Plant Regeneration Through Direct Somatic Embryogenesis from Leaf Explants of *Paraphalaenopsis Labukensis* P. S. Shim

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Abstract. *Paraphalaenopsis* is a small genus of the tribe Vandeae, the genus comprises 4 species: *Paraphalaenopsis denevei, Paraphalaenopsis laycockii, Paraphalaenopsis labukensis* and *Paraphalaenopsis serpentilingua*. All are known endemic to Borneo, and also rare due to over exploitation, therefore requires rescue efforts. In vitro culture is one of effort that can be done to regenerate a large number of seedlings and to ex situ conservation. Optimal growth and morphogenesis of tissues may vary for different plants according to their nutritional requirement. Auxin (NAA) is an essential factor for root growth in tissue culture work. Activated charcoal is often used in tissue culture to improve cell growth and development. This research was conducted to find out the in vitro growth effect from leaf explants of *P. labukensis* with plant growth regulators (NAA) and activated charcoal on ½ MS medium as basal medium. The treatment with NAA 2 ppm and 1 g/l activated charcoal showed the highest percentage of life (50 %) and percentage of leaf (57.1 %). The largest percentage of roots showed in 2 g/l activated charcoal (35.7 %). The death of leaf explants *P. labukensis* was caused by browning. The phenolic compound which accumulated during wounding is causing browning.

1. Introduction

Orchids, part of Orchidaceae family, there are more than 25,000 species of orchids notified entire the world. Indonesia is a home to a large amount of biodiversity, has more than 5,000 species of orchid [1]. Orchidaceae are among the most evolutionarily and ecologically significant plants known for two major growth forms and hardiness, epiphytic and terrestrial; and also successfully colonize almost every habitat on earth, including lithophytic (rock surfaces) [2]. Epiphytic orchids attach on host tree trunk for living, meanwhile terrestrial orchids grow on soil, humus or litter.

Mainly, orchids are pollinated by insects or birds which are attracted to the flowers, have various shapes, colours, beautiful flower characteristic, and/or a great variety of fragrances. Orchid is one of the ornamental plants which have been commercial value. Many orchids are threatened or even endangered by extinction because of over collection and habitat destruction, capture of wild orchids especially without considering sustainability, which led to the extinction of certain orchids. *Paraphalaenopsis labukensis* in the natural habitat is becoming rare, which have been high commercial value. In situ and/or ex situ conservation is the most suitable way for sustaining these endangered species, one of the methods is through in vitro culture.

The successful of in vitro culture method is influenced by various factors, such as the culture media used, lighting (dark and or bright), and temperature conditions during culture [3]. The composition of
culture media affects the growth of plant tissues and organs, not only contains macro and micro nutrients, but also sources of carbohydrates as a source of carbon, vitamins, and growth regulators). The addition of growth regulating substances (auxins and/or cytokinins) is particularly effective and indicated for the success of the in vitro orchid propagation.

A wide variety of research on in vitro orchids propagation have been reported by using various types of explants materials (seeds, leaves, protocorm-like bodies, shoots, nodes, plantlets), in various medium culture (such as Murashige and Skoog, Vacin and Went, Knudson C) with and/or without addition of organic supplement (bananas, tomatoes, bean sprouts, coconut water, potatoes, sweet potatoes), growth regulators (auxin, cytokinin, gibberellins), with addition of sucrose as a source of carbohydrates and activated charcoal [4, 5, 6, 7, 8, 9, 10]. Growth regulator substances which are often used in in vitro culture are auxin and cytokinin. Auxins, in tissue culture, are usually used to stimulate callus production and cell growth, to initiate shoots and rooting, to induce somatic embryogenesis, to stimulate growth from shoot apices and shoot stem culture [11]. Auxins play roles in directly affect processes of stimulating cell elongation and cell division, whereas cytokinin involved in cell growth and differentiation, apical dominance, axillary bud growth and leaf senescence.

The addition of activated charcoal (AC) into medium orchid in vitro propagation is to improve cell growth and development, by adsorptions of toxic and inhibitory substances in the culture medium, decrease in the phenolic oxidation or brown exudates accumulation, alteration of medium pH and establishment of a darkened environment in the medium [12, 13, 14, 15, 16]. The concentration of AC differed widely in plant tissue culture from a range of 0.002 g/l to 150 g/L [16], this is maybe related to different plant species, medium, explants, purpose etc. This study aims to determine the explants response to the addition of NAA concentration and AC concentration in ½ Murashige and Skoog (MS) media, and also to determine the best concentration of AC to reduce the browning effect of the leaves culture growth of *P. labukensis* as explants.

2. Method

2.1. Materials

The leaf explants of *P. labukensis* originated from in vitro collections at the Bogor Botanic Gardens, Indonesia, with 0.7 – 1 cm length. The leaf explants were cultured in jars containing basal medium, half-strength Murashige and Skoog medium, with 30 g/l sucrose and solidified with 8 g/l agar. The cultures were incubated at a temperature of 25 ± 2 oC and a 16-h photoperiod. The media were variously supplemented with NAA alone, AC alone, the combination of NAA (1 ppm, 2 ppm) and AC (1 g/L, 2 g/L). The pH was adjusted to 5.8 before adding agar with KOH and/or HCl. The media culture for leaf explants of *P. labukensis* are (1) ½ MS; (2) ½ MS + NAA 1 ppm; (3) ½ MS + NAA 2 ppm; (4) ½ MS + AC 1 g/l; (5) ½ MS + AC 2 g/l; (6) ½ MS + NAA 1 ppm + AC 1 g/l; (7) ½ MS + NAA 2 ppm + AC 1 g/l; (8) ½ MS + NAA 1 ppm + AC 2 g/l; (9) ½ MS + NAA 2 ppm + AC 2 g/l.

2.2. Procedure

Observation has been done every 4 weeks until three months after planting. Parameters that observed were an emergence percentage number of leaves, percentage number of roots, and percentage of browning.

3. Result and discussion

There are several factors that can influence success in in vitro culture, likes culture media, organic supplement, growth regulators substance, carbon source, explants and the environment. Generally, in vitro culture media contains macronutrients, micronutrients, vitamin, growth regulators, amino acids and also agar as solidifier. MS media are often used in almost all types of plants. Proportion among endogenous growth regulators and exogenous growth regulator will influence growth explants and organ formation, caused by the addition of exogenous growth regulator will change the level of
endogenous growth regulator then will affect the process of growth and development of explants. Addition of AC to culture medium is a recognised practice in plant tissue culture to improve growth and/or promote morphogenesis in a wide variety of plant species [14]. The advantage of application auxin in tissue culture work on orchid culture medium was much done to promote the growth of callus, cell suspensions or organs, and to regulate morphogenesis. The effects responses of cells, tissues and organs of callus, cell suspensions or organs in vitro can vary according to culture conditions, the type of explants and the plant genotype [14]. In the present study, after 8 weeks of culture, about 66.67 % of leaf explants were lost due to browning process. The percentage of life was achieved in half-strength MS with 2 ppm NAA with 1 g/L activated charcoal (50 %) after 3 months in culture (Figure 3). A problem with orchid micropropagation is lethal browning when dealing with explants isolated. Orchid cells in tissue culture exude a large quantity of phenolics into the medium that become toxic to the cells when oxidized [4]. Addition of activated charcoal to the medium can help overcome the inhibitory effects of phenolic released into the medium. Activated charcoal will release and/or adsorbs substances that might be produced by cultures or be present in medium, which promote and/or inhibit the in vitro growth of plants or explants.

Browning caused by some phenolic compound which accumulated during wounding or when the plant parts become senescence. The phenolic compounds usually released into medium from the cut surfaces of explants, and caused the culture medium turns to dark brown in colour due to oxidation. The browning of tissue culture can cause the cells fails to grow even become death.

Browning caused death in P. labukensis leaf culture. The leaf explants of P. labukensis exudate of phenol that can cause browning after isolation. The browning process begins with an explants discoloration from green to brown on the wounded area. The phenolic compounds are highly toxic to explants which can inhibit growth and differentiation cells and also ultimately causing death. The browning of the cells occurs by the activity of copper-containing oxidase enzymes such as polyphenol oxidase and tironase which are released or synthesized when the cells are wound [17]. The oxidation of phenolic compounds can inhibit enzyme activity and cause the media to blacken. The addition of activated charcoal to the culture media is expected to be able to absorb the compounds of the phenol compound.

The present investigation examined specific requirements for leaf explants of P. labukensis, leaf initiation was formed, 71.43 %, after 8 weeks on in vitro culture. Percentage of leaf initiation after 3 months of culture was achieved more rapidly in half-strength MS with 2 ppm NAA and conjunction with 1 g/L activated charcoal (Figure 4). The composition of culture medium can affect the process of growth and development of leaves which require a number of other compounds in media, such as growth regulators (NAA) and activated charcoal, also energy derived from the process respiration. Exogen growth regulators can be reaches growth primordial of the leaf.

After 3 months in culture, a higher percentage of root initiation occurred in half-strength MS with 2 g/L activated charcoal (35.7 %) and in half-strength MS with 2 ppm NAA and conjunction with 1 g/L activated charcoal (28.6 %) (Figure 5). In this study, rooting occurred in the ½ MS medium supplemented with NAA in the presence of AC. Roots were not observed in ½ MS media without supplemented NAA and AC.

Growth regulator substance Naphthalene Acetic Acid (NAA) from auxin group is an essential factor for stimulated initiation primordial of root. Activated charcoal in culture medium can provide a dark environment and the amount of light passing through a solidified medium is reduced and/or light can be kept away from the rooting zone, therefore, darkness is generally beneficial for rooting, especially during the inductive phase [14]. Low light intensity could promote some physiology reactions which occur in the dark, such as can stimulate endogenous growth regulator more optimally in the process of growth and development roots. Auxins are metabolised less rapidly in the dark than in the light [14]. The addition of 2 ppm NAA and 1 g/L AC to culture media enhanced rooting ability, this suggests that AC might affect auxin transport. The largest number of buds and roots hybrid ‘BLC Pastoral Innocence’ was in medium with 4.5 g/L AC into Knudson C Medium [3].
The level of auxin and cytokinin is important to determine the formation of roots and buds during in vitro culture. Whereas, roots are formed under conditions of high auxin and low cytokinin levels, buds are formed under conditions of low auxin and high cytokinin. If both are low, no growth or development [3]. The addition of AC into medium might alter the interaction between endogenous auxin and cytokinin.

![Figure 1](image1.png)

**Figure 1.** *Paraphalaenopsis labukensis* (a) plant; (b) flowers; (c) fruits

![Figure 2](image2.png)

**Figure 2.** *P. labukensis* culture from leaf explants: (a) explants; (b) leaf explants; (c) leaf explants in media 1, 3 months old; (d) leaf explants in media 2, 3 months old; (e) leaf explants in media 3, 3 months old; (f) leaf explants in media 4, 3 months old; (g) leaf explants in media 5, 3 months old; (h) leaf explants in media 6, 3 months old; (i) leaf explants in media 7, 3 months old; (j) leaf explants in media 8, 3 months old; (k) leaf explants in media 9, 3 months old

![Figure 3](image3.png)

**Figure 3.** Effect of NAA and activated charcoal (AC) in leaf explants of *P. labukensis* for 3 months on percentage of life.
Figure 4. Effect of NAA and activated charcoal (AC) in leaf explants of *P. labukensis* for 3 months on a percentage number of leaves.

Figure 5. Effect of NAA and activated charcoal (AC) in leaf explants of *P. labukensis* for 3 months on a percentage number of roots.

Conclusion

Based on the results, showed that half-strength MS with 2 ppm NAA and conjunction with 1 g/L activated charcoal culture medium was the best media for leaf induction (57.1 %) and life percentage (50 %), whereas half-strength MS with 2 g/L activated charcoal culture medium was the best media for root induction (35.7 %).

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