EFFECT OF BASAL MEDIA AND CARBON SOURCES ON CALLUS CULTURE MAINTENANCE OF *Vanda dearei*

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ABSTRACT. *Vanda dearei is an endemic orchid of Borneo and has been listed as an endangered orchid in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Vanda dearei has beautiful pale yellow-flowers, large petals and strongly scented. Therefore, in vitro micropropagation has been applied in order to develop a novel micropropagation method to mass produce this species. Through callus culture techniques, orchids with limited resources can be mass propagated in a shorter period. However, callus culture in orchid is hardly maintain due somatic embryogenesis properties and easily regenerated to plantlets. Thus, this study aims to develop an efficient protocol for callus cultures of V. dearei by manipulating basal media strengths and carbon sources. Callus induced from the leaf segments of V. dearei were used as explants and were cultured on KC, Mitra, MS and VW basal medium at different nutrient strengths (1/4, 1/2, 1 and 2x) added with 1.0:0.1 mg/l TDZ:NAA and 1 to 4% (w/v) of sucrose, glucose or fructose, respectively. All cultures were incubated in the dark with temperature of 25±2°C. Results showed that callus growth has improved with decreased nutrients strength of basal media. Quarter strength of Mitra medium promotes the best condition for callus maintenance to approximately 8.00±17.89% at 8 weeks of culture. This is followed by the ½ strength of MS and ¼ strength of VW with 8.00±10.95% and 5.00±10.00%, respectively. Callus grown on the other basal strengths are mainly differentiated and developed into protocorm like bodies (PLBs), especially at double strengths (100±0% explants turn into PLBs). In addition, low percentage of necrosis (less than 28%) was also observed on Mitra basal medium compared to the other media (more than 36%). Sucrose has been identified as the best carbon source to support callus growth followed by glucose and fructose. Addition of 1% (w/v) sucrose increased callus maintenance up to 32±17.9%, promote cell differentiation and increased average size of callus (1.52±0.63 callus score). This treatment also support the longest retention time of explant maintained in callus for 5 weeks and has the lowest percentage of callus necrosis (20±24.5%).*

KEYWORDS. *Vanda dearei*; Orchid; Micropropagation; Callus Maintenance

INTRODUCTION

Vanda dearei is an endemic orchid species of Borneo and has been listed as an endangered orchid species in Appendix II of the *Convention on International Trade in Endangered Species of Wild Fauna and Flora* (CITES). *V. dearei* has beautiful, large, strongly scented flowers and is one of the main sources of yellow colour in *Vanda* hybrids (Motes & Hoffman, 1997) which is commercially admired by many orchid enthusiasts. Plant tissue culture techniques have been proven to conserve rare and endangered orchid species. Therefore, *in*

vitro micropropagation has been applied in order to develop a novel micropropagation method to mass produce this species.

One of the most challenging micropropagation methods is an involvement of intermediary callus phase, which is considered a rather difficult morphogenetic pathway for mass plantlets production in orchids. Through intervening of callus phase, production can be scaled up manifold (Sha *et al*. 2002) from limited resources and it will produce larger number and uniform planting material in a relatively short period of time (Firoozabady & Gutterson, 2003). Even though callus induction of various orchid species have been reported using many types of explants, there are very few reports on callus maintenance as orchid callus has been considered difficult to be induced and maintained in culture (Kerbauy, 1984; Arditti & Ernst, 1993). Callus maintenance is crucial as it is used for initiating suspension cultures for rapid cell proliferation and plantlets production (George *et al*., 2008). Cell suspension cultures contain a relatively homogeneous cell population, allowing rapid and uniform access to nutrition, precursors, growth hormones and signal compounds for the cells (Mustafa *et al*., 2011). Long-term maintenance of callus cultures is very useful in plant tissue culture to facilitate the year round availability of somatic embryos and ready to be regenerated at any time without any difficult limitations (Pola *et al*., 2009).

Past studies indicated that the chemical composition of the medium is one of the factors determining the success of *in vitro* cultures (Kozak *et al*., 2007; Faedi *et al*., 2002). In addition, a wide variety of sugars may act as a carbon source for callus growth, where some species and hybrids showed preferences for specific sugar (Arditti, 1967). Therefore, this study is focused on the effect of basal media type and strengths and carbon sources on the maintenance and development of *V. dearei* callus.

MATERIALS AND METHODS

Preparation of Culture Material

Sixty-day old calli induced from leaf segment of *V. dearei* were excised into 2-4 mm^2 of size and were used as source material. Calli were induced on Mitra (Mitra *et al*., 1976) basal medium supplemented with 1.0 mg/L TDZ x 0.1 mg/L NAA (Suaiba, 2012).

Effect of Basal Media Strengths

Sixty-day old calli induced from leaf segment of *V. dearei* were used as explants and were cultured on KC (Knudson, 1946), Mitra (Mitra *et al*., 1976), MS (Murashige & Skoog, 1962) and VW (Vacin & Went, 1949) basal medium of different strengths (1/4, 1/2, 1 and 2x) added with 1.0 mg/L TDZ and 0.1 mg/L NAA**.** All cultures were incubated at 24 hours in the dark with temperature of 25±2°C.

Effect of Carbon Sources

Sixty-day old calli induced from leaf segment of *V. dearei* were used as explants and were cultured on 1/4 strength of Mitra (Mitra *et al*., 1976) basal medium added with 1.0 mg/L TDZ x 0.1 mg/L NAA supplemented with 0, 1, 2, 3 and 4% (w/v) of sucrose, glucose and fructose respectively. All cultures were incubated at 24 hours in the dark with temperature of 25±2°C.

Experimental Design and Data Analysis

Experiments were performed in a Completely Randomized Design (CRD). Each treatment was conducted in 5 replicates and each replicate consisted of five calli. All the cultures were examined weekly for 8 weeks. Percentages of callus maintenance, callus score, callus colour, callus necrosis and development of callus to protocorm-like bodies (PLBs) were observed

and recorded weekly for 8 weeks. Data were statistically analyzed using SPPS (Statistical Package for Social Science) version 16.0 and subjected to analysis of variance (ANOVA). Duncan Multiple Range Tests were conducted for mean comparisons of data collected at $p<0.05$.

RESULTS AND DISCUSSION

Maintenance and Development of Callus

Mitra (Mitra *et al*., 1976) basal medium was suitable for maintenance and development of *V. dearei* callus followed by MS (Murashige & Skoog, 1962), VW (Vacin & Went, 1949) and KC (Knudson, 1946). KC was not suitable for callus maintenance where all explants developed into PLB at the final week of observation. Maintenance of callus was improved with the decrease in strength of basal media. As for effect of carbon sources, the most effective type of carbon source for callus maintenance was sucrose, followed by glucose and fructose. Lower concentration (1 and 2%, w/v) of all carbon sources (sucrose, glucose and fructose) have been identified as the best carbon sources to support callus maintenance compared to higher concentrations of carbon sources.

Effect of Basal Media Strengths on Callus Maintenance

Basal media with lower strengths (1/4x and 1/2x) were suitable for callus maintenance and explants most likely to form PLB on higher strengths of basal media. Results show that Mitra basal medium at 1/4 strength medium was a suitable medium for maintenance and development of *V. dearei* callus with 8.00±17.89% callus remained until the final week of observation (Table 1). This is followed by the MS basal medium at 1/2 strength medium and VW at 1/4 strength medium with 8.00±10.95% and 5.00±10.00% callus maintained respectively. In hybrid *Cymbidium*, VW basal medium used for PLBs formation effectively while 1/2x MS basal medium used for callus induction and formation (Teixeira, 2012). Callus of *Vanda coerulea* was successfully maintained for a month on 1/4x strength of media (Lang & Hang, 2006) and callus of *Cymbidium ensifolium* was also effectively maintained on 1/2x MS media (Lee & Lee, 2003). There was no callus maintain observed on KC basal medium. Basal media with higher strength (2x) was ineffective for callus maintenance where all explants (100±0%) turned into PLBs. Previous study showed that KC basal medium were most suitable for PLBs development of *Vanda* genus. Growth and development of *Vanda tessellate* was at optimum using KC media (Roy & Banerjee, 2002). All treatments showed high formation of PLBs at the final week of observation ranging between 92-100% of explants developed into PLBs. According to Pola *et al*. (2009), MS medium was efficient for callus induction and plant regeneration. In addition, low percentage of necrosis in the range of 8 - 28% was also observed on Mitra basal medium compared to the other media that reach up necrosis range of 36 - 100%.

Table 1. Effect of different types and strengths of basal Media on maintenance and development of *V. dearei* callus after 8 weeks of culture.

Treatments (w/v)		Percentages of callus maintain (± SD)	No. of explant develop into PLB (Mean ± SD)	Percentages of explant necrosis (± SD)	Average size of explants (mm ± SD)	Callus colour	Callus texture
KC	1/4x	0±0	100±0^{a}	36.00±32.86^{ef}	4.87±0.31^{bcd}	Y	PLB
	1/2x	0±0	100±0^{a}	85.00±19.15^{abc}	5.93±2.00^{ab}	Y	PLB
	1x	0±0	100±0^{a}	55.00±37.86^{de}	6.33±3.18a	Y	PLB
	2x	0±0	100±0^{a}	68.00±43.81^{cd}	4.10±1.25^{de}	Y	PLB
Mitra	1/4x	8.00±17.89^{a}	92.00±17.89^{a}	28.00±33.47^{fg}	4.65±0.72^{bcde}	Y	C
	1/2x	4.00±8.94^{a}	96.00±8.94^{a}	24.00±26.08^{g}	5.70±1.18^{abc}	Y	C
	1x	0±0	100±0^{a}	8.00±10.95^{fg}	6.40±1.98^{a}	W	PLB
	2x	0±0	100±0^{a}	20.00±20.00^{fg}	5.92±0.92^{ab}	W	PLB
MS	1/4x	0±0	100±0^{a}	56.00±28.08^{de}	4.68±0.73^{bcde}	Y	PLB
	1/2x	8.00±10.95^{a}	92.00±10.95^{a}	72.00±33.47^{bcd}	4.12±1.18^{de}	Y	C
	1x	4.00±8.94^{a}	96.00±8.94^{a}	88.00±10.95^{abc}	3.84±0.62^{de}	B	C
	2x	0±0	100±0^{a}	100±0^{a}	3.32±0.30^{e}	B	PLB
VW	1/4x	5.00±10.00^{a}	95.00±10.00^{a}	70.00±25.82^{bcd}	4.20±0.28^{de}	Y	C
	1/2x	0±0	100±0^{a}	64.00±32.86^{cd}	4.15±0.87^{de}	Y	PLB
	1x	0±0	100±0^{a}	56.00±29.66^{de}	4.32±0.41^{cde}	Y	PLB
	2x	0±0	100±0^{a}	96.00±8.94^{ab}	3.44±0.33^{de}	B	PLB

Note: Data obtained from twenty five replicates. Data followed by the same letters are not significantly different ($p< 0.05$), based on Duncan's Multiple Range Test. SD- Standard deviation. B-Brown; Y-Yellowish; W-White; C-Compact; PLB-Protocorm-like body.

Effect of Carbon Sources on Maintenance and Development of Callus

Callus maintenance of *V. dearei* was observed on 1/4x Mitra medium added with 1.0 mg/L NAA+1.0 mg/L BAP and supplemented with various carbon sources (sucrose, glucose and fructose) with 1, 2, 3 and 4% (w/v) concentrations. The percentages of callus maintain, number of explant develop into PLB, percentages of explant necrosis, average score of callus size, callus color and callus texture were shown in Table 2 for effect of sucrose, glucose and fructose at different concentrations. Sucrose has been identified as the best carbon source to support callus maintenance followed by glucose and fructose. Highest proliferation of callus was also observed in media containing sucrose maybe due to easy utilization of sucrose in metabolism (Abu *et al*., 2005). Sucrose contributes to the osmotic potential of the medium (Nowak *et al*., 2004) which would permit the absorption of mineral nutrients present in medium, essential to the cells growth and so the optimal osmotic pressure required for optimal proliferation, may vary from culture to culture. Results showed that low concentration of carbon sources was more effective on callus maintenance.

Best carbon source treatment was observed on 1/4x Mitra medium supplemented with 1% (w/v) sucrose which maintained callus up to 32.00±17.9% after 8 weeks of culture as shown in Figure 1(b). It also has the lowest percentage of explant necrosis (20±24.5%). In the present study, low percentages of callus maintain obtained from media added with higher concentrations of carbon sources. This was in contrast to the observations on callus maintenance by Priyanka *et al*. (2011) where 2% (w/v) sucrose reduced the callus growth while medium contained 3% sucrose was defined as the best conditions for long term maintenance and proliferation of callus cultures. Treatment with glucose at 1 to 4% (w/v) succeeded in maintaining callus up to week 4 of culture however callus further developed

into PLB (Figure 1c). The percentage of shoot-redifferentiated callus clumps was also highest in glucose and lowest in sucrose (Harada *et al*., 1983). Treatment with 1% (w/v) glucose has the highest percentage of callus maintained (16.00±17.9%) and lowest percentage of explant necrosis (12.00±0.6%) compared to treatment with 2 to 4% (w/v) glucose. Callus was maintained up to week 3 in treatment with fructose however callus was further developed into PLB. Callus formed PLB was highly observed on treatment with 2% (w/v) fructose which was 64.00±21.9% and followed by 1% (w/v) fructose (52.00±10.95%). Media added with fructose followed by glucose also shown highest growth of PLBs of *Dendrobium* Alya Pink (Nambiar *et al*., 2012). There was no callus maintained on treatments with 3 and 4% (w/v) fructose after final week of observation. Highest rate of necrosis was also observed on treatments with 3 and 4% (w/v) of all carbon sources range between 52-100% (Figure 1d). Callus was also maintained on treatment without carbon sources and according to Giladi *et al*. (1977), stress conditions such as sucrose starvation have been shown to produce a rise in endogenous plant growth regulators which may also be involved in callus formation. In addition, highest average score of callus size recorded on treatment with 1% (w/v) glucose (2.0±1.0) followed by 1% (w/v) sucrose (1.5±0.6) and 1% (w/v) fructose (1.5±0.7). Recent study on PLBs size also recorded larger PLB sizes with the addition of glucose, sucrose and fructose compared to other carbon sources treatment (Nambiar *et al*., 2012). This was because both carbon sources especially glucose and sucrose play an essential role in acceleration cell division process by specifically enhancing cell expansion (Borisjuk *et al*., 2003).

Table 2. Effect of carbon sources on maintenance and development of *V. dearei* callus on 1/4x Mitra (Mitra *et al*., 1976) after 8 weeks of culture.

Treatments (w/v)		Percentages of callus maintain (± SD)	No. of explant develop into PLB (Mean ± SD)	Percentages of explant necrosis (± SD)	Average score of callus size (Mean ± SD)	Callus colour	Callus texture
Control		12±26.83^{bc}	28±17.89^{bcd}	68±17.9^{ab}	1.2±0.6^{bc}	B	PLB
Sucrose	1%	32±17.9^{a}	52±10.9^{a}	20±24.5^{ef}	1.5±0.6^{ab}	Y	Compact
	2%	4±8.9^{bc}	56±21.9^{a}	40±24.5^{de}	1.2±0.3^{b}	Y	PLB
	3%	0±0^{c}	48±22.8^{abc}	52±22.8^{cd}	1.1±0.4^{bc}	Y	PLB
	4%	0±0^{c}	8±10.9^{abc}	92±10.95^{ab}	0.5±0.8^{d}	B	PLB
Glucose	1%	16±16.7^{b}	72±22.8^{a}	12±17.9^{f}	2.0±1.0^{a}	Y	Compact
	2%	12±10.9^{bc}	56±21.9^{a}	32±30.3^{def}	1.6±0.7^{ab}	Y	PLB
	3%	4±8.9^{c}	24±8.9^{cde}	72±17.9^{bc}	1.2±0.5^{bc}	Y	PLB
	4%	0±0^{c}	8±10.9^{de}	92±10.9^{ab}	0.7±0.6^{cd}	B	PLB
Fructose	1%	8±10.9^{bc}	52±10.9^{ab}	40±14.1^{de}	1.5±0.7^{ab}	Y	PLB
	2%	4±8.9^{bc}	64±21.9^{a}	32±22.8^{def}	1.3±0.5^{b}	Y	PLB
	3%	0±0^{c}	0±0^{e}	100±0^{a}	0.8±0.5^{cd}	B	PLB
	4%	0±0^{c}	8±10.9^{de}	92±10.9^{ab}	0.5±0.6^{d}	B	PLB

Note: Data obtained from twenty five replicates. Data followed by the same letters are not significantly different ($p< 0.05$), based on Duncan's Multiple Range Test. SD- Standard deviation. Callus size increment scoring: 0-No size increase (0mm); 1-Size increased less than 0.5mm; 2-Size increased 1mm; 3-Size increased more than 1mm. B-Brown; Y-Yellowish; W-White; C-Compact; PLB-Protocorm-like body.

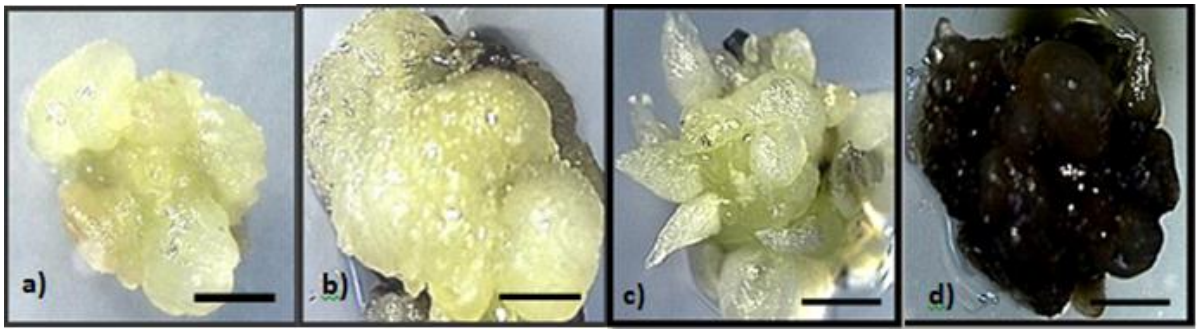

Figure 1. Maintenance and development of *V. dearei* callus (a) Sixty-day old callus induced from leaf segment of *V. dearei* were used as explants; (b) Explant maintained in callus formed after 8 weeks of culture; (c) Explant developed into PLB; (d) Explant experienced necrosis. (Bar= 1mm).

CONCLUSION

In conclusion, optimization of different types and strengths of basal media and carbon sources on maintenance and development of *V. dearei* callus had been achieved on 1/4 strength of Mitra basal medium supplemented with 1% (w/v) of sucrose, 1.0 mg/L TDZ and 0.1 mg/L NAA. This condition promotes highest percentages value of callus maintain and the lowest percentage of necrosis callus for up to 8-weeks of culture. The callus maintenance study is an important work as orchid callus culture tends to form protocorm-like body for long term maintenance and further used to initiate suspension cells culture.

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