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Effect of growth regulator on shoot induction from protocorm of *Dendrobium* Anna

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Abstract Result showed that after 8 weeks of culture, treatment which supplemented with BA 2 mg/l was revealed to be suitable as a medium in tissue culture of *Dendrobium*. It showed the highest number of shoot (19.6 shoots), shoot length (1.30 cm), fresh weight (1.82 g), dry weight (0.28 g), chlorophyll A and chlorophyll B (0.19 and 0.11 mg/g FW, respectively) in treatment which supplemented with BA 2 mg/l. Total non-structural carbohydrate (TNC) showed the highest value in treatment which supplemented with NAA 0.5 and BA 0 mg/l (5.54 mg/FW). Therefore, the supplemented with BA 2 mg/l enhanced shoot induction of *Dendrobium* from protocorm culture and recommended for commercial production.

Keywords: Tissue culture, Plant growth regulator, Dendrobium, Orchid

Introduction

Orchid is a monocotyledon in the family Orchidaceae. It is a popular cut flower due to the beautiful flowers and beautiful patterns. There are good as cut flower that has a long flowering. Orchids are an important economic plant of Thailand. Because it is exported to foreign countries, making annual income of hundreds millions baht. In 2009 the department of international trade promotion ministry of commerce, royal Thai government report that the Export orchid species of Thailand, for example: Dendrobium, Aranda, Arachnis, Oncidium and Vanda. (Prangnuch, 2019). There are many varieties of orchids grown in Thailand especially, Dendrobium orchids. It is the largest orchid genus among various orchids. Thailand is a tropical country where produce many kinds of orchids in nature. We cultured the orchids for conservation and propagation. Because of the orchid in nature have been decrease rapidly, especially the wild orchids that the market has high demands. So, many species of orchids in Thailand are endangered species (Thammasiri, 2015). Potted *Dendrobium* orchids are produced in a large scale in many countries. *Dendrobium* is the second-largest orchid genus which consist more than 1,000 natural species

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(Puchooa, 2004). Thailand is one of the most important area for tropical orchids in the world. There are many varieties of orchids grown in Thailand especially, *Dendrobium* orchids. It is the largest orchid genus among various orchids. Currently plays a role in the Thailand orchid industry more than other orchids and export to many countries around the world. *In vitro* culture techniques have been successfully adapted for orchid plantlets mass propagation (Arditti, 2008). Nowadays, tissue culture technique has been used in commercial propagation in orchid industrial. Protocom-like body formation, shoot multiplication and callus culture had been used in conventional tissue culture protocols for orchid mass propagation (Gow *et al*., 2009). There are many factors that lead to the success of tissue culture technique for example; seed maturity, genotype, media, plant growth regulators, carbohydrates and type of explants (Pati *et al.*, 2005; Nhut *et al.*, 2010). The medium in tissue culture in a sterile condition, based on the formulas of medium and plant regulator that are put into the medium, which the groups that are commonly used are the auxin and cytokinin groups to stimulate cell division and increase the number of cells. Plant regulator in auxin group which is used in orchids such as naphthalene acetic acid (NAA), 2,4-dichloro-phenoxy acetic acid (2,4-D), indole-3- Acetic acid (IAA) and indole-3-butyric acid (IBA). The group of cytokinin includes Benzyladenine (BA), benzylaminopurine (BAP) and 6-furfurylaminopurine (kinetin) (Tawatchai *et al*., 2013). Previous research reported that PLBs of *Dendrobium* were induced from the vertically-cultured of leaf explants in medium containing with 3 mg/L NAA or 5 mg/L BAP (Tee *et al*., 2010). This research interested in studying the plant regulators which are BA and NAA that effect to induce shoot from callus of *Dendrobium* spp. Due to BA which in cytokinin plant regulator can increase cell division faster and produce more calluses. Moreover, cytokinin when working with auxin, will encourage cell division of root and also increase protein, RNA and DNA. For naphthalene acetic acid (NAA) in plant tissue cultures normally use to induce roots. Thus, the objectives of this study were studied the effect of MS medium supplemented with different concentrations of BA and NAA that effects to induce shoot from callus of *Dendrobium* Anna. and find the suitable medium formula for induce shoot from callus.

Materials and methods

Plant materials

Protocorm of *Dendrobium* Anna. were selected in the same size which are 0.3 cm. (Figure. 1) The experiment was carried out by studying the induction of shoot from *Dendrobium* Anna. callus on MS medium (Murashige and Skoog, 1962) supplemented with NAA and BAA with different concentrations, consisting of 9 treatments, with 5 replications, each

replication contained 5 calluses. The pH was adjusted to 5.6. The MS medium supplemented with 30 g/l of sugar and 8 g/l of agar was added. The concentration of NAA was 0, 0.1 and 0.5 mg/l and BA was 0, 2 and 3 mg/l. The following experiments:

Treatment T1: MS medium with NAA 0 mg/l and BA 0 mg/l
Treatment T2: MS medium with NAA 0 mg/l and BA 2 mg/l
Treatment T3: MS medium with NAA 0 mg/l and BA 3 mg/l
Treatment T4: MS medium with NAA 0.1 mg/l and BA 0 mg/l
Treatment T5: MS medium with NAA 0.1 mg/l and BA 2 mg/l
Treatment T6: MS medium with NAA 0.1 mg/l and BA 3 mg/l
Treatment T7: MS medium with NAA 0.5 mg/l and BA 0 mg/l
Treatment T8: MS medium with NAA 0.5 mg/l and BA 2 mg/l
Treatment T9: MS medium with NAA 0.5 mg/l and BA 3 mg/l

All flasks were transferred to growth room at 25 ± 2 ℃ under white florescent light with intensity 2000 lux for the growth and development.

Figure 1. The appearance of protocorm were selected for culture on the medium with difference concentration of NAA and BA

Data collection

The number of root, number of shoot, shoot length, fresh weight, dry weight, Chlorophylly A content, Chlorophyll B content and Total non-structural carbohydrates (TNC) were check and recorded.

Chlorophyll content

After 8 weeks of culture, the fresh leave from each treatment were selected to analyze chlorophyll content. Chlorophyll analysis was check. The method is modified from Arnon *et al.* (1954), fresh leaves were cut from seedling into small pieces. Weight the sample 1 g per treatment and soaked with 80% acetone. Keep the sample overnight in the darkness. Samples were homogenized and filtered into 50 ml volumetric flask through filter paper (Whatman No.2). Add 5-10 ml 80% acetone and homogenized

the sample again for 2-3 times. Then add 80% acetone until 50 ml. The absorbance of extract solution at 440.5 nm, 645 nm, and 663 nm were measured by using spectrophotometer. The formulation for calculated the chlorophyll content follow by:

- Chlorophyll a (mg) = (12.7 x 633 nmOD - 2.69 x 645 nmOD) x V/ (1000 x W)
- Chlorophyll b (mg) = (22.9 x 645 nmOD – 4.68 x 663 nmOD) x V/ (1000 x W)
- Total Chl (mg) = (20.2 × 645 nmOD＋8.02 × 663 nmOD) × V / (1000 ×W)

V= Volume of 80% acetone (50 ml)
W = Sample weight (1 g)

Total non-structural carbohydrate

After 8 weeks of culture, the plantlets from each treatment were selected to analyze Total non-structural carbohydrates (TNC). Analysis of TNC were used dried plantlets samples 0.05 g then add 0.2 N H_2SO_4 40 ml. Cover the container with aluminum foil and put in oven at 70 ℃ for 2 hours. Waiting until it cool and adjust the pH to 7.00, then add 50 ml of distilled water, filtered with whatman No.1 filter paper for further analysis (Smith, 1969).

First preparation of standard curve by using glucose solutions (D-Glucose) 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml. Use 1 ml of standard solution add to 1 ml of Nalson's alkaline copper regent, cover with aluminum foil and soak in water bath for 20 minutes. After cool down, add 1 ml of arsenomolybdic acid regent with 7 ml of distilled water. The absorbance was determined by a spectrophotometer at 540 nm.

To determined TNC by the Nelson reducing sugar procedure method described by Hodge and Hofreiter (1962). To analyze the amount of TNC used 1 ml of the extraction solution following to the preparation of standard curve compared to the standard graph. To calculate TNC using Equation:

TNC = [(mg) glucose equivalent * 50 ml] / (weight of sample * vol.)

Statistical analysis

The experiment was performed in Completely Randomized Design (CRD) with 9 treatments, and 5 replications. Each replication contained 5 call. Data were analyzed by analysis of variance and treatments were compared using the least significance difference (LSD) test at P=0.05 using SPSS software program (SPSS Inc.; Chicago, IL, USA).

Results

Effect of NAA and BA concentration on shoot induction

When the protocorm were cultured on MS media supplemented with 0, 0.1, and 0.5 mg /l of BA and 0, 2, 3 mg/l of NAA for 8 weeks, it was found that the highest number of shoot showed treatment 2 (0 mg/l of NAA and 2 mg/l of BA) following by treatment 1(0 mg/l of NAA and 0 mg/l of BA), 4 (0.1 mg/l of NAA and 0 mg/l of BA) and 9 (0.5 mg/l of NAA and 3 mg/l of BA). The number of shoot in treatment 2, 1, 4 and 9 were 19.6, 11.4, 6.6 and 5.5 shoots, respectively which show in table 1. The treatment that showed the lowest number of shoot was treatment 6 (0.1 mg/l of NAA and 3 mg/l of BA), which were 3.6 shoots (Table 1).

Table 1. The effect of different concentration of NAA and BA on shoot induction of *Dendrobium* Anna after 8 weeks of culture

Treatment	NAA concentration (mg/l)	BA concentration (mg/l)	No. of shoot	Shoot length (cm)	No. of Root
T1	0	0	10.8 ± 0.89^{b}	1.15 ± 0.03$^{a1/}$	2.40 ± 0.55^{c}
T2	0	2	19.6 ± 1.34^{a}	1.30 ± 0.10^{a}	3.60 ± 0.89^{ab}
T3	0	3	7.8 ± 0.84^{bc}	1.26 ± 0.08^{a}	2.20 ± 0.83^{c}
T4	0.1	0	5.6 ± 0.55^{c}	0.92 ± 0.10^{b}	1.80 ± 1.09^{d}
T5	0.1	2	4.2 ± 0.84^{cd}	1.01 ± 0.09^{b}	3.40 ± 1.14^{b}
T6	0.1	3	3.6 ± 1.14^{d}	1.02 ± 0.09^{b}	3.60 ± 0.54^{ab}
T7	0.5	0	11.4 ± 1.14^{b}	1.23 ± 0.09^{a}	3.40 ± 1.14^{b}
T8	0.5	2	9.0 ± 0.76^{bc}	0.99 ± 0.15^{b}	5.80 ± 0.83^{a}
T9	0.5	3	6.6 ± 0.89^{c}	1.18 ± 0.14^{a}	3.30 ± 0.70^{b}

1/:Values showing the mean ± SD in a column followed by similar letters do not different significantly at $p < 0.05$.

Effect of NAA and BA concentration on shoot length

After 8 weeks of cuture, the results indicated that the appropriate concentration of NAA (0 mg/l) and BA (2 mg/l) have the effect to increase the shoot length. Treatment 2 (0 mg/l of NAA and 2 mg/l of BA) showed the highest shoot lenght which was 1.30 cm following by treatment 3 (0 mg/l of NAA and 3 mg/l of BA), treatment 7 (0.5 mg/l of NAA and 0 mg/l of BA), treatment 9 (0.5 mg/l of NAA and 3 mg/l of BA) and treatment 1 (0

mg/l of NAA and 0 mg/l of BA). The lowest shoot lenght was showed in treatment 4 (0.1 mg/l of NAA and 0 mg/l of BA) which was 0.92 cm (Table 1).

Effect of NAA and BA concentration on number of root

The highest number of roots (5.80 roots) was recorded at treatment 8 (0.5 mg/l of NAA and 2 mg/l of BA) 8 week of culture following by treatment 2 (0 mg/l of NAA and 2 mg/l of BA) and treatment 6 (0.1 mg/l of NAA and 3 mg/l of BA) which had 3.60 roots per shoot. While the lowest number of root (2.20 and 2.40 roots per shoot) was record at treatment 3 (0 mg/l of NAA and 3 mg/l of BA) and control, respectively (Table 1).

Effect of NAA and BA concentration on fresh weight

The highest result of fresh weight showed in treatment 2 (0 mg/l of NAA and 2 mg/l of BA) following by treatment 1 (0 mg/l of NAA and 0 mg/l of BA) and treatment 9 (0.5 mg/l of NAA and 3 mg/l of BA) which are 1.83, 1.54 and 1.12 g, respectively. The treatment that showed the lowest fresh weight are treatment 4 (0.1 mg/l of NAA and 0 mg/l of BA), treatment 3 (0 mg/l of NAA and 3 mg/l of BA) and treatment 6 (0.1 mg/l of NAA and 3 mg/l of BA). The fresh weight of these 3 treatments are 0.57, 0.56 and 0.52 g, respectively (Table 2).

Table 2. The effect of different concentration of NAA and BA on fresh and dry weight of *Dendrobium* Anna after 8 weeks of culture

Treatment	NAA concentration (mg/l)	BA concentration (mg/l)	Fresh weight (g)	Dry weight (g)
T1	0	0	$1.54 \pm 0.23^{b1/}$	0.18 ± 0.06^{d}
T2	0	2	1.83 ± 0.55^{a}	0.28 ± 0.03^{a}
T3	0	3	0.57 ± 0.03^{d}	0.26 ± 0.07^{b}
T4	0.1	0	0.56 ± 0.06^{d}	0.24 ± 0.02^{c}
T5	0.1	2	0.67 ± 0.10^{cd}	0.19 ± 0.01^{d}
T6	0.1	3	0.52 ± 0.16^{d}	0.14 ± 0.02^{e}
T7	0.5	0	1.44 ± 0.69^{bc}	0.23 ± 0.03^{c}
T8	0.5	2	0.77 ± 0.20^{cd}	0.24 ± 0.08^{c}
T9	0.5	3	1.12 ± 0.67^{c}	0.24 ± 0.02^{c}

1/:Values showing the mean ± SD in a column followed by similar letters do not different significantly at $p < 0.05$.

Effect of NAA and BA concentration on dry weight

The highest dry weight was achieved on treatment 2 (0 mg/l of NAA and 2 mg/l of BA) following by treatment 3 (0 mg/l of NAA and 3 mg/l of BA) which are 0.28, 0.26 g, respectively. On the other hand, the lowest dry

weight was showed in treatment 5 (0.1 mg/l of NAA and 2 mg/l of BA) and control (0 mg/l of NAA and 0 mg/l of BA) which are 0.19, 0.18 g, respectively (Table 2).

Effect of NAA and BA concentration on the appearance of plantlets

After 1 week of culture, the protocorm of *Dendrobium* Anna had developed initial shoot in all treatment. Root developed on regenerated shoots after 2 weeks of culture, treatment 2 (0 mg/l of NAA and 2 mg/l of BA) is the first treatment followed by treatment 1 (0 mg/l of NAA and 0 mg/l of BA), treatment 7 (0.5 mg/l of NAA and 0 mg/l of BA) and treatment 4 (0.1 mg/l of NAA and 0 mg/l of BA). After 8 weeks of culture, the highest shoot induction was obtained from the medium supplemented with NAA 0 mg/l and BA 2 mg/l (treatment 2). The color of shoot was dark green and have longest roots (Figure 3). The result of the great appearance and dark green shoots showed in treatment 1 (0 mg/l of NAA and 0 mg/l of BA) and treatment 7(0.5 mg/l of NAA and 0 mg/l of BA). While, other treatment showed the light green shoots and small roots (Figure 2).

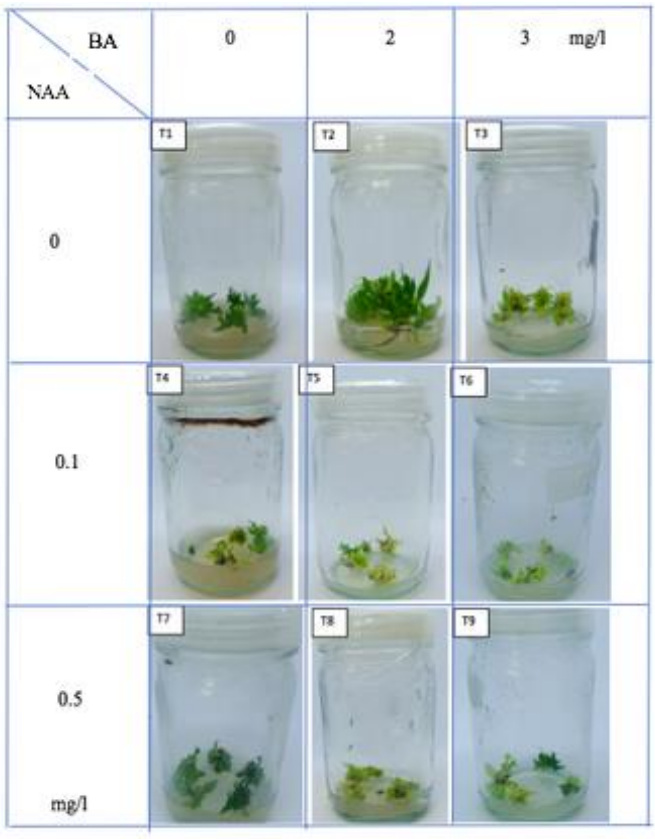

Figure 2. The small plantlets after 8 weeks of culture on MS medium supplemented with different concentration of BA and NAA

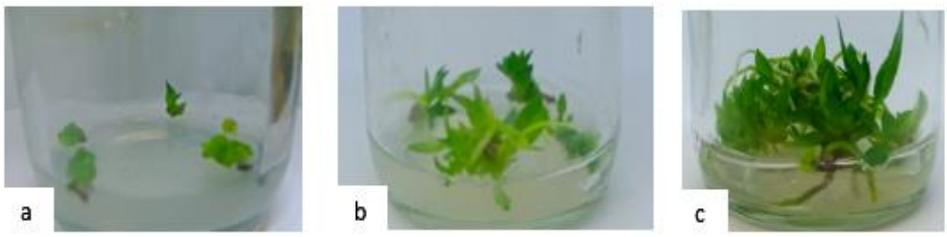

Figure 3. Shoot initiation and plantlet regeneration of *Dendrobium* Anna on MS medium supplement with 0 mg/l of NAA and 2 mg/l of BA after (a) 1 week of culture (b) 4 weeks of culture (c) 8 weeks of culture

Effect of NAA and BA concentration on chlorophyll content

After 8 weeks of culture, the result showed that treatment 2 (0 mg/l of NAA and 2 mg/l of BA) had the highest chlorophyll A content (0.186 mg/gFW) and chlorophyll B content (0.112 mg/100gFW). The following result also showed in treatment 1(0 mg/l of NAA and 0 mg/l of BA) that have chlorophyll A content (0.184 mg/gFW) and chlorophyll B content (0.103 mg/gFW). On the other hand, treatment 6 (0.1 mg/l of NAA and 3 mg/l of BA), 5 (0.1 mg/l of NAA and 2 mg/l of BA) and 8 (0.5 mg/l of NAA and 2 mg/l of BA) were showed the lowest content of chlorophyll A content (0.049, 0.048 and 0.048 mg/gFW). The lowest chlorophyll B content were showed in treatment 5 (0.1 mg/l of NAA and 2 mg/l of BA), 6 (0.1 mg/l of NAA and 3 mg/l of BA), 3 (0 mg/l of NAA and 3 mg/l of BA) and 8 (0.5 mg/l of NAA and 2 mg/l of BA) (0.032, 0.032, 0.030 and 0.028 mg/gFW) (Table 3).

Table 3. The effect of different concentration of NAA and BA on chlorophyll content of *Dendrobium* Anna after 8 weeks of culture

Treatment	NAA concentration (mg/l)	BA concentration (mg/l)	Chlorophyll A (mg/gFW)	Chlorophyll B (mg/gFW)
T1	0	0	$0.184 \pm 0.002^{a1/}$	0.103 ± 0.003^{b}
T2	0	2	0.186 ± 0.001^{a}	0.112 ± 0.003^{a}
T3	0	3	0.054 ± 0.001^{e}	0.030 ± 0.003^{e}
T4	0.1	0	0.118 ± 0.002^{b}	0.068 ± 0.002^{c}
T5	0.1	2	0.048 ± 0.002^{f}	0.032 ± 0.004^{e}
T6	0.1	3	0.049 ± 0.002^{f}	0.032 ± 0.003^{e}
T7	0.5	0	0.095 ± 0.002^{d}	0.055 ± 0.003^{d}
T8	0.5	2	0.048 ± 0.001^{f}	0.028 ± 0.003^{e}
T9	0.5	3	0.107 ± 0.001^{c}	0.059 ± 0.002^{d}

1/:Values showing the mean ± SD in a column followed by similar letters do not different significantly at $p < 0.05$.

Effect of NAA and BA concentration on total non-structural carbohydrates content

When the protocorm were cultured on MS media supplemented with 0, 0.1, and 0.5 mg /l of NAA and 0, 2, 3 mg/l of BA for 8 weeks, it was found that the highest TNC content showed in treatment 7 (0.5 mg/l of NAA and 0 mg/l of BA). It was recorded that TNC content of treatment 7 was 5.54 mg/gFW. Follow by treatment 5 (0.1 mg/l of NAA and 2 mg/l of BA) and treatment 2 (0 mg/l of NAA and 2 mg/l of BA) which was the lowest TNC was showed in treatment 1 (0 mg/l of NAA and 0 mg/l of BA) which was 2.34 mg/gFW (Table 4).

Table 4. The effect of different concentration of BA and NAA on TNC content of *Dendrobium* Anna after 8 weeks of culture

Treatment	NAA concentration (mg/l)	BA concentration (mg/l)	TNC (mg/gFW)
T1	0	0	$2.34 \pm 0.04^{h1/}$
T2	0	2	4.77 ± 0.02^{c}
T3	0	3	3.73 ± 0.03^{f}
T4	0.1	0	4.46 ± 0.15^{d}
T5	0.1	2	5.08 ± 0.01^{b}
T6	0.1	3	4.31 ± 0.01^{e}
T7	0.5	0	5.54 ± 0.18^{a}
T8	0.5	2	3.63 ± 0.02^{f}
T9	0.5	3	3.12 ± 0.01^{g}

1/:Values showing the mean ± SD in a column followed by similar letters do not different significantly at $p < 0.05$.

Discussion

According to the result, it showed that the positive result of BA on shoot induction. The result showed that after 8 weeks of culture, the treatment which supplement with BA 2 mg/l without NAA showed the highest number of shoot, shoot length, fresh weight and dry weight. Cytokinins have proficiency to enhance cell division and also have been affected to shoot and root development (Moubayidin *et al*., 2009). BA supplemented medium also showed the highest response of shoot induction which can use to induce shoot from shoot tip of *Ruta graveolens*. The result showed the highest number of shoots (9.4) and shoot length (4.3 cm) on MS medium supplemented with 10 μM of BA (Faisal *et al*., 2018). Erisen *et al*. (2010) have also reported the highest shoot formation in the treatment 0.2 mg/l BA after 6 weeks of culture. Similarly, with Riva *et al.* (2016) reported the highest percentage of shoot induction was found in MS treatment that supplement with 0.2 mg/l BA in *Dendrobium bensoniae*. Kim (2003) also reported the effect of BA in the culture medium is important for shoot regeneration. This result also similar to previous work of several researches

on *Dendrobium densiflorum* (Roy, 2003). Shoot induction may be varied because of plant growth regulator concentration especially cytokinin.

The effect of root formation showed that the highest number of roots was recorded at treatment 8 (0.5 mg/l of NAA and 2 mg/l of BA) 8 week of culture which is 5.8 roots. NAA is a hormone in the auxin group that has the effect of stimulating the rooting well especially, in orchids. The previous report also showed the similar results that auxin can stimulate the root of Dendrobium callus. Kong *et al*. (2007) studied the effect of NAA on root induction. The result showed that ½ MS medium supplemented with 0.2 mg/l NAA enhanced the protocorm of *Dendrobium strongylanthum* Rchb.f. orchids from seed explant. While, ½ MS medium supplemented with 0.5 mg/l NAA with mashed banana promoted root formation. The combination of auxin and cytokinin ratio is the effect that potential signaling molecule to control plant growth and development. The medium that supplement with high ratio of auxin than cytokinin usually induce root formation. While, the medium that supplement with high ratio of cytokinin than auxin usually induce shoot formation. From our result also show that the medium that supplement with high BA (Treatment 2) showed the highest number of shoot and shoot length. Moreover, the medium that supplement with high NAA (Treatment 5) showed the highest number of root.

Chlorophyll is a photosynthetic pigment which found in plants and green algae. Two types of chlorophyll which are chlorophyll, a and b in the ratio approximately 3:1 in higher plants have the propoties to absorb light mainly in two different wavelength (650 – 700 nm and 400 – 500 nm) of the visible spectrum. (Aminot and Ray, 2000; Arnon, 1954). It is essential for photosynthesis which is the important physiological processes in plants, hence influencing growth and development. In this study, the result of the highest chlorophyll a and chlorophyll b content also show in the treatment that supplement with BA 2 mg/l without NAA. Nikolić *et al.* (2006) and Patil *et al.* (2012) reported that the exogenous cytokinin can stimulate specific metabolic activity for seed germination and growth. Moreover, BA is an extremely active cytokinin that improve efficiency of photosynthesis mechanism of Feba beans tissue culture (Ron'zhina, 2003). Our results supported the experiment of Akram and Aftab (2015) which found that the highest amount of chlorophyll a (1.86 mg/gFW) and chlorophyll b (1.90 mg/gFW) by supplement with BA.

Total non-structural carbohydrates or TNC are organic compounds that are important to all plants. These compounds are derived from photosynthesis. Most of them are single molecule sugars such as glucose, fructose, etc. These plants are used for growth and reproductive development and are stored in the form of starch in different parts of the plant such as branches, stems or roots, etc. In our experiment, it was found that the highest TNC content showed in treatment that supplement with 0.5 mg/l of NAA and 0 mg/l of BA. From result it was showed that auxin

effected to TNA content. To determine TNC result also showed the highest value in the treatment supplement with NAA 0.5 mg/l. TNC include starch and sugar but starch is the major from of TNC (Pisamai *et al.*, 2007).

Therefore, which supplemented with NAA 0 and BA 2 mg/l could enhance shoot induction of *Dendrobium* ANNA from protocorm culture faster and can be recommended for commercial production.

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