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RESPONSE OF TREHALOSE AS A CARBON SOURCE ON ORGANOGENESIS OF PROTOCORM-LIKE BODIES (PLBS) IN *Dendrobium kingianum* CULTURED *IN VITRO*

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Abstract

Plant growth and development are largely dependent on the availability of carbohydrates as well as to serve as energy and carbon source *in vitro* culture. The aim of these studies was to know the response of trehalose on organogenesis of protocorm-like bodies (PLBs) in and selection of satisfactory application procedure in Murashige and Skoog (MS) medium for getting better response in this aspect. First experiment results revealed that there were no significant differences among treatments. In case of second experiment, maximum number of PLBs (19.4) was produced in the medium where PLBs were treated in 5 g/L trehalose for 2 days and then transferred to the sucrose (20 g/L) medium and were kept for five weeks when compared with the control (10.1). Third experiment showed PLBs formation rate was 100% at blue and red LED light treatments whereas shoot formation was highest (3.8) at red LED light.

INTRODUCTION

Dendrobiums are highly valued in the flower industry as potted plant and cut flower (Khosravi et al., 2008). Micropropagation provided an important breakthrough for mass propagation of many orchid species which have highly heterozygous genotype and have extremely slow sexual reproduction capability (Kanjilal et al., 1999). Over the years, Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) widely used in many orchid's micropropagation protocol including *Dendrobium* genus (Martin and Madassery, 2006; Fadel et al., 2010). Optimization of medium compositions was an important approach to fasten the micropropagation process and improve the quality of regenerated plantlets through culturing callus, adventitious shoots or protocorm-like bodies (PLBs) (Ichihashi, 1992; Chen et al., 2000; Park et al., 2002). Among them, different carbon sources such as sucrose, glucose, maltose, fructose and sorbitol were proven to have effects on PLB growth (Islam and Ichihashi, 1999; Tokuhara and Mii, 2003).

Sucrose has been accounted to be superior to other sugars for *in vitro* orchid growth (Jawan et al., 2010). Sucrose is easily available to cell and directly participate in glycolytic and pentose phosphate pathways for cell growth and was also found to act as osmotic role to the culture system (Zha et al., 2007). However, few studies have been done on using trehalose (a-D-glucopyranosyl-(1,1)-a-D-glucopyranoside) as a substitute for carbon source for the proliferation of PLBs. Trehalose is a nonreducing sugar, not easily hydrolyzed by acid, and the glycosidic bond is not cleaved by alpha-glucosidase. Trehalose was previously reported to be used on orchid seed propagation (Ernest, 1967; Liu et al., 2006; Smith, 1973) when compared with different sugars as energy source for asymbiotic germination. In addition, trehalose has been used on the preservation of somatic embryos (Ryan et al., 1999) and maintenance of algae suspension cultures (Malik, 1995). This work was undertaken in order to highlight the effect of trehalose on PLBs proliferation of *Dendrobium kingianum*. The influence of using different culture systems was taken into consideration too.

Light is the major energy source for photosynthesis and regulates plant morphogenic and gene expression (Ma et al., 2001; Mark et al., 2000). Plants respond to a wide spectrum of light ranging from ultraviolet (UV) to far-red light. *In vitro* culture, fluorescent white light which having different spectral emission composed of many wavelengths from 350-750 nm has been used as a light source for maintaining tissue cultured plants (Lian et al., 2002). Based on to increase the efficiency of *in vitro* techniques, environmental factors in particular medium composition, temperature and light are considerable important for modification and innovation of cultivation technologies for orchid plant.

MATERIALS AND METHODS

Plant and culture conditions

PLBs of *D. kingianum* were proliferated on modified MS (Shimasaki and Uemoto, 1990) medium supplement with 412.5

mg/L ammonium nitrate, 950 mg/L potassium nitrate, 20 g/L sucrose, and 2.2 g/L phytagel (Sigma). Modified MS medium was adjusted to pH 5.8 with 1 mM 2-(N-morpholino) ethanesulfonic acid sodium salts (MES-Na) before autoclaving at 121°C for 15 min at 1.5 kg f cm^{-2}. 250 ml of UM culture bottles (AsOne, JAPAN) with plastic caps were used; each bottle received 30 ml of medium. Five PLBs explants were put in each culture vessel and three culture vessels were used for each treatment.

Carbohydrate sources

Two kinds of carbohydrate sources: sucrose (Sigma, USA) and trehalose (Hyashibara, Japan) were used in different ways. In experiment 1, sucrose (0, 5, 10, 15, and 20 g/L) was combined with trehalose (0, 5, 10, 15, and 20 g/L). In experiment 2, trehalose (5 g/L) was used as pulse treatments and PLBs were treated for 1, 2, 3, 4, and 5 days, and then transferred to 20 g/L sucrose treatment. In experiment 3, sucrose (15g/L) combined with trehalose (5g/L) to elucidate the effect of trehalose on different LED light conditions on the in vitro PLBs growth of *Dendrobium kingianum* , the cultures were established and grown under different light conditions of photon flux density (PFD) of 54 µmol m^{-2} s^{-1}.

Data collection and statistical analysis

Visual observations were carried out weekly. The numbers and percentage of PLBs, the numbers and percentage of shoots, and fresh mass of PLBs + shoot were recorded after five weeks of culture. The experiment was a completely randomized design with 3 repetitions and each repetition contained 5 PLBs. The data were subjected to a one-way analysis of variance (ANOVA) and differences between means were tested using Tukey's honestly significant different test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Effect of trehalose and sucrose on organogenesis of PLBs in *D. kingianum*

Data in **Table 1** shows that there were no significant differences found among treatments in all traits. However, maximum number of PLBs (13.5) and shoot (1.9) were found in the medium containing 5 g trehalose combined with 15 g/L sucrose. The percentage of PLBs formation were 100% in all treatments whereas maximum percentage of shoot formation (80%) was found in 10 g/L trehalose combined with 10 g/L sucrose treatment. The highest fresh weight (0.293 g) was found in 20 g/L trehalose treatment.

Table 1. Effect of Trehalose and Sucrose on organogenesis of PLBs in *Dendrobium kingianum* after 35 days

Trehalose+Sucrose	Number of PLB	% of PLB	Number of Shoot	% of shoot	Fresh mass (g)
0 + 20 g	10.1[a]	100	1.8[a]	66.7	0.254[a]
5 + 15 g	13.5[a]	100	1.9[a]	73.3	0.265[a]
10 + 10 g	10.5[a]	100	1.9[a]	80.0	0.265[a]
15 + 5 g	9.90[a]	100	1.3[a]	46.7	0.229[a]
20 + 0 g	12.0[a]	100	1.8[a]	73.3	0.293[a]

Values represent means followed by the different letters show significant differences by Turkey HSD test (P≤0.05).

Another experiment was done to see the effects of 5 g/L trehalose used as pulse treatment. The highest number of PLBs (19.4) was found in the medium where PLBs were treated in two days which was significantly different when compared with control (**Table 2**). But the rate of shoot formation (66.7) was maximum at control (**Figure 1**).

Table 2. Response of trehalose (5 g/L) as pulse treatment on organogenesis of PLBs in *Dendrobium kingianum* after 35 days.

Trehalose (5 g/L)	Number of PLB	Number of shoot	Fresh mass (g)
0 day	10.1[b]	1.6[a]	0.124[b]
1 day	14.2[ab]	1.7[a]	0.133[b]
2 day	19.4[a]	1.7[a]	0.407[a]
3 day	16.8[a]	1.1[b]	0.390[a]
4 day	15.8[a]	2.2[a]	0.365[a]
5 day	14.1[ab]	1.9[a]	0.289[a]

Values represent means followed by the different letters show significant differences by Turkey HSD test (P≤0.05)

Effects of trehalose (5g) and sucrose (15g) on growth and development of PLBs *in Dendrobium kingianum* cultured *in vitro* under different light emitting diode

Effects of trehalose in modified MS under different light quality on organogenesis in PLB of *Dendrobium kingianum* after 35 days of culture is shown in **Table 3**. The highest number of PLBs (7.2) was recorded in the media grown under blue LED light and had negative effect on shoot formation compared with other light. Meanwhile, PLBs formation rate showed 100% at blue and red LED light treatments whereas shoot formation was highest (3.8) at red LED light. In case of PLBs formation green LED showed near same effect like blue LED when 5 g trehalose used with 15 g sucrose.

Table 3. Effects of trehalose (5g) and sucrose (15g) on growth and development of PLBs *in Dendrobium kingianum* cultured *in vitro* under different light emitting diode

Light Quality	Avg No. of PLBs	Avg No. of Shoots	Fresh Wt. (g)
White Fluor. Lamp	6.6±0.03	3.2±0.01	0.31
Blue LED light	7.2±0.02	0.7±0.04	0.19
Red LED light	6.3±0.03	3.8±0.04	0.33
Green LED light	7.0±0.01	2.1±0.02	0.22
Blue+Red (1:1)	4.6±0.04	1.5±0.01	0.12
Red+ green (1:1)	5.7±0.02	3.0±0.01	0.26
Green+Blue (1:1)	5.3±0.02	2.7±0.03	0.22

Values represent meas ± SE value.

Carbohydrates are very important energy source for growth and developments of plant cells. Accumulation of the trehalose plays an important role to protect the cell composition from damage by the oxidation reaction under the stress condition (Tokuhara and Mii, 2003). Trehalose itself could affect development by acting as a signal molecule in carbohydrate metabolism. For example, trehalose induces enzymes of fructan synthesis in barley (Wagner et al., 1986; Muller et al., 2000) and sucrose synthase activity in soybean (Muller et al., 1998). Wingler et al. (2000) suggested that trehalose interferes with carbon allocation to the *Arabidopsis* sink tissues by inducing starch synthesis in the source tissues. Sucrose (10 g/L) was extremely inefficient for production of PLBs of *Dendrobium huoshanense* as compared to 35 g/L sucrose (Zha et

al., 2007). In *Cymbidium* species, trehalose supposed to be signals for organogenesis *in vitro* cultures.

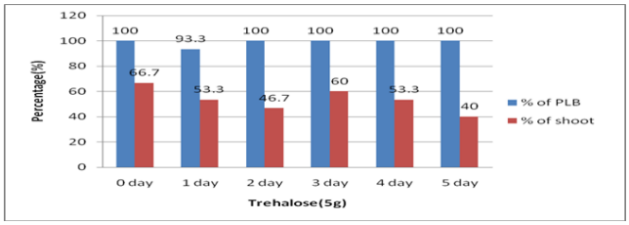

Figure 1. Response of trehalose (5g/L) as pulse treatment on PLB and shoot formation rate (%) of *Dendrobium kingianum.* Percentage of PLB/Shoot formation (%) = (Number of cultured explants with new PLBs or shoot/Total number of cultured explants) × 100.

Pulse treatments using trehalose and sucrose were considered to be useful for plantlet production in *Cymbidium* spp. (Shimasaki et al., 2003). Using the fungal carbohydrates trehalose and mannitol, as well as glucose, Smith (1973) demonstrated that seed of the terrestrial orchids *Dactylorhiza purpurella* and *Bletilla striata* utilized trehalose and glucose as suitable primary carbohydrate sources during asymbiotic germination. Hew and Mah (1889) reported that carbohydrate hydrolysis by extracellular hydrolytic enzymes is possible, as demonstrated with PLBs of *Dendrobium*.

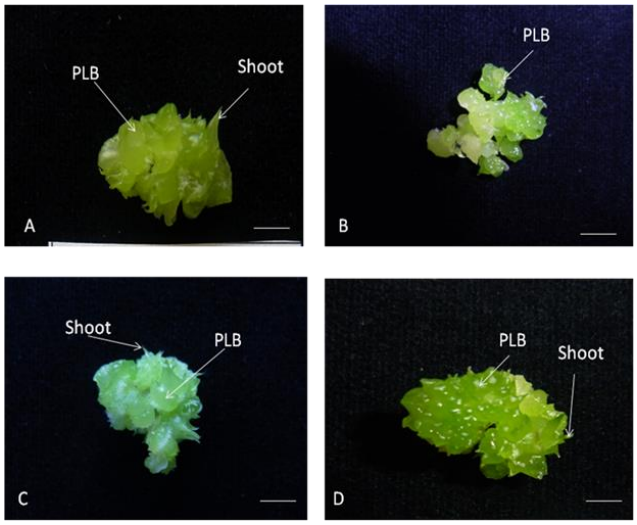

Figure 2. Effects of trehalose on organogenesis of PLBs in *Dendrobium kingianum;* A.Control; B. 5g/L Tre + 15g /L Suc ; C. 10g/L Tre + 10g /L Suc; D. 20g/L Tre + 0g /L Suc; Bars: 1cm.

Deficiency of sucrose supply to *in vitro* orchid is detrimental for cell growth rate. Sucrose is specifically needed in plant embryo to increase cell division by cell encouraging cell expansion and reserve accumulation (Borisjuk et al., 2003). However, increasing sucrose over the threshold concentration could lead to excessive carbohydrate accumulation and hinder photosynthesis which eventually impairs cell growth of rose plant (Capellades et al., 1991). Optimum media salt concentration is essential for *in vitro* orchid to provide sufficient nutrient required to promote metabolism and cell growth and to prevent toxic effect of media salt (Fadel et al., 2010). Jheng et al. (2006) demonstrated that trehalose at concentration of 20 g/L is a superior carbohydrate to expedite converting PLBs to plants. For orchid crops, the system of callus cultures and regeneration is rather time-consuming. In addition to high efficiency of PLB production, how to shorten the culture time is also an important task. In previous studies demonstrated that the combination of red and blue light was an effective light source for several crops (Kim et al., 2004). Orchid PLBs cultured under red LED showed the lowest differentiation rate, while using blue LED resulted in the highest differentiation rate in cultures of *Oncidium* and *Dendrobium officinale in vitro* (Xu et al., 2009; Lin et al., 2011). In contrast, with *Cymbidium* orchid cultures, a mixture of red plus blue light, and red LED alone, enhanced both plant growth and development by increasing the net photosynthesis (Tanaka et al., 1998; Huan and Tanaka, 2004). This is because the spectral energy distribution of red and blue light coincided with that of chlorophyll absorption (Goins et al., 1997). Sucrose is specifically needed in plant embryo to increase cell division by cell encouraging cell expansion and reserve accumulation (Borisjuk et al., 2003). However, increasing sucrose over the threshold concentration could lead to excessive carbohydrate accumulation and hinder photosynthesis which eventually impairs cell growth of rose plant (Capellades et al., 1991). This study reveals that trehalose is a suitable carbohydrate source as an alternative of sucrose for large amount of PLB formation and for high efficiencies of plant regeneration in *D. kingianum* culture *in vitro* and the contribution of LED lights, sucrose and trehalose (combined) can induce PLB and shoot formation of *Dendrobium kingianum* tissue culture without use any plant growth regulator and blue and red light showed best formation rate on PLB and shoot accordingly compare with other qualities of lights.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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