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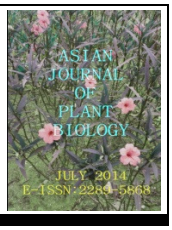


# Asymbiotic Germination and Seedling Development of *Dimorphics lowii* (Orchidaceae)

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## ABSTRACT

*Dimorphorchis lowii*, a threatened Borneo endemic epiphytic orchid, is gradually becoming rare due to over collecting and habitat disturbance. Therefore this study was carried out to obtain *in vitro* propagation through asymbiotic seed germination and seedling development by optimizing capsule maturity, nutrients requirements and light conditions before being introduced back to its natural habitat for conservation purposes. Capsules were collected at 100, 150 and 170 days through hand-pollination. The seeds were germinated on Murashige and Skoog (MS), Knudson C (KC) and Vacin and Went (VW) media added with 10% additives (coconut water, potato homogenate and tomato juice) under light and dark conditions. Seeds from 150 days old capsule grow on VW medium added with potato homogenate under light condition were observed to be an optimum condition with higher germination percentage as compare to other treatments. Seeds started to germinate by swollen of embryo (8 days) before the testa were ruptured at 23 days and further developed into mature protocorm at 33 days. Seeds with 4 leaves and 5 roots were ready for hardening process within 200 days. A successful developed system for *in vitro* propagation will contribute to the development of a sustainable management program for D. *lowii* in Sabah, Malaysia.

## INTRODUCTION

Orchidaceae belongs to the largest flowering plants in the world, with an estimated 800 genera and 20 000 species. Borneo orchids comprising around 3000–4000 species which contribute up to 12 - 16% of the flora diversity (Chan *et al*., 1994). However, many of these Borneo orchids become depleted due to habitat loss. One of the factors is deforestation. The ultimate driving force behind all deforestation is increasing of human population leading to human activities such as logging, agriculture and urbanization. Another factor is the illegal collection from local people that result to large number of species become exceedingly rare, and some have been driven to extinction. One of the most affected species is *Dimorphorchis*, which is belonging to *Vandaeae* tribe and subtribe *Aeridinae. Dimorphorchis is* recognised for its dimorphic flowers. Borneo populations of *Dimorphorchis* consist of two species which are D. *lowii* and D. *rossii*. *Dimorphorchis lowii* is distributed only in Kalimantan Tengah, Sabah and Sarawak (Chan *et al*., 1994; Cribb and Bell, 2008). D. *lowii* is a monopodial and epiphytic growth of orchid and has been listed on Appendix II of the Convention on International Trade in Endangered Species (CITES), which restricts trade in wild plants. Seed germination is one of significant method to conserve plants by maintaining its genetic diversity following the Mendes law of genetic inheritance. Seed germination and development of orchids started when an embryo enlarged to form protocorm and further develop into new seeds (Jaime *et al*., 2005). Unfortunately, seed germination of orchid is considered difficult in nature because of a symbiosis relationship (Salifah *et al*., 2011). Mycorrhiza that acts as a carbon source is required to stimulate germination and can affect growth and development due to lack of endosperm and membrane tissue intact with embryo (Arditi, 1967; Rao, 1977; Rasmussen, 1995; Khor and Chiang, 2005; Stewart and Kane, 2006; Johnson *et al*., 2011; Mahendran *et al*., 2012). Symbiotically, only 2% to 5% seeds can be germinated in nature (Luan *et al*., 2006; Dutta *et al*., 2011). Since conventional ways of propagating the species are slow, hence a biotechnological approach is considered. Asymbiotic propagation has been proved to be an efficient approach which can germinate up to 100% without wasting the function of seeds (Jaime *et al*., 2005; Stewart and Kane, 2006; Kauth *et al*., 2006; Kim *et al*., 2007; Dutra *et al*., 2008; Mahendran and Narmatha Bai, 2009). The success of asymbiotic

germination is controlled by several factors such as maturity of capsules, basal media, complex additives and lights (Kauth *et al*., 2011; Parthibhan *et al*., 2012; Sonia *et al*., 2012). Therefore, this study has been conducted to optimize these factors for efficient asymbiotic germination of D. *lowii* seeds.

## MATERIALS AND METHODS

### Seed Source and Capsule Sterilization

Seeds were obtained from artificially hand-pollinated by transferring pollen onto stigma of the same flower when they became fully opened. The development of capsules had been observed and the collection of capsules was carried out at three different harvest times, which are 100 days, 150 days and 170 days after pollination. The capsules then washed thoroughly under running tap water and brushed to remove solid particles that adhere on the surface. Next, the capsules were surface sterilised by dipping into 30% (v/v) Clorox® (5.25 % (w/v) sodium hypochlorite) solution with two drops of Tween-20 for 20 minutes. The capsules were then rinsed three times with sterile distilled water under laminar flow. The sterilised capsules were cut longitudinally into two and the seeds were taken out and sowed immediately onto culture media.

### Basal Media Selection and Culture Condition

Three basal media that reported can successfully promote seed germination in orchids were used for the media screening which are; MS media (Murashige & Skoog, 1962), KC media (Knudson, 1946) and VW media (Vacin & Went, 1949). All media were prepared and pH adjusted to 5.6- 5.8 before added 9$gl^{-1}$ of agar Sigma prior to autoclaving at 121°C for 20 minutes. Media were poured and seeds (150 days after pollination) were inoculated evenly on the culture media to examine the seed germination and protocorm development. Culture media were incubated under continuous light photoperiod provided by cool white inflorescent.

### Seed Maturity and Additives Complex on Germination

Effective media (VW media) from previous experiment had been selected to examine the effect of three different maturity of capsules (100, 150 and 170 days after pollination (DAP)) and supplemented with three types of complex additives such as; 10% (v/v) coconut water (CW), 10% (w/v) potato homogenate (PH) and 10% (w/v) tomato juice (TJ). CW was obtained from young coconut and was taken directly after being filtered. Potato and red tomato juice were prepared fresh to avoid oxidation by peeling off the skin and cut in small pieces, weighed and blend without adding water in a mixer. The homogenate was added with 20 $gl^{-1}$ sucrose and 9 $gl^{-1}$ Sigma agar into the media before autoclaving. The seeds were cultures onto media treatment were incubated at 25±2°C under 24 hour light conditions (≈32.4 µmol $m^{-2}$ $s^{-1}$).

### Effect of Light Germination *in vitro*

Seeds from 150 DAP capsules were cultured on VW (Vacin and Went, 1949) medium supplemented with 10% (w/v) potato homogenate. The cultures were incubated at 24 hours light conditions (≈ 32.4 µmol $m^{-2}$ $s^{-1}$) and 24 hours dark conditions.

### Seedling Development

Observation was done for 60 days after culture and the cultures were observed every week to monitor the development of seeds and protocorm. Developmental stages of seeds were adopted and referred from Arditti (1967) and Johnson & Kane (2007). Individual seeds were observed using stereomicroscope and image analyzer to count and evaluate. When testa seeds were ruptured due to enlarging of embryo (stage 2), the seeds were considered as germinate. The growing protocorm were subcultured every four weeks to promote seedling development.

### Statistical Analysis

There are six replicates for each treatment, and experiments were carried out in a completely randomised design (CRD). The data were subjected to Analysis of Variance (ANOVA) and T-test. Subsequently, the Duncan Multiple Range Test (DMRT) was used to determine significance different at $p<0.05$. The percentages of germinated seeds were calculated by dividing the number of seed in each stage with the total number of seed in each plate.

## RESULTS AND DISCUSSION

### Effect of Basal Media on Germination of D. *Lowii*

In the present study, germination evidence by ruptured testa was shown up to 28.00±0.20% on VW media after 20 days of culture (Table 1). It was proved to have significantly different in MS (18.65±0.22%) and KC medium (0%) that give the lowest germination. The percentages of seeds germination were increased rapidly after 40 days of culture. VW media accelerated the germination up to 87.89±0.06% follow by MS media, 82.02±0.07%. After DMRT test was determined, there is no significant different in both of the basal media. The seeds continued to germinate up to 100% and produce green mature protocorm on both media after 60 days of culture (Figure 2A-B). KC media was proved to inhibit the germination process of D. *lowii*. The seeds died and failed to germinate after 60 days of culture (0%).

The seed germination of D. *lowii* on VW and MS media has been influenced by the suitability and presence of major elements as compare to KC media. Although VW and KC media are categorize as a simple media, whereas MS media as a complex media, the differences in concentration of major elements has been recognised. All tested media contain variation of mineral salts, not only in terms of its concentrations, but also the mineral forms such as variation of nitrogen (Sonia *et al*., 2012). KC and VW media contain only inorganic nitrogen (ammonium and nitrate), while MS media contain both inorganic (ammonium and nitrate) and organic sources (glycine). The results may related to the preferences of D. *lowii*'s seeds in lowest concentration of VW media in term of nitrate (5.19 mM), ammonium (7.57 mM), phosphorus (3.77 mM) and potassium (7.03 mM) compared with MS media (nitrate; 39.4 mM; ammonium: 20.62 mM; phosphorus: 0.63 mM; potassium: 21.75 mM) and KC media (nitrate; 10.49 mM; ammonium: 13.82 mM; phosphorus: 1.84 mM; potassium:5.19 mM). Seeds of *Cyrtopodium punctatum* also show the highest germination percentage cultured on VW media compared to KC and MS media (Dutra *et al*., 2009). Nitrogen is

Table 1: Effect of basal media on seed germination of D. *lowii*.

| Basal media | Germination of seeds (mean,%±SD) by days of culture | | |
|---|---|---|---|
| | 20 | 40 | 60 |
| KC | 0c | 0b | 0b |
| VW | 28.00±0.20a | 87.89±0.06a | 100±0a |
| MS | 18.65±0.22b | 82.02±0.07a | 100±0a |

Different letter in same row shows significantly different at $p<0.05$. KC; Knudson C media. VW; Vacin & Went media. MS; Murashige & Skoog media. SD; standard deviation.

important in developing the cell wall. Lack of nitrogen ion can inhibit the growth of seeds. Inorganic nitrogen is able to reduce the germination rates due to the decreasing activity of enzyme nitrate reductase (Dutra *et al*., 2008). But, nitrogen in a form of ammonium was reported more effective in germination of orchid seeds as compare to nitrate and nitrite (Kauth *et al*., 2006; Suzuki *et al*., 2012). Basically, low concentration of nitrogen can stimulate germination, thus organic nitrogen from fungi can be replaced with nutrient element that contains in culture media (Arditti, 1967; Anderson, 1996; Kiran *et al*., 2012). Absorption of nutrient is depending on the species, base on to the flexibility of orchid cells. However, the germination process can be failed due to lack of nutrient intake (Shiau *et al*., 2005; Lauzer *et al*., 2007; Dutra *et al*., 2008). Green protocorm indicates the protocorm grow healthy. This is due to presence of magnesium and potassium that functions to stimulate the development of chlorophyll. In the case of D. *lowii*, low concentration of nutrient preferred to support germination of seeds.

**Effect of Capsule Age and Complex Additives on Germination of D. *lowii***

Immature capsule is widely used prior to easy-surface sterilize and embryos become viable after capsule ripening (Lo *et al*., 2004; Thakur and Dongarwar, 2012). Achieving full maturity takes it times according to genus and species of orchids such as *Phragmipedium humboldtii* (70 days of pollination), P. *longifolium* (112 days of pollination) and P. *pearcei* (224 days of pollination) (Munoz and Jimenez, 2008). Determining proper harvesting time and optimum age capsules is very useful to avoid seed dormancy, in order to develop efficient protocol on germination. Variation of age capsule that supplemented with complex additives can be study to perform a short period of germination process. Unsuitability to each factor can cause inhibition of seeds germination (Kauth *et al*., 2008). Thus, the best selection of complex additives can contributes to successful study and development of an efficient and low-cost method. Based on the result, ANOVA shows that there were significantly different among capsule age, complex additives and the interaction on both factors. Overall, seeds were swollen and germinated within 2 weeks after culture. The seeds totally develop into protocorm at stage 3 up to 60 days of culture. By 20 days of culture, germination occurs significantly with interaction between 150 days of capsule age and 10% (w/v) potato homogenate media (85.34±3.73%). The same interaction factor would increase the germination rates up to 98.02±0.93% when the culture past to 40 days, as well as another four interaction which is not different significantly based on DMRT test (Table 2). Seeds almost completely developed into protocorm after 60 days and formed shoot apex with rhizoids regardless of the capsule age.

The benefits of complex additives added to media on germination have been reported in many orchid species such as *Dendrobium tosaense* (Lo *et al*., 2004). Nutrients from the complex additives positively influence to induce the germination of D. *lowii*'s seeds. Thus, it is important to identify the content of the additives. Potato homogenate is less being used as compare to coconut water. It contains sucrose, dipotassium phosphate, potassium chloride, ferrous sulphate, nitrogen source, protein, carbohydrate, Vitamin B and C (Thompson, 1929; Gavronsky, 1945; Andreas and Marleny, 2009). Lo *et al*. (2004) reported that the seed's age for Dendrobium tosaense from 8 to 14 weeks after pollination, germinates well in media with addition of potato homogenate. Coconut water is widely known as growth regulators that can induce cell growth. It contains inorganic ions, nitrogen source, amino acid, enzyme, organic acid, vitamin, sucrose and plant hormones (Goh, 1981; Arditti and Ernst, 1993). It was proved that coconut water facilitates the germination of *Paphiopedilum villosum* var. *densissimum* (Long *et al*. 2010) and *Encyclia* aff. *oncidioides* (Znaniecka *et al*. 2005). Tomato juice also can induce germination due to presence of protein, carbohydrate, potassium, phosphorus, magnesium, calcium, ferum, copper, zinc, manganese, vitamin A, vitamin E and niacin (Hernandez *et al*. 2005; Prasertsongskun and Awaesuemae, 2009). The high acidity content can lead to addition of sodium hydroxide as neutralizing agent and cause accumulation of sodium in media that can affects seeds germination. However, it is depending on the species.

**Effect of Light Exposure**

Light is essential to enhance the germination rates in orchid seeds. Generally, germination of seeds require incubation under 16/8 hrs light/dark exposure (Kauth *et al*., 2008; Parthiban *et al*., 2012). However, continuously exposing the seeds under light for 24 hours can produce greener protocorm such as *Cattleya* protocorm (Arditti, 1967). By 20 days of culture, the seeds germinate rapidly under light exposure (85.33±0.04%) as compare to dark light condition (0%). Slowly, seeds under dark exposure become

Table 2: Effect of capsule age and complex additives on seeds germination of D. *lowii* on VW media.

| Capsule age | Treatment | Germination of seeds (mean,%±SD) by days of culture | | |
|---|---|---|---|---|
| | | 20 days | 40 days | 60 days |
| 100 days | Control | 28.92±13.49e | 81.19±3.63f | 100±0 |
| | 10% (v/v) CW | 41.41±7.11cd | 81.64±1.39ef | 100±0 |
| | 10% (w/v) PH | 43.22±7.90cd | 89.52±3.63cd | 100±0 |
| | 10% (w/v) TJ | 27.53±13.30e | 84.73±3.70ef | 100±0 |
| 150 days | Control | 7.29±1.98f | 91.79±2.71bc | 100±0 |
| | 10% (v/v) CW | 16.30±1.68f | 97.23±1.41a | 100±0 |
| | 10% (w/v) PH | 85.34±3.73a | 98.02±0.93a | 100±0 |
| | 10% (w/v) TJ | 6.83±2.32f | 93.71±3.31abc | 100±0 |
| 170 days | Control | 47.78±14.39c | 90.98±4.85c | 100±0 |
| | 10% (v/v) CW | 59.00±6.68b | 95.80±0.90ab | 100±0 |
| | 10% (w/v) PH | 62.03±4.42b | 96.75±2.10a | 100±0 |
| | 10% (w/v) TJ | 35.01±12.92de | 86.06±8.96de | 100±0 |
| Capsule age | | F =39.53* | F =53.82* | |
| Complex additives | | F =74.86* | F =12.98* | |
| Interaction | | F =26.04* | F =3.47* | |

Different letter in same row shows significantly different at $p<0.05$. CW; coconut water. PH; potato homogenate. TJ; tomato juice. SD; standard deviation. *significantly different.

Table 3: Effect of light exposure on germination of D. *lowii* and VW as a basal media supplemented with 10% (w/v) potato homogenate.

| Light exposure | Germination of seeds (mean,%±SD) by days of culture | | |
|---|---|---|---|
| | 20 days | 40 days | 60 days |
| 24 hrs light | 85.33±0.04 | 98.01±0.01 | 100±0 |
| 24 hrs dark | 0±0 | 72.34±0.03 | 100±0 |

SD; standard deviation.(T-test value, p= 0.00)

enlarged and started to germinate for 72.34±0.03% after 40 days whereas seeds under light exposure increased up to 98.01±0.01% significantly. Seeds were fully germinated and completely develop into protocorm after 60 days of culture (100%) in both conditions with a different morphology.

Seeds under light exposure germinates greenly but under dark exposure, seeds germinated without chlorophyll pigment and white in colour, relate to phytochrome I; dominant to plant which need less light, and II; plants which sensitive to light such as *Odontoglossum glosarium*. When sensitive plants exposed to dark condition, the phytochrome types I quantity is increasing 100 times, therefore cell actively divided and grow due to phototrophic effects (Jaime and Yolima, 2006; Ingrid and Andrea, 2011; Johnson *et al*., 2011). *Vanilla* sp., *Cyprepedium* sp., *Dendrobium fimbriatum* and *Cephalanthera falcata* can germinates well under dark condition but needs light for further growth and seed development (Arditti, 1967; Sharma *et al*. 2005; Yamazaki and Miyoshi, 2006) In addition, certain species can germinates in both conditions such as *Cattleya*, *Epidendrum*, *Oncidium*, *Goodyera oblongifolia* and *Calopogon tuberosus* (Arditti, 1967; Kauth *et al*. 2006; Dutra *et al*. 2008).

**Seedling Development**

Based on the efficiency of medium, VW basal medium that supplemented with 10% (w/v) potato homogenate and kept in culture room with light exposure successfully develop a complete seed development. It takes approximately 300 days of culture. Starting with 0 days, seeds started to germinate and form protocorm structure with appointed shoot apex with rhizoid up to 47 days. The first leaf was emerged between 48 to 55 days of culture and more leaves were produced with roots up to 200 days. The seeds were considered mature and grow uniformly after produce more than six leaves and four roots, basically up to 260 days. The acclimatization process has been conducted and the seedlings were proved to be survived even after 40 days left at the green house.

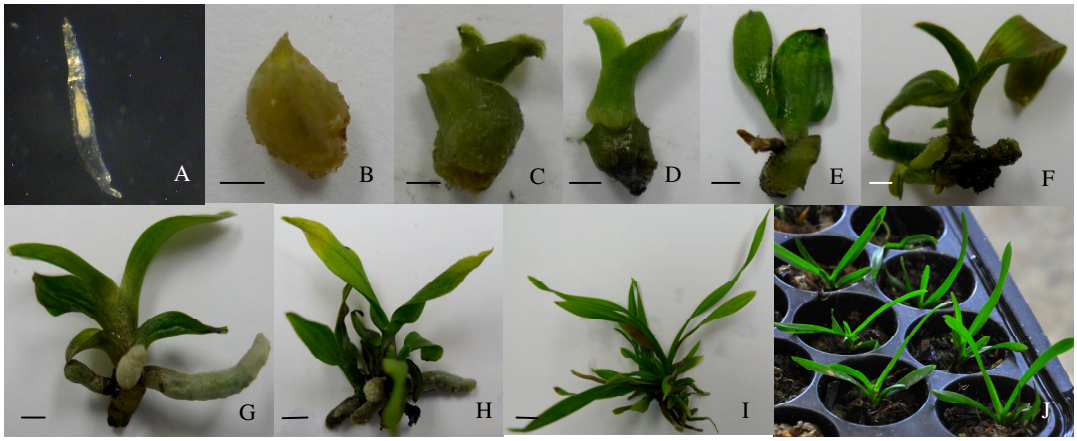


Figure 2: The seedling development of D. *lowii*. Bar = 1mm

Table 4: The seedling development of D. *lowii*.

| No. | Description | Age |
|---|---|---|
| A | Ungerminated seed. | 0 days |
| B | Protocorm with appointed shoot apex and rhizoid | 34-47 days |
| C | Protocorm with emergence primary leaf | 48-55 days |
| D | Seedling with two leaves | 75-85 days |
| E | Seedling with two leaves and one root | 95-100 days |
| F | Seedling with three leaves and two roots | 110- 120 days |
| G | Seedling with four leaves and three roots | 150-170 days |
| H | Seedling with six leaves and four roots | 180-200 days |
| I | Mature seedling | 240-260 days |
| J | Survival seedling | 300 days |

## CONCLUSION

An efficient protocol for germination and seedling development of D. *lowii* was established. The maximum numbers of germinated seeds were determined. It was recommended to use 150 days of capsule age and cultured on VW basal media supplemented with 10% (w/v) potato homogenate for better seed germination and seedling development.

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