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Full Length Research Paper

The production of a plant regeneration system from callus of *DENDROBIUM* cv. Serdang Beauty

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An *IN VITRO* propagation protocol was established for the *DENDROBIUM* Serdang Beauty orchid. The propagation protocol utilized calli tissues that were successfully initiated from protocorm-like bodies (PLBs) explants, while the leaf and root tip explants died. The percentage of protocorm-like bodies explants responding to calli formation was 100% in all tested levels of IAA, IBA and NAA auxin treatments. The highest amount of calli (49.59 gram) proliferated on MS medium containing 1.5 mg/L IBA. These calli successfully regenerated on media supplemented with either KIN or BAP cytokinins and combined treatments of KIN and IAA (4 mg/L) or NAA (1.5 mg/L). However, media supplemented with only 1 mg/L KIN was sufficient to produce significantly high percentage of plantlet formation (80%), high number of planlets per explant (4-5 plantlets) and high mean fresh weight per plantlet (11.128 g). These plantlets were acclimatized on all tested media and obtained satisfactory rate of plantlet survival (80-100%), mean number of leaves per plant (4-6 leaves), and mean leaf length (4 - 5 cm). Among these media, charcoal was considered the most economical and available material in the local market. During the development of this protocol, substantial necrosis of calli were observed when cultures were treated with 2,4-D and BAP. It was proposed that the presence of ethylene within the cultures, which is known to be emitted by plant growth regulators into the micro-climate of *IN VITRO* culture vessels, is the determining factor of a suitable plant growth regulator for the survival and growth of the *DENDROBIUM* Serdang Beauty calli cultures in our study.

Keyword: Auxin, cytokinin, callus, Plbs regeneration, orchid.

INTRODUCTION

The Orchidaceae is a large flowering family that is undoubtedly recognized as an economically important commodity in the international floriculture industry, both as cut flowers and potted plants (Arditi, 1992; Kuehnle, 2007). Among various orchid categories in the family, the *Dendrobium* orchid have become increasingly popular due to its floriferous flower sprays, wide range of colours, sizes and shapes, year-round availability, and long flowering life of several weeks to months (Kuehnle, 2007).

In the US alone, about 90% of the 52 million imported orchid stems were *Dendrobium* orchids which valued a total of USD 3.6 million, in 2005. In the same year, potted *Dendrobium* orchids reached a production value of USD 5.8 million out of total potted orchids of USD 122 million (Jerardo, 2007). In Malaysia orchid exports value about RM 40 million annually, of which 11.7% comprise the *Dendrobium* production. Due to growing domestic and export markets, Malaysia has recorded increasing floricultural products from RM 0.73 million in 1992 to RM 2.77 million in 1995. At the continued growth rate of 6% per annum, productions are expected to reach RM 36 billion by the year 2010 (Jong et al., 1998).

Recognizing the fact that such growing interest and demand for flowers of the Orchidaceae would follow the issue of the sufficient supply of planting materials, *in vitro*

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Abbreviations: IAA, Indol-3-acetic acid; **IBA**, indoe-3-butyric acid; **NAA**, α-naphthalenacetic acid; **2,4-D**, 2,4-dicolophenoxyactic acid; **BAP**, N^6-benzylaminopurine; and **KIN**, 6-fururylaminopurine.

mass propagation methods have been developed and established for various orchids using various orchid explants (reviewed in Zhao et al., 2008). Furthermore, the conservation and preservation of wild and endangered orchids (Weatherhead, 1995; Lo et al., 2004) and the production of improved varieties via genetic transformation (reviewed in Kuehnle, 2007), sincerely requires the advantage of such efficient mass propagation methods.

The asexual propagation of commercial *Dendrobium* was generally low as 2-4 plants are produced per year. Based on various reports, established *in vitro* propagation of the *Dendrobium* genera utilized shoot buds (Kim and Kim, 2003; Talukder et al., 2003; Ferreira et al., 2006) and protocorm like bodies (PLBs) (Zhao et al., 2008; Roy et al., 2007; Yu et al., 2001). In the most recent reports, callus tissues were induced from PLBs cultures for long-term maintenance (Roy et al., 2007) and plantlet regeneration (Zhao et al., 2008).

Therefore, this study investigated the establishment of an *in vitro* plant regeneration system of a local *Dendrobium* cultivar, the 'Serdang Beauty'.

MATERIALS AND METHODS

This study was conducted in the Laboratory of Agrobiotechnology, Faculty of Agriculture, University of Putra, Malaysia.

Callus induction

Callus induction from various explants of the *Dendrobium* Serdang Beauty was investigated in the first study. Excised leaves (2 x 2 cm), root tips (2 cm in length) and PLBs (0.5 cm in radius) were cultured in MS media (Murashige and Skoog, 1962) supplemented with various auxins [indol-3-acetic acid (IAA), indoe-3-butyric acid (IBA), α-naphthalenacetic acid (NAA), 2,4-dicolophenoxyactic acid (2,4-D)] and treatment levels (0, 0.5, 1.0, 1.5, 2.0, 4.0, 8.0 and 16.0 mg/L). All media contained 3% (w/v) of sucrose, was solidified with 0.4% (w/v) agar (Gelrite, USA) and adjusted to pH 5.8 with 1 N NaOH or HCl before autoclaving at 121°C for 15 min. Each treatment was prepared in 15 replicates in a Randomized Complete Block Design (RCBD). Incubation conditions were maintained at 25 ±1°C under cool fluorescent light at 40 µmol m^{-2} s^{-1} of light of 16-h photoperiod per day. At the end of 16 weeks of culture, the percentage of explants responding with callus formation and mean fresh weight of the calli were recorded. Recorded data were analyzed using ANOVA and the comparison of treatment means were analyzed using Duncan New Multiple Range Test (DNMRT) at α = 5%.

Plantlet regeneration from callus

Calli induced from the second study were subsequently used for the study of plant regeneration. Calli (2 g) were cultured in MS media supplemented with treatment combinations of cytokinin [N^6-benzyla-minopurine (BAP) and 6-fururylaminopurine (KIN)] and auxin (NAA and IAA). BAP and KIN at concentrations 0, 0.5, 1, 2, 4, 8, and 16 mg/L, were combined with 4 mg/L IAA or 1.5 mg/L NAA. At the end of 16 weeks of culture, the percentage of explants regenerating p lantlets, mean number of plantlets produced per explant and mean fresh weight of the plantlets were recorded. Media preparation, incubation conditions and data analysis were conducted according to the first study.

Acclimatization of plantlets

Successfully regenerated *in vitro* plantlets from the second study were used in the acclimatization study. *In vitro* plantlets with vigorous growth and uniform size were transferred to various acclimatization media consisting of: charcoal (M1); charcoal mixed with broken rock (M2); broken rock (M3); sawdust (M4); and sawdust mixed with charcoal (M5). Each treatment had 10 replicates. The cultures were kept in a misting chamber with incubation conditions of 28 ± 1°C, 60 ± 10% humidity, 700-1000 Lux of light intensity for 16 h and irrigated with sprinklers twice a day for 20 min each time. At the end of 16 weeks of culture, the percentage of plant survival, mean number of leaf and roots produced per plant, and mean leaf length were recorded. Data analysis was conducted according to the first study.

RESULTS AND DISCUSSION

The effect of various auxins on callus induction from leaf, root and PLBs explants

Only the PLB explants produced callus, while the leaf and root explants failed to form callus and gradually became necrotic. Calli formation was observed on all treatments including control, from 30 days after culture (Figure 1). This observation indicated that the PLB explants contained sufficient endogenous auxin for the induction of calli, and for the proliferation of calli which produced mean fresh weights that were not significantly different from calli induced by IAA, IBA and NAA auxins until the 4th subculture), except for treatment 1.5 mg/L IBA which gave significantly the highest mean fresh weight of 49.59 g (Table 1; Figure 1). The feasibility to induce and proliferate calli on hormone-free media is an important advantage in clonal propagation efforts, as cultures exposed to continuous exogenous plant growth regulators may cause unwanted somaclonal variation, which also allows the maintenance of calli cultures for an extended period to ensure the availability and mass production of various propagating materials (Vázquez, 2001; Lim and Loh, 2003).

Although 2,4-D did initiate callus formation from the PLB explants, however the cultures became necrotic by the 3rd subculture (Figure 1) and produced significantly lower percentage of responding explants and mean calli fresh weights compared to control (Table 1). Similarly, in a study that used *Dendrobium candidum,* the highest percentage of calli induction among the 2,4-D treatments was 32% at 1.5 mg/L (Zhao et al., 2008).

The effect of cytokinin and auxin combinations on the regeneration of plantlets from PLB explants

In contrast to the calli induction study, calli explants on control treatment gave the lowest percentage of response, with the lowest number of plantlets produced per explant, and mean plantlet fresh weight (Table 2). This indicated that endogenous auxin in the proliferating calli (as observed in the control of the first study) was not able

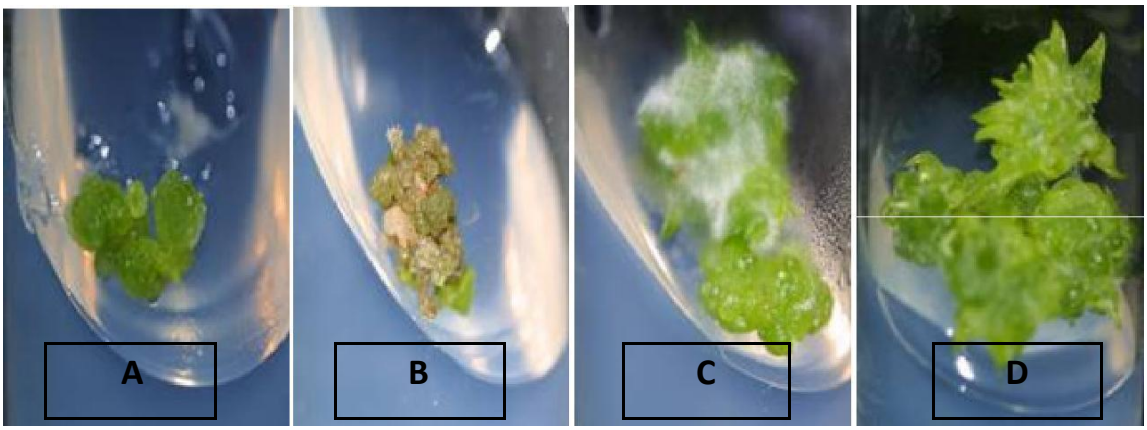

Figure 1. Callus formation on PLB exlants: Callus formation on PLB explant after 30 days of culture (A); necrotic calli on 2,4-D treatments after the 3rd subculture (B); callus proliferation after each subculture (C); proliferated callus after the 4th subculture (D).

Table 1. The effect of various auxins on callus induction from PLBs explants.

Plant Growth Regulators (µM)				Explants producing callus (%)	Callus fresh weight (grams)
IAA	IBA	NAA	2,4-D		
-	-	-	-	100^{a}	26.92^{bcd}
0.5	-	-	-	100^{a}	29.04^{bcd}
1.0	-	-	-	100^{a}	36.72^{b}
1.5	-	-	-	100^{a}	36.72^{b}
2.0	-	-	-	100^{a}	27.69^{bcd}
4.0	-	-	-	100^{a}	36.00^{b}
8.0	-	-	-	100^{a}	25.72^{bcd}
16.0	-	-	-	100^{a}	26.98^{bcd}
-	0.5	-	-	100^{a}	17.24^{ed}
-	1.0	-	-	100^{a}	30.86^{bcd}
-	1.5	-	-	100^{a}	49.59^{a}
-	2.0	-	-	100^{a}	16.84^{ed}
-	4.0	-	-	100^{a}	22.60^{bcd}
-	8.0	-	-	100^{a}	17.84^{cde}
-	16.0	-	-	100^{a}	24.66^{bcd}
-	-	0.5	-	100^{a}	14.83^{ed}
-	-	1.0	-	100^{a}	14.75^{ed}
-	-	1.5	-	100^{a}	22.02^{bcd}
-	-	2.0	-	100^{a}	34.87^{bc}
-	-	4.0	-	100^{a}	30.07^{bcd}
-	-	8.0	-	100^{a}	20.67^{bcd}
-	-	16.0	-	100^{a}	35.12^{bc}
-	-	-	0.5	40^{b}	13.40^{ed}
-	-	-	1.0	20^{b}	2.00^{e}
-	-	-	1.5	20^{b}	2.00^{e}
-	-	-	2.0	20^{b}	2.00^{e}
-	-	-	4.0	20^{b}	2.00^{e}
-	-	-	8.0	20^{b}	2.00^{e}
-	-	-	16.0	20^{b}	2.00^{e}

Means followed by the same letter are not significantly different at $p \leq 0.05$. Data represent mean value of 15 replicates. Mean callus fresh weight includes inoculum weight. Incubation period was 16 weeks.

Table 2. The effect of cytokinin and auxin on the regeneration of plantlets from calli after 16 weeks of culture.

Plant growth regulators				Responding explants (%)	Mean number of plantlets per explant	Plantlet mean fresh weight (gram)
KIN	BAP	IAA	NAA			
-	-	-	-	10^{ef}	0.4^{ef}	0.982^{ef}
-	-	-	1.5	50^{abcde}	1.6^{cdef}	3.909^{bcdef}
-	-	4	-	20^{def}	0.5^{ef}	1.814^{def}
0.5	-	-	-	30^{cdef}	1.0^{def}	6.255^{abcde}
1	-	-	-	8.0^{ab}	4.9^{a}	11.128^{a}
2	-	-	-	62.50^{abcd}	3.0^{bc}	7.582^{abc}
4	-	-	-	60^{abcd}	2.3^{bcde}	4.710^{bcdef}
8	-	-	-	30^{cdef}	1.1^{def}	2.436^{cdef}
16	-	-	-	10^{ef}	0.2^{f}	0.325^{f}
0.5	-	-	1.5	80^{ab}	3.0^{bc}	3.376^{abcde}
1	-	-	1.5	90^{a}	4.0^{a}	9.264^{ab}
2	-	-	1.5	60^{abcd}	1.8^{cdef}	4.628^{bcdef}
4	-	-	1.5	30^{cdef}	1.1^{def}	3.056^{cdef}
8	-	-	1.5	30^{cdef}	1.0^{def}	3.053^{cdef}
16	-	-	1.5	30^{cdef}	0.6^{ef}	1.676^{ef}
0.5	-	4	-	50^{abcde}	2.3^{bcde}	5.503b^{cdef}
1	-	4	-	50^{abcde}	2.7^{bcd}	7.188^{abcd}
2	-	4	-	50^{abcde}	1.7^{cdef}	5.343^{bcdef}
4	-	4	-	40^{bcdef}	1.4^{cdef}	1.946^{def}
8	-	4	-	40^{bcdef}	0.6^{ef}	1.585^{def}
16	-	4	-	20^{def}	0.4^{ef}	0.969^{ef}
-	0.5	-	-	50^{abcde}	1.5^{cdef}	5.625^{bcdef}
-	1	-	-	60^{abcd}	1.6^{cdef}	6.484^{abcde}
-	2	-	-	70^{abc}	2.7^{bcd}	6.659^{abcd}
-	4	-	-	30^{cdef}	0.8^{def}	4.944^{bcdef}
-	8	-	-	30^{cdef}	0.9^{def}	4.704^{bcdef}
-	16	-	-	30^{cdef}	0.5^{ef}	3.593^{cdef}
-	0.5	-	1.5	0.0^{f}	0.0^{f}	0.000^{f}
-	1	-	1.5	0.0^{f}	0.0^{f}	0.000^{f}
-	2	-	1.5	0.0^{f}	0.0^{f}	0.000^{f}
-	4	-	1.5	0.0^{f}	0.0^{f}	0.000^{f}
-	8	-	1.5	0.0^{f}	0.0^{f}	0.000^{f}
-	16	-	1.5	0.0^{f}	0.0^{f}	0.000^{f}
-	0.5	4	-	0.0^{f}	0.0^{f}	0.000^{f}
-	1	4	-	0.0^{f}	0.0^{f}	0.000^{f}
-	2	4	-	0.0^{f}	0.0^{f}	0.000^{f}
-	4	4	-	0.0^{f}	0.0^{f}	0.000^{f}
-	8	4	-	0.0^{f}	0.0^{f}	0.000^{f}
-	16	4	-	0.0^{f}	0.0^{f}	0.000^{f}

Means followed by the same letter are not significantly different at $p \leq 0.05$. Data represent mean value of 15 replicates.

to support the regeneration of plantlets, as it was also observed that the addition of exogenous auxins of 4 mg/L of IAA and 1.5 mg/L of NAA did not significantly increase any of the measured variables from control. However, significant increase was observed in the presence of cytokinins KIN or BAP, and in combinations of cytokinin KIN and auxins NAA or IAA (Table 2, Figure 2).

Among the treatments that were significantly higher than control, treatment KIN (1 and 2 mg/L) or BAP (2 mg/L) alone was sufficient to produce more than 50% of regeneration response from the calli explants with significantly high number of plantlets per explant (2-5 plantlets) and mean fresh weight per plantlet (6.659 - 11.128 g) (Table 2). This indicated that a favourable ratio was achieved for plantlet regeneration between the exogenous cytokinin supplied and endogenous auxin of

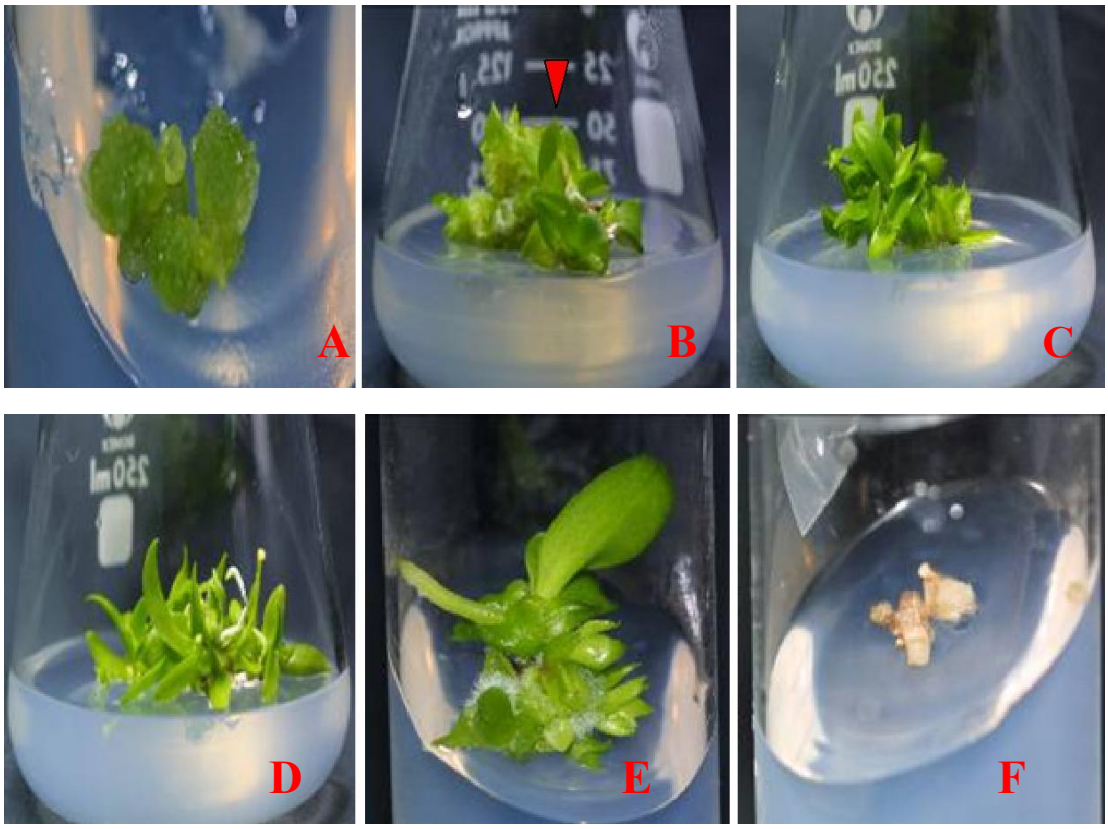

Figure 2. Plantlet regeneration from calli: PLBs explants used for calli induction (A); formation of primordial shoots from callus (red arrow) (B); plantlet developed after 6 weeks of culture in BAP (C); plantlet developed after 8 weeks of culture in Kinetin either alone or with 1.5 mg/L NAA or 4 mg/L IAA (D); plantlet development interspersed with callus after 16 weeks of culture (E); necrotic callus in combined BAP with NAA or IAA treatment (F).

the calli, as it was also observed that the explant regeneration response was significantly different from control under exogenously supplied KIN (0.5, 1 and 2 mg/L) and NAA (1.5 mg/L). However, calli under the combined supply of KIN and auxin IAA did not produce regeneration response that was significantly higher than control, except for treatment 1 mg/L KIN with 4 mg/L IAA that produced significantly high mean number of plantlets (2-3 plantlets) and mean plantlet fresh weight (7.188 g).

An important observation was also noted for combined BAP and auxin treatments in our study. Calli explants in all the combined treatments became necrotic and failed to produce any regeneration response, but responses were observed (although not significantly different from control) when BAP or either auxin (IAA or NAA) were supplied alone (Table 2, Figure 2). Orchid calli cultures are generally susceptible to necrosis, which have led many propagation protocols to utilize other tissue organs as explants for micropropagation (Zhao et al., 2008; Roy et al., 2007).

Among the various *in vitro* culture conditions manipulated for suitable growth of the cultured tissues, the micro-climate changes within the culture vessels are unavoidable. The presence of volatile ethylene gas from cultured tissues under the influence of plant growth regulators within the media has been reported in various plant cultures (Imakawa et al., 2002; Kumar et al., 1998). In rice callus cultures, the combined effect of 2,4-D and BAP had a negative effect on callus growth due to increased ethylene production within the cultures, but promoted soybean calli growth (Imakawa et al., 2002). This combination also strikingly produced more ethylene compared to IBA (1 mg/L) and BAP (1.1 mg/L) in the soybean cultures. Generally, ethylene production under combined auxin and cytokinin treatment was greater than that induced by each hormone (reviewed in Imakawa et al., 2002).

Although the ethylene content of the cultures was not quantified, our study suggests that the *Dendrobium* Serdang Beauty calli necrosis was due to their sensitivity to the presence of ethylene within the cultures, induced by the combined BAP and auxin treatments. This sensitivity was similarly implied to PLBs explants that gave low calli induction response and mean fresh weights on 2,4-D treatments (Table 1), since a positive correlation between increased 2,4-D concentrations and ethylene production

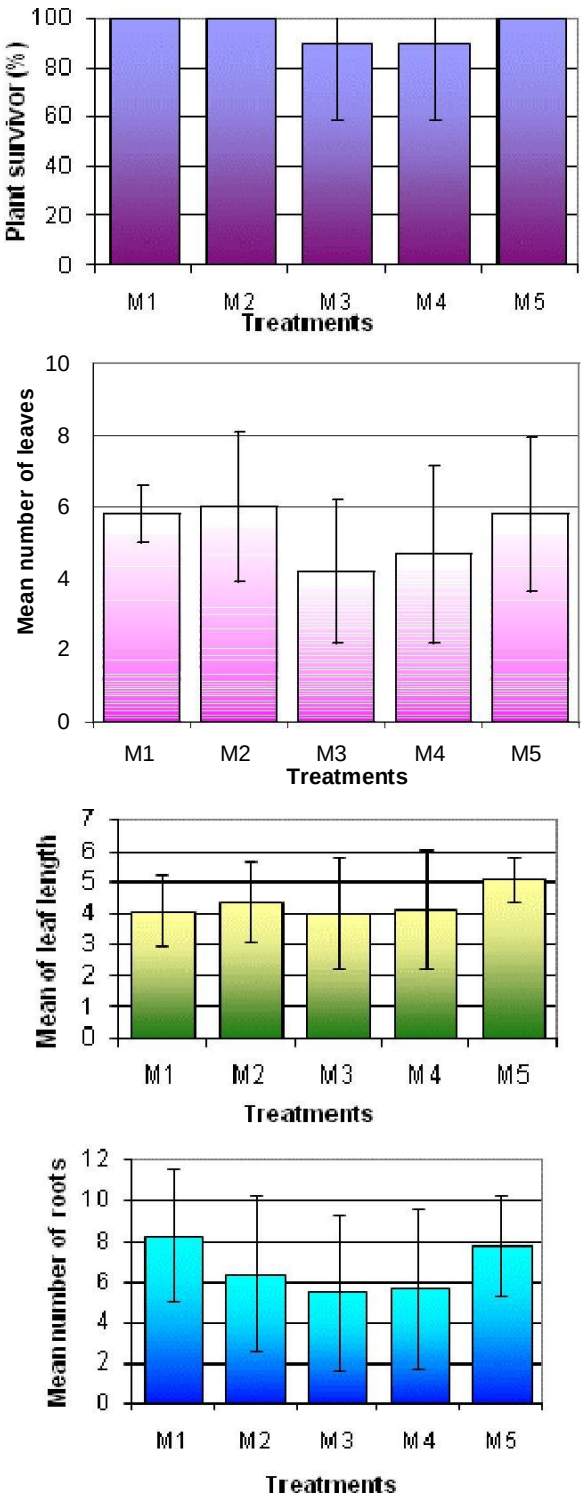

Figure 3. Effect of various acclimatization media on plant survival, number of leaves and roots produced at the end of 16 weeks of cultivation. All treatments are not significantly different at p=0.05 (Bar=Standard deviation, n=10).

also showed a negative effect on both rice and soybean calli growth (Imakawa et al., 2002). Nonetheless, it is important to investigate the effects of ethylene on the *Dendrobium* Serdang Beauty calli growth and its plantlet regeneration response. Otherwise, the tissue differential response to various exogenous cytokinins is generally suggested to be due to the cytokinin type-specific metabolic activity of the tissues (Horgan 1987; De Pauw et al., 1995), whereby the effect of ethylene as the determining factor of favourable cytokinin and auxin, remains to be illucidated.

Media supplemented with 1 mg/L KIN was considered the most favourable treatment for high percentage of plantlet regeneration response from calli explants (80%), high number of planlets per explant (4-5 plantlets) and high mean fresh weight per plantlet (11.128 g).

Plantlet acclimatization

All acclimatization media produced high rate of plantlet survival, mean number of leaves per plant (4-6 leaves), and mean leaf length (4 - 5 cm) (Figure 3). However, plants on charcoal media were vigorous in growth and produced roots that strongly attached to the charcoal, with bright leaf colour, indicating healthy growth. Furthermore, charcoal is the most economic media compared to the other media, and can be easily obtained at local markets. Generally, charcoal and/or brick pieces provide maximum water holding capacity, porosity and drainage is essential for proper growth and development of *in vitro* raised plantlets, as required by epiphytes such as the *Dendrobium* (Lo et al., 2004; Saiprasad et al., 2004).

Conclusion

Among the leaf, root tip and PLBs explants used for calli induction, the PLBs explants survived and initiated calli formation, while the rest died. Auxins IAA, IBA and NAA in all concentrations tested induced 100% calli induction from the PLBs explants in our study, while 1.5 mg/L of IBA was the best supplement for calli proliferation as the highest mean calli fresh weight was produced. Auxin 2,4-D was favourable for initial calli induction from the PLBs (100%), but these calli gradually became necrotic by the 3rd subculture, which significantly reduced surviving calli to less than control (20-40%). The regeneration of plantlets from these calli were successful on cytokinin supplemented media of KIN or BAP and combined KIN and NAA or IAA treatments; but treatment 1 mg/L KIN was considered the best plantlet regeneration media as significantly high percentage of responding explants, number of plantlets produced per explant and fresh weight per plantlet were produced. These plantlets were then successfully acclimatized in all acclimatization media. However, charcoal was considered the most suitable media as it was the most economical and available in the local market.

Despite the successful regeneration protocol developed in our study, the *in vitro* differential growth and death of calli tissues exhibited in response to specific plant growth regulators applied, which is generally explained by the cytokinin-type specific metabolic activity, remains to be investigated in the presence of ethylene within the cultures.

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