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Effect of edible sugar on *in vitro* growth and organogenesis of *Dendrobium bigibbum* x *Dendrobium* Thailand Black

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Abstract

The most popular propagation method of *Dendrobium* is *in vitro* micropropagation. However, using laboratory-grade sucrose as the carbon source in micropropagation is expensive for small-scale producers. Present study is aimed to assess the performance of edible sugar as an alternative carbon source to develop an effective, low-cost medium. Protocorm like bodies (PLBs) and plantlets of *Dendrobium* hybrid (*Dendrobium bigibbum* x *Dendrobium* Thailand Black) were cultured on basal MS medium containing different concentrations of sugar; 0.0, 15.0, 30.0, 45.0, 60.0, 75.0 and 90.0 g/L, (T_1-T_7). A modified MS medium (T_8) containing sugar (30.0 g/L), BAP (2.5 mg/L) and NAA (0.5 mg/L) was also used to determine whether there is a comparable effect of sugar individually and when combined with plant growth regulators (PGRs). Growth performance was evaluated in regular intervals. MS medium containing sugar (45.0 g/L) was identified as the best medium for the growth and organogenesis of PLBs resulting in the highest weight accumulation of 20.31 g and 35 plantlets regeneration from 1.00 g of PLBs after two months of incubation. MS medium supplemented with 60.0 g/L of sugar was identified as the most successful medium for the plantlet growth with 17 of mean leaf generation, 33 of mean root generation and an average root length increment of 1.5 cm after four months of incubation. In conclusion, edible sugar can be recommended to use as a sucrose supplement for a cost-effective medium to promote successful *in vitro* growth and development of the *Dendrobium* hybrid even with the absence of PGRs.

Keywords: Dendrobium, Edible sugar, *In vitro*, Organogenesis, Plant growth regulators

Introduction

Orchids have a high floricultural and economic value in the global floriculture industry. It is mostly because of their long-lasting shelf life, attractive diverse floral morphology (cut flower and pot plant) and herbal value. Among thousands of orchids, *Dendrobium* is the most popular floriculture product in the global market with high consumer demand (Khuraijam et al., 2017). Naturally, Dendrobiums are propagated sexually through seeds and asexually through vegetative propagation techniques as division, back bulbs and offshoots. However, it is essential to develop a suitable method for quick regeneration to address the demand in the market. *In vitro* plant regeneration offers a feasible propagation method for orchids and can be utilized for the year-round and rapid propagation of orchids. Therefore, growers use tissue culture as a rapid micro propagation technique to overcome this challenge in the mass production of selected high-quality traits (Young et al., 2011).

Among different types of culture media used in *in vitro* propagations, Murashige and Skoog (MS) medium, developed in 1962 (Murashige and Skoog, 1962) is one of the most efficient medium used in *Dendrobium* production with better performance (Teixeira et al., 2015). The appropriate nutrient composition in the medium is the most critical factor in plant tissue culture, while the carbon source is one of the major components required for high energy demanding developmental processes like embryogenesis and organogenesis (Yaseen et al., 2012). Several studies have been done to evaluate the performance of different carbohydrate types that are used to fulfill the carbon requirement of plants in tissue culture. Nambiar and Maziah (2012) reported that glucose, fructose and sucrose produced a higher fresh weight of protocorm like bodies (PLBs) of *Dendrobium* Alya Pink variety among six types of sugar tested (galactose, mannitol, sorbitol, glucose, sucrose and fructose). Further, Zahara et al. (2017) has reported that sucrose is mostly used as the carbon source instead of glucose, fructose or dextrose as alternatives. In most studies, a sucrose concentration of 20-30 g/L is used as the standard concentration of carbon source for the successful growth of plantlets *in vitro* (Ferreira et al., 2010). However, several studies have reported that concentrations of sucrose in *in vitro* media affect differently on growth parameters. While, some studies have reported that production of PLBs is inhibited by higher concentrations of sucrose (Udomdee et al., 2013). Further, in most modified tissue culture media, plant growth regulators are used to achieve unique performances throughout the production. Concentrations of naphthalene acetic acid (NAA) and 6-benzyl amino purine (BAP) individually and as a combination has shown varied effects on different stages of tissue culture (Maharjan et al., 2020).

Therefore, the present research aimed to study the effect of edible sugar as the sucrose component in MS medium on growth performance, effective growth and organogenesis of Dendrobiums. Further, it was aimed to find whether there is a comparable effect of sugar individually and in combination with plant growth regulators on the growth and development of Dendrobiums.

Methodology

Plant material

Seeds of a *Dendrobium* hybrid (*Dendrobium bigibbum* x *Dendrobium* Thailand Black) were grown on basal MS medium for 06 months under *in vitro* conditions. The regenerated PLBs were separated and used for the experiment to evaluate the effect of sugar on organogenesis. Regenerated plantlets were transferred to basal MS medium supplemented with NAA (0.5 mg/L) and BAP (2.5 mg/L) and maintained for 03 months under 20 mol/m^2/sec 14 h photoperiod at 26±4 ˚C. Plantlets with 2-3 cm height containing 2-3 nodes were used to evaluate the effect of sugar on the growth and development of plantlets.

Culture media preparation

Basal MS medium (T_1 – T_7) supplemented with different concentrations of edible sugar (0.0, 15.0, 30.0, 45.0, 60.0, 75.0, 90.0 g/L) and a modified MS medium (T_8) containing sugar (30.0 g/L), BAP (2.5 mg/L) and NAA (0.5 mg/L) were used as eight different treatments. The MS medium (T_1) without sugar (0.0 g/L) was used as the control. The pH of the media was adjusted to 5.8.

Evaluation of the growth of PLBs

Six months old PLBs (1.00 g per replicate) were cultured in the replicates of all the eight treatments (T_1 – T_8). The cultures were maintained under 20 mol/m^2/sec 14 h photoperiod at 26±4 °C. After one month of culturing, growth parameters were observed and recorded as, fresh weight of PLBs and the number of plantlets per PLB that have been regenerated. Cultures were maintained for two months and growth parameters were evaluated as fresh weight of PLBs, number of plantlets per replicate PLB, number of leaves per plantlet and number of roots per plantlet. Sub culturing was carried out at every 04 weeks interval.

Evaluation of plantlet growth

In each treatments (T_1 – T_8), 15 plantlets having 2-3 nodes and 2-3 cm height were cultured in five culture bottles (three plantlets per bottle). The cultures were maintained under 20 mol/m^2/sec 14 h photoperiod at 26±4 °C. After one month and four months period of culturing, growth parameters; plantlet height, number of leaves, number of roots, leaf diameter and root length were recorded. Sub culturing was carried out at every 04 weeks interval.

Statistical analysis

Completely Randomized Design (CRD) method was used and five replicates per treatment were used for both PLBs and plantlets. The results were analyzed using one-way ANOVA and Turkey's mean comparison test using the Minitab (19.0) statistical software.

Results and Discussion

After one month of incubation in MS media supplemented with different concentrations of edible sugar as the sucrose supplement, the highest increment of fresh weight of PLBs was observed in four different treatments which were T_3, T_4, T_5 and T_8. But after two months of incubation, the highest weight accumulation of 20.31 g from 1.00 g of PLBs was observed in MS medium supplemented with 45.0 g/L of sugar followed by the treatment T_8 which contained sugar 30.0 g/L, BAP (2.5 mg/L) and NAA (0.5 mg/L) (Table 1).

Table 1. *Effect of different concentrations of edible sugar on the growth and organogenesis of PLBs of the Dendrobium hybrid.*

Treatment	Sugar Concentration (g/L)	After 30 days		After 60 days			
		Fresh Weight (g)	No. of plantlets	Fresh Weight (g)	No. of plantlets	Mean no. of leaves per plantlet	Mean no. of roots per plantlet
T_1	0.0	1.40 ^{b}	1.2 ^{ab}	1.30 ^{c}	0.2 ^{b}	0.2 ^{c}	0.0 ^{a}
T_2	15.0	3.80 ^{ab}	8.6 ^{ab}	12.41 ^{b}	16.2 ^{ab}	2.8 ^{a}	0.0 ^{a}
T_3	30.0	5.60 ^{a}	11.2 ^{a}	17.10 ^{ab}	25.6 ^{ab}	4.0 ^{a}	2.6 ^{a}
T_4	45.0	5.40 ^{a}	11.2 ^{a}	20.31 ^{a}	35.2 ^{a}	2.8 ^{a}	5.6 ^{a}
T_5	60.0	4.00 ^{a}	10.8 ^{a}	13.55 ^{ab}	36.2 ^{a}	2.2 ^{ab}	5.2 ^{a}
T_6	75.0	1.40 ^{b}	1.2 ^{ab}	3.11 ^{c}	3.8 ^{b}	0.8 ^{bc}	0.0 ^{a}
T_7	90.0	1.40 ^{b}	0.2 ^{b}	1.46 ^{c}	0.0 ^{b}	0.0 ^{c}	0.0 ^{a}
T_8	30.0+PGR	5.00 ^{a}	11.0 ^{a}	19.04 ^{ab}	33.0 ^{a}	2.6 ^{ab}	0.0 ^{a}

PGR – Plant Growth Regulators - BAP (2.5 mg/L) & NAA (0.5 mg/L)
Values within the same column followed by the same letter are not significantly different as determined by Tukey's mean comparison test ($p \leq 0.05$).

According to Rafique et al. (2020), 5% (50.0 g/L) sucrose level was found to be the best in all the cases followed by 3% (30.0 g/L) and 7% (70.0 g/L) after 60 days of incubation for *Dendrobium sabin* H. variety. Same study reported that zero growth of fresh weight after 20, 40 and 60 days of incubation were observed in MS medium containing sucrose at the levels of 0.0 g/L and 130.0 g/L. In the current study, almost no growth of PLBs was observed in MS medium containing sugar at the concentrations of 0.0, 75.0 and 90.0 g/L after one and two months of incubation. With the absence of sucrose in the culture media, there was no carbon source to support the growth of calluses during the photosynthesis and it would result in a lack of adaptability to be autotrophic. Meanwhile, high sucrose concentrations could also have created a hypertonic environment in culture media, adversely affecting the availability of other required nutrients for the growth of calluses (Rafique et al., 2020 and Zahara et al., 2017).

When considering the organogenesis from PLBs of the *Dendrobium* hybrid, the highest number of plantlets (11.0) was observed in treatments T_3 (sugar, 30.0 g/L), T_4 (sugar, 45.0 g/L), T_5 (sugar, 60.0 g/L) and T_8 (sugar, 30.0 g/L + plant growth regulators) after 30 days of incubation while after 60 days of incubation, that ranking could be observed in the treatments T_4, T_5 and T_8. Meanwhile, no rooting was observed in any treatment after 30 days of incubation. After 60 days of incubation the best rooting was observed in the treatment T_4 supplemented with 45.0 g/L of sugar and it was not significantly different from the values of treatments T_3 and T_5. The highest average number of leaves per plantlet was observed in MS medium supplemented with 30.0 g/L sugar (treatment T_3) and that was not significantly different from the observation of treatments T_2 (sugar, 15.0 g/L) and T_4 (sugar, 45.0 g/L) (Figure 1). All the above results showed that treatment T_4 (45.0 g/L) has shown the best observations in all the tested parameters including fresh weight accumulation of PLBs, regeneration of plantlets, leaf formation and root formation resulting in the best overall organ development from PLBs after two months of incubation in *in vitro* conditions. Similar observations concerning the number of plantlets were reported by Rafique et al. (2020) with the highest number of plantlets in MS media containing 5% (50 g/L) sugar followed by 0%, 1.5%, 3%, 7%, 9%, 11% and 13%. However, in contrast to that, there was no effect of sucrose concentration on root formation of *Dendrobium* during *in vitro* propagation according to Faria et al. (2004).

Talukder et al. (2003) have reported that the best shoot proliferation, root formation, leaf formation and increment of shoot length and the least time requirement for regeneration were obtained from MS medium supplemented with a combination of BAP (2.5 mg/L) and NAA (0.5 mg/L) for orchids. Therefore, in this study, the above combination of plant growth regulators was used to compare the effect of growth hormones and the optimized sugar level on the organogenesis of PLBs of the *Dendrobium* hybrid. After 60 days of incubation, T_8 resulted in successful organogenesis similar to treatment T_4 only in the fresh weight accumulation, shoot generation and leaf formation but T_8 did not induce the root formation as the T_4 (sugar, 45.0 g/L). It can be due to the inhibition effect of auxins on root formation. The results showed that an optimized amount of edible sugar (45.0 g/L) would induce/motivate more organogenesis including both shooting and rooting in PLBs of the selected *Dendrobium* hybrid when compared to the plant growth regulators.

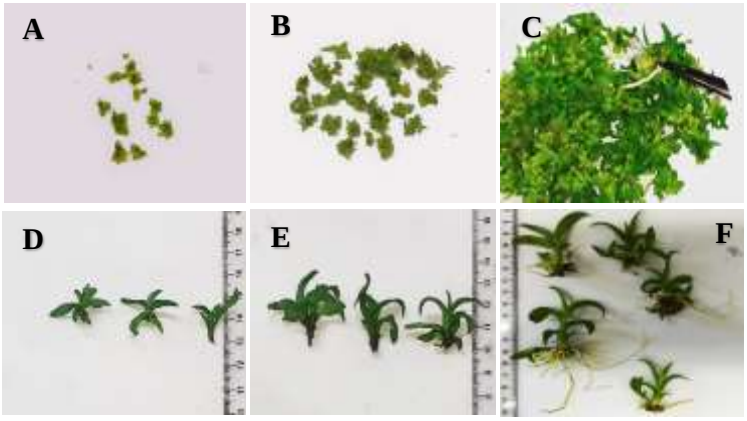

Figure 1. *In vitro growth and organogenesis of Dendrobium hybrid. PLBs grown in MS medium containing 45.0 g/L sugar; A) at initial stage, B) after one month of incubation and C) after two months of incubation. Plantlets grown in MS medium containing 60.0 g/L sugar; D) at initial stage, E) after one month of incubation and F) after four months of incubation (1 unit of scale = 1.0 cm).*

Referring to the effect of edible sugar on *in vitro* plantlet growth of the *Dendrobium* hybrid, no significant difference in any measured parameter relevant to the development of shoots and leaves was observed between treatments after one month of incubation (Table 2).

Table 2. *Effect of different concentrations of edible sugar on the growth and development of plantlets of the Dendrobium hybrid.*

		After One Month					After Four Months				
Treatment	Sugar concentration (g/L)	Mean difference of plantlet height (cm)	Mean difference of number of leaves per plantlet	Mean difference of number of roots per plantlet	Mean difference of leaf diameter (cm)	Mean difference of root length (cm)	Mean difference of plantlet height (cm)	Mean difference of number of leaves per plantlet	Mean difference of number of roots per plantlet	Mean difference of leaf diameter (cm)	Mean difference of root length (cm)
T_1	0.0	-0.5 ^{a}	-5.4 ^{b}	-0.9 ^{b}	-0.1 ^{a}	-0.1 ^{a}	-1.5 ^{c}	-7.6 ^{b}	-3.1 ^{c}	-0.5 ^{c}	-0.6 ^{b}
T_2	15.0	0.1 ^{a}	1.7 ^{a}	0.6 ^{ab}	0.0 ^{a}	0.0 ^{a}	0.0 ^{bc}	3.9 ^{ab}	3.3 ^{bc}	0.0 ^{bc}	0.2 ^{ab}
T_3	30.0	0.2 ^{a}	2.6 ^{a}	2.2 ^{ab}	0.1 ^{a}	0.0 ^{a}	1.1 ^{a}	9.7 ^{a}	17.2 ^{abc}	0.4 ^{a}	1.1 ^{ab}
T_4	45.0	0.3 ^{a}	3.6 ^{a}	3.4 ^{a}	0.0 ^{a}	0.3 ^{a}	0.8 ^{ab}	10.1 ^{a}	17.1 ^{abc}	0.3 ^{ab}	1.5 ^{a}
T_5	60.0	0.0 ^{a}	1.1 ^{a}	0.9 ^{ab}	0.1 ^{a}	0.0 ^{a}	0.6 ^{ab}	16.7 ^{a}	32.5 ^{a}	0.4 ^{a}	1.5 ^{a}
T_6	75.0	0.0 ^{a}	1.3 ^{a}	2.0 ^{ab}	0.0 ^{a}	0.0 ^{a}	0.2 ^{ab}	3.0 ^{ab}	19.2 ^{abc}	0.2 ^{ab}	1.5 ^{a}
T_7	90.0	0.1 ^{a}	0.8 ^{a}	1.4 ^{ab}	0.1 ^{a}	0.2 ^{a}	0.6 ^{ab}	7.1 ^{ab}	26.9 ^{ab}	0.2 ^{ab}	1.4 ^{a}
T_8	30.0 +PGR	0.1 ^{a}	4.1 ^{a}	0.6 ^{ab}	0.1 ^{a}	0.1 ^{a}	0.8 ^{ab}	14.6 ^{a}	2.8 ^{bc}	0.6 ^{a}	0.3 ^{ab}

PGR – Plant Growth Regulators - BAP (2.5 mg/L) & NAA (0.5 mg/L)
Values followed by the same letter are not significantly different as determined by Tukey's mean comparison test ($p \leq 0.05$).

However, after four months of incubation, significant differences in all the parameters were observed when all the treatments were compared. Among them, the significantly highest increment of shoot length (1.1 cm) was observed in treatment T_3. However, the other two parameters relevant to the development of shoots; leaf formation and the increment of leaf diameter, have shared the significantly highest values with different treatments. The best leaf formation was observed in treatments T_3 (9.7/plantlet), T_4 (10.1/plantlet), T_5 (16.7/plantlet) and T_8 (14.6/plantlet) while the best growth of leaf blades could be observed in treatments T_3 (0.4 cm), T_5 (0.4 cm) and T_8 (0.6 cm). When considering the leaf formation and the increment of leaf diameter together, treatments T_3, T_5 and T_8 had a similar effect. Therefore, the best treatment for the development of shoots and leaves of the *Dendrobium* hybrid could not be determined. Also, the difference between the effect of sugar and plant growth regulators on the development of shoots and leaves could not be identified. However, according to Talukder et al. (2003), the best shoot proliferation (1.90/explant), leaf formation (4.25/plantlet) and the increment of shoot length (0.472 cm) and the least time requirement for regeneration were observed with the supplement of BAP (2.5 mg/L) combined with NAA (0.5 mg/L). Further, MS medium supplemented with BAP (0.5 mg/L) and NAA (0.5 mg /L) has given the optimum seedling growth and shoot formation (Pant and Thapa, 2012) in *Dendrobium* cultures.

When considering the root formation, after one month of incubation, the highest difference in the number of roots (3.4/plantlet) was observed in MS medium supplemented with sugar at a level of 45.0 g/L. After four months of incubation, the best root formation (32.5/plantlet) was observed in treatment T_5 supplemented with 60.0 g/L of sugar. Further, the significantly highest increment of root length (1.5 cm) was observed in treatments T_4, T_5, T_6 and T_7 after four months of incubation though there was no significant difference of this parameter between different treatments after 30 days of incubation. However, when considering both root formation and root length increment together, only the treatment T_5 (sugar, 60.0 g/L) has performed well after four months of incubation. Therefore, the sugar concentration of 60.0 g/L in MS medium is more suitable to generate and develop roots from plantlets of the *Dendrobium* hybrid under *in vitro* conditions. Similar observations have been reported by Ferreira et al. (2010) by showing the highest root formation in Vaccine and Went medium containing sucrose at a concentration of 60.0 g/L after five months of incubation.

According to the observations, treatments T_3, T_5 and T_8 have affected similarly on the development of shoots and leaves while the treatment T_5 had the best performance on rooting. By considering these two major observations, it is suggested that the treatment T_5 can be used for the better growth and development of shoots and roots together (Figure 1). Further, it can be suggested that an optimized level of sugar (60.0 g/L) would induce more growth and development including shooting and rooting in plantlets of the *Dendrobium* hybrid when compared to the plant growth regulators.

Since, plantlets in the T_1 were dying due to the absence of a carbon source in the medium, no growth and only decaying was observed. Therefore, minus values were observed with all the parameters of the T_1 and the values increased with time as decaying continued with time.

Conclusion

According to the findings of the present research, basal MS medium containing 45.0 g/L of edible sugar has performed well for efficient growth and organogenesis of PLBs of the *Dendrobium* hybrid (*Dendrobium bigibbum* x *Dendrobium* Thailand Black), while MS

medium with an edible sugar concentration of 60.0 g/L showed the best *in vitro* growth and development of plantlets of the hybrid. Further, edible sucrose (sugar) can be used as an alternative carbon source to replace laboratory-scale sucrose and also a balanced level of edible sugar can be used as an alternative for plant growth regulators in a low-cost MS medium for successful *in vitro* growth and development of the *Dendrobium* hybrid.

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