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In Vitro Germination and Flowering of *Dendrobium capra* J.J. Smith, An Endemic Orchid of Java

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ABSTRACT

***Dendrobium capra* is an Indonesian endemic orchid species that live in Java. It grows on low altitude with warm climate. *D. capra* has beautiful small yellow greenish flower that grow in raceme inflorescence. This orchid faces a threat in its natural habitat due to having a long life cycle and a forestry main commodity as a main host thus categorized as Appendix II on CITES list. To address that problem, ex situ conservation approach using *in vitro* culture method is necessary. Germination enhancement effort using complex organic substances found that 200 ml/l tomato extract gave best germination result. Analysis on *D. capra* plantlet growth also showed that MS medium produced better plantlet size than NP, VW and KC medium. Supplementing medium with a combination of NAA and TDZ has also successfully induced early flowering within 11 month of culture period. This information is important to achieve successful *in vitro* culture of *D. capra* for various purposes.**

1. Introduction

Indonesia is known as one of world's biodiversity hotspots and home for around 5,000 orchid species. Among those species, some are known as endemic due to various barrier of limiting their distribution. *Dendrobium capra* is one of Indonesia endemic orchid species that found in Java, especially in Central to East Java area. This species inhabits low elevated area and grew on the branches of teak tree as its main host (Yulia and Ruseani 2008). Having teak tree as its main host, *D. capra* faced huge threat on its existence in nature due to its host being one of main commodity in Indonesian forestry industry. According to Comber (1990), *D. capra* has epiphyte form with slender pseudobulbs. Its leaves and inflorescence grow from lateral meristem near apical area of the pseudobulb. The inflorescence grows in straight raceme with small yellow greenish flower around 3 cm in diameter. The labellum has similar color with 3 lobes and brown striated lamella (Figure 1). This orchid has been categorized in Appendix II based on CITES list from UNEP-WCMC (Anonymous 2014).

In vitro culture is a powerful method to germinate endosperm-less seed of orchids. It utilizes culture medium that will provide nutrients for seed germination and development. There are various culture medium that are often used for orchid culture such as MS, NP, VW, and KC medium (Knudson 1946; Vacin and Went 1949; Murashige and Skoog 1962; Ichihashi 1992). Different orchid species may have different medium preference therefore testing different medium on desired species is necessary to obtain best result. The medium performance could be improved through addition of complex organic substance such as coconut water or tomato extract. Using said substances have successfully enhanced various orchid species growth (Zhang *et al.* 2015; Dwiyani *et al.* 2015; Setiari *et al.* 2016; Utami *et al.* 2017; Utami and Hariyanto 2019).

Another approach to obtain desired growth and development is by utilizing plant growth regulator (PGR). PGR presence at precise concentration in the medium could help improve explant growth or even induce early flowering. Known PGR that could help induce early flowering in orchid are from auxin, cytokinin and gibberellin group (Goh 1979; Ferreira *et al.* 2006; Wang *et al.* 2009; da Silva *et al.* 2014; Novak *et al.* 2014). Precise combination of those PGR has helped various orchids to reach early flowering. Until recently, there are only a few publications have been made about *D. capra*. Those publications gave us information about their root and leaf anatomy, habitat and carbohydrate influence on *in vitro* culture (Yulia and Ruseani 2008;

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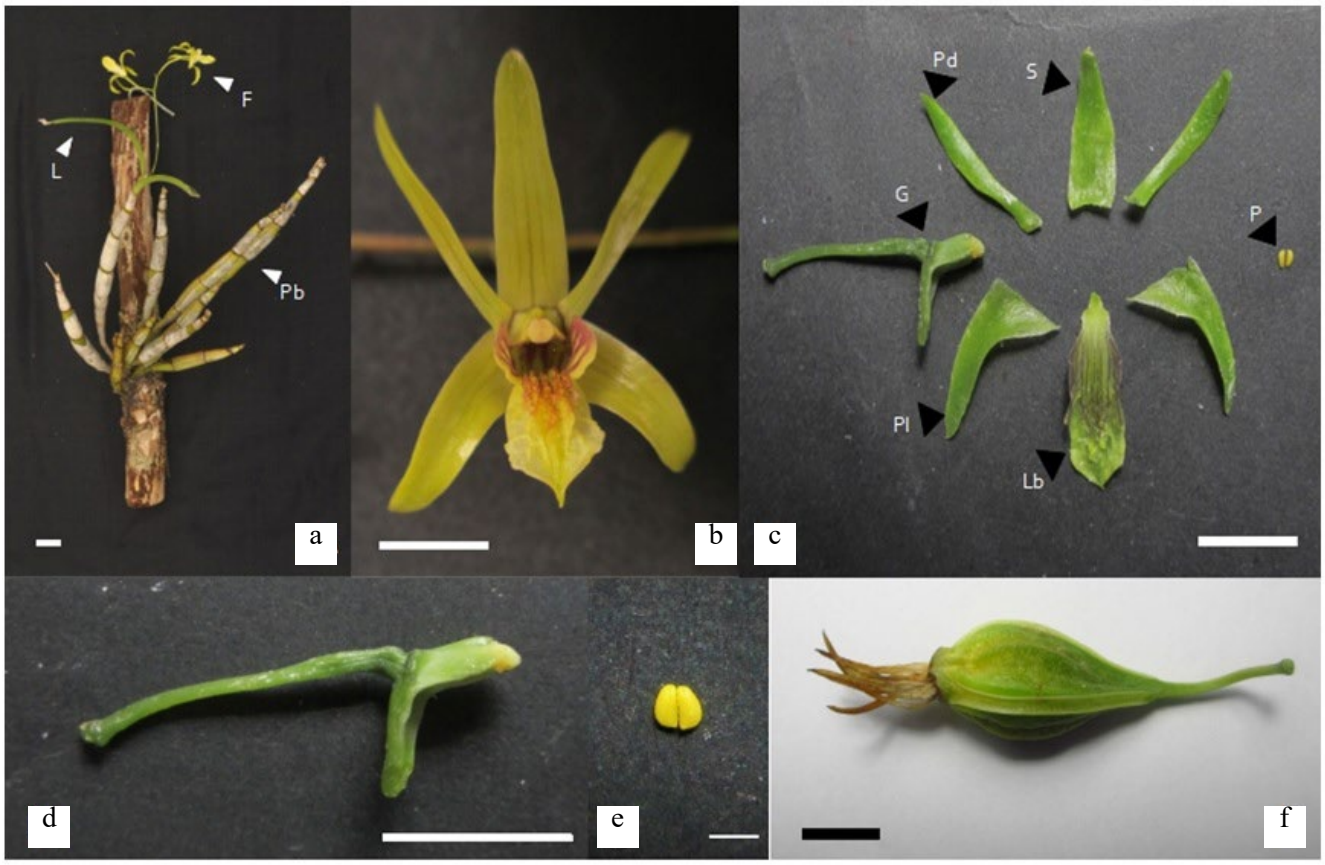


Figure 1. Morphology of *D. capra*. (a) Habitus of adult *D. capra*, (b) one-week old fully bloomed flower of *D. capra* ready for pollination, (c) flower organs of *D. capra*, (d) gynostemium of *D. capra*, (e) pollinia of *D. capra* have no viscidium disc, (f) ten-weeks old ripened capsule of *D. capra* (L: leaf, F: flower, Pb: pseudobulb, S: sepals, P: pollinia, Pd: dorsal petals, Pl: lateral petals, Lb: labellum, G: gynostemium; bars A-D, F: 1 cm; E: 1 mm)

Fadhilah *et al.* 2012; Metusala *et al.* 2017). Therefore, there are many things that has yet to be discovered.

2. Materials and Methods

2.1. Plant Material

Flowering *D. capra* plants from "Puri Anggrek" nursery are used to obtain fruits. The flowers were self-pollinated and kept in greenhouse for 9-10 weeks until the capsule ripened. Ripened capsule is marked with dried floral accessory and faint yellowish color on its skin. Ripened capsule was used as seed source for *in vitro* culture.

2.2. Culture Medium Preparation

New Phalaenopsis (NP) medium was used for germination of *D. capra* seeds. The medium was supplemented with 3 gr/l gellan gum (PhytoTech, US) and pH value of the medium was set at 5.6-5.8. The medium also supplemented with either coconut water or tomato extract (Table 1).

Development of *D. capra* plantlets were carried out by utilizing four different culture media to find out the most suitable culture medium for plantlet growth. The culture media used were MS, VW, NP, and KC medium. The medium was supplemented with 3 gr/l gellan gum and pH value were set as the same as germination medium. All culture mediums were autoclaved at 121°C, 15 psi for 15 minutes.

Table 1. Concentration of coconut water and tomato extract used in this study. Each of them were supplemented separately

Complex organic substance	Concentration
Coconut water (ml/l)	100
	200
	300
	400
Tomato extract (ml/l)	100
	200
	300
	400

Flowering induction of *D. capra* plantlets were done *in vitro*. Plantlets were planted on modified MS medium supplemented with various plant growth regulators in different concentration (Table 2) based from

Table 2. PGR combination for flowering induction of *D. capra*

Treatment	PGR	Concentration (mg/l)				
1	GA_3	0.10	0.50	1.00	2.00	3.00
	NAA	0.50	0.50	0.50	0.50	0.50
2	GA_3	0,10	0,50	1.00	2.00	3.00
	TDZ	0.05	0.05	0.05	0.05	0.05
3	GA_3	0.10	0.50	1.00	2.00	3.00
	NAA	0.50	0.50	0.50	0.50	0.50
	TDZ	0.05	0.05	0.05	0.05	0.05
4	NAA	0.05	0.10	0.50	1.00	2.00
	TDZ	2.00	1.00	0.50	0.10	0.05

Cen *et al.* (2010). The plantlets were incubated at 25±1°C under constant white light illumination.

2.3. *In Vitro* Culture of *D. capra*

D.capra capsule was sterilized using physical sterilization method. The capsule was washed by tap water and detergent then dried using paper towel. Clean capsule was brought to clean bench and sterilized using 96% ethyl alcohol solution and flame. The sterilization procedure was repeated 3 times. Sterile capsule was opened and the seeds were sowed on the medium surface. After germination process, *D. capra* seedlings were sub-cultured on four different media (MS, VW, NP, and KC). For flower induction, plantlets were sub cultured on modified MS medium supplemented with various PGR (Table 2). All cultures were incubated in a room under constant white light illumination at 25±1°C.

2.4. Germination and Growth Observation

Seeds germination and growth were observed every week for 5 weeks using stereo microscope (Eschenbach) equipped with OptiLab microscope camera (Miconos). Some seeds were also taken to make anatomy slides. The slides were observed using light microscope (Olympus) at 40x magnification. *D. capra* plantlets growth were observed for 8 weeks. Their height, leaves, roots, and bulbs were measured every week. Flowering induced plantlets were observed for 11 months to flowering.

3. Results

3.1. Seed Germination and Embryo Development

D. capra seeds were fully germinated and developed in 5 weeks. The development stages could be differentiated into 5 phases (Figure 2). The initial stage when seeds are recently scraped from capsule and planted onto medium is assigned as phase 0 (Figure 2 A1 and B1). First phase was achieved in second week when embryo became swollen and started to break the testa (Figure 2 A2 and B2). Second phase was achieved in third week when swollen embryo became greenish and globular shaped (Figure 2 A3 and B3). Third phase was achieved in fourth week marked with formation of shoot apical meristem and absorbing hair on embryo (Figure 2 A4 and B4). Fourth phase was achieved in fifth week with shoot formation and marked with leaf formation (Figure 2 A5 and B5). Plantlet then became fully formed with developed root marked its sixth phase (C).

Addition of complex organic substances such as coconut water or tomato extract to germination medium improved seed germination and embryo growth. Coconut water and tomato extract were able to increase germination quality, growth rate and embryo size of *D. capra* significantly (Figure 3). Embryos that were grown on tomato extract supplemented medium have grown better than the ones on coconut water supplemented medium.

Among various concentration that were used to supplement embryo growth, 200 ml/l tomato extract is considered to be the best concentration to enhance *D. capra* embryo growth. Tomato extract with 200 ml/l concentration and above have no significant difference on embryo size while there is a slight significant difference with 100 ml/l concentration. On the other side, embryos that were grown on coconut water supplemented medium have significantly smaller size.

Tomato extract was also capable to enhance *D. capra* growth rate. The growth rate difference was noticeable in third week after sowing, in which seeds grown on tomato extract supplemented medium were able to achieve phase 4 one week earlier than their counterpart that were grown on coconut water supplemented medium (Figure 4). These results suggest that tomato extract has better advantage than coconut water on *D. capra* seed germination and growth.

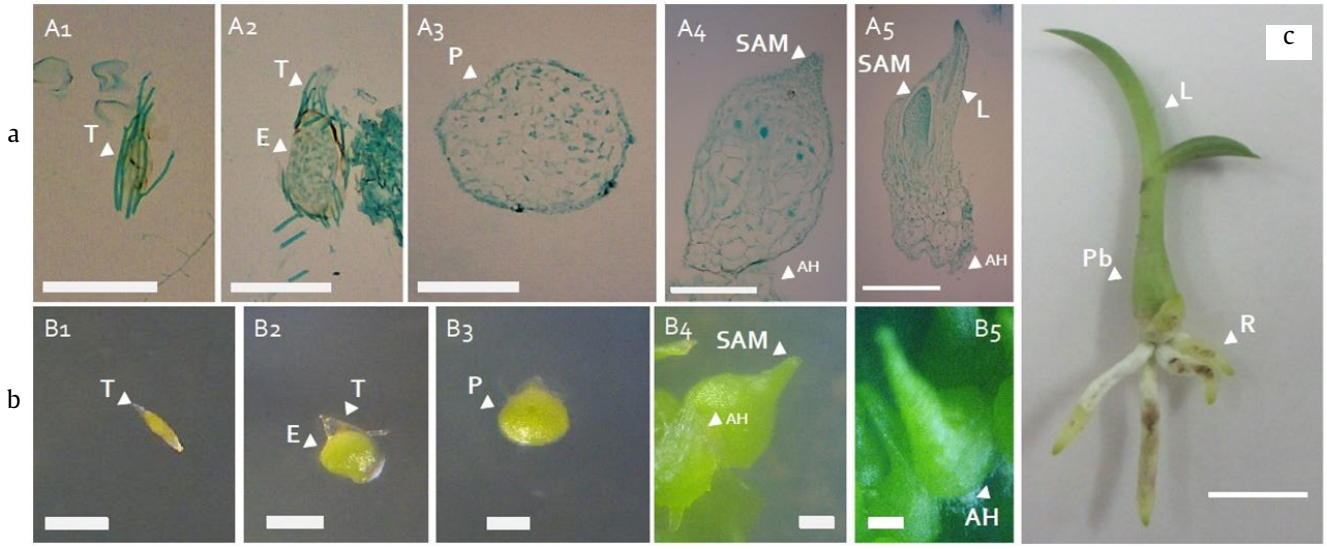


Figure 2. Phase changes of *D. capra* embryo development. (a) Histology and (b) morphology change of *D. capra* embryo throughout developmental process during 5 weeks culture. (c) Fully formed *D. capra* plantlet with developed leaf, pseudobulb and root. Phase changes occured during one week for each phase (T: testa, E: embryo, P: protocorm, SAM: shoot apical meristem, AH: absorbing hair, L: leaf, Pb: pseudobulb, R: root; bar A1-A3, A6-A10: 100 µm, A4-A5: 500 µm, C: 1 cm)

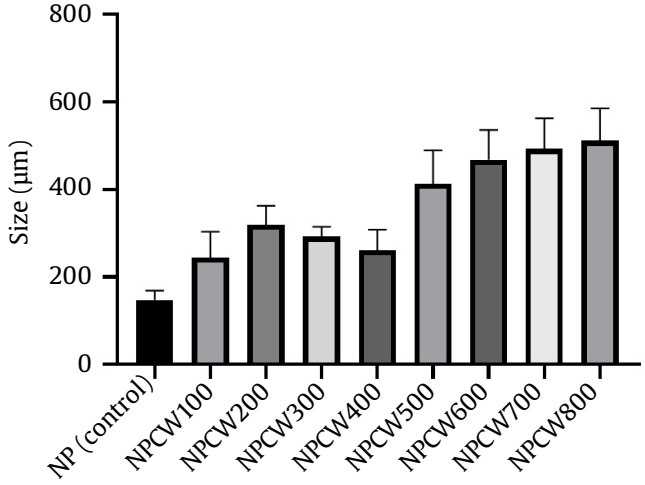


Figure 3. Size comparison of *D. capra* embryos grown on complex organic substances supplemented medium. Embryos grown on tomato extract supplemented medium have significantly bigger size

3.2. *D. capra* Plantlet Growth and Development

Germinated *D. capra* were sub cultured to induce further growth and development. Various media were tested to discern which medium is best suited for *D. capra* growth. Height, root length and leaf length were used to measure *D. capra* growth for 8 weeks. Our observation result showed that MS medium has able to induce better growth of *D. capra* plantlets compared to other mediums. Plantlets that were grown on MS medium have similar root and leaf length compared to the ones that were grown on the other three mediums but they have grown significantly taller (Figure 5).

3.3. Flowering Induction of *D. capra*

Plantlets that have grown were subjected to various PGR treatments to induce flowering (Table 3). After 11 months of treatment, plantlets that were grown on NAA0.05TDZ2 have initiated floral development. From all plantlets that able to develop floral organ, none of them achieved fully developed flower (Figure 6). Furthermore, all of the floral organs were withered after one week before they were able to fully develop.

4. Discussion

During 5 weeks observation, *D. capra* seeds were able to fully germinated and developed into plantlets. The development pattern of *D. capra* is similar with development pattern on *Phalaenopsis amabilis* and *D. phalaenopsis* seeds (Semiarti *et al.* 2010; Setiari *et al.* 2016). In three weeks, *D. capra* seeds were able to achieve protocorm stage as a unique development stage known to orchids. After protocorm stage, SAM was further developed and leaves were formed on fifth week. Before root developed, *D. capra* formed a structure known as absorbing hair on the opposite side of the SAM. This structure helped

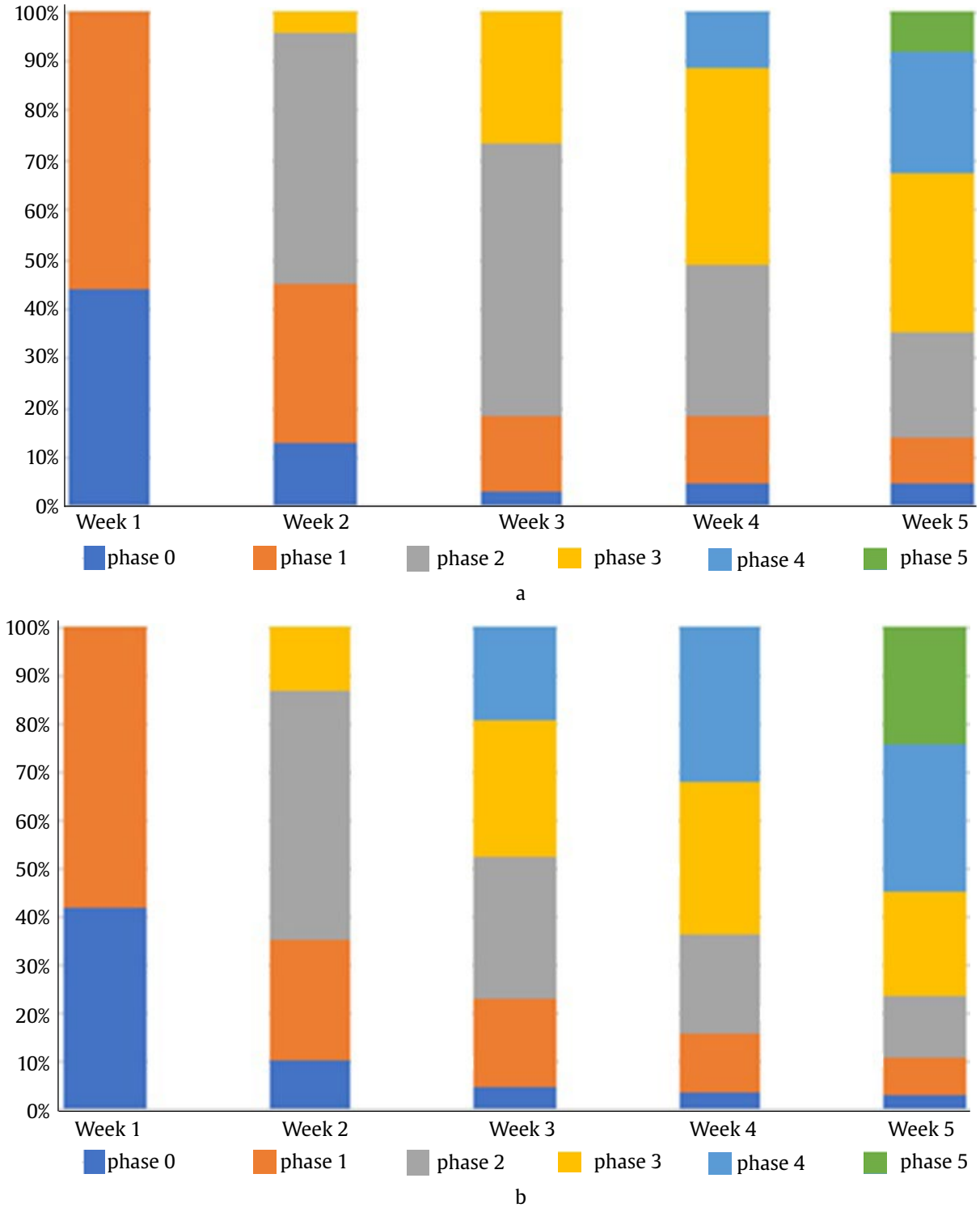


Figure 4. Comparison of growth phase distribution of *D. capra* embryo on (a) coconut water and (b) tomato extract supplemented medium. Tomato extract gave faster growth showed start from third week when the embryos already reached phase 4

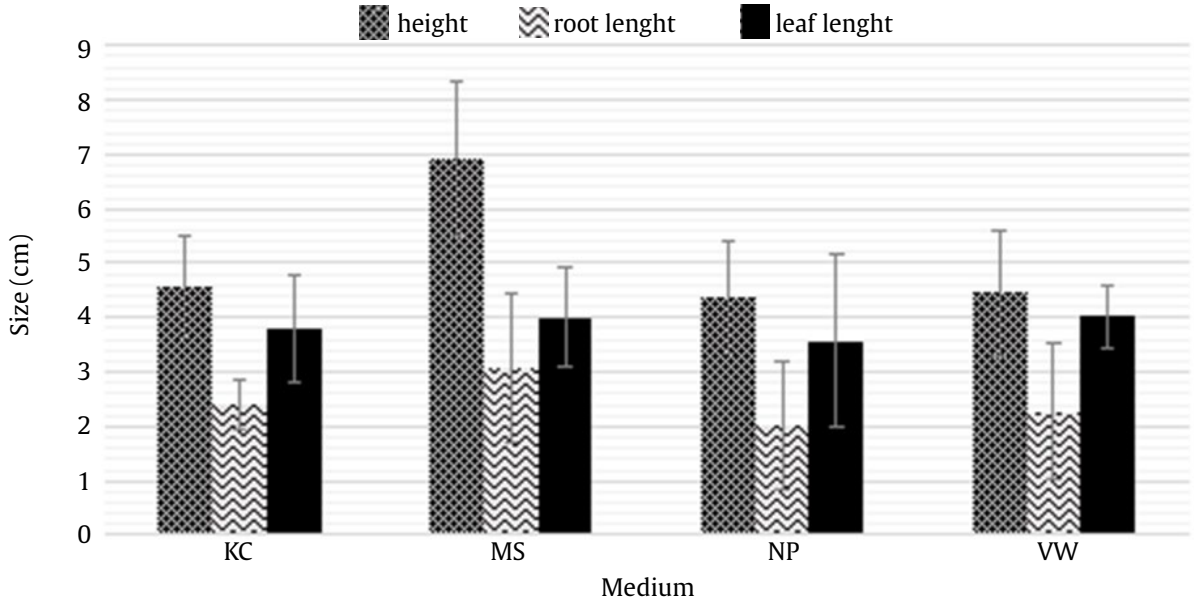
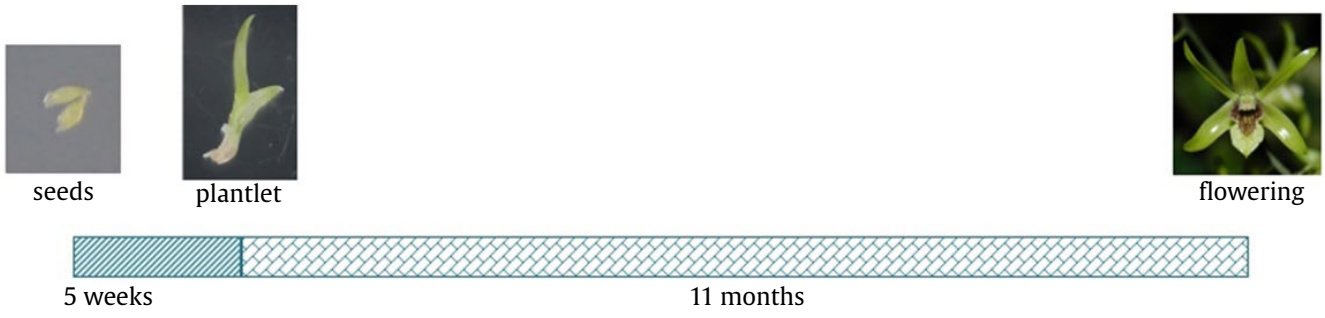


Figure 5. *D. capra* plantlet on various medium size comparison. This data shows that no significant difference in root and leaf length among different medium but MS medium gave better plantlet height than others

Table 3. *D. capra* plantlets after 11 months culture on PGR supplemented medium

PGR combination (mg/l)	Height (cm)	Note
Control	1.28±0.62^{cdef}	
GA0.10 NAA0.50	1.26±0.29^{cde}	
GA0.50 NAA0.50	1.36±0.22^{cdef}	
GA1.00 NAA0.50	1.60±0.44^{ef}	
GA2.00 NAA0.50	1.40±0.12^{def}	
GA3.00 NAA0.50	0.98±0.68^{bcde}	
GA0.10 TDZ0.05	1.90±0.33^{fg}	
GA0.50 TDZ0.05	0.72±0.22^{abc}	
GA1.00 TDZ0.05	0.32±0.406a	
GA2.00 TDZ0.05	1.22±0.50^{cde}	
GA3.00 TDZ0.05	0.32±0.29^{a}	
GA0.10 NAA0.5 TDZ0.05	0.72±0.19^{abc}	
GA0.50 NAA0.5 TDZ0.05	1.30±0.45^{cdef}	
GA1.00 NAA0.5 TDZ0.05	0.96±0.29^{bcde}	
GA2.00 NAA0.5 TDZ0.05	1.20±0.51^{cde}	
GA3.00 NAA0.5 TDZ0.05	1.32±0.56^{cdef}	
NAA0.05 TDZ2.00	0.84±0.49^{abcd}	80% flowering
NAA0.10 TDZ1.00	1.36±0.44^{cdef}	
NAA0.50 TDZ0.50	2.32±0.68^{g}	
NAA1.00 TDZ0.10	0.46±0.17^{ab}	
NAA2.00 TDZ0.05	0.34±0.42^{a}	



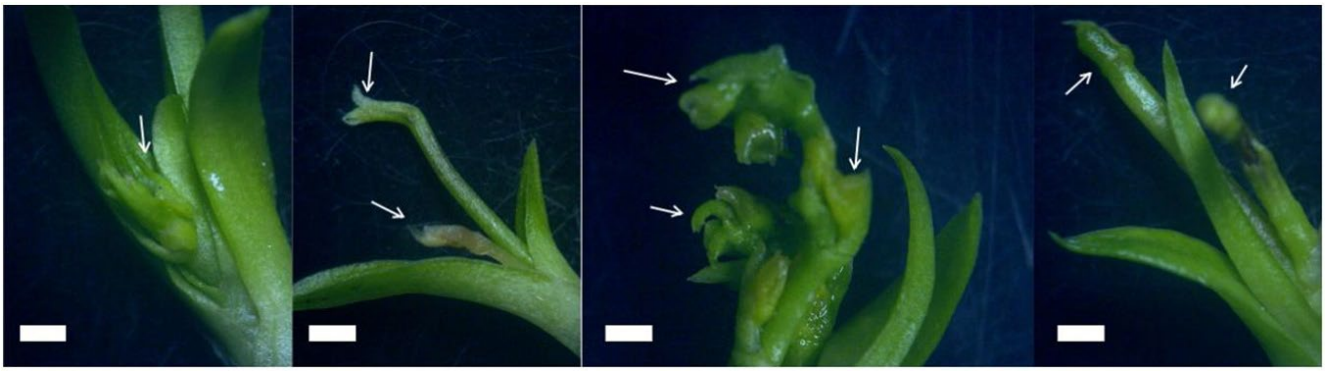


Figure 6. Early development of *D. capra* flower organ. (a) Flowering was achieved in 11 months after sub cultured plantlets in the PGR-added medium, (b) all flowers were underdeveloped with no specific flowering organs (bar 1 cm)

embryo to absorb more nutrients from medium (Chang *et al.* 2005; Novak and Whitehouse 2013). The development of absorbing hairs and SAM development were simultaneously on fourth week indicate that increasing in cell activity for SAM development were escalated nutrient needs.

Presence of an additive supplement could make a difference in embryo growth and development. Coconut water and tomato extract which is added to the medium caused *D. capra* embryo grew significantly better compare to the control medium. Those two complex organic substances are well recognized additives for enhancing plant culture growth. Coconut water contains various nutrients and phytohormones, especially cytokinin that make it an ideal additive supplement for plant tissue culture (Yong *et al.* 2009). On the other hand, tomato extract is known to possess various vitamins and antioxidant lycopene (Semiarti *et al.* 2010; Dwiyani *et al.* 2015). Tomato extract is also known as a good buffer to stabilize pH value and has the ability to prevent iron precipitation in *in vitro* culture medium (Vacin and Went 1949). Application on those two additives for *D. capra* seed germination showed that tomato extract gave better yield than coconut water possibly due to buffer effect of tomato extract in culture medium and its antioxidant properties. These were most likely also contributed to *D. capra* embryos growth rate, enabled them to grow faster than the coconut water-supplemented ones.

Utilizing a suitable medium is also an important factor for orchid culture. There were various culture medium that are widely used for orchid propagation such as MS, VW, NP, and KC medium. Among those medium, only MS that was not initially developed for orchid but is used widely in various publications (da Silva *et al.* 2014). Comparison of those medium on *D. capra* plantlet growth gave an interesting result. Eight weeks observation showed that there was no significant difference in root and leaf growth among *D. capra* plantlets grown on those four mediums. On the other hand, the height achieved by plantlets grown on MS medium is significantly higher than other plantlets grown on VW, NP, and KC medium. Among the medium used, MS and NP medium are composed with more complex composition with almost similar components. The differences between those two are their macronutrient composition and

micronutrient concentration. MS medium has higher micronutrient concentration and NP medium has more complex macronutrient composition. On that note, MS medium has given *D. capra* plantlet more favorable condition for enhanced metabolism that lead to better plantlet's height. Therefore, it will be necessary to conduct further research to ascertain which medium component gave such result.

Flower development is normally taken years to achieve on orchid. However, early flowering in orchid is possible through supplementing a precise combination of plant growth regulator. NAA, TDZ, and GA_3 are plant growth regulators that were used to induce early flowering in various orchids (Ferreira *et al.* 2006; Matsumoto 2006; Wang *et al.* 2009; Cen *et al.* 2010; Semiarti *et al.* 2013). Among all combinations, only NAA0.05TDZ2 combination that was able to induce flowering on *D. capra* plantlet. This implied that manipulating auxin-cytokinin ratio in *D. capra* has better chance to induce flowering in *D. capra* rather than using GA_3. Unfortunately, there are no fully developed-flowers observed on developed plantlets. All flowers that obtained have no distinct sepals, petals, labellum or gynostemium. Furthermore, those flowers were wilted around one week after formation. This peculiar phenotype could be resulted from TDZ application. Some research suggested that TDZ could affect flower morphology, inhibit flower organ development and reduce flower life span (Zhao *et al.* 2013). Based on this result, further analysis using molecular approach will be necessary to determine TDZ impact on orchid flowering.

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