In vitro propagation protocol of Dendrobium 'Gradita 31' via protocorm like bodies

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Abstract

Preparing good quality protocorm like-bodies (PLBs) in-vitro culture of orchids, their regeneration, regeneration, and root formation are important steps in establishing an-vitro protocol of orchids. Murashige and Skoog (MS) medium as a basic medium in the in-vitro culture of the plant was generally applied, utilization of fertilizer as a basic medium for the purpose has few reports. The in-vitro propagation protocol of Dendrobium 'Gradita 31' via PLBs, using varied-Growmore (GM) media, was successfully established. Half-strength MS medium containing 1 mg/l Thidiazuron (TDZ) and 0.5 mg/l N^6-benzyladenine (BA) was applied in obtan ining high PLB formation and initial proliferation of Dendrobium using shoots as donor explants via repeated subcultures. GM-6 medium (1.6 g/l of 32N:10P:10K) containing 100 ml/l coconut water (CW), was an appropriate medium for growth, proliferation, and regeneration of D. 'Gradita 31' PLBs. The medium the stimulated growth rate of PLBs up to 309 mg per month, or equal with 17 PLBs per month, and induced PLB regeneration up to 76% with a high number of regenerated PLBs (5.6 regenerated-PLBs per cluster of PLBs), 3.5 leaves per shoot and 1.19 cm leaf length. The GM-3 medium (200 mg/l 32N:10P:10K+50 mg/l 20N:20P:20K+100 mg/l 6N:30P:30K), augmented with 100 ml/l CW, was a suitable medium for root formation with a shorter period needed for root formation (22.8 days), and 2.4 roots per shoot and 0.76 cm root length, compared to other media. Plantlets were easily acclimatized in ex-vitro conditions with 100% of survivability using Cycas rumphii bulk, and grew well individually after repotting in a mixture medium of C. rumphii bulk and wood charcoal.

Keywords: *In-vitro culture, growth, Growmore, proliferation, protocorm like-bodies (PLBs), regeneration, acclimatization and Dendrobium 'Gradita 31'.*

1. Intoduction

Dendrobium 'Gradita 31' is a promising *Dendrobium* cut-flower derived from breeding activities in the Indonesian Ornamental Crops Research Institute (IOCRI). The cultivar was resulted from hybridization between Dendrobium Sonia Deep Pink × *Dendrobium* 1265 accession. The variety has flower stalk length 24-32 cm, 7 cm flower diameter, 8-12 flower buds per stalk with 1.5 months of vase-life [1] ; and based on a consumer preference test, *D*. 'Gradita 31' flower has a high potential for substituting *D*. 'Sonia' as a cut flower product in Indonesia. Furthermore as a promising cut flower, the availability of the

qualified-planting materials was an important aspect. It has to be prepared sufficiently for commercial purposes and *in-vitro* mass propagation is the best solution to manifest the availability of the qualified-planting materials for the orchid.

Several *in-vitro* culture protocols for *Dendrobium*, prepare good qualified-seedlings via protocorm-like bodies (PLBs), were published. In *D. nobile*, the protocol was successfully established using thin cross sections of plbs as donor explants by, Murashige and Skoog [2] medium supplemented with zeatin riboside (ZR) at 14.0 μM for initiation, 9.8 μM indole-3-butyric acid (IBA) for proliferation and regeneration [3]. Half-strength MS medium containing 44.4 mM BA for plb formation and proliferation, 6.97 mM kinetin (Kin) for regeneration and 2 g/l activated charcoal (AC) for *in-vitro* rooting were proved effective for *Dendrobium* hybrids [4]. For MS medium with macronutrients at half strength, micronutrients at full strength, 2% sucrose and 8.8 μM BA for initiation, the medium was hormone free for PLBs formation. Half-strength MS medium with *2.7* μM α-naphthalene acetic acid (NAA) for plantlet preparation was established for *D. candidum* [5]. In *D. densiflorum*, the highest percentage of explants producing plbs (72%), with an average of 15 PLBs per explant, was induced by culturing stem segments on MS medium supplemented with 5.0 mg/l 6-benzylaminopurine (BAP). PLBs proliferated well on the basal MS medium and completely converted into shoots on MS medium containing 2.0 mg/l BAP [6]. All above protocols were successfully established using MS medium with varied-application of hormones, while utilization of compound fertilizers as basic medium in *in vitro* culture of *Dendrobium* has not been reported yet.

Application of the compound fertilizer reported previously in *in-vitro* culture of orchids is still very limited. In *Phalaenopsis*, application of Hyponex medium for proliferation and regeneration of PLBs and plantlet preparation was reported [7, 8]. The 5N:10P:5K medium was applied for callus proliferation, PLB formation and shoot differentiation of *D. crumenatum* [9]. A combination of Viking Ship, Saturn, and Hyponex fertilizer was applied in PLB proliferation of *Phalaenopsis* 'Silky Moon' [10]. Hyponex N026 medium supplemented with 1.0 mg·L^{-1} NAA, 50 g·L^{-1} banana homogenate (BH), and 0.1% AC was suitable for plantlet formation and growth of 95.5% of plantlets that developed from PLBs of *Nothodoritis zhejiangensis* [11]. The medium supplemented with 1.0 mg/l NAA, 1.0 g/l peptone, 100 g/l BH, and 1.0 g/l AC was suitable for the growth of *in vitro* seedlings of *Paphiopedilum* wardii [12]. Gaviota™ was applied in *in-vitro* culture of *Cattleya* for seedling growth [13]. A application of GM fertilizer for growth, proliferation, and regeneration until root formation of regenerated-PLBs, especially for *Dendrobium* 'Gradita 31' has not been published yet.

In the study, an *in-vitro* propagation protocol *of Dendrobium* 'Gradita 31' via PLB formation using varied-GM media for growth, proliferation, regeneration, and root formation was successfully established. Interesting results of the study were due to successful application of GM medium for substituting of MS medium with almost similar results in PLB proliferation, regeneration, and plantlet preparation.

2. Materials And Methods

2.1 Plant material and preparation

The study was conducted at the tissue culture laboratory of IOCRI (1100 m asl) from January to December 2012. Shoot tips and axillary shoots harvested from *Dendrobium* 'Gradita 31' were used as initial donor explants for inducing PLBs. The explants were sterilized gradually under tap water for 2 h for pretreatment, and then disinfected with 0.05% mercury chloride for 5 min, and rinsed 6 times using sterile water (each time was 5 min). The leaves of a pseudo-bulb were removed carefully using a tissue culture blade (BB510, Aesculap AG & CO. KG AM Aesculap-Platz 78532, Tutlingen, Germany). Each node was cut and re-disinfected using 0.01% mercury chloride ($HgCl_2$) for 2 min and rinsed 6 times with sterile water (each time was 5 min). The nodes containing dormant axillary shoots were cultured in the culture initiation medium to induce shoots to increase the number of donor explants.

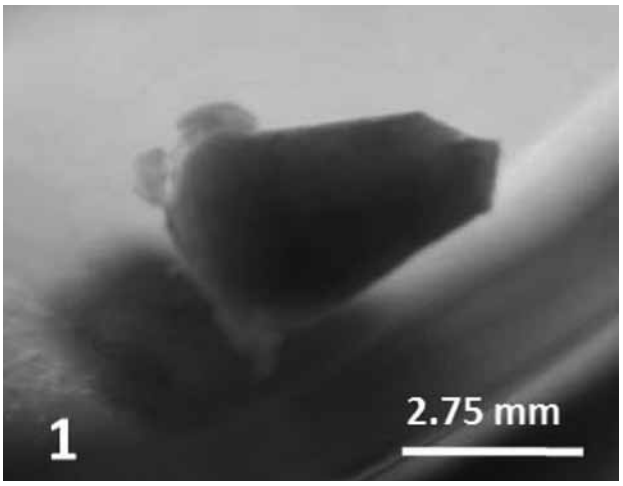

Fig. 1. Shoot tip as explant source.

Small shoots (± 0.3 cm ; Fig. 1) as donor explants were cultured on semi-solid of half-strength MS, containing 1 mg/l TDZ and 0.5 mg/l BAP. Total shoots cultured in the experiment were 20 shoots. The cultures were incubated for a 12 h photoperiod under cool fluoresent lamp with~13.5 μmol/m^2/s light intensity and 23.5 ± 1.1°C, and 60.6 ± 3.8% relative humidity until PLB initiation was clearly observed. The explants were subcultured periodically for 15 days each in the same medium. After PLB initiation, the explants were cultured in liquid half-strength MS, supplemented with 0.3 mg/l TDZ and 0.1 mg/l NAA. In the medium, periodical subcultures (4-5 times, each subculture period was 15 days) were carried out in the same medium for PLB proliferation. PLBs, resulting from the stage, were used as donor material for the experiment.

All components of MS medium used pro-analysis chemicals from Merk, Darmstadt- Germany. TDZ, BAP, and NAA were from Sigma-Aldrich, Germany. All media used in these studies contained 2% sucrose (Merck, Darmstadt, Germany) and 1.8 g/l Gelrite (Duchefa-Biochemie, RV Harleem, The Netherlands) for semi-solid conditions. Growmore (32N:10P:10K, 20N:20P:20K, 6N:30P:30K; New Century Drive, Gardena, CA-USA) was used in this study. The pH of all media was adjusted to 5.8 (Model 420A pH meter, Thermo Orion, Beverly, USA) using 1 N HCl or NaOH. The media was sterilized for 20 min at 121°C and 15 kPa atm (Pressure Sterilizer Model No. 1941X, Foundry Co. Inc. 838 South 16th. St. Monitowoc, Wisconsin-USA).

All cultures were placed on an orbital shaker (GFL 3017, Geselischaft fűr Labortechnik mbH, D-30938 Burgwedel-Germany) with 100 rpm in liquid culture during proliferation stage and on incubation racks under fluorescent lamps (TL-Philips, The Netherlands) under ~13.5 μmol/m^2/s (light intensity measured with a Digital Lux Meter, Lutron LX 101-Lutron Electronic Enterprise Co., Ltd., Taiwan) for a 12-h photoperiod, 23.5 ± 1.1°C, and 60.6 ± 3.8% relative humidity (temperature and relative humidity measured by a Haar-Synth-Hygro thermo-hygrometer-Germany) for semi solid culture, for regeneration and rooting. Measurement data resulting from the Lutron LX 101 was in lux and then converted to μmol/m^2/s by multiplying each datum with the conversion factor for cool white fluorescent lamps, i.e. 0.0135 [14].

2.2 Effect of varied-GM media on growth and proliferation of *Dendrobium* 'Gradita 31' PLBs

Six GM media studied in the experiment were 50 mg/l 32N:10P:10K + 12.5 mg/l 20N:20P:20K (GM-1), 100 mg/l 32N:10P:10K+25 mg/l 20N:20P:20K (GM-2), 200 mg/l 32N:10P:10K+50 mg/l 20N:20P:20K+100 mg/l 6N:30P:30K (GM-3), 400 mg/l 32N:10P:10K + 100 mg/l 6N:30P:30K (GM-4), 800 mg/l 32N:10P:10K (GM-5), 1600 mg/l 32N:10P:10K (GM-6),and half-strength MS medium supplemented with 0.3 mg/l TDZ and 0.1 mg/l NAA as control. All Growmore media were supplemented with 100 mg/l myo-inositol, 100 ml/l coconut water, and 20 g/l sucrose. The pH of media were adjusted to 5.8. Fifteen milliliter of each media

was poured in 100 ml Erlenmeyer flasks (Pyrex, IWAKI TE-32, ASAHI Glass Licency, PT. Anugerah Niaga Mandiri, Jakarta-Indonesia). The media were then autoclaved at 121°C, 15 kPa for 20 min.

The experiment was arranged in a completely randomized design with 4 repetitions. Each treatment consisted of 2 Erlenmeyers. Each Erlenmeyer contained 1 g of PLBs ± 55 PLBs. All cultures were placed on an orbital shaker at 100 rpm and under fluorescent lamps of ~13.5 μmol/m2/s, for a 12-h photoperiod, 23.5 ± 1.1°C, and 60.6 ± 3.8% relative humidity.

Parameters observed in the experiment were (1) fresh weight added of PLBs after one month incubation (mg), (2) added number of PLBs, (3) percentage of necrosis (%) and (4) color of PLBs. Daily observation was conducted to check for each alteration PLB during incubation. All data were collected one month after culture. Fresh weight added of PLBs was calculated by subtracting total fresh weight at the end of observation with total fresh weight in the initial stage of culture; added number of PLBs was counted by timing fresh weight added with 51, as the initial number of PLBs in the initial culture; while the percentage of necrosis was calculated by counting the number of necrosis PLBs divided by the total amount of PLB culture time by 100%.

2.3 Effect of varied-GM media on regeneration of *Dendrobium* 'Gradita 31' PLBs

Six GM media applied in the previous experiment were also used in the experiment. The experiment was arranged in a completely randomized design with 4 repetitions. Each treatment consisted of 2 jam bottles. Each Erlenmeyer contained 4 clusters of PLBs (± 10 PLBs). All cultures were placed under fluorescent lamps of ~13.5 μmol/m^2/s in a 16-h photoperiod, 23.5 ± 1.1°C, and 60.6 ± 3.8% relative humidity.

Parameters observed in the experiment were (1) percentage of regenerated PLBs (%), (2) number of regenerated-PLBs per cluster, (3) leaf number and (4) leaf length (cm). Daily observation was conducted to check each alteration occurred during incubation. All data were collected one month after culture. Percentage of regeneration was calculated by counting the number of germinated-PLBs, divided by the total amount of PLB cultur time by 100%.

2.4 Root formation of regenerated-PLBs

Root formation of regenerated-PLBs derived from the study was carried out by culturing the regenerated-PLBs with 3-4 leaves in varied-GM media, as utilized in the first and second experiment. The experiment was arrang in a completely randomized design with four replications. Each treatment consisted of four bottles. Each bottle contained 5 regenerated-PLBs. All cultures were incubated under fluorescent lamps of ~13.5 μmol/m2/s in a 16-h photoperiod, 23.5 ± 1.1°C, and 60.6 ± 3.8% relative humidity.

Parameters observed in the experiment were (1) root initiation time (days), (2) number of roots per regenerated PLBs and (3) root length (cm). Periodical observation was carried out daily to know each alteration, especially in comparison to initial root formation. Number of roots per regenerated-PLBs and root length was recorded 3 months after culture incubation.

2.5 Acclimatization of plantlets

Plantlets with 3-5 leaves and 2-4 roots were selected and prepared for acclimatization purposes. The plantlets were taken out from the culture vessels and then cleaned from remains of agar by washing them with sterile distilled water. The plantlets were then immersed in 1% fungicide and bactericide for 3 min to reduce root rot due to plant pathogen attack. The small plants were then cultivated in plastic pots (30 cm in diamater) containing *Cycas rumphii* bulk. Each plastic pot was planted with ± 35 plantlets. Total plantlets acclimatized in the step were 210 plantlets in six plastic pots. The plastic pots were covered with transparent plastic for 7 days and placed in a glass house, especially in a reduced-light intensity area (37–74 μmol/m^2/s). In the study, the total number of survived and dead-plantlets were recorded after 2 months of acclimatization.

2.6 Data Analysis

All data collected from the experiments were analyzed using analysis of variance, with SAS program Release Windows 6.12. Significant differences between means were assessed by Tukey's Studentized Range at $P = 0.05$ [15].

3. Results and Discussion

3.1 Initiation and initial proliferation of PLBs

Periodic subculture of shoot explants twice a month maintained the explants in a condition that allowed them to remain green and vigorous, leading to easily producing PLB. Callus initiation was clearly observed in wounded areas in the basal part of shoots ± 2.5 months after culture on half-strength MS containing 1 mg/l TDZ and 0.5 mg/l BAP. The percentage regenerated-explants reached 87%. The continuing callus growth actively produced callus clusters ± 3.5 months after culture (Fig. 2). Continued-periodical subcultures of the PLBs on half-strength MS medium supplemented with 0.3 mg/l TDZ and 0.1 mg/l NAA, resulted in an increased number of PLBs (Fig. 3). In this stage, 3-5 PLBs produced 5-12 new PLBs after 4.5 months of cultureing. A high number of PLBs sufficient for the study was established ± 8 months after culturing.

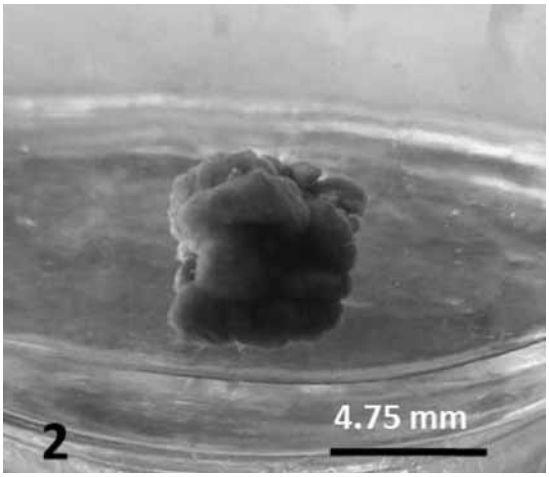

Fig. 2. Shoot tip explant with several initial PLBs, 3.5 months after culturing.

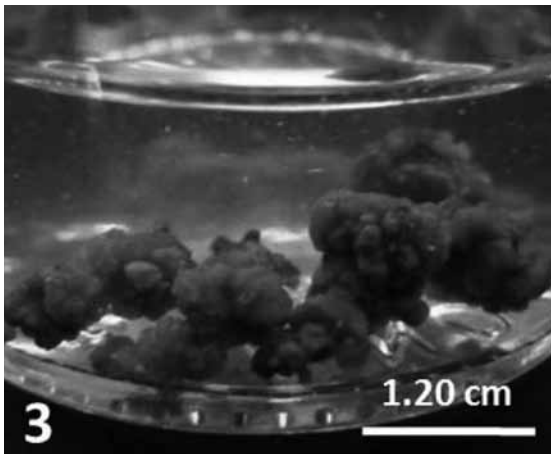

Fig. 3. Initial proliferation of PLBs in third subculture.

3.2 Effect of GM media on growth and proliferation of *Dendrobium* 'Gradita 31' PLBs

There was a gradual alteration of PLBs during the experiment performed. The alteration was clearly observed in changing PLB color and the presence of necrosis. The PLB color changed from dark green to light green in all GM media, while necrosis took place normally 10-15 days after culturing. The necrosis was initiated by altering PLB tips from light green to pale green and from pale green to white. The high necrosis indicated a high percentage of necrosis up to 12.3% and 11.7% was recorded in PLBs cultured in GM-5 and GM-1 medium, respectively.

GM media gave a significant effect on growth and proliferation of PLBs statistically. The GM-6 (1600 mg/l 32N:10P:10K) was the most appropriate GM medium indicating higher results with 308.8 mg fresh weight added of PLBs per month, equal to 17 added PLBs and 6.7% grorth of PLB necrosis, compared to other GM media (Table 1 and Fig. 5). Although the GM-6 medium could not reach a similar result compared to PC-1 medium as control (Fig. 4), the medium has a high potential to substitute for application of PC-1 medium in the growth and proliferation of PLBs. PLBs harvested from GM-6 medium were generally light green, varied-size and rounds (Fig. 6). While other GM media exhibited low results with the lowest results showed by GM-1.

Table 1. Effect of GM media on growth and proliferation of PLBs.

Growmore medium (GM)	Fresh weight added of PLBs (mg)	Added number of PLBs	Percentage of necrosis (%)	Color of PLBs
GM-1	82.3 e	4.5 e	11.7 a	Light green
GM-2	268.8 c	14.8 c	6.7 c	Light green
GM-3	93.5 e	5.5 e	10.6 ab	Light green
GM-4	94.8 e	5.2 e	8.3 bc	Light green
GM-5	196.5 d	10.8 d	12.3 a	Light green
GM-6	308.8 b	17.0 b	6.7 c	Light green
PC-1 (Control)	561.3 a	30.9 a	0.0 d	Dark green
Coefficient of variation (%)	5.78	5.21	7.61	

Note: GM-1, 50 mg/l 32N:10P:10K + 12.5 mg/l 20N:20P:20K; GM-2, 100 mg/l 32N:10P:10K+25 mg/l 20N:20P:20K; GM-3, 200 mg/l 32N:10P:10K+50 mg/l 20N:20P:20K+100 mg/l 6N:30P:30K; GM-4, 400 mg/l 32N:10P:10K + 100 mg/l 6N:30P:30K; GM-5, 800 mg/l 32N:10P:10K; GM-6, 1600 mg/l 32N:10P:10K and PC-1, half-strength MS medium supplemented with 0.3 mg/l TDZ and 0.1 mg/l NAA as control. All GM media were supplemented with 100 ml/l coconut water. Means followed by the same letter in the same column are not significantly different, based on Tukey test ($p=0.05$)

3.3 Effect of GM media on regeneration of *Dendrobium* 'Gradita 31' PLBs

Regeneration of *D*. 'Gradita 31' PLBs was significantly affected by application of the GM media. The regeneration of the PLBs was initiated by immersing the growing point, accompanied by altering the point from green, to greener 9-18 days after culture. Formation of the growing point was followed by producing the first initial leaf. Developing the first, leaf leading to forming the perfect leaf (23-29 days after culture), was followed by raising the second leaf (Fig. 7), and the second leaf to third leaf, etc. Regenerated PLBs with 2-3 perfect leaves wear easily recorded ± 2.5 months after cultureing. Regeneration percentage of PLBs varied from 25-90%.

The GM media gave varied-results on the PLB regeneration. GM-6 medium also gave high potential results on the regeneration of PLBs. The medium stimulated percentage of PLB regeneration up to 76% with 5.6 regenerated-PLBs, 3.5 leaves per regenerated-PLBs and 1.19 cm leaf length (Table 2, Fig. 8). The GM-6 also indicated almost similar results, compared to PC-1 medium as control. The results also gave evidence that the GM-6 hads a high potential to replace PC-1 medium for high quality of regeneration. The second best GM media was indicated by GM-5, with the lowest results exhibited by GM-1.

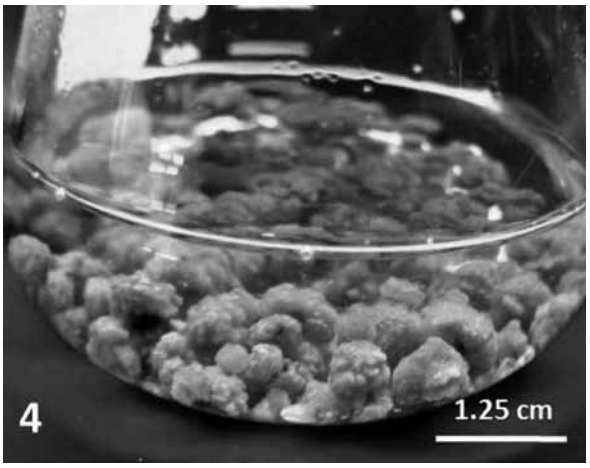

Fig. 4. Vigorous and greener PLB performance in half-strength MS medium with 0.3 mg/l TDZ and 0.1 mg/l NAA after the sixth subculture.

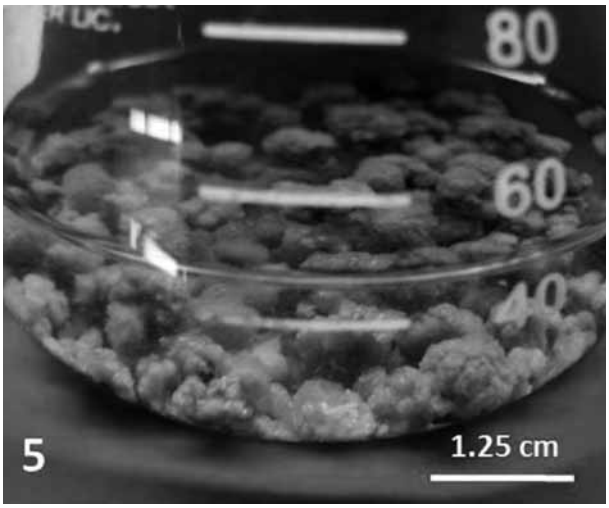

Fig. 5. Light green PLB performance in GM-6 medium after the sixth subculture.

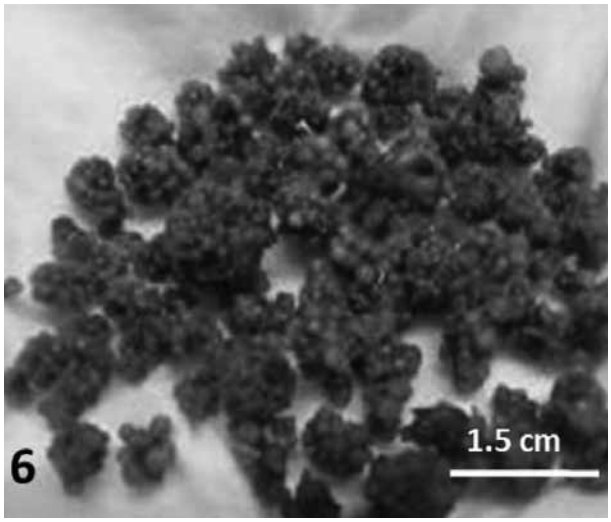

Fig. 6. Performance of PLBs harvested from GM-6 medium.

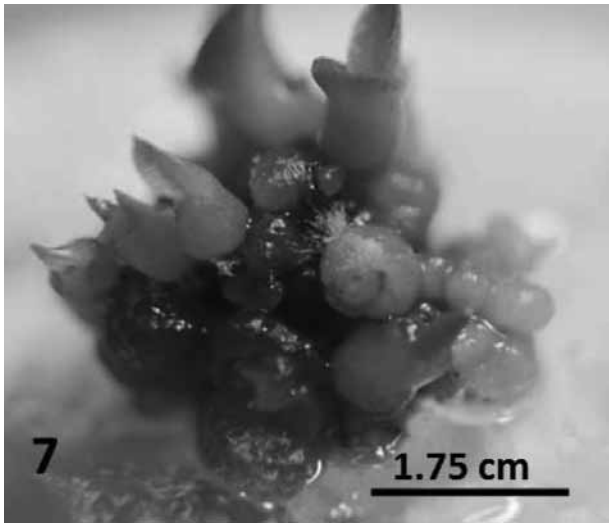

Fig. 7. Initial germination of PLBs on GM-6 medium.

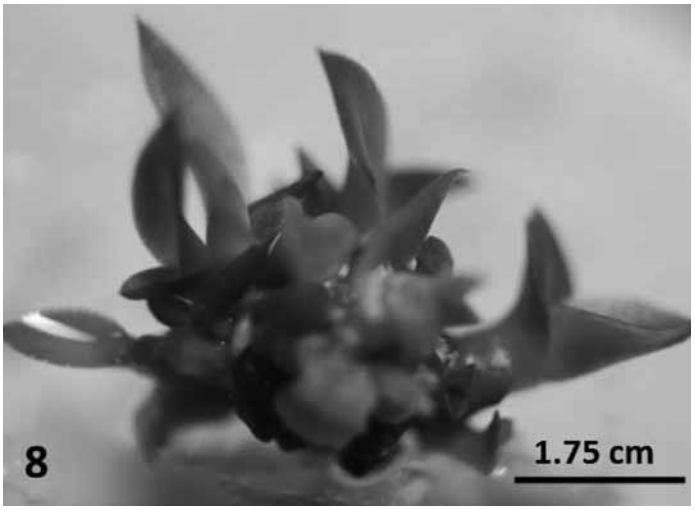

Fig. 8. Regenerated-PLBs, 2 months after culture on GM-6 medium.

Table 2. Effect of GM media on growth and proliferation of PLBs.

Grow-more medium (GM)	Percentage of regeneration (%)	Number of regenerated-PLBs	Number of leaves per shoot	Leaf length (cm)
GM-1	33.2 d	2.1 c	1.3 d	0.41 e
GM-2	47.7 c	2.3 c	1.9 c	0.61 d
GM-3	49.9 c	3.3 bc	2.0 c	0.63 d
GM-4	52.3 c	3.3 bc	2.3 c	0.61 d
GM-5	66.5 b	4.1 b	3.0 b	0.80 c
GM-6	76.3 a	5.6 a	3.5 a	1.19 b
PC-1 (Control)	83.9 a	4.1 b	3.6 a	1.37 a
Coefficient of variation (%)	5.63	19.69	7.43	6.08

Means followed by the same letter in the same column are not significantly different, based on Tukey test ($p=0.05$)

3.4 Effect of GM media on root formation of regenerated-PLBs

Root formation of regenerated-PLBs was also influenced by the GM media. Initial root formation was clearly observed from 18-25 days after culture. The initial roots continued to grow in number, size, and length. Two to four roots per shoot were successfully produced using the GM media 3 months after culture (Fig. 9). The GM media generally gave better results for root formation, compared to PC-1 medium as control. The GM-3 (200 mg/l 32N:10P:10K+50 mg/l 20N:20P:20K+100 mg/l 6N:30P:30K) medium was appropriate medium for root formation. The medium induced initial root formation in a shorter period down the to 22.8 days with 2.4 roots per shoot and 0.76 cm in length. The second best GM media um was GM-5 with the lowest results exhibited by the GM-1 medium. From the study it was clearly shown that application of the GM media, especially the GM-3, gave better results than the PC-1 medium for root formation.

3.5 Acclimatization of plantlets

Transferring plantlets from *in vitro* to *ex vitro* condition via removing them from culture vessel, immersing in 1% fungicide and bactericide solution, culturing on *C. rumphii* bulk, covering the plastic box with transparent plastic, and placing under reduced-light intensity area of glass house, resulted in high survivability of them as reported in another orchid study previously. In the experiment all plantlets acclimatized had 100% survivability (no dead, plantlets, Fig. 10). The survived-plantlets continually grew, increased in leaves, root, number, and length, and 2 month after acclimatization, the acclimatized-plantlets were ready to be planted individually in bigger plastic pots, containing a mixture of wood charcoal and *C. rumphii* bulk. Transferring the acclimatized-plantlets to bigger plastic pots followed by watering and fertilizing regularly accelerated their vegetative growth. Seven months after repotting them, good-growth of the individual plants was established (Fig. 11).

Achievement in establishment of an *in-vitro* propagation protocol of *Dendrobium* 'Gradita 31' via PLB formation opens another way for mass propagation of the *Dendrobium*. Due to increasing potentiality of the plant for commercial purposes, availability of qualified-seedlings at the right time and number is important and the protocol will help its commercialization. Utilization of shoot tip as an explant source and half strength MS medium containing 1.0 mg/l TDZ and 0.5 mg/l BA and 0.3 mg/l TDZ and 0.1 mg/l NAA as initiation medium was successfully applied for PLB formation. Initial proliferation percentage of regeneration was up to 87%. In another study with *Dendrobium* 'Zahra FR 62' the percentage of explant regeneration indicated almost similar result with 85% of the shoot tips producing initial callus [16]. The high potential of the shoot tips to form PLBs was also reported previously. Culturing the shoot tip explants on New Dogashima medium [17], containing 0.1 mg/l NAA and 1 mg/l BAP [17]; 0.5 μM NAA), 4.4 μM BAP and 29.2 mM sucrose [18, 19] induced callus and PLB formation; Knudson C [20] used a medium of 0.5 mg/l NAA and 1 mg/l BAP for *D. fimbriatum* Lindl. var. *oculatum* Hk.f. [21]. The medium with 1.0 mg/l NAA, 5% CW and 0.1% AC, induced 84% callus forming PLBs of *N. zhejiangensis* [11]. The medium of 44.4 μM BA for *Dendrobium* hybrids Sonia 17 and 28 [4, 22], 1 mg/dm^3 TDZ for *Dendrobium* 'Chiangmai Pink' [23]. Other examples are: 2 μM TDZ or BAP for *D. chrysotoxum* Lindl [24], 8.8 µM BAP for *D. candidum* [5], 5.0 mg/l BAP for *D. densiflorum* [6], 2.0μM/l NAA and 1.2 µM/l kin for *D. nanum* [25], 18 μM TDZ for dwarf *Dendrobium* [26], 1.5 mg/l BAP, 15% CW and 3% sucrose for *D. aggregatum* [27].

The percentage of the shoot tip explant regeneration up to 86%, leading to success in initial PLB proliferation, was afftected by subculturing the explants periodically every 15 days in half-strength MS medium, supplemented with 0.3 mg/l TDZ and 0.1 mg/l NAA. The periodical subculture increased totipotency of explants in producing callus with greener and vigorous performance. The periodical subculture of explant was also successfully applied in other studies. In *D. crumenatum,* increasing callus formation and its totipotence in forming PLBs were determined by subculturing explants every month on Vacin and Went medium [28], supplemented with 20 g/l' sugar, combination of 1 mg/l BA and 0.1 mg/l NAA, 2 g/l

peptone, 2 g/l Gelrite and 2 g/l activated charcoal at pH 5.3 [9]. Periodical subculture of explants, two times every 40 days on Murashige and Skoog medium with macronutrients at half strength, micronutrients at full strength, 2% sucrose, and with 8.8μM BAP successfully induced callus formation for *D. candidum* [5]. Using a 3-month subculture period, a 69-fold increase in callus weight was achieved with 0.5 μM NAA, while as many as 133 PLBs could be obtained per 100 mg callus in the presence of 1 μM NAA [24].

In *in-vitro* propagation of *Dendrobium,* so far, there is no information published previously on applications of fertilizer medium for proliferation and regeneration of PLBs and plantlet preparation. Application, of fertilizer medium, with easily found and cheap material, will lead to increasing *in-vitro* propagation efficiency cost. Substitution of pro-analysis MS medium with GM medium for proliferation, regeneration of PLBs, and plantlet preparation for acclimatization purposes, was successfully applied in the study. GM-6 medium (1.6 g/l of 32N:10P:10K) containing 100 ml/l CW was the optimal substitution medium for proliferation and regeneration of PLBs, compared to others (Table 1 and 2). In other orchid studies, application of the fertilizer medium for proliferation of PLBs was reported. PLB proliferation, up to 13–18 PLBs developed from single PLB sections of different cultivars of *Phalaenopsis,* was achieved on a modified Hyponex medium (1 g/l 6.5N+4.5P+19K and 1 g/l 20N+20P+20K) with 2 g/l peptone, 3% (w/v) potato homogenate, 0.05% activated 1 g/1 charcoal [8]. The hyponex medium (1 g/l 6.5N – 4.5P – 19K dan 1 g/1 20N – 20P – 20K + 1% potato homogenate) was also successfully applied for PLB proliferation up to 18,000 PLBs from 20 g of inoculum for *Phalaenopsis* [7].The medium containing 1.0 g/l peptone, 30 g/l sucrose, 1.0 g/l AC, 1.0–2.0 mg/l NAA ,and 100 g/l BH induced PLB differentiation of *Paphiopedilum hangianum,* up to 28% [29]. While 0.75 g/l Viking Ship (10N-20P-30K) with 1.0 g/l Saturn fert provided significant higher number of PLBs per explant, up to 24.4 PLBs of for *Phalaenopsis*'Silky Moon' [10].

GM-3 medium (200 mg/l 32N:10P:10K+50 mg/l 20N:20P:20K+100 mg/l 6N:30P:30K) containing 100 ml/l CW was a suitable medium for rooting regenerated-PLBs. In *Phalaenopsis*, Hyponex medium (1 g/l 6.5N – 4.5P – 19K and 1 g/1 20N – 20P – 20K + 1% potato homogenate) was also found to be suitable for conversion of PLBs into plantlets, and 83% of PLBs transformed into plantlets on this medium [7]. The same Hyponex medium (1 g/l 6.5N+4.5P+19K and 1 g/l 20N+20P+20K) with 2 g/l peptone, 3% (w/v) potato homogenate, and 0.05% activated 1 g/1 charcoal, was also suitable for plantlet preparation [8]. The medium with 1.0 mg/l NAA, 50 g/l BH, and 0.1% AC induced 95.5% of PLBs producing plantlets of *N. zhejiangensis* [11], and 1.0 mg/l NAA and 100 mg/l BH were suitable for plantlet preparation of P. *hangianum* [29]. While Viking Ship medium [1 g/l (10N-20P-30K), Viking Ship (without Saturn fert) and Hyponex medium [1 g/l (6.5N-6P-19K), and Hyponex with (20N-20P-20K) Saturn fert] containing yeast extract or biotin or folic acid, enhanced, the number of plantlets [10].

Plantlets derived from the previous experimant were successfully transferred in *ex-vitro* conditions with 100% survivability using a combination potting medium of wood charcoal and *C. rumphii* bulk (1:1, v/v). High results on transferring plantlets in *ex-vitro* environment was also achieved in other studies. *D. nobile* plantlets with up to 95% of survivability were successfully tranferred in small plastic pots (7.5 cm in diameter) containing peat moss, wood charcoal, and bricks (1:1:1) [3], and 92% in a mixture medium of charcoal chips, coconut husk and broken tiles (2:2:1) [30]. In *D. candidum,* 95% survivability of plantlets were established on vermiculite medium [5], a mixture of sand, brick or tile, charcoal pieces and coir fiber (1:4:4:2) for *D.* 'Sonia', with 80% of plantlets survive [4]. Charcoal for *D.* 'Serdang Beauty' gave 80-100% survivability of plantlets [31]. All the acclimatization results indicated that tranferring plantlets to *ex-vitro* conditions as not a problem in establishing *in-vitro* propagation protocol of *Dendrobium*.

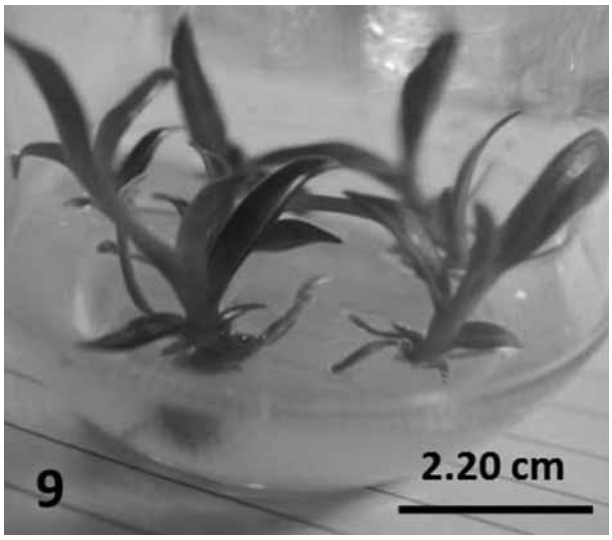

Fig. 9. Rooting of regenerated-PLBs on GM-3, 1.5 months after culturing.

Fig. 10. Acclimatized-plantlets, 15 days after acclimatization on *C. rumphii* bulk.

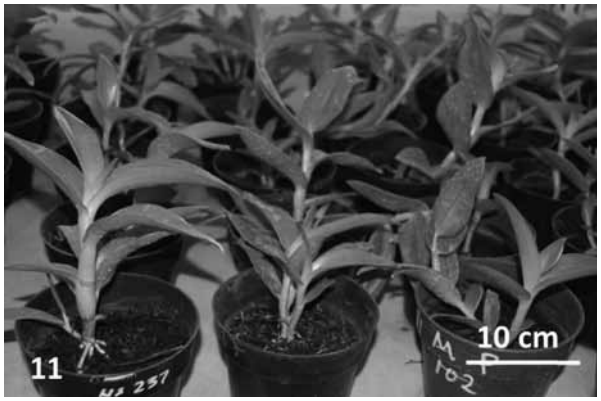

Fig. 11. Good growth of individual plants after seven months of repotting on a mixture of wood charcoal and *C. rumpii* bulk (1:1, v/v).

4. Conclusion

From the study it can be concluded, an *in-vitro* propagation protocol of *Dendrobium*, especially for *D*. 'Gradita 31', using GM medium for proliferation, that regeneration of PLBs and plantlet preparation was successfully established. Half-strength MS, medium containing 1 mg/l TDZ and 0.5 mg/l BA, was applied in obtaining high PLB formation, and initial proliferation used shoots as donor explants and repeated subcultures. GM-6 (1.6 g/l of 32N:10P:10K) media was the most appropriate medium for growth, proliferation, and regeneration of PLBs, with the GM-3 (200 mg/l 32N:10P:10K+50 mg/l 20N:20P:20K+100 mg/l 6N:30P:30K) medium for root formation. Plantlets were easily acclimatized in *ex-vitro* conditions with 100% survivability using *Cycas rumphii* bulk, and grew well individually after repotting in a mixture medium of the *C. rumphii* bulk and wood charcoal (1:1, v/v).

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Table 3. Effect of GM media on root formation of regenerated-PLBs.

Grow-more medium (GM)	Period of initial root formation (day)	Number of roots per shoot	Root length (cm)
GM-1	45.8 a	1.3 c	0.71 ab
GM-2	33.3 b	1.4 c	0.59 ab
GM-3	22.8 d	2.4 a	0.76 a
GM-4	31.5 bc	1.7 bc	0.54 b
GM-5	27.3 cd	2.0 ab	0.58 ab
GM-6	34.5 b	1.5 bc	0.60 ab
PC-1	47.5 a	0.1 d	0.05 c
Coefficient of variation (%)	6.05	16.90	16.65

Means followed by the same letter in the same column are not significantly different, based on Tukey test ($p=0.05$)

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