Asymbiotic Seed Germination and in vitro Seedling Development of Paphiopedilum liemianum Fowlie, an Endangered Terrestrial Orchid in Northern Sumatra, Indonesia

by Edy Setiti Wida Utami

Submission date: 31-May-2018 10:26AM (UTC+0800)
Submission ID: 970544711
File name: C_1..Journal_of_Plant_Sciences.pdf (546.85K)
Word count: 4814
Character count: 25681

Journal of Plant Sciences

Volume 10 (1): 25-34, 2015

Research Article

Asymbiotic Seed Germination and *in vitro* Seedling Development of *Paphiopedilum liemianum* Fowlie, an Endangered Terrestrial Orchid in Northern Sumatra, Indonesia

Edy Setiti Wida Utami, Hery Purnobasuki, Thin Soedarti and Sucipto Hariyanto

Abstract

Paphiopedilum liemianum Fowlie, is a terrestrial orchid species and endemic in Northern Sumatra, Indonesia. However, due to a deep dormancy that the seed display at dispersion and the difficulty to obtain uniform plant in a short time period, micropropagation may be a feasible alternative. Micropropagation by *in vitro* seed germination techniques have been applied to the conservation of endangered orchid. Four months old seeds of *P. liemianum* germinated on five different basal media. All media were supplemented with 2.5 µM α-napthalleneacetic acid (NAA) and cultures were incubated in the dark for 4 weeks followed by protocorm development at condition a 16/8 h L/D photoperiod. Germination percentage was 78.8% in Vacin and Went (VW) medium were significantly higher than other basal media. To evaluated the effect of organic nutrient additives on seed germination and protocorm development, the seed were cultured on VW medium amended with different of organic nutrient. Additives, especially 10% Coconut Water (CW) to VW medium improved the protocorm development well, with 33.3% the protocorm development to stage 5 (seedling). The seedlings were cultured on VW medium supplemented with different concentrations (0.0, 1.0, 2.0, 3.0 and 4.0 µM) of thidiazuron (TDZ). Healthy plantlets with developed leaves and roots were planted in pots with sphagnum moss and grown under *ex vitro* condition and the result was 76% survival rate after 4 weeks.

How to cite this article:

Edy Setiti Wida Utami, Hery Purnobasuki, Thin Soedarti and Sucipto Hariyanto, 2015. Asymbiotic Seed Germination and *in vitro* Seedling Development of *Paphiopedilum liemianum* Fowlie, an Endangered Terrestrial Orchid in Northern Sumatra, Indonesia. *Journal of Plant Sciences, 10: 25-34.*

DOI: 10.3923/jps.2015.25.34

URL: https://scialert.net/abstract/?doi=jps.2015.25.34

INTRODUCTION

Paphiopedilum is a genus of the flowering plant family Orchidaceae, well known as Slipper Orchids, is characterized by resemblance of the pouch-shaped lip to a lady's slipper (Lin *et al*., 2000; Chen *et al*., 2004). This genus is terrestrial orchids with about 70 species that are native to South and South East Asia and a distribution that extends from the ASEAN region up to Papua New Guinea (Teob, 1989). Their flowers are variety of shapes, size, colors and the most popular commercially available orchids among the Orchidaceae family and a highly valued ornamental plant (Cribb, 1998; Ng and Saleh, 2011; Hong *et al*., 2008).

Although, *Paphiopedilum* produce abundant seeds, this orchid is relatively rare in the wild due to the absence of endosperm in their seed (Arditti and Ernst, 1993; Pierik *et al*., 1988; Long *et al*., 2010). In nature, the *Paphiopedilum* are endangered population because of destruction and very restricted habitat and all species were included to the list of endangered and threatened species in Appendix I of the convention on international trade in endangered species of wild fauna and flora (CITES, 2011).

Paphiopedilum liemianum Fowlie, is a terrestrial orchid species which is endemic in Northern Sumatra, Indonesia. It was found growing among rocks or at the root of the tree at the elevation of 500-1200 m. This species is characterized by pouch shaped-lip, rounded at the ends, pink and fade towards the edge, leathery and ribbon leaves with a prominent middle rib, leaf color dark green at upper surface and green with purple grape spotting at lower surface with trichomes at the edge leaves. Inflorescence emerges from the tip of the stem, supporting approximately to 8 flowers. The flowers stay fresh for up to 3 weeks. They are marketed as a pot plant with high value because of its unique and exotic flowers. This species is unique because of spiral corolla. For the micropropagation and conservation of this endangered species, information concerning its developed an effective protocol for the asymbiotic production of the terrestrial orchid *P. liemianum* is thus of great importance to study. Knowledge of the factors that affect asymbiotic seed germination required in the design of experiments for ***in vitro*** propagation studies. Micropropagation by ***in vitro*** seed germination techniques have been applied to the conservation of endangered and threatened orchid and may be useful in the re-introduction of *P. liemianum*.

There are many reports of the micropropagation of some *Paphiopedilum* species and their hybrids by the ***in vitro*** seed germination (Pierik *et al*., 1988; Long *et al*., 2010; Ding *et al*., 2004; Lee, 2007; Tay *et al*., 1988; Stimart and Ascher, 1981; Nagashima, 1982; Zeng *et al*., 2012, 2013). However, there is still lack of information about the asymbiotic seed germination on *P. liemianum*.

In this study, the effects of germination media and organic nutrient additives on asymbiotic mature seed germination of *P. liemianum* were evaluated. The effects of growth regulator on ***in vitro*** seedlings development were also investigated. The aim of this study was to formulate the simple protocol for propagation of threatened orchid species by ***in vitro*** in large scale.

MATERIALS AND METHODS

Seed source and sterilization procedure: The mature capsules that are 4 months old after hand pollination were collected from DD Orchid Nursery, Junrejo Village, East Java, Indonesia. The capsules were soaked in commercial detergent liquid (Sunlight, Unilever, Indonesia) for 4 min for eliminated the dust, followed was washed 3 times with sterile-distilled water and drained. The capsules surfaces were disinfected using 70% ethanol and then flamed for 3 times in a laminar air flow cabinet. The capsules were sliced transversally and longitudinally into four parts with a sterile surgical blade in a sterile petri dish. The mature seeds from the capsules were removed and pooled into the petri dish with the help of a sterilized spatula.

Basal media effect on asymbiotic seed germination: To identify the suitable medium for seed germination and protocorm development, the seed were sowing onto five basal media: (1) Vacin and Went (Vacin and Went, 1949), (2) Quarter-strength Murashige and Skoog: 1/4 Murashige and Skoog macro-and micro-nutrient (Murashige and Skoog, 1962), (3) Half-strength MS (1/2 MS macro-and micro-nutrient), (4) Knudson C orchid medium (Knudson, 1946) and (5) Robert and Erast (Arditti, 1982). All media were supplemented with 2.5 µM α-napthalleneacetic acid (NAA, from Merck-Schuchardt), 3% sucrose and solidified with 0.2% gellan gum (Phytagel; Sigma Chemical Co., St. Louis, MO) and adjusted to a pH of 5.6 with NaOH and HCl prior to autoclaving at 120°C and 105 kg cm^{-2} for 15 min.

Table 1: Seed germination and protocorm development stages of *Paphiopedilum liemianum* fowlie modified from Yamazaki and Miyoshi (2006) and Miyoshi and Mii (1995)

Stages	Description
0	Ungerminated: No growth of embryo occurs
1	Enlarged embryo: Covered by testa
2	Testa ruptured: Embryo emerges from the testa (germination)
3	Embryo is discharged from the testa: Appearance of the shoot apex and or rhizoids (protoco
4	Emergence of first leaf
5	Presence of two or more leaves, roots primordial present (seedling)

For each treatment, approximately 200 seeds were cultured in a 150 mL culture flask containing 25 mL of medium which were covered with alumunium foil. All experiments consisted of three independent replicates with 5 cultures flasks per replication. All the cultures were incubated in the dark for 4 weeks followed by protocorm development under a 16/8 h L/D photoperiod at 23±2°C. Culture were observed at 12 weeks after inoculation to determinate the effect of basal medium on seed germination and protocorm development using a Tension stereomicroscope, Nikon SMZ-1, Japan. The process of seed germination to protocorm development was classified into the following six stages according to the developmental stages of embryos (Table 1), which were modified from Yamazaki and Miyoshi (2006) and Miyoshi and Mii (1995). Germination was consideret to have occured only if testa ruptured and if embryo emerges from the testa (Stage 2, Fig. 1d). The persentage of seed at the different developmental stage was counted by dividing the number of seed in each stage by the total number of seedx100 (including viable and non viable seed).

Organic nutrient additives effect on asymbiotic seed germination: To evaluate the effect of organic nutrient additives on seed germination and protocorm development, the seed were cultured on VW medium which is the most suitable medium concluding from the first experiment result. The seeds were placed on VW medium amended with 10% Coconut Water (CW), 100 g L^{-1} tomato mash, 100 g L^{-1} potato mash, 2 g L^{-1} peptone and without organic nutrient additives as control treatment. The source of these additives were fresh tomato *Lycopersicum esculentum* Mill, potato *Solanum tuberosum* L, that were skinned and homogenized in a blender. All the cultures were incubated in the dark for 4 weeks followed by protocorm development under a 16/8 h L/D photoperiod. All the cultures were maintened for 12 weeks at 23±2°C. The percentage of seed at the different developmental stage was counted by dividing the number of seed in each stage by the total number of seedx100 (including viable and non viable seed).

Evaluation of the effects of growth regulator on seedling development: After 12 weeks of culture, seedling derived from seeds of mature capsules were sub cultured to fresh media. Seedlings, 4-6 mm in length were grown on VW medium supplemented with Plant Growth Regulators (PGRs) with 1, 2, 3 and 4 µM thidiazuron (TDZ) and without PGRs as control treatment. Each treatment consisted of three independent replicates with five culture flasks per replicate containing four to five seedlings in each flask. After 16 weeks culture, weight and length of plantlet, number of leaves and roots, length and maximum width of leaf, length and maximum diameter of root were recorded. Following *P. liemianum* plantlets with 2-3 leaves bearing 2-3 roots (approximately 3-4 cm in height) were taken out from the culture flask and washed with running water to remove any residual agar.

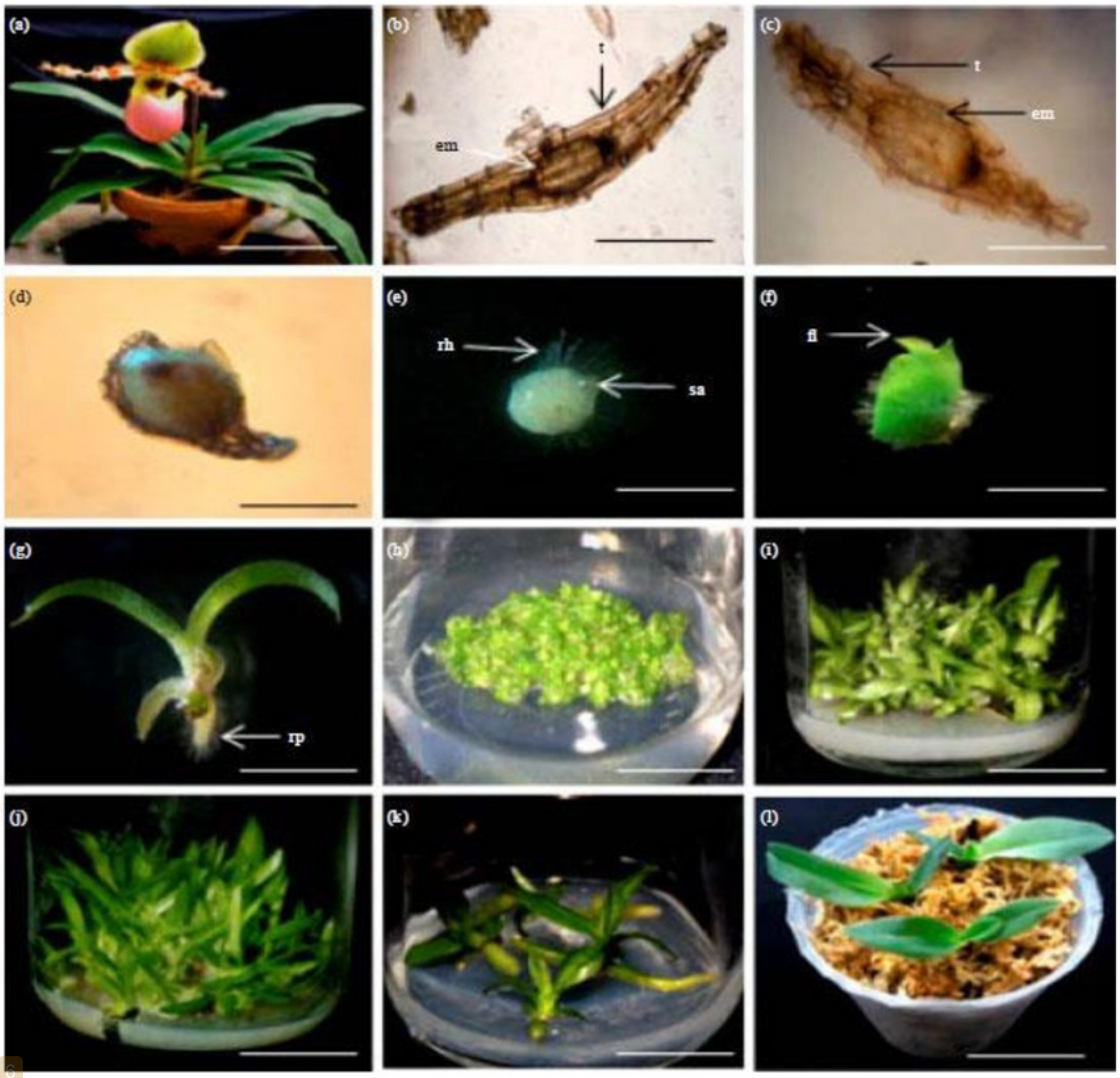

Fig. 1(a-l): Asymbiotic seed germination and seedlings development of Paphiopedilum liemianum Fowlie, (a) Flowering plant of Paphiopedilum liemianum fowlie, (b) Stage 0, seed, no growth of embryo occurs, (c) Stage 1, enlarged embryo, (d) Stage 2, testa ruptured (= germinated), (e) Stage 3, embryo is discharged from the testa (= protocorm), (f). Stage 4, emergence of first leaf, (g) Stage 5, present of two or more leaves, roots primordial present (= seedling), (h) Asymbiotic seed germination and protocorm development on VW medium supplemented with 2.5 µM NAA, (i) Seedlings **in vitro** on VW medium supplemented with 2.5 µM NAA and 2 g LG1 peptone, (j) Seedlings growth on VW medium supplemented with 2.5 µM NAA and 10% coconut water (CW: v/v), (k) Developed plantlet with 3-4 leaves and roots after 16 weeks of culture on VW supplemented with 4 µM thidiazuron and (l) Transplanted plantlets after 4 weeks acclimatize in the greenhouse. em: Embryo, fl: First leaf, rh: Rhizoids, rp: Root primordial, sa: Shoot apex, t: Testa. Scale bars: (a) 5 cm, (b) 177 µm, (c) 230 µm, (d)

240 µm, (e) 870 µm, (f) 2.5 mm, (g) 3.5 mm, (h) 1.5 cm, (i) 1.6 cm, (j) 1.6 cm, (k) 1.25 cm and (l) 3.5 cm

The plantlets were then placed into 10 cm diameter plastic pots containing shagnum moss. All the pots were then kept to the greenhouse (temperature 26±2°C, light intensity 3700 lux, 14 h photoperiod and sprayed with water twice a day) for acclimatization.

Experimental design and statistical analysis: This experiment was arranged in a Completely Randomized Design (CRD). The data of all the experiments was analyzed using one-way analysis of variance (ANOVA) with SPSS 14. Subsequent mean separation was followed by the Duncan's Multiple Range Test (DMRT) with level of significance at p = 0.05 (Duncan, 1955).

RESULTS

Asymbiotic seeds germination and protocorm development: Dry seeds of *P. liemianum* were used as explants of this study that have brownish yellow are 816 µm longs, 142 µm wide and the embryos consist only undifferentiated cells surrounded by a testa (Fig. 1b). The morphological development stage of *P. liemianum* from seed to seedling was documented (Fig. 1b-g). The seed germination stages starts about 3 weeks after inoculation with an enlarged embryo (Fig. 1c). Six weeks after inoculation, the embryos become swollen, the testa begins ruptured and embryo so white (Fig. 1d). Following embryos is discharged from the testa, shoot apex become able to be seen at one side of protocorm and appear rhizoids at the other (Fig. 1e). When protocorm reaches a size of about 2.5 mm, the protocorms fastly turn green, followed by the first leaf forms respectively (Fig. 1f). Generally, the roots primordial presence when the second leafs present (Fig. 1g).

Basal medium effect on asymbiotic seed germination and development of *Paphiopedilum liemianum* fowlie: The effect of five different basal media on asymbiotic seed germination of *P. liemianum* after 12 weeks in culture is shown in Table 2. The results show that type of basal media significantly affected on asymbiotic seed germination and development of *P. liemianum*. The highest percentage seed germination was obtained on VW medium (78.8%). The seed germination rate in the RE medium (63.9%) was lower than in VW medium, but higher than in half-strength MS (60.6%), quarter-strength MS (58.2%) and KC (55.6%). VW medium was also found suitable for protocorms development, 28.6% protocorms developed to stage 5 seedlings, were significantly higher than other basal media.

Organic nutrient additives effect on asymbiotic seed germination: The results of Table 3 show that seeds, cultured on VW medium supplemented with 2 g L^{-1} peptone had highest percent germination among all treatment (72.8%, respectively) when compared VW containing 10% CW (66.7%), tomato 100 g L^{-1} (53.3%), whereas both VW medium containing potato 100 g L^{-1} and without any organic amendments (control) showed seed **germination percentage** are same (49.7%).

Table 2: Basal medium effect containing 2.5 µM NAA on asymbiotic seed germination and development of *Paphiopedilum liemianum* fowlie for 12 weeks culture

Basal medium	Seedling developmental stage (%)						
	Stage 0	Stage 1	Stage 2 (germinated)	Stage 3 (protocorm)	Stage 4	Stage 5 seedling	Total germination (stage 2
KC	36.8±0.9^{a}	7.6±0.4^{a}	6.3±0.9^{a}	10.2±0.5^{a}	14.4±0.6^{b}	24.7±1.5^{c}	55.6±1.1^{a}
RE	24.0±0.7^{b}	12.1±0.7^{b}	15.6±1.0^{a}	13.5±0.9^{d}	15.0±0.7^{c}	19.8±1.8^{a}	63.9±0.8^{d}
VW	13.8±0.6^{a}	7.4±0.6^{a}	11.9±1.0^{c}	16.5±0.7^{a}	21.8±0.6^{d}	28.6±1.4^{d}	78.8±0.8^{a}
1/2 MS	24.9±0.7^{c}	14.5±0.7^{d}	13.8±0.6^{d}	12.6±0.5^{c}	14.3±0.5^{ab}	19.9±1.3^{a}	60.6±0.7^{c}
1/4 MS	29.1±0.7^{d}	12.7±0.6^{c}	9.2±0.6^{b}	11.7±0.7^{b}	13.8±0.5^{a}	23.5±1.0^{b}	58.2±0.7^{b}

Values followed by same the letters within a column are not significantly different at p≤0.05 according to DMRT. Each mean is based on microscopic observation

Table 3: Organic nutrient additives effect on asymbiotic seed germination and development of *Paphiopedilum liemianum* fowlie on VW medium containing 2.5 µM NAA for 12 weeks culture

Organic nutrient	Seedling development stage (%)						
	Stage 0	Stage 1	Stage 2 (germinated)	Stage 3 (protocorm)	Stage 4	Stage 5 (seedling)	Total germinat (stage 2-5)
Control	39.8±0.6^{d}	10.5±0.8^{c}	9.4±0.5^{b}	8.5±0.5^{c}	8.6±0.6^{a}	23.2±1.1^{a}	49.7±1.0^{a}
Tomato 100 g L^{-1}	37.9±1.0^{c}	8.8±0.5^{b}	13.0±0.7^{c}	6.4±0.4^{a}	9.1±0.6^{ab}	24.8±1.1^{b}	53.3±0.8^{b}
Potato 100 g L^{-1}	39.4±0.7^{d}	10.9±0.6^{c}	8.4±0.7^{a}	7.5±0.5^{b}	9.3±0.8^{b}	24.5±1.1^{b}	49.7±1.0^{a}
Peptone 2 g L^{-1}	20.9±1.2^{a}	6.3±0.5^{a}	16.9±0.8^{d}	14.1±0.6^{a}	14.6±0.5^{d}	27.2±0.8^{c}	72.8±1.2^{d}
10% CW	24.9±0.7^{b}	8.4±1.0^{b}	8.4±0.9^{a}	11.0±1.0^{d}	14.0±1.1^{c}	33.3±1.3^{d}	66.7±1.1^{c}

Values followed by same the letters within a column are not significantly different at p≤0.05 according to DMRT. Each mean is based on microscopic observation

Table 4: Effects of growth regulator on seedlings development of *Paphiopedilum liemianum* fowlie on VW medium for 16 weeks culture

TDZ (µM)	Plantlet		Leaf (mm)			Root (mm)		
	Weight (g)	Length (mm)	No.	Length	Maximum width	No.	Length	Maximum diame
0	16.8±0.2^{a}	31.5±1.1^{a}	2.3±0.4^{a}	25.6±1.9^{a}	7.3±0.6^{a}	2.0±0.5^{a}	7.7±0.9^{a}	1.1±0.2^{a}
1	17.9±0.2^{b}	34.3±0.8^{c}	2.6±0.4^{b}	27.5±1.0^{b}	8.9±0.8^{c}	2.2±0.4^{ab}	9.5±0.6^{b}	1.2±0.3^{a}
2	38.9±0.1^{d}	32.3±0.7^{b}	3.5±0.5^{c}	29.3±0.7^{c}	8.1±1.0^{b}	3.3±0.4^{c}	9.6±0.4^{b}	1.6±0.3^{b}
3	23.9±0.1^{c}	43.4±1.0^{d}	2.4±0.5^{ab}	30.3±0.7^{d}	9.5±0.5^{d}	2.4±0.4^{b}	11.6±1.4^{c}	1.8±0.2^{c}
4	64.6±0.1^{e}	47.9±0.8^{e}	4.6±0.5^{d}	30.2±0.9^{d}	12.5±0.6^{e}	4.1±0.5^{d}	11.5±0.7^{c}	2.3±0.4^{d}

Values followed by same the letters within a column are not significantly different at p≤0.05 according to DMRT

Effects of growth regulator on seedling development: Table 4 shows the effects of growth regulator (PGRs) TDZ on seedling development. Seedling measuring about 4-6 mm in length were cultured on VW medium supplemented with various concentration of TDZ (0-4 µM). The presence of PGRs in the VW medium had significantly resulted in a better response than without PGRs. In the treatment without PGRs showed a low leaf number and root number. Here also

slow leaf and root growth were observed. The maximum response was obtained on VW medium supplemented with 4 µM TDZ with an average weight 64.6 g per plantlet, an average length 47.9 mm per plantlet, an average maximum width 12.5 mm per leaf and an average maximum diameter 2.3 mm per root. Among the four TDZ concentration tested, the 4 µM TDZ also proved to be more effective in increasing the leaf and root formation. In 3 µM TDZ-containing medium also showed more growth induction with an average length 30.3 mm per leaf and an average length 11.6 mm per root, however there is no significant difference with 4 µM TDZ.

DISCUSSION

We found the germination occurred on all media testing, but **germination percentage** in VW medium were significantly higher than other basal media and only VW medium supported high number of advanced stage 5 seedlings. The stimulate effect of the VW medium on asymbiotic seed **germination percentage** and protocorm development, which possible be due to the VW medium contained phosphate at a relatively higher concentration (4.74 mM) compared to RE (2.21 mM), KC (1.84 mM), half-strength MS (0.62 mM) and quarter-strength MS (0.32 mM). This result is similar to Dutra *et al*. (2008) working with *Bletia purpurea* terrestrial orchid cultured on VW medium more optimal for asymbiotic seed germination than on KC, ½ MS, MM, BM-1, P723. Phosphate availability in the culture media also had significantly effect to growth and development of *Bletia purpurea* seedlings. Teixeira Da Silva *et al*. (2005) reported the source and concentration of phosphate plays a decisive role in the success of protocorm-like body and **callus induction** and development in hybrid *Cymbidium*. In different, Zeng *et al*. (2012) reported a higher percentage of germination for *Paphiopedilum wardii* on ½ MS than ¼ MS, MS, KC, VW, RE, Thomale GD, Hyponex. Tay *et al*. (1988) reported that seeds of *Paphiopedilum sukhakulii* cultured on Norstog medium to be optimal for ***in vitro*** germination than on Burgeff EG-1 medium. Johnson *et al*. (2007) reported P723 medium to be most suitable for germination of *Eulopia alta* terrestrial orchid than on ½ MS, KC, MM, VW, while Stewart and Kane (2006) working with *Habenaria macroceratitis* terrestrial orchid obtained on LM and KC medium, significantly higher than on ML, MS, VW, MM media. In addition, same medium the ideal for seed germination of the differ *Paphiopedilum* species; for example: *Paphiopedilum ciliolare*, *Paphiopedilum delenatii*, *Paphiopedilum villosum* var. *densisimum* have been shown that on KC medium to be most suitable for germination of these species (Pierik *et al*., 1988; Nhut *et al*., 2005; Long *et al*., 2010).

Effect of organic nutrient additives on seed germination and protocorm development had already tested in a large number orchids such as *Epidendrum ibaguense* Kunth (Hossain, 2008); *Ophrys spuneri* (Kitsaki *et al*., 2004); *Rhychostylis retusa* Blume (Thomas and Michael, 2007); *Cymbidium findlaysonianum* Lindl (Towaro *et al*., 2008); *Cleisostoma racemiferum* Lindl (Temjensangba and Deb, 2006); *Grammatophyllum scriptum* (Abbas *et al*., 2011); *Grammatophyllum speciosum* (Khampa *et al*., 2010); *Acampe papillosa* (Piri *et al*., 2013). We therefore, investigated the effects of organic nutrient on seed germination and protocorm development in *P. liemianum*. Organic nutrient additives in culture media as CW, tryptone, peptone, banana homogenates, potato homogenates, apple mash, lactalbumin hydrolysate, chayote mash for seed germination and protocorm development in some terrestrial orchids has been (Pierik *et al*., 1988; Long *et al*., 2010; Zeng *et al*., 2012), however showed different results.

As shown in Table 3, we found the germination occured on all organic nutrient additives evaluated. Although seed **germination percentage** was maximum on VW medium supplemented with 2 g L^{-1} peptone and 14.1% protocorm remained in stage 3, however only 27.2% protocorm development to stage 5, but rather on 10% CW (33.3%). Our results indicated that for asymbiotic seed germination and subsequent protocorm development, VW supplemented with 10% CW was found to be most suitable medium for *P. liemianum*. This facilitating effect of CW may be because CW contain carbohydrate, vitamine, **amino acid**, **organic acid**, organic ion and enzyme which are usually important to plant cell development (Nitsch, 1951). Shantz and Steward (1952) and Shantz and Steward (1955) showed CW at concentration of 10-15%, it enhanced seed germination of many orchid species ***in vitro***. This result of the study supported by Zeng *et al*. (2012) reported seed germination and protocorm development percentages of *Paphiopedilum wardii* Sumerh to be ca 65% highest with 10% CW than potato and banana homogenate, peptone and tryptone. Coconut water induced seed germination has been reported by Long *et al*. (2010) with *Paphiopedilum villosum* var. *dentissimum*. Of the five organic nutrient used, 10% CW was found to be most potential in which highest frequency of seed germination ca 10%, germination using potato and apple homogenates were <3%, while those with lactalbumin hydrolysate and chayote mash were 0%. In contrast, Pierik *et al*. (1988) reported seed **germination percentage**s of *Paphiopedilum ciliolare* to be ca 80% higher with tryptone than banana homogenates.

In this study, the maximum seedling growth of *P. liemianum* were obtained when they were cultured onto VW medium containing the highest level of TDZ (4 µM). The highest number of leaf and root was also observed when seedling grown on this medium. Thomas and Michael (2007) reported that addition of TDZ showed maximum response on seedling growth and development of *Rhynchostylis retusa*. This result is also partially agreed with Shiau *et al*. (2005) where they found cytokinins including TDZ, promoted the seedling growth of *Haemaria discolor*. In contrast, Vogel and Macedo (2011) observed that the addition of high concentration of TDZ abnormal shoot formation in *Cyrtopodium glutuniferum*. This indicated that effects of cytokinin supplementation to the culture medium on the seedling development of orchids varies with the species.

Healthy plantlets with developed leaves and roots were planted in pots sphagnum moss and grown under greenhouse condition. These plantlets adapted well in the *ex vitro* condition and the result was recorded 76% survival rate after 4 weeks (Fig. 1l).

CONCLUSION

The conclusion of this study, asymbiotic seed germination techniques can be used for producing large numbers of *P. liemianum* seedlings and the plantlets can be succesfully *ex vitro* acclimatized.

AUTHOR'S CONTRIBUTIONS

The work presentated here was carried out in collaboration between all authors. ESWU and HP designed the experiment, TS and SH hand pollination, collected the data and analyzed the data,

ESWU carried out the laboratory experiments and wrote the manuscript, HP revised the manuscript. All the authors agreed on the contents of the paper and post no conflicting interest.

References

Abbas, B., F.H. Listyorini and B. Amriati, 2011. *In vitro* seeds germination and plantlets development of *Grammatophyllum scriptum* Lindl. (Orchidaceae). Int. Res. J. Plant Sci., 2: 154-159.
Direct Link |

Arditti, J. and R. Ernst, 1993. Micropropagation of Orchids. John Wiley and Sons, New York.

Arditti, J., 1982. Orchid Biology: Reviews and Perspectives, II. Cornell University Press, Ithaca, NY., ISBN-13: 978-0801412769, Pages: 368.

CITES, 2011. Checklist of CITES species-2011: CITES appendices I, II and III. Convention on International Trade in Endangered Species of Wild Fauna and Flora, CITES Secretariat, Geneva, Switzerland. http://www.cites.org/eng/app/2011/E-Dec22.pdf.

Chen, T.Y., J.T. Chen and W.C. Chang, 2004. Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids. Plant Cell Tissue Organ Cult., 76: 11-15.
CrossRef |

Cribb, P., 1998. The Genus Paphiopedilum. 2nd Edn., Natural History Publication (Borneo), Kota Kinabalu, Malaysia, ISBN-13: 9789838120234, Pages: 427.

Da Silva, J.T., T. Yam, S. Fukai, N. Nayak and M. Tanaka, 2005. Establishment of optimum nutrient media for *in vitro* propagation of *Cymbidium* Sw. (Orchidaceae) using protocorm-like body segments. Propag. Ornam. Plants, 5: 129-136.
Direct Link |

Ding, C.C., H. Wu and F.Y. Liu, 2004. Factors affecting the germination of *Paphiopedilum armeniacum*. Acta Botanica Yunnanica, 26: 673-677.

Duncan, D.B., 1955. Multiple range and multiple F tests. Biometrics, 11: 1-42.
CrossRef | Direct Link |

Dutra, D., T.R. Johnson, P.J. Kauth, S.L. Stewart, M.E. Kane and L. Richardson, 2008. Asymbiotic seed germination, *in vitro* seedling development and greenhouse acclimatization of the threatened terrestrial orchid *Bletia purpurea*. Plant Cell Tissue Organ Cult., 94: 11-21.
CrossRef |

Hong, P.I., J.T. Chen and W.C. Chang, 2008. Plant regeneration via protocorm-like body formation and shoot multiplication from seed-derived callus of a maudiae type slipper orchid. Acta Physiologiae Plantarum, 30: 755-759.
CrossRef | Direct Link |

Hossain, M.M., 2008. Asymbiotic seed germination and *in vitro* seedling development of *Epidendrum ibaguense* Kunth. (Orchidaceae). Afr. J. Biotechnol., 7: 3614-3619.
Direct Link |

Johnson, T.R., S.L. Stewart, D. Dutra, M.E. Kane and L. Richardson, 2007. Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)-preliminary evidence for the symbiotic culture advantage. Plant Cell Tissue Organ Cult., 90: 313-323.
CrossRef |

Khampa, S., P. Wangsomnuk and P. Wangsomnuk, 2010. Factors affecting seed germination of *Grammatophylum speciosum* cultured *in vitro*. Asia-Pac. J. Mol. Biol. Biotechnol., 18: 193-197.
Direct Link |

Kitsaki, C.K., S. Zygouraki, M. Ziobora and S. Kintzios, 2004. *In vitro* germination, protocorm formation and plantlet development of mature versus immature seeds from several *Ophrys* species (Orchidaceae). Plant Cell Rep., 23: 284-290.
CrossRef | PubMed | Direct Link |

Knudson, L., 1946. A new nutrient solution for germination of orchid seed. Am. Orchid Soc. Bull., 15: 214-217.

Lee, Y.I., 2007. The asymbiotic seed germination of six *Paphiopedilum* species in relation to the time of seed collection and seed pretreatment. Acta Hort., 755: 381-385.
Direct Link |

Lin, Y.H., C. Chang and W.C. Chang, 2000. Plant regeneration from callus culture of a *Paphiopedilum* hybrid. Plant Cell Tissue Organ Cult., 62: 21-25.
CrossRef |

Long, B., A.X. Niemiera, Z.Y. Cheng, C.L. Long, 2010. *In vitro* propagation of four threatened *Paphiopidilum* species (Orchidaceae). Plant Cell Tissue Organ Cult., 101: 151-162.
Direct Link |

Miyoshi, K. and M. Mii, 1995. Enhancement of seed germination and protocrom formation in *Calanthe discolor* (Orchidaceae) by NaOCl and Polyphenol adsorbent treatments. Plant Cell Tissue Organ Cult., 12: 267-272.
Direct Link |

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Planta., 15: 473-497.
CrossRef | Direct Link |

Nagashima, T., 1982. Studies in the seed germination and embryogenesis in *Goeringii* Rchb. F. and *Paphiopedilum insigne* var. sanderae Rchb. J. Jpn. Soc. Hort. Sci., 51: 94-105.

Ng, C.Y. and N.M. Saleh, 2011. *In vitro* propagation of *Paphiopedilum* orchid through formation of protocorm-like bodies. Plant Cell Tissue Organ Cult., 105: 193-202.
CrossRef | Direct Link |

Nhut, D.T., P.T.T. Trang, N.H. Vu, D.T.T. Thuy, D. Van Khiem, N. Van Binh and K.T.T. Van,

2005. A wounding method and liquid culture in *Paphiopedilum delenatii* propagation. Propag. Ornam. Plants, 5: 158-163.

Nitsch, J.P., 1951. Growth and development *in vitro* of excised ovaries. Am. J. Bot., 38: 566-571.

Pierik, R.L.M., P.A. Sprenkels, B. van der Harst and Q.G. Van der Meys, 1988. Seed germination and further development of plantlets of *Paphiopedilum ciliolare* Pfitz. *in vitro*. Scient. Hortic., 34: 139-153.
CrossRef | Direct Link |

Piri, H., P. Pathak and R.K. Bhanwra, 2013. Asymbiotic germination of immature embryos of a medicinally important epiphytic orchid *Acampe papillosa* (Lindl.) Lindl. Afr. J. Biotechnol., 12: 162-167.
Direct Link |

Shantz, E.M. and F.C. Steward, 1952. Coconut milk factor: The growth-promoting substances in coconut milk. J. Am. Chem. Soc., 74: 6133-6135.
CrossRef | Direct Link |

Shantz, E.M. and F.C. Steward, 1955. The identification of compound a from coconut milk as 1, 3-diphenylurea. J. Am. Chem. Soc., 77: 6351-6353.
CrossRef | Direct Link |

Shiau, Y.J., S.M. Nalawade, C.N. Hsai and H.S. Tsay, 2005. Propagation of *Haemaria discolor* via *in vitro* seed germination. Biol. Plant., 49: 341-346.
CrossRef | Direct Link |

Stewart, S.L. and M.E. Kane, 2006. Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. Plant Cell Tissue Organ Cult., 86: 147-158.
CrossRef | Direct Link |

Stimart, D.P. and P.D. Ascher, 1981. *In vitro* germination of *Paphiopedilum* seed on a completely defined medium. Sci. Horticult., 14: 165-170.
CrossRef |

Tay, L.J., K. Takeno and Y. Hori, 1988. Culture conditions suitable for *in vitro* seed germination and development of seedling in *Paphiopedilum*. J. Jpn. Soc. Hort. Sci., 57: 243-249.
Direct Link |

Temjensangba and C.R. Deb, 2006. Effect of different factors on non-symbiotic seed germination, formation of protocorm-like bodies and plantlet morphology of *Cleisostoma racemiferum* (Lindl.) Garay. Indian J. Biotechnol., 5: 223-228.
Direct Link |

Teob, E.S., 1989. Orchids of Asia. Times Books International, Singapore, pp: 125-134.

Thomas, T.D. and A. Michael, 2007. High-frequency plantlet regeneration and multiple shoot induction from cultured immature seeds of *Rhynchostylis retusa* Blume., an exquisite orchid.

Plant Biotechnol. Rep., 1: 243-249.
CrossRef | Direct Link |

Towaro, S., P. Suraninpong and S. Chanprame, 2008. Germination and regeneration of *Cymbidium findlaysonianum* Lindl. on a medium supplemented with some organic sources. Walailak J. Sci. Tech., 5: 125-135.
Direct Link |

Vacin, E.F. and F.W. Went, 1949. Some pH changes in nutrient solutions. Bot. Gaz., 110: 605-613.
Direct Link |

Vogel, I.N. and A.F. Macedo, 2011. Influence of IAA, TDZ and light quality on asymbiotic germination, protocorm formation and plantlet development of *Cyrtopodium glutiniferum* Raddi., A medicinal orchid. Plant Cell Tissue Organ Cult., 104: 147-155.
CrossRef |

Yamazaki, J. and K. Miyoshi, 2006. *In vitro* asymbiotic germination of immature seed and formation of protocorm by *Cephalanthera falcata* (Orchidaceae). Ann. Bot., 98: 1197-1206.
CrossRef | Direct Link |

Zeng, S., J. Wang, K. Wu, J.A.T. da Silva, J. Zhang and J. Duan, 2013. *In vitro* propagation of *Paphiopedilum hangianum* Perner and Gruss, Sci. Hortic., 151: 147-156.
CrossRef | Direct Link |

Zeng, S., K. Wu, J.A.T. da Silva, J. Zhang, Z. Chen, N. Xia and J. Duan, 2012. Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. Sci. Hort 138: 198-209.
CrossRef |

Asymbiotic Seed Germination and in vitro Seedling Development of Paphiopedilum liemianum Fowlie, an Endangered Terrestrial Orchid in Northern Sumatra, Indonesia

ORIGINALITY REPORT

42%	27%	39%	0%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Songjun Zeng, Kunlin Wu, Jaime A. Teixeira da Silva, Jianxia Zhang, Zhilin Chen, Nianhe Xia, Jun Duan. "Asymbiotic seed germination, seedling development and reintroduction of Paphiopedilum wardii Sumerh., an endangered terrestrial orchid", Scientia Horticulturae, 2012 Publication	10%
2	A.R. Roy, R.S. Patel, V.V. Patel, S. Sajeev, Bidyut C. Deka. "Asymbiotic seed germination, mass propagation and seedling development of Vanda coerulea Griff ex.Lindl. (Blue Vanda): An in vitro protocol for an endangered orchid", Scientia Horticulturae, 2011 Publication	3%
3	www.academicjournals.org Internet Source	2%
4	academicjournalsinc.com Internet Source	2%

5	www.nature.com Internet Source	2%
6	Songjun Zeng, Jia Wang, Kunlin Wu, Jaime A. Teixeira da Silva, Jianxia Zhang, Jun Duan. "In vitro propagation of Paphiopedilum hangianum Perner & Gruss", Scientia Horticulturae, 2013 Publication	1%
7	ir.kib.ac.cn:8080 Internet Source	1%
8	Daniela Dutra. "Asymbiotic seed germination, in vitro seedling development, and greenhouse acclimatization of the threatened terrestrial orchid Bletia purpurea", Plant Cell Tissue and Organ Culture, 07/2008 Publication	1%
9	images3.wikia.nocookie.net Internet Source	1%
10	T. Dennis Thomas. "High-frequency plantlet regeneration and multiple shoot induction from cultured immature seeds of Rhynchostylis retusa Blume., an exquisite orchid", Plant Biotechnology Reports, 11/15/2007 Publication	1%
11	"Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols", Springer Nature, 2018	1%

Publication

12	academicjournals.org Internet Source	1%
13	Ribeiro, MÃ¡rcia de NazarÃ© Oliveira, Moacir Pasqual, FabÃola Villa, Leila Aparecida Salles Pio, and Henk William Maria Hilhorst. "In vitro seed germination and seedling development of Annona crassiflora Mart.", Scientia Agricola, 2009. Publication	1%
14	www.globalsciencebooks.info Internet Source	1%
15	www.bioversityinternational.org Internet Source	1%
16	Pierik, R.L.M.. "Seed germination and further development of plantlets of Paphiopedilum ciliolare Pfitz. in vitro", Scientia Horticulturae, 198801 Publication	1%
17	Ting-Yu Chen. "Multiple shoot formation and plant regeneration from stem nodal explants of Paphiopedilum orchids", In Vitro Cellular & Developmental Biology - Plant, 11/2002 Publication	1%
18	journals.modares.ac.ir Internet Source	1%

19	Chyuam-Yih Ng. "In vitro propagation of Paphiopedilum orchid through formation of protocorm-like bodies", Plant Cell Tissue and Organ Culture, 09/28/2010 Publication	1%
20	J. YAMAZAKI, K. MIYOSHI. "In vitro Asymbiotic Germination of Immature Seed and Formation of Protocorm by Cephalanthera falcata (Orchidaceae)", Annals of Botany, 2006 Publication	1%
21	link.springer.com Internet Source	<1%
22	www.arcjournals.org Internet Source	<1%
23	rd.springer.com Internet Source	<1%
24	horticulturejournal.usamv.ro Internet Source	<1%
25	e-journal.biologi.lipi.go.id Internet Source	<1%
26	recentscientific.com Internet Source	<1%
27	globalscienceresearchjournals.org Internet Source	<1%
	Pou-leng Hong. "Plant regeneration via	

28	protocorm-like body formation and shoot multiplication from seed-derived callus of a maudiae type slipper orchid", Acta Physiologiae Plantarum, 09/2008 Publication	<1%
29	Scott L. Stewart. "Asymbiotic seed germination and in vitro seedling development of Habenaria macroceratitis (Orchidaceae), a rare Florida terrestrial orchid", Plant Cell Tissue and Organ Culture, 08/2006 Publication	<1%
30	Bo Long. "In vitro propagation of four threatened Paphiopedilum species (Orchidaceae)", Plant Cell Tissue and Organ Culture, 02/02/2010 Publication	<1%
31	Kee-Hwa Bae, Myoung Suk Ko, Mi Hyun Lee, Nam Young Kim, Jae Mo Song, Gwanpil Song. "Effects of NaOCl treatment on in vitro germination of seeds of a rare endemic plant, Oreorchis coreana Finet", Journal of Plant Biotechnology, 2013 Publication	<1%
32	Toshinari Godo. "Germination of mature seeds of Calanthe tricarinata Lindl., an endangered terrestrial orchid, by asymbiotic culture in vitro", In Vitro Cellular & Developmental	<1%

Biology - Plant, 02/05/2010
Publication

33	Irene Ávila-Díaz. "In vitro propagation of the endangered orchid Laelia speciosa", Plant Cell Tissue and Organ Culture, 09/23/2009 Publication	<1%
34	Guohua Ma. "Shoot organogenesis and plant regeneration in Metabriggsia ovalifolia", Plant Cell Tissue and Organ Culture, 11/20/2010 Publication	<1%
35	M.-C. CHUNG. "Embryo Development in the Lady's Slipper Orchid, Paphiopedilum delenatii, with Emphasis on the Ultrastructure of the Suspensor", Annals of Botany, 06/27/2006 Publication	<1%
36	Li Zhu, Liangqiong Ma, Feng Xu, Weiwei Zhang. "Molecular Cloning and Expression of a Squalene Epoxidase Gene from Ilex cornuta", Biotechnology(Faisalabad), 2018 Publication	<1%
37	www.frontiersin.org Internet Source	<1%
38	"Thidiazuron: From Urea Derivative to Plant Growth Regulator", Springer Nature, 2018 Publication	<1%
39	banglajol.info Internet Source	<1%

40	Kunlin Wu, Songjun Zeng, Jaime A. Teixeira da Silva, Zhilin Chen, Jianxia Zhang, Yuesheng Yang, Jun Duan. "Efficient regeneration of Renanthera Tom Thumb ‘Qilin’ from leaf explants", Scientia Horticulturae, 2012 Publication	<1%
41	www.e-sciencecentral.org Internet Source	<1%
42	M. Monteiro do Rêgo, E. Ramalho do Rêgo, H.C. Carvalho dos Santos. "MICROPROPAGATION OF AN AMAZONIAN TERRESTRIAL ORCHID (BRASSIA BIDDENS) FROM RORAIMA STATE, BRAZIL", Acta Horticulturae, 2009 Publication	<1%
43	www.scielo.br Internet Source	<1%
44	Mahendran, G., and V. Narmatha Bai. "An efficient in vitro propagation, antioxidant and antimicrobial activities of Aphyllorchis montana Rchb.f.", Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, 2015. Publication	<1%
45	www.mspp.org.my Internet Source	<1%

46	worldwidescience.org Internet Source	<1%
47	Ruangwut Chutima. "Endophytic fungi from Pecteilis susannae (L.) Rafin (Orchidaceae), a threatened terrestrial orchid in Thailand", Mycorrhiza, 07/09/2010 Publication	<1%
48	Wang, C. X., and M. Tian. "Callus-mediated and direct protocorm-like body formation of Bletilla striata and assessment of clonal fidelity using ISSR markers", Acta Physiologiae Plantarum, 2014. Publication	<1%
49	Sonia Abraham. "Asymbiotic seed germination and in vitro conservation of Coelogyne nervosa A. Rich. an endemic orchid to Western Ghats", Physiology and Molecular Biology of Plants, 05/30/2012 Publication	<1%
50	hort.ufl.edu Internet Source	<1%
51	Teixeira da Silva, Jaime A., Elena A. Tsavkelova, Tzi Bun Ng, S. Parthibhan, Judit Dobránszki, Jean Carlos Cardoso, M. V. Rao, and Songjun Zeng. "Asymbiotic in vitro seed propagation of Dendrobium", Plant Cell Reports, 2015.	<1%

Publication

52	Kaur, S., and K. K. Bhutani. "In vitro conservation and asymbiotic propagation of Coelogyne flaccida (Lindl.): A threatened orchid", Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, 2014. Publication	<1%
53	www.pomics.com Internet Source	<1%
54	Saha, Soumen, Chandan Sengupta, and Parthadeb Ghosh. "Molecular and phytochemical analyses to assess genetic stability in alginate-encapsulated microshoots of Ocimum gratissimum L. following in vitro storage", The Nucleus, 2014. Publication	<1%
55	journals.tubitak.gov.tr Internet Source	<1%
56	springerplus.springeropen.com Internet Source	<1%
57	Wu, Kunlin, Songjun Zeng, Danni Lin, Jaime A. Teixeira da Silva, Zhaoyang Bu, Jianxia Zhang, and Jun Duan. "In Vitro Propagation and Reintroduction of the Endangered Renanthera imschootiana Rolfe", PLoS ONE, 2014. Publication	<1%

58	etd.lib.npust.edu.tw Internet Source	<1%
59	aob.oxfordjournals.org Internet Source	<1%
60	www.researchgate.net Internet Source	<1%
61	Methods in Molecular Biology, 2011. Publication	<1%

Exclude quotes	Off	Exclude matches	Off
Exclude bibliography	On		

Asymbiotic Seed Germination and in vitro Seedling Development of Paphiopedilum liemianum Fowlie, an Endangered Terrestrial Orchid in Northern Sumatra, Indonesia

GRADEMARK REPORT

FINAL GRADE

/0

GENERAL COMMENTS

Instructor

PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7

PAGE 8

PAGE 9

PAGE 10

PAGE 11

PAGE 12

PAGE 13