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Ex-situ conservation of the native orchid *Coelogyne rochussenii* de Vriese from the Bukit Rimbang and Baling Wildlife Reserve Areas

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ABSTRACT. The native orchid *Coelogyne rochussenii* de Vriese is critically endangered, hence, conservative measures are needed to prevent extinction. Meanwhile, in-situ conservation is constrained by time, resources and costs. Therefore, this study aims to identify the most effective method for ex-situ conservation, especially with basal medium and activated charcoal, to determine the fastest germination. The completely randomized factorial design of four different basal media treatments including Knudson C, Vacin and Went, Murashige and Skoog, and Hyponex + vitamin medium were used. These treatments were combined with the application of activated charcoal at four different levels, with concentrations of 0, 1, 2, and 3 g/L^{-1}, meanwhile, the parameters observed include germination day and percentage, as well as contamination percentage. The results showed that the conservation of native orchid *C. rochussenii* de Vriese using various growing media and activated charcoal had a significant effect on the growth of the embryo culture. Based on the results, the Hyponex + vitamin medium with 3 g/L^{-1} accelerated germination days to 29.33, increased germination percentage to 92.06%, and reduced contamination to 0%.

Keywords: activated charcoal; basal medium; completely randomized factorial design; orchid conservation; tissues culture

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INTRODUCTION

The orchid *Coelogyne rochussenii* de Vriese is a widely distributed plant across Southern Thailand, Peninsular Malaysia, Singapore, Kalimantan, Sumatra, and Java Island, Sulawesi, Maluku, and Palawan in the Philippines (Lok *et al*., 2011).

Native orchids are threatened with extinction both locally and globally, this condition is triggered by the plundering of the plants for commercial purposes (Ticktin *et al*., 2020). The trade of protected species in 2015 reached 7000 cases in the world (UNODC, 2016), while illegal logging and forest land conversion from 2000 to 2010 was up to 6.6 Mha in Indonesia, particularly in Kalimantan, Sumatra, Papua, Sulawesi, and Maluku (Abood *et al*., 2015). In addition, native orchid extinctions are also caused by the dependence of the plant on mycorrhizal fungi and insect pollinators which are disturbed by the imbalance in the ecosystem (Kolanowska *et al*., 2020).

The habitat of this plant has also been eroded by the increasingly disrupted forest conditions. There is degradation of the buffer areas in the Bukit Rimbang and Baling Wildlife Reserve areas up to 82.25% (Suandy *et al*., 2014), therefore, conservative measures are needed to prevent orchids from extinction.

Conservation is a strategic measure to save the orchid *C. rochussenii* de Vriese from extinction locally. This is carried out by saving existing genetic resources, which are then reintroduced into the habitat (Crouzeilles *et al*., 2021). Furthermore, conservation is performed in two ways namely in-situ and ex-situ. In-situ conservation produces great results but it is time-consuming and resource-intensive, hence, the most effective method is ex-situ (Leclère *et al*., 2020).

Ex-situ conservation of orchids is carried out using tissue culture techniques (Lin *et al*., 2020). This technique was chosen because it has many advantages compared to conventional techniques (Agrawal *et al*., 2019; Ayuso *et al*., 2019; Muñoz *et al*., 2019; Streczynski *et al*., 2019) and produces plant seeds in large quantities within a short time (Heriansyah & Marlina, 2021). The in-vitro conservation of *C.*

rochussenii orchid is influenced by various factors, including the type of explants used in tissue culture techniques. Besides, the use of explants depends on the number of orchid populations in the habitat. Hence, there is a need to consider the type of explants used. Potential explants include the use of seeds (Utami & Hariyanto, 2019) as previously studied by Adhikari & Pant (2019), Dulić *et al*. (2019), Godo *et al*. (2020), and Manokari *et al.* (2021). However, this study differs from previous studies, we provided *C. rochussenii* orchid seeds taken directly from the wildlife reserve area of Bukit Rimbang and Baling wildlife reserve areas.

Embryo growth in orchid seed culture is strongly influenced by the type of medium used (Barrientos & Fang, 2019), each orchid has a different response to medium formulations. The Knudson C (KC) medium is more suitable for planting *C. ovalis* Lindl (Singh & Kumaria, 2020), while Murashige and Skoog (MS) medium is more suitable for *Dendrobium officinale* orchids (Gao *et al*., 2020), and *Gastrochilus matsuran* (Makino) Schltr (Kang *et al.*, 2020). Furthermore, the Vacin and Went medium is more suitable for planting *Phalaenopsis amboinensis* JJ Sm orchid (Utami & Hariyanto, 2019). Therefore, there is need to determine the most suitable media for *C. rochussenii* orchids seed culture.

The germination of seeds via in-vitro propagation is still low with a range of 30-45%, meanwhile, the germination percentage is influenced by the presence of light in the medium (Sorgato *et al*., 2020). Besides, the various types of orchids respond differently to the percentage of light in the media, hence, it is necessary to add a light-reducing ingredient in the form of activated charcoal. A previous study reported that the addition of activated charcoal produced a good effect on the germination percentage (Kim *et al*., 2019). Therefore, this study aims to determine the best basal medium and concentration of activated charcoal for conservation of native orchids *C. rochussenii* de Vriese from Bukit Rimbang and Baling Wildlife Reserves Areas.

MATERIALS AND METHODS

Plant collection. The adult orchid capsules (*Coelogyne rochussenii* de Vriese) were collected from Bukit Rimbang and Baling Wildlife Reserves Area at coordinate points 00°18'59.61"S 101°11'03.75"E, elevation 92 m above sea level. The capsules were dried at 280°C for 48 h to remove excess moisture, placed in a petri dish on a silica gel base and kept at 40°C in continuous darkness until use. The collection protocol was following our previous studies (Heriansyah & Marlina, 2021).

Capsule sterilization. After a storage period of 1 day, the capsule surface was sterilized with 7% (w/v) calcium hypochlorite containing 0.1% (v/v) Tween 20 (Acros Organics, Geel, Belgium) for 20 min. The seeds were further rinsed with distilled water three times, sterilized in a laminar flow hood with 70% (v/v) ethanol for 2 min, and then rinsed three times in sterile distilled water (Vasil, 1984; Bello *et al*., 2018).

The treatment of various basal media and the addition of activated charcoal. To evaluate the optimal medium composition for germination, four basal media were used including Knudson C (KC), Vacin and Went (VW), Murashige and Skoog (MS), and Hyponex + vitamin medium (Oktafiani *et al.*, 2011). This treatment was combined with the addition of activated charcoal at four different levels of 0, 1, 2, and 3 g/L^{-1}. All tested media contained 2% (w/v) sucrose, 7% (w/v) agar (Biolife Italiana), while the pH of all medium was adjusted to 5.80 ± 0.02 with 0.1 M HCl or 0.1 M NaOH. Furthermore, the medium was heated to 100°C and then placed into a culture glass with a thickness of 2 cm and then covered with aluminum foil and plastic. The media was then autoclaved at 121°C for 20 min, and stored in the culture room until the seeds were ready for planting.

Planting explants. The sterilized capsules in a laminar airflow cabinet were placed in a petri dish, split using a scalpel, sprinkled into a sterilized solid media and stored for one week. After sowing the seeds, the medium was then covered with aluminum foil and plastic, stored at 23 ± 20°C with a light photoperiod of 16 h, while 40 μmol $m^{-2}s^{-1}$ photosynthetically active radiation (PAR) was provided by a white fluorescent lamp (Philips, Saint-Priest, France) (McKendrick, 2000).

Observations. The embryo culture results were observed before subculturing, given that the parameters of leaves, stems, and roots are not observable after subculturing due to the small size of the embryo. Therefore, the following parameters were observed including germination day (day) and percentage (%), and contamination percentage (%). The embryos were also observed using a microscope.

Data analysis. Data processing and statistical analysis were performed on the data observed in this study. The data obtained were analyzed using ANOVA and the mean values were separated using Tukey's HSD test at 5% level. All data analysis were performed with SPSS version 26 (IBM Corp., Armonk, New York, USA).

RESULTS AND DISCUSSION

Germination day. The fastest germination day (29.33) was found in the Hyponex + vitamin medium combined with 3 g/L^{-1} activated charcoal media. Furthermore, the combination of activated charcoal with Knudson C (KC), Vacin & Went (VW), and Murashige & Skoog (MS) media, showed that the 3 g/L^{-1} activated charcoal concentration produced the fastest germination compared to the control (Fig. 1).

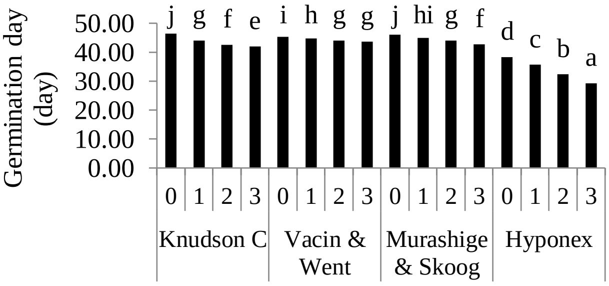

Fig. 1. The effect of various media and activated charcoal with concentrations of 0, 1, 2, and 3 g L–1 on germination day of *Coelogyne rochussenii* De Vriese. Different letters indicate significant differences according to Tukey's HSD (P<0.05) test.

Observations on the germination day of the *C. rochussenii* de Vriese orchid are presented in Fig. 1. Hyponex medium has a very simple compound similarly provided by the plant's natural habitat. Meanwhile, the most important factor needed for embryo development is the water content in the medium. Compared to Puspitaningtyas & Handini (2014), this study obtained different results, thus the growth regulators used in the initial medium were not added. Furthermore, the 3 g/L^{-1} activated charcoal concentration produced the fastest germination, because activated charcoal reduces light in the medium. This is in line with Kim *et al.* (2019) that light affects the speed of plant germination.

Percentage of germination. The highest germination percentage (92.06%) was found in the Hyponex + vitamin medium combined with 3 g/L^{-1} activated charcoal. Furthermore, the combination of activated charcoal with KC, VW, and MS medium showed higher yields. The 3 g/L-1 activated charcoal concentration produced the highest germination percentage (Fig. 2).

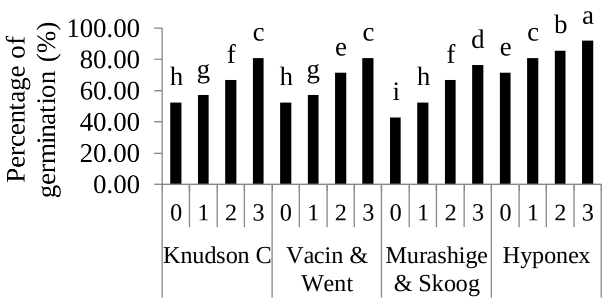

Fig. 2. Percentage yield of germination with various medium treatments and activated charcoal concentration. The same capital or non-capital letters in the same diagram show no significant difference according to the honest real difference test (Tukey test) at the level 5%.

The Hyponex medium treatment produced the best germination percentage because this medium effectively supports orchid embryo germination. In line with Ren (2020) which reported that Hyponex medium containing simple nutrients in the form of N, P, and K supports *Paphiopedilum wardii* growth. Furthermore, the 3 g/L^{-1} activated charcoal concentration produced the highest germination percentage due to the limited light provided to the medium. According to Sorgato *et al*. (2020), germination was accelerated under light limitation on the medium. The development stages of *C. rochussenii* de Vriese with four basal media are visualized in Fig. 3.

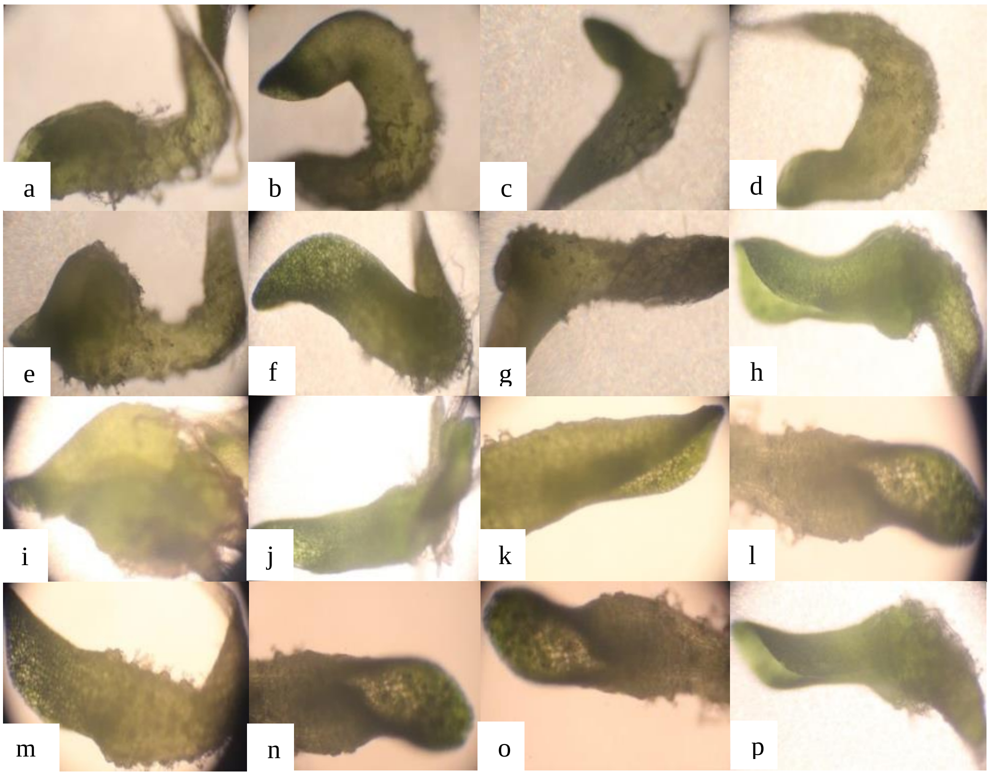

Fig. 3. Development stages of *Coelogyne rochussenii* de Vriese with various basal media: a-d. Knudson C; e-h. Vacin and Went; i-l. Murashige and Skoog; m-p. Hyponex + vitamin. Each treatment was combined with the addition of activated charcoal sequentially (0, 1, 2, and 3 g/L^{-1}).

Percentage of contamination. The lowest contamination percentage (0%) was found in the Hyponex + vitamin medium combined with 3 g/L^{-1} activated charcoal. Furthermore, the combination of activated charcoal with KC, VW, and MS media showed that the 3 g/L^{-1} activated charcoal concentration produced the lowest contamination percentage (Fig. 4).

The Hyponex medium treatment produced the lowest percentage of contamination. The addition of Hyponex required a short time, hence, suppressing the opportunity for contaminants to enter the culture medium. Meanwhile, a longer time is needed in other media to weigh and insert the stock solution sequentially, thereby providing opportunities for the entry of contaminants. The technique for making a medium affects the level of contamination of explants, the longer the medium container is open, creating air currents, the greater the possibility of contamination (Sanders, 2012; Prihastanti *et al.*, 2020). Furthermore, treatment with activated charcoal produced a significant effect on the percentage of contamination, with the lowest percentage found at a concentration of 3 g/L^{-1}. Activated charcoal is an absorbent, plays a role in absorbing contaminants. The activated charcoal in the media acts as an absorbent, absorbs contaminants as well as exudates released by explants, and improve growth and development (Prizão *et al.*, 2012; Warakagoda & Subasinghe, 2013).

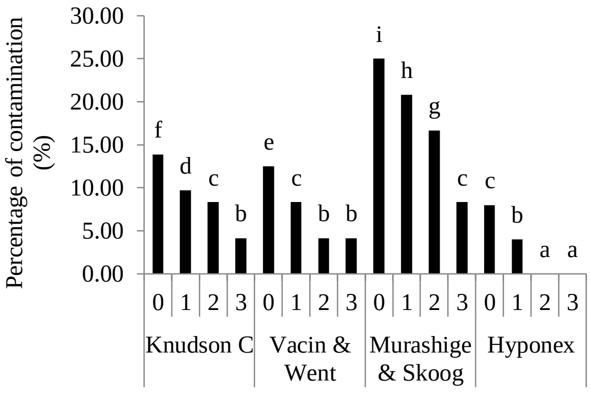

Fig. 4. Average percentage of contamination with various medium treatments and activated charcoal concentration. The same capital or non-capital letters in the same diagram show no significant difference according to the honest real difference test (Tukey test) at the level = 5%.

Based on QS. Al-Mulk verse 3 (Kementerian Agama RI, 2019a), Allah swt created the earth and everything in it in a balanced state. The balance shows that the components of the ecosystem are perfectly regulated by Allah swt. However, damages to forests which lead to the potential extinction of organisms is caused by human activities. An example of plant which is critically endangered is orchid due to logging and looting activities carried out by humans, according to Allah QS Ar-Rum verse 41 (Kementerian Agama RI, 2019b) which says, it is human hands that cause destruction on Earth.

Humans appointed by Allah swt as *Khalifah* (leader) on earth need to pay attention to Allah's prohibition in QS Al-Qashash verse 77 (Kementerian Agama RI, 2019c) which forbids humans from causing damage to the earth, including carrying out logging and looting activities which leads to the potential extinction of orchids in the forest. Furthermore, humans as *Khalifah* have an obligation to maintain and restore the balance of nature, in other words, humans are obliged to carry out conservation measures to prevent the extinction of organisms on earth.

CONCLUSION

The conservation of the native orchid *Coelogyne rochussenii* de Vriese using various growing media and activated charcoal had a significant effect on the growth of the embryo culture. The Hyponex + vitamin medium with 3 g/L^{-1} accelerated germination days to 29.33, increased germination percentage to 92.06%, and reduced contamination to 0%.

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