Vol.7, No.2 pages 35-46 Science Technology and Engineering Journal (STEJ) Research Article

Effective Protocol for Rapid and Mass Micropropagation of *Cymbidium aloifolium* (L.) Sw. Protocorms Using Different Carbohydrate and Plant Growth Regulator

Khoirista Noor Rohmah[1] and Worasitikulya Taratima[2*]

[1] *Faculty of Science, Khon Kaen University, 40002, Thailand*
[2] *Salt-tolerant Rice Research Group, Department of Biology, Faculty of Science, Khon Kaen University, 40002, Thailand*
[*] *Name Corresponding Author: worasitikulya@gmail.com*

(Received: 18th May 2021, Revised: 7th July 2021, Accepted: 8th July 2021)

Abstract-Efficient *in vitro* propagation of *Cymbidium aloifolium* protocorm-like-bodies (PLBs) using carbohydrate sources and hormones as a medium supplement was established. Twelve-week-old protocorms were used as explants. Experiment 1 ; protocorms were propagated on solidified New Dogashima (ND) medium supplemented with various concentrations of glucose and sucrose (0, 20, 25, 30, 35 and 40 g/l) as carbohydrate sources. Experiment 2 ; protocorms were cultured on the same medium added 2,4-dichlorophenoxyacetic acid (2,4-D ; 0, 0.5, 1 and 1,5 mg/l) combination with kinetin (0, 1 and 2 mg/l) as plant growth regulators. Survival, response, shoot number and length, root number and length, leaf number, and fresh and dry weight were measured after 12 weeks of culture. Results revealed that sucrose as the appropriate carbohydrate source for promoting shoot and leaf development while glucose was better for stimulating weight and root development. Optimal concentration of sucrose was 20-35 g/l with highest leaf number obtained at 20 g/l, shoot number at 25 g/l and shoot length at 35 g/l. The highest fresh weight, dry weight, root number and length were recorded at 35 g/l glucose treatment. Supplementation of hormone 2,4-D as auxin, and kinetin as a cytokinin produced highest growth of protocorm at 0.5 mg/l 2,4-D and 2 mg/l kinetin treatment. Different supplements and concentrations had diverse affected on root, shoot and leaf growth. These results provided valuable knowledge for future orchid conservation studies.

Keywords: 2,4-D, carbohydrate source, kinetin, orchid *in vitro* culture, protocorm

1. Introduction

Orchids are globally well-known as beautiful ornamental flowers (Pornpienpakdee *et al*., 2010). Many people are attracted to cultivate orchids because of their pretty, long-lasting blooms. Some countries are orchid producers such as Thailand (Worrachottiyanon & Bunnag, 2018). In Thailand, orchids grow naturally in all habitats, including evergreen forest hills at 2,565 m. *Cymbidium aloifolium* is a very popular orchid that can be found in Thailand (Pant, 2013). This species has many benefits and is often used for floriculture and also traditional medicine (Regmi *et al*., 2017). Populations of *C. aloifolium* require conservation to maintain their existence. Nowadays, market demand has greatly increased, while orchid abundance has decreased because of environmental destruction (Pant, 2013). Plant tissue culture can assist plant production in high numbers by providing prominent nutrients to stimulate plant growth (Goswami *et al*., 2015). *In vitro* is a plant propagation technique that can be used as an effective solution to produce plants in large numbers within a short time period (Asghar *et al*., 2011). Using this technique, organic and inorganic nutrition is provided to support growth and development of plants so that decline in orchids numbers can be prevented (Goswami *et al*., 2015).

Plant tissue or cell culture needs a carbohydrate source to support energy demands (Al-Khateeb, 2008). Carbohydrate sources play a prominent role as the foremost energy suppliers to support the growth of plant tissues. Sugars usually used for orchid culture are sucrose, glucose, maltose and fructose (Sopalun *et al*., 2010). Carbohydrate sources such as glucose and sucrose have been shown to have a great impact on shoot development (Luo *et al*., 2009). Hormones such as auxin and cytokinin are essential factors to improve orchid growth from protocorm to plantlet (Parthibhan *et al*., 2015). Auxin and cytokinin enhance protocorm growth, stimulate protocorm multiplication and increase shoot and root size. One of the most important classes of plant growth hormones are cytokinins that can increase protocorm-like-bodied (PLB) regeneration, such as zeatin riboside (Parthibhan *et al*., 2015), 6-Benzylaminopurine (BAP) in combination with 1-Naphthaleneacetic acid (NAA) in *C. aloifolium* cultures (Trunjareun & Taratima, 2018). Media containing 2, 4-D (2,4-dichlorophenoxyacetic acid) and kinetin are essential for inducing shoot growth (Haque & Ghosh, 2017). Protocorm treatment with auxin or cytokinin is a vital factor in supporting plant development (Parthibhan *et al*., 2015). Effects of auxin, cytokinin and carbohydrates as media supplements on shoots depend on their concentrations and types (Prasertsirivatna & Koolpluksee, 2011). Different species may react differently to different concentrations, types and combinations of hormones. Appropriate concentrations of medium supplements highly impact orchid quantity, either alone or in combination. Here, the effects of glucose and sucrose as carbohydrate sources, and 2,4-D and kinetin as hormones were studied on *C. aloifolium in vitro* propagation.

2. Materials and Methods

2.1 Plant Materials

For protocorm induction, capsules of *C. aloifolium* were sterilized using 5% (v/v) sodium hypochlorite (Clorox) with

two drops of Tween 20 for 15 min and then dissected into two pieces. The mature seeds were transferred and cultured on solid New Dogashima (ND) medium supplemented with 20 g/l sucrose and pH was adjusted to 5.4. The cultures were incubated at 25 ± 2 °C with a 16/8 h light/dark cycle providing 40 μmol.m-2.s-1 for 12 weeks.

2.2 Glucose and Sucrose Treatment

Twelve-week-old *C. aloifolium* protocorms were used as explants. Protocorms were propagated on solidified New Dogashima (ND) medium supplemented with various concentrations of glucose in combination with sucrose (0, 20, 25, 30, 35 and 40 g/l) as carbohydrate sources. The cultures were incubated at 25 ± 2 °C with a 16/8 h light/dark cycle providing 40 $\mu mol.m^{-2}.s-1$. Shoot survival percentage, shoot response percentage, shoot number per plantlets, shoot length, root number per plantlets, root length, leaf number per plantlets and fresh weight and dry weight were measured after culture for 12 weeks.

2.3 Kinetin and 2,4-D Treatment

Twelve-week-old protocorms were propagated on solidified ND medium with 20 g/l sucrose and supplemented with various concentrations of 2,4-D (0, 0.5, 1 and 1,5 mg/l) in combination with kinetin (0, 1 and 2 mg/l). The cultures were incubated at 25 ± 2 °C with a 16/8 h light/dark cycle providing 40 $\mu mol.m^{-2}.s^{-1}$. Shoot survival percentage, shoot response percentage, shoot number per plantlets, shoot length, root number per plantlets, root length, leaf number per plantlets and fresh weight and dry weight were measured after culture for 12 weeks.

2.4 Statistical Analysis

Both experiments were conducted using completely randomized design (CRD) with five replications. Data were analyzed by one-way analysis of variance (ANOVA) with difference between means separated by Duncan's multiple range test ($p \leq 0.05$). All data were presented as mean value ± standard error (SE).

3. Results

Table 1. Survival percentage, response percentage, shoot number, shoot length, root number, root length, and leaf number of regenerated plants after cultured on ND medium supplemented with difference carbohydrate sources

Treatments	Survival percentage	Response percentage	Growth performance (mean ± SE)				
			Shoot number/ PTC	Shoot length (cm)	Root number/ PTC	Root length (cm)	Leaf number/ PTC
Glucose (g/l)							
0	100	100	0.64 ± 0.07^{d}	0.61 ± 0.09^{e}	0.02 ± 0.02^{f}	0.01 ± 0.01^{d}	1.34 ± 0.17^{e}
20	100	100	1.02 ± 0.02^{c}	1.92 ± 0.15^{a}	1.22 ± 0.12^{c}	1.31 ± 0.13^{c}	2.16 ± 0.14^{ab}
25	100	100	0.92 ± 0.04^{c}	1.60 ± 0.19^{bc}	1.24 ± 0.11^{c}	1.42 ± 0.18^{c}	1.86 ± 0.12^{cd}
30	100	100	1.12 ± 0.09^{bc}	1.47 ± 0.13^{c}	1.30 ± 0.11^{bc}	1.90 ± 0.18^{ab}	2.06 ± 0.15^{c}

Table 1. Survival percentage, response percentage, shoot number, shoot length, root number, root length, and leaf number of regenerated plants after cultured on ND medium supplemented with difference carbohydrate sources (cont.)

Treatments	Survival percentage	Response percentage	Growth performance (mean ± SE)				
			Shoot number/ PTC	Shoot length (cm)	Root number/ PTC	Root length (cm)	Leaf number/ PTC
35	100	100	1.08 ± 0.04^{bc}	1.57 ± 0.13^{bc}	1.56 ± 0.13^{a}	2.21 ± 0.21^{a}	2.14 ± 0.10^{b}
40	100	100	0.98 ± 0.05^{c}	1.00 ± 0.08^{d}	1.02 ± 0.10^{d}	1.30 ± 0.16^{c}	1.62 ± 0.12^{d}
Sucrose (g/l)							
20	100	100	1.24 ± 0.09^{b}	1.78 ± 0.12^{b}	0.96 ± 0.10^{de}	1.51 ± 0.21^{bc}	2.58 ± 0.20^{a}
25	100	100	1.62 ± 0.10^{a}	1.28 ± 0.11^{cd}	0.82 ± 0.08^{e}	1.22 ± 0.19^{c}	2.48 ± 0.15^{ab}
30	100	100	1.02 ± 0.03^{c}	1.91 ± 0.19^{a}	1.16 ± 0.12^{cd}	1.17 ± 0.15^{c}	2.10 ± 0.11^{bc}
35	100	100	1.12 ± 0.05^{bc}	2.01 ± 0.15^{a}	1.40 ± 0.11^{b}	1.71 ± 0.18^{b}	2.44 ± 0.14^{ab}
40	100	100	1.08 ± 0.06^{bc}	1.99 ± 0.16^{a}	1.58 ± 0.12^{a}	1.70 ± 0.19^{b}	2.16 ± 0.11^{ab}

Mean ± SE followed by the different letter are significantly different according to ANOVA and Duncan's Multiple Range Test ($p < 0.05$), PTC=protocorm

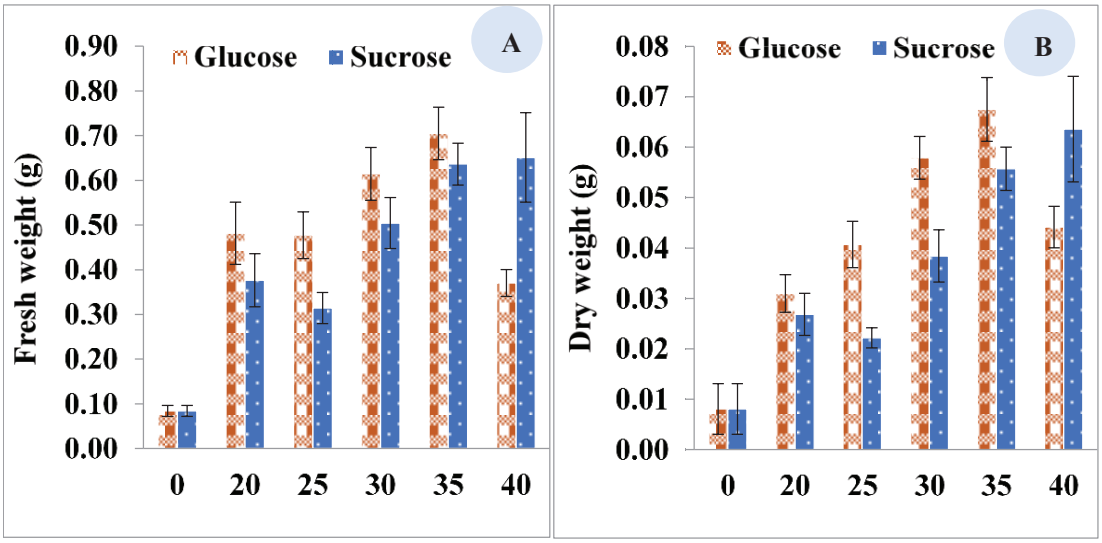

Figure 1. Biomass of *C. aloifolium* plantlets after cultured on ND medium with various concentrations of glucose and sucrose; (A) fresh weight, and (B) dry weight. Data are shown as mean ± SE.

Appropriate concentration of glucose and sucrose stimulated protocorm growth. Survival and response of protocorm-like-bodies (PLBs) were 100% in all treatments. The appropriate sucrose to stimulate shoot and leaf development was 20-35 g/l, while glucose best stimulated root and weight at 35 g/l. The highest shoot growth resulted from sucrose treatment with 1.62 ± 0.10 shoot per protocorm at 25 g/l sucrose

treatment and 2.01 ± 0.15 cm shoot length at 35 g/l treatment. The highest leaf number per protocorm was obtained at 20 g/l sucrose treatment which significantly difference from other treatments and control (Table 1). Treatment at 35 g/l glucose resulted in highest PLBs weight, fresh weight (0.70 ± 0.06 g) and dry weight (0.07 ± 0.01 g) (Figure 1). The roots achieved high growth at 35 g/l glucose treatment, with 1.56 ± 0.13 root per protocorm and 2.21 ± 0.21 cm length. All treatments showed plantlets with roots, except for the control that presented only shoots (Figure 2).

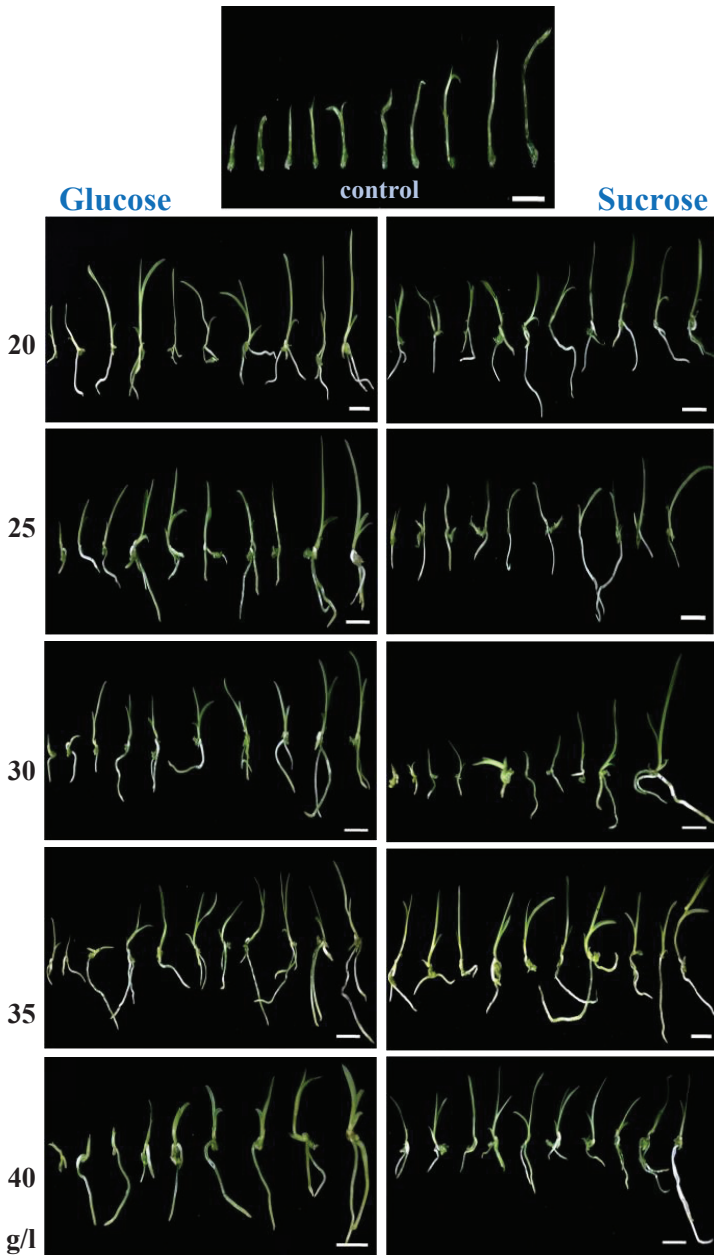

Figure 2. Plantlets of *C. aloifolium* after treated with different concentrations of glucose and sucrose (scale=1 cm).

Table 2. Survival percentage, response percentage, shoot number, shoot length, root number, root length, and leaf number of regenerated plants after cultured on ND medium supplemented with various concentrations of Kinetin and 2,4-D.

Treatments		Survival percentage	Response percentage	Growth performance (mean ± SE)				
Kin (mg/l)	2,4-D (mg/l)			Shoot number/ PTC	Shoot length (cm)	Root number/ PTC	Root length (cm)	Leaf number/ PTC
0	0	100	98	1.02±0.03^{b}	2.14±0.19^{a}	2.04±0.14^{ab}	1.88±0.14^{ab}	2.72±0.13^{ab}
0	0.5	46	46	1.29±0.18^{ab}	1.49±0.45^{a}	1.71±0.29^{ab}	0.49±0.12^{c}	2.57±0.20^{ab}
0	1	22	22	nd	nd	nd	nd	nd
0	1.5	30	30	nd	nd	nd	nd	nd
1	0	100	100	1.50±0.13^{ab}	2.40±0.23^{a}	2.28±0.14^{ab}	1.97±0.14^{ab}	3.88±0.31^{ab}
1	0.5	38	38	1.00±0.00^{b}	0.93±0.35^{a}	1.00±0.00^{b}	0.38±0.09^{c}	1.50±0.29^{b}
1	1	48	48	1.00±0.00^{b}	1.85±0.65^{a}	1.50±0.50^{ab}	0.35±0.05^{c}	2.50±0.50^{ab}
1	1.5	40	40	1.00±0.00^{b}	1.23±0.12^{a}	1.00±0.00^{b}	1.10±0.06^{abc}	1.67±0.33^{b}
2	0	52	52	1.56±0.15^{ab}	1.88±0.15^{a}	1.84±0.12^{ab}	1.84±0.11^{ab}	3.26±0.18^{ab}
2	0.5	100	100	2.50±0.73^{a}	2.46±0.48^{a}	2.50±0.33^{a}	2.30±0.40^{a}	4.13±0.79^{a}
2	1	40	40	1.00±0.00^{b}	1.47±0.20^{a}	1.33±0.33^{ab}	0.87±0.24^{bc}	2.33±0.67^{ab}
2	1.5	26	26	1.00±0.00^{b}	0.80±0.20^{a}	1.50±0.50^{ab}	0.50±0.10^{c}	0.50±0.50^{b}

Mean ± SE followed by the different letter are significantly different according to ANOVA and Duncan's Multiple Range Test ($p < 0.05$), Kin=Kinetin ; 2,4-D=2,4-dichlorophenoxyacetic acid ; nd=no data ; PTC=protocorm

Hormones play an important role in plant growth and development especially auxin and cytokinin. Protocorm treated with 0.5 mg/l 2,4-D and 2 mg/l kinetin resulted in highest growth of PLBs which significantly difference from other treatments and control (Table 2). The highest shoot number per protocorm was 2.50 ± 0.73, with 2.46 ± 0.48 cm shoot length. Root number per protocorm was 2.50 ± 0.33, root length was 2.30 ± 0.40 cm, leaf number per protocorm was 4.13 ± 0.79, while fresh weight was 0.93 ± 0.09 g and dry weight was 0.50 ± 0.10 g (Figure 3). The lowest growth rate was observed at maximum concentrations of 2,4-D and kinetin (1.5 mg/l 2,4-D and 2 mg/l kinetin). Protocorms supplemented with 1 mg/l and 1.5 mg/l of 2,4-D only showed enlargement without producing shoots (Figure 4).

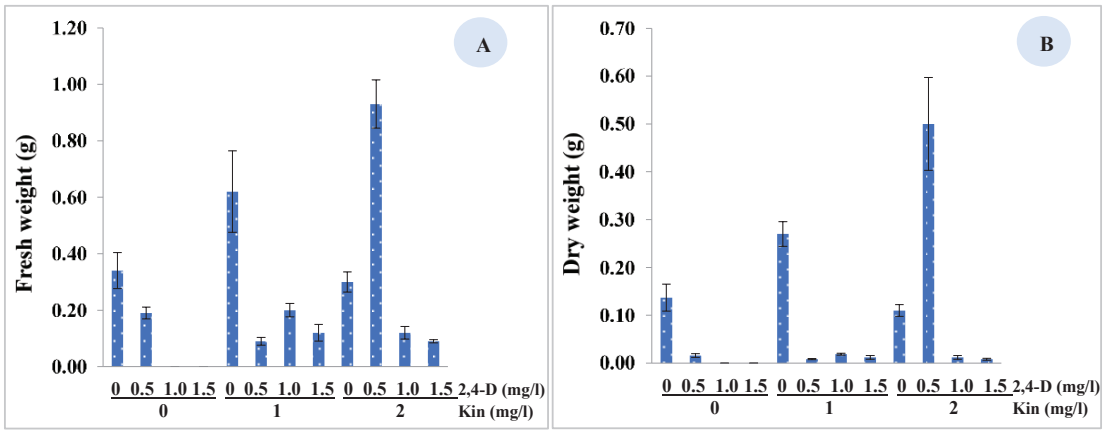

Figure 3. Fresh weight (A), and dry weight (B) of *C. aloifolium* plantlets after various concentrations of 2,4-D and kinetin treatments. Data are shown as mean ± SE.

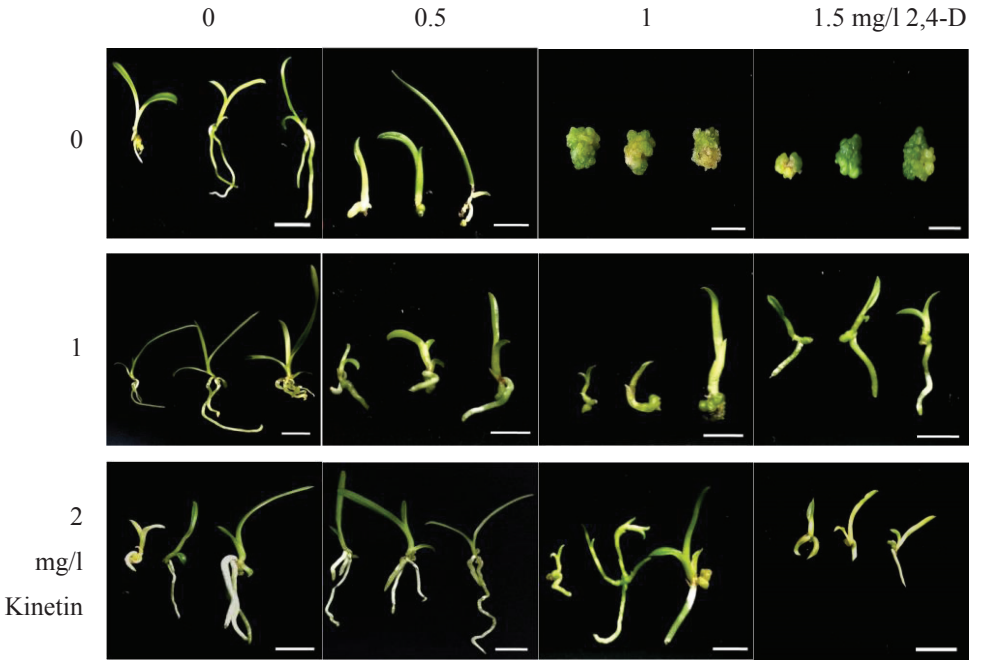

Figure 4. Plantlets of *C. aloifolium* derived from protocorms after 2,4-D and kinetin treatment. (scale=1 cm).

4. Discussion and Conclusion

Carbohydrate sources are considered to be important factors in shoot development from PLBs. Carbohydrates provide energy for biosynthetic and growth processes. Carbohydrates also play a role in osmotic pressure regulation and have a major impact on orchid seed development (Huh *et al*., 2016). Different carbon sources had a major impact on protocorm to plantlet conversion in *Phalaenopsis* sp. (Luo *et al*., 2009). Carbohydrates in the medium as carbon sources influenced root quality and frequency (Martins *et al*., 2015). Glucose, sucrose, fructose and maltose are

carbohydrates commonly used in plant tissue culture (Sopalun *et al*., 2010). Novotna *et al*. (2007) discovered that adding glucose and sucrose to *Dactylorhiza majalis* improved protocorm, shoot, and root growth rates. However, both glucose and sucrose improved protocorm growth, depending on species. Glucose was effective in producing PLBs in some species such as *Phalaenopsis* sp. (Pimsen & Kanchanapoom, 2011), while sucrose resulted in the highest enhancement of PLBs development in *Dendrobium* Second Love (Orchidaceae) (Ferreira *et al.*, 2011). The impact of glucose and sucrose on plantlets are different depending on the species. Different carbohydrate sources result in different impacts on shoot growth and development (Luo *et al*., 2009).

Sucrose was found to be appropriate for shoot and leaf growth, while glucose was found to be appropriate for root and weight development in this study. Sucrose produced a large number of shoots and a long shoot length, while glucose produced a large number of root and root length. When compared to glucose, sucrose resulted in a greater shoot number and length. In line with these results, Swamy *et al*. (2010) revealed that addition of 2% sucrose yielded longer shoots and higher shoot number compared to glucose and fructose on *Pogostemon cablin* Benth. A better impact of sucrose was also shown by Luo *et al*. (2009). They revealed that glucose produced lower shoot number than sucrose on *Dendrobium huoshanense*. Sucrose is considered as an optimal carbon source, and commonly used because of its effectiveness in inducing shoots (Al-Khateeb, 2008). In this study, high leaf number was yielded from glucose and sucrose at the same concentration (20 g/l) but sucrose showed higher leaf number than glucose. High growth using sucrose was also reported by Zahara *et al*. (2017). They found that 20 g/l sucrose was optimal in improving leaf number, width and length, and plant height on *Phalaenopsis* sp. Sucrose is recognized as the foremost carbon energy supplier because it is found in phloem and involved in developmental process regulation (Huh *et al*., 2016). Similarly, in sago palm plant, Sumaryono *et al*. (2012) showed that sucrose at 30 g/l was better than other carbohydrate sources, and produced highest plantlet size and leaf number of *Metroxylon sagu*.

Glucose, as another carbohydrate source, also has a great impact on plant growth through *in vitro* propagation (Swamy *et al*., 2010). Based on this study, glucose created high root growth at 35 g/l compared to sucrose at 40 g/l, while highest root number and length were obtained from glucose. Similarly, Junior *et al*. (2018) reported that among glucose, fructose and sucrose, higher root number and root length of *Cattleya schilleriana* Reichb.f. were produced by glucose but at a different concentration (45 g/l). In this study, both glucose and sucrose produced highest plant weight at the same concentration of 35 g/l but glucose gave higher dry and fresh weight than sucrose. This outcome agreed with Nambiar *et al*. (2012) who supplemented the medium with 2% glucose, fructose and sucrose in *Dendrobium* Alya Pink. Results showed that glucose created highest fresh weight. Experiments by Al-Khateeb (2008) also found similar results, with glucose generating higher dry weight than sucrose on date palm (*Phoenix dactylifera* L.). Experiments on potato plant (*Solanum tuberosum* L.) by Rahman *et al*. (2010) revealed that glucose treatment achieved higher fresh weight than maltose and

sucrose. Generally, sucrose is widely used in plant tissue culture because it has high solubility in water and can easily pass plasma membrane (Sumaryono *et al*., 2012). This study showed that both glucose and sucrose play important roles in enhancing protocorm development. Rahman *et al*. (2010) indicated that carbohydrate sources such as glucose, sucrose and fructose have a prominent role as energy providers for cell division, and also as osmotic agents and stress regulators that may relate to plant morphology. However, different types of sugars play diverse roles in regulating physiological processes (Lastdrager *et al*., 2014). However, various types of carbohydrates, concentrations, and plant species can have different reactions.

Regeneration of PLBs in many orchid species through *in vitro* multiplication also requires a supply of hormones such as auxins and/or cytokinins (Parthibhan *et al*., 2015). Cytokinin hormones have been reported as the most prominent factors in improving plant regeneration, for example, triacontanol in *D. nobile* and zeatin riboside in *C. aloifolium* (Luo *et al*., 2009). In *Dendrobium* sp. micropropagation, cytokinins are widely used to stimulate shoot development of PLBs. Kinetin actively induces the mobilization of necessary nutrients from source area to sink as a supporting element to enlarge shoot and root size (Asghar *et al*., 2011). In this study, optimal concentration producing high protocorm growth was 0.5 mg/l 2,4-D and 2 mg/l kinetin. These results concurred with Hossain *et al*. (2010) who demonstrated that a combination of 2,4-D and kinetin at 1 mg/l produced high protocorm multiplication on *C. giganteum* Wall. ex Lindl. Similarly, Liu *et al*. (2018) showed that supplementation of 2,4-D on *Rosa hybrida* L. generated long and high numbers of explants. Prasertsirivatna and Koolpluksee (2011) revealed that addition of 2,4-D to *D. friedericksianum* Rchb.f. produced slightly better results in terms of root number, shoot height, and fresh weight, but not in terms of root length or shoot number. Luo *et al*. (2009) reported that the highest shoot formation of *D. huoshanense* was created from kinetin (20 μM) treatment. Similarly, Parthibhan *et al*. (2015) stated that kinetin at 5 mg/l produced higher shoot size and number compared to 6-benzylaminopurine (BA) on *D. aqueum* Lindley. 2,4-D and kinetin promoted plant improvement either alone or in combination. The efficacy of 2,4-D and kinetin treatment varies by species, and results are dependent on concentration ; however, both of these hormones are equally essential for protocorm growth induction.

5. Acknowledgement

This work was financially supported by the Salt-tolerant Rice Research Group, Department of Biology, Faculty of Science, Khon Kaen University and KKU Scholarship for ASEAN and GMS Countries' Personnel, Academic Year 2019. The authors would like to acknowledge assistance from the Department of Biology, Faculty of Science, Khon Kaen University, Thailand for facility support.

6. References

Al-Khateeb, A.A. (2008). Regulation of *in vitro* bud formation of date palm (*Phoenix dactylifera* L.) cv. Khanezi by different carbon sources. *Bioresource Technology, 99*, 6550-6555. doi: 10.1016/j.biortech.2007.11.070.

Asghar, S., Ahmad, T., Hafiz, I.A. & Yaseen, M. (2011). In vitro propagation of orchid (*Dendrobium nobile*) var. Emma White. *African Journal of Biotechnology, 10*, 3097-3103. doi: 10.5897/AJB10.401.

Ferreira, W.M., Suzuki, R.M., Pescador, R. Figueiredo-Ribeiro, R.C.L. & Kerbauy, G.B. (2011). Propagation, growth, and carbohydrates of *Dendrobium* Second Love (Orchidaceae) *in vitro* as affected by sucrose, light, and dark. *In Vitro Cellular & Developmental Biology, 47*, 420-427.

Goswami, K., Yasmin, S., Nasiruddin, K., Khatun, F. & Akte, J. (2015). *In vitro* regeneration of *Dendrobium* sp. of orchid using leaf tip as explant. *Journal of Environmental Science and Natural Resources, 8*, 75-78. doi: https://doi.org/10.3329/jesnr.v8i2.26869.

Haque, S.M. & Ghosh, B. (2017). Regeneration of cytologically stable plants through dedifferentiation, redifferentiation, and artificial seeds in *Spathoglottis plicata* Blume. *Horticultural Plant Journal, 3*, 199-208. doi: 10.1016/j.hpj.2017.10.002.

Hossain, M.M., Sharma, M., Silva, J.A.T. & Pathak, P. (2010). Seed germination and tissue culture of *Cymbidium giganteum* Wall. ex Lindl. *Scientia Horticulturae, 123*, 479-487. doi: 10.1016/j.scienta.2009.10.009.

Huh, Y.S., Lee, J.K., Nam, S.Y., Hong, E.Y., Paek, K.Y. & Son, S.W. (2016). Effects of altering medium strength and sucrose concentration on *in vitro* germination and seedling growth of *Cypripedium macranthos* Sw. *Journal Plant Biotechnology, 43*, 132-137. doi: 10.5010/JPB.2016.43.1.132.

Junior, R.F.G., Mantovani, C. & Lemos, E.G.M. (2018). Carbohydrates on the growth of *Cattleya schilleriana* Reichb.f. seedlings (Orchidaceae). *Ciencia Rural, 48*, e0977.

Lastdrager, J., Hanson, J. and Smeekens, S. (2014). Sugar signals and the control of plant growth and development. *Journal of Experimental Botany, 65*, 799-807. doi: 10.1093/jxb/ert474.

Liu, J., Feng, H., Ma, Y., Zhang, L., Han, H. & Huang, X. (2018). Effects of different plant hormones on callus induction and plant regeneration of miniature roses (*Rosa hybrida* L.). *Horticulture International Journal, 2*, 201-206. doi: 10.15406/hij.2018.02.00053.

Luo, J.P., Wawrosch, C. & Kopp, B. (2009). Enhanced micropropagation of *Dendrobium huoshanense* C.Z. Tang et S.J. Cheng through protocorm-like bodies: The effects of cytokinins, carbohydrate sources and cold pretreatment. *Scientia Horticulturae, 123*, 258-262. doi: 10.1016/j.scienta.2009.08.008.

Martins, J.P.R., Pasqual, M., Martins, A.D. & Ribeira, S.F. (2015). Effects of salts and sucrose concentrations on *in vitro* propagation of *Billbergia zebrina* (Herbert) Lindley (Bromeliaceae). *Australian Journal of Crop Science, 9*, 85-91.

Nambiar, N., Tee, C.S. & Maziah, M. (2012). Effects of organic additives and different carbohydrate sources on proliferation of protocorm like bodies in *Dendrobium* Alya Pink. *Plant Omics Journal, 5*, 10-18.

Novotna, K.W., Vejsadova, H. & Kindlmann, P. (2007). Effects of sugars and growth regulators on *in vitro* growth of *Dactylorhiza* species. *Biologia Plantarum, 51*, 198-200.

Pant, B. (2013). Medicinal orchids and their uses: Tissue culture a potential alternative for conservation. *African Journal of Plant Science, 7*, 448-467. doi: 10.5897/AJPS2013.1031.

Parthibhan, S., Rao, M.V. & Senthil Kumar, T. (2015). *In vitro* regeneration from protocorms in *Dendrobium aqueum* Lindley-An imperiled orchid. *Journal of Genetic Engineering and Biotechnology, 13*, 227-233. doi: 10.1016/j.jgeb.2015.07.001.

Pimsen, M. & Kanchanapoom, K. (2011). Effect of Basal Media and Sugar Types on *in vitro* Regeneration of *Grammatophyllum speciosum* Blume. *Notulae Scientia Biologicae, 3*, 101-104. doi: 10.15835/nsb336153.

Pornpienpakdee, P., Singhasurasak, R., Chaiyasap, P., Pichyangkura, R., Bunjongrat, R., Chadchawan, S. & Limpanavech, P. (2010). Improving the micropropagation efficiency of hybrid *Dendrobium* orchids with chitosan. *Scientia Horticulturae, 124*, 490-499. doi: 10.1016/j.scienta.2010.02.008.

Prasertsirivatna, S. & Koolpluksee. (2011). The study on optimization of growth conditions for *Dendrobium friedericksianum* Rchb.f. seedlings in aseptic culture. *Journal of Agricultural Technology, 7*, 739-749.

Rahman, M.H., Islam, R., Hossain, M. & Islam, M.S. (2010). Role of sucrose, glucose and maltose on conventional potato micropropagation. *Journal of Agricultural Technology, 6*, 733-739.

Regmi, T., Pradhan, S. & Pant, B. (2017). *In vitro* mass propagation of an epiphytic orchid, *Cymbidium aloifolium* (L.) Sw., through Protocorm Culture. *Biotechnology Journal International, 19*, 1-6. doi: 10.9734/BJI/2017/34891.

Sopalun, K., Thammasiri, K. & Ishikawa, K. (2010). Micropropagation of the Thai orchid *Grammatophyllum speciosum* blume. *Plant Cell Tissue Organ Culture, 101*, 143-150. doi: 10.1007/s11240-010-9671-2.

Sumaryono, Muslihatin, W. & Ratnadewi, D. (2012). Effect of Carbohydrate Source on Growth and Performance of *In Vitro* Sago Palm (*Metroxylon sagu* Rottb.) Plantlets. *Journal of Biosciences, 19*, 88-92. doi: 10.4308/hjb.19.2.88.

Swamy, M.K., Sudipta, K.M., Balasubramanya, S. & Anuradha, M. (2010). Effect of different carbon sources on *in vitro* morphogenetic response of patchouli (*Pogostemon cablin* Benth). *Journal of Phytology, 2*, 11-17.

Trunjareun, A. & Taratima, W. (2018). An effective micropropagation of *Cymbidium aloifolium* (L.) Sw. *Thai Journal of Botany, 1*, 77-91.

Worrachottiyanon, W. & Bunnag, S. (2018). Cryopreservation of *Cymbidium finlaysonianum* lindl. by encapsulation-dehydration method. *Songklanakarin Journal of Science and Technology, 40*, 682-691. doi: 10.14456/sjst-psu.2018.69.

Zahara, M., Datta, A., Boonkorkaew, P. & Mishra, A. (2017). The effects of different media, sucrose concentrations and natural additives on plantlet growth of *Phalaenopsis* Hybrid 'Pink'. *Brazilian Archives of Biology and Technology, 60*, doi: 10.1590/1678-4324-2017160149.