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Conservation of Gayo's Endemic Orchid (*Paphiopedilum primulinum*) Through In Vitro Seed Germination and Development with Coconut Water

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Abstract: *Paphiopedilum primulinum* is one of endemic orchids in Gayo, Aceh, Indonesia. This terrestrial orchid is registered as endangered species due to IUCN. The low availability of this orchids in nature is due to habitat lost, over-collection for commercial purpose, and low seed germination rate which affected by unidentified factors, yet the study of propagating this orchid is rarely discussed. This experimental research is aim to see the effect of coconut water (CW) concentration to *P. primulinum* seed germination and development through in vitro culture. The sample of the study used the seed of *P. primulinum* which age 14 weeks after pollination. The research use ½ Murashige and Skoog media (MS) with four different coconut water (CW) concentration (0,10%, 20%, 30%) and incubated for 90 days. The seed germination rate is calculated due to the percentage of its total germinate seed and seed development indicator divided into 5 stages (0-5). The result shows that the seed of *Paphiopedilum primulinum* germinate best at 30% CW concentration and only in this concentration the seedling stage reach stage 5 (4.8%). This result implies that 30% CW concentration in ½ MS medium is the best medium to germinate this seed.

Keywords: Coconut water; In vitro; *Paphiopedilum primulinum;* Seed Germination

Introduction

Paphiopedilum is a soil orchid genus with elliptical oval-shaped leaves and is patterned with an exotic marsupial flower structure (Indah, 2013). Because of its beauty, this orchid is in great demand by flower collectors and causes over-exploitation and being threatened (Khamchatra et al., 2016; Zeng *et al*, 2016). In nature, this type of orchid is only spread in a small population because of its low breeding rate and many unidentified factors (Kartikaningrum et al., 2021; Luan et al., 2019). Habitat destruction without conservation efforts for orchids can cause them to become extinct (Salsabila et al., 2022). Aceh province is geographically located on the eastern tip of Sumatra and the central Aceh region, Gayo has one endemic species of *Paphiopedilum*, namely *Paphiopedilum primulinum*. According to the Republic of Indonesia's Minister of Environment and Forestry's directive, *Paphiopedilum primulimun* is an endangered and protected species (Ministry of Environment and Forestry, 2018). It is essential to propagate this species for conservation and reintroduction goals. Yet, the study about propagating *Paphiopedilum primulinum* is rarely discussed.

In Indonesia, orchids have long been a commercial business because there are many enthusiasts of orchid plants. To meet market demand, massive and fast production is needed. In a fast and rapid growth of

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technology, invitro culture are a promising solution for plant propagation (Handini, 2008). In general, orchid propagation is done by germinating seeds in vitro to produce same results. Germination by in vitro culture can be used to increase the viability and germination of orchid seeds (Sari et al, 2023). Success in germinating orchid seeds is influenced by several factors such as seed maturity, basic media and the addition of organic matter (Apriliyania et al, 2021). Several commercial and conservation efforts with in vitro culture have been carried out on *Paphiopedilum* orchids, but *Paphiopedilum* cultures experience a fairly long germination rate (Zeng et al, 2012). One identified factor is the *asymbiotic* orchid seed and its lack of *endosperm*. Moreover, structure of the seed coat (testa) of *Paphiopedilum* is thicker and impermeable (Handini et al, 2016). It is supported by Handini's report who conducted in vitro culture with 0.8 Knudson C media on several *Paphiopedilum* seeds and monitored the length of germination time (*P. Suerbiens* 124 days, *P. primulinum* 60 days, *P. glaucophyllum* 67 days). But among the cultured variations, *P. primulinum* has a small percentage of germination (5%).

Medium is an important thing in tissue culture, the medium is a must be able to meet the needs of explants in order to live optimally. Various standard medium compositions have been formulated to optimize plant's growth, one of which is MS medium. The most used medium for in vitro cultivation is MS media. Medium generally contains macronutrients and micronutrients in the form of organic salts in certain levels and ratios, sources of carbohydrates, water, amino acids, vitamins, and growth regulators (ZPT) (Kultura, 2020). Essential amino acids, inorganic salts, vitamins, buffer solutions, and an energy source—typically glucose—are all components of in vitro growth conditions. The success of in vitro plant propagation is greatly influenced by this media. Therefore, in making media, the right and appropriate dosage is needed to maximize the results. Coconut water (CW) has been commonly used in in vitro techniques. Coconut water is an additional organic substance that can increase the rate of seed germination due to the presence of organic components like amino acids, sucrose, mineral salts and several hormones such as auxin, gibberellins and cytokinins (Sumantra, Widnyana, 2011).

Based on the described background, the experimental research was carried out to see the effect of coconut water concentration to the germination of *P. primulinum* seed as a way of conservation and determine the best concentration for seed culturing.

Method

Preparation Stage of Plant and Germinate Media

The *P. primulinum (*PM.W. Wood & P. Taylor) was obtained from its natural habitat in Takengon, Central Aceh Regency. The plant then was placed in ALIFA Laboratory of Tissue Culture. The part of the plant that used is the 14 weeks seed of *P. primulinum* after pollination.

Coconut water was sieved twice to three times to remove foreign objects. Various amounts of coconut water (0%, 10%, 20%, and 30%) were added to the ½ MS medium, which was used as the base medium. After media preparation, the pH of the medium was adjusted using NaOH and HCl to balance it within the range of 5.6 (Kamaruzaman et al., 2018).

Table 1. Various concentration of coconut water in MS media

Treatment	Code	Media Concentration
Treatment 1	K0	½ MS
Treatment 2	K1	½ MS + 10% CW
Treatment 3	K2	½ MS + 20% CW
Treatment 4	K3	½ MS + 30% CW

Culture Initiation Stage

The seed sample was put in a bottle containing sterile aquadest and added 3 drops of tween, then vacuumed for 1 hour (until the seeds sank). After that, activities are carried out in laminar air flow. The seeds were washed with 10% Clorox for 10 minutes, then the seeds were washed again with 5% Clorox for 5 minutes. The seeds were then thoroughly washed three times with sterile aquadest. After that, the seeds were opened using a sterile scalpel and distributed in the prepared seedling medium containing 0%, 10%, 20% and 30% of CW.

Incubation Stage

The seed of *Paphiopedilum primulinum* is incubated for 90 days at 26^{o}.

Seed Germination and Development

The seedling development of orchid seed generally divided into 6 stages (Table 2.)

Table 2. Seedling Development Stage

Stage	Characteristics
0	Seed ungerminated; no growth of embryo.
1	Enlarge embryo
2	Germinated
3	Embryo discharge from testa (PLB)
4	onset and extension of the first leave
5	At least has two leaves

(Utami, et al., 2015)

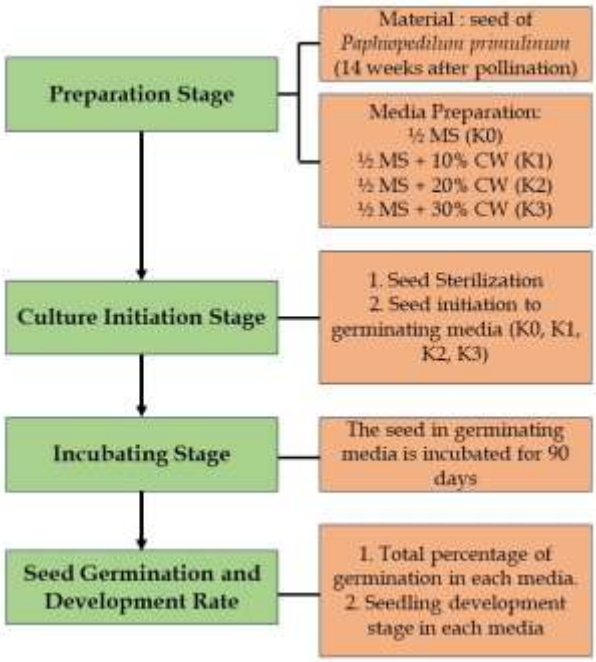

Figure 1. Research Procedure

Result and Discussion

One of popular orchids genera is *Paphiopedillum*. *Paphiopedillum* is an exotic terrestrial orchid with marsupial-shape flower. Its unique characteristics makes this plant become favored and sold for commercial purpose. In nature, the population is rare and only in small population (Diengdoh et al., 2023). It is threatened because of over-collection and habitat lost (Rahmiati et al., 2021). This typical of orchid has low reproduction rate due to its morphological and physiological traits which contributes to its amount in wild. The propagation of *Paphiopedillum* in nature need long time consuming and less success for undetermined factors (Luan et al., 2019). The asymbiotic seed characteristic claimed be one of factors for this low reproduction rate. *Paphiopedillum primulinum* is an endemic orchid in Gayo, Aceh Indonesia. It is yellow-flowering terrestrial orchid and recently mark as endangered species due to IUCN. The research of *P. primulinum* propagation also still restricted now. So, it is important and challenging to carried out research to conserve this species.

The plant of *P. primulinum* is obtained from Takengon, Central Regency of Aceh. The seed used was age 14 weeks after pollination. It is suitable with Handini (2008) that state the seed of *Paphiopedilum* will mature at 14 weeks after pollination. Due to their completely developed testa and reduced water content, mature orchid seeds may be more viable for propagation and storage (Diengdoh et al, 2017; Feng et al, 2022). Success of asymbiotic germination is dependent on several factors, including physical germination circumstances, growth media composition, and seed characteristics including seed capsule origin and maturity (Chen et al, 2015; Khamcatra et al, 2016). The germination of seeds and the growth seedlings of *Paphiopedilum primulinum* are listed in Figure 2.

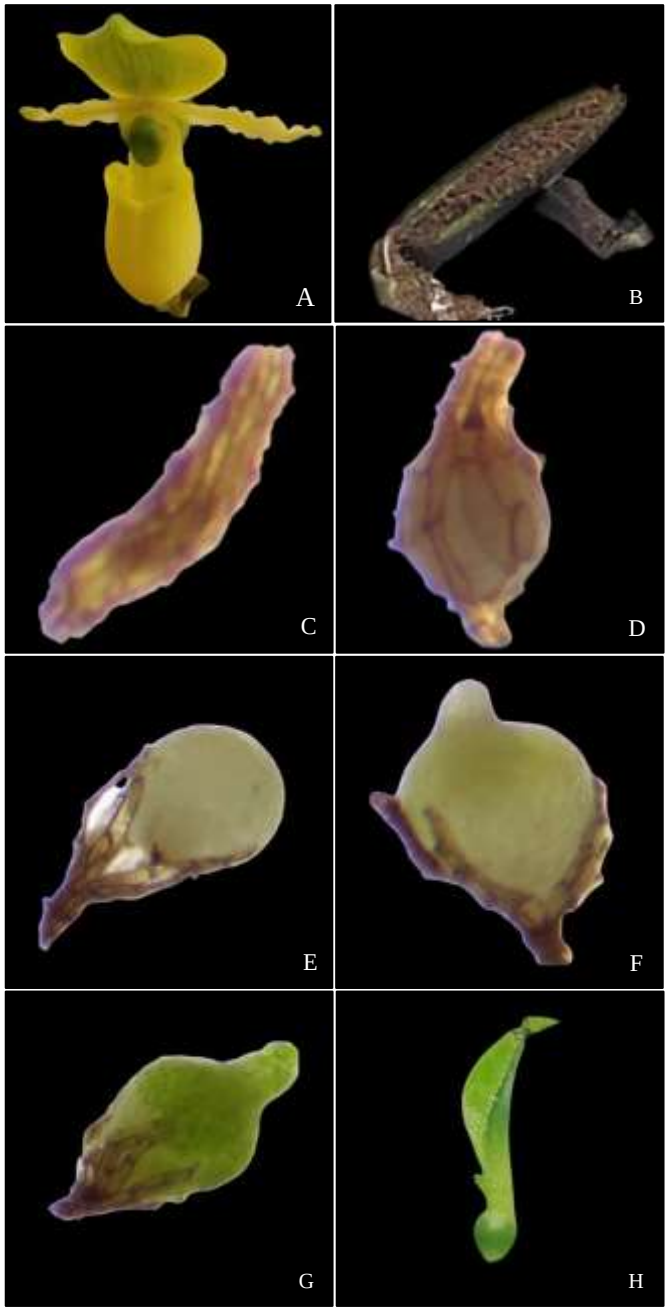

Figure 2. The germination of seeds and the growth of seedlings of *Paphiopedilum primulinum.* flower of *Paphiopedilum primulinum* (A); the 14 weeks seed of *Paphiopedilum primulinum* (B); stage 0 : ungerminated seed (C); Stage 1 : embryo enlargement (D); stage 2: the cleave of testa/germinate (E); stage 3: Protocorm (F); stage 4: onset and extension of the first leave (G); stage 5: At least has two leaves (H).

Figure 2 (D) presented the growth of the seed which reach the first stage of germination. the seed became swollen and absorbs nutrients from the growth media. It makes the structure of the seed become bigger in size and caused the seed coat become ruptured (E). The protocorm grow (F) and the shoot elongate which will differentiate into first leaves (G). the Figure 2 (H) shows the seed grow and has two leaves (Utami, et al., 2015). The ideal pH for its growth is 5.2-6.0 (Zeng et al, 2016), while in this research the media has pH of 5.6 which suitable for seed growth. Knudson C (KC), Vacint Went (VW) media are commonly used in in vitro orchid propagation (Sari, et al, 2023), but Murashige and Skoog (MS) media widely used in tissue culture and yield good result for *Paphiopedilum* seed germination (Chen et al, 2015; Diengdoh et al, 2023; Santika et al, 2023).

Table 3. Percentage of Seedling development

Media	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
K0	43	64	3	0	0	0
K1	28.5	48.5	19.2	3.8	0	0
K2	41	19.2	8.3	18.5	13	0
K3	2.9	6.7	11.4	49.5	24.8	4.8

Table 3 represent the percentage of seedling development. Each developmental stage's percentage of seed germination and seedling development is calculated by dividing it by the total number of seeds x100 (Utami, et al., 2015). 30% CW concentration yield the best seed germination and only this concentration stimulates seed growth into the maximum stage (stage 5; 4.8%). The result of this study shows that the media sulemented with CW give better result than without CW in ½ MS media.

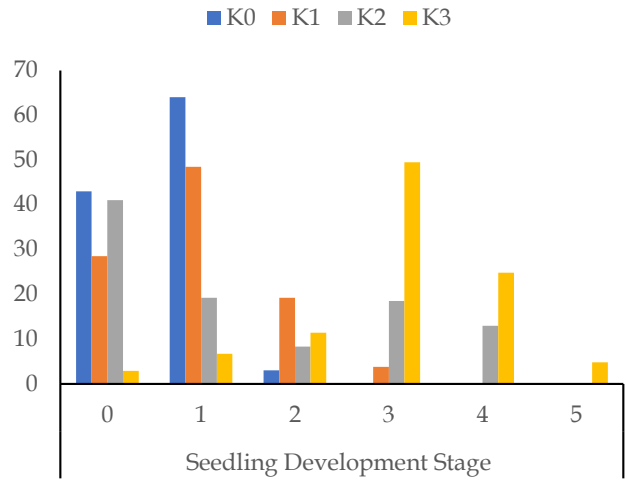

Figure 2. seedling development stage of *Paphiopedilum primulinum*

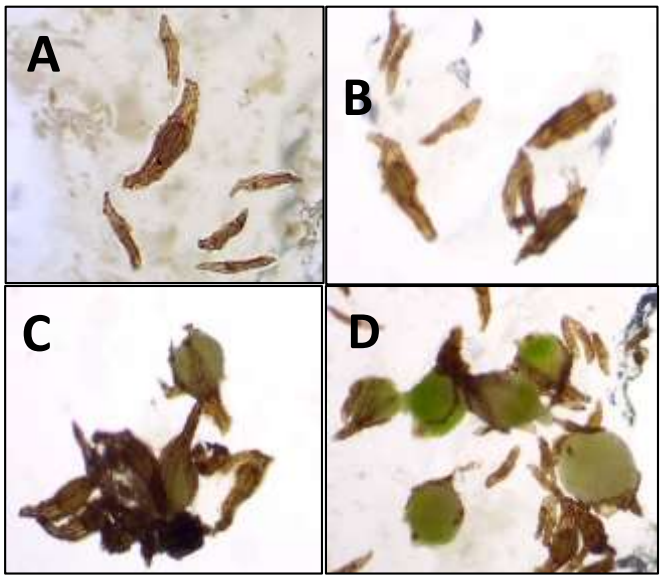

Figure 3. seed germination and development in each CW concentration (A) 0%; (B) 10%; (C) 20%; (D) 30%

The majority of *Paphiopedilum* species need a low mineral media for seed germination, whereas the Murashige and Skoog medium's high total mineral concentration inhibits *Paphiopedilum* germination, while such that 1/2, 1/4, 1/6, 1/5 or 1/8 have been shown to be more suitable (Zhou et al.,2013). *P. wardii* also demonstrated considerably less seed germination on MS compared to ½ MS media. (Zeng et al., 2012). So in this research the media is formulated with ½ MS. It has been claimed that adding organic nutrients such apple extract, banana extract, potato extract, and coconut water will accelerate the germination of several orchid species (Long et al., 2010; Zeng et al., 2012; Shekarriz et al., 2014). Organic amendments can either promote or prevent protocorm growth and seed germination in orchids (Zeng et al., 2012). Several fruit juices, including coconut water, are employed as organic amendments (CW). According to Zeng et al. (2012), P. wardii seeds cultivated on 1/2 MS medium supplemented with 7.5, 10 and 15% CW (but not 5%) germination percentage increased significantly compared to controls. It related to the result of this research were the seeds grow optimally with the additional of CW to the media (30% CW). According to Winarto and Silva (2015), Since the early 1940s, coconut water has been utilised as an organic addition and has shown to be successful in promoting and increasing the growth and profusion of numerous varieties of orchids. Furthermore, natural additives like coconut water can promote the growth of explants due to the high nutritional and hormonal content of the beverage (Abbaszadeh & Naderi, 2018; Lubis et al, 2023). Diphenyl urea plays the role of cytokinin and auxin that aid in promoting the growth of explants. According to Yong et al. (2009), CW contains a

wide range of biochemicals that may affect seed germination or seedling development, including amino acids, vitamins, sugar, minerals, phytohormones, and their natural inhibitors and regulators such as ethylene, ABA, phenols, and flavonols. Inorganic ions such phosphorus, magnesium, potassium, and sodium are also present in CW and are helpful for orchid seed germination (Hossain et al., 2013). Other research also reveal that the Vacint and Went media with 15% and 20% CW speed up the germination of orchid seed (Sumantra, Widnyana, 2011).

Conclusion

The result shows that the seed of *Paphiopedilum primulinum* grow best at CW concentration 30%. Only this concentration stimulates seed growth into the maximum stage (stage 5; 4.8%). The result of this study shows that the media sulemented with CW give better result than without CW in ½ MS media.

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Conflicts of Interest
The authors declare no conflict of interest.

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