

## Exquisite protocol of callus induction and protocorm-like bodies (PLBs) regeneration of *Dendrobium sonia-28*

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### Abstract

Protocorm-like bodies (PLBs) of *Dendrobium sonia-28* were used as explant for callus induction. The PLBs segments were cultured on half strength MS semi-solid medium supplemented with different concentrations of 1-naphthaleneacetic acid (NAA) (0.05, 0.1, 0.25, 0.5, 0.75, 1.0 and 2.0 mg/L) or/with 2,4-dichlorophenoxyacetic acid (2,4-D) (0.001, 0.005, 0.01, 0.05, 0.1, 0.25 and 0.5 mg/L) alone or in combinations for six weeks. The medium contained with the combination of 1.0 mg/L NAA and 0.1 mg/L 2,4-D was optimal for callus induction. After six weeks of culture, whitish yellow and friable callus were obtained. Callus proliferated very well on the optimal medium without tryptone treatment after six weeks of culture. The calluses were also regenerated into PLBs from the medium with 20 g/L sucrose and without plant growth regulators after eight weeks of culture.

**Keywords:** *Dendrobium sonia 28*, Protocorm-like bodies (PLB), Callus, Regeneration.

**Abbreviation:** MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, benzlamino-purine; ANOVA, One-Way Analysis of Variance.

### Introduction

In family Orchidaceae, *Dendrobium* orchids have been the major orchid cut flowers export for Malaysia, Thailand, and Philippines. In Malaysia, the export value of total orchids is estimated at RM40 million per year and about 11.7% of the export value is comprised of the *Dendrobium* orchid's production (as been reviewed by Khosravi et al., 2009). Callus is an undifferentiated and non-organized tumor tissue that arises from the wound sites of the differentiated plant tissues and organs (George and Sherrington, 1984). Many types of explants tissues are used as starting materials for callus induction such as leaves, flowers, roots, and others. The pseudobulb sections, rhizomes and roots of *Cymbidium ensifolium* seedlings have shown to induce formation of callus (Chang and Chang, 1998). Callus formation occurs when plant growth regulators added in the nutrient medium. Ishii et al. (1998) was the first to show that callus of *Phalaenopsis* can be induced from PLB segments on the culture medium containing sucrose. In addition, PLBs were reportedly to be induced from the vertically-cultured of leaf explants of *Dendrobium* in medium containing with 3 mg/L NAA or 5 mg/L BAP (Tee et al., 2010). In this study, 1-naphthaleneacetic acid (NAA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) are used for induction of callus from PLBs of *Dendrobium sonia-28*. Protocorm-like bodies (PLBs) were selected as the choice of explants in this research because it can be grown through *in vitro* culture from various organs and tissues (Kuehnle, 2006). In this study, we reported on the best medium formulation for callus induction from PLBs, the effect of tryptone on callus proliferation and the effect of coconut water and sucrose for regenerations of callus to PLBs.

### Results

#### *Callus induction from PLBs of Dendrobium sonia-28*

#### *Effects of NAA on callus induction*

After two weeks of culturing, white granules emerged from the surface PLBs segments. Subsequently, after six weeks, whitish yellow and friable callus were formed. The callus were induced from PLBs segments in different treatments with different frequencies rate. Figure 2 shows the percentage of PLBs segments formed into callus, callus/PLBs, PLBs and died PLBs segment. The medium which yielded the highest percentage of callus formation was in the medium containing with 0.1 mg/L of NAA, at the percentage of 17.14%. The lowest percentage of callus formation from PLBs segments were obtained from the medium supplemented with 0.5, 0.75, 1.0 and 2.0 mg/L NAA respectively, at 2.86%. The frequency of callus formation increased with NAA concentration when reaching 0.1 mg/L. With higher concentration than 0.1 mg/L NAA was noticed a reducing of frequency of callus formation. The highest percentage of callus/PLBs formation from PLBs segments was on medium contained with 1.0 mg/L NAA, resulted at 22.86%. For the lowest percentage of formation of callus/PLBs, it was on the medium contained 0.75 mg/L NAA with 2.86%. The medium with 0.5 and 0.75 mg/L NAA respectively, showed the highest percentage of PLBs formation at 91.43%, meanwhile the medium supplemented with 0.1 mg/L NAA produced the lowest percentage of PLBs formation (71.43%). In addition, the medium contained 0.25

**Table 1.** Effects of tryptone on callus proliferation on half strength MS semi-solid medium supplemented with 1.0 mg/L NAA, 0.1 mg/L 2,4-D and various tryptone concentrations (0, 2, and 4 g/L) respectively after six weeks of culture.

Tryptone (g/L)	Mean Ratio Weight (Final weight/Initial weight)	Percentage Explants Proliferated Well (%)	Morphogenesis
0	6.494 ab	100.00±0 a	Callus
2	8.714 a	68.00±10.95 b	Embryogenic callus
4	5.500 b	28.00±17.89 c	Embryogenic callus

Means followed by different letters are significantly different at p=0.05 according to Tukey Test

**Table 2.** Percentage (%) of explants formed PLBs, shoot/PLBs, no response on half strength MS semi-solid medium contained with 20 g/L sucrose, 200 ml/L coconut water, 20 g/L sucrose and 200 ml/L coconut water respectively after two months of culture.

Treatment	Percentage of Explants formed PLBs (%)	Percentage of Explants formed shoots/PLBs (%)	Percentage of Explants showed No Response (%)
Medium with Sugar	92.00±10.95 a	0 b	8.00±10.95 a
Medium with Coconut Water	88.00±17.89 a	0 b	12.00±17.89 a
Medium with Sugar and Coconut Water	32.00±10.95 b	52.00±10.95 a	16.00±8.94 a

Means within a column followed by same letters are not significantly different with Tukey Test at p=0.05.

mg/L and 0.75 mg/L NAA respectively were found 2.86% of death PLBs segments.

#### *Effects of 2, 4-D on callus induction*

Whitish yellow callus were formed from the edge of cut PLBs segments after six weeks. The calluses induced were friable in structure. Figure 3 shows the percentage of PLBs segments formed into callus, callus/PLBs, PLBs and died PLBs segment. The highest percentage of callus formation were on the medium contained with 0.001 mg/L and 0.25 mg/L 2,4-D respectively was at 17.14%. The lowest percentage of callus formation were on the medium supplemented with 0.05 mg/L and 0.5 mg/L 2,4-D was at 2.86%. For the concentration between 0.001 mg/L to 0.05 mg/L 2,4-D, the percentage of callus formation had decreased from 17.14% to 2.86%. For concentration between 0.05 mg/L to 0.25 mg/L, the percentage of formation of callus had increased again from 2.86% to 17.14%. Meanwhile, the callus formation's frequency had reduced on medium with 0.5 mg/L 2,4-D treatment. The highest percentage of callus/PLBs formation was on the medium with 0.01 mg/L 2,4-D that was yielded 31.43%, while the lowest percentage of callus/PLBs formation was on the medium with 0.5 mg/L 2,4-D which was at 2.86% (Fig. 3). Besides that, the medium with 0.1 mg/L and 0.5 mg/L 2,4-D respectively were produced the highest percentage of PLBs that could induced into callus formation at 85.71%. The lowest percentage of PLBs formed was the medium supplemented with 0.01 mg/L and 0.25 mg/L 2,4-D (54.29%). Moreover, the medium with different concentrations of 0.001, 0.01, 0.25 and 0.5 mg/L yielded brownish death PLBs segments. For the medium with 0.001, 0.01, and 0.25 mg/L respectively, the percentage of PLBs segment died was at 2.86%.

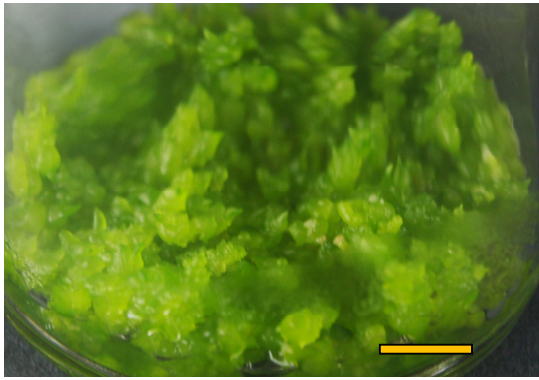
#### *Effects of combinations of different concentrations NAA with constant 0.01 mg/L 2, 4-D*

Calluses were observed to be induced at the cut edges of the explants. All of the callus formed on different combination of treatments were whitish yellow in colour and friable in structure. Figure 4 highlighted the percentage of formation of callus, callus/PLBs, PLBs, and died from the PLBs segments cultured on medium. The medium with the highest

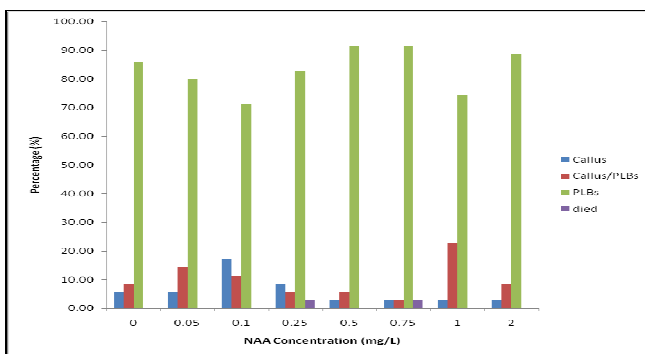
percentage of formation of callus was the medium with 0.1 mg/L and 0.5 mg/L NAA respectively which was at 20%. The second highest was the medium contained with 1.0 mg/L NAA and 0.01 mg/L 2,4-D at 14.29%. The medium supplemented with 0.05 mg/L and 2.0 mg/L NAA with constant 0.01 mg/L 2,4-D respectively, yielded the lowest percentage of callus formation at 5.71%. For callus/PLBs formation, the medium with 2.0 mg/L NAA and 0.01 mg/L 2,4-D produced the highest percentage at 42.86%. The lowest percentage was on medium with 0.25 mg/L NAA and 0.01 mg/L 2,4-D, with the percentage of 11.43. Medium with 0.05 and 0.25 mg/L NAA and 0.01 mg/L 2,4-D produced highest percentage of PLBs formation at 80%, meanwhile the medium with 0.5 and 2.0 mg/L NAA and 0.01 mg/L 2,4-D displayed the lowest percentage which was at 51.43%. In addition, 2.86% of death PLBs segment was found on medium with 0.5 mg/L NAA and 0.01 mg/L 2,4-D.

#### *Effects of combinations of different concentrations NAA with constant 0.1 mg/L 2,4-D*

All the combinations of treatments were displayed with different frequency of callus formation. After six weeks, calluses were induced from edges of cut PLBs segments and covered the whole PLBs segments. Callus formed were in friable structure and whitish yellow in colour. The percentage of formation of callus, callus/PLBs, PLBs and died from PLBs segment cultured on the medium was shown in Figure 5. With cultured on half strength MS semi-solid medium with 1.0 mg/L NAA and 0.1 mg/L 2,4-D, the percentage of callus induced was the highest among all in the other combinations. The highest percentage of callus formation was 34.29%. Then, the second highest percentage were the medium with 0.75 mg/L and 2.0 mg/L NAA respectively with 0.1mg/L 2,4-D which were 28.57%. The lowest percentage of callus formation with 5.71% was on the medium with 0.1mg/L NAA and 0.1mg/L 2,4-D. The medium with 0.05 mg/L NAA and 0.1 mg/L 2,4-D was obtained with the highest percentage of callus/PLBs formation (34.29%), meanwhile the lowest percentage was at 22.86% on medium supplemented with 0.1, 0.25, 1.0 and 2.0 mg/L NAA with 0.1 mg/L 2,4-D, respectively. In addition, medium with 0.1 mg/L NAA and 0.1 mg/L 2,4-D had formed 71.43% of PLBs, which was the highest percentage. For the lowest percentage, there were



**Fig 1.** Four weeks old *in vitro* culture of PLBs *Dendrobium sonia-28* on half strength MS semi-solid medium. The bar represents 1cm.



**Fig 2.** Percentage (%) of explants formed callus, callus/PLBs, PLBs and died PLBs segment on half strength MS semi-solid medium supplemented with different concentrations of NAA (0, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, and 2.0 mg/L ) after six weeks of culture.

40% of PLBs formation on medium with 0.75 mg/L NAA and 0.1 mg/L 2,4-D. However, total of 2.86% death with brownish PLBs segment appearance obtained on medium with 0.05 and 0.75 mg/L NAA and 0.1mg/L 2,4-D.

#### **Effects of tryptone on callus proliferation**

The callus explants continued to proliferate and the weight of callus were measured after six weeks. For the control treatment without addition of tryptone, the mean weight of callus was  $0.649 \pm 0.130$  g (Fig. 6). It was about 6.5-fold increase of weight which the initial weight of callus, 0.100 g. For treatment with 2 g/L tryptone, the mean weight of callus was  $0.871 \pm 0.257$  g, which was about 9-fold increase of weight and the highest mean weight obtained. For the treatment with 4 g/L tryptone, the mean weight of callus formed was  $0.550 \pm 0.082$  g and was the lowest mean weight of callus, with about 5.5-fold of weight increment. Based on Tukey Test at  $p = 0.05$ , the control treatment was significantly different with 2 g/L and 4 g/L tryptone treatments. The colour of callus for the control treatment were white yellowish and friable in structure. For the treatment with addition of 2 g/L tryptone, the callus explants were formed into embryogenic callus forming shoots which were whitish in colour. Moreover, for the treatment with 4

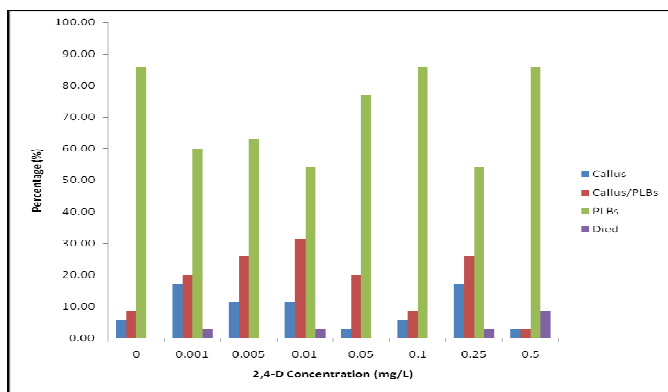
g/L tryptone, the callus explants were also produced embryogenic callus that were whitish in colour. For the percentage of explants proliferated well in medium, there were 100% of explants proliferated well for control treatment. For treatment with 2 g/L and 4 g/L tryptone, there were 68 % and 28 % of explants survived well respectively (Table 1). Based on Tukey Test at  $p = 0.05$ , the percentage of explants well proliferated for control treatment was significantly different from the treatment with tryptone.

#### **Effects of coconut water and sucrose on PLBs formation from callus**

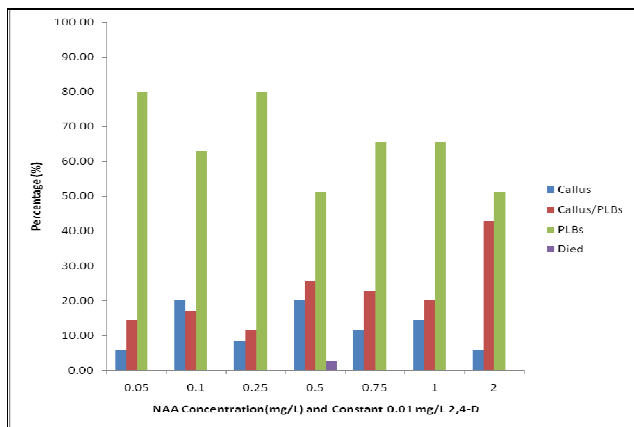
The callus were continued to proliferate and after three weeks of culture, the surface appearance of callus were changed to more compact structure and yellowish in colour. There was also formation of small protuberances. Within two months, the protuberances continued to grow and gradually turned into green colour and formed PLBs. From Figure 7, the highest mean weight of PLBs formation was on the medium with sucrose, which was  $3.467 \pm 1.111$  g. The second highest mean weight of PLBs developed was on the medium contained with coconut water but without sucrose, which was at  $1.600 \pm 0.238$  g. For the medium contained with sucrose and coconut water obtained the lowest mean of weight that was  $1.095 \pm 0.135$  g. Based on Tukey Test at  $p = 0.05$ , the mean weight of PLBs in medium contained with sucrose was significantly different from the medium with coconut water and medium with coconut water and sucrose. The PLBs formed on medium with sucrose was globular in shape, clumped together and greenish in colour. Besides that, there were also some regenerated PLBs with cotyledon structure. For the medium contained with coconut water only, the PLBs formed were also green in colour but the PLBs size were smaller compared with the size of PLBs formed in medium contained with sucrose. The shape of PLBs formed were also not uniform. There were also formation of some regenerated PLBs with cotyledon structure. The size of PLBs formed in medium contained with sucrose and coconut water were in big clumping size and not globular in shape. The colour of PLBs were greenish yellow in colour. There were also shoots and PLBs formation from the callus. From Table 2, the highest percentage of explant formed PLBs for medium contained with sucrose was  $92.00 \pm 10.95$ . For the medium contained with coconut water, the percentage was  $88.00 \pm 17.89$  while the lowest percentage of explant formed PLBs was on the medium with sucrose and coconut water which was  $32.00 \pm 10.95$ . The percentage of PLBs formation on medium with sucrose and the medium with coconut water were not significantly different. But, they were significantly different with medium contained with sugar and coconut water. On medium with sugar and coconut water, there was formation of long shoots/PLBs which the percentage was  $52.00 \pm 10.95$  while the medium with sugar and medium with coconut water had no shoots/PLBs formation. Besides that, there were also explants that showed no response. For medium with sugar and medium with coconut water, the percentage explants showed no response were  $8.00 \pm 10.95$  and  $12.00 \pm 17.89$  respectively. The medium with sugar and coconut water were  $16.00 \pm 8.94$ . They all were not significantly different from each others.

#### **Discussion**

In orchids, the growth of callus is slow and the maintenance of callus is often very difficult (Huan et al., 2004). The callus



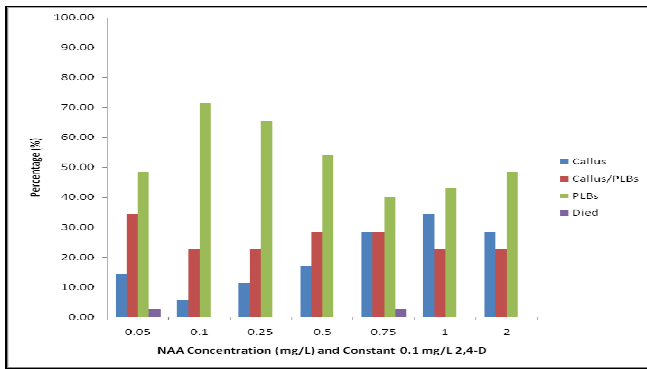
**Fig 3.** Percentage (%) of explants formed callus, callus/PLBs, PLBs and died PLBs segment on half strength MS semi-solid medium supplemented with different concentrations of 2,4-D (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.25, and 0.5 mg/L ) after six weeks of culture.



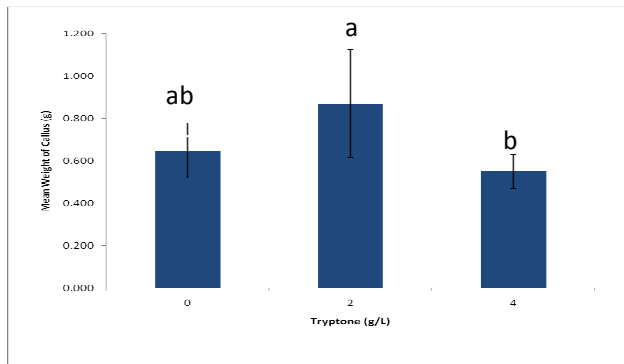
**Fig 4.** Percentage (%) of explants formed callus, callus/PLBs, PLBs and died PLBs segment on half strength MS semi-solid medium supplemented with different concentrations of NAA (0.05, 0.1, 0.25, 0.5, 0.75, 1.0, and 2.0 mg/L ) and constant value of 0.01 mg/L 2,4-D after six weeks of culture.

induced from the inner tissue of PLBs of Cymbidium orchids were turned brown and died after 2 months of incubation (Begum et al., 1994). In recent years, callus lines had been established successfully such as *Cyripedium formosanum* (Lee and Lee, 2003), *Paphiopedilum* orchid (Lin et al., 2000) and *Pleione formosana* Hayata (Lu, 2004). Totipotent callus of *Cyripedium formosanum*, *Paphiopedilum* hybrid and *Pleione formosana* Hayata induced from seed-derived protocorm segments respectively had proliferated well and formed PLBs. The PLBs formed were then regenerated into well-developed plantlets. The callus induction from PLBs segments of *Dendrobium sonia-28* were carried out on the half strength MS semi-solid medium contained with different concentrations NAA, 2,4-D alone or with different combinations treatments. The frequency of callus formation was varied with different types and concentration of plant growth regulators. Callus could be induced from PLBs segments in most treatments including control treatment at different frequencies. The results obtained in the experiment indicated that the exogenous plant growth regulators are

important for callus induction from PLBs segment of *Dendrobium sonia-28*. This is in agreement with other published works on callus formation in orchid species such as *Oncidium*, *Paphiopedilum* orchid, *Dendrobium fimbriatum* Lindl. (Chen and Chang, 2000; Lin et al., 2000; Roy and Banerjee, 2003). The NAA had no significant effect on the induction of callus from shoot-tips of *Dendrobium chrysotoxum* Lindl. The callus formation was noted only on medium with 2  $\mu$ M NAA (0.38 mg/L) and was not significantly different from the control treatment (Roy et al., 2007). Similarly in this experiment, the percentage of callus formation on medium with different concentrations NAA was low. The highest percentage of callus formation was noted only in medium contained with 0.1mg/L NAA (Fig.2). The percentage of callus induced was increased from medium with 0 to 0.1 mg/L, and then decreased from 0.1 mg/L to 2.0 mg/L (Fig. 2). Similarly, Zhao et al. (2008) reported that when the concentration of NAA increased from 0 to 0.2 mg/L, the frequency of callus induction in *Dendrobium candidum* Wall ex Lindl. increased while when NAA concentration increased from 0.2 mg/L to 2 mg/L, the frequency of callus formed were decreased. For the induction of shoot from PLBs of *Encyclia mariae* on the medium supplemented with different NAA, IAA and IBA (0.5, 1.0, 2.0 mg/L) respectively, Maria and Candy (2009) reported that low percentage of callus were obtained on the medium with NAA. However, they had obtained high frequencies of PLBs differentiated into shoots on medium contained with 2.0 mg/L NAA. Similarly in this work, the medium with different concentrations of NAA obtained high percentage of PLBs formation that could subsequently forming shoots (Fig. 2). 2,4-D is one of the most important plant growth regulator for callus induction and formation of callus culture. At higher concentration of 2,4-D, it will become toxic and suppress the cell growth which damage the chromosomes (Fabio et al., 2007). For the different concentrations of 2,4-D treatments, the highest percentage of callus induction from *Dendrobium candidum* Wall ex Lindl. was on the medium with 1.5mg/L 2,4-D meanwhile lowest was on medium with 0.5 mg/L (Zhao et al., 2008). Similar result were observed in this experiment, where medium contained with 0.5 mg/L 2,4-D that had induced lowest percentage of callus formation. At higher concentration of 0.25 mg/L 2,4-D, the percentage of callus obtained was increased to 17.14%. This may due to the PLBs segments that cultured on medium were contained with endogenous hormone which can have synergistic effect with the exogenous plant growth regulators (such as 2,4-D) and thus induce more callus formation. MS medium with lower concentration of 2,4-D at lower than 5 mg/L induced the better callus formation for *Cyperus aromaticus* compared with MS medium supplemented with higher concentration of NAA that is more than 5 mg/L (Chan, 2005). By comparing the medium with NAA and 2,4-D alone respectively, the percentage of callus formation on medium with 2,4-D alone was higher than the medium with NAA alone (Fig. 2 and 3). However, there is an agreement that there was no significant difference for the callus formed on the medium contained with NAA and also the medium contained with 2,4-D (Huan et al., 2004). Janarthanam and Seshadri (2008) also reported that the 2,4-D generated higher frequencies of callus formation of *Vanilla planifolia* Andr. compared with NAA and the percentage response was also not significantly different between the two types of auxin. For the treatments with different combinations of NAA and 2,4-D, different frequencies of callus was induced. There were higher percentage of callus induced while lower percentage of PLBs



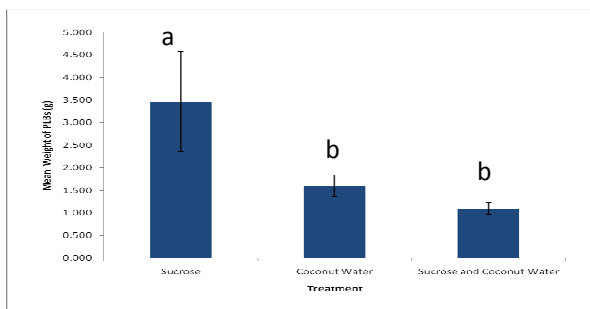
**Fig 5.** Percentage (%) of explants formed callus, callus/PLBs, PLBs and died on half strength MS semi-solid medium supplemented with different concentrations of NAA (0.05, 0.1, 0.25, 0.5, 0.75, 1.0, and 2.0 mg/L ) and constant 0.1 mg/L 2,4-D after six weeks of culture.



**Fig 6.** Mean weight (g) of callus proliferated on half strength MS semi-solid medium supplemented with 1.0 mg/L NAA, 0.1 mg/L 2,4-D and various tryptone concentrations (0, 2, and 4 g/L) after six weeks of culture. Means followed by different letters are significantly different at  $p=0.05$  according to Tukey Test.

formation obtained in combinations treatments compared with NAA or 2,4-D alone (Fig. 4 and 5). This may due to the combination of NAA and 2,4-D act synergistically with each other and induce the cells to dedifferentiate to form callus. With combination of higher concentration of 2,4-D and lower concentration of NAA give better effect for inducing callus formation compared with lower concentration 2,4-D and higher concentration NAA (Kayani, 2008). This showed contradict with the experiment in which the higher concentration of NAA (1.0 mg/L) combined with lower concentration of 2,4-D (0.1mg/L) had yielded the highest percentage of callus. However, this may due to different plant species and also different plant parts were used for callus induction. Different plant species and different plant parts may react differently in different types, concentration and combinations of hormone. The frequencies of callus induction may be varied due to the endogenous hormone contents in plants, their uptake, type of auxins and cytokinins supplemented and also their mode of action (Gupta et al., 2010). The addition of organic nitrogen compounds into basal medium contained with inorganic salts is widely used

in *in vitro* propagation of orchid species (Arditti, 2008). These organic nitrogen compounds generally consist of proteins with low molecular weight, amino acids, vitamins and substances for plant growth. The organic nitrogen compounds have been reported as supplement to proliferate the callus (Chen and Chang, 2000; Meesawat and Kanchanapoom, 2002; Huan et al., 2004). PLBs formation from callus and regeneration of PLBs (Sinha and Roy, 2004), enhance the multiple shoots formation (Ng et al., 2010). There were presence of different types of organic nitrogen compounds, which were casein hydrolysate, peptone and tryptone-peptone (0.5, 1.0 and 2.0 g/L) in the culture medium to induce the multiple shoots formation. The addition of organic nitrogen compounds into the basal medium slightly enhanced the number of multiple shoots formed compared to MS medium without organic nitrogen compounds (Ng et al., 2010). In this experiment, the half strength MS medium contained with tryptone concentration (2 and 4 g/L) had induced the formation of embryogenic callus which showed shooting formation. Meesawat and Kanchanapoom (2002) had concluded that in callus proliferation stage of *Dendrobium crumenatum*, medium supplemented with suitable concentration of plant growth regulators and peptone are required to proliferate the callus vigorously. For *Dendrobium crumenatum*, the callus were proliferated well in Vacin and Went medium with 0.1 mg/L NAA, 1 mg/L BA and 1 g/L peptone (Meesawat and Kanchanapoom, 2002). Embryogenic callus were formed from root tips, stem and leaf segments of *Oncidium* on half strength MS basal medium supplemented with TDZ, 2,4-D and peptone (1 g/L) for 4 to 7 weeks. Embryogenic callus were subcultured on the same medium for callus induction and proliferated well in 1 month of incubation (Chen and Chang, 2000). There are at least two proteins needed to be produced and stored during the development of seed and then they will be metabolized quickly in protocorm development (Meesawat and Kanchanapoom, 2002). The proteins also provide the nutrition source during embryo development into seedling of *Dendrobium crumenatum* as been reported by Meesawat and Kanchanapoom (2002). In this study, the treatment with 2 g/L and 4 g/L tryptone had showed the formation and proliferation of embryogenic callus which are whitish in colour compared with the control treatment, the callus proliferated were white yellowish in colour. Huan et al. (2004) had reported that after four weeks in culture, there were about 10-fold increase of fresh weight of callus which was cultured on the medium supplemented with 0.1 mg/L NAA, 0.01 mg/L TDZ and 2 g/L tryptone. In this study, there were about 9-fold increase of weight of embryogenic callus for treatment with 2 g/L tryptone. However, only 68 % of explants proliferated well and other explants had showed brownish in colour and necrosis. By comparing with control treatment, there were 100 % of callus explants well proliferated. Carbon sources are used to provide energy and osmotic agents in order to support the growth of plant tissue and are used predominantly for the development of somatic embryogenesis. In plant tissue culture, the most popular carbohydrate source used is sucrose with 2 to 5%. The sucrose acts as the sole carbon source this is due to the sucrose can be uptake across the plasma membrane efficiently (Balasubramanya and Anuradha, 2010). Coconut water contains various kinds of substances that can promote the growth of cells. The substances are amino acids, organic



**Fig 7.** Mean weight (g) of PLBs formation on half strength MS semi-solid medium with sucrose, coconut water, combination of sucrose and coconut water after two months of culture. Means followed by different letters are significantly different at  $p=0.05$  according to Tukey Test.

acids, inorganic ions, vitamins, sugars, lipids, nitrogenous compounds and hormones (Yong et al., 2009). From Figure 7, the medium contained with sucrose was obtained the highest mean weight of PLBs formation from callus,  $3.467 \pm 1.111$  g. The second highest mean weight was on the medium contained with coconut water,  $1.600 \pm 0.238$  g while the medium contained with coconut water and sucrose was the lowest mean weight of PLBs formation,  $1.095 \pm 0.135$  g. Huan et al. (2004) reported that the PLBs formation from callus was the best on basal medium without plant growth regulators. In this research work, the half strength MS medium with sucrose only was obtained the highest mean weight of PLBs compared with others. Naing et al. (2010) reported that the addition of organic supplements to the medium had generated effects on the growth and development of PLBs then subsequent regeneration of plantlets. Basal medium added with coconut powder was suitable to regenerate shoots from PLBs segment of endangered medicinal orchid, *Coelogyne cristata* (Naing et al., 2010). The addition of coconut water had also improved the rate on somatic embryo induction and also increased the germinate percentage of date palm (Al-Khayri, 2010). For medium contained with coconut water in absence of sugar, callus culture turned into many PLBs which were greenish in colour. This was supported by Ishii et al. (1998), who had also reported that the callus cultured on medium without sucrose but with coconut water turned green in colour and produced many PLBs. There were many PLBs formed which were irregular in shape and small size. Similarly, Huan et al. (2004) reported that the medium supplemented with coconut water without sucrose produced many small and irregular shaped PLBs. For the treatment with coconut water and sucrose, PLBs formed were in bigger clump size and only 5 to 8 PLBs were produced. From Table 2, there was 52% of explants showed shoots/PLBs formation. The PLBs formed had continued regenerated into shoots. The coconut water known with various kinds of hormones such as auxin, cytokinin and gibberellins (Yong et al., 2009). Hormones are able to induce PLBs regeneration. The medium added with 15 % coconut water was able to enhance the early germination and the PLBs differentiation into healthy plantlets for *Coelogyne suaveolens* (Lindl.) Hook which is a sympodial epiphytic orchid (Sungkamlong and Deb, 2008). In addition, Sheelavanthmath et al. (2005) had reported that the medium contained with sucrose and coconut water was not suitable for PLBs induction from protocorm and leaf explants of *Aerides crispum*.

## Materials and methods

### Plant material for callus induction

*In vitro* cultures of protocorm-like bodies (PLBs) of *Dendrobium sonia-28* were used as the starting plant material (Fig. 1). The PLBs were subcultured every 4 weeks on half strength MS semi-solid medium (Murashige and Skoog, 1962). For further experimentation, half strength MS semi-solid medium was prepared. The half strength MS semi-solid medium were prepared and supplemented with 20 g/L sucrose and 1 mg/L of benzlaminopurine (BAP).

### Callus induction from PLBs of *Dendrobium sonia-28*

#### NAA treatments on callus induction

PLBs with the size of 3-4 mm in diameter were grown on half strength MS semi-solid medium were used as the starting material for the induction of callus. The upper part and lower part of the PLBs were cut transversely and were again subjected to longitudinal cut into 2 segments. The PLBs segments were used as explants. Five PLBs segments were cultured on a culture jar containing 40 ml of half strength MS semi-solid medium supplemented with 20 g/L sucrose, with different concentration of NAA (0, 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, and 2.00 mg/L) and 2.75 g/L Gelrite. Each treatment was done in 7 replicates. The cultures were kept under dark condition in culture room maintained at  $25 \pm 2^\circ\text{C}$ . The data were analysed by using One-Way Analysis of Variance (ANOVA) and statistical significance was determined using Tukey Test at  $p=0.05$  using SPSS program to determine the effects of NAA on callus induction.

#### 2, 4-D treatments on callus induction

The PLBs with the size of 3-4 mm in diameter which grown on half strength MS semi-solid medium and was used as the starting material for the induction of callus. The upper part and lower part of the PLBs were cut transversely and were again subjected to longitudinal cut into 2 segments. The PLBs segments were used as explants. Five PLBs segments were cultured on a culture jar containing 40 ml of half strength MS solid medium supplemented with 20 g/L sucrose, with different concentrations of 2,4-D (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.25 and 0.5 mg/L) and 2.75 g/L Gelrite. Each treatment was done in 7 replicates. The cultures were kept under dark condition in culture room maintained at  $25 \pm 2^\circ\text{C}$ . After six weeks of culture, the percentage of explants forming callus were recorded. The data were analysed by using One-Way Analysis of Variance (ANOVA) and statistical significance was determined using Tukey Test at  $p=0.05$  using SPSS program to determine the effects of 2,4-D on callus induction.

#### Combination of NAA and 2,4-D treatments on callus induction

The PLBs with the size of 3-4 mm in diameter which grown on half strength MS semi-solid medium and was used as the starting material for the induction of callus. The upper part and lower part of the PLBs were cut transversely and were again subjected to longitudinal cut into 2 segments. The PLBs segments were used as explants. Five PLBs segments were cultured on a culture jar containing 40 ml of half strength MS solid medium supplemented with 20 g/L sucrose,

different concentrations of NAA (0.05, 0.1, 0.25, 0.5, 0.75, 1.0, and 2.0 mg/L) and fixed concentration of 2,4-D (0.01 and 0.1 mg/L) respectively and 2.75 g/L Gelrite. Each combination treatment was done in 7 replicates. The cultures were kept under dark condition in culture room maintained at  $25 \pm 2^\circ\text{C}$ . After six weeks of culture, the percentage of explants forming callus were recorded. The data were analysed by using One-Way Analysis of Variance (ANOVA) and statistical significance was determined using Tukey Test at  $p=0.05$  using SPSS program to determine the effects of combination of NAA and 2,4-D on callus induction.

#### ***Tryptone treatments on callus proliferation***

The best callus induction medium, half strength MS semi-solid medium supplemented with 1.0 mg/L NAA and 0.1 mg/L 2, 4-D was used. The half MS semi-solid medium was prepared and supplemented with 1.0 mg/L NAA, 0.1 mg/L 2,4-D, tryptone (0, 2, and 4 g/L), 20 g/L sucrose and 2.75 g/L Gelrite. Total of 20 mg of callus pieces induced from PLBs segments was weighed by using digital weighing balance (Shimadzu TX323L). Five (5) of 20 mg callus pieces were cultured in a culture jar contained with 40 ml of half strength MS semi-solid medium. Each treatment was completed with 5 replicates. The cultures were kept under dark condition in culture room maintained at  $25 \pm 2^\circ\text{C}$ . After six weeks of culture, the fresh weight of callus proliferated was weighed using the digital weighing scale. The data results recorded were analysed by using One-Way Analysis of Variance (ANOVA) and statistical significance was determined using Tukey Test at  $p=0.05$  using SPSS program to determine the effects of tryptone on callus proliferation.

#### ***Coconut water and sucrose treatments on PLBs formation from callus***

The half strength MS semi-solid medium without plant growth regulators was used. The basal medium was supplemented with coconut water in the presence or absence of sucrose to examine the effect of coconut water and sucrose on the formation of PLBs from the callus. Coconut water (200 ml/L) was added directly into the basal medium. Five (5) of 20 mg callus pieces were cultured in a culture jar containing half strength MS semi-solid medium with a single supplement or combinational supplement like sucrose or coconut water or sucrose and coconut water respectively. Each treatment was done in 5 replicates. The cultures were incubated at  $25 \pm 2^\circ\text{C}$  in a 16-h photoperiod under cool white fluorescent lamps (Philips TLD, 36 W) at  $150 \mu\text{mol m}^{-2}\text{s}^{-1}$ . After two months of culture, the fresh weight of PLBs formed was weighed. The data results recorded were analysed by using One-Way Analysis of Variance (ANOVA) and statistical significance was determined using Tukey Test at  $p=0.05$  using SPSS program to determine the effects of coconut water and sucrose on PLBs formation from callus.

#### **Conclusion**

As a conclusion, the effect of auxins (NAA and 2,4-D) with various concentrations and different combinations of both auxins on the callus induction of protocorm-like bodies (PLBs) of *Dendrobium sonia-28* in half strength Murashige and Skoog (MS) semi-solid medium supplemented with 1.0 mg/L NAA and 0.1 mg/L 2,4-D was the optimal medium for callus production. Whitish yellow and friable callus were induced from PLBs segments. The calluses proliferated very well on the optimal medium compared with the medium

supplemented alone with 2 g/L and 4 g/L of tryptone, respectively. Besides that, the callus were regenerated into globular and greenish PLBs and obtained the highest mean weight of PLBs on the medium without plant growth regulators but with 20 g/L of sucrose.

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