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In vitro propagation of the endangered medicinal orchid, *Dendrobium lasianthera* J.J.Sm through mature seed culture

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ABSTRACT

Objective: To study asymbiotic seed germination and mass propagation of *Dendrobium lasianthera* which is one of the endangered medicinal orchids using seeds.
Methods: The 14 weeks old hand pollinated seeds were sown on Vacin and Went (VW) solid medium supplemented with various concentrations of peptone (1, 2, 3 g/L) and without peptone which was used as control treatment. At the 4, 8, and 12 weeks after the seeds were sown, seed germination and shoot formation were investigated. To evaluate the role of organic nutrient additives on subsequent shoot development and root formation, particular shoots with about 1 cm length contains 1–2 leaves obtained from the seeds germination was cultured on VW medium additives with different of organic nutrient: 15% coconut water, 2 g/L peptone, 150 g/L banana homogenate, and without organic nutrient was used as control. After 16 weeks of culture, the plantlet height, number of leaves, number of roots, leaf length and root length were recorded.
Results: The supplementation of 2 g/L peptone in VW medium was proven to be suitable concentration for seed germination (100%) and shoot formation with 84.0% the protocorm development to phase 5 (shoot). VW medium containing 15% coconut water was effectively improved the shoot development, with well developed roots and leaves compared to the other treatment and 95% of acclimatized plantlets survived.
Conclusions: This protocol is an efficient way for the *in vitro* mass propagation of this *Dendrobium lasianthera*.

1. Introduction

Dendrobium lasianthera J.J.Sm. (*D. lasianthera*) is an endemic epiphytic orchid species in Papua Island, Indonesia. This species typically grows in lowland areas (0–500 m above sea level) and thrives in temperatures of 16–19 °C at night and 24–32 °C during the day, with a humidity range between 50% and 80% and the degree of acidity natural media (pH) 7–7.5. This species is a very large plant with nearly 3 m long, cane-like stem. The flowers are about 7 cm across, fascinating and attractive with the combination of red, purple pink, maroon, and white [1]. It is medicinally important for its vegetative organs (roots, stems, and leaves) are toxic and contain anti-cancer of breast T47D with LC50 (μg/mL) = 117 ± 6.35. However, the presence of these orchids in the natural habitat is categorized as susceptible because of inevitable forest exploitation.

The main problems in the development of orchid plants as raw material for medicine are: 1) the mass propagation technique is relatively formidable, 2) the vegetative phase in its life cycle is lengthy (1–2 years) and 3) the genetic stability of the plant. Orchids can be generatively propagated through seed culture and vegetation. Orchids produce seeds in large quantity (2–3 million seeds/capsule), however they do not have functional endosperm. Only 0.2%–0.3% of them is able to germinate seeds in nature, hence the quantity is limited [2]. Vegetative propagation of

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orchids can be conducted in three ways; cutting, separating shoots and separating clumps, however this method has several setbacks. It demands a long period and it is effortful to obtain enough tillers. This orchid requires another efficient propagation method.

Some propagation methods have been done for *Dendrobium* through *in vitro* culture from different explants including shoot tip [3], protocorms and protocorm-like bodies [4–6], nodal segments [7], seeds [8] and callus [9]. However, propagation through the seed culture in *D. lasianthera* J.J.Sm. *in vitro* has not yet been accomplished.

In this research, we evaluated the role of peptone supplemented on seed germination and shoot formation of *D. lasianthera*. The role of organic nutrient additives on root formation and shoots development were also examined subsequently. We discussed the important role of a reproducible technique for the establishment of plantlet from seeds via *in vitro* culture in this orchid species.

2. Materials and methods

2.1. Plant materials and process of sterilization

D. lasianthera J.J.Sm. used in this study was obtained from DD Orchids Nursery, Junrejo Village, East Java, Indonesia. The 14-week old yellowish green hand pollinated capsule (Figure 1A) was rinsed using 10% sunlight detergent solution (sunlight is a commercial brand from Unilever, Indonesia) for 5 min to eliminate the dust, and then washed under tap water. This process was followed by sterilization using 1% sodium hypochlorite (Na, Cl, O) solution (Bayclin, Johnson, Indonesia) for 3 min with occasional stir, and then it was rinsed three times using sterile-distilled water. After the capsule surface was disinfected using 70% alcohol, it was put on a Petri dish, placed into a laminar flow and flamed 3 times. The capsule was sliced into four parts transversely and longitudinally using a sterile scalpel in a sterile Petri dish. Using a sterile spatula, the mature seeds of *D. lasianthera* were removed from the capsule and pooled.

2.2. FDA staining

The mature seeds were soaked in fluorescein diacetate (FDA, HIMEDIA, India) solution with equal volume of distilled water and FDA stain (0.5 g in 100 mL of absolute acetone) for 15 min and examined under an Olympus CX41 (UV light) fluorescence microscope. Seeds with completely stained embryos (fluorescent) were considered viable.

2.3. Asymbiotic seed germination and shoot formation

To evaluate the effect of peptone on seed germination and shoot formation, the seeds were sowed on Vacin and Went [10] medium supplemented with 1–3 g/L peptone (Difco Laboratories Detroit, USA), as well as medium without peptone used as control treatment. All media were supplemented with 30 g/L sucrose (Merck, Made in Germany), solidified with 2 g/L gellan gum (Phytagel: Sigma Chemical Co., St. Louis, MO) and set into pH 5.6 before sterilized at 120 °C for 15 min. For each treatment, about 300 seeds were cultured in culture tube filled with 25 mL of medium. All experiments were triplicated with 5 cultures tube per replication. All the cultures were maintained under 16/8 h day/night, respectively at (23 ± 2) °C. After 4, 8, and 12 weeks of inoculation, the cultures were examined under Tension stereomicroscope, Nikon SMZ-1, Japan, to define the role of peptone on seed germination and shoot formation. The processes of seed germination until shoot formation were classified into six groups according to embryo development phases which were adapted from [11]. The phases are: Phase 0: Seed with embryo, seed coat intact; Phase 1: Swollen embryo, covered by seed coat; Phase 2: Embryo continues to grow larger, seed coat bursts; Phase 3: Embryo is released from the seed coat (protocorm); Phase 4: The emerge of the first leaf; Phase 5: Protocorm is continuously elongated and followed by the formation of a second leaf. Germination is considered to occur only if the seed coat bursts and embryo emerges from the seed coat (Phase 2, Figure 1E). The percentage of seed in different

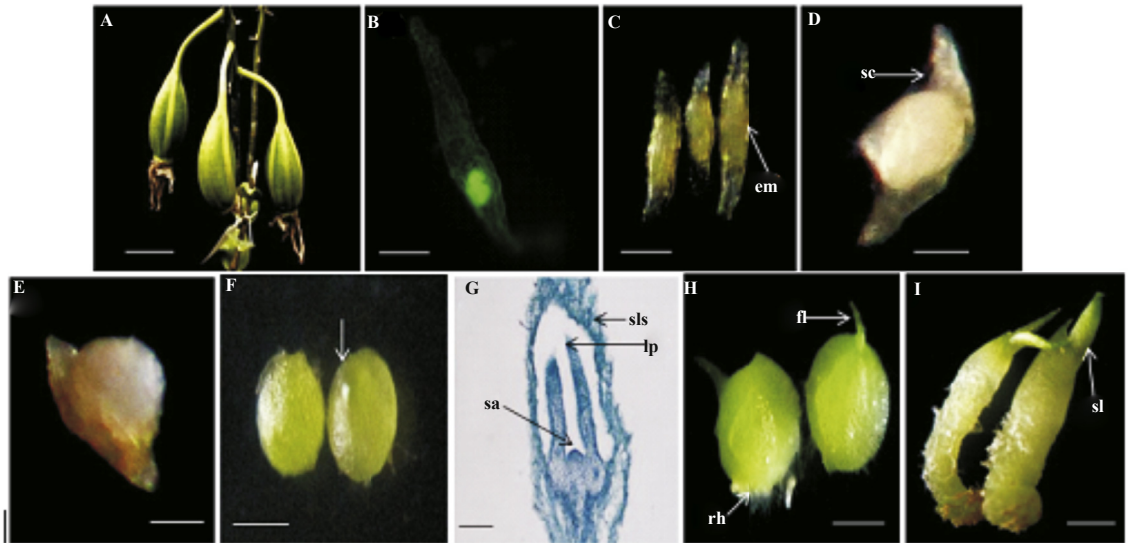

Figure 1. Asymbiotic seed germination and shoot formation of *D. lasianthera* J.J.Sm.
A: Seed capsules; B: Viable seeds stained with FDA; C: Phase 0, seed with embryo, seed coat intact; D: Phase 1, embryo swells, covered by seed coat; E: Phase 2, enlarged embryo, seed coat burst; F: Phase 3, embryo is released from the seed coat, with pointed appendicle (arrow); G: Long section of protocorm showing shoot apex; H: Phase 4, appearance of the first leaf; I: Phase 5, continuously elongated protocorm and followed by the formation of a second leaf. em: Embryo, fl: First leaf, lp: Leaf primordium, rh: Rhizoid, sl: Second leaf, sa: Shoot apex, sc: Seed coat, sls: Scutellum-like structure. Scale bars: (A) 2.15 cm, (B) 150 μm, (C) 164 μm, (D) 133 μm, (E) 250 μm, (F) 2.0 mm, (G) 75 μm, (H) 2.4 mm, (I) 8 mm.

developmental phase was calculated by dividing the amount of seed in each phase by the total amount of seed ×100.

2.4. Histology analysis

For histological observation, microscope slides were made by employing paraffin method. The protocorms were fixed in FAA (70% ethyl alcohol:glacial acetic acid:formaldehyde, 90:5:5 v/v/v), dehydrated in ethyl alcohol series and embedded in paraffin wax for 24 h. Next, longitudinal sections were made at 10 µm thickness using a rotary microtome (Shibuya, Japan), stained with 1.0% safranin and 1.0% fast green, and mounted with Canada Balsam Synthetic in xylene (Aldon, USA). Microscopic slides were examined under light microscope (Olympus FSX100, Japan).

2.5. Root formation and subsequent shoot development

After 12 weeks of culture, the shoots obtained from the seeds germination were used for inducing root. Particular shoots characterized by 1 cm long with 1–2 leaves were cultured individually on VW medium supplemented with organic nutrients *i.e.* 15% coconut water (CW), 2 g/L peptone, and 150 g/L banana homogenate (BH). A medium without organic nutrient was used as control treatment. Coconut water was obtained from fresh green coconuts, and filtered. Ripe banana was obtained from market, peeled, and homogenized in a mix. Each treatment was triplicated with five culture tubes per replication. Each culture tube consists of four to five shoots. All the cultures were maintained at (23 ± 2) °C under 16/8 h day/night photoperiods. After 16 weeks of culture, plantlet height, the number of leaves and roots, leaf length and root length were recorded. Subsequently, *D. lasianthera* plantlets with 4–5 leaves bearing 4–6 roots (approximately 2–3 cm in height) were removed from the culture tube, rinsed under tap water to wipe off the agar, and transplanted into plastic pots which contain a mixture of coconut fibre and sphagnum moss (3:1 v/v). Potted plants were grown in the greenhouse under 30%–40% natural light and sprayed two times a day for acclimatization.

2.6. Statistical analysis

The experimental units were set up in a completely randomized design. The data were analysed with SPSS (Version-17) using ANOVA. The mean values were separated using Duncan's multiple range test (DMRT) with level of significance at $P < 0.05$ [12].

3. Results

3.1. Asymbiotic seeds germination and shoot formation

The mature seeds of *D. lasianthera* used as explants had light yellow colour and the embryos consist only of a clump of undifferentiated cell enclosed by the seed coat (Figure 1B, C). The morphological development phases of *D. lasianthera* from seed to shoot were documented (Figure 1C–I). The germination process started approximately about 2 weeks after inoculation by swollen embryo (Figure 1D). Six weeks after inoculation, embryo continued to grow larger, seed coat bursted and embryo emerged from the seed coat (Figure 1E), following the light green embryo, appendicle (arrow) then became visible in one side of the protocorm (Figure 1F). Protocorm with apex shoot and leaf primordial continued to grow (Figure 1G). When the protocorm reached about 2.4 mm length, the protocorm turned into green, the first leaf as well as rhizoids appeared (Figure 1H), and second leaf was formed, respectively (Figure 1I).

3.2. The effect of peptone on seed germination and shoot formation of D. lasianthera J.J.Sm in vitro

The effect of peptone on asymbiotic seed germination of *D. lasianthera* after 4 weeks, 8 weeks, and 12 weeks were shown in Figure 2. After 4 weeks of culture, the percentage of maximum seed germination in 3 g/L peptone was 84.0%, followed by 2 g/L peptone with 79.3%, whereas 1 g/L peptone and without peptone (control) was 67.3% and 66.6%. At 8 weeks of culture, it can be seen that peptone supplemented in the VW medium significantly affected seed germination and shoot formation of *D. lasianthera*. The seed germination rate in the treatment without peptone was lower (78.9%) compared to VW containing 1 g/L (84.0%), 2 g/L (95.4%) and 3 g/L peptone (99.0%). Phase 5, shoot with true leaf were present only in the VW medium supplemented with 1 g/L (2.6%), 2 g/L (2.3%), and 3 g/L peptone (3.7%). By the 12th week, the percentage result showed that seeds culture on all germination treatments are the

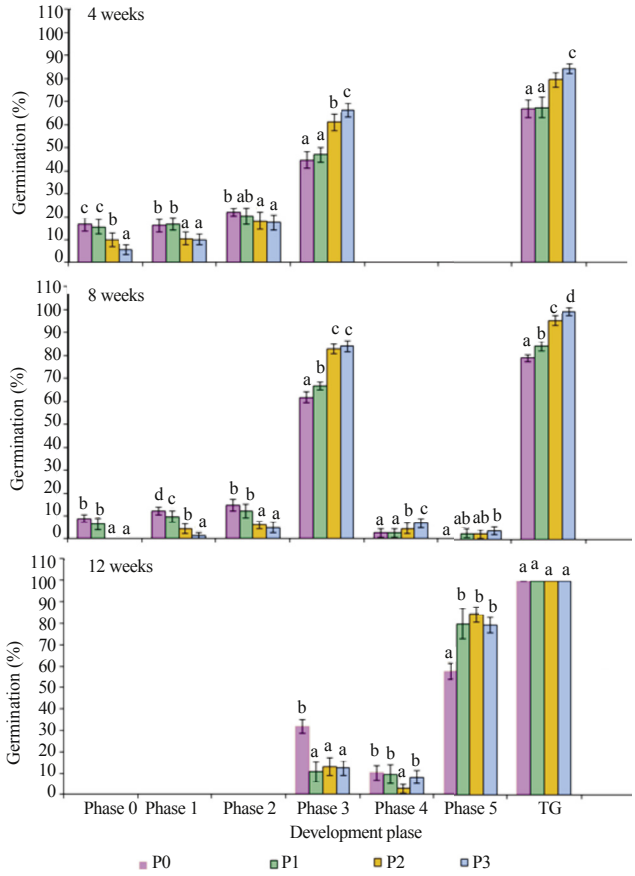

Figure 2. The effect of peptone on the seed germination and shoot formation of *D. lasianthera* 4, 8, and 12 weeks after *in vitro* culture.
P0: VW medium without peptone; P1: VW medium supplemented with peptone 1 g/L; P2: VW medium supplemented with peptone 2 g/L; P3: VW medium supplemented with peptone 3 g/L; TG: Total germination. Means ± SD in each phase followed by the different letter are significantly different at the $P = 0.05$ by Duncan's multiple range test.

same (100%). Phase 5, existed in all treatments; but a higher percentage of phase 5 (84.0%) was observed on VW medium containing 2 g/L peptone.

3.3. Organic nutrient additives effect on root formation and subsequent shoot development

The role of organic nutrient additives on root formation and subsequent shoot development of *D. lasianthera* is presented in Table 1, and the performance of *in vitro* regeneration is shown in Figure 3. The presence of organic nutrient additives in VW medium, showed significant effect on the root formation and shoot development. At 16 weeks after inoculation, the highest length of plantlet, leaves, and root were 3.4 cm, 1.9 cm, and 1.8 cm, respectively, in addition to that, the number of leaf was 5.2. All of those were obtained from VW medium with coconut water, while the lowest length of plantlets, leaves, and root were obtained from control treatment. On another note, both VW medium containing peptone and VW medium containing banana homogenate showed less significant difference. Coconut water also increased the root formation, six roots/shoots were significantly higher than those of other treatments.

Table 1

The effects of organic nutrient on root formation and subsequent shoots development *D. lasianthera* J.J.Sm on VW medium for 16 weeks culture.

Organic nutrient	Plantlet	Root		Leaf	
	Length (cm)	No	Length (cm)	No	Length (cm)
Control	2.8 ± 1.1^{a}	0.1 ± 0.6^{a}	0.4 ± 0.1^{a}	3.8 ± 1.6^{a}	1.0 ± 0.2^{a}
15% CW	3.4 ± 1.7^{b}	6.0 ± 3.6^{c}	1.8 ± 0.4^{c}	5.2 ± 2.1^{b}	1.9 ± 0.5^{c}
Peptone 2 g/L	2.9 ± 1.0^{ab}	3.3 ± 1.9^{b}	1.8 ± 0.4^{c}	5.1 ± 2.8^{b}	1.4 ± 0.3^{b}
150 g/ LBH	2.9 ± 0.9^{ab}	4.7 ± 2.5^{b}	1.1 ± 0.4^{b}	5.1 ± 2.8^{b}	1.3 ± 0.1^{b}

Means ± SD followed by the different letter within a column are significantly different at the $P = 0.05$ by Duncan's multiple range test.

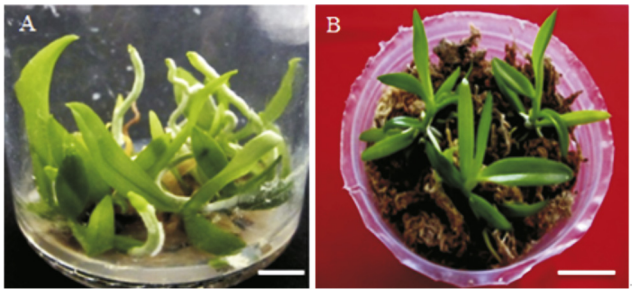

Figure 3. Developing shoots and establishment of *D. lasianthera* J.J.Sm. plantlets.

A: Shoots of *D. lasianthera* J.J.Sm. after 16 weeks cultured on VW medium supplemented with 15% CW. Some of the roots have been appearing on the basal part of the shoot; B: Plants approximately 4 cm height, on 5 weeks after transplanted to plastic pots loaded a mixture of coconut fibre and sphagnum moss (3:1 v/v). Scale bars: (a) 8 mm, (b) 1.3 cm.

4. Discussion

The use of peptone as an element to increase the growth of plant tissue *in vitro* has already tested in large plant such as to stimulate shoot and root regeneration of *Persea americana* [13], somatic embryo production of *Oncidium* [14], and hairy root formation of ginseng [15]. Peptone is also known to have supported the *in vitro* seed germination and protocorm like body formation of *Phalaenopsis hybrid* [16]. It also stimulated seed germination and advanced protocorm development in *Calopogon tuberosus* [17]. In *Cymbidium pendulum*, peptone induced multiplication of protocorm-like bodies (PLBs) [18]. We therefore, investigated the role of peptone on seed germination and shoot formation of *D. lasianthera*. At 4 weeks culture, as shown in Figure 2, some embryos were in phase 3 protocorm and had not developed yet into phase 4 and phase 5. At 8 weeks culture (Figure 2) some embryos were in phase 3 protocorm and only protocorm in VW medium with peptone supplementation developed into phase 5. At 12 weeks culture (Figure 2), we found that the germination 100% occurred in all treatment (1 g/L, 2 g/L, 3 g/L peptone and without peptone), hence the seed germination percentage on VW medium supplemented with 1 g/L, 2 g/L, 3 g/L peptone and without peptone are the same (100%). However only 57.6% of protocorm developed in phase 5 in VW medium without peptone supplementation, compared to the medium with peptone 1 g/L (79.9%), peptone 2 g/L (84.0%), and peptone 3 g/L (79.2%). Our results indicated that 2 g/L peptone in VW medium was the most sufficient for seed germination and the early shoot formation *D. lasianthera*. This facilitating effect of peptone may be because peptone contains amino acid, protein [13] and vitamin: biotin, pyridoxine, thiamin and nitrogen, [2] and [19] can increase the growth and the development of explants. The results of this study is supported by Hossain and Dey [20] who reported that Murashige and skoog (MS), phytamax (PM), and P723 media containing peptone supported the seed germination in *Spathoglotis plicata* better than without peptone. The supplementation of peptone in KC basal medium reported by David *et al.* could increase the rapid development of protocorm to seedling in native orchid *Vanda belvola* [21].

Several kinds of organic additives have been utilized in plant tissue culture to support the development of the plants such as coconut water, banana homogenate, and potato homogenate [22–26]. In these studies, three organic additives (15% coconut water, 2 g/L peptone and 150 g/L banana homogenate) were assayed for their effectiveness in root formation and subsequent shoot development of *D. lasianthera*. As shown in Table 1, we found that the presence of supplement organic additives in the VW medium resulted in a better response than control treatment. Supplement organic additives were added to increase root formation and shoot development. The maximum response was obtained from VW medium supplemented with 15% CW. Here 100% culture responded with average number of 6.0 roots/shoots and it was significantly different from other treatments. The increased length of root, length of leaf and length of plantlet were also observed when shoots grew on this medium. Beneficial effect of CW in enhancing shoot development and root formation may be correlated to the fact that the CW contains sugars, vitamins, amino acids, minerals and phytohormones which promote the growth of the cultures [27]. Jualang *et al.* reported that the addition of CW (20%) to the Knudson C medium increased protocorm development and shoot growth of *Vanda dearei* [28]. Prando *et al.* found that CW (20%) was the best for increasing the number of adventitious shoots of *Corylus avellana* [29]. Plantlet development from the protocorm of *Vanda roxburghii* was exposed by Islam *et al.* at MS medium which contain 15% coconut water [30]. Kaur *et al.* also found that CW (20%) was the best for regeneration and protocorm-like bodies

formation of *Dendrobium nobile*, better than CW (10%), CW (30%) and without CW [31]. Rooted plantlets (Figure 3A) were washed and planted in the mixture of coconut fibre and sphagnum moss (3:1) and acclimated in mist house. The surviving rates of *D. lasianthera* were more than 90%.

Conflict of interest statement

We declare that we have no conflict of interest.

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