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Research Article

Optimizing the potential utilization of bioreactors for the mass propagation of Indonesian *Dendrobium* varieties

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Abstract

The study focuses on two Indonesia *Dendrobium* varieties, *D*. Dian Agrihorti (DDA) and *D*. Syifa Agrihorti (DSA), which have potential to be commercialized, but face limitations on the availability of qualified seedlings sustainably. The research aimed to establish an *in vitro* propagation protocol using a Temporary Immersion System (TIS) to produce high-quality seedlings efficiently. Various factors, including varieties, media, plant growth regulators, anti-phenol compounds, organic additives, and TIS settings, were investigated using the basal part of the plantlet as the explant source. Key findings revealed that DDA outperformed DSA across all observed variables. In the initiation phase, basal plantlets cultured in Murashige and Skoog (MS) medium supplemented with 1.0 mg L^{-1} thidiazuron and 0.5 mg L^{-1} N-6 benzylaminopurine enhanced embryogenic callus (EC) formation, with a 13.5-day initiation period, 72% potential explant growth, 0.41 cm callus size, and a 3.45 rate of multiplication. During the proliferation stage, the addition of 150 mg L^{-1} ascorbic acid (AC) and the application of a TIS with a 30-minute dry period and a 15-minute wet period resulted in a 515.5% increase in EC fresh weight for DDA accompanied by a 6.16 multiplication rate. Regeneration of shoots was achieved using a Vacin and Went medium with 150 g L^{-1} banana extract, yielding 29.2 shoots per clump. Subsequent rooting of the shoots in 2 g L^{-1} Hyponex® medium with 20 g L^{-1} sugar and 2% AC proved successful. Acclimatization of plantlets with *Cycas rumpii* bulk demonstrated a 100% survivability rate. The established propagation protocol for DDA holds significant potential for application to other Dendrobium varieties, offering a sustainable and efficient method for meeting commercial demands in the Indonesian market.

Keywords: anti-phenol compounds; callus; media; orchid; organic additive; proliferation; temporary immersion system (TIS)

Introduction

Orchids are an important ornamental commodity in Indonesia, especially for *Dendrobium*. The *Dendrobium* is widely cultivated mainly in West Java, and West Kalimantan, with total production of up to

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6.79 million stems, 16.27 ha total cultivation area, US$ 75.4 thousand export value, US$ 371.75 thousand import value (Setiawati, 2022). The *Dendrobium* cut flowers were sold for IDR 34,000 - 42,000 per stem depending on their quality (Ganiyyu, 2023; Khanza, 2023) and dominantly derived from imported hybrids. The national *Dendrobium* cultivars such as *D.* Dian Agrihorti and *D*. Syifa Agrihorti possess commercial potential. However, their penetration into the local market is constrained by the scarcity of sustainably qualified seedlings. Therefore, to increase domestic production and reduce imported, substantial support was provided for accelerating the commercialization of *D.* Dian Agrihorti and *D*. Syifa Agrihorti varieties, especially in preparing sustainable qualified seedlings.

Traditionally, *Dendrobium* is propagated by seed, separating tillers, and keikies with limited offspring (Kilpatrick, 2012; Nasirudin *et al*., 2003; Martin *et al*., 2005; Teixeira da Silva *et al*., 2015). These methods did not support the industrial scaling up for the *Dendrobium*. Therefore, developing a suitable *in vitro* mass propagation protocol to accelerate the commercialization of *Dendrobium* is important. Effective micropropagation protocols were reported using different explants, media, and liquid culture systems with varied results. Winarto (2012) and Winarto and Rachmawati (2013) used shoot tip as an explant source, half-strength Murashig and Skoog (MS), and Growmore as basal medium and liquid culture. Orbital shaker was used as culture system for *D.* Gradita 31, and *D*. Indonesia Raya (Rachmawati *et al*., 2015). The combination of shoot tips explant, half strength MS medium, and airlift bioreactor were applied for *D.* Zahra FR 62, *D*. Gradita 10, *D*. Indonesia Raya Ina, and *D*. Balithi CF22-58 (Winarto *et al*., 2013; Rachmawati *et al*., 2014, 2016a, 2019, 2022). While, the combination of basal part of plantlets as an explant, half strength MS medium, and airlift bioreactor for *D. lasianthera* x *D. antennatum* (Sasmita *et al.*, 2022). However, there is no report on *D.* Syifa Agrihorti and *D*. Dian Agrihorti. Furthermore, while *in vitro* propagation through liquid culture accelerated the proliferation of embryogenic callus better than solid culture, browning incidence frequently occurred as the main problem (Amente and Chimdessa, 2021). The issue was reported in the *in vitro* culture of *D*. Gradita 31 (Winarto, 2012; Winarto and Rachmawati, 2013), *D.* Zahra FR 62 (Winarto *et al*., 2013), and *D. crumenatum* (Kaewubon et al., 2015). Therefore, the establishment of an *in vitro* propagation protocol for *D.* Syifa Agrihorti and *D*. Dian Agrihorti is explored in the research placing a heightened focus on optimizing embryogenic callus (EC) initiation, addressing challenges related to browning, and successfully regenerating EC.

Culture initiation is the initial stage of starter culture preparation, a crucial step in mass multiplication using a bioreactor system. Several critical aspects existed in establishing a mass propagation protocol for *Dendrobium* via somatic embryogenesis in the initiation stage. Optimal EC initiation using a basal part explant was determined in a half-strength MS medium containing 1.5 mg L^{-1} TDZ and 0.5 mg L^{-1} BAP (Rachmawati *et al*., 2019, 2022). Overcoming browning incidence while maintaining and improving EC growth under a liquid culture system is a crucial issue tackled during the proliferation stage. According to Amente and Chimdessa (2021), the incidence can be reduced by incorporating anti-phenolic compounds into the medium. (Rittirat *et al*., 2012) successfully applied 0.2% activated charcoal (AC) for *Phalaenopsis cornucervi (Breda) Blume and Rchb. f.* and 0.2 % AC for *Cymbidium aloifolium* (Nongdam and Chongtham, 2011). Ascorbic acid (AA) in 10 mg L^{-1} with 2 g L^{-1} of AC was applied in *Paphiopedilum insigne* (Poniewozik *et al*., 2022). Though the application of polyvinylpyrrolidone (PVP) was proved to reduce browning in sugarcane at 0.2-0.3 g L^{-1} (Shimelis *et al*., 2015), 0.3 g L^{-1} for Hongyang kiwi fruit and 0.5 g L^{-1} for *Paeonia lactiflora* (Amente and Chimdessa, 2021), however, there was no report of PVP application in orchids. Furthermore, the application of amino acids like glutamine can stimulate embryogenic callus formation and development (Daniel *et al.*, 2018) was also noted for *Phalaenopsis* Raiza Agrihorti at 150 mg L^{-1} (Rachmawati *et al*., 2021). In addition, using a temporary immersion bioreactor system (TIS-bioreactor) provides an opportunity to overcome the browning problem due to continuous immersion in the airlift bioreactor. Reducing browning explants and improving EC proliferation under a temporary immersion system (TIS) bioreactor were successfully carried out by applying 1 min/4 h dried and immersion frequencies for *C. forbesii* Lindl (Ekmekçigil *et al*., 2019); 5 min/4 h for *Epipactis flava* Seidenf (Kunakhonnuruk *et al*., 2019); 5 min/6 h for *Dendrobium nobile* Lindl (Zhang *et al*., 2020); and 1 min/2 h for *Mochara* orchid (Minh, 2022). Banana extract (BE) noted had optimal effect on regeneration and preparation of well rooted shoots (Nurfadilah *et al*., 2018; Sidhu and Zafar, 2018; Novita *et al*., 2022) with 50 g L^{-1} for *Cymbidium pendulum* (Kaur and Bhutani, 2012), 100 ml L^{-1} for *Dendrobium* sp. (Islam *et al*., 2016), 150 g L^{-1} for *D. lasianthera* (Utami *et al*., 2016), and 100 g L^{-1} for *D. lineale* (Mustika and Semiarti, 2021). These results confirmed that AC, AA, PVP, Glutamine, and BE had high potential applied in establishing *in vitro* protocol for *D.* Syifa Agrihorti and *D*. Dian Agrihorti dealing with browning and EC regeneration.

Therefore, the objective of the research was to establish an *in vitro* mass propagation protocol for *D.* Syifa Agrihorti and *D*. Dian Agrihorti by examining the effect of genotypes, basic media, plant growth regulators, anti-phenolic compounds, organic additives, and utilizing bioreactor system in proliferation stage. We expected an effective *in vitro* mass propagation protocol for *D.* Syifa Agrihorti and *D*. Dian Agrihorti to be determined and potentially applied to other varieties.

Materials and Methods

Preparation of plantlets as an explants source

Two Indonesian *Dendrobium* varieties, i.e., *D.* Syifa Agrihorti (Figure 1A) and *D*. Dian Agrihorti (Figure 1B), were used in the experiments. Healthy and vigorous shoots (7-10 cm) were harvested from the two donor plants (Figure 1C). The preparation of plantlets as an explants source followed the method described by Rachmawati *et al*. (2019). Periodical subcultures were carried out until the number of explant sources for the next experiment was available (Figure 1D).

Embryogenic callus initiation of two Indonesian Dendrobium varieties on different initiation media

Embryogenic callus (EC) was initiated by isolating the basal part of the plantlet under a stereo microscope (Stemi SV8 Zeiss, Germany) (Figure 1E). Randomized Completely Block Design (RCBD) with four replications was used in the experiment. The first factor was two Indonesian *Dendrobium* varieties, i.e. (1) *D*. Syifa Agrihorti and *D.* Dian Agrihorti. The second factor was initiation medium e.i. IM-1: ½ MS supplemented with 0.75 mg L^{-1} TDZ, and 0.25 mg L^{-1} BAP; IM-2: ½ MS containing 1.0 mg L^{-1} TDZ and 0.5 mg L^{-1} BAP; IM-3: MS fortified by 0.75 mg L^{-1} TDZ and 0.25 mg L^{-1} BAP; and IM-4: MS augmented with 1.0 mg L^{-1} TDZ and 0.5 mg L^{-1} BAP. All media were added with 20 g L^{-1} sucrose and 2 mg L^{-1} gelrite.

The callus regenerated in the experiment was observed four weeks after culture (Figure 1F). Variables observed were : (1) explant browning (%), by calculating the number of explants browning divided by the total cultured explants multiplied by 100% (2) callus initiation times (days), calculated from the start of the explant being cultured until EC is formed; (3) explant grow potential (%), by calculating the number of explants that form EC divided by the total cultured explants multiplied by 100% ; (4) callus growth in size, EC diameter was measured one month after EC was initiated, and (5) EC multiplication rate (times), calculated by dividing the average of final EC fresh weight with an initial EC fresh weight. Fast growth, green, and friable EC (Figure 1G) established from the stage were chosen as culture materials for the next experiment. One gram of this EC was periodically subcultured in 25 mL of liquid ½ MS medium fortified by 0.5 mg L^{-1} TDZ and 0.1 mg L^{-1} BAP (Figure 1H).

The proliferation of embryogenic callus in airlift bioreactor systems with different anti-phenolic compounds

The starter culture used for the proliferation stage was EC-derived and prepared from the previous experiment (Figure 1H). The experiment used a two-factor RCBD with three replications. The first factor was the EC of two *Dendrobium* varieties. The second factor was the application of anti-phenolic compounds of (1) 150 mg L^{-1} proline (as control) (Merck, Germany), (2) 1.5 g L^{-1} activated charcoal (AC) (Merck, Germany), (3) 150 mg L^{-1} ascorbic acid (AA) (Merck, Germany) and (4) 150 mg L^{-1} polyvinyl pyrrolidone (PVP) (Merck, Germany) in ½-MS medium containing 0.5 mg L^{-1} TDZ, 0.1 mg L^{-1} BAP, and 20 g L^{-1} sucrose. About 10 g of EC were cultured in a 500 mL airlift bioreactor (Favorit 3000 mL Schott Duran, Germany), *air flow meter* (Dwyer RMA-14-SSV, USA), compressor (Rocker 320, USA) filled with a 150 mL treatment media.

Proliferation of embryogenic callus on modification of bioreactor culture systems

The experiment used a two-factor RCBD with three replications. The first factor was two *Dendrobium* varieties, and the second was five bioreactor systems, i.e., airlift bioreactor with 10 g per 150 mL^{-1} media and five vessel volume $minute^{-1}$ (vvm) aeration rate as control (Bios-1); temporary immersion system (TIS) with 15 minutes dry period (MDP) and 15 minutes wet period (MWP) (Bios-2), TIS with 15 MDP and 30 MWP (Bios-3), TIS with 30 MDP and 15 MWP (Bios-4), and TIS with 30 MDP and 30 MWP (Bios-5) (modification of *airlift bioreactor* Favorit 3000 mL Schott Duran, Germany), *air flow meter* (Dwyer RMA-14-SSV, USA), compressor (Rocker 320, USA). About 20 g of EC were cultured in a bioreactor with a capacity of 500 mL using ½-MS medium containing 0.5 mg L^{-1} TDZ, 0.1 mg L^{-1} BAP, 150 mg L^{-1} L-Proline, and 20 g L^{-1} sucrose.

The variables observed at the EC proliferation in the bioreactor were: (1) explant browning (%); (2) increased EC fresh weight (g); (3) EC weight improvement (%), calculated by decreasing the average of final EC fresh weight with initial EC fresh weight timed by 100%; (4) EC multiplication rate (times), calculated by dividing the average of final EC fresh weight with an initial EC fresh weight. EC was cultured in a bioreactor for four weeks, and the growth response was recorded.

Regeneration of D. Dian Agrihorti embryogenic callus on different basic media and organic additives

Callus was derived from the proliferation stage in the TIS bioreactor used as material for this study. Two basic media were tested, i.e., ½ MS and Vacin and Went (VW). The organic additives investigated in the experiment were banana extract (BE) and corn extract (CE) in different concentrations, i.e., (1) 150 g L^{-1} of BE, (2) 150 g L^{-1} CE, (3) 100 g L^{-1} BE, and 50 g L^{-1} CE, (4) 50 g L^{-1} BE and 100 g L^{-1} CE. 20 g L^{-1} sugar and 7 g L^{-1} agar were added to all media. The experiment used a two-factor RCBD, and each treatment consisted of three bottles with ten callus clumps (± 0.5 cm in size) per bottle. Variables observed were: (1) clump regeneration (%), calculated by the number of growing callus clumps (new callus and initial shoots) divided by the number of cultivated clumps times by 100%; (2) regenerated-leaf length (cm); (3) initial shoots per clump; and (4) morphogenesis of a clump (%), calculated by the number of callus clumps that regenerate into shoots divided by the number of cultured callus clumps times by 100%. The Growth and development of the culture and any changes that occurred during the incubation period were noted every week. The mean of each observed data was calculated from every observation period of 12 weeks.

Incubation of cultures

Throughout all stages of the experiment, all cultures were subjected to illumination from a fluorescent lamp with an intensity of 2407 Lux for 12 hours daily (SL-Shinyoku, Indonesia; measured using a lux meter (Lutron LX 101, Taiwan). The environmental conditions maintained included a temperature of 23.5 ± 1 °C and a relative humidity of 60% to 70%.

Plantlet acclimatization

Germinated ES with 2-3 leaves and ± 1 cm in height derived from the previous experiment were sub-cultured onto 2 g L^{-1} Hyponex® (20N-20P-20K-TE) (Nusa Tani Ltd., Indonesia) medium containing 20 g L^{-1} sugar, 7 g L^{-1}, agar and 2% AC for plantlet preparation (Figure 1K). Plantlets with 2-4 roots, 3-5 leaves, and 4-5 cm height were hardened in a greenhouse for four weeks before acclimatization (Figure 1L). Plantlets were then taken out from the bottles. Roots were washed using tap water, immersed in 2% fungicide, and bactericide solutions (Benlox®50 WP, Dharma Guna Wibawa Ltd. and Agrept®20WP, Mastalin Mandiri Ltd., Jakarta, Indonesia) for 5 minutes, and air-dried. About 30 plantlets were planted in a compot style in a plastic tray containing *C. rumpii* media. After a week, acclimatized plantlets were watered every morning and NPK (20:20:20) (Nusa Tani Ltd, Indonesia) was applied once a week. The number of survival plantlets was noted eight weeks after acclimatization (Rachmawati *et al*., 2019) (Figure 1M).

Data analysis

Data were analysed using analysis of variance (ANOVA) with SAS version 9.4 (SAS, 2023). The significant difference in the mean value of the treatment was further tested using Tukey's Honestly Significant Difference (HSD) with a 95% confidence level.

Results

Embryogenic callus initiation of two Indonesian Dendrobium varieties on different initiation media

Based on periodic observation, there were differences in the growth response of the basal part of plantlets from the two Indonesian *Dendrobium* varieties cultured in different initiation media (Table 1). The percentage of browning explant varied from 22-46% with 54-78% explant growth. Initiation of EC was observed at 13.5-24.3 days after culture with 0.16-0.49 cm in callus size and 2.43-3.45 multiplication rate. The best results were obtained from the basal part explant sliced from *D*. Dian Agrihorti plantlets cultured in IM-4 medium (Figure 1F). The basalt part explant was the bottom part of plantlets after removing all leaves and roots (Figure 1E). The part looks like a disc, where the outer layer easily browns, and the inner layer produces EC (Figure 1D). Basal part explant produced 74-93% EC with 5-16% of embryos and 1-10% shoots.

Figure 1. *In vitro* propagation protocol of *D.* Syifa Agrihorti and *D.* Dian Agrihorti from initiation to acclimatization stages. (A) Inflorescence of *D.* Syifa Agrihorti, (B) Inflorescence of *D.* Dian Agrihorti, (C) *Dendrobium* lateral shoot used as explant source, (D) 24 weeks after culture (WAC) plantlet from initiation stage, (E) basal part explant (0.3-0.5 cm in size) from 24 WAC plantlet, (F) eight WAC EC on MS medium containing 1.0 mg L^{-1} TDZ and 0.5 mg L^{-1} BAP, (G) initial proliferation of ECs by subculture periodically once month on ½ MS medium containing 0.75 mg L^{-1} TDZ and 0.25 mg L^{-1} BAP, (H) proliferation and adaptation of EC in liquid ½-MS fortified by 0.5 mg L^{-1} TDZ and 0.1 mg L^{-1} BAP (MP) for four weeks, (I) EC proliferation in *airlift* bioreactor containing MP medium with 150 mg L^{-1} ascorbic acid for four weeks of culture, (J) germinated EC of *D.* Dian Agrihorti four weeks after subculture, (K) eight WAC germinated embryos on VW supplemented with 150 g $L^{-1}$of banana extract, (L) eight WAC plantlets on 2 g L^{-1}Hyponex® containing 20 g L^{-1} sugar, 7 g L^{-1}agar and 2 % activated charcoal, and (M) eight weeks after acclimatization plants in *C. rumpii* medium planted in plastic tray. Scale bar: A-D, H-I, and L-M= 1 cm; E-G and J-K= 0.5 cm.

The first experiment revealed that the two Indonesian *Dendrobium* varieties and four IMs had a high effect on the EC initiation in all variables observed; however, there is no interaction effect between the two treatments. The basal parts harvested from *D.* Dian Agrihorti have better responses in the EC initiation. The explant produced less explant browning explant (22%), faster EC initiation (14.3 days after the initiation period), a higher potential explant growth (78%), bigger size of EC (0.49 cm on average), and a higher rate of multiplication (3.3 times) compared to the explant from *D.* Syifa Agrihorti (Table 1; Figure 1G). While IM-4 was the most suitable medium for EC initiation with 28% explant browning, 13,5 days EC initiation time, 72% explant growth, 0,41 cm EC size, and 3.45 rate of EC multiplication (Table 2).

Table 1. Initiation responses of basal part explants derived from *D*. Syifa Agrihorti dan *D*. Dian Agrihorti

Varieties	Explant browning (%)	EC initiation times (days)	Explant growth potential (%)	EC growth in size (cm)	EC multiplication rate (times)
D. Syifa Agrihorti	46.00±5.01 a	21.80±4.67 a	54.00±5.10 b	0.16±0.10 b	2.60±0.49 b
D. Dian Agrihorti	22.00±5.06 b	14.30±4.57 b	78.00±4.84 a	0.49±0.10 a	3.33±0.52 a
Tukey's value	3.14	1.43	3.30	0.04	0.29
p	<.0001**	<.0001**	<.0001**	<.0001**	<.0001**

Means followed by the same letter in the same column for varieties are not different based on Tukey's; $p<0.05$. *Abbreviations: ECs= embryogenic callus.*

Table 2. Responses of basal part explants derived from *D*. Dian Agrihorti cultured on different initiation media

Initiation Media	Explant browning (%)	EC initiation times (days)	Explant growth potential (%)	EC growth in size (cm)	EC multiplication rate (times)
IM-1	38.00±14.04 a	24.30±4.28 a	62.00±13.52 b	0.28±0.18 b	2.43±0.40 c
IM-2	34.00±13.45 ab	15.00±4.60 c	66.00±13.58 ab	0.42±0.18 a	3.18±0.37 ab
IM-3	36.00±13.22 a	19.57±4.16 b	64.00±13.42 b	0.21±0.19 b	2.80±0.75 bc
IM-4	28.00±13.52 b	13.50±4.19 c	72.00±13.62 a	0.41±0.18 a	3.45±0.44 a
Tukey's value	6.01	2.74	6.33	0.07	0.57
p	<.0001**	<.0001**	<.0001**	<.0001**	<.0001**

Note: IM-1, ½ MS supplemented with 0.75 mg L^{-1} TDZ, and 0.25 mg L^{-1} BAP; IM-2, ½ MS containing 1.0 mg L^{-1} TDZ and 0.5 mg L^{-1} BAP; IM-3, MS fortified by 0.75 mg L^{-1} TDZ and 0.25 mg L^{-1} BAP; and IM-4, MS augmented with 1.0 mg L^{-1} TDZ and 0.5 mg L^{-1} BAP. Means followed by the same letter in the same column for varieties and initial media are not different based on Tukey's; $p<0.05$. Abbreviations: EC= embryogenic callus.

The proliferation of embryogenic callus in airlift bioreactor systems with different anti-phenolic compounds

Applying anti-phenolic compounds for the two Indonesian *Dendrobium* varieties in the proliferation stage influenced EC's behaviour and growth pattern in an airlift bioreactor. The two treatments also exhibited interaction effects in all variables observed. High browning EC remained the main problem during their proliferation. The percentage of EC browning varied from 33-59% for *D.* Syifa Agrihorti and decreased to 27-40% for *D*. Dian Agrihorti. Applying PVP and AA successfully reduced the percentage of browning up to 31% and 20%, respectively. AA in 150 mg L^{-1} for *D.* Dian Agrihorti proliferated EC with 26.8% browning, 31 g increase of EC fresh weight, 310% EC weight improvement, and 4.10 times multiplication rate. The 150 mg L^{-1} PVP for *D.* Syifa Agrihorti resulted in lower proliferation, with the lowest results noted on medium-free anti-phenolic compounds for both varieties (Table 3).

Table 3. The effect of anti-phenolic compounds on embryogenic callus (ECs) proliferation of *D*. Syifa Agrihorti and *D*. Dian Agrihorti in airlift bioreactor after four weeks in culture

Varieties	Anti-phenolic compounds (APC)	Observation variables			
		Browning (%)	Increased of EC fresh weight (g)	Increased of EC weight (%)	Multiplication rate of EC
D. Syifa Agrihorti	APC-0	59.20±1.00 a	8.43±2.00 e	84.3±1.00 e	1.84±0.02 e
	APC-1	35.50±3.55 cd	14.80±2.00 cd	148.0±6.00 cd	2.48±0.30 cd
	APC-2	38.50±1.50 c	10.50±1.50. e	105.0±5.00 e	2.05±0.35 e
	APC-3	46.70±3.35 b	11.44±1.00 de	114.4±10.00 de	2.14±0.03 de
	APC-4	33.30±3.00 d	16.55±2.00 c	165.5±10.00 c	2.66±0.29 c
D. Dian Agrihorti	APC-0	39.80±1.00 c	22.72±2.00 b	227.2±5.00 b	3.27±0.26 b
	APC-1	23.70±3.00 d	26.24±1.53 ab	262.4±20.00 ab	3.62±0.25 ab
	APC-2	30.50±1.50 d	24.25±2.15 b	245.5±6.00 b	3.43±0.16 b
	APC-3	26.80±2.00 de	30.96±2.00 a	309.6±5.00 a	4.10±0.23 a
	APC-4	20.90±2.00 e	29.84±1.00 a	298.4±5.00 a	3.98±0.24 a
Tukey's value		5.92	5.08	28.84	2.19
p		<.0001**	<.0001**	<.0001**	0.0142*

This means following the same letter in the same column is not different based on Tukey's HSD at *p=0.05. Note: Half-strength MS medium (½ MS) containing 0.5 mg L^{-1}thidiazuron (TDZ) and 0.1 mg L^{-1} 6-benzyl amino purine (BAP) used as a basic medium with the different anti-phenolic compound (APC) applied: APC-0 (free APC) as control, APC-1 (150 mg L^{-1}Proline), APC-2 (1.5 g L^{-1}activated-charcoal), APC-3 (150 mg L^{-1} ascorbic acid), and APC-4 (150 mg L^{-1} polyphenyl-pyrrolidone (PVP)). Abbreviations: EC= embryogenic callus.*

The proliferation of embryogenic callus on modification of bioreactor culture systems

Modifying the airlift bioreactor system for the two Indonesian *Dendrobium* varieties brought distinct changes in EC growth. The two treatments also induced significant interaction effects in all variables observed. However, browning was still a problem in the proliferation of ECs under a bioreactor system. The browning percentage of *D*. Syifa Agrihorti ECs was 22.5-48.5% and lowered to 18.5-37.5% for *D*. Dian Agrihorti ECs. TIS with a 30-minute dry period (MDP) and 15-minute wet period (MWP) (Bios-4) was the improved airlift bioreactor system in obtaining optimal results for both Indonesian *Dendrobium* varieties in most observed variables (Table 4). The use of Bios-4 for the proliferation of ECs resulted in 18.5% browning ECs, 51 g increased fresh weight, 515.5% increased ECs fresh weight, and 6.2 times multiplication rate (Table 4) and stimulated lower results for *D*. Syifa Agrihorti.

Table 4. The effect of bioreactor system modification on EC proliferation of *D*. Syifa Agrihorti and *D*. Dian Agrihorti in a bioreactor culture system after four weeks in culture

Varieties	Modification of bioreactor systems	Observation Variables			
		Browning (%)	Increased of EC fresh weight (g)	Increased of EC weight (%)	Multiplication rate of EC (times)
D. Syifa Agrihorti	Bios-1	48.50±2.00 a	22.52±2.00 f	225.2±2.00 f	3.25±0.31 d
	Bios-2	31.00±1.00 c	39.58±1.00 cd	395.8±1.00 cd	4.96±0.27 b
	Bios-3	38.50±1.50 b	32.54±1.00 e	325.4±2.00 e	4,25±0.28 c
	Bios-4	22.47±1.66 d	40.90±2.00 cd	409.0±2.00 cd	5.09±0.27 b
	Bios-5	30.50±2.00 c	35.96±1.00 de	359.6±20.00 de	4.60±0.02 bc
D. Dian Agrihorti	Bios-1	37.50±2.50 b	33.55±1.00 e	335.5±9.00 e	4.36±0.05 c
	Bios-2	21.00±1.00 d	49.35±1.00 a	493.5±20.00 ab	5.94±0.06 a
	Bios-3	28.50±1.50 c	44.64±2.00 b	446.4±1.53 bc	5.46±0.05 ab
	Bios-4	18.50±1.00 d	51.55±1.00 a	515.5±10.00 a	6.16±0.05 a
	Bios-5	20.50±2.00 d	50.98±2.00 a	509.8±9.00 ab	6.10±0.05 a
Tukey's value		5.08	3.85	30.52	0.57
p		0.0167*	<.0001**	0.0009**	0.0111*

Note: The basalt medium used was ½-MS medium containing 0.5 mg L^{-1} TDZ, 0.1 mg L^{-1} BAP, 150 mg L^{-1} L-Proline, and 20 g L^{-1} sucrose. *Bios-1 (control treatment) = airlift bioreactor with 10 g 150 mL^{-1} explant density and 5 vvm aeration rate; Bios-2= temporary immersion system (TIS) with 15 minutes dry period (MDP) and 15 minutes wet period (MWP); Bios-3= TIS with 15 MDP and 30 MWP; Bios-4: TIS with 30 MDP and 15 MWP; and Bios-5= TIS with 30 MDP and 30 MWP*. Means followed by the same letter in the same column is not different based on Tukey's; *p=0.05*. *Abbreviations: EC= embryogenic callus.*

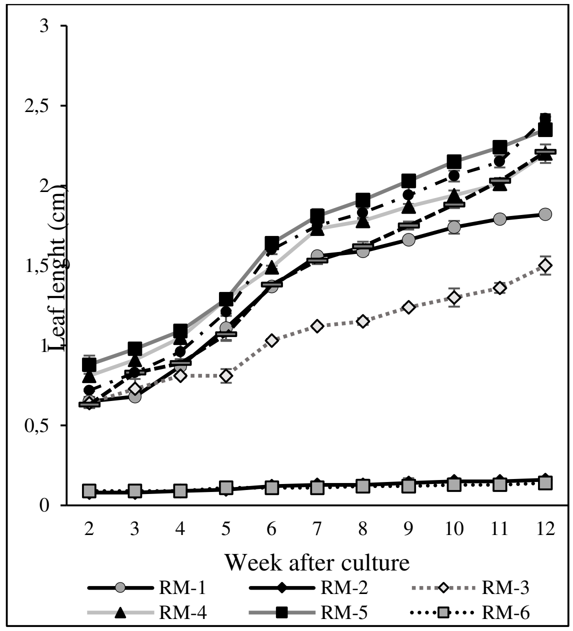
Regeneration of D. Dian Agrihorti embryogenic callus on different basic media and organic additives

Based on periodic data measurement, the regeneration of leaves can be an indicator of embryo germination. We also found that regenerated shoots increased during the data measurement period (Figures 2A and 2B). Significant results were proved on RM-5 (VW supplemented with 150 g L^{-1} of BE) produced plantlets with 2.4 cm in leaf length and 29.2 shoots per clump 12 weeks after culture (Figure 1J and Figure 2A, 2B). Other media stimulated lower results, with the lowest results noted at RM-2 medium (½ MS with 150 g L^{-1} corn extract) (Tabel 5).

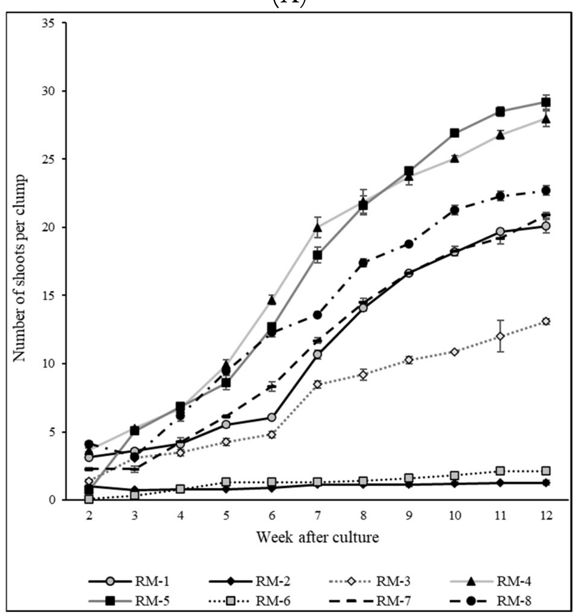
Table 5. The effect of basic media and organic additives on the regeneration of *D. Dian Agrihorti ECs*

Treatments	Clump regeneration (%)	Callus morphogenesis (%)	Shoot morphogenesis (%)	Leaf length (cm)	Shoots number per clump
RM-1	93.30±1.22 b	46.70±0.15 b	53.30±0.25 b	1.82±0.01 c	20.10±0.85 c
RM-2	26.70±0.80 c	0.00±0.00 c	100.00±0.00 a	0.16±0.03 e	1.27±0.29 e
RM-3	100.00±0.00 a	0.00±0.00 c	100.00±0.00 a	1.50±0.10 d	13.10±0.36 d
RM-4	100.00±0.00 a	53.30±0.58 a	46.70±0.75 c	2.20 ±0.10 b	28.00±1.00 a
RM-5	100.00±0.00 a	0.00±0.00 c	100.00±0.00 a	2.35±0.04 ab	29.20±0.92 a
RM-6	13.30±0.30 d	0.00±0.00 c	100.00±0.00 a	0.14±0.02 e	2.13±0.13 e
RM-7	100.00±0.00 a	0.00±0.00 c	100.00±0.00 a	2.21±0.01 b	20.09±0.32 c
RM-8	100.00±0.00 a	0.00±0.00 c	100.00±0.00 a	2.42±0.02 a	22.70±0.61 b
Tukey's value	1.57	0.61	0.79	0.15	1.92
p	<.0001**	<.0001**	<.0001**	<.0001**	<.0001**

Note: The data were derived from 30 clumps in each treatment. *RM-1= ½ MS supplemented with 150 g L^{-1} of banana extract (BE); RM-2= ½ MS fortified by 150 g L^{-1} corn extract (CE); RM-3= ½ MS containing 100 g L^{-1} BE and 50 g L^{-1} CE; RM-4= ½ MS augmented with 50 g L^{-1} BE and 100 g L^{-1} CE; RM-5= Vacin and Went (VW) supplemented with 150 g L^{-1} of BE; RM-6: VW fortified by 150 g L^{-1} CE; RM-7= VW containing 100 g L^{-1} BE and 50 g L^{-1} CE; and RM-8= VW augmented with 50 g L^{-1} BE and 100 g L^{-1} CE.* Means followed by the same letter in the same column is not different based on Tukey's; *p=0.05*.



(A)



(B)

Figure 2. Growth and regeneration of embryogenic callus clump *D*. Dian Agrihorti on different basic media and organic additives (RM-1 to RM-8). (A) Leaf length growth from second to 12th weeks after culture (WAC), and (B) shoots number per clump from second to 12th WAC. The means are shown ± standard error

Abbreviations: RM-1= ½ MS added with 150 g L^{-1} of banana extract (BE); RM-2= ½ MS fortified by 150 g L^{-1} corn extract (CE); RM-3= ½ MS containing 100 g L^{-1} BE and 50 g L^{-1} CE; RM-4= ½ MS augmented with 50 g L^{-1} BE and 100 g L^{-1} CE; RM-5= VW supplemented with 150 g L^{-1} of BE; RM-6: VW fortified by 150 g L^{-1} CE; RM-7= VW containing 100 g L^{-1} BE and 50 g L^{-1}; and RM-8= VW augmented with 50 g L^{-1} BE and 100 g L^{-1} CE

Plantlets acclimatization

In the acclimatization stage, plantlet enlargement and rooting of *D*. Dian Agrihorti were cultured in 2 g L^{-1} Hyponex® medium containing 20 g L^{-1} sugar, 7 g L^{-1} agar, and 2% AC. This protocol successfully produced highly qualified performances of plantlet with 100% survivability.

Discussion

Developing suitable mass propagation of the Indonesian *Dendrobium* varieties based on a bioreactor culture system was successfully established in this study. Our research indicates that *D.* Dian Agrihorti has a better growth response than *D.* Syifa Agrihorti in the initiation and proliferation of the EC. The differences revealed the existence of genotype dependence. Under greenhouse observation, *D*. Dian Agrihorti had faster growth, a higher number of pseudobulbs, vigour, healthier performances, and more inflorescences than *D*. Syifa Agrihorti. Genetic different responses and behaviour in *in vitro* culture also occurred in each genotype specifically (Gudeva and Trajkova 2012; Hesami *et al*., 2023). Thus, IM4 (MS + 1.0 mg L^{-1} TDZ + 0.5 mg L^{-1} BAP) was the most suitable medium for high EC formation. Our results showed that genotypes and the media play a significant role in EC initiation and proliferation. The TDZ and BAP are important in stimulating cell division and callus formation in the initiation and proliferation of EC (De Klerk, 2012). Similar outcomes were observed in local clones and various varieties of *Dendrobium.* For instance, clone NS 001-31 had better organogenesis ability compared to clone NS 022/62, which in turn had a higher ability to form EC than eight of the other clones (Rachmawati *et al.* 2010). *D.* Indonesia Raya Ina had the best response to initiation and proliferation of EC compared to *D.* Sonia Earsakul and *D.* Gradita 10 with 89.1% to 99.7% regeneration of explants and 87.5% to 98.7% formation of EC (Rachmawati *et al.* 2016b). Additionally, *D.* CF22-58 produced a higher EC proliferation improvement up to 356.7% with a 4.6 multiplication rate than *D.* Dian Agrihorti (Rachmawati *et al*., 2022). Thus, *D.* Jayakarta had a better embryogenesis response than *D.* Zahra FR 62 and *D.* Gradita 31 (Winarto, 2012). Other studies also reported *D.* Sonia 17 has better results on inducing protocorm like-bodies (plbs) and plbs number per clump on ½ MS containing 10.0 mg L-1 BA compared to the one in *D.* Sonia 28 (Martin *et al*., 2005; Martin and Madassery, 2006). These results confirmed that each variety or clone in the optimal culture medium produced a high response in initiating EC.

The proliferation of EC using a bioreactor system presents particular challenges. In the case of an airlift bioreactor, the continuous mixing of callus and media over prolonged periods leads to the onset of environmental stress, resulting in decreased growth rates, morphological deformities, and cell death. Common issues that frequently arise encompass EC browning, vitrification (hyperhydricity), the formation of cell aggregates (compact EC), foam formation, reduced growth rates, and cell death (Ziv, 2005; Esyanti and Muspiah, 2006; Celiktas *et al*., 2010). In this study, the most significant contributor to EC malformations was EC browning, accounted for 48.50% of cases in BIOS-1 (an airlift bioreactor with 10 g 150 mL^{-1} explant density and 5 vvm aeration rate). Callus browning occurs due to stressing of callus tissue friction and callus deposition, causing an imbalance between the aeration rate and the callus density used (Rachmawati *et al*., 2016, 2022). The friction between callus causes the rupture of vacuoles and stimulates the production of phenol compounds. Organ injury can lead to a disruption in metabolic balance involving Reactive Oxygen Species, peroxidation of lipid membranes, and the compromise of cell membrane integrity. This, in turn, can instigate an excessive accumulation of phenolic compounds, ultimately resulting in browning. The compounds were generated due to the reaction between oxygen and phenol oxidase enzymes to form toxic quinone compounds that cause cell death (Pirttilä *et al*., 2008; Rittirat *et al*., 2012; Ru *et al*., 2013).

Our study revealed that genotypes, anti-phenolic compounds, and bioreactor systems strongly influenced EC growth and proliferation in bioreactors. In the research, 150 mg L^{-1} AA effectively reduced the browning problem on *D.* Dian Agrihorti ECs in an airlift bioreactor down to 18.5%. Though there are no reports of other orchids, other studies reported that immersing the callus for 30 minutes in the 100 mg L^{-1} AA before planting successfully reduced callus browning of *Hevea Brasiliensis* of clone PB 330 down to 7.5% (Admojo and Indrianto, 2016), 13.6% browning and 74.5% survival rate of shoot tip explants of *Persian walnut* Sulaiman was established when the explants were treated with 550 mgL^{-1} AA (Bhat et al., 2022), treating the explants of *Musa spp.* Mzuzu with 1.2 g L^{-1} AA for explants preparation suppressed browning explants down to 35.75 (Ngomuo *et al*., 2014). Adding 200-250 mg L^{-1} AA can reduce browning in *Brahylaena huillensis* by up to 77% (Ndakidemi *et al*., 2014). The antioxidant compounds capture free oxygen radicals, causing de-compartmentation by activating the polyphenol oxidase (PPO) enzyme. Ascorbic acid (AA) can convert tocopheryl radicals into semi-dehydroascorbic acid instead of forming toxic hydrogen peroxide compounds (Veltman and Peppelenbos, 2003; Ionita, 2013). Ascorbic acid (AA), including ascorbate, can directly inactivate PPO, scavenge oxygen radicals, prevent the oxidation of phenolic compounds in damaged tissues, and convert the colorless o-quinones produced by PPO back to diphenols. The process ultimately reduces the occurrence of tissue browning (Martinez and Whitaker, 1995; Titov *et al*., 2006; Ndakidemi *et al*., 2014). Application of AA, in fact, also enhances cell division, differentiation, elongation, and survivability (Irshad *et al*., 2018; Kaviani, 2014). In the study, 150 mg L^{-1} AA also improved EC fresh weight and multiplication of EC up to 31.3% and 21.7%, respectively. Meanwhile, 100 mg L^{-1} AA in the medium resulted in the highest survival of *Musa* spp Mzuzu explants up to 64.3% (Ngomuo *et al*., 2014).

This research demonstrates that the TIS-Bioreactor offers advantages such as reduced browning and enhanced multiplication compared to the airlift bioreactor. The immersion frequency in a temporary immersion system (TIS) bioreactor significantly impacts bioactive compounds' proliferation, regeneration, and accumulation (McAlister *et al*., 2005; Jesionek *et al*., 2017; Ruta *et al*., 2020). Modifying immersion frequencies in TIS can reduce browning incidence and increase the multiplication rate of *Dendrobium's* EC proliferation. Reducing browning incidence and increasing EC growth is due to the balance of direct exposure of EC in better gas exchange with nutrient medium volume and container volume (Etienne and Berthouly, 2002; Georgiev *et al*., 2014). Each TIS with an explant culture has a specific condition that differs from one another. However, when TIS was imbalanced with explant culture, browning incidence, inhibiting EC growth, and malformation were found. PLBs proliferation of *C. forbesii* in RITA® bioreactor with 1 min drying/4 h immersing produced 3998 shoots per RITA and 199.9 shoots per clump, 95 times higher than semi-solid culture (Ekmekçigil *et al*., 2019). Plantlets of *E. flava* grew in TIS (5 min/4 h), resulting in 1.5 shoots per explant, 4.4 roots per plantlet, 29.4 mm shoot height, 4.4 leaves per shoot (Kunakhonnuruk *et al*., 2019). The best multiple-shoots of *Mokara* Orchid were conducted in TIS (1 min/2 h) with 14.8 shoots per cluster, 2.6 cm height shoot, 2.1 cm diameter shoots clusters, and 0,0 number of dead shoots per cluster (Minh, 2022). TIS (5 min/6 h) on *D. nobile* produced 349.23 g L^{-1} fresh and 54.48 g L^{-1} dry weight (B. Zhang *et al.*, 2022). While in the study, high EC proliferation with 18.5% EC browning, 51.6 g increased EC fresh weight, 515.5% increased EC weight, and 6.2 multiplication rate of *D.* Dian Agrihorti was recorded in the TIS (15 MWP and 30 MDP) than the airlift bioreactor.

The combination of VW and 150 g L^{-1} of the banana extract (BE) was the best regeneration medium for *D*. Dian Agrihorti EC to produce the best performances of plantlets. Optimal regeneration indicated via better shoot and root growth was due to banana nutrition contents, i.e., glucose, carbohydrate, hormones, and vitamins of A, B, C, E, and K involving B1 or thiamine (De Souza *et al*., 2013; Bayraktar *et al*., 2015; Hapsari and Lestari, 2016; Mustika and Semiarti, 2021). Thiamine is a type of vitamin B1 that can stimulate cell division in the shoot and root so that the shoots and roots can grow faster due to its important role as a cofactor in metabolism activities and regulatory system (Subki *et al*., 2010; De Souza *et al*., 2013; Humaira *et al*., 2015). Vitamins are also necessary for the growth of cells that are still actively dividing because they act as catalysts in metabolism (Humaira *et al*., 2015; Hapsari and Lestari, 2016). High carbohydrates and sugars can help facilitate plant metabolism and increase cell growth and differentiation (Sianipar *et al*., 2019; Nuryadin *et al*., 2020). Other studies reported that 150 g L^{-1} BE in a similar medium increased the root number of *D. lasianthera* (Utami *et al*., 2016). The combination of 2.5% BE and 10^{-6} M BAP stimulated shoot emergence shortly with high number of shoots and leaves of *Coelogyne pandurata* (Nurfadilah *et al*., 2018), 75 g $L^{-1}$of BE induced 3.2 mm root length, 6.7 leaves per shoot and 2.3 roots per shoot in *D.* Gatton Sunray (Herawati *et al*., 2021), 100 ml L^{-1} of BE for *Dendrobium* sp. Suitable for PLBs multiplication and plantlet regeneration (Islam *et al*., 2016), 100 g L^{-1} BE increases the growth rate of *D. lineale* with 1.58 mg g^{-1} chlorophyll content (Mustika and Semiarti, 2021), 40 g L^{-1} BE and 20% coconut water (CW) induced high seed germination rate of *Cattleya maxima* up to 97.1% (Vilcherrez-Atoche *et al*., 2020), 50 g L^{-1} BE resulted in high regeneration frequency of *C. pendulum* (Kaur and Bhutani, 2012), and 20 gr L^{-1} BE was the best concentration for *P. amabilis* embryo regeneration (Nuryadin *et al*., 2020). The low effect of CE on the regeneration of *D.* Dian Agrihorti was presumably due to the optimal concentration applied, as reported by Kasutjianingati *et al*. (2019) for *Vanda foetida*. The results confirmed that using BE in a medium greatly affected EC regeneration and plantlet growth of orchids.

In the last stage, acclimatization of *Dendrobium* plantlets to *ex vitro* condition is not a critical problem (Winarto and Rachmawati, 2013; Vijayakumar *et al*., 2013; Rachmawati *et al*., 2015, 2016; Teixeira da Silva *et al*., 2017). Under simple treatment and media, high survivability plantlets in *ex vitro* conditions were noted. In the study, applying pesticide solution in combination with the utilization of *C. rumphii* bulk as acclimatization media resulted in 100% survivability of *D.* Dian Agrihorti plantlets. Successful application of the treatment also increased the survivability of plantlet *D. palpebrae* plantlets up to 74% (Bhowmik and Rahman, 2020), 95% on *D. agregatum* (Vijayakumar *et al*., 2013), 100% on *D.* Gadita-31 and *D.* Zahra FR 62 (Winarto and Rachmawati, 2013; Teixeira da Silva *et al*., 2017), 91.6% on *D.* Indonesia Raya Ina (Rachmawati *et al*., 2015, 2016), 88.3% on *Phalaenopsis* (Winarto, 2016). These results showed that utilization of *C. rumphii* bulk was suitable for acclimatizing orchid plantlets.

While we emphasize the genotype-dependent nature of initiation and proliferation stages in selected *Dendrobium* varieties in this report, our findings also highlight the feasibility of utilizing these results as a foundation for a standardized culture approach and basic media when cultivating different *Dendrobium* varieties. It's important to note that further genotype-specific optimization may still be required.

Conclusions

Mass propagation of Indonesian *Dendrobium* varieties, namely *D.* Syifa Agrihorti and *D.* Dian Agrihorti, based on the bioreactor culture system, was successfully determined from initiation to acclimatization. The EC initiation was resulted by *D.* Dian Agrihorti, which was cultured on IM4 medium (MS + 1.0 mg L^{-1} TDZ + 0.5 mg L^{-1} BAP) with the lowest browning, the fastest EC initiation, and the highest explant growth, morphogenesis, size, and multiplication rate of EC. At the EC proliferation stage, adding 150 mg L^{-1} ascorbic acid to media in the airlift bioreactor reduced browning incidence and increased EC fresh growth, weight, and multiplication rate. In addition, the TIS Bioreactor with an immersion frequency of 15 min/30 min reduced the EC browning problem and increased EC proliferation. The best plantlet regeneration was obtained from VW medium added with 150 g L^{-1} of banana extract. Plantlets were successfully acclimatized to *C. rumpii* bulk medium with high survivability. Though a genotype-dependent approach was performed in the study, the established protocol for *D.* Dian Agrihorti has a high potential to be applied to other varieties.

Authors' Contributions

Conceptualization: FR; Methodology: FR; Formal analysis: FR, HS, TH, DP; Investigation: FR, HS, SR,TH; Validation: FR, DP, SR, BW; Writing - original draft: FR, DP, BW; Writing – review and editing: FR, DP, SR,TH, BW; Supervision: SR, BW. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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