Biosaintifika 8 (2) (2016) 165-171

Biosaintifika
Journal of Biology & Biology Education

http://journal.unnes.ac.id/nju/index.php/biosaintifika

The Effect of Organic Nutrient and Growth Regulators on Seed Germination, Embryo and Shoots Development of *Dendrobium antennatum* by *In Vitro*

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DOI: 10.15294/biosaintifika.v8i2.5165

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History Article

Received 21 February 2016
Approved 11 June 2016
Published 1 September 2016

Keywords:

coconut water; *Dendrobium antennatum*; 1-Naphthylacetic acid; seed germination; embryo and shoot development

Abstract

Dendrobium antennatum has high economic value as cut flowers and flowerpots. Like orchid seeds in general, *D. antennatum* is difficult to germinate under natural conditions. This study aimed to determine the effect of coconut water on seed germination and embryo development, as well as the effect of NAA on shoots development of *D. antennatum*. This study consisted of two stages. In the first stage, the 12 weeks-old seeds after pollination were sown on MS medium containing 2 g/L peptone + 0%; 5%; 10%; and 20% coconut water. After 8 weeks of culture, the seeds germinated and the shoot form were recorded. The highest seed germination (92.2%) and the formation of shoots (51.4%) were obtained when seeds were cultured on MS medium containing 2 g/L peptone + 20% coconut water. In the second stage, the shoots were sub-cultured on MS medium containing 1 mg/L thidiazuron + 0 mg/L; 1 mg/L; 2 mg/L; and 3 mg/L NAA. After 16 weeks of sub-culture, the height of *plantlets*, the length of the roots and leaves, number of leaves and roots formed were recorded. MS medium containing 1 mg/L thidiazuron + 1 mg/L NAA was the most suitable for the shoots development of *D.antennatum*. The embryo development of *D.antennatum in vitro* begans with the enlargement of embryo, with further it emerged from the seed coat (germinated) followed by the formation of the apical meristems to form the shoots and the roots.

How to Cite

Utami, E. S. W., & Hariyanto, S. (2016). The Effect of Organic Nutrient and Growth Regulators on Seed Germination, Embryo and Shoots Development of *Dendrobium antennatum* Lindl. Orchid by *In Vitro*. *Biosaintifika: Journal of Biology & Biology Education*, 8(2), 165-171.

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p-ISSN 2085-191X
e-ISSN 2338-7610

INTRODUCTION

Dendrobium antennatum Lindl. is known as the antelope orchid, commonly used as an ornamental plant in the pot, complementary bouquets, and cut flowers. The flower is fragrant thus has a potential as a producer of fragrances and medicinal plants (Fanfani & Rossi, 1989). Moreover, *D. antennatum* is flowering more frequent and more durable. Its flowers appear between March and December with the type inflorescence of 9-21 flowers on each stalk (Jones, 2006).

The biological aspects of *D. antennatum* is not yet widely known, especially in terms of seed germination, embryo and shoots development *in vitro*. The knowledge in seed germination, embryo development and regeneration may reflect the degree of ease in propagation of plants that could affect the development of the population. This considered as very useful for conservation and cultivation for ornamental plant, especially for hobbyists and entrepreneurs, in order to estimate the length of time needed to produce *plantlets* ready to acclimatize. The study of embryo development and regeneration of *plantlets D. antennatum* from embryo was expected to provide useful scientific information for its cultivation and conservation. Therefore, this study aimed to understand the seed germination, embryo and shoots development of *D. antennatum in vitro*.

In general, the success in plant tissue culture is influenced by growth regulators and nutrients that are added to the media. The medium used for propagation of orchids, in general contains salt, vitamins, minerals, carbon source, and growth regulators (Bektas et al., 2013). Plant growth regulators which are frequently used in plant tissue culture are a group of auxin and cytokinin. The combination of the type and concentration of these growth regulators added to the culture media have been widely used to enhance the development of shoots in many species of hybrid orchid. Wu et al. (2014) reported that the MS medium containing banana extract 100g/L, peptone 1g/L and 10% coconut water added NAA 1mg/L was found to be the best for the formation of *Renanthera imschootiana plantlets*. Similarly, Long et al. (2010) from his study reported that Knudson medium containing BA 5mg/L added by NAA 0.5 mg/L was able to increase the frequency organogenesis of shoots of *Paphiopedilum villosum var. densissimum* and *Paphiopedilum insigne*. Another important component in plant tissue culture is its organic nutrient (Murdad et al., 2010; Zhang et al., 2013; Zeng et al., 2013). The addition of organic nutrient such as apple extract, banana extract, potatos extract, and coconut water have been reported capable to increase germination in some species of orchids (Long et al., 2010; Zeng et al., 2012; Shekarriz et al., 2014; Kaur & Bhutani, 2012). This study evaluated the effect of the addition of coconut water on seed germination and development of the embryo as well as the effect of 1-Naphthylacetic acid (NAA) on the development of *D. antennatum* shoot.

METHODS

Preparation and sterilization of green capsules

The 12 weeks-old capsules after pollination (Figure 1) were washed using a detergent solution for 5 minutes to remove dust particles and were rinsed three times with sterile distilled water. The capsules were put in a laminar flow and they were soaked with 1% sodium hypochloride solution for 10-15 minutes, while they were shaked during this process, and then they were rinsed three times with sterile distilled water. The capsules surfaces sprayed with 70% alcohol and they were flamed and repeated two times. The capsules were put in sterile Petri dish and they were cut into 4 parts. Seeds released from the capsules and were collected with the help of a sterile spatula.

The effect of organic nutrient on seed germination and embryo development of *D. antennatum* Lindl (First stage: Experiment I)

To determine the effect of coconut water in the media on seed germination and embryo development, the seeds were sown on Murashige and Skoog (MS: Murashige & Skoog, 1962) which contained 2g/L peptone (Difco Laboratories Detroit, USA), and was being treated with 0%; 5%; 10%; and 20% coconut water. Sucrose (30g/L) was added into all media (Merck, Made in Germany) and the medium was adjusted to pH 5.6. Media was solidified with 2g/L gellan gum (Phytagel: Sigma Chemical Co., St. Louis, MO) and was sterilized at 120^0C for 15 minutes. Each treatment consisted of about 200 seeds, were cultured in flasks contained medium. All treatments were repeated for three times with 5 culture bottles for in each repetition. After 8 weeks of culture, observations of seed germination and embryo development using Stereomicroscope (SM-1 Nikon, Japan). Process in germination seed until the embryo developed into shoot was classified into 6 phases according to the phases of embryo development *D. antennatum* observed, namely: Phase 0, seed, the growth of the embryo was not yet visible. Phase 1, the embryo enlarged. Phase

2, the embryo enlarged and filled the seed coat. Phase 3, ruptured seed coat, embryo emerged from the seed coat (germination). Phase 4, the embryo separated from the seed coat, the apical meristems was visible. Phase 5, the first leaves and/or second leaves appeared (shoots). Percentage at any phase of embryo development were calculated by dividing the number of embryos in each phase divided by the total number of seeds were observed and multiplied by 100% (including seeds that were viable and not viable).

Evaluation the effect of growth regulators on the shoot development of *D. antennatum* (Second stage: Experiment II)

After 8 weeks of culture, shoots obtained from the first experiment were subcultured to new media. Shoots with length 3-5 mm and with 1-2 leaves grown on MS medium containing 1mg/L thidiazuron (Phyto Technology Laboratories, United States) was treated with different concentrations of 0; 1; 2; and 3mg/L 1-Naphthylacetic acid (NAA, MERCK-Schuchardt). Each treatment repeated three times with four culture bottles for each replication. Each culture bottles consisted of 4-5 shoots. The development of shoot covers two aspects: growth and differentiation. For growth, height of the *plantlets*, the length of root and the leaves were observed. For differentiation, the number of roots and leaves were observed. After 16 weeks of culture, *plantlets* were released from the flask and washed with running water to remove the agar. The *plantlets* height, leaf length, root length, number of leaves and roots were recorded.

Data analysis

These experiments were designed using Completely Randomized Design. The data were analyzed using analysis of variance with SPSS (Version 14), then the mean values were separated by Duncan's Multiple Range Test (DMRT) at level of significance $\alpha = 0.05$.

RESULTS AND DISCUSSION

Phases in seed germination and embryo development of D. antennatum Lindl .

The seeds of *Dendrobium antennatum* used as explants in this study had an elongated-oval shape with a narrow end and the other is rounded end, 663µm in length, and 153µm in wide, composed of embryos which were protected by the seed coat. Seed coat looked white, thin, patterned like arrangement of nets. Embryos *D. antennatum* located at the end of the narrowed seed, yellow-colored, small and undifferentiated (Figure 2A). Phases of seed germination began with changes in the embryo; embryo that was originally yellow-colored and was in size of about 80µm (Figure 2A) turned green and shiny and growing embryos enlarged from the previous size which was about 87µm (Figure 2B). After 4 weeks of culture, the embryo still growing (Figure 2C), as observed from the size of the embryo increased (188µm) and as it filled the seed coat. Furthermore, the embryo was continuously enlarged, followed by the ruptured of the seed coat and embryo emerged from the seed coat (germination phase, Figure 2D). The embryo continued to grow and increased in length followed by the appearance of a protrusion as apical meristems at one end of the embryo (Figure 2E). Once the embryo has reached 5mm in length, the first leaf and the second leaf appeared (Figure 2F).

Figure 1. The 12 weeks-old capsules of *D. antennatum* Lindl after pollination. Bar = 1.4 cm

***Evaluation the effect of organic nutrient on seed germination and embryo development of D. antennatum* Lindl**

The effect of the addition of organic nutrient (coconut water) on seed germination and embryo development of *D. antennatum* Lindl for 8 weeks after cultured, presented in Table 1.

The results of this study (Table 1) showed that adding coconut water and its concentrattion have effect on seed germination and embryo development of *D. antennatum*. The highest percentage of total seed germination (92.2%) was obtained when the seeds germinated on MS media given in combination of organic nutrient, namely 2g/L peptone + 20% coconut water and was significantly different with the control (2g/L peptone only) and with other treatments. The lowest seed germination percentage (77.33%) was obtained when the seeds germinated on MS media given with only a single organic nutrient that was 2g/L

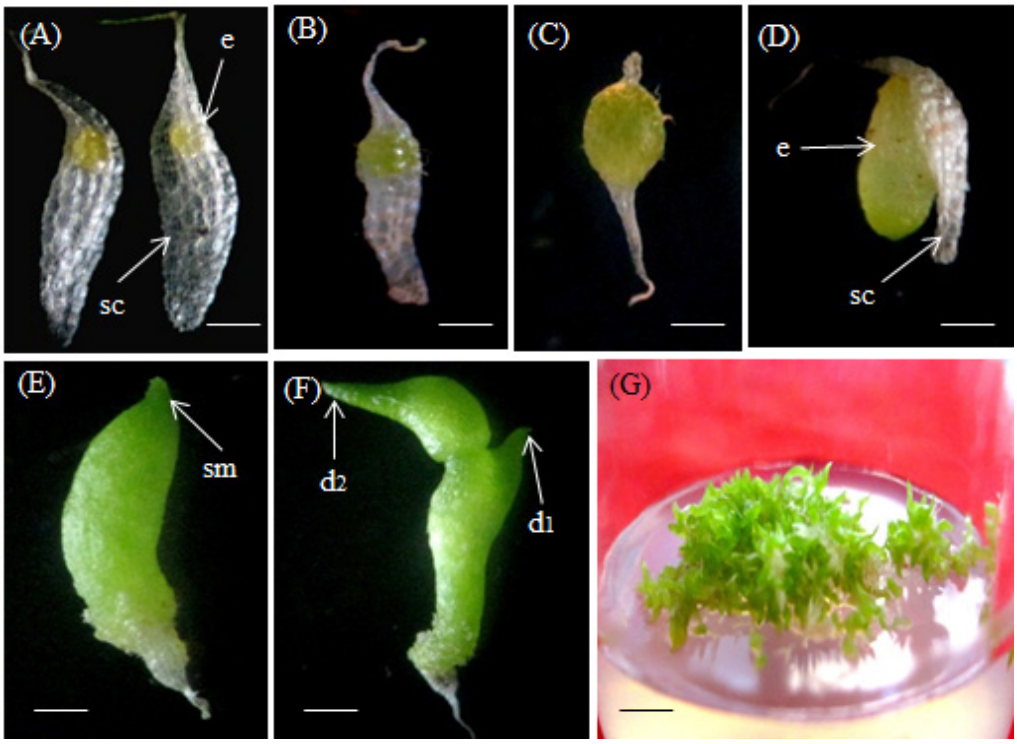

Figure 2. Seed culture and embryo development of *Dendrobium antennatum*. *in vitro*. (A) Phase 0, seed, the growth of the embryo was not yet visible. (B) Phase 1, the embryo enlarged. (C) Phase 2, the embryo enlarged and filled the seed coat. (D) Phase 3, ruptured seed coat and embryo emerged from the seed coat (germinated). (E) Phase 4, the embryo separated from the seed coat and the apical meristems was visible. (F), Phase 5, the first leaves and second leaves appeared (shoots). (G). Shoot growth on MS medium containing peptone 2g /L+ 20% coconut water. e: Embryo, sc: Seed coat, sm: Shoot meristem, d_1: First leaf, d_2: Second leaf. Bars: (A) 80µm, (B) 87µm, (C) 188µm, (D) 210µm, (E) 350µm, (F) 500µm, (G) 0.6 cm.

Table 1. The effect of coconut water with different concentration on seed germination and embryo development of *D. antennatum* Lindl at MS medium containing 2g/L peptone, 8 weeks after culture.

Coconut water concentration (%)	Phases of embryo development					
	Phase 1	Phase 2	Phase 3 (germinated)	Phase 4	Phase 5 (shoot)	Total germinated (Phase 3-5)
0	12,1±2,6^{d}	10,6±1,2^{bc}	42,5±2,4^{c}	19,8±2,0^{c}	14,9±1,8^{a}	77,3±2,1^{a}
5	10,6±1,2^{c}	9,7±1,4^{b}	35,3±2,0^{b}	20,4±2,3^{c}	23,9±1,6^{b}	79,7±2,1^{b}
10	8,3±1,4^{b}	11,6±2,6^{c}	27,8±2,0^{a}	17,1±1,5^{b}	35,3±2,0^{c}	80,2±2,8^{b}
20	2,7±1,6^{a}	5,1±1,6^{a}	26,3±1,1^{a}	14,4±1,9^{a}	51,4±1,5^{d}	92,2±1,5^{c}

The mean ± SD followed by different letters in the same column indicate significant difference according to Duncan's test at a significance level α = 0.05.

peptone (control). Similarly, the observation in the percentage of shoot formed (phase 5), which indicated that there was an effect of coconut water as organic nutrient at various concentrations on the shoot development of *D. antennatum*. The highest percentage of shoot formation (51.44%) was found when the seeds germinated on MS medium given with a combination of organic nutrient 2g/L peptone + 20% coconut water and was significantly different from the control treatment (2g/L peptone). The lowest percentage of shoot formation (14.99%) was found when the seeds germinated on MS medium were given only with a single organic nutrient that was 2g/L peptone, this shows that the seeds were able to germinate and to grow into shoots on MS medium using only a single organic nutrient, but the percentage is lower when compared with provided with combination of organic nutrient. The same results had been reported as well by Utami et al., (2015) that

the seeds of *Paphiopedilum liemianum* cultured in VW media given only with 2g/L of peptone were also able to germinate and to develop into *plantlets*. This is possible because peptone contains vitamin ie: biotin, pyridoxine, thiamin, nitrogen (Dutra et al., 2008) and amino acids, proteins (Nhut et al., 2008) which are able to increase the growth and development of the explants. The results also indicated that MS medium provided with 2g/L peptone + 20% coconut water as organic nutrient was suitable for seed germination and for further embryo development in order to form shoot. Hasanah et al. (2014) reported that the addition of 15% coconut water into the MS medium containing Hyponex as fertilizers is able to increase the growth of *Dendrobium kelemense plantlets*. Enhancement in percentage of seed germination and embryo development on such treatment is possible because in MS medium other than by peptone, was also being provided with coconut water that contains carbohydrates, vitamins, amino acids, organic acids, enzymes that play an important roles for the development of plant cells (Yong et al., 2009; Nambiar et al., 2012). The percentage of germinated seeds (Phase 3) was 26.3% and 14.4% (Phase 4), where it was found that the seeds cultured in the media provided with 2g/L peptone + 20% coconut water was lower than other treatments. This is because the treatment caused the embryo further developed into phase 5 (shoot).

Evaluation the effect of growth regulators (NAA) on shoot development of *D. antennatum* Lindl

To determine the effect of various concentrations of NAA as growth regulators on the growth and differentiation of shoot, 70 days-old shoots obtained from the first experiment with, 3-5 mm long, consisted 1-2 leaves, and without root were being transferred on MS medium containing 1mg/L thidiazuron + NAA with different concentration: 0mg/L; 1mg/L; 2mg/L; 3mg/L. The result (Figure 3 and 4) showed that the addition of NAA in MS medium resulted to a positive response compared to the control treatment (1mg/L thidiazuron without NAA). The maximum of growth and differentiation response was observed when the shoots were sub-cultured on MS medium containing thidiazuron at a concentration of 1mg/L added with low concentrations of NAA (1mg/L), which gained an average of the height of plantlets 44,5mm, length of the leaf 19mm, length of the root 18mm, consisted of 7.2 leaves and 8 roots/*plantlets*. This was possible because the medium, provided with cytokinin (thiadiazuron) and auxin (NAA), which plays roles in differentiation of cell and plant tissue as well as in the formation of xylem and roots (Vogel and Mucedo, 2011). The results (Figure 3 and 4) also showed that the presence of plant growth regulator cytokinin, ie. 1mg/L thidiazuron without NAA, can increase the shoot differentiation,

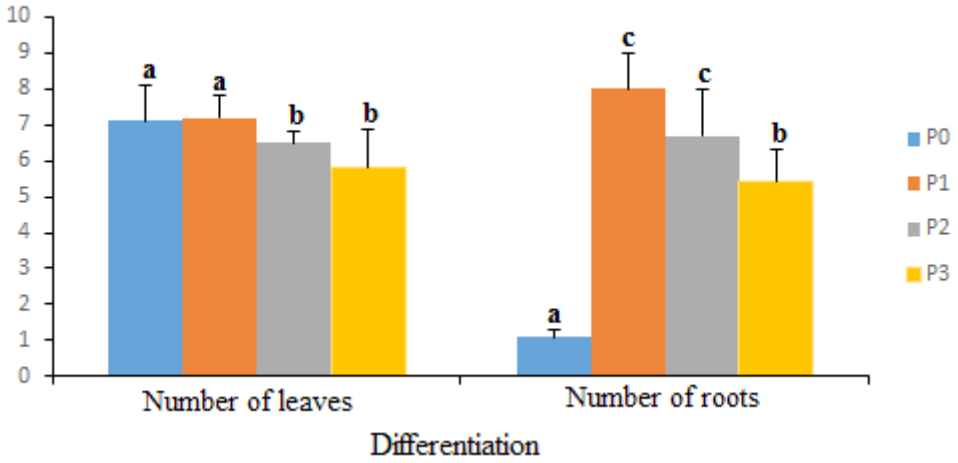

Figure 3. The effect of growth regulators NAA on shoot differentiation of *D. antennatum* at 16 weeks after the culture. P0 = MS medium containing 1mg/L thidiazuron, without NAA, P1 = MS medium containing 1mg/L thidiazuron + 1mg/L NAA, P2 = MS medium containing 1mg/L thidiazuron + 2mg/L NAA, P3 = MS medium containing 1mg/L thidiazuron + 3mg/L NAA.

The mean number of leaves in treatment P0 = 7.1 ± 1.0; P1 = 7.2 ± 0.6; P2 = 6.5 ± 0.3; and P3 = 5.8 ± 1.1. The mean number of roots in the treatment of P0 = 1.1 ± 0.2; P1 = 8.0 ± 1.0; P2 = 6.7 ± 1.3; and P3 = 5.4 ± 0.9. The mean ± SD followed by different letters indicate significant difference according to Duncan's test at a significance level $\alpha = 0.05$

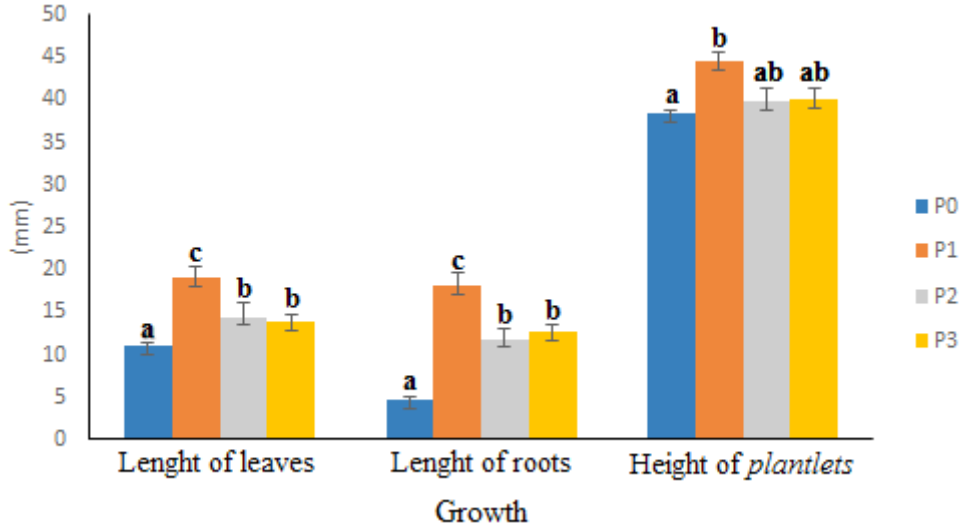

Figure 4. The effect of growth regulators NAA on shoot growth of *D. antennatum* at 16 weeks after the culture. P0 = MS medium containing 1mg/L thidiazuron, without NAA, P1 = MS medium containing 1mg/L thidiazuron + 1mg/L NAA, P2 = MS medium containing 1mg/L thidiazuron + 2mg/L NAA, P3 = MS medium containing 1mg/L thidiazuron + 3mg/L NAA.

Average length of leaves (mm) in the treatment P0 = 10.9 ± 0.4; P1 = 19.0 ± 1.3; P2 = 14.4 ± 1.6; and P3 = 13.8 ± 0.9. The mean of length of the roots (mm) in the treatment P0 = 4.6 ± 0.5; P1 = 18.0 ± 1.6; P2 = 11.8 ± 1.2; and P3 = 12.7 ± 0.9. The mean of the height of *plantlets* (mm) in the treatment P0 = 38.3 ± 0.5; P1 = 44.5 ± 1.0; P2 = 39.8 ± 1.5; and P3 = 39.9 ± 1.5. The mean ± SD followed by different letters indicate significant difference according to Duncan's test at a significance level α = 0.05.

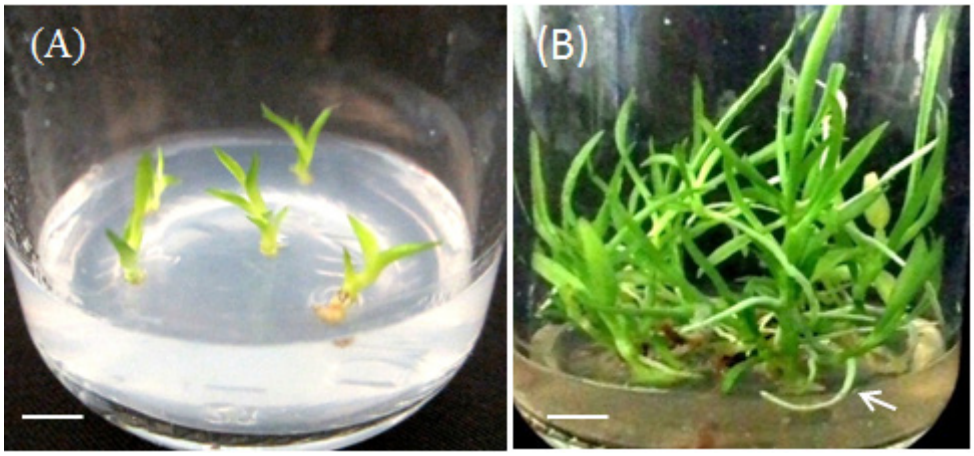

Figure 5. The shoots development of *D. antennatum* Lindl. (A). 2 weeks-old shoots were cultured on MS medium containing 1mg/L thidiazuron + 1mg/L NAA. (B). *Plantlets* resulted from the growth and differentiation of shoots on MS medium containing 1mg/L thidiazuron + 1mg /L NAA after 16 weeks of culture. The roots (arrow) appeared at the bottom of the *plantlet*. Bars:(A) 0.75cm, (B) 0.83cm.

forming the leaf which was 7.1 leaves/*plantlet* in average; higher when compared with using combination of 1mg/L thidiazuron + 2mg/L NAA (6.5 leaves/*plantlet*) and 1mg/L thidiazuron + 3mg/L NAA (5.8 leaves/*plantlet* in average). This was possible because thidiazuron plays roles in cell division and organ regeneration (Guo et al., 2011; Oluk & Orthan, 2010). Enhanced shoot formation of *Astragalus cariensis* with thidiazuron had been reported by Erisen *et al.*, (2011) as well as in growth in seedling *Paphiopedilum liemianum* (Utami et al., 2015).

CONCLUSION

An increase in the percentage of seed germination and embryo development of *D. antennatum* on MS medium treated with the combination of organic nutrient compared to a single treatment. The percentage of seed germination was 92.22 % and the highest shoot formation 51.44% was found when the seeds were cultured on MS medium containing 2g/L peptone + 20% coconut water. The addition of NAA in MS medium containing 1mg /L thidiazuron increased the differentiation and growth of shoot. The highest formation of roots (8 root/*plantlet*), the formation of leaves (7 leaves/*plantlet*) as well as in term of the length of roots (18mm), height of *plantlets* (44.5mm), and length of leaves (19mm) obtained when shoots were sub-cultured on MS medium containing 1mg/L thidiazuron + low concentration of NAA (1mg/L).

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