

Original Article

In vitro Embryogenesis Derived from Shoot Tips in Mass Propagation of Two Selected-Clones of *Phalaenopsis*

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Abstract

Phalaenopsis is of high economic value and market demand in Indonesia; however, orchid products are mostly imported from other countries. 'Kristina Dwi' (KD) 69.274 and 'Dedeh' (D) 802.28 are two selected clones with high potential utilized and developed commercially. To support their commercialization, a reliable *in vitro* propagation protocol is essential. In the current study, an *in vitro* mass propagation protocol for KD 69.274 and D 802.28 clones was successfully established using shoot tips as explant sources. A high number of embryos, up to 8.2 embryos per explant, with 58.5% explant regeneration, and 3.5 regenerated-explants in average were regenerated from shoot tips of KD 69.274 clone cultured on half-strength Murashige and Skoog (MS) medium, with full strength micro, Fe-chelate and vitamin containing 0.5 mg/L thidiazuron (TDZ) and 0.25 mg/L N6-benzyladenine (BA). The initial embryos were proliferated by culturing embryos individually on half-strength MS medium with 0.13 mg/L TDZ and 0.25 mg/L BA and resulted in high embryo regeneration up to 91.4%, with 10.2 embryos per explant and no embryo browning. The embryos were multiplied under periodical subcultures of 3 months each, resulting in gradual increasing number of embryos from the first subculture till the fifth subculture, with 23.6 embryos produced, then declined afterward. The embryos were easily germinated on half-strength MS medium with full strength of vitamin and hormone free, with 73.9% embryo germination and 14.9 germinated embryos. Healthy plantlets were stimulated on the same medium with 2 g/L activated charcoal (AC) and successfully acclimatized on *Cycas rumphii* bulk, with 88.3% survival plantlets. Finally, it can be summarized that a new *in vitro* mass propagation protocol, as new alternative choice for *Phalaenopsis* propagation, was successfully established.

Keywords: initiation, orchid, micropropagation, multiplication rate, proliferation, acclimatization

Introduction

Phalaenopsis (Orchidaceae), commonly known as moth orchids (Chen *et al.*, 2009; Gow *et al.*, 2009; 2010), is one of the most popular and high economical orchid in the world for floriculture market. The *Phalaenopsis* is generally sold commercially as pot plant and cut flowers with the Netherland (70%) and Taiwan (23%) as the main producers of orchids (Griesbach, 2002; Liu *et al.*, 2006; Plasmeijer and Yanai, 2012). The orchid, involving in a monopodial orchid, is difficult to propagate vegetatively (Ishii *et al.*, 1998), while generative propagation derived from seeds do not produce uniform regenerants (Park *et al.*, 2002), thus propagation through tissue culture is desirable. However, utilization of shoot tips harvested from mother plants as explant source for *in vitro* culture of *Phalaenopsis* may lead to the loss of the mother plants (Ishii *et al.*, 1998), therefore alternative explant sources shall be addressed for the purpose.

Recent *in vitro* culture protocols for *Phalaenopsis* were successfully developed via protocorm-like body (plb) formation, shoot multiplication and callus culture using plb,

entire shoots, shoot tips, stem nodes, leaf segments and root tips (Gnasekaran *et al.*, 2010; Murdad *et al.*, 2010; Niknejad *et al.*, 2011; Tavares *et al.*, 2012; Samarfard *et al.*, 2013; Rittirat *et al.*, 2014). These explants were generally cultured on Murashige and Skoog (MS) ((Murashige and Skoog, 1962) modified by adding 2.0 mg/L thidiazuron (TDZ) (Rittirat *et al.*, 2014), 10% of Mas (AA) banana pulp (Gnasekaran *et al.*, 2010), 150 mg/L glutamine and 1 g/L activated-charcoal (AC) (Sinha and Jahan, 2011), 10 mg/L chitosan (Samarfard *et al.*, 2013), 30 g/L sucrose (Antensari *et al.*, 2014), applying trimmed bases protocorm technique (Murdad *et al.*, 2006), etc. However, in fact, each propagation method can not be easily applied for different types of *Phalaenopsis* due to different behavior and response of each type of *in vitro* culture (Winarto *et al.*, 2013), involving embryogenesis method.

Recently works on the *in vitro* embryogenesis of *Phalaenopsis* were successfully established previously. Kuo *et al.* (2005) used entire leaf surface cultured on half-strength MS medium containing 1.0 mg/L TDZ to produce high embryos (55 embryos per explant). Cut end from leaf explant cultured

on the half-strength modified MS supplemented with 3.0 mg/L N⁶-benzyladenine (BA) and 1.0 mg/L N⁶-(2-isopentenyl) adenine (2-iP) was the most effective for embryogenesis of *P. amabilis* and *P. 'Nebula'* respectively (Gow et al., 2008) and 3.0 mg/L TDZ with sucrose free for *P. amabilis* and *P. 'Nebula'* (Gow et al., 2010). Sub-culturing embryos in the half-strength MS medium fortified with 3.0 mg/L TDZ were successfully applied for secondary embryogenesis in *P. amabilis* var. *formosa* (Chen and Chang 2006). Two months old seedlings of *P. aphrodite* easily produced embryos in the distal part of shoots on modified-half-strength MS augmented with 1.0 mg/L TDZ (Feng and Chen, 2014). All this being considered, it was noted that there is no *in vitro* mass propagation method established for Indonesian *Phalaenopsis*, especially selected clones of 'Kristina Dwi' (KD) 69.274 and 'Dedeh' (D) 802.28, both having high potential to produce commercially and substituting import products. Establishment of the *in vitro* mass propagation protocol for the clones has important effect on commercialization of the Indonesian *Phalaenopsis*.

A reliable *in vitro* mass propagation via embryogenesis of KD 69.274 and D 802.28 clones involving induction and proliferation of embryos derived from shoot tips, followed by embryo germination, plantlet preparation and acclimatization, were the main objectives of the study. Interesting findings in each stage are discussed in this paper.

Materials and Methods

Plant material and explant preparation

Inflorescence flowers were harvested from the *Phalaenopsis* mother plants of two selected-clones (KD 69.274 and D.802.28; of ±1.5 years old) and used as explant sources. The inflorescence surfaces were then pre-treated by gently wiping all surfaces with wetted-cotton using 96% alcohol. After the pre-treatment, all nodus without flower buds were cut at ± 3 cm length (2.5 cm bottom and 0.5 upper part of nodus). The explants were put under tap water for 1 h, soaked in 1% Tween 20, then in 1% pesticide solution of benomil 50% and 20% streptomycin

Table 1. Interaction effect of clone and media on the number of embryos per explant

Clone	Initiation medium (IM)				
	M-1	M-2	M-3	M-4	M-5
KD 69.274	5.2 a	8.2 a	4.8 a	1.9 a	0.8 a
D 802.28	5.3 a	3.3 b	2.5 b	0.8 a	0.4 a

Means followed by the same letter in the same column are not significant different based on HSD (p=0.05).

sulphate, followed by manual shaking for 30 min, then finally rinsed with distilled water 4-5 times (@ 3 min each). The explants were then sterilized by immersing 0.05% mercury chloride (HgCl₂) for 10 min. followed by rinsing 5-6 times using sterile distilled water (@ 3 min. each) and then put on sterile Petri dish with sterile towel tissue.

After sterilization, damage tissue in each nodus was sliced using tissue culture blade. The nodus was then cultured on half-strength MS medium containing 1.5 mg/L TDZ, 0.25 mg/L BA, 20 g/L sucrose and 2 g/L gel rite, for 1.5 months for shoot initiation, under light incubation with 16 h photoperiod under cool fluorescence lamp with light intensity of ~13 μmol/m²/s and 24 ± 1 °C (Fig. 4A). After initiated-shoots in ± 0.5 cm in length, the shoots were used as explant source of shoot tips (Fig 4B and 4C).

Isolation of shoot tips was carried out with less than 40 × magnification of Wild Herbrugg stereo microscope using blunt pinset and sharp Aesculap tissue culture blade. Using the tools, the 4-5 scale like leaves covered the shoot tip were carefully removed one by one. After all the scale like leaves were removed, the shoot tip was sliced vertically in four positions as close as possible to the tip position using the blade, with final size of ± 0.3 × 0.3 × 0.3 mm in length-width and height (Fig. 4D). The shoot tips were then cultured on initiation media (Fig. 4E).

Induction of embryos derived from shoot tips

Induction of embryos was carried out by culturing shoot tips (Fig. 4F) of the two selected-clones KD. 69.274 and D. 802.26 (as main plot) on different initiation media (IM, as sub-plot) of (1) half-strength MS containing 0.75 mg/L TDZ and 0.25 mg/L BA (IM-1), (2) half-strength macro MS and full strength

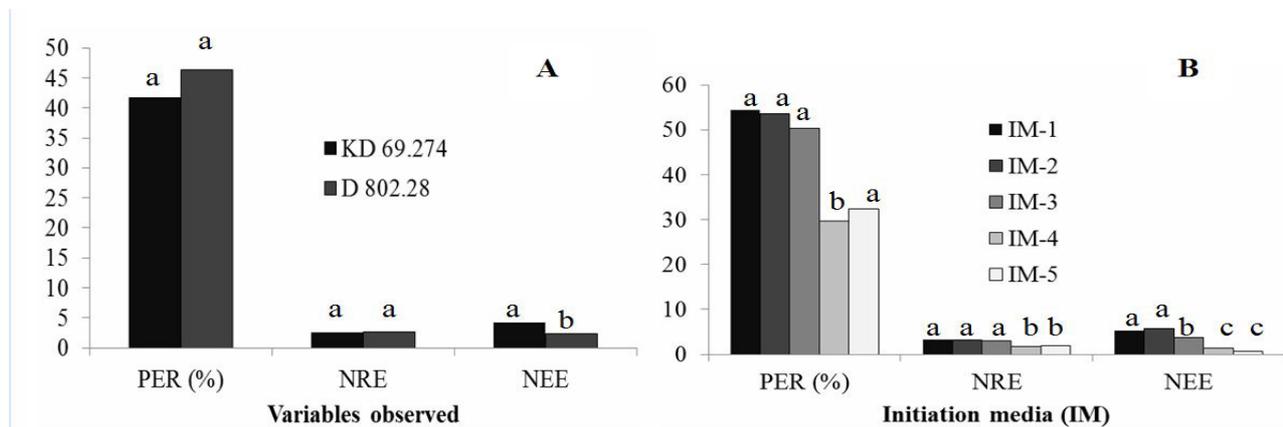


Fig. 1. Effect of clones (A) and initiation media (B) on embryo formation. PRE– percentage of explant regeneration (%); NRE– number of regenerated-explants; NEE– number of embryos per explant; IM-1– half-strength MS medium supplemented with 0.75 mg/L TDZ and 0.25 mg/L BA; IM-2– half-strength MS medium with full micro, Fe and vitamin containing 0.5 mg/L TDZ and 0.25 mg/L BA; IM-3– half-strength MS with full vitamin supplemented with 0.5 mg/L TDZ and 0.25 mg/L BA; IM-4 – half-strength MS medium with micro, Fe-chellate and vitamin augmented with 150 ml/L CW; IM-5– half-strength MS medium with full vitamin containing 150 ml/L CW. Means followed by the same letter in the same column were not significant different based on HSD (p=0.05)

for micro, Fe-chellate and vitamin supplemented with 0.5 mg/L TDZ and 0.25 mg/L BA (IM-2), (3) half-strength MS for macro, micro and Fe-chellate and full strength for vitamin augmented with 0.5 mg/L TDZ and 0.25 mg/L BA (IM-3), (4) half-strength MS for macro and full strength for micro, Fe-chellate and vitamin containing 150 ml/L coconut water (CW) (IM-4) and (5) half-strength MS for macro, micro and Fe-chellate and full strength for vitamin supplemented with 150 ml/L CW (IM-5).

The split-plot experiment was arranged in a randomized complete block design with four replications. Each treatment consisted of 2 bottles and each bottle contained 5 explants. Cultures were incubated in dark condition for 1.0-1.5 months. Embryos successfully regenerated were then transferred to light till 3 months of incubation.

Proliferation of embryos derived from shoot tips

Proliferation of embryos derived from shoot tips was carried out by culturing three types of explants as main plot and using different proliferation media as sub-plot. Three different types of explants applied in the step were (1) single embryo, (2) transversal Thin Cell Layer (tTCL) and longitudinal TCL (ITCL) of single embryo in ± 1 mm thickness. Proliferation media (PM) selected in the experiment were: PM-1, half-strength MS medium supplemented with 1.5 mg/L TDZ and 0.25 mg/L BA; PM-2, half-strength MS medium augmented with 0.75 mg/L TDZ and 0.25 mg/L BA; PM-3, half-strength MS medium containing 0.5 mg/L TDZ and 0.25 mg/L BA; PM-4, half-strength MS medium containing 0.25 mg/L TDZ and 0.25 mg/L BA; PM-5, half-strength MS medium added with 0.13 mg/L TDZ and 0.25 mg/L BA; PM-6, half-strength MS medium with full strength of vitamin containing 200 ml/L CW; PM-7, half-strength MS with full strength of vitamin augmented with 150 ml/L CW; PM-8, half-strength MS medium with full strength of vitamin containing 100 ml/L CW; PM-9, half-strength MS medium with full strength of vitamin supplemented with 50 ml/L CW; PM-10, half-strength MS medium with full strength of vitamin and hormone free.

The split-plot experiment was arranged in a randomized

complete block design with 3 replications. Each treatment consisted of three bottles. Each bottle contained 5 explants cultured. The cultures were incubated in dark for 1.0-1.5 months and then transferred to light condition in 16 h photoperiod under cool fluorescent lamp with ~13 mmol/m²/s and 24 ± 2 °C till 3 months of incubation.

Multiplication of embryos derived from shoot tips

Multiplication of embryos derived from shoot tips was carried out by individually culturing the embryos on two multiplication media (MM): half-strength MS medium supplemented with 0.13 mg/L TDZ and 0.25 mg/L BA (MM-1) and half-strength MS medium with full strength of vitamin containing 150 ml/L CW (MM-2).

Each treatment consisted of 10 bottles; each bottle contained 10 embryos and was replicated 5 times. The cultures were incubated in dark for 1.0-1.5 months and light condition till 3 months of incubation. The periodical subculture of embryos was done till optimal multiplication rate of them was noted, and declined thereafter.

Germination of embryos and plantlet preparation

Germination of embryos and plantlet preparation were carried out by culturing clusters of embryos on half-strength MS medium with full vitamin hormone free. In the experiment, the treatment consisted of ten bottles and was replicated 4 times. Each bottle contained 4 clusters of embryos. Each cluster had ± 20 embryos.

Variables observed in this experiment were percentage of germination and number of germinated-embryos per cluster. Plantlet preparation was done by culturing germinated-embryos individually in half-strength MS medium with full vitamin containing 2 g/L AC. In the experiment, the treatment consisted of ten bottles and was replicated 4 times. Each bottle contained 5 germinated-embryos.

Acclimatization of plantlets

Well-rooted plantlets (90 days old) with 4-6 leaves and 3-4 roots were selected and prepared for acclimatization. Plantlets were carefully removed from culture vessels and remaining agar

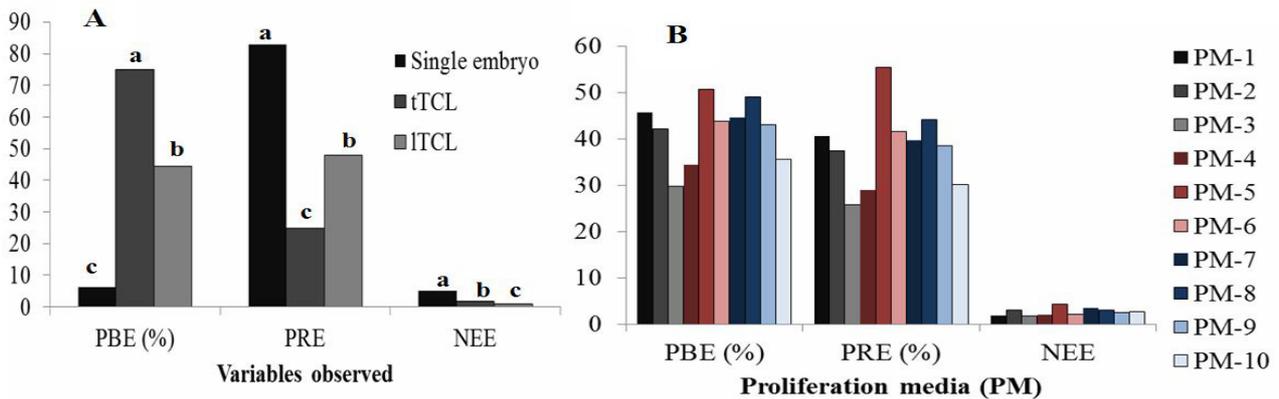


Fig. 2. Effect of explant types (A) and proliferation media (B) on embryogenesis. PM-1– half-strength MS medium supplemented with 1.5 mg/L TDZ and 0.25 mg/L BA; PM-2– half-strength MS medium augmented with 0.75 mg/L TDZ and 0.25 mg/L BA; PM-3 – half-strength MS medium containing 0.5 mg/L TDZ and 0.25 mg/L BA; PM-4– half-strength MS medium containing 0.25 mg/L TDZ and 0.25 mg/L BA; PM-5– half-strength MS medium added with 0.13 mg/L TDZ and 0.25 mg/L BA; PM-6– half-strength MS medium with full strength of vitamin containing 200 ml/L CW; PM-7– half-strength MS with full strength of vitamin augmented with 150 ml/L CW; PM-8– half-strength MS medium with full strength of vitamin containing 100 ml/L CW; PM-9– half-strength MS medium with full strength of vitamin supplemented with 50 ml/L CW; PM-10– half-strength MS medium with full strength of vitamin hormone free. Means followed by the same letter in the same column are not significant different based on HSD (p=0.05)

Table 2. Interaction effect of explant types and proliferation media on the number of embryos produced per explant

Proliferation medium (PM)	Explant type		
	Single embryo	τ-TCL	l-TCL
PM-1	3.2 de	0.5 d	1.7 b
PM-2	5.2 bcd	0.6 d	3.3 a
PM-3	1.6 e	0.7 cd	3.2 a
PM-4	1.6 e	0.8 bcd	3.5 a
PM-5	10.8 a	0.6 d	1.4 bc
PM-6	4.2 cde	1.4 abc	0.7 cd
PM-7	7.9 ab	1.6 a	0.6 cd
PM-8	7.0 bc	0.9 bcd	1.2 bcd
PM-9	5.3 bcd	1.4 ab	0.4 d
PM-10	5.1 bcd	1.3 abc	1.8 b

Means followed by the same letter in the same column are not significant different based on HSD ($p=0.05$).

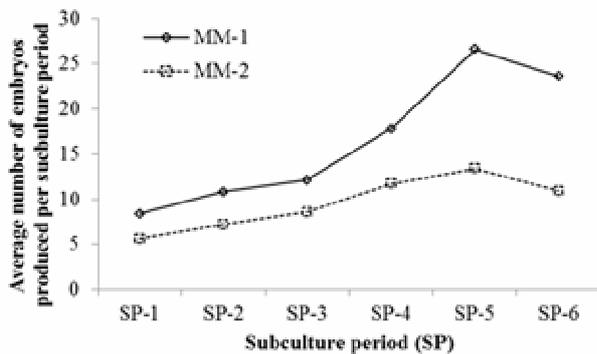


Fig. 3. Development pattern of secondary embryos produced gradually in each subculture period in two selected-media of MM-1 and MM-2. MM-1, half-strength MS medium supplemented with 0.13 mg/L TDZ and 0.25 mg/L BAP; MM-2, half-strength MS medium with full strength of vitamin containing 150 ml/L CW.

was washed off gently with tap water. Plantlets were then immersed in a 1% pesticide solution of 50% benomyl and 20% streptomycin sulphate for 3 min to reduce root rot caused by *Erwinia* sp. Small plants 4-6 cm in canopy height were cultivated in plastic pots (30 cm in diameter) containing *Cycas rumphii* bulk. Approximately 35 plantlets were planted in each plastic pot. A total of 210 plantlets derived from the root formation experiment were acclimatized in six plastic pots, which were covered with transparent plastic foil for 7 days and placed in a glass house under reduced light intensity (100–120 $\mu\text{mol}/\text{m}^2/\text{s}$) using 50% shading net. Relative humidity during plantlet acclimatization was 85–95% when plantlets in plastic pots were covered by transparent plastic and 70–90% after removing the plastic.

Variables or parameters

Variables observed in these experiments were (1) percentage of explant browning (%), calculated by counting number of browned-explants divided by total explant cultured time by 100%, (2) percentage of explant regeneration (%), calculated by counting number of regenerated-explants divided by total explant cultured time by 100%, (3) number of regenerated-explants, (4) number of embryos per explant, (5) multiplication rate of embryos, total number of embryos-regenerated per explant in the end of culture divided by number of embryos culture in the initial culture, (6) percentage of germination (%),

calculated by counting number of germinated-embryos divided by total embryos cultured times by 100%, (7) number of germinated embryos per cluster, (8) percentage of survivability (%), calculated by counting number of survival plantlets divided by total plantlets cultured time by 100%, and (9) number of survival plantlets. Periodical observation was carried out to note all alteration occurred during culture incubation. Data were recorded \pm 3 months after culture for initiation till multiplication experiment and \pm 2 months after culture for others.

Data analysis

All data collected from the experiments were analyzed by analysis of variance (Anova) with SAS program Release Windows 9.12. Significant differences between means were assessed by Tukey's Studentized Range (HSD) at $p = 0.05$ (Mattjikand Sumertajaya, 2006).

Results and Discussion

Induction of embryos derived from shoot tips

Under periodical observation it was known that initial embryo formation was taken place 15-20 days after culture (Fig. 4G). The initial embryos continually grew and were easily observed 30-40 days after culture (Fig. 4H). After transferring embryos derived from shoot tips under light incubation, the white-yellow embryos turned green 5-10 days after transferring culture under light. After 2.5-3.0 months of culture, number of embryos produced per explant varied between 1-9 embryos (Fig. 4I).

From statistical data analysis it was clearly known that the type of clones and initiation media gave significant effect on embryo regeneration in all variables observed ($p=0.05$). The two treatments also induced high significant interaction effect, whereas the type of media had higher effect compared to clones. KD 69.274 was the responsive clone in embryo formation and gave significant effect, especially in number of embryos regenerated, compare to D 802.28 clone. Though the clone had lower percentage of explant regeneration and number of regenerated-explant, it produced a number of embryos per explant up to 4.2 embryos (Fig. 1A). IM-2 (half-strength MS medium with full strength micro, Fe-chelatte and vitamin containing 0.5 mg/L TDZ and 0.25 mg/L BA) was the most appropriate medium for embryo formation, though there was no significant different compared to IM-1 (Fig. 1B). The medium induced higher number embryos up to 5.7



Fig. 4. Development of *in vitro* propagation protocol via shoot tip explant embryogenesis of *Phalaenopsis* selected-clones. A. Inflorescent node in the initial culture, selected to regenerate shoot as explant source; B. Regenerated-shoot 1.5 months after culture; C. Cross section longitudinally of shoot isolated as explant source; D. Initial isolated-shoot tip prepared and used as explant source; red circle indicate area where the shoot tip explant isolated; E. Size of shoot tip ($\pm 3 \times 3 \times 3$ mm; length, width and height) used for explant source; F. Initial condition of shoot tip cultured on initiation medium; G. Initial embryogenic callus derived from shoot tip on half-strength Murashige and Skoog (MS) medium with full strength micro, Fe-chelate and vitamin containing 0.5 mg/L thidiazuron (TDZ) and 0.25 mg/L M6-benzyladenine (BA) ± 15 days after culture; H. Development of initial embryos 1.5 months after culture; I. Mature embryos derived from shoot tip ± 3 months after culture; J. Single embryo used as explant source in proliferation and multiplication step; K. Single embryo with initial secondary embryos on half-strength MS medium supplemented with 0.13 mg/L TDZ and 0.25 mg/L BA 1.5 months after culture; L. Mature embryos derived from single embryo culture on the selected proliferation medium ± 3 months after culture; Black arrow indicates the originated embryo; M. Germinated embryos on half-strength MS medium with full strength of vitamin and hormone free ± 2 months after culture; N. Plantlets prepared for acclimatization on half strength MS medium with full strength of vitamin, 2 g/L AC and hormone free ± 2 months after culture; O. Plantlet performance prepared for acclimatization; P. Well growth performance of acclimatized plantlets on *C. rumphii* bulk 3 months after acclimatization; Q. Performance of individual acclimatized-plant ± 4 months after repotting on the same medium.

embryos per explant and the value indicated significant effect compared to IM-3 to IM-5, while the lowest medium effect on embryo regeneration was exhibited by IM-5.

Treatments of clones and media also gave significant interaction effect on embryo formation in all variables observed. Clone of KD 69.274 cultured on IM-2 medium was the most suitable combination, though it was not statistically different compared to others in percentage of explant regeneration and number of regenerated-explant. The combination successfully induced higher embryo regeneration up to 8.2 embryos per explant (Table 1, Supplementary Tables 1 and 2). The second best combination was performed on D 802.28 and KD 69.274 clones cultured on IM-1, while the lowest combination effect was indicated by D 802.28 cultured on IM-5 or IM-4, along with KD 69.274 cultured on IM-5.

Proliferation of embryos derived from shoot tips

Based on periodical observation it was known that initiation of embryos was recorded within 13-21 days after culture. In single embryo culture, especially, embryo formation was initiated by immersing protrusions in middle to basal part of single embryo body (Fig. 4K). Number on initial embryos varied among 4-25 embryos per explant (Fig. 4L) that were regenerated faster (13-15 days) than initial embryos derived from τ TCL and ITCL (15-21 days). Number of embryos induced from τ TCL and ITCL was only 1-6 embryos per explant.

In the experiment, types of explant and proliferation media gave significant effect on embryo formation. The two treatments also exhibited significant interaction effect, whereas types of explant stimulated higher effect compared to proliferation media. τ TCL and ITCL stimulated higher number of embryos initiated per explant; the two treatments caused lowering number of regenerated-embryos and stimulating high explant browning. Percentage of explant regeneration derived from the treatments was approximately 20-50%, with higher percentage of explant browning up to 75% and number of embryos produced per explant of 1.0-1.8 embryos (Fig. 2A). Culturing single embryo individually led to reducing explant browning down to 6%, increasing percentage of explant regeneration up to 83% and inducing high number of embryos regenerated up to 5.2 embryos per explant. Whereas PM-5 medium (half-strength MS medium supplemented with 0.13 mg/L TDZ and 0.25 mg/L BA) was a suitable medium for embryo proliferation. Though there was no significant different of percentage of explant browning and regeneration, the medium stimulated higher number of embryos per explant up to 4.3 embryos (Fig. 2B).

Interaction effect of explant types and proliferation media was also significantly revealed in the experiment. Single embryo cultured in PM-5 (half-strength MS medium added with 0.13 mg/L TDZ and 0.25 mg/L BA) was the most suitable combination treatment compared to other combinations. The combination reduced explants' browning down to 0%, increased percentage of explant regeneration up to 91.4% with 10.2 embryos regenerated per explant (Table 2, Supplementary Tables 3 and 4). The second best combination was single embryo cultured on PM-7 (half-strength MS with full strength of vitamin augmented with 150 ml/L CW). Combination of τ TCL and ITCL expected could produce high results, which were due to leading high

explant browning with lower regeneration of embryos. Potential embryo formation up to 3.2 embryos per explant was performed by ITCL cultured on PM-4.

Multiplication of embryos derived from shoot tips

Interested-information was clearly revealed in the stage under periodical subculture of single embryo in two multiplication media [half-strength MS medium supplemented with 0.13 mg/L TDZ and 0.25 mg/L BAP (MM-1), and half-strength MS medium with full strength of vitamin containing 150 ml/L CW (MM-2)] with different results. Number of new embryos derived from single embryo cultured increased gradually in each subculture period from first subculture till the fifth subculture and declined thereafter (Fig. 3). In MM-1 medium, single embryo in the first subculture period (SP-1) successfully produced secondary embryos up to 8.4 embryos, increased to be 10.8 embryos the SP-2, 12.1 embryos in the SP-3, 17.8 embryos in the SP-4, 26.6 embryos in the SP-5, then lowered to 23.6 embryos in the SP-6. While in the MM-2, secondary embryos produced in each subculture period were less than in MM-1. From the study, it was also clearly observed that application of synthetic hormone, TDZ and BAP in the experiment, was pre-requisite culture medium condition needed to produce higher secondary embryos for *Phalaenopsis* clones, while application of natural hormone derived from CW induced lower secondary embryos compared to the synthetic one.

Germination of embryos, plantlet preparation and its acclimatization

Germination of embryos was successfully carried out by culturing embryos in half-strength MS medium with full strength of vitamin and hormone free. The treatment induced high percentage of embryos germination from 65-89% (73.9% in average) with 13-18 numbers of germinated embryos (14.9 germinated-embryos in average) (Fig. 4M). Well plantlets were successfully prepared by culturing germinated-embryos individually in half-strength MS medium with full strength of vitamin, 2 g/L AC and hormone free. Under the treatment, plantlets had 4-6 leaves, 0.5 - 4.1 cm leaf length (1.94 cm in average), 0.4 - 1.6 cm in width (0.93 cm in average), 3 - 4 number of roots and 1.3 - 4.2 cm root length (2.47 cm in average) (Fig. 4N and 4O). Whereas in acclimatization stage of plantlets, percentage of plantlet survivability was 74.3 - 91.4% with 88.3% in average and number of survival plantlets were 26-32 plantlets, with 29.2 plantlets in average (Fig. 3P). After repotting individually, healthy and well growth of plantlets was established (Fig. 4Q).

From the study, it was clearly revealed that germination of embryos, plantlet preparation and acclimatization were easily and successfully carried out with promising results. Each step of *in vitro* plant culture needs specific and unique pre-requisite condition (Chen *et al.*, 2007; Winarto *et al.*, 2013). High responsive genotype or explant supported by optimal medium in initiation till well plantlet preparation will produce a reliable *in vitro* propagation protocol (Kuo *et al.*, 2005; Sinha and Jahan, 2011; Winarto and Teixeira da Silva, 2015). From the hereby research, it was successfully proved an *in vitro* propagation protocol for two selected *Phalaenopsis* clones. The protocol can enrich alternative choices of protocol in producing high quality and productivity of

qualified seeds in *Phalaenopsis*.

Initiation of embryo is the most critical step for *in vitro* plant culture (Sinha and Jahan, 2011; Winarto *et al.*, 2013). This phase can be affected by several factors and some of them are genotype or explant, culture medium and incubation culture (Gow *et al.*, 2009; 2010; Winarto *et al.*, 2013). Culturing small shoot tips on the half-strength MS medium with full strength of micro elements, Fe-Chellate and vitamin containing 0.5 mg/L TDZ and 0.25 mg/L BA and incubating in dark condition for one month, then transferring to light incubation, produced high embryos up to 8.2 per explant, with 58.5% explant regeneration for \pm 2.5 months. While Tokuhara and Mii (2001) found 44.4% plb formation with 24.4% callus formation by culturing shoot tips of *P.* [(Baby Hat \times Ann Jessica) \times Equestris] on New Dogashima medium (NDM) containing 0.1 mg/L α -naphthaleneacetic acid (NAA), 1.0 mg/L 6-benzylaminopurine (BAP) and 10 g/L sucrose under light incubation with 14 h photoperiod provided by fluorescent lamp at 33 μ mol/m²/s for 4 months. The plb formation was enhanced by substituting 10 g/L sucrose with 10.5 g/L glucose (Tokuhara and Mii, 2003). In other studies, utilization of apical meristem of ginger cultured on MS medium containing 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 3.0 mg/L BA resulted in high embryogenic callus formation up to 93.3% with 32.8 embryos per g of callus (Rostiana and Syahid, 2008); the similar explant of *Vitis vinifera* cultured on MS medium fortified with 1.0 mg/l indole-3-butyric acid (IBA) and 1.5 mg/l BA successfully regenerated 5.4 shoots per explant (Aazami, 2010); the meristem of *Aloe barbadensis* cultured on MS medium supplemented with 0.25 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and 1.0 mg/L Kinetin (Kin) induced embryogenic callus formation up to 21% (Garro-Monge *et al.*, 2008) and lateral meristem of *Manihot esculenta* 'Kibaha' cultured on MS medium containing 10 mg/L BAP stimulated 77.6% embryo formation with 5.8 embryos per explant (Opabode *et al.*, 2013).

Those above studies revealed that though meristematic cells having high totipotency and competency to be initiated and stimulated to form embryogenic callus and/or embryos (Henry *et al.*, 1994; Gaj, 2004), embryogenesis derived from the cells, in fact, was varied in results. High compatibility and balance effect between the explant, culture medium, culture condition and other factors kept playing important roles in supporting *in vitro* plant culture success. The optimal condition successfully induced high number of embryos up to 12.8 embryos per explant in peagon pea (Krishna *et al.*, 2011), inversely it unsuccessfully regenerated embryo formation in the most of cassava varieties (Opabode *et al.*, 2013). Thus it is worth mentioning that in the current study, high-moderate result of embryos was noted.

Successful in regeneration of embryos in initial stage of plant tissue culture will not give high effect on production and preparation of qualified-seeds, when the initial regenerated-embryos fails to be proliferated. Proliferation of the embryos or repetitive embryogenesis was significantly affected by media and/or explant type (Chen and Chang, 2007; Gow *et al.*, 2010; Feng and Chen, 2014). Chen and Chang (2007) reported that high proliferation of embryos up to 13.8 embryos per explant was established on half-strength

MS supplemented with 3.0 mg/L TDZ on *P. amabilis*. Abaxial side down of *P. amabilis* embryos on half-strength MS containing the similar TDZ concentration induced high repetitive embryos up to 15.6 embryos per explant (Gow et al., 2010), then improved by incubation of embryos in dark condition for 60 days (Gow et al., 2010). The half-strength MS medium augmented with 0.5 mg/L TDZ stimulated embryos up to 19.2 embryos (Feng and Chen, 2014). Whereas in the hereby study, it was revealed that high repetitive embryogenesis up to 10.2 embryos per embryo cultured with 91.4% explant regeneration were established by culturing ad axial side down of single embryos in half-strength MS medium supplemented with 0.13 mg/L TDZ and 0.25 mg/L BAP in dark condition for 30 days then transferred to light incubation for \pm 1.5 months. Furthermore, TCL, either τ TCL or ITCL, successfully applied and was reported on *Aranda* 'Deborah' (Lakshmanan et al., 1995), *Rhynchostylis gigantean* (Bui et al., 1999), *Cymbidium* Twilight Moon 'Day Light' (Teixeira da Silva et al., 2005); Teixeira da Silva and Tanaka, 2006) induced low embryo proliferation and high explant browning for *Phalaenopsis* clones studied in the research.

Browning explants is due to several factors and in the study it was presumably induced by stress of explant after slicing and lateness culturing them on culture media. The condition let occurring phenolic compounds easily released and in the existence of oxygen the phenolic compounds were oxidized by phenoloxidase (Ndakidemi et al., 2014), then produced quinone compounds that have negative effect on cell growth and can result in death/necrosis of cells (Ozygit et al., 2008; Rittirat et al., 2012). In other studies, browning explants were occurred due to surface damage caused by liquid culture rotation, as reported on *Dendrobium* 'Zahra FR 62' (Winarto et al., 2013), by unsuitable culture medium as reported on *Phalaenopsis cornu-cervi* on New Dogashima medium (Tokuhara and Mii, 1993) supplemented with 4% sucrose and without sucrose that induced plb browning up to 30% (Rittirat et al., 2012), or by condition of culture incubation as reported in culturing leaf explants in the dark for 15 days followed by 45 days under light which induced browning of the explants up to 90% of embryos after 60 days (Gow et al., 2009).

Multiplication of embryos under periodical subcultures on selected medium generally indicates different growth patterns (from acceleration till deceleration) and capacities in each subculture. In the hereby study, it was noted that production of embryos increased gradually starting from the first, second, third, and fourth subculture, then declined afterwards with 16.6 multiplication rate (MR), especially on half-strength MS medium containing 0.13 mg/L TDZ and 0.25 mg/L BA. Other studies showed similar trend, as recorded in *D. 'Zahra FR 62'* plb proliferation on half-strength MS medium containing 0.05 mg/L BAP with 11.99 MR (Winarto et al., 2013), in *D. 'Gradita 31'* 2.9 MR established on half-strength MS medium supplemented with 150 ml/L CW (Winarto and Teixeira da Silva, 2015). Different growth pattern and capacity were also reported by Winarto et al. (2015) on multiplication of *Eustoma grandiflorum* adventitious shoots, *Rumohra adiantiformis* (Winarto and Teixeira da Silva, 2012) and *Ruscus hypophyllum* (Winarto and Setyawati, 2014). From those studies, it was revealed that each explant and plant has unique and specific growth pattern and capacity in the multiplication explant

derived from initiation step.

High germination of embryos (73.9%) and well, healthy and vigor plantlet performance led to high survival rate of plantlets (88.3%) in transferring them in *ex vitro* condition as established in the current studym, using half-strength MS medium with full strength of vitamin, 2 g/L AC and hormone free, and *C. rumphii* bulk. The healthy and vigor plantlets were presumably become important key factor leading to high acclimatization results. The condition was also evident and reported in: *P. cornu-cervi* with high germination rate, well plantlet performance and 100% survival plantlets using MS medium supplemented with 150 ml/L CW and 2.0 g/L AC for development plbs into complete plantlets of *Sphagnum* moss (Rittirat et al., 2014); *P. amabilis* 'Golden Horizon', 100% plb conversion and 85% survival plantlets, half strength MS medium supplemented with 2 g/L peptone, 2 g/L banana pulp powder, 20 g/L sucrose, 100 ml/L CW, 1 g/L activated charcoal and coconut husk (Sinha and Jahan, 2011); *Aerides odorata*, well plantlets performance, 95% survivability of plantlets, half-strength MS medium containing 0.5 mg/L NAA and mixture of brick chip and charcoal pieces (1:1, v/v) (Devi et al., 2013); *Cymbidium lowianum*, 100% plantlet formation, 92% of survivability, half-strength MS medium 1.5 mg/L NAA, 0.1 mg/L BA, 3.0 g/L AC, and moss (Wang et al., 2013); *Orchis catasetum*, high plb germination, plantlet preparation, 100% of plants survived, MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L NAA, perlite + wood pieces + ionolite + mineral cartridge shell (1:1:1:1, v/v/v/v) (Fakouri-Ghazani et al., 2014); *Coelogyne flaccida*, high plb conversion, well plantlet performance, 80% of survival plantlets, MS medium with 2 mg/L NAA and 2 mg/L Kin, and coconut husk (De and Sil, 2015). These results indicated that plb germination, plantlet preparation and high acclimatization were easily established for orchid *in vitro* propagation protocols.

Conclusions

Finally, it can be summarized that a new *in vitro* mass propagation protocol, as new alternative choice for *Phalaenopsis* propagation, was successfully established. High embryo formation was determined by culturing shoot tips on half-strength MS medium with full strength micro, Fe-chelate and vitamin containing 0.5 mg/L TDZ and 0.25 mg/L BA. High proliferation of embryos was proved by culturing embryos individually on half-strength MS medium supplemented with 0.13 mg/L TDZ and 0.25 mg/L BA. Under periodical subcultures, the embryos were successfully multiplied on half-strength MS medium supplemented with 0.13 mg/L TDZ and 0.25 mg/L BA; number of embryos increased gradually from the first subculture till the fifth subculture, then decreased thereafter. The embryos were easily germinated on half-strength MS medium with full strength of vitamin and hormone free. Well and healthy plantlets were stimulated on the same medium with 2 g/L AC and successfully acclimatized on *C. rumphii* bulk with high survival plantlets.

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Table 1. Interaction effect of clones and media on percentage of explant regeneration (%)

Clone	Initiation medium (IM)				
	IM-1	IM-2	IM-3	IM-4	IM-5
KD 69.274	50.0 a	58.5 a	58.5 a	21.0 b	21.0 b
D 802.28	58.8 a	48.8 a	42.0 b	38.3 a	43.8 a

Means followed by the same letter in the same column are not significant different based on HSD ($p=0.05$).

Table 2. Interaction effect of clones and media on number of regenerated-explant

Clone	Initiation medium (IM)				
	M-1	M-2	M-3	M-4	M-5
KD 69.274	3.0 a	3.5 a	3.5 a	1.3 b	1.3 b
D 802.28	3.5 a	2.9 a	2.5 b	2.3 a	2.6 a

Means followed by the same letter in the same column are not significant different based on HSD ($p=0.05$).

Table 3. Interaction effect of explant types and proliferation media on percentage of explant browning (%)

Proliferation medium (PM)	Explant type		
	Single embryo	t-TCL	l-TCL
PM-1	15.5 ab	77.5 ab	43.8 ab
PM-2	4.2 ab	85.0 ab	37.5 ab
PM-3	0.0 b	52.0 b	37.5 ab
PM-4	4.2 ab	80.0 ab	18.8 b
PM-5	0.0 b	87.5 a	43.8 ab
PM-6	20.9 a	68.8 ab	62.5 a
PM-7	0.0 b	71.3 ab	62.5 a
PM-8	8.4 ab	82.5 ab	56.3 a
PM-9	4.2 ab	81.3 ab	43.8 ab
PM-10	4.2 ab	65.0 ab	37.5 ab

Means followed by the same letter in the same column are not significant different based on HSD ($p=0.05$).

Table 4. Interaction effect of explant types and proliferation media on percentage of explant regeneration (%).

Proliferation medium (PM)	Explant type		
	Single embryo	t-TCL	l-TCL
PM-1	75.5 ab	22.5 ab	51.8 ab
PM-2	81.8 ab	15.0 ab	57.5 ab
PM-3	85.0 b	48.0 a	56.5 ab
PM-4	85.8 ab	20.0 ab	76.8 a
PM-5	91.4 a	12.5 b	51.8 ab
PM-6	78.1 ab	31.8 ab	32.5 a
PM-7	89.3 b	28.3 ab	35.5 a
PM-8	71.6 b	17.5 ab	38.3 a
PM-9	87.1 ab	18.3 ab	56.8 ab
PM-10	83.8 ab	35.0 ab	57.5 ab

Means followed by the same letter in the same column are not significant different based on HSD ($p=0.05$).