In vitro regeneration of Borneo endemic orchid *Vanda hastifera* Rchb.f through protocorm like-bodies

Devina David[1], Bong Chung Shyan[2], Roslina Jawan[2], Hartinie Marbawi[2], Jualang Azlan Gansau[2#]

1 Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Sandakan Campus, Mile 10, Sg. Batang, 90000, Sandakan, Sabah, MALAYSIA.
2 Faculty of Science and Natural Resources, Universiti Malaysia Sabah, 88450 Kota Kinabalu Sabah, MALAYSIA.
#Corresponding author. E-Mail: azlanajg@ums.edu.my; Tel: +6088-320000

ABSTRACT A protocol for in vitro regeneration of *Vanda hastifera* via protocorm-like bodies (PLBs) induction from the leaf section was established. In this study, young leaves from the in vitro-grown seedlings of *V. hastifera* were divided into apical and basal segments and cultured on Mitra basal media. The effect of individual and combinations of plant growth regulators (PGRs) including 6-benzylaminopurine (BAP), kinetin, α-naphtaleneacetic acid (NAA), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) at different concentrations were studied. PLBs were initiated at the cut-end surfaces after 35 days of culture via direct or indirect regeneration pathways. The addition of PGRs singly promoted the formation of (PLBs) from both leaf segments, however, the combination of 4.0mg/L of BAP + 4.0mg/L of NAA obtained the highest PLBs formation (10.45 ± 2.47) from the leaf base explant. The present finding holds significant importance as it not only promotes large-scale cultivation but also contributes to the conservation of this native orchid species.

KEYWORDS: Micropropagation, Leaf explant, PLBs, NAA, BAP
Received 20 October 2023 **Revised** 8 November 2023 **Accepted** 22 November 2023 **In press** 14 December 2023 **Online** 31 December 2023

Original Article

INTRODUCTION

Orchidaceae is among the largest and most diverse families of flowering plants, estimated to contain 28, 000 species from 736 genera (Christenhusz & Byng, 2016). *Vanda hastifera* is an epiphyte orchid endemic to Borneo which can be found distributed in Mt. Kinabalu and Tambunan areas in Sabah. The flowers of this orchid are large and sweetly scented, with colour ranges from white to cream with reddish-brown blotching, and it flowers throughout the year (Chan *et al.,* 1994). Illegal collection and habitat destruction for land development have posed a significant threat to the wild orchid population (Besi *et al*., 2020; 2021; Juiling *et al*., 2020). Due to these harmful activities, many wild orchid species including *V. hastifera* have become extremely rare with some have been driven to extinction. Hence, the development of an effective conservation strategy becomes crucial to preserving and propagating orchids, not only for conservation purposes but also for horticultural exploitation, which can be achieved through techniques like plant tissue culture.

Despite the large number of orchid species in Borneo, efforts to conserve these native species through biotechnology approaches remain limited. For *Vanda* orchids, the application of plant tissue culture has been manipulated for years to conserve several rare species including *V. dearei* (Jualang *et al*., 2014), *V. helvola* (David *et al*. 2015), *V. brunnea* (Nowakowska *et al*., 2022), and *V. tessellata* (Manokari *et al*., 2022). Micropropagation through leaf sections or whole leaves has been identified as a promising approach for regeneration. This method is preferred because leaf explants are readily obtainable, less costly to the mother plant, and accessible throughout any season, in contrast to inflorescence explants (Chookoh *et al*., 2019). The in vitro regeneration from the leaf culture could be achieved via protocorm-like bodies (PLBs) formation. The term "PLBs" refers to the specific type of protocorm-producing germination, which is a characteristic feature of orchids (Cardoso *et al*., 2020). The incorporation of various types of PGRs in the culture media was beneficial to stimulate the PLBs

formation from leaf explant culture (Manokari *et al*., 2021). This approach was successfully demonstrated in *V. cristata* (Pathak *et al*., 2022) and *V. coerulea* (Jitsopakul *et al*., 2013) enabling the large-scale regeneration from leaf explants while conserving the mother plant. Considering the endangered status of the native *Vanda* spp. in Borneo, we aimed to establish a suitable protocol for in vitro regeneration of *V. hastifera* via PLBs induction from the leaf segment culture. The established protocol may be exploited for large-scale propagation of this endogenous orchid as well as to reintroduce the in vitro regenerated plants into their natural habitats.

METHODOLOGY

Plant materials

Young leaves from the in vitro-grown *V. hastifera* seedlings were sectioned into apical and base segments under sterile conditions, each measuring 0.5 cm^2 in size as explants.

Effect of singly and in combinations of PGRs on PLBs induction

In this study, the leaves segments (apical and base) were cultured on Mitra basal medium (Mitra *et al*., 1976) supplemented with individual PGRs including BAP, kinetin, NAA, IBA and IBA at 1.0, 2.0, 4.0 and 6.0 mg/l of concentrations. To see the effect of PGRs in combination, NAA and BAP were selected and tested at various concentrations. All treatments were fortified with 2 g/L of peptone and 2% (w/v) of sucrose before being adjusted to pH 5.6. Finally, the media was solidified with 0.8% (w/v) of agar (Sigma), followed by sterilization at 121 °C for 20 minutes. All cultures were maintained at the temperature of 25 ± 2 °C under 16 h of photoperiods (20-50 μmol m^{-2} s^{-1}) provided by cool white florescent tubes (Philips, Malaysia).

Data collection and statistical analysis

Five leaf explants were cultured on every plate, with each treatment consisting of five replicates. Subculturing into the fresh medium was performed at four-week intervals. After 60 days of culture, data including the days to PLBs initiations, the percentage of explant producing PLBs, the average number of PLBs produced per responsive explant, as well as the PLBs generation pathways were recorded. All data were analyzed using IBM SPSS Statistics and subjected to analysis of variance (ANOVA). Mean comparisons of the data were conducted using Duncan's multiple range tests with a significance level set at $p < 0.05$.

RESULT AND DISCUSSION

Effect of individual PGR on PLBs induction

The effect of individual PGRs including BAP, kinetin, NAA, IAA, and IBA was tested on the leaf explants (apical and base segments) at different concentrations specifically 1, 2, 4, or 6 mg/L. Medium devoid of any PGRs served as control. During this study, two regeneration pathways for protocorm-like bodies (PLBs) induction were observed (Figure 1). The first pathway is the direct formation of PLBs without the callus phase. The second pathway is the indirect pathway which involves an intermediary callus phase. When treated with BAP alone, the cut end of the leaf explant was observed to be swollen, and it was later developed into PLBs within 37 days of culture. Adding 2mg/L of BAP has promoted the highest PLBs formation at an average of three PLBs per responded explant (32%) (Table 1). Meanwhile, the addition of kinetin at 1, 2, and 4 mg/L only promoted an average of one PLB per responded explant. Treatment with individual auxin (NAA at 1 or 2 mg/L) promoted an

average of one PLB per responsive explant. Meanwhile, the addition of IAA and IBA at all concentrations failed to stimulate the formation of any PLBs.

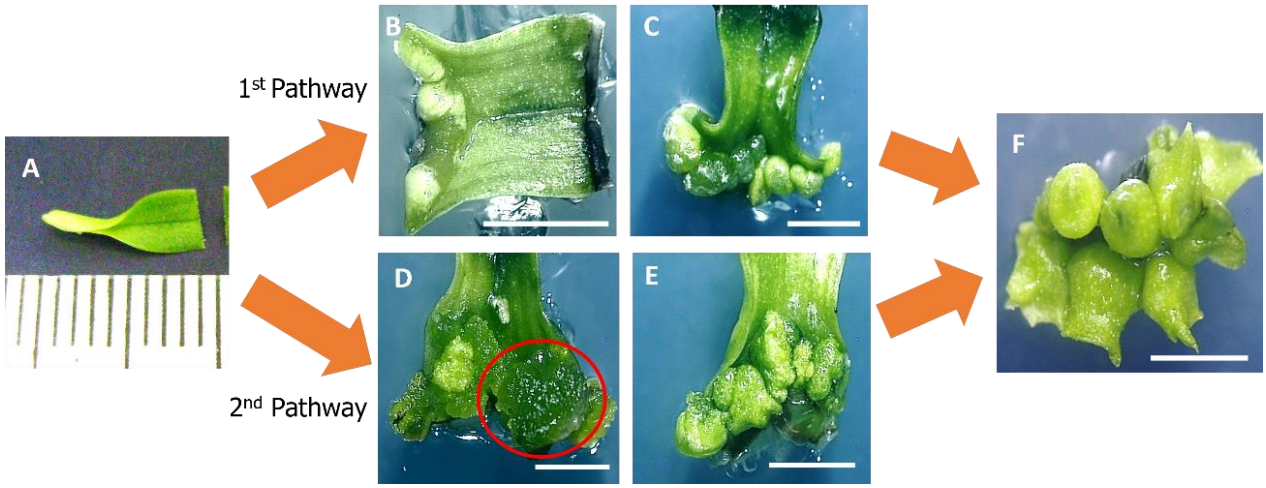

Figure 1. PLBs induction pathways from leaf base explant. A, B, C, F: Direct pathway (1st pathway); A, D, E, F: Indirect pathway with intermediary callus phase (2nd pathway). Bar: 0.5 cm

In this study, PLBs were predominantly initiated on the cut-end surface of the leaf base explant of *V. hastifera* rather than on the leaf tip explant. According to Antony *et al.* (2014), this phenomenon was attributed to the activation of previously inactive cells near the cut surface, leading to cell proliferation. The current findings are consistent with previous studies that have reported the effectiveness of leaf base section in promoting PLBs induction in certain orchid species including *V. cristata* (Pathak *et al*., 2022), *Rhynchostylis retusa* (Kumari & Pathak, 2021), and *Tolumnia* orchids (Chookoh *et al*., 2019).

Effect of PGRs in combinations

To increase the PLBs induction from both leaf segments of *V. hastifera*, the combination of BAP and NAA was tested at different concentrations of 1, 2, 4, and 6 mg/L. The result revealed that, although PLB initiation was observed in both leaf segments, it was found that the culture of the leaf base yielded more favourable results compared to the use of leaf tip explants. When using leaf bases as explants, the combination of 4.0 mg/L NAA + 4.0 mg/L BAP promoted an average of 10 PLBs within 60 days of culture (Table 2). On the other hand, the culture of leaf apical explant only produced an average of six PLBs on the same treatment (4.0 mg/l NAA + 4.0 mg/l BAP). All treatments have promoted the PLBs formation via an indirect regeneration pathway which involved an intermediary callus phase.

The concentration range between auxin and cytokinin is essential for the success of PLBs induction (Zakaria *et al*., 2021). The current finding is in line with Pathak *et al*. (2022) that the combination of 8.8 µM of BAP + 10.6 µM of NAA has successfully promoted in vitro regeneration from the leaf base culture of *V. cristata*. Previously, the combination of BAP and NAA was also reported beneficial in promoting PLBs induction from the leaf culture of *Rhynchostylis gigantea* (Pathak *et al*., 2017), *Aerides multiflora* (Bhowmik & Rahman, 2020) and *Vanilla planifolia* (Malhotra *et al*., 2021).

Table 1. Effects of individual PGR on PLBs induction from leaf base and apical segments of *V. hastifera*, cultured on Mitra (Mitra *et al*., 1976) medium under 16 h photoperiod at 25±2 °C for 60 days.

PGRs concentration (mg/L)		Explant-producing PLBs (%)		PLBs per responsive explant (Mean ± SD)		PLBs induction (days)		PLBs induction pathway	
		apical	base	apical	base	apical	base	apical	base
Control	0	0	0	0	0	-	-	-	-
BAP	1.0	16^{abc}	20^{abc}	2.20 ± 2.05^{a}	1.70 ± 1.48^{ab}	37	37	1st	1st
	2.0	16^{abc}	32^{a}	0.90 ± 0.89^{ab}	3.15 ± 2.98^{a}	37	37	1st	1st
	4.0	0	24^{ab}	0	1.70 ± 1.20^{ab}	-	37	-	1st
	6.0	0	12^{abc}	0	2.20 ± 2.05^{ab}	-	40	-	2nd
Kinetin	1.0	12^{abc}	12^{abc}	1.00 ± 0.9^{ab}	1.20 ± 1.10^{ab}	40	37	2nd	1st
	2.0	8^{bc}	16^{abc}	1.00 ± 0.9^{ab}	1.50 ± 1.50^{ab}	40	37	2nd	1st
	4.0	0	8^{bc}	0	1.60 ± 1.41^{ab}	-	40	-	2nd
	6.0	0	4^{c}	0	0.40 ± 0.29^{b}	-	37	-	2nd
NAA	1.0	16^{abc}	16^{abc}	1.20±1.10^{ab}	1.10±1.02^{ab}	45	45	2nd	2nd
	2.0	8^{bc}	12^{abc}	0.60±0.55^{b}	0.60±0.55^{b}	45	45	2nd	2nd
	4.0	0	4^{c}	0	0.40±0.39^{b}	-	45	-	2nd
	6.0	0	0	0	0	-	-	-	-
IAA	1.0	0	0	0	0	-	-	-	-
	2.0	0	0	0	0	-	-	-	-
	4.0	0	0	0	0	-	-	-	-
	6.0	0	0	0	0	-	-	-	-
IBA	1.0	0	0	0	0	-	-	-	-
	2.0	0	0	0	0	-	-	-	-
	4.0	0	0	0	0	-	-	-	-
	6.0	0	0	0	0	-	-	-	-

Mean values within a column followed by the same letters are not significantly different at $p<0.05$ according to Duncan's Multiple Range Test; n=5 per treatment, conducted in 5 replicates. SD= Standard Deviation. 1st pathway: Direct pathway; 2nd pathway: Indirect pathway

CONCLUSION

Traditionally, plant tissue culture techniques have been used for asymbiotic seed germination in many orchid species, leading to the development of heterozygous plants. Therefore, the establishment of in vitro regeneration protocols for orchids from various vegetative explants is essential to maintain genetic stability. The current study has established a straightforward and efficient method for inducing PLBs from *V. hastifera* leaf segments. Both leaf segments responded to PLBs initiation, however, the leaf base culture produced better results. The incorporation of 4.0 mg/L of BAP + 4.0 mg/L of NAA in Mitra basal medium has resulted in the highest number of PLBs generated from the leaf base culture of *V. hastifera* after 60 days maintained under 16 h photoperiod. These findings underscore the effectiveness of leaf base culture and the fortification of NAA and BAP for the large-scale propagation of this native orchid.

Table 2. Effects of BAP and NAA in combinations on PLBs induction from leaf base and apical segments of *V. hastifera*, cultured on Mitra (Mitra *et al*., 1976) medium under 16 h photoperiod at 25±2 °C for 60 days.

Treatment (mg/L)		Explant-producing PLBs (%)		No. PLBs per responsive explant (mean ± SD)		Days to PLBs formation (days)		PLBs induction pathway	
NAA	BAP	Apical	Base	Apical	Base	Apical	Base	Apical	Base
1.0	1.0	5de	45^{b}	1.50±1.00^{bcd}	4.46±1.73^{b}	39	39	2nd	2nd
1.0	2.0	5de	43^{b}	1.50±1.00^{bcd}	4.13±2.85^{b}	39	35	2nd	2nd
1.0	4.0	12^{cde}	64^{ab}	3.00±2.83^{abcd}	8.03±2.68^{ab}	38	35	2nd	2nd
1.0	6.0	16^{bcde}	48^{b}	4.20±3.90^{abc}	8.22±3.30^{ab}	38	35	2nd	2nd
2.0	1.0	12^{cde}	44^{b}	3.60±3.36^{abcd}	4.37±2.53^{b}	38	35	2nd	2nd
2.0	2.0	4de	48^{b}	0.40±0.29^{cd}	6.03±3.41^{ab}	38	35	2nd	2nd
2.0	4.0	8^{cde}	68^{ab}	0.60±0.50^{cd}	7.90±2.54^{ab}	38	35	2nd	2nd
2.0	6.0	20^{abcde}	72^{a}	3.40±2.70^{abcd}	8.40±2.88^{ab}	38	35	2nd	2nd
4.0	1.0	40^{a}	55^{ab}	4.29±1.16^{abc}	7.73±4.65^{ab}	38	35	2nd	2nd
4.0	2.0	16^{bcde}	60^{ab}	2.70±2.54^{abcd}	6.05±5.35^{ab}	38	35	2nd	2nd
4.0	4.0	36^{ab}	64^{ab}	6.27±3.59^{a}	10.45±2.47^{a}	38	35	2nd	2nd
4.0	6.0	8^{cde}	60^{ab}	1.80±1.69^{bcd}	7.90±2.96^{ab}	38	35	2nd	2nd
6.0	1.0	28^{abc}	44^{b}	5.30±3.67^{ab}	8.43±2.76^{ab}	38	35	2nd	2nd
6.0	2.0	20^{abcde}	40^{b}	1.90±1.82^{bcd}	6.90±2.04^{ab}	38	35	2nd	2nd
6.0	4.0	24^{abcd}	48^{b}	3.40±1.14^{abcd}	6.17±3.07^{ab}	38	35	2nd	2nd
6.0	6.0	40^{a}	68^{ab}	2.93±1.49^{abcd}	7.93±1.69^{ab}	38	35	2nd	2nd

Mean values within a column followed by the same letters are not significantly different at $p<0.05$ according to Duncan's Multiple Range Test; n=5 per treatment, conducted in 5 replicates. SD= Standard Deviation. 1st pathway: Direct pathway; 2nd pathway: Indirect pathway

ACKNOWLEDGEMENTS

We would like to express our gratitude to Mr. Jain Linton and Mr. Samuel Tihoi from Taman Pertanian Sabah Lagud Sebrang, Tenom, for providing the orchid pods. The in vitro-grown seedlings of *V. hastifera* featured in this study were derived from this invaluable resource.

REFERENCES

[1] Antony, J. J. J., Sundarasekar, J., Rathinam, X., Subramaniam, S. & Marimuthu, K. 2014. Microscopical analysis of in vitro Mokara Broga giant orchid. *Emirates Journal of Food and Agriculture*, 26(1), 73-81.

[2] Besi, E. E., Nikong, D., Justine, V. T., Nordin, F. A., Mus, A. A., Nelson, H. V., Mohamad, N. N., Rusdi, N. A. & Go, R. 2020. Preliminary Checklist of Orchids in the Sungai Kangkawat, Imbak Canyon Conservation Area (ICCA), Sabah, Malaysia. *Journal of Tropical Biology and Conservation*, 17, 49–63.

[3] Besi, E. E., Nikong, D., Mus, A. A., Nelson, H. V., Mohamad, N. N., Ombokou, R., Rusdi, A., David, D., Aziz, Z. A. & Go, R. 2021. A Species Checklist of Wild Orchids in Selected Sites in Kadamaian, Kota Belud, Sabah. *Journal of Tropical Biology and Conservation*, 18, 131–147.

[4] Bhowmik, T. K. & Rahman, M. M. 2020. In vitro study of medicinally important orchid *Aerides multiflora* Roxb. from nodal and leaf explants. *Journal of Pharmacognosy and Phytochemistry*, 9(4), 179-184.

[5] Cardoso, J. C., Zanello, C. A. & Chen, J. T. 2020. An overview of orchid protocorm-like bodies: Mass propagation, biotechnology, molecular aspects, and breeding. *International Journal of Molecular Sciences*, 21(3), 985.

[6] Chan, C. L., Lamb, A., Shim, P. S. & Wood, J. J. 1994. *Orchids of Borneo. Vol. 1. Introduction and a Selection of Species.* The Sabah Society, Kota Kinabalu and Royal Botanic Gardens Kew, England.

[7] Christenhusz, M. J. & Byng, J. W. 2016. The number of known plants species in the world and its annual increase. *Phytotaxa*, 261(3), 201-217.

[8] Chookoh, N., Chiu, Y. T., Chang, C., Hu, W. H. & Dai, T. E. 2019. Micropropagation of Tolumnia orchids through induction of protocorm-like bodies from leaf segments. *Hortscience*, 54(7), 1230-1236.

[9] David, D., Jawan, R., Marbawi, H. & Gansau, J. A. 2015. Organic additives improves the in vitro growth of native orchid *Vanda helvola* Blume. *Notulae Scientia Biologicae*, 7(2), 192-197.

[10] Jitsopakul, N., Thammasiri, K., Ishikawa, K., Wannajuk, M., Sangthong, P., Natapintu, S. & Won-In, K. 2013. Efficient adventitious shoot regeneration from shoot tip culture of *Vanda coerulea*, a Thai orchid. *ScienceAsia*, 39(5), 449-455.

[11] Jualang, A. G., Devina, D., Hartinie, M., Sharon, J. S. & Roslina, J. 2014. Asymbiotic seed germination and seedling development of *Vanda dearei*. *Malaysian Applied Biology*, 43(2), 25-33.

[12] Juiling, S., Lee, Y. L. & Khoo, E. 2020. Conservation assessment and spatial distribution of endemic orchids in Sabah, Borneo. *Nature Conservation Research*, 5(1), 136–144.

[13] Kumari, A. & Pathak, P. 2021. De novo plantlet regeneration from leaf explant of *Rhynchostylis retusa* (l.) Blume: a study in vitro. *Journal of Orchid Society India*, 35, 47-53

[14] Malhotra, E. V., Bansal, S., Pathania, P., Mali, S. C. & Agrawal, A. 2021. Regeneration of plantlets through protocorm-like bodies: An alternative method for vanilla micropropagation. *Indian Journal of Biotechnology*, 20(4), 388-395

[15] Manokari, M., Priyadharshini, S., Cokulraj, M., Dey, A., Faisal, M., Alatar, A. A., Alok, A. & Shekhawat, M. S. (2022). Assessment of cell wall histochemistry of velamentous epiphytic roots in adaptive response of micropropagated plantlets of *Vanda tessellata* (Roxb.) Hook. ex G. Don. *Plant Cell, Tissue and Organ Culture*, 149(3), 685-696.

[16] Manokari, M., Priyadharshini, S. & Shekhawat, M. S. 2021. Direct somatic embryogenesis using leaf explants and short term storage of synseeds in *Spathoglottis plicata* Blume. *Plant Cell, Tissue and Organ Culture*, 145(2), 321-331.

[17] Mitra, G. C., Prasad, R. N. & Roychowdhury, A. 1976. Inorganic salts and differentiation of protocorms in seeds callus of orchids and correlative changes in its free amino acid content. *Indian Journal Experimental Biology*, 14, 350-351.

[18] Nowakowska, K., Marciniak, P. & Pacholczak, A. 2022. A protocol for efficient micropropagation of rare orchid *Vanda brunnea* Rchb. f. *South African Journal of Botany*, 150, 233-239.

[19] Pathak, P., Verma, S., Prakash, A. & Mahant, K. C. 2017. Regeneration competence of an ornamentally important epiphytic orchid, *Rhynchostylis gigantea* (Lindl.) Ridl. through leaf segments: A study in vitro. *The Journal of the Orchid Society India*, 31, 97-101.

[20] Pathak, P., Kumari, A. & Thakur, B. 2022. Regeneration competence of an endangered orchid, *Vanda cristata* Wall. ex Lindl. using leaf explants: A study in vitro. *South African Journal of Botany*, 151, 1018-1024.

[21] Zakaria, S., Antony, J.J.J., Chin, C.K., Zakaria, R. & Subramaniam, S. 2021. Development of in vitro culture system for proliferation of protocorm-like bodies of *Oncidium* Golden Anniversary orchid. *Songklanakarin Journal of Science & Technology*, 43(6), 1542-1549.