was obtained from EuE_1 (BAP 0.1 mg L^{-1}) followed by 0.32 cm from EuE_3 (BAP 0.1 + GA_3 0.5 mg L^{-1}). The lowest leaf width (0.19 cm) was produced from EuE_5 (Control). By statistics, the treatment means were highly significant from each other. It was found that BAP alone support leaf expension but the addition of GA_3 inhibit leaf width expension (Table 7).

Table 7. Effect of BAP and GA_3 on leaf width of *Eucalyptus citriodora* Hook.

Treatment	Leaf width (cm)				
	14 DAI	28 DAI	42 DAI	56 DAI	Mean
EuE_1 (BAP 0.1 mg L^{-1})	0.26	0.29	0.34	0.43	0.33
EuE_2 (BAP 0.1 + GA_3 0.1 mg L^{-1})	0.21	0.21	0.26	0.29	0.24
EuE_3 (BAP 0.1 + GA_3 0.5 mg L^{-1})	0.24	0.26	0.33	0.46	0.32
EuE_4 (BAP 0.1 + GA_3 1.0 mg L^{-1})	0.13	0.20	0.21	0.24	0.20
EuE_5 (Control)	0.13	0.14	0.23	0.26	0.19
F-test	**	**	**	**	-
5%LSD	0.43	0.51	0.62	0.47	-
cv %	20.1	21	20.6	12.7	-

DAI = Days after inoculation EuE = Elongation of Eucalypt ** = highly significant

Leaf length

The longest leaf length (0.45 cm) was observed from EuE_3 (BAP 0.1 + GA_3 0.5 mg L^{-1}) followed by 0.42 cm from EuE_1 (BAP 0.1 mg L^{-1}). The shortest leaf length (0.32 cm) was achieved from EuE_4 (BAP 0.1 + GA_3 1.0 mg L^{-1}). The shortest leaf length (0.32) was also obtained from EuE_5 (Control). By statistics, the treatment means were highly significant from each other (Table 8).

Table 8. Effect of BAP and GA_3 on leaf length of *Eucalyptus citriodora* Hook.

Treatment	Leaf length (cm)				
	14 DAI	28 DAI	42 DAI	56 DAI	Mean
EuE_1 (BAP 0.1 mg L^{-1})	0.36	0.40	0.46	0.47	0.42
EuE_2 (BAP 0.1 + GA_3 0.1 mg L^{-1})	0.31	0.37	0.44	0.44	0.39
EuE_3 (BAP 0.1 + GA_3 0.5 mg L^{-1})	0.37	0.39	0.47	0.56	0.45
EuE_4 (BAP 0.1 + GA_3 1.0 mg L^{-1})	0.24	0.29	0.36	0.37	0.32
EuE_5 (Control)	0.24	0.27	0.34	0.41	0.32
F-test	**	**	*	**	-
5%LSD	0.74	0.77	0.91	0.79	-
cv %	21.9	20.3	20	15.9	-

DAI = Days after inoculation, EuE = Elongation of Eucalypt, * = significant, ** = highly significant

Study 3. Rooting of multiplicated shoots of *Eucalyptus citriodora* Hook. using IBA and sucrose

Rooting of multiplicated shoots by IBA treatments

Number of shoot

Thirty days after cultured number of shoot, shoot length, number of root and root length were recorded. The maximum number of shoot (17.40) was obtained from EuR_2 (IBA 0.5 mgL^{-1}) followed by 15.40 from EuR_4 (IBA 1.5 mg L^{-1}). The minimum number of shoot (14.20) was produced from EuR_3 (IBA 1.0 mg L^{-1}). The maximum number of shoot (21.70) was also obtained from EuR_1 (Control).

Number of root

The maximum number of root (12.20) was obtained from EuR_3 (IBA 1.0 mg L^{-1}). The second highest number of roots (10.90) was produced from EuR_4 (IBA 1.5 mg L^{-1}) followed by 7.80 from EuR_2 (IBA 0.5 mg L^{-1}). EuR_1 (Control) did not produce roots.

Shoot length

The longest shoot length (6.10 cm) was obtained from EuR_4 (IBA 1.5 mg L^{-1}) followed by 2.70 cm from EuR_2 (IBA 0.5 mg L^{-1}). The shortest shoot length (2.20 cm) was produced from EuR_3 (IBA 1.0 mg L^{-1}). EuR_1 (Control) possessed 4.70 cm which was shorter than EuR_4 but longer than EuR_2 and EuR_3.

Root length

The longest root length (1.20 cm) was obtained from EuR_4 (IBA 1.5 mg L^{-1}) followed by 1.00 cm from EuR_2 (IBA 0.5 mg L^{-1}) and EuR_3 (IBA 1.0 mg L^{-1}). EuR_1 (Control) did not produce roots.

Rooting of multiplicated shoots by Sucrose treatments

Thirty days after cultured, number of shoot, number of root, shoot length and root length in each treatment were collected. The maximum root length (2.48 cm) was observed in 4% sucrose treatment. The maximum number of root (1.67) was occurred in concentration of sucrose 2%.

Number of shoot

The maximum number of shoot (21.50) was obtained from EuC_2 (Sucrose 2%) followed by 10.30 from EuC_5 (Sucrose 8%). The minimum number of shoot (4.70) from EuC_3 (Sucrose 4%). EuC_1 (Control) had 13.00 which was lower than EuC_2 but higher than other treatments.

Number of root

The highest number of root (1.70) was obtained from EuC_2 (Sucrose 2%) followed by 1.20 from EuC_3 (Sucrose 4%). EuC_5 (Sucrose 8%) had the lowest number of roots (0.30). EuC_4 (Sucrose 6%) and EuC_1 (Control) have not developed any roots.

Shoot length

The longest shoot length (2.80 cm) was obtained from EuC_2 (Sucrose 2%). The second longest shoot length (1.30 cm) was observed from EuC_5 (Sucrose 8%) followed by 1.10 cm from EuC_3 (Sucrose 4%). The shortest shoot length (0.90 cm) was produced from EuC_4 (Sucrose 6%). EuC_1 (Control) had 2.70 cm which was lower than EuC_2 but higher than other treatments.

Root length

The longest root length (2.50 cm) was obtained from EuC_3 (Sucrose 4%) followed by (1.40 cm) from EuC_2 (Sucrose 2%). The shortest root lengths (0.30 cm) was produced from EuC_5 (Sucrose 8%). EuC_1 (Control) and EuC_4 (Sucrose 6%) were not produced root.

Table 9. Effect of IBA and sucrose on number of roots, root length, shoot length and number of shoots of *Eucalyptus citriodora* Hook.

Rooting	Shoot length (cm)	No. shoot	No. root	Root Length (cm)
EuR_1 (Control)	4.7	21.7	-	-
EuR_2 (IBA 0.5 mg L^{-1})	2.7	17.4	7.8	1
EuR_3 (IBA 1.0 mg L^{-1})	2.2	14.2	12.2	1
EuR_4 (IBA 1.5 mg L^{-1})	6.1	15.4	10.9	1.2
Rooting	**Shoot length (cm)**	**No. shoot**	**No. root**	**Root Length (cm)**
EuC_1 (Control)	2.73	6.33	-	-
EuC_2 (Sucrose 2%)	2.78	10.17	1.67	1.37
EuC_3 (Sucrose 4%)	1.13	4.67	1.17	2.48
EuC_4 (Sucrose 6%)	1.07	3.33	-	-
EuC_5 (Sucrose 8%)	1.33	6.00	0.33	0.27

Figure 1. One hundred and fourteen days after culture of *Eucalyptus citriodora* Hook. on MS medium supplemented with various concentrations and combination of NAA and BAP for multiplication

Figure 2. Fifty-six days after inoculation of *Eucalyptus citriodora* Hook. on MS medium supplemented with various concentrations and combination of BAP and GA_3 for elongation

Figure 3. Thirty days after cultured, *in vitro* rooted plantlets of *Eucalyptus citriodora* Hook. on half MS medium supplemented with various concentrations of IBA for rooting

Figure 4. Thirty days old cultures of *Eucalyptus citriodora* Hook. on half MS medium supplemented with various concentrations of sucrose for rooting

Discussion and Conclusion

The 1 cm size 30 days old shoot apex and nodal segment of *in vitro* raised seedlings were excised in the culture chamber. These segments were cultured in modified MS medium containing various concentrations and combination of NAA and BAP. One hundred and fourteen days after inoculation (114 DAI), EuS_6 (NAA 0.75 + BAP 3.0 mg L^{-1}) possessed the longest shoot length (2.25 cm) but maximum number of shoot (8.97), and maximum number of leaves (16.74) were obtained from EuS_5 (NAA 0.15 + BAP 3.0 mg L^{-1}). In micropropagation, the responses of *Eucalyptus* species were varied according to concentration of plant growth regulators and also

depended on culture conditions. Gomes and Canhoto (2003 ***In:*** Brondani *et al.,* 2010) verified that 0.9 µM BAP (0.20 mg L^{-1}) combined with 0.05 µM NAA (0.01 mg L^{-1}) on half MS medium was the optimum concentration for *E. nitens* bud proliferation. Joshi *et al.* (2003 ***In:*** Brondani *et al.,* 2010) observed that *E. tereticornis* x *E. grandis* produced 20 to 25 buds per explant 150 days after cultured in MS medium supplemented with 1 mg L^{-1} BAP and 1 mg L^{-1} NAA. Similar results were obtained by Bisht *et al.* (1999 ***In:*** Brondani *et al.,* 2010) for *E. tereticornis* x *E. camaldulensis*, where the highest multiplication, regardless of clone, occurred after 120 days in MS medium supplemented with 1 mg L^{-1} BAP and 1 mg L^{-1} NAA. George *et al.* (2008 ***In:*** Brondani *et al.,* 2010) emphasized that excess cytokinin in the culture medium may be toxic to the explant, causing severe problems in the subsequent stages. Inclusion of BAP (1.0 - 10.0 mg L^{-1}) in the culture medium was essential for bud break and shoot multiplication of *R. hybrida* .

Bennett *et al.* (1994 ***In:*** Brondani *et al.,* 2010) recorded that BAP concentrations above 2.5 µM (0.56 mg L^{-1}) in MS medium reduced the average number of buds per explant of *E. globules*. Similar effects were verified by Bisht *et al.* (1999 ***In:*** Brondani *et al.,* 2010) for *E. tereticornis* x *E. camaldulensis*, who reported multiplication followed by elongation was occurred 120 days after cultured in MS medium supplemented with BAP and NAA. After 180 days in MS medium, all BAP and NAA treatments had elongation, producing shoots of 2.0 cm to 3.5 cm in length. During establishment, the lowest number of shoot bud production was 3 per explant and the highest 9 was obtained from EuS_5 (NAA 0.15 + BAP 3.0 mg L^{-1}). This treatment also had the highest number of shoot (8.97). It is therefore concluded that EuS_5 (NAA 0.15 + BAP 3.0 mg L^{-1}) can be assumed as the suitable treatment for shoot and leaves multiplication.

In the next step, the culture was carried out to find out the effect of various concentrations and combinations of BAP and GA_3 on *in vitro* shoot elongation of *Eucalyptus*. Micro shoots (1 - 1.5 cm) were sub-cultured on MS medium having BAP (0.1 mg L^{-1}) combined with various concentrations of

GA_3 (0.1, 0.5 and 1.0 mg L^{-1}). The study was monitored 14 days after cultured and measurement was done with respect to shoot length, number of shoots, number of leaves, leaf width and leaf length. The optimum shoots length response to the applied BAP and GA_3 were observed in pure BAP 0.1 mg L^{-1} treatment. Brondani (2011) reported that *Eucalyptus* hybrid H12 had the longest shoot length with medium supplemented with 0.07 mg L^{-1} BAP and 0.22 mg L^{-1} $GA_{3,}$ with shoots on average of 1.25 cm in length when grown on half-strength MS medium. When full-strength MS medium was used, *Eucalyptus* hybrid H12 clones were 1.13 cm long with medium containing 0.08 mg L^{-1} BAP and 0.16 mg L^{-1} GA_3 . In this study, the longest shoot length was obtained from the explants cultured on the MS medium containing BAP (0.1 mg L^{-1}). After 56 DAI on MS medium containing BAP 0.1 mg L^{-1} had elongation, producing the shoot length of 3.01 cm. The maximum number of shoot was 26.81, the maximum number of leaves was 40.43 and the maximum leaf width was 0.33 cm and leaf length was 0.45 cm. Joshi *et al.* (2003) observed that the mean length of elongated shoots varied from 2.5 to 3 cm in length during 30 days when grown on MS medium supplemented with 1 mg L^{-1} BAP and 0.04 mg L^{-1} GA_3. Similar effects were verified by Bisht *et al.* (1999) for *E. tereticornis* x *E. camaldulensis*, who reported that multiplication followed by elongation at 120 days after culture on MS medium supplemented with BAP and NAA. After 180 days on MS medium, all BAP and NAA treatments had elongation, producing shoots of 2.0 cm to 3.5 cm in length. However, Graca and Mendes (1989) reported that shoot elongation was greatest when cultured on half-strength MS medium containing 0.1 mg L^{-1} BAP and 0.01 mg L^{-1} IBA. Addition of $GA_{3,}$ at both concentrations, did not induce as much growth as culturing on half-strength MS medium alone. In my experiment, using the combination of BAP and GA_3 did not give the superior result as in the medium supplemented only BAP. Nevertheless, shoots were longer when cultured on medium containing $GA_{3,}$ compared to those grown on MS medium alone. Le Roux and Van Staden (1991) reported that Gibberellic acid has been added to media to obtain shoot elongation. Franclet and Boulay (1982) the addition of activated charcoal to a mediu m containing GA_3 and

other growth regulators was reported to cause elongation and to recover the morphological characteristics of the species. Graca and Mendes (1989) the addition of activated charcoal and growth regulators reduced shoot elongation and caused leaf browning. The greater reduction of shoot growth was also observed when shoots were cultured on Goncalves medium containing 0.5 mg L^{-1} BAP and 1.0 mg L^{-1} IAA. Joshi *et al.* (2003) reported that in *Eucalyptus* F_1 hybrid (*Eucalyptus tereticornis* × *Eucalyptus grandis*), the elongation of shoots was achieved within 15 days when such rosette clump of buds were cultured on MS medium supplemented with Kn and GA_3 along with BAP. In the present study, individual BAP treatment to *Eucalyptus citriodora* Hook. gave the longer shoot length than the combination of BAP and GA_3. It is therefore concluded that BAP treatment was suitable for *Eucalyptus* shoot elongation.

The rooting experiment was carried out to find out the effect of various concentrations of IBA (0, 0.5, 1.0 and 1.5 mg L^{-1}) and sucrose (0%, 2%, 4%, 6% and 8%) on elongated shoots (1.5 - 2.5 cm). The maximum number of roots was observed in half strength MS medium supplemented with 1.5 mg L^{-1} IBA where the small calli are observed in IBA treatments. The same was also observed in 2% sucrose supplemented medium. For root initiation IBA, NAA and IAA were used at a level of 0.5 mg L^{-1} each (Joshi *et al;* 2003). Joshi *et al;* 2003 mentioned that highest number of roots (3.22 ± 0.32) was observed in 1 mg L^{-1} IBA while the lowest number of roots (1.16 ± 0.15) in 0.1 mg L^{-1} IBA. The maximum root length (5.66 ± 0.69 cm) was observed in lower concentration (0.1 mg L^{-1}) of IBA (Joshi, 2003). In the present study, the maximum number of root (12.2) was obtained from 1.0 mg L^{-1} IBA and longest root length (1.2 cm) from IBA 1.5 mg L^{-1}.

Forty days after cultured, the problem of micro propagation of *Eucalyptus* by IBA treatment were: firstly browning of explants from the cut ends, however, the callus formation was still developed. At subsequent days, leaf drying of the explants was observed. Finally, wilting and drying of the whole explants were resulted this IBA experiment. Therefore the application

of the different concentration of sucrose was carried out in this experiment. Sucrose is a necessary component in medium because explants *in vitro* are unable to photosynthesize effectively. Twenty to 60 g L^{-1} sucrose is most commonly used concentrations. Alternative sugars could be glucose, maltose, or lactose (Coffin *et al*., 1976). The results showed that the maximum root length (2.48 cm) was observed in concentration of 4% sucrose. The maximum number of root (1.67) was occurred in concentration of sucrose 2%.

Conclusion

NAA 0.15 + BAP 3.0 mg L^{-1} was suitable for shoot and leaf multiplication. BAP 0.1 mg L^{-1} gave the optimum shoot elongation. GA_3 has not significant effect on shoot elongation compared to BAP. IBA 1.0 mg L^{-1} gave the multiplicated roots but 1.5 mg L^{-1} of IBA gave the optimum root length. Sucrose 2% had the effect on number of root but 4% sucrose on root length.

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