



The Alternative Media Supporting the Protocorm and Plantlet Growth of the Indonesian Black Orchid (*Coelogyne pandurata* Lindl.) Grown *In Vitro*

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Abstract

Due to the high cost of the most often used basic media in tissue culture, it is vital to identify more affordable alternatives. This research aimed to determine the best alternative culture media for the protocorm and plantlet growth of *Coelogyne pandurata* Lindl. It employed a completely randomized design, eight treatments and four replications. The treatments referred to the different types media and consisted of M1 = New Phalaenopsis (NP) medium, M2 = a medium made from foliar fertilizer (FFM), M3 = NP + 2 cc L⁻¹ AB mix solution (a media for hydroponics), M4 = FFM + 2 cc L⁻¹ AB mix solution, M5 = NP + 50 cc L⁻¹ of tomato extract, M6 = NP + 50 cc L⁻¹ of bean sprout extract, M7 = FFM + 50 cc L⁻¹ of tomato extract and M8 = FFM + 50 cc L⁻¹ of bean sprout extract. The M4 medium exhibited the best results in terms of average leaves count (4.80), average shoot length (2.68 cm), average root length (4.35 cm), the average fresh weight per plantlet (214.5 mg) and dry weight of plantlets (73.1 mg). The average number of roots per plantlet was 4.25, acquired using the less expensive M8 treatment, which also produces a negligible number of leaves (4.50). In conclusion, the M4 medium is the most appropriate medium for growing protocorm and plantlet of *C. pandurata*. The experiment also found that the FFM basic medium combined with 50 cc L⁻¹ of bean sprout extract can be used as another cheaper alternative for growing protocorms of *C. pandurata*.

Keywords: foliar fertilizer media; *in vitro* propagation; medium selection; orchid culture

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INTRODUCTION

Coelogyne pandurata Lindl. or 'Anggrek Hitam' is an endangered wild orchid native to Kalimantan Island, Indonesia (Semiarti et al., 2010; Puspitaningtyas, 2020). The black orchid is also widespread on other Indonesian islands, including Sulawesi (Semiarti et al., 2011), which used in this research (Figure 1). The black orchid of *C. pandurata* experience sympodial growth and the pseudobulbs have two leaves each. Flowers are arranged in inflorescence,

fragrant, with green perianth and black labellum. The species are rare due to deforestation caused by natural phenomena or human activity. However, Wahyudiningsih et al. (2018) stated that *C. pandurata* is an Indonesian orchid that the government protects according to "PP RI No 7 Tahun 1999". Besides that, Arditti (1992) mentioned that propagation through a conventional method is one of the difficulties in cultivating this orchid. Therefore, it is vital to obtain an alternative method to propagate this species.

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Seeds can propagate orchids originated from artificial pollination, i.e., selfing. The perianth withers and collapses three to seven days after pollination. The ovary expands and develops a fruit known as a pod or capsule, containing an enormous number of tiny orchid seeds (Dwiyani, 2014). Orchid seeds have little or no endosperm (Arditti, 1992; Yeung, 2017), therefore, the seed germination is relatively low, less than 5% (Zeng et al., 2015; Vudala and Ribas, 2017). Under natural conditions, germination of mature orchid seeds and their early development depend on compatible mycorrhizal fungi (Long et al., 2010; Zhang et al., 2018; Sousa et al., 2019). As a result, Sousa et al. (2019) grew seeds of *Cyrtopodium saintlegerianum* orchid with several fungal isolates. The fungi Rhizoctonia complex can be in symbiosis with *C. saintlegerianum* seeds *in vitro* to assist in seed germination and protocorm growth. However, orchid seed germination and protocorm growth can also be carried out asymbiotically on culture media containing macro and micro-nutrients, vitamins and sucrose through an *in vitro* method in a laboratory.

There are several basic media used for *in vitro* culture and the most commonly used for the orchid seed culture are Murashige Skoog/MS (Murashige and Skoog, 1962), New Phalaenopsis/NP (Islam et al., 1998), Vacin and Went/VW (Vacin and Went, 1949), etc. The composition of these culture media contains macro-nutrients, micro-nutrients and vitamins. Sucrose and growth regulators for plants are added during the production process of the media. These media are available in markets, but their prices are relatively high (Chen et al., 2019). Therefore, this research tried alternative media to substitute.

Orchid seed culture is commonly supplemented with organic materials, such as tomato extract, coconut water, bean sprout extract, potato extract, banana homogenate, etc. The addition of organic supplements is an excellent technique for the mass propagation and efficient acclimatization of orchids for reintroduction to natural habitats, facilitating the conservation and sustainable utilization of endangered orchid species (Utami and Hariyanto, 2020). Furthermore, these organic materials have many roles in orchid seed germination and protocorm development due to their content. Protocorm is a structure resulting from germinated orchid seed. For example, tomato juice, containing carbohydrates, vitamins,

minerals and antioxidants, promotes germination and protocorm growth. In contrast, banana homogenate, containing carbohydrates, minerals, vitamins, natural growth regulator improves germination and increases plantlet formation (Utami and Hariyanto, 2020).

Dwiyani et al. (2015) reported that adding 150 to 200 g L⁻¹ of tomato extract into the NP medium with or without coconut water provides the best growth for seeds of *Vanda tricolor* orchid from Bali. Dwiyani (2013) also reported that protocorm of *V. tricolor* of 7 month-aged pods grown on MS medium required 150 g L⁻¹ tomato extract for the best growth. Utami and Hariyanto (2019) stated that the most vigor plantlets *Phalaenopsis amboinensis* were obtained from seeds on the VW medium with 15% (v/v) of coconut water and 10 g L⁻¹ of banana juice. Furthermore, Untari and Puspitaningtyas (2006) reported that adding 150 g of sweet potato on a VW medium generated the most significant growth of *C. pandurata* seeds. Mustika and Semiarti (2021) found that the addition of 100 g L⁻¹ bananas as organic substances in an *in vitro* medium culture increases the growth rate of *Dendrobium lineale* orchids. Meanwhile, Huh et al. (2016) found that 100 mL of coconut water added to 1 L of MS media had the highest germination rate and the formation of the protocorm of the orchid *Cypripedium macranthos* Sw.

The current research also tested the addition of natural organic substances such as tomato and bean sprout extract as alternative media tissue culture to promote sustainable production of orchid protocorms and plantlets (Evangelista et al., 2021). Besides being cheaper, the use of organic materials to promote plant tissue growth in the *in vitro* culture is much more environmentally friendly than growth regulators.

This also utilized NP medium, an alternative basic media manufactured from inexpensive compounds, to grow orchids in a tissue culture laboratory. The medium with foliar fertilizers (named FFM) and the relatively cheap hydroponic medium have not yet been reported for orchid seed germination. However, many orchid enthusiasts in Bali (Indonesia) are interested in such media because it is cheap, but no result was reported. Therefore, this research investigated the impact of such cheap additives on the *C. pandurata* seed germination *in vitro*. Several alternative culture media, including commercial medium as control, were

assessed to develop the most suitable protocol for obtaining *C. pandurata* plantlets *in vitro*. This also aimed to determine the best alternative culture media for the protocorm and plantlet growth of *C. pandurata* Lindl.

MATERIALS AND METHOD

This research was conducted from January to June 2020 in the Laboratory of Plant Tissue Culture, the Faculty of Agriculture, Universitas Udayana, Bali Province of Indonesia. The orchid of *C. pandurata* plant was obtained from Sulawesi and already cultivated in Denpasar for a year (Figure 1).



Figure 1. *C. pandurata* orchid (Personal documentation); red bar = 1 cm

The flower of *C. pandurata* orchid was self-pollinated and then the ovary swelled three days after pollination to form a pod. The pod was harvested five months after pollinating and sterilized in the following steps. First, it was washed clean by brushing with detergent and running water, then rinsed and air-dried. After that, the pod was dipped in rubbing alcohol and exposed to the fire. This step was repeated up to three times and the capsule was then placed on a sterile petri dish in a laminar flow cabinet. In the laminar, the capsule was dissected and seeds were then taken out with a spatula. About half of the spatula (250 mg) of seeds were sown at the prepared treatment media in each bottle. The culture was performed at a room temperature of 20°C and 85% relative humidity (RH). Four weeks after seed sowing, protocorms were sub-cultured to the same fresh

media. Some variables were observed to occur after 16 weeks of the first sub-culture or 20 weeks after seed sowing.

The experiment was conducted using a completely randomized design, eight treatments and eight replications, where 64 experimental units were represented by 1 culture bottle and each bottle consisted of eight plantlets. The treatments referred to the types of culture media (M) and consisted of M1 = NP medium, M2 = FFM, M3 = NP + 2 cc L⁻¹ AB mix solution (NP + AB), M4 = FFM + 2 cc L⁻¹ AB mix solution (FFM + AB), M5 = NP + 50 cc L⁻¹ of tomato extract (NP + TE), M6 = NP + 50 cc L⁻¹ of bean sprout extract (NP + SE), M7 = FFM + 50 cc L⁻¹ of tomato extract (FFM + TE) and M8 = FFM + 50 cc L⁻¹ of bean sprout extract (FFM + SE). The basic FFM media was produced with the following formulation: 2 g L⁻¹ of foliar fertilizer + 1 cc L⁻¹ fish emulsion + 2 cc L⁻¹ of Atonik + 2 cc L⁻¹ of vitamin B1. Furthermore, the AB mix solution is a nutrient commonly used for hydroponic cultivation. A set of AB mix solutions used consisted of fertilizer A and B that contained 9.90% of NO₃, 0.48% of NH₄, 4.83% of P₂O₅, 16.50% of K₂O, 2.83% of MgO, 11.48% of CaO, 3.81% of SO₃, 0.013% of B, 0.025% of Mn, 0.015% of Zn, 0.002% of Cu, 0.003% of Mo and 0.037% of Fe. Fish emulsion is an organic garden fertilizer made from whole or part of fish. It provides an NPK ratio of 4-1-1 and is most often used as a foliar feed to provide a quick nitrogen boost. Meanwhile, atonik is a synthetic biostimulant manufactured in Japan and contains water as well as three phenolic compounds: sodium para-nitrophenolate (PNP) for 0.3%, sodium ortho-nitrophenolate (ONP) for 0.2% and sodium 5-nitroguaiacolate (5NG) for 0.1% (Przybysz et al., 2014). All products included in FFM media and AB mix solutions are commercially available and easily obtained at a low price. The media was solidified with 7 g L⁻¹ of bio agar and 2 g L⁻¹ of active charcoal. Then, 20 g L⁻¹ of sucrose was added by adhering to the amount of sucrose added per liter on NP media (Islam et al., 1998).

The observation was conducted destructively by taking out the plantlets from the bottle at 20 weeks after seed sowing. Variables observed were the number of leaves, shoot length, number of roots, root length, fresh and dry weight of plantlet. In addition, it was performed on each plantlet grown in a bottle

and then averaged. The number of leaves was calculated by counting the total leaves present in each plantlet. Shoot length was measured from the base to the growth point (base of the youngest leaf). Also, the root number was calculated by counting all roots that emerged from each plantlet. The fresh weight represents that of the whole plantlet, and to obtain the dry weight, plantlets were then oven-dried (80°C) for three days. The data were then analyzed using Analysis of Variance

(ANOVA). Finally, the average treatment differences were analyzed by the Least Significant Difference (LSD) of 5%.

RESULTS AND DISCUSSION

Table 1 represents the influence of the treatments on the variables of the number of leaves, shoot length, the number of roots, root length, fresh weight and dry weight of plantlets at 20 weeks after seed sowing.

Table 1. The effects of the treatments on the number of leaves, shoot length, the number of roots, root length, fresh weight and dry weight of plantlet at 20 weeks after seed sowing

Treatments	Number of leaves	Shoot length (cm)	Number of roots	Root length (cm)	Fresh weight plantlet ⁻¹ (mg)	Dry weight plantlet ⁻¹ (mg)
M1 (NP)	4.30 ^{ab}	1.79 ^{bc}	4.05 ^a	1.54 ^d	127.0 ^b	40.9 ^b
M2 (FFM)	4.30 ^{ab}	2.11 ^b	2.95 ^b	1.08 ^d	25.5 ^c	14.1 ^{bc}
M3 (NP + AB)	3.55 ^b	1.73 ^{bc}	3.85 ^{ab}	3.04 ^b	103.0 ^b	19.7 ^{bc}
M4 (FFM + AB)	4.80 ^a	2.68 ^a	4.05 ^a	4.35 ^a	214.5 ^a	73.1 ^a
M5 (NP + TE)	4.10 ^b	1.29 ^c	3.75 ^{ab}	1.44 ^d	21.5 ^c	10.7 ^{bc}
M6 (NP + SE)	3.50 ^b	0.91 ^c	3.50 ^{ab}	2.26 ^c	17.0 ^c	8.5 ^c
M7 (FFM + TE)	3.55 ^b	1.43 ^c	2.70 ^b	1.45 ^d	12.0 ^c	10.0 ^{bc}
M8 (FFM + SE)	4.50 ^{ab}	1.99 ^b	4.25 ^a	2.05 ^{cd}	93.5 ^b	23.8 ^{bc}

Notes: The same letter behind mean values at the same column indicates no significant differences among the mean treatment based on the Least Significant Difference (LSD) at 5% level of probability and vice versa for different letters. The numbers written in bold show the best result

Table 1 shows that the highest number of leaves was obtained from M4 treatment (4.80), followed by M8 (4.50), M1 and M2 (4.30 each). The most extended shoot was reached with M4 treatment and was significantly different from other treatments. Therefore, M4 was the most appropriate media for the growth of the upper part of the plantlet.

The highest number of the root was achieved by M8 treatment (4.25), however, it was not significantly different with M1 and M4 treatment (4.05 each). Even though the M4 media resulted in the highest value for root length, it was significantly different from other treatments. In conclusion, the most appropriate media for supporting the root growth of *C. pandurata* plantlet was M4 media.

The success of M4 media in supporting the growth for the upper organ of plantlet and roots also resulted in the highest value for the fresh and dry weight of the plantlet (Table 1). Therefore, the modified culture medium where fertilizer (FFM) was added with hydroponic media is the most appropriate medium among media treated.

Figure 2 shows the phenotypes of plantlets obtained 20 weeks after seed sowing. The effect of the treated medium clearly showed that M4 and M8 support better phenotypes for *C. pandurata* plantlets than others. Plantlets were visually more vigor and sturdy with quicker growth than other treatments. This is in line with the data from Table 1, which shows that the M4 and M8 treatments had more leaves, longer roots and more numbers of roots than other treatments. This more vigor condition will support plantlet growth when acclimatized, which is the final propagation stage through *in vitro* culture. Finally, plantlets are removed from the *in vitro* environment to adapt to the *ex vitro* environment. The environmental condition for *ex vitro* growth was very different from *in vitro* cultivation (Cha-um et al., 2009). It was a crucial phase because many plantlets did not survive (Ehirim et al., 2014). Therefore, more vigor plantlets were required from M4 or M8 treatments.

The results showed that good root growth supports upper part organ development (Table 1). This suggests that in plant propagation through tissue culture, promoting

root growth is important because those with good growth absorb nutrients from the culture media to stimulate the development of leaves, shoots and overall plantlet. Root absorbs nutrients from the culture media to grow upper plant organs (plantlets). Elongation and branching determine the development of roots (Nibau et al., 2008; Atkinson et al., 2014). With the *ex vitro* research, Gruber et al. (2013) described that these processes result in differences

in root morphologies, including the length, number and diameter of roots in root systems. This current research revealed that the variety of compounds contained in each media treatment led to variations in root formation, therefore, the length and number of roots varied. The data showed that the plantlets on M4 medium had the longest roots and a reasonably high number of leaves, shoot length, fresh weight and dry weight.

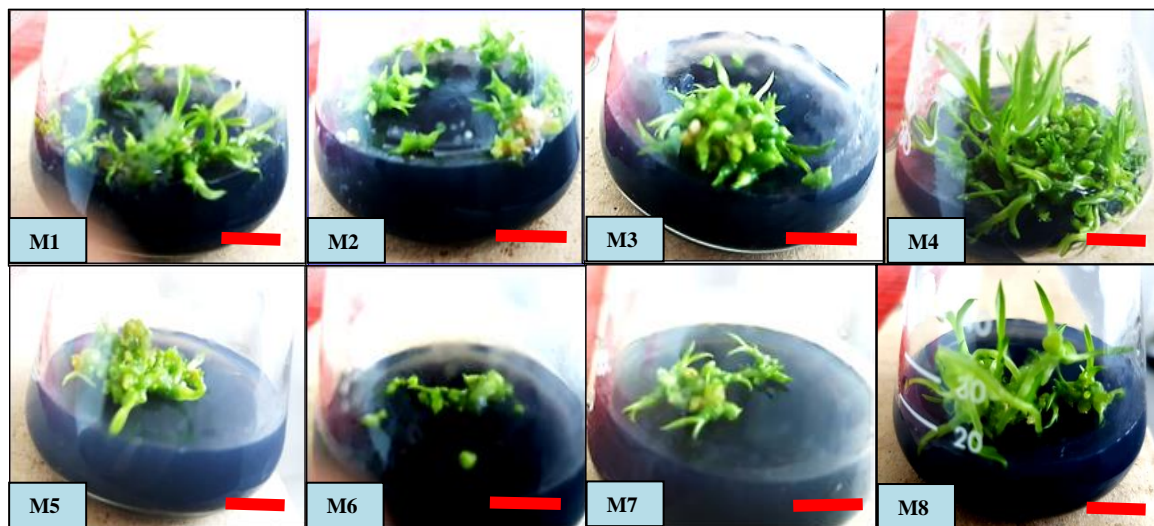


Figure 2. The plantlet phenotypes of *C. pandurata* orchid on different types of culture media. Description: the diameter of culture bottle is 4.5 cm; M1 = NP medium (NP); M2 = FFM; M3 = NP + 2 cc L⁻¹ AB mix solution; M4 = FFM + 2 cc L⁻¹ AB mix solution; M5 = NP + 50 cc L⁻¹ of tomato extract; M6 = NP + 50 cc L⁻¹ of bean sprout extract; M7 = FFM + 50 cc L⁻¹ of tomato extract; and M8 = FFM + 50 cc L⁻¹ of bean sprout extract; red bar = 1 cm

Table 1 shows that three treatment media provided relatively good growth and applied to protocorm growth of *C. pandurata* orchid. The best treatments are M4 (FFM + AB), M1 (NP) and M8 (FFM + SE). As seen in Figure 1, phenotypes of these media are better than other treatments. In addition, they provided relatively the best results of the fresh and dry weights. NP media was created to grow the orchids from the genus *Phalaenopsis* (Islam et al., 1998). However, it was reported that the media is good to cultivate the genus of the *Dendrobium* orchid (Akter et al., 2007). Semiarti et al. (2010) also found that after 12 weeks of seed sowing, NP medium was the best medium for shoot induction to obtain plantlets of *C. pandurata* with three leaves. This current research proved that the NP medium is suitable for the protocorm and plantlet growth of *C. pandurata* orchid as experimented

with several alternative media. First, the foliar fertilizer media added with hydroponic media (FFM + AB) or M4 showed better growth and significant difference in shoot length, root length, fresh and dry weight of plantlets than the relatively expensive NP media. Second, the foliar fertilizer media added with the bean sprout extract (FFM + SE) was applicable to grow the protocorm of *C. pandurata* orchid.

M4 and M8 medium consisted of the basic media with foliar fertilizer (FFM), including 2 g L⁻¹ of foliar fertilizer + 1 cc L⁻¹ of Atonik (a brand) + 2 cc L⁻¹ of fish emulsion + 2 cc L⁻¹ of vitamin B1. The basic media for FFM consisted of several cheap components available in markets. Moreover, with the FFM media, protocorms of *Coelogyne asperata* orchid and *V. tricolor* orchid were successfully grown in the laboratory of Plant Tissue Culture of the Faculty of Agriculture

Universitas Udayana (data not published). The basic media for foliar fertilizer (FFM) contain similar macro nutrients, micro-nutrients and vitamins to basic standard commercial media such as NP, MS and VW media in the organic form of diverse compounds. However, FFM used Atonik as one of the compounds in its composition. Therefore, the phenomenon is different because Atonik is not found in commercial culture media. It is a synthetic biostimulant manufactured in Japan that contains substances of three phenolic compounds: sodium para-nitrophenolate (PNP) for 0.3%, sodium ortho-nitrophenolate (ONP) for 0.2% and sodium 5-nitroguaiacolate (5NG) for 0.1%. These substances are not applied in the basic culture media commonly used in the laboratory of plant tissue cultures such as NP and MS.

The biostimulant is a product consisting of diverse formulations that positively affect the vital processes in the plant body (Calvo et al., 2014). The effects are more visible when plants face stress because they can increase their tolerance to unfavorable conditions (Przybysz et al., 2014). Research investigating the use of Atonik on several plant species have been successfully reported. For example, Aksona and Ůnay (2019) reported that Atonik combined with amino acids and administered through leaves increases crop yields and fiber quality (*Gossypium hirsutum* L.). A similar result was obtained when Covařa et al. (2019) treated Atonik on the legume plant. Furthermore, the treatment on *Arabidopsis thaliana* plants positively influenced the growth (Przybysz et al., 2014), even though the use of Atonik in *in vitro* culture has not been reported. The use of FFM alone (M2) or together with tomato extract (M7) provided fewer good results. This statement indicates that the best growth of plantlets produced by the M4 treatment was caused by Atonik and a favorable combination of the compounds contained in the media. FFM composed of 2 g L⁻¹ of foliar fertilizer + 1 cc L⁻¹ of Atonik (brand) + 2 cc L⁻¹ of fish emulsion + 2 cc L⁻¹ of vitamin B1, in combination with 2 cc L⁻¹ of AB solution showed the best results. This finding is signified by the highest result of the fresh and dry weight (Table 1) and the best appearance of the phenotype of plantlets (Figure 2).

The compounds existing in the FFM can interact synergically with hydroponic media (AB mix solution). The existing compounds

provided the best results for the protocorm growth of *C. pandurata* orchid and promoted the best plantlets growth. Furthermore, FFM can be combined with bean sprout extract as an inexpensive alternative medium to grow protocorms of *C. pandurata*. It was emphasized that M8 is a cheaper alternative media than M4 because the price of bean sprouts is lower than AB mix solution. It yielded no significant differences in the number of leaves and roots compared to M4 media using AB mix solution. The positive effect of adding organic matter for orchid seed germination and its continued growth has also been reported by Parthibhan et al. (2015) on *Dendrobium aqueum* Lindley orchids. The highest number of shoots was produced in banana pulp with a concentration of 3% or coconut water at 3%. Then the number decreased when the banana pulp or coconut water was increased. In this research, the addition of 50 cc L⁻¹ (5%) of bean sprout extract positively affected the growth of the protocorm and plantlets of *C. pandurata*.

However, this research proved that the addition of tomato extract had an inhibitory effect on the growth of the protocorm and plantlets of *C. pandurata* when the tomato extract was added to FFM or NP medium. The number of leaves, shoot length, roots, fresh weight and dry weight of plantlets decreased when tomato extract was added to the media (FFM + TE or NP + TE) compared to the treatment of FFM or NP alone. The effect of tomatoes as organic matter added *in vitro* to the culture media is closely related to the variety used. According to Mladenovic et al. (2014), the biologically active (bioactive) compounds in tomato (*Lycopersicon esculentum* Mill.) differ depending on the variety. The inhibitory effect of the addition of organic material on seed germination and protocorm growth of orchids was also reported by Calevo et al. (2020), where almond milk inhibited seed germination and growth protocorm of *Cymbidium tracyanum*.

Astarini et al. (2015) reported that the best media for growing protocorms of *C. pandurata in vitro* was the commercial media of W3, while Kartiman et al. (2018) used commercial media of MS (half strength of MS) and plant growth regulator (0.2 mg L⁻¹ BAP) to obtain the highest number of shoots in *in vitro* propagation of *C. pandurata* through seeds. The current finding stresses the exclusion of costly commercial

media for plant tissue culture or synthetic plant growth regulators. It proved that protocorms of *C. pandurata* can grow well in cheaper media, as described above.

CONCLUSIONS

In conclusion, the best alternative media for growing protocorm and plantlets of *C. pandurata* orchid is medium using foliar fertilizer, known as FFM basic media. It was formed using 2 g L⁻¹ of foliar fertilizer + 1 cc L⁻¹ of Atonik + 2 cc L⁻¹ of fish emulsion + 2 cc L⁻¹ of vitamin B1 combined with 2 cc L⁻¹ of AB solution, media for hydroponic cultivation. Furthermore, it provided better growth of protocorms of *C. pandurata* orchid compared to those of NP as a commercial tissue culture media. The experiment also found that the FFM basic medium combined with 50 cc L⁻¹ of bean sprout extract can be used as another cheaper alternative for growing protocorms of *C. pandurata*.

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