Identification of suitable culture media for stimulation of
*Dendrobium* ‘Earsakul’ growth

S Hualsawat¹, K Ngoenyu¹, K Phanya¹, S Inthaisong¹, S Moranok¹, S Promaunand¹ and P A Tantasawat¹,²

¹School of Crop Production Technology, Suranaree University of Technology, Muang District, Nakhon Ratchasima, Thailand

E-mail: piyada@sut.ac.th

**Abstract.** *Dendrobium* is one of the most important economic cut-flowers in Thailand because of its attractive floral characteristics and popularity among consumers worldwide. However, its slow growth has prevented rapid mass micropropagation. The objective of this work was to stimulate *Dendrobium* growth *in vitro* using supplements, plant growth regulators and natural extracts. *Dendrobium* plantlets were cultured on VW0 media supplemented with 3 doses each of the energy drink Ready™ (5, 10 and 15 mL/L), 6-benzylaminopurine (BAP; 1, 2 and 3 mg/L), V8™ juice (100, 200 and 300 mL/L) and shrimp paste (2.5, 5 and 7.5 g/L) for 90 days. Plain VW0 media were used as control. The results showed that at 90 days the highest fresh weight (0.39 ± 0.05 g), plant height (3.99 ± 0.23 cm) as well as total root length (7.08 ± 1.30 cm) was obtained on VW0 media supplemented with 1 mg/L BAP. These were 1.8, 1.5 and 2.5-fold higher than those observed on plain VW0 media, respectively. However, the number of roots and number of leaves of plantlets cultured on this medium were not statistically significant. Results suggest that this culture medium is useful in promoting *D. ‘Earsakul’* growth *in vitro* and may be applied for any future commercial micropropagation.

1. **Introduction**

*Dendrobium* is one of the largest genera of orchids and is widely used in commercial cut-flower production. It has become popular worldwide because of its floral characteristics i.e., color, size, and shape [1]. To enhance its production, improved techniques/methods of micropropagation are important because of their ability to produce a significant number of quality plants throughout the year. Additionally, micropropagated plants are uniform in age, size, growth and maturity which in turn increase their economic value. They can also be disease-free and genetically true to type [2,3]. However, micropropagation of orchids in general is limited due to slow growth and thus necessitates the development of culture media to stimulate orchid growth *in vitro*. The success of tissue culture depends on the components and state of culture media, namely, macronutrients, micronutrients, plant growth regulators, vitamins, carbon sources as well as organic compounds [4], which are necessary for the growth and development of *in vitro* cultured plantlets.

Plant growth regulators (PGRs) affect plant growth and cell differentiation. They have been supplemented in culture media to induce the physiological processes of plant tissues, allowing them to grow and develop *in vitro* [5]. Generally, cytokinin is the most common PGR group that is widely used in micropropagation. 6-Benzylaminopurine (BAP) is also largely used because of its high efficiency in stimulating cell elongation, shoot proliferation and growth of lateral buds. Previous studies showed that BAP was efficient for stimulation of growth and development of orchids, namely,
Cymbidium finlaysonianum, Vanda tessellate and Violet phalaenopsis [6-8]. In addition to BAP, shrimp paste derived from either shrimp or krill contains kinetin which is the essential precursor of chitosan helps in increasing the cell division of plants [9,10]. There are a few reports describing the effectiveness of shrimp paste in accelerating the root formation of ‘Nam Dok Mai’ rose apple ex vitro [9]. However, it has not been used in the tissue culture of orchids as a supplement.

Vitamins are essential components for plant growth and development when they are directly or indirectly combined with other media components [11]. This study aimed to reduce the costs of micropropagation by using additives in the form of common products available on the market, e.g. the energy drink Ready® containing goji berry and mixed berry juices, V8® and shrimp paste. Ready® possesses vitamins, especially vitamin C (ascorbic acid), which has antioxidant effects on scavenging free radicals, i.e. hydroxyl radicals (•OH), hydrogen peroxide (H2O2), and singlet oxygen (‘O2) [12-15]. Therefore, plant tissues are protected from high light intensity stress by vitamin C. In addition, it helps preserve nitrate which is an important nutrient for growth [16]. Chaikhiri and Chouychai [17] reported that 10 mL/L of Ready boot® was the most appropriate supplement for in vitro growth of Cymbidium finlaysonianum, especially for shoot and root length, number of leaves and roots as well as fresh weight. Vegetable juice ‘V8™’ is also considered appropriate because it contains water and the concentrated juices of tomatoes, carrots, celery, beets, parsley, lettuce, water cress and spinach including vitamin C, beta carotene as well as citric acid, which are precursors that participate in various biological processes in plants such as photosynthesis, photomorphogenesis, photoprotection, and development [18-20]. Both products are rich in vitamins and are cheaper than PGRs and therefore considered as alternative additives in the culture media of orchids. The objective of the present study is to investigate the effects of various culture media for the stimulation of D. ‘Earsakul’ growth, especially during a culture period of 90 days. The knowledge gained can be applied to provide maximum benefits to farmers and entrepreneurs for the micropropagation of orchids and other plant species for commercial purposes.

2. Materials and methods

2.1. Plant materials

Lengths of approximately 2.4 - 2.7-cm plantlets of D. ‘Earsakul’ were initially grown on Vacin and Went 0 medium (VWO); VW basal salts, 100 g/L potato (Solanum tuberosum), 200 mL/L coconut water, 50 g/L ‘Hom Thong’ banana, 2% (w/v) sucrose and 0.2% (w/v) activated charcoal [21] for the present experiment.

2.2. Culture media for growth stimulation

The VW0 medium was developed for the in vitro culture of many orchid species [21-23]. This medium was supplemented with the energy drink Ready® (5, 10 and 15 mL/L; T.C. Pharmaceutical Industries Company Limited, Thailand), 6-benzylaminopurine (BAP) (1, 2 and 3 mg/L; Acros Organics, New Jersey, USA), V8® juice (100, 200 and 300 mL/L; Campbell Soup Company, Camden, New Jersey, USA) and shrimp paste (2.5, 5.0 and 7.5 g/L; SGS (Thailand) Limited, Thailand). Plain VW0 medium was used as control. The pH of the culture media was adjusted to 5.0 before gelling with agar (0.7% w/v). All culture media were dispensed in volume of ca.15 ml into glass bottles (4 oz.). The media were autoclaved at 121°C for 20 min at 103.4 KPa pressure. Roots were cut from plantlets under aseptic conditions, and the plantlets were transferred to various media randomly. Cultures were maintained at 25±2°C during a 16/8 h photoperiod.

2.3. Measurement of growth parameters

The following growth parameters were measured and recorded at 0, 45 and 90 days: fresh weight (g), height (cm), number of leaves, number of roots and total root length (cm). To determine the fresh weight, the plantlets were removed from the media, thoroughly washed in distilled water and dried with paper before measuring with a 4 digit balance. The plant height was measured from the base to
the top of the pseudobulbs. The numbers of leaves and roots were manually counted. The total root length was measured from all of the roots from the base to the longest point.

2.4. Statistical analysis
The effects of culture media on stimulation of D. ‘Earsakul’ growth were statistically analyzed using the analysis of variance (ANOVA) and a completely randomized design (CRD) with 15 replications. The means among all treatments were compared by Duncan’s new multiple range test (DMRT). All statistical analyses were performed using SPSS version 16.0 [24].

3. Results and discussion
When the effects of the culture media on the growth and development of D. ‘Earsakul’ plantlets were evaluated based on various growth parameters (fresh weight, plant height, number of leaves, number of roots and total root length) at 0, 45 and 90 days, it was found as expected that all growth parameters of the plantlets at the initial stage (0 day) were not significantly different among various culture media. After 45 days of the culture period, significant differences were observed on most growth parameters of the plantlets cultured on different media (p<0.05). The differences for all growth parameters were highly significant (p<0.01) at 90 days among the different media.

- **Fresh weight**: At 45 days, plantlets that were cultured on 1 and 2 mg/L BAP had the highest total fresh weight, however, no significant difference was observed among these media and those supplemented with 5 and 10 mL/L Ready® and VW0 control. After 90 days of culture on different media, this parameter also differed significantly (p<0.01) among the culture media. *Dendrobium* plantlets had a tendency to grow and develop best when they were cultured on the medium supplemented with 1 mg/L BAP. The total fresh weight of plantlets grown on this medium was highest (1.8-fold higher than VW0 control), but it was not significantly different from those supplemented with 2 mg/L BAP and 10 mL/L Ready® (figure 1A). The culture media supplemented with V8® and shrimp paste negatively affected the fresh weight of *D. ‘Earsakul’* plantlets. Plantlets grown on these media were small and had yellowish-green pseudobulbs and leaves (figure 2: H-M).

- **Plant height**: At 45 days, plantlets grown on the culture media supplemented with 1 mg/L BAP and 10 mL/L Ready® showed highest plant height (1.2 and 1.3-fold) and this was significantly greater (p<0.05) than those plantlets which were cultured on VW0 (control). Nevertheless, no significant differences among these media and those supplemented with 2 mg/L BAP and 15 mL/L Ready® were observed. After 90 days of culture on different media, the highest plantlets were obtained in the medium supplemented with 1 mg/L BAP (1.5-fold higher than VW0 control), followed by 2 mg/L BAP, 10 and 15 mL/L Ready® respectively, which were significantly higher (p<0.05) than those grown on other media (1.2-fold higher than VW0 control) (figure 1B). On the other hand, plantlets cultured on the medium supplemented with 300 mL/L V8® had the lowest height and this medium also caused yellowish-green pseudobulbs and leaves (figure 2J).

- **Number of leaves**: At 45 days of the culture period, no significant differences among the culture media were observed for the number of leaves. However, *Dendrobium* plantlets cultured on different media began to exhibit significant differences in the number of leaves (p<0.01) after 90 days of culture. The highest number of leaves was achieved in plantlets cultured in the media containing 10 at (p<0.05) and 15 mL/L Ready®, but these were not significantly different from those grown on the media supplemented with 1 and 2 mg/L BAP, 5 g/L shrimp paste and VW0 as control (figure 1C). By contrast, plantlets cultured on the media containing 2.5 and 7.5 g/L shrimp paste had fewer leaves with the lower leaves turning brown (figure 2: K and M).

- **Number of roots**: At 45 days of the culture period, plantlets showed the highest number of roots when they were cultured in media supplemented with 1, 2 and 3 mg/L BAP. However, they were not significantly different from those cultured on VW0 control and many other
Figure 1. Effects of culture media on (A) fresh weight, (B) plant height, (C) number of leaves, (D) number of roots and (E) total root length of D. ‘Earskul’ plantlets. Bars represent standard error (SE) of the mean. Different letters (a, b, c, d, e, f and g) show statistically significant differences among the treatments (Duncan’s new multiple range test (DMRT)).
Figure 2. The effects of culture media on growth and development of *D. ’Earsakul’* in vitro at 90 days (A) VW0 control, (B) 1 mg/L BAP, (C) 2 mg/L BAP, (D) 3 mg/L BAP, (E) 5 mL/L Ready®, (F) 10 mL/L Ready®, (G) 15 mL/L Ready®, (H) 100 mL/L V8®, (I) 200 mL/L V8®, (J) 300 mL/L V8®, (K) 2.5 g/L shrimp paste, (L) 5 g/L shrimp paste and (M) 7.5 g/L shrimp paste.

media. After 90 days of culture on different media, plantlets cultured on the medium supplemented with 2 mg/L BAP tended to have the highest number of roots (figure 1D), but with no significant difference from those cultured on media supplemented with 1 mg/L BAP, 15 mL/L Ready® and VW0 control. By contrast, culture media supplemented with shrimp paste and high concentrations of V8® adversely influenced root numbers. Plantlets grown on these media had the lowest numbers of roots (significantly lower than those cultured on VW0 control; p<0.05).

- **Total root length**: At 45 days of the culture period, total root length of *D. ’Earsakul’* plantlets was highest in media supplemented with 1 and 2 mg/L BAP (1.8 and 2.0-fold significantly higher than VW0 control), but these were not significantly different from the medium supplemented with 3 mg/L BAP. After 90 days, the total root length of *D. ’Earsakul’* plantlets increased maximally when they were cultured on the medium supplemented with 1.0 mg/L BAP (2.5-fold significantly longer than VW0 control) but they were not significantly different from those cultured on 2 mg/L BAP (Figure 1E). Media containing 200 and 300 mL/L V8® (figure 2: I and J) as well as 5 and 7.5 g/L shrimp paste (Figure 2: L and M) had significantly inhibited the root length of *D. ’Earsakul’* plantlets and also induced yellowish-green pseudobulbs and leaves.

When the results were taken together, it was observed that *D. ’Earsakul’* plantlets cultured on media supplemented with 1 mg/L BAP had the highest growth and were healthier and more vigorous (figure 2B). These results are in agreement with previous studies in which it was reported that 1 mg/L BAP induced the growth of orchids (*Vanda tessellate, Dendrobium* and *Violet phalaenopsis*) [6-25-27]. In addition, BAP promoted the expansion of leaves, roots and promoted chloroplast maturation.
However, the increasing concentrations of BAP may induce programmed cell death (PCD) in plants by accelerating senescence, and causing slow and stunted growth [31,32]. In addition, the supplementation of 10 and 15 mL/L Ready® also produced relatively better growth of D. ‘Earsakul’ plantlets than those of V8® and shrimp paste and VW0 control. These may result from Ready® containing goji berry and mix berries juices which have high vitamin C and have acted as major redox buffers by regulating various physiological processes controlling growth and development [33]. Chaikhiri and Chouychai [17] reported that 10 mL/L Ready boot® was the most appropriate supplement for growth of Cymbidium finlaysonianum in vitro, especially with respect to shoot length, number of leaves, roots and fresh weight. By contrast, culture media supplemented with V8® and shrimp paste negatively affected D. ‘Earsakul’ plantlets in vitro, possibly due to the inappropriate concentrations used, or the possible toxicity of some components. Therefore, D. ‘Earsakul’ plantlets grown on these media were small and had yellowish-green pseudobulbs and leaves as well as root inhibition (Figure 2 H-M). However, previous reports showed that shrimp paste can accelerate ex vitro root formation in rose apple, Isorachinensis, Carmona retusa (Vahl) Masam and Duranta erecta L. [9,10].

4. Conclusion
In summary, our results suggest that the medium supplemented with 1 mg/L BAP was the most appropriate culture medium for stimulation of D. ‘Earsakul’ plantlets growth in vitro, especially with regard to fresh weight, plant height and total root length.

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References
[1] Kuehnlle A R 2006 Orchids: Dendrobium Flower Breeding and Genetics ed N O Anderson (Hawaii: University of Hawaii Springer) chapter 20 pp 539-60
[2] Pillay M and Tenkouano A 2011 Banana Breeding: Progress and Challenges (New York: CRC Press)
[3] Pragya, Singh S K, Misra R L and Ranjan J K 2012 In vitro shoot regeneration from cormel derived callus of gladiolus and bio-hardening of plantlets Indian J. Biotechnol. 11 99-104
[4] Tantasawat P A and Woranyuwat A 2008 Plant Tissue Culture Laboratory Manual (Bangkok: Agentex) p 109
[5] Davies P J 2010 Their nature, occurrence, and functions The Plant Hormones ed P J Davies (Dordrecht: Springer) pp 1-15
[6] Rahman M S, Hasan M F, Das R, Hossain M S and Rahman M 2009 In vitro micropropagation of orchid (Vanda tessellata L.) from shoot tip explant J. Bio-Sci. 17 139-44
[7] Pradhan S, Puadal Y P and Pant B 2013 Efficient regeneration of plant from shoot tip explants of Dendrobium densiflorum Lindl., a medicinal orchid Afr. J. Biotechnol. 12 1378-83
[8] Poonpipat N, Soipetkasem P and Chaiprasart P 2018 Effect of NAA and BA on micropropagation of Dendrobium dixathun Lcb.f., Dendrobium signatum Rchb.f. and Dendrobium tortile Lindl Agricultural Sci. J. 49(S) 230-3
[9] Chumpookam J, Weeraratprapa N and Pichakum N 2015 Effects of auxin and shrimp paste on layering of ‘Nam Dok Mai’ rose apple Agricultural Sci. J. 46(S) 669-72
[10] Homjan T, Komen A, Kaeawprasong K and Nabhadalung N 2011 Study on effects of shrimp paste on root growth of cutting Isora chinensis, Carmona retusa (Vahl) Masam and Duranta erecta L. Proc. Int. Conf. on Science and Agricultural Technology 1st (Bankkok, Thailand) pp 1–6
Abrahamian P and Kantharajah A 2011 Effect of vitamins on in vitro organogenesis of plant *AIPS*. 2 669-74

TCP 2019 Winning power momentum products [on-line] Available: https://www.tcp.com/en/product/products-ed/ready

Hacısevki A 2009 An overview of ascorbic acid biochemistry *J. Fac. Pharm.* 38 233-55

Arrigoni Ond De Tellio M C 2002 Ascorbic acid: much more than just an antioxidant *Biochim. Biophys. Acta*. 1569 1-9

Pehliván F E 2017 Vitamin C: An Antioxidant Agent ed Amal H Hamza *IntechOpen* [on-line] Available: https://www.intechopen.com/books/vitamin-c/vitamin-c-an-antioxidant-agent

King Y 2019 The effects of vitamin C & folic acid on the growth of plants [on-line] Available: https://www.hunker.com/13429040/the-effects-of-vitamin-c-folic-acid-on-the-growth-of-plants

Chaikhiri A and Chouychai W 2017 Effect of media on growth of *Cymbidium finlaysonianum* in vitro and effect of fertilizer on acclimatization *Khon Kaen A. J.* 45(S) 1197-202

Veggies for all 2016 V8® Vegetable juice original [on-line] Available: https://www.campbells.com/v8/vegetable-juice/v8-original/

Borlongan J 2017 What effects does citric acid have on plant life [on-line] Available: https://www.gardenguides.com/92182-effects-citric-acid-plant-life.html

Nisar N, Li L, Lu S, Chi Khin N and Pogson B J 2015 Carotenoid metabolism in plants *Cell Press*. 8 68-82

Tantasawat P A, Khairum A, Arsakit K, Poolswat O, Pornbunkerd P and Kativat C 2015 Effects of different culture media on growth and proliferation of *Dendrobium* ‘Earsakul’ protocorm-like bodies *HortTechnology* 25 681-6

Sebastianraj J, Britto S J, Kumar D V, Robinson J P and Thangavel P 2014 Rapid propagation of *Vanda testacea* (Lindl.) Rchb.F. – A highly medicinal value epiphytic orchid of India *WSRJ*. 10 223-30

Akter S, Nasiruddin K M and Hossain K 2008 Effects of different media and organic additives interaction on in vitro regeneration of *Dendrobium* orchid *J. Agric. Rural. Dev.* 6 69-74

Levesque R and SPSS Inc 2006 SPSS *Programming and Data Management: A Guide for SPSS and SAS* 4th ed SPSS (Chicago: SPSS Inc.) p 540

Gansau J A, Indan H, Abdullah S N, David D, Marbawi H and JawanR 2016 Effects of organic additives and plant growth regulators on protocorm development of *Dendrobium lowii* *Transactions on Science and Technology* 3 462-8

Gnasekaran P, Rathinam X, Sinniah U R and Subramaniam S 2010 A study on the use of organic additives on the protocorm-like bodies (PLBs) growth of *Phalaenopsis violacea* orchid *J. Phytol*. 2 29-33

Nambiar N, Tee C S and Maziah M 2012 Effects of organic additives and different carbohydrate sources on proliferation of protocorm-like bodies in *Dendrobium Alya Pink Plant Omics J.* 5 10-8

Chory J, Aguilar N and Peto C A 1991 The phenotype of *Arabidopsis thaliana det1* mutants suggests a role for cytokinins in greening *Symp. Soc. Exp. Biol.* 45 21-9

Chory J, Reinecke D, Sim S, Washburn T and Brenner M 1994 A role for cytokinins in de-etiolation in *Arabidopsis: det* mutants have an altered response to cytokinins *Plant Physiol.* 104 339-47

Spiro M D, Torabi B and Cornell C N 2004 Cytokinins induce photomorphogenetic development in dark-grown gametophytes of *Ceratopteris richardii* *Plant Cell Physiol.* 45 1252-60

Carimi F, Terzi M, Michele R D, Zottini M and Schiavo F L 2004 High levels of the cytokinin BAP induces PCD by accelerating senescence *Plant Sci*. 166 963-9

Otroshey M, Khalili Z, Ebrahim M A, Negou M K and Moradi K 2013 Effect of growth regulators and explant on plant regeneration of *Solanum lycopersicum* L. var. *cerasiforme*.
[33] Hossain M A, Bosch S M, Vivancos P V, Burritt D, Fujita M and Lorence A 2018 *Ascorbic Acid in Plant Growth, Development and Stress Tolerance* (Switzerland: Springer International Publishing AG)