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In vitro seed germination of *Paphiopedilum lowii*, an endangered slipper orchid in North Borneo

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Abstract. *Ragu VS, Ombokou R, Repin R, Molidin D, Miadin R, Aziz ZA. 2022. In vitro seed germination of* Paphiopedilum lowii, *an endangered slipper orchid in North Borneo. Biodiversitas 23: 5687-5694. Paphiopedilum lowii* (Lindl.) Stein is alarmingly decreasing in numbers due to habitat destruction and over-collection. Propagation through seed is one effort to conserve the species. In vitro method offers better results for seed germination in orchids, but the success of germination is species-specific. Hence, this research aimed to evaluate different types of media [(½ strength Murashige & Skoog and Orchimax Medium (Duchefa Biochemie)], and to determine the effects of sucrose [0% 1%, 2% and 3% (w/v)], peptone [0%, 0.1% and 0.2% (w/v)], coconut water [0%, 10%, 20% and 30% (v/v)] and fertilizer [Orchid Focus - 0%, 0.1% and 0.2% (v/v)] on seed germination. The protocorm size was also determined after 12 weeks of observation. Half-strength MS media consistently gave higher seed germination, ½ MS supplemented with 1% (w/v) sucrose, 0.1% (w/v) peptone, 0% (v/v) coconut water and 0.1 % (v/v) fertilizer resulted in the highest seed germination at 5.32% ± 5.9, 19.27% ± 9.34, 11.33% ± 3.80 and 19.31% ± 9.03 respectively. Despite being 12 weeks in culture, the sizes of the protocorms are small (0.222±0.089 mm diameter, 0.703±0.280 mm circumference, and 0.045±0.043 mm^2 area); this requires future investigation. These findings can serve as base information for further enhancement of seed germination and development of propagation methods of *P. lowii* for use in a conservation program.

Keywords: Orchimax medium, *Paphiopedilum lowii*, protocorm size, seed germination

INTRODUCTION

Paphiopedilum lowii (Lindl.) Stein is a slipper orchid with multiflowered inflorescence growing as an epiphyte on trees or in rock crevices filled with organic materials (Cribb 2014). It is one of the species in the genus having multiflowered inflorescence with 3-7 flowers on a peduncle. The flowers are a mixture of light green and purple with several maroon spots, and flowering is between April and June. The species is found in the Malay Peninsula, Sumatera, Borneo, Java, and the Celebes (Cribb 1998). In North Borneo, the species grows wild in limited areas near Mount Kinabalu.

Paphiopedilum lowii is highly desired and sold illegally in Sunday markets, supplies for these markets are from the wild population, together with habitat destruction for land development these had resulted in a dwindling of the population size. Local and international policies are available to protect the species in the genus *Paphiopedilum* (Guo et al. 2012; Warren 2012; Romadlon et al. 2021). Unfortunately, these have not deterred poachers from extracting the species from its native environment. The *P. lowii* has been classified as endangered under International Union for Conservation of Nature (IUCN) Red List (Rankou 2015). Similarly, Convention on International Trade in Endangered Species (CITES) protects it under Appendix I, under this category, any kind of trading is prohibited (Govaerts et al. 2018).

For conservation purposes, a few individual plants of *P. lowii* are kept in the nursery at the Kinabalu National Park. To conserve endangered orchids, it is important that wild plants are not extracted to avoid a dwindling population number. Therefore, propagating the species is beneficial for protecting the species as the propagated individuals can be used to supply the markets hence avoiding the wild plant from being extracted. Generally, orchids from this genus are propagated through lateral bud division (Zeng et al. 2013). However, the propagation rate using this technique is very slow and inefficient, as only one new growth per year is obtained (Liao et al. 2011; Kaur and Buthani 2016).

To assist in the conservation programs of orchids, seed germination is important as orchids propagated from seeds are genetically diverse (Diengdoh et al. 2017). In a natural setting, seeds of *Paphiopedilum germinate* very slowly and in low numbers (Zeng et al. 2015). Hence, asymbiotic seed germination has been suggested to propagate orchids for conservation efforts as the technique has resulted in more reliable germination and propagation of many orchid taxa (Fu et al. 2016). In vitro seed germination methods can produce many seedlings in a shorter time. The in vitro propagated orchids can reduce collection pressure on the wild population by flooding the market, allowing the wild population to thrive (Lal and Sing 2021), and in more severe situations, in vitro materials can be used to reintroduce endangered species to their natural habitats (Hossin et al. 2013; Wu et al. 2014). Therefore, a reliable

seed germination protocol for *P. lowii* would assist in the conservation programs of the species.

Paphiopedilum is one of the four genera of Cypripedioideae, which is hard to culture in vitro, especially via seeds (Cribb 2014; Zeng et al. 2014). Many studies have reported in vitro seed germination of *Paphiopedilum* species. Chen et al. (2015) developed a protocol for the in vitro germination and development of *P. spicerianum.* Zeng et al. (2012) reported asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii*. While Deb and Jahka (2019) developed a protocol for in vitro culture of immature embryos and propagation of *P. villosum* var. *Boxallii*. Most recently, Yao et al. (2021) reported a protocol for asymbiotic seed germination and seedling development for *P. tigrinum. Paphiopedilum* seed germination is influenced by factors that include capsule maturity, medium composition, culture conditions and culture methods (Zeng et al. 2015; Khamchtra et al. 2016). Each species and variety have a different requirement for seed germination (Aewsakul et al. 2013). In those previous reports, factors such as seed maturity, medium composition, growth conditions, and methods of culture have a significant influence on seed germination and protocorm development. To date, there are no reports and studies regarding in vitro seed germination for *P. lowii*. Thus, this study was conducted to evaluate the effect of medium dan additives on the seed germination of the species.

MATERIALS AND METHODS

Plant material and surface sterilization

Flowers of *Paphiopedilum lowii* (Figure 1.A) at the nursery of Kinabalu National Park (Sabah, Malaysia) were hand-pollinated by transferring the pollen to the stigma of the same flower. The resulting capsules (Figures 1.B and 1.C) containing seeds were harvested at 121 DAP (4 months) and 172 DAP (5 months). The capsules were washed under running tap water for 1-2 minutes to remove any visible dirt and then immersed in 70% (v/v) ethanol for 1 minute in a universal bottle. Next, the ethanol was replaced with 25% (v/v) Clorox with two drops of Tween 20 for 30 minutes with regular bottle shaking. To complete the sterilization, the capsules were rinsed three times with sterile distilled water. The capsules were dried using sterilized tissues on a Petri dish and dissected vertically using a sterile blade to expose the seeds for culturing purposes.

Viability of seeds

Viability was assessed by 2,3,5-triphenyltetrazolium chloride (TTC) reduction assay (AOSA 2000; Vujanovic et al. 2000) and germination of seeds. For the TTC reduction, seeds were incubated in a 1% (w/v) TTC solution for 24 h at 25°C in the dark. The number of seeds with TTC-stained embryos was counted, and the viability was defined as the percentage of seeds showing staining. At least 100 seeds were counted.

Effect of media and sucrose on germination

After surface sterilization, the seeds were cultured on half-strength Murashige and Skoog (½ MS) medium (Murashige and Skoog 1962) or Orchimax Medium (OM) (Duchefa, Biochemie) with different concentrations of sucrose [0, 1, 2, and 3% (w/v)]. Both media were supplemented with 0.1% (w/v) activated charcoal and 0.8% (w/v) agar. The pH of all media was adjusted to 5.2 and was autoclaved at 121°C, 120 p.s.i for 15 to 20 minutes. All media were dispensed in 5 mm diameter Petri dishes.

Effect of additives on seed germination

To evaluate the effect of additives, both media were used, and this study was carried out concurrent with evaluating the effect of sucrose. The media were supplemented with 2% (w/v) sucrose, 0.1% (w/v) activated charcoal and 0.8% (w/v) agar plus additives at different concentrations. Coconut water was added at concentrations of 0, 10, 20, and 30% (v/v). Coconut water from a green coconut, aged between 8-9 months old when the meat was jelly-like, was filtered through muslin cloths before being added to the media. Peptone at the concentrations of 0, 0.1 and 0.2% (w/v) was tested. Fertilizer (Orchid Focus) was added at the concentrations of 0, 0.1 and 0.2% (v/v). The nitrogen, phosphorus and potassium values for the fertilizer were 1.6:2.4:2.6, and the detailed nutritional compositions of the fertilizer (% w/v) are nitrogen (1.56), nitrate nitrogen (1.56), phosphorus pentoxide (2.36), potassium oxide (2.65), calcium oxide (1.37), copper chelated by EDTA (0.0002), iron chelated by DTPA (0.04), manganese chelated by EDTA (0.010), molybdenum (0.001), zinc chelated by EDTA (0.0025) (Growth Technology 2018). After the organic supplements or fertilizer were added, the pH of the media was adjusted to 5.2 and was autoclaved at 121°C, 120 p.s.i for 15 to 20 minutes. All media were dispensed in 5 mm diameter Petri dishes.

Germination of seeds

Seeds from sterilized capsules of *P. lowii* were scattered randomly on top of membranes placed on each medium. The *P. lowii* seeds were allowed to germinate in a plant growth chamber under controlled temperature and light intensity. For the first seven weeks, the cultures were kept in the dark. After seven weeks, to avoid the protocorms turning brown, the seeds were left to germinate further under light (16-hour photoperiod). The temperature was maintained at 25±2°C throughout the study. Germination and seedling development were scored on a scale of 1-5, as described in Table 1. Seeds were considered germinated once the embryos ruptured the testa (Stage 2).

Determination of the size of protocorms

After 12 weeks, the seeds were cultured, the measurements of the resulting protocorms were taken using a Dino-Lite Edge Microscope. Protocorms on the medium that induced the highest seed germination were chosen, and the protocorms were randomly chosen. About 100 protocorms were measured in each replicate (a total of 300 seeds for three replicates) for their diameter, circumference, and area.

Table 1. Seed germination and seedling developmental growth stages of *Paphiopedilum lowii*

Stage	Description
1	Seed with swollen embryo and intact testa
2	Swelling of embryo followed by rupturing of testa
3	Early protocorm with a pointed vegetative apex
4	Protocorm showing the first leaf primordium or shoots
5	Plantlet showing two spreading leaflets.
6	Plantlet with two or more shoots and roots

Note: Adapted from Arditti (1967)

Data collection and analysis

This study was carried out in a Completely Randomized Design (CRD). Three replicates were prepared for each type of formulation, with at least 100 seeds scattered randomly in each. Changes in the growth and development of seeds were observed and recorded weekly for 90 days (12 weeks). During this period, the percentage of seed germination was recorded by the following formula:

$$\text{Seed germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \; x\; 100\%$$

These data were subjected to Analysis of Variance (ANOVA) using IBM SPSS Version 21.0. Duncan Multiple Range Test (DMRT) was employed to determine a significant difference at $p<0.05$.

RESULTS AND DISCUSSION

Seed viability

The capsules of *P. lowii* were elliptic in shape, the 5-month-old capsule (Capsule 1) was 5.9 cm in length and 1.85 g in weight, while the 4-month-old capsule (Capsule 2) was 6.8 cm in length and 4.37 g in weight. The seeds from Capsule 1 were light brown in color, dry and separated from each other. In contrast, the seeds from Capsule 2 were white, moist, and attached to each other. The embryos of viable seeds stained red upon testing with TTC (Figure 1.D). The viability of seeds based on the TTC test differed at the different ages of the capsules. Only the seeds from the 5-month-old capsule (Capsule 1) were viable (54.59%) (Table 2).

When evaluating the conditions for germination, it is important that only viable seeds are used; this is to ensure that non-germinating seeds are due to unfavorable conditions and not because of seeds that are not viable. The viability of seeds is affected by the age of the capsules; this present study revealed that viable seeds could only be obtained when the capsule was harvested 5 months post-pollination. Further investigation needs to be done to test the viability of seeds from capsules at the age beyond 5 months. However, due to a limited number of capsules, it is not known if the 5-month-old seeds gave the maximum germination capability for *P. lowii*. Timing in collecting seeds is very important as it affects germination and is species dependent. For slipper orchids, various timing in capsule collection has been reported; for *Paphiopedilum wardii*, only seeds that were collected 90 days after pollination (DAP) germinated and the maximum germination was observed from those seeds collected at 180 DAP (Zeng et al. 2012); for *Paphiopedilum hangianum*, the minimum age for seeds to germinate was 60 DAP and maximum germination was from seeds aged 180 DAP (Zeng et al. 2013). *Cypripedium lentiginosum*, a species in the Lady's Slipper orchid family, germination was observed when seeds were collected at 45 DAP and optimum germination was reported at 90-105 DAP (Jiang et al. 2017). This present study also revealed that the viability based on the TTC test does not reflect the germination capability of the seeds. The seed viability based on the TTC test was higher than the seed germination in all treatments. Different results between the TTC test and germination were observed because, for TTC test, any living tissues can reduce the colorless tetrazolium chloride to a red compound, indicating viable seeds, but for germination, only seeds with embryos that had developed completely can germinate (Zeng et al. 2012). In addition, dormancy can be the reason for low seed germinability (Fu et al. 2016). Different results of TTC staining tests and germination tests were reported for *Bletilla formosana* seeds (Wu et al. 2018).

Influence of media and sucrose on seed germination

Throughout the 12-week study, seeds of *P. lowii* had successfully germinated on both media, but the percentages were different in each medium (Table 2). Embryos started to swell (Stage 1) as early as Week 1 and broke out of the testa (germinated - Figure 1.E) as the cultures entered Week 2.

Table 3. Effect of media and sucrose on in vitro seed germination of *Paphiopedilum lowii* upon 7 weeks of incubation in 24hr dark and additional 5 weeks of incubation in 16 hr photoperiod at 25±2ºC

Sucrose concentration (% w/v)	Seed germination (%±S.D.)	
	Half-strength MS Medium (½ MS)	Orchimax Medium (OM)
0	8.04±5.82^{a}	11.62±4.11^{b}
1	15.32±5.97^{c}	11.95±4.50^{b}
2	14.70±7.85^{c}	7.12±1.16^{a}
3	11.32±2.18^{b}	6.27±0.76^{a}

Note: Data was obtained from a total of three replicates. Mean followed by the same letters did not differ significantly at $p<0.05$ according to Duncan's Multiple Range Test (DMRT). SD-Standard deviation

Table 2. Viability of seeds from two capsules at different ages based on TTC test

Capsule	Capsule age	Length (cm)	Weight (g)	Viability (%)
Capsule 1	5 months (172 DAP)	5.90	1.85	54.59
Capsule 2	4 months (121 DAP)	6.80	4.37	0

Figure 1. Seed germination of *Paphiopedilum lowii*. (A) Flower of *P. lowii* Bar =1.8 cm; (B) A 5-months-old capsule; (C) A 4-month-old capsule with undeveloped seeds, Bar = 1.7 cm; (D) Seeds of *P. lowii* stained with TTC, X100 magnification; (E) Germinating seeds (arrow) - embryo breaks out of testa, Bar = $4.5x10^{-4}$mm; (F) Protocorms on ½ MS medium - round and green, Bar = 8.5×10^{-5} mm; (G) Protocorms on OM medium - oval and cream, Bar = 1.0×10^{-4} mm

Embryos that were free from the testa continued to swell in size and become protocorms (Stage 3). However, the growth was very slow, by Week 11 some protocorms on ½ MS started to turn green. Regardless of the concentrations of sucrose, ½ MS medium showed higher percentages of germination (Table 3) as compared to OM medium. Both media showed similar germination profiles; germination increases as the concentration of sucrose increases and reach the maximum at 1% (w/v) sucrose (15.32%±5.97 and 11.32%±2.18 germinations on ½ MS and OM media, respectively), beyond this concentration, germination started to decline, and at the highest sucrose concentration of 3% (w/v) the germinations had dropped to 11.32%±2.18 and 6.27%±0.76 for ½ MS and OM media respectively. Importantly, on the control media (0% sucrose), germination was higher on OM medium

(11.62%±4.11) as compared to ½ MS (8.04%±5.82). Protocorms on ½ MS medium were bright green and round and had tiny rhizoids with the appearance of the first shoot (Figure 1.F) compared to those on OM media, which were white and slightly oval with the first shoot appearance (Figure 1.G).

The outcomes of this study proved that ½ MS medium is the best for in vitro seed germination of *P. lowii*. This outcome is in accordance with a study conducted to study the asymbiotic germination of *Paphiopedilum exul*, a rare and endangered lady's slipper orchid. The study reported that the species germinated well in ½ MS medium at different pH levels (Imsomboon et al. 2017). Similarly, Munoz and Jimenez (2008) stated that ½ MS medium was comparatively better in enhancing seed germination of *Phragmipedium longifolium* and *Phragmipedium pearcei* than that of Knudson C medium. This outcome is in line with the results of this study.

The suitability of ½ MS medium in vitro seed germination of orchids is due to the low salt or mineral concentration. If a full-strength MS medium was used, this would have detrimental effects on seed germination (Zeng et al. 2015). In general, seeds of several species of orchids germinate and develop better in low-salt and nitrogen mediums (Munoz and Jimenez 2008; Arditti and Pridgeon 2013). Studies report successful in vitro seed germination of orchids in the quarter, one-fifth, half and other lower-strength MS medium (Chen et al. 2004; Ding et al. 2004; Liu et al. 2012; Huh et al. 2016).

However, this present study is in contradiction to that of Aricidiacono et al. (2021), where OM medium resulted in very high or better seed germination for *Anacamptis longicornu* and *Ophrys panormitana*. OM medium contains macronutrients and micronutrients like that of ½ MS. However, the medium is enriched with tryptone and a higher level of vitamins (Nicotinic acid at 1.0 mg/L; Pyridoxin HCl at 1.0 mg/L and Thiamine HCl at 10 mg/L). The medium also contains MES buffer to prevent acidification. This present study indicated that the enhancement of OM with these components did not enhance the germination of *P. lowii*. This present study proves that the suitability of the medium depends on the species being cultured (Utami and Hariyanto 2019).

Based on the results obtained, a low concentration of sucrose [1% (w/v)] had the maximum promoting effect on the germination of *P. lowii* seeds in vitro, regardless of the media used. However, the absence of sucrose and concentrations of sucrose beyond 1% (w/v) led to a reduction of germination. In previous studies involving *Dendrobium nobile* hybrids and *Cypripedium macranthos*, sucrose concentrations of 1% (w/v) was proven to be the best (Udomdee et al. 2014; Huh et al. 2016). This is in accordance with the results obtained in this study. The reduction in seed germination and protocorm development at a high level of sucrose concentrations are due to osmotic stress or the growth and development were inhibited by the products of sucrose hydrolysis during the autoclaving procedure (Udomdee et al. 2014). Sucrose acts as an energy source for seeds so that in the absence of photosynthesis, they can still utilize it for their primary growth. Due to light energy deficiency and decreased concentrations of carbon dioxide, the addition of sucrose in the correct concentration may aid in vitro germination of seeds as well as the growth of plantlets (Puspitaningtyas and Handini 2021).

Influence of peptone, coconut water and fertilizer on seed germination

Throughout the study, ½ MS media consistently produced higher germination frequencies than OM media. The germination profiles in both media were similar for treatments with coconut water and fertilizer but different for peptone (Table 4). On ½ MS media, peptone had caused an increase in seed germination but not significantly. Peptone at 0.1% (w/v) recorded the highest percentage of seed germination (19.27%±9.34), whereas 0.2% (w/v) peptone had the lowest germination (13.63%±6.43). While on OM media, as the concentration of peptone increases, the germination decreases, OM medium without peptone (control) gave the highest seed germination (9.59%±4.47) while 0.2% (w/v) peptone gave the lowest germination (9.28%±4.04).

The optimal peptone concentration obtained through this study is 0.1% (w/v). This concentration was also proven to be the best for the germination of *Paphiopedilum insigne*, *Paphiopedilum hirsutissimum* and *Paphiopedilum wardii* seeds (Zeng et al. 2012). Peptone consists of proteins of low molecular weight, amino acids, vitamins, and various plant growth substances. It helps to promote the growth of in vitro plant cultures due to its ability to hydrolysate with very high amino acid content (Kaur and Bhutani 2012). It is also a ready source of nitrogen, an important nutrient in the germination and growth of in vitro cultures (Shekarriz et al. 2014; Darmawati et al. 2021).

The addition of coconut water reduces germination, the medium without coconut water gave the highest germination (11.33%±3.80 for ½ MS and 8.07%±2.42 for OM medium), increasing the coconut water content to 20% (v/v) and 30% (v/v) in ½ MS media significantly ($p<0.05$) resulted in the drop of germination to 8.26%±2.00 and 8.24%±1.66 respectively. While on OM medium, a significant ($p<0.05$) reduction in germination was seen only on the medium with 30% (v/v) coconut water (10.49±4.56). Coconut water was seen to have caused ungerminated seeds to shrink before turning black and eventually dying. The fertilizer induced germination in both media; however, the increment was insignificant.

The absence of coconut water had a promoting effect on *P. lowii* seed germination. On the other hand, coconut water was seen to be inhibiting the optimal seed germination of *P. lowii*. This outcome contradicted most studies that reported the beneficial effects of coconut water on the growth and development of in vitro grown plant materials (Zeng et al. 2011; Zeng et al. 2012; Utami and Haryanto 2020). Like this study, the proliferation and regeneration of Phalaenopsis hybrid 'Pink' were reduced in coconut water (Zahara et al. 2017). The inhibitory effects of coconut water might be due to the stage of maturity of the coconut used which influences the composition of coconut water (Zeng et al. 2015). Moreover, the presence

of phytohormones in coconut water might be the cause of the negative response in *P. lowii* seed germination (Zahara et al. 2017).

Fertilizer at the concentration of 0.1% (v/v) resulted in the highest germination for both media. Germination was increased by 0.3% on ½ MS and almost 1.7% on OM medium compared to the control medium. The orchid focus used in this study is a fertilizer specifically used for orchids. The purpose of using orchid focus was to evaluate the effectiveness in promoting in vitro seed germination of *P. lowii* because the percentage of germination for the species was low in all treatments. Although it was not significant, the fertilizer at the concentration of 0.1% (v/v) caused an increment in germination. This promoting effect may be due to the beneficial interactions of nutrients in the basal medium with fertilizer components. The fertilizer contains nitrogen, phosphorus, potassium, calcium, boron, cobalt, copper, manganese, molybdenum dan zinc. The addition of the fertilizer into both media further elevated the concentrations of the components in the media, this did not enhance seed germination. Previous work by Chen et al. (2015) on *Paphiopedilum spicerianum* reported that high solute concentrations in the medium might have led to solute leakage from seeds causing shrinkage of seeds, which could have influenced seed germination.

Protocorm size distribution

Upon 12 weeks of observation, ½ MS medium with 0.1% (w/v) peptone gave the highest germination percentage (Table 3). Size measurements of a total of 300 protocorms were randomly taken. The mean diameter for the protocorms was 0.22±0.09 mm (Table 5), with the majority of the protocorms having diameters between 0.100 mm and 0.230 mm (Figure 2.A) and having mean circumferences of 0.70±0.28 mm which was distributed in the range of 0.400 mm and 0.800 mm (Figure 2.B). It is also evident that most protocorms have mean areas of 0.05±0.04 mm^2 distributed between 0.010 mm^2 and 0.050 mm^2 (Figure 2.C).

The average sizes of protocorms after 12 weeks of in vitro culture were generally small. Indirectly, this conveys the suitability of the protocorms to be transferred to the multiplication medium for further growth and development. Normally, a multiplication and development medium will contain plant growth regulators (PGRs), unlike most germination medium that only contains complex additives as used in this study and this depends on the species of orchid that is being cultured. For an orchid protocorm to withstand the strength of PGRs, it must be of a suitable size. However, the protocorms of *P. lowii*, upon 12 weeks of culturing, were not suitable for multiplication due to their small sizes. Our previous experience with other orchids revealed that small protocorms usually have a low surviving rate on the multiplication or seedling development media.

Protocorm sizes are also treatment dependent. This means that the sizes of protocorm vary depending on the medium with respective additives and carbohydrate concentrations. This is due to the promoting or inhibitory effect of the respective medium with its respective contents. A study conducted by Hossain (2008) on in vitro seed germination of *Epidendrum ibaguense* reported that sizes of protocorms vary depending on the content of the media, and in his report, activated charcoal resulted in the largest protocorms (1.63±18 cm diameter). Therefore, further investigations are necessary to promote the growth of *P. lowii* protocorms to be suitable for subsequent use in the multiplication stage.

Table 5. Mean values for diameter, circumference, and area of 300 protocorms measured using Dino Light Edge Microscope

Type of measurement	Mean ± SD
Diameter	0.22±0.09 mm
Circumference	0.70±0.28 mm
Area	0.05±0.04 mm^2

Note: SD-Standard deviation

Table 4. Effect of additives in half-strength Murashige and Skoog (1962) (½ MS) and Ochimax (OM) media on seed germination of *Paphiopedilum lowii*

Additive	Concentration	Seed germination (%±S.D.)	
		Half-strength MS Medium (½ MS)	Orchimax Medium (OM)
Peptone	0% (w/v)	16.00±10.11^{ab}	9.59±4.47^{a}
	0.1% (w/v)	19.27±9.34^{b}	9.37±4.03^{a}
	0.2% (w/v)	13.63±6.43^{a}	9.28±4.04^{a}
Coconut Water	0% (v/v)	11.33±3.80^{b}	8.07±2.42^{c}
	10% (v/v)	10.30±3.39^{b}	6.74±1.71^{ab}
	20% (v/v)	8.26±2.00^{a}	7.62±1.70^{bc}
	30% (v/v)	8.24±1.66^{a}	6.40±1.80^{a}
Fertilizer	0% (v/v)	19.03±8.65^{a}	10.49±4.56^{a}
(Orchid Focus)	0.1% (v/v)	19.31±9.03^{a}	12.18±7.31^{a}
	0.2% (v/v)	19.27±9.15^{a}	11.53±5.14^{a}

Note: Data was obtained from a total of three replicates. Mean followed by the same letters did not differ significantly at $p<0.05$ according to Duncan's Multiple Range Test (DMRT). SD-Standard deviation

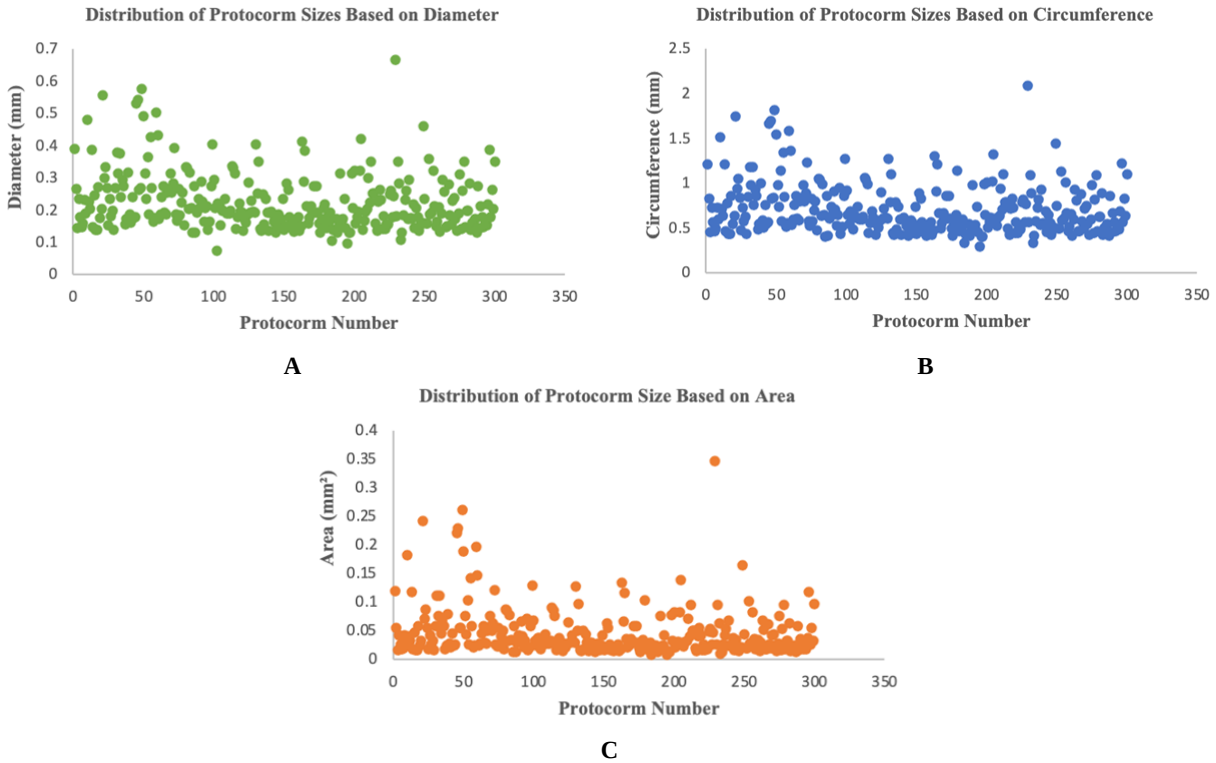

Figure 2. Distribution of 300 random protocorm sizes in ½ MS medium supplemented with 0.1% (w/v) peptone upon 7 weeks of incubation in 24hr dark and additional 5 weeks of incubation in 16hr photoperiod. (A) The protocorms are having diameter with a mean of 0.22±0.09 mm; (B) The circumference of the protocorms is in the average of 0.70±0.28 mm; (C) The mean area of the protocorms is 0.05±0.04 mm

In conclusion, this study successfully identified conditions for seed germination and obtained protocorms of *P. lowii*. The information would be the basis for further investigating the species to conserve the orchid. The best medium for in vitro germination of *P. lowii* was ½ MS. Regardless of the type of media, sucrose, and peptone at the concentrations of 1% (w/v) and 0.1% (w/v), respectively, had a promoting effect on in vitro seed germination of *P. lowii*. However, the absence of coconut water [0% (v/v)] gave the maximum germination percentage. The fertilizer Orchid Focus at 0.1 % (v/v) was beneficial on OM media but not on ½ MS. In general, *P. lowii* has small sizes of protocorms after 12 weeks of culture, for these protocorms to be suitable for multiplication or seedling development further treatments need to be done as the size of a protocorm does affect the response on a medium.

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