

Embryogenic Callus Induction of Pencil Orchid (*Papilionanthe hookeriana* Rchb.f.) Through in Vitro Culture

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Abstract— *Papilionanthe hookeriana* Rchb.f. (Orchidaceae), popularly known as ‘Pencil orchid’ in Indonesia, is a perennial epiphyte orchid, found only at Dendam Tak Sudah Lake in Bengkulu. The aims of this research were to find the best sterilization technique of *P. hookeriana* explant and to induce maximum formation of embryogenic calli. Rapid multiplication of this orchid was achieved through culture of shoot tips and young leaf segments of mature plants by in vitro cultured on Murashige and Skoog (MS) basal medium enriched with 50 g L⁻¹ banana pulp. The experiment used Completely Randomized Design (CRD) with three replications. In the first stage, the explants were sterilized using three compositions of sterilization material. In the second stage, the explants were planted on the MS basal medium with addition of five levels of 2,4-Dichlorophenoxy Acetic Acid concentrations, namely 0, 0.25, 0.50, 0.75, and 1.00 mg L⁻¹. The result showed that the best sterilization method to reduce explant contaminant was method 3, in which the explant was washed with detergent, rinsed with flowing water, soaked in 0.1% (v/v) HgCl₂ solution for 30 minutes, soaked again in the 10% (v/v) Sodium hypochloride solution for 20 minutes, rinsed three times with sterile water before planted and then soaked in sterile water added 10% (v/v) betadine before planted on treated medium. This method was able to reduce contamination levels up to 70% from explants cultured for 5 months on MS medium. MS medium added with 1.00 mg L⁻¹ 2,4-D produced the highest number of embryogenic calli, and the biggest callus diameter (3.5 cm), characterized by transparent green color and friable callus structure.

Keywords— 2,4-D; *Papilionanthe hookeriana*; orchid; sterilization; callus; in vitro

I. INTRODUCTION

Bengkulu province has abundant orchid germplasm source. The source of germplasms are in protected forest, National park and nature reserve. Based on Romeida’s result research [1], among 164 wild and domesticated species of Bengkulu nature orchid, there was Pencil orchid (*Papilionanthe hookeriana*) which has been rare in its habitat (Dusun Besar Nature Reserve). Now *P. hookeriana* is one of orchid species protected by Indonesian government regulation no 7, 1999 regarding preservation of wild plant and animal species.

Unfortunately, many orchids are threatened with extinction because of environmental degradation, human occupation of natural habitats, and over exploitation for horticultural and ethnobotanical uses [2]. Habitat destruction due to road construction, fire, urbanization, drainage, and other anthropogenic influences can also directly affect these plants [3]. The number of orchid species is rapidly and steadily declining because of their low rate of natural propagation and the ongoing collection from nature [4], [5], and [6]. Careless collection of these species has led to

serious genetic and ecological erosion; many have already been listed as endangered species [7], [8], and [9].

P. hookeriana orchid is an epiphyte orchid. The plant growth type is monopodial. This orchid is very similar to *P. Teres* from Thailand, so that is difficult to distinguish between the two orchids if there are seen from appearance only. In the natural habitat, the orchid grows well until 2.5 m high. The stem is cylindrical-rounded, segmented and covered with thin sheath. The stem diameter is around 0.5 – 1.2 cm. The leaf is a cylindrical with acute tip and stand-up similar to pencil. The surface of stem and leaf are green. The roots appear at the node of stem. So, the orchid can be reproduced with stem cutting [3].

P. hookeriana scarcely produces fruits in the nature habitat. Fertilization can occur when it is assisted by insects or human. If the seeds are produced, they can not germinate in the natural habitat, because they do not have endosperm in their seeds, so, the germination rate is only around 1% in their habitats [10].

Propagation of this orchid through in vitro technique is one alternative to overcome the low germination rate in the natural habitat. In vitro culture can produce a lot of seedlings within short time period. For in vitro propagation,

we can use seeds and young stem leaf or shoot tip explant sources [11]. In its habitat, *P. hookeriana* needs *Crinum aciaticum* as a wet stem plant support so it can grow up right, but it does not take up the mineral nutrient from the *Crinum*.

In Sumatera island this orchid is found only in Bengkulu and Bangka Belitung [12]. In the Bengkulu province, it is found only in Dusun Besar Nature Reserve (DBNR) registered No. 602/Kpts-II/1992 by Forestry Ministerial decree which is established as orchid conservation area. However, this species is threatened to the brink of extinction because of habitat degradation, theft and encroachment. It is listed in Appendix 1 of CITES in 2013 and The IUCN Red List of Threatened Species. For breeding, Natural Resources Conservation Center (NRCC) has difficulty to find this orchid in their habitat. At the end of 2002, only one plant was found which was used as source for propagation. In 2005, 36 more plants were found. Bengkulu province NRCC was able to propagate the plants into 365 plants until 2008 and then they were planted outside its natural habitat. However, because the climate and habitat for nursery were not suitable for growing this orchid, the population decreased continuously. So, in 2011 only 15 plants survived. In the end of 2011 NRCC returned 10 plants to their natural habitat.

According to Bengkulu Antara News [13], *Vanda* Miss Joaquim (the Singapore's national flower) is crossing between *P. hookeriana* (from NRCC Bengkulu, Indonesia) with *V. teres* (from Thailand). Another famous breed of *P. hookeriana* is *V. cooperi* which is the result of back cross between *V. Miss Joaquim* with *V. hookeriana*. This variety of this orchid has not been studied comprehensively, because it needs long time for research and costumers prefer hybrid orchids that have many variations.

The success of in vitro orchid propagation depends on sterilization methods used. This orchid grows above the surface of swamp so the plants have many disease agents such as fungi and bacteria. To be used the in vitro explant, the plants must be sterilized using some techniques and stages.

Seed sowing on in vitro culture medium is an orchid propagation standard. Each species of orchid requires different medium composition, depending on the type of explants used. To accelerate and direct the growth and multiplication of explants, addition of plant growth regulator (PGR) into the planting medium is needed. The use of 2,4-D has been reported for embryogenic callus growth induction. The highest callus weights on Sorbon Lili variety which used the flower stalk explants was produced on the MS + 0.2 mg L⁻¹ TDZ + 0.05 mg L⁻¹ + 2,4-D + 0.3 mg L⁻¹ kinetin medium [14]. The highest multiplication crossing *Vanda tricolor* x *Vanda Pteroceras* var. *Validum* seeds is generated on MS medium adding 2% activated charcoal, 150 g L⁻¹ banana Ambon pulp, 5 ppm kinetin and 1 ppm 2,4-D [15]. (Romeida and Yuliasari. 2003)15.

According to [16] the highest embryogenic calli can be formed from root tips and cut of stem tip and leaf segments on 1/4 MS basal medium supplemented with 0.1- 3 mg L⁻¹ TDZ and 3 - 10 mg L⁻¹ 2,4-D for 4 weeks.

Some of *Vanda*'s genus need different types of plant regulator to propagate their explants. Shoot tips (0.5 – 0.8 cm in length) of Blue *Vanda* (*Vanda coerulea*) cultured on

Mira media responded with direct formation of 3-12 shoot buds per explant in 12 weeks. The highest number of shoot (70 -100 shoots per explant) had pruced on medium Mira enriched with 35 g L⁻¹ banana pulp, 30% coconut water and 1,08 µM NAA after 30 weeks of culture [25]. The endangered Blue *Vanda* is specific *Vanda* from located in Sri Lanka that has a beautiful flower (The IUCN Red List of threatened species too) [17].

Embryogenic callus of *Hibiscus sabdariffa* was produced from 0.01 to 0.05 mg L⁻¹ of 2,4 - D concentration [18]. The enhanced 2,4-D and Banana pulp on medium Vacin and Went (VW) was able to induce embryogenic callus on many species of orchids especially for *Phalaenopsis* sp. [19].

This experiment was done in two stages. The first stage of experiment aimed to get the best sterilization method to effectively reduce many contaminants from young leaf segment of *P. hookeriana* used as explants. The second one was induced the embriogenic calli using five levels of 2,4-D concentration that can be developed to protocorm Like Bodies (PLBs).

II. MATERIALS AND METHODS

This research was done in two stages. The first stage was the effect of several sterilization methods to reduce *P. hookeriana* explants' contaminant level. The second one was in vitro embryogenic calli induction of *P. hookeriana* through several 2,4-D concentrations.

A. The effect of several sterilization methods to reduce *P. hookeriana* explants' contaminant level

This research was done in the Tissue Culture Laboratory, Agriculture Faculty, Bengkulu University, Indonesia in 2013. The explant used in this research was collected from plant growing at the orchid plant breeding nursery and farm located near the DBNR Bengkulu Province.

In the first stage, the explants were sterilized using three compositions of sterilant. The young leaves of *P. hookeriana* were sterilized with three sterilization methods. The chemical compound used in that methods consisted of Agrept, previcur N, Benlate, HgCl₂, Sodium hypochloride, and Bethadine. They were combined into three compositions and stages of sterilizations (Table 1). The young leaves of 0.5 cm in length used as explants had been sterilized then planted on MS Medium that was supported by 20 g L⁻¹ sucrose without PGR (Fig. 1). Data collected were contaminant percentage, types of contaminated microorganism and survival rate of explant.

B. In Vitro Embryogenic Calli Induction of *P. hookeriana* Using Several 2,4-D Concentrations

In the second stage, the sterile explants (resulted from the first experiment) were planted on surface of Murashige and Skoog (MS) basal medium supplemented with myoinositol (100 mg L⁻¹), pyridoxine HCl (5 mg L⁻¹) thiamine HCl (1 mg L⁻¹), glycine (2 mg L⁻¹), coconut water (15% v/v), and sucrose (20 g L⁻¹) recommended by [20] and [21], The pH of media was adjusted to 5.8 with 1N NaOH and HCl. Medium was sterilized using autoclave for 20 minutes at 121°C and 15 psi at pressured. The experimental used Completely Randomized Design (CRD) with five levels of 2,4-Dichlorophenoxy acetic acid (2,4-D) concentrations, namely 0,

0.25, 0.50, 0.75, and 1,00 mg L⁻¹. Every treatment consisted of three replications. Three explants were planted in each culture bottle, so the total experimental unit was 45 bottles. Data observed were explant color, explant development, callus quality, callus quantity, callus diameter, callus color and callus proliferation.

TABLE I
STAGES OF STERILIZATION METHODS USING SEVERAL STERILANT COMPOSITION

Methods	Specification of compositions and stages of sterilization
M1	The explant was washed with detergent, rinsed with flowing water, soaked in 2 ml L ⁻¹ Previcurl N + 2 g L ⁻¹ Agrept for 1 hour, soaked with 10% (v/v) Sodium hypochloride solution for 20 minutes, rinsed three times with sterile water before planted then soaked in sterile water added 10% (v/v) betadine before planted on treated medium.
M2	The explant was washed with detergent, rinsed with flowing water, soaked overnight 2 ml L ⁻¹ Previcur N + 2 g L ⁻¹ Benlate, rinsed three times with sterile water before planted then soaked in sterile water added 10% (v/v) betadine before planted on treated medium.
M3	The explant was washed with detergent, rinsed with flowing water, soaked on 0.1% (v/v) HgCl ₂ solution for 30 minutes, soaked again on 10% (v/v) Sodium hypochloride solution for 20 minutes, rinsed three times with sterile water before planted, then soaked in sterile water added 10% (v/v) betadine solution before planted on treated medium.



Fig. 1 Explant used (young leaf segment of *P. hookeriana* : (a) plant *P. hookeriana* orchid 2 years old, (b) young leaves, (c) explant (segment of leaf 0.5 cm in length)

III. RESULTS AND DISCUSSION

A. The effect of several sterilization methods to reduce *P. hookeriana* explants' contaminant level

The observation at 5 months after planted on MS medium, young leaf explants orchid *P. hookeriana* previously sterilized using three methods produced different responses depending on the methods which were used (Table 2).

The observation of the three methods of sterilization used, sterilization gradually used method 3 (The explant was washed with detergent, rinsed with flowing water, soaked on 0.1% (v/v) HgCl₂ solution for 30 minutes, soaked again on 10% (v/v) Sodium hypochloride solution for 20 minutes, rinsed three times with sterile water before planted, then soaked in sterile water added 10% (v/v) betadine solution before planted on treated medium). This method has produced the highest number of sterile explants and percentage of explants survival up to 70% (Table 2).

The results of LSD test at 5% level showed that the percentage of contaminated explants and the percentage of survival explants were significantly different among the three methods used. Although the third method has produced the best response, which was declined in the percentage of explants contaminated but the method 3 contained dangerous chemicals compound that is HgCl₂. The use of the sterilant continuously can cause accumulation of heavy metal (Hg) in the plant tissues or plant cells. The use of this chemical compound has been banned especially for edible plants, because it can give the negative effect on human health. HgCl₂ sterilant can be used only for crops that are not to be consumed, including the ornamental plants.

TABLE II
THE EFFECT OF STERILIZATION METHODS ON PERCENTAGE OF EXPLANT CONTAMINATION AND PERCENTAGE OF EXPLANT SURVIVAL.

Sterilization Methods	Contamination Percentage (%)			Survival explant (%)
	Bacterium	Fungi	Total	
M1	53,34	13,33	66,67 b	33,33 b
M2	53,33	26,67	80,00 a	20,00 c
M3	20,00	10,00	30,00 c	70,00 a

Note : The number that was followed by the same letters in the same column are not significantly different of LSD at 5% levels

Bacterial contamination occurred from day 3 until 2 months after planting, while fungi contamination occurred after 1 to 4 months after planting. Bacterial contamination had caused white mucus symptoms around the explants, whereas contamination by fungi had caused symptoms such as threads (hyphae/mycelium) and flour (spores). The hyphae and spores color of a fungus infected *P. hookeriana* explant orchids were white, grey and black (Fig.2).

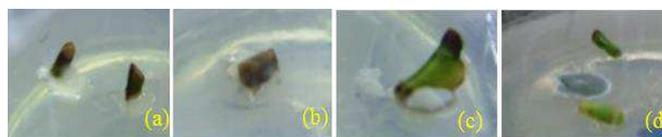


Fig. 2 Contaminants have infected explant young leaf segment of *P. hookeriana* caused by a. bacterium (white mucus), b. Fungi (white hyphae/mycelium), c. Fungi (grey hyphae/mycelium), d. Fungi (black hyphae/mycelium)

The highest level of contamination on this research was detected by bacteria, because the *P. hookeriana* orchid had been grown in a high humidity medium, part of the stems submerged in the water. In its habitat, *P. hookeriana* needs *Crinum aciaticum* that grows in the NRCC area in Bengkulu city as a wet plant support, so it can grow upright.

Water is the best medium for bacterial growth. Contamination by bacteria is more difficult to eliminate than the contamination caused by the fungi. It is difficult to control bacteria caused by bacteria living inside the cell, while the fungi generally lives on the epidermal layer of leaf tissue and part of the hyphae enters the plant tissue (surface contaminant).

The use of 0.1% HgCl₂ was effective to reduce contamination of explants caused by bacteria and fungi up to 70% (only 30% of explants were contaminated). While the use of method 1 caused contamination level higher than method 3 (66.67%). Application of method 2 was ineffective

to control the bacteria and fungi that contaminated young leaves of explants because the contamination level was very high, up to 80%.

HgCl₂ is a kind of poison that can eliminate contaminant directly in case of direct contact with contaminants. Besides eradicating the contaminants, the use of high level HgCl₂ concentration can influence the plant cells. Lethal concentrations ranging from 0.60 to 3.519 mg L⁻¹ can kill 95% of the population *Clarias gariepinus* [22].

B. In Vitro Embriogenic Calli Induction of *P. hookeriana* using Several Levels of 2,4-D Concentrations

The observations of explant development after five months sub-cultured to MS medium enriched with 2,4-D treatment were presented in Table 3.

TABLE III.
EFFECT OF 2,4-D CONCENTRATION TO EXPLANT GROWTH OF *P. HOOKERIANA* YOUNG LEAF EXPLANT AT 5 MONTHS AFTER SUB CULTURE

2,4-D Concentration (mg L ⁻¹)	explant color	Explant development	Callus explant	Callus Diameter (cm)	Callus Quantity	Callus Quality	Callus Proliferation to PLBs (%)
0.00	brown	expanded	No callus	0.00	No callus	No callus	0
0.25	green	callus	white	1.31	few	friable	43
0.50	green	callus	Transparent green	2.46	moderate	friable	53
0.75	green	callus	Transparent green	2.70	moderate	friable	42
1.00	green	callus	Transparent green	3.03	many	friable	11

Note : PLBs (Protocorm Like Bodies)

The explants grown on MS medium without PGR, expanded 2 months after the sub-cultured had no response yet. At the third month the explants color changed into yellowish, finally the explants became brownish after 5 months. The explant could not develop. The adding of 2,4-D around 0.25 to 1.00 mg L⁻¹ was able to maintain the green color of explants, friable callus quality and transparent green color (Fig. 3). These characteristics showed the embryogenic callus characteristic. The same research result was reported by [23] who have found embryogenic calli on *Vanda tessellata* orchid.

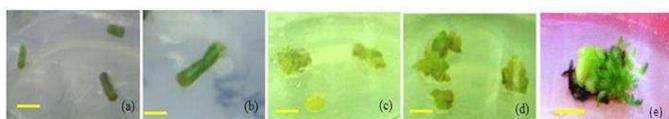


Fig. 3 Callus Quantity . (a) explant (b). No callus formed, (c). a few callus, (d). moderate callus, (e) The biggest callus with PLBs.

Explants development into callus started from the third month after sub-cultured. The quantity and diameter of callus formed were positively correlated with 2,4-D concentration increased up to 1.00 mg L⁻¹. Callus quantity formed following a linear curve ($y = 0.745x - 0.334$) and $R^2 = 0.897$. The resulted of research mean the higher the concentration of 2,4-D applied, the bigger the callus formed. The biggest callus diameter (3.03 cm) resulted from explants grown on MS medium enriched with 1.00 mg L⁻¹ 2,4-D (Fig. 4).

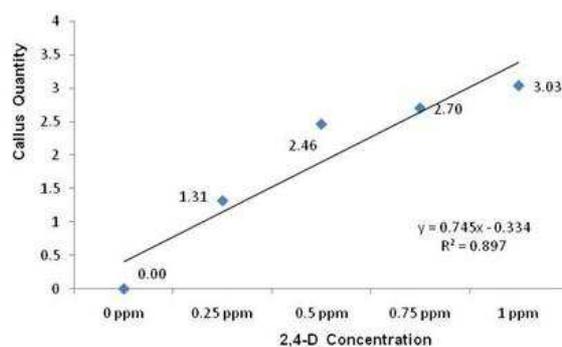


Fig. 4 Effect of 2,4-D Concentrations on *P. hookeriana* callus diameter

Callus proliferation into Protocorm Like Bodies (PLBs) formed following a quadratic curve ($y = -12.07x^2 + 74.52x - 61$) and $R^2 = 0.991$. The highest proliferation rate (53%) was produced at the explants grown on MS medium supported with 0.5 mg L⁻¹ of 2,4 - D concentration (Fig. 5).

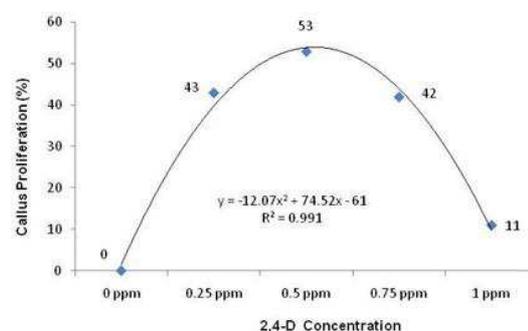


Fig. 5. Effect of 2,4-D Concentration on Proliferation Callus Percentage of *P. hookeriana* developed to PLBs

2,4-D is one kind of auxin group plant growth regulator that is very good for growing callus and root crops, especially when it is used on in vitro culture, but at high concentrations it can cause poisoning as an active ingredient with a systemic herbicide that inhibits the effect of weed growth by accelerating respiration [24] and [25].

This research result is consistent with [18] and [26]. They have studied the regeneration of Red Vanda orchid using leaf explants (*Rhenanthera imschootiana*) and Orchid Blue Vanda (*Vanda coerulea*). Embryogenic callus of *Dendrobium microbullon* was formed from 4 to 8 months after planted on in vitro [27].

This study is also in line with research conducted by [17] on the *Vanda spatula* orchid which is a specific Sri Lanka's orchid and has a very beautiful flower. Callus growth from the flower stalk was produced after 6 months. Multiple shoot formations (6.1 shoot per explant) was produced after 3 to 9 weeks later at the Mira medium added with 75 g L⁻¹ banana pulp + 22.2 to 44.4 μM BA + 5.7 to 28.5 μM IAA. While the root formation was obtained after shoots were sub-cultured to Mira medium supported with 75 g L⁻¹ banana pulp + 5.7 μM IAA after 9 weeks after sub cultured.

IV. CONCLUSIONS

The best sterilization method to reduce the explant contamination level was method 3, in which explants were washed with detergent, rinsed with flowing water, soaked in a solution of 0.1 % v/v HgCl₂ solution for 30 minutes, soaked again in a solution of 10 % v/v sodium hypochloride for 20 minutes, then rinsed 3 times with sterile water, before planting, soaked in sterile water added 10% v/v bethadine. This method was able to reduce contamination levels up to 70% from explants cultured on 5 months after sub cultured on MS medium.

Bacterial contaminants level (52.22 %) which infected the young leaves of orchid used as explant was higher than fungi contaminant level (30%). Contamination caused by bacteria was more difficult to eliminate than that caused by fungus.

MS medium supported with 1.00 mg L⁻¹ of 2,4-D concentration generated the highest number of embryogenic callus and the biggest callus diameter (3.5 cm). The embryogenic callus was characterized by transparent green color callus and friable callus structure.

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