Effects of Green LED Lighting on Organogenesis and Superoxide Dismutase (SOD) Activities in Protocorm-like Bodies (PLBs) of *Cymbidium* Cultured in vitro

Narumol Kaewjampa\(^1\) and Kazuhiko Shimasaki\(^2\)

\(^1\) The United Graduate School of Agricultural Sciences, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8556, Japan
\(^2\) Faculty of Agriculture, Kochi University, B200 Monobe, Nankoku, Kochi 783-8502, Japan

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Cymbidium protocorm-like bodies (PLBs) were cultured in modified MS (1962) medium (Shimasaki and Uemoto, 1990) to investigate the effect of light quality on organogenesis in PLBs, and the changes in superoxide dismutase (SOD). Of the different light treatments used in the research, six times of weekly light breaking by one day of green LED lighting (green LED interval lighting) during red LED illumination showed optimal number (14.1) and formation rate (93.1\%) of PLB cultures. Optimal shoot formation of PLB cultures obtained at treatments of fluorescent lamp + interval lighting of green LED and blue LED + interval lighting of green LED. The highest fresh weight was achieved under fluorescent lamp condition. Except for red LED lighting treatment, interval lighting of green LED enhanced increase in antioxidant enzyme SOD activity of PLB cultures, and showed the highest SOD activity at blue LED + green LED interval lighting treatment. The study concludes that except for red LED lighting condition, interval lighting using green LED could stimulate proliferation, shoot formation in PLB cultures which accompanying with increase in SOD enzymes.

Keywords: *Cymbidium*, green light, protocorm-like body (PLB), superoxide dismutase (SOD)

INTRODUCTION

*Cymbidium* is an important genus in Orchidaceae, with 52 documented species. *Cymbidium* species have been hybridized for over a century to produce plants with flowers of rich texture, color and size that have formed the basis of a worldwide flower. With their many desirable features, hybrid *Cymbidiums* has high value among orchid flowers in auction markets around the world. In order to meet the needs of the flower market, new ways have been sought to improve the reproduction speed of hybrid *Cymbidium*. Thus, many attempts have been made to develop better methodologies for *Cymbidium* micropropagation. Currently, these hybrids are gaining in popularity in many countries, especially in Japan. To increase the efficiency of *in vitro* techniques, cultivating conditions such as light, temperature, and medium composition must be optimized.

Light quality (spectral quality), quantity (photon flux) and photoperiod have a profound influence on the morphogenesis of propagules cultured *in vitro*, and also on further growth of the organs.
initiated from the cultures (Exonomou and Read, 1987). The common light for in vitro culture is provided by fluorescent lamp. Light emitting diode (LED) as a new light source have many advantages compared with fluorescent light, namely longer life, wavelength specificity and narrow bandwidth (Hoenecke et al., 1992; Brown et al., 1995). Red and/or Blue light provided by LED lamps have been applied for plant growth promoting (Lee et al., 2007; Kim et al., 2004; Baque et al., 2010). In orchid cultures in vitro, combination lighting with red and blue LEDs were effective for the growth and development of PLBs in Cymbidium, Doritaenopsis, Phalaenopsis, and Cataphene (Huan and Tanaka, 2004; Shin et al., 2008; Wongnak et al., 2008; Baque et al., 2011). Single lighting by blue LED also increased shoot formation of PLB cultures, in Dendrobium officinale (Lin et al., 2011). However, there is little information available for effect of green-LED lighting on growth and environmental factors of plants (Islam et al., 1999). Kudo et al. (2011) showed that interval lighting of green light could induce pathogen resistance by giving stress to plants which were accompanied by activation of elicitor activities. In plant tissue culture, light is one of the abiotic stress factors that can induce oxidative stress, which formation of reactive oxygen species (ROS) molecules such as superoxide anion O₂⁻, hydrogen peroxide H₂O₂ and hydroxyl ion OH⁻. ROS can cause damage to the cellular structure and eventually lead to cell death (Asada, 1999; Mittler, 2002). Plants have evolved efficient enzymatic antioxidant systems to scavenge ROS. Superoxide dismutase (SOD) is the most effective intracellular enzymatic antioxidant, and is ubiquitous in all aerobic organisms and in all subcellular compartments prone to ROS mediated oxidative stress. The SODs convert superoxide to H₂O₂ and O₂ by catalyzing its dismutation (Ali et al., 2005). Gill and Tuteja (2010) proposed that SOD is important in plant stress tolerance and provides the first line of defense against the toxic effects of elevated levels of ROS. Red and blue lights affected on growth and development of plant tissue and organ cultures which were accompanied by changing SOD activity in cultures (Shohael et al., 2006; Baque et al., 2010). In orchid, SOD activity increased with higher light intensities (Ali et al., 2005; Li et al., 2004; Li et al., 2001). However, the effects of green-LED lighting on organogenesis and SOD activities in tissue and organ cultures of orchid PLBs are not well understood.

Therefore, this study investigated the effect of interval light of green LED lighting during illumination provided by different types of lamps on organogenesis and changes in SOD antioxidant enzymes of PLB cultures of Cymbidium.

MATERIALS AND METHODS

Plant material and culture medium

Approximately 5 mm in diameter PLBs derived from meristem cultures of Cymbidium Waltz ‘Idol’ which proliferated in modified MS medium (Shimasaki and Uemoto, 1990) were served for explants. MS medium with 412.5 mg l⁻¹ ammonium nitrate, 950 mg l⁻¹ potassium nitrate, 20 g l⁻¹ sucrose, and 2 g l⁻¹ Phytagel (Sigma) was adjusted to pH 5.5-5.8 before autoclaving. 250 ml of UM culture bottles (AsOne, JAPAN) with plastic caps were used, each bottle receiving 30 ml of medium. Five explants were put in each culture vessel and three culture vessels were used for each treatment.

Lighting conditions

To elucidate the effect of different light conditions on the in vitro PLBs growth of C. Waltz ‘Idol’, the cultures were established and grown under different light conditions of photon flux density (PFD) of 50 µmol m⁻²s⁻¹. There were seven radiation treatments: (1) fluorescent lamp (National FL20SS as control), (2) red LEDs (Jecom, P18W-E1701-R, peak wavelength: 640 nm), (3) blue LEDs (Jecom, P18W-E1701-B, peak wavelength: 450 nm), (4) green LEDs (Jecom, P18W-E1701-G, peak wavelength: 510 nm), (5) fluorescent plus green LEDs, (6) red LEDs plus green LEDs and (7) blue LEDs plus green LEDs. In addition treatments 5-7 were subjected to
effects of green LED lighting on cybridium organogenesis

additional green LEDs exposure for one day in the 7 day cycle (i.e. 6 days under one type of light quality, then 1 day under green LEDs) repeated over a 6 weeks period.

All treatments were maintained at 25±2°C with a 16 h photoperiod for 6 weeks (42 days). The number of new PLBs, number of shoots per explants, PLB formation, shoot formation and fresh weight of PLBs were recorded.

Antioxidant enzyme assay

To discriminate which antioxidant enzymes activities were superoxide dismutase (SOD; EC 1.15.1.1) activity, the fresh PLBs of C. Waltz ‘Idol’ (2 g FW) were homogenized in 8 ml of 50 mM phosphate buffer at pH 7.0 in a pre-chilled mortar and pestle by liquid nitrogen. The homogenate was filtered through two layers of cheesecloth and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was centrifuged an additional 2–3 times to clear all the debris. The supernatant was then transferred into microtube, stored at −20°C, and centrifuged again before use. SOD activity (U/ml) was tested with the SOD Assay Kit. The procedure was carried out in accordance with the protocol in the kit used. Plant extracts (20 μl) at a concentration of 0.25 g ml⁻¹ were added to 200 μl of the kit working solution. After gently agitating the mixture, 20 μl of the kit enzyme working solution was added and this was incubated at 37°C for 20 min. The absorbance of the mixtures was measured at 450 nm using a microplate reader (BIO-RAD iMark™, JAPAN) and the SOD activity was calculated using the following equation (Xing et al., 2010): Percentage of inhibition (SOD activity) = \( \frac{[(A_{\text{blank} 1} - A_{\text{blank} 2}) - (A_{\text{sample} 1} - A_{\text{sample} 2})]}{(A_{\text{blank} 1} - A_{\text{blank} 2})} \times 100 \), where \( A_{\text{blank} 1} \) was a mixture of the working solution (200 μl) and enzyme working solution (20 μl) containing 20 μl double distilled water (ddH₂O). \( A_{\text{blank} 2} \) contained the plant extract (20 μl) with working solution (200 μl) and dilution buffer (20 μl), while ddH₂O (20 μl) was added to the plant extract in the \( A_{\text{blank} 1} \).

One unit of SOD activity was defined as the amount of enzyme in 20 μl of sample solution that inhibits the reduction reaction of WST-1 with superoxide anion by 50%

Statistical analysis

The experiment was a completely randomized design with 3 replications and each replicate contained 5 PLB. The data were subjected to a one-way analysis of variance (ANOVA) and differences between means were tested using Turkey’s honestly significant different test (P ≤ 0.05).

RESULTS

Effect of different light conditions on the multiplication of PLB

The growth and development of protocorm-like bodies (PLBs) of C. Waltz ‘Idol’ were significantly affected by different light treatments in vitro. The resulting PLBs were green-yellow when grown under red LED, red+ green LED and green LED.

PLBs showed normal chlorophyll formation when grown under blue LED, blue+ green LED, fluorescent lamp and fluorescent + green LED (Fig. 1). As shown in Table 1, after 42 days of culture, the maximum percentage of PLB formation was 93.1% – an average of 14.1 PLBs per explant – under red + green LED. By contrast, the minimum percentage of PLB formation was 88.7% – an average of 7.8 PLBs per explant – under fluorescent lamp + green LED. Except for the treatment of red + green LED, all treatments subjected to green LED inhibited PLB formation. On the other hand, red + green LED treatment significantly enhanced both number and percentage of PLB formation.

Of the seven different light conditions, fluorescent lamp + green LED showed the maximum effect on percentage of PLBs shoot formation – 93.3% after 42 days of culture. Meanwhile, PLBs averaging more than 3 shoots were produced under blue + green LED, blue LED and fluorescent + green LED, and PLBs averaging less than 3 shoots were produced under green LED followed by red + green LED, red LED, and fluorescent lamp conditions.
Fig. 1 PLB Cultures of *Cymbidium* Waltz ‘Idol’ after 42 days of culture under different light treatments. a: Red LED, b: Red + green LED, c: Green LED, d: Blue LED, e: Blue + green LED. f: Fluorescent lamp. g: Fluorescent + green LED. Bars = 10 mm.

Table 1 Effect of different light conditions on organogenesis in PLB cultures of *Cymbidium* Waltz ‘Idol’

<table>
<thead>
<tr>
<th>Lighting treatments</th>
<th>PLB</th>
<th>Shoot</th>
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<tbody>
<tr>
<td></td>
<td>Average Number</td>
<td>Formation Rate (%)</td>
</tr>
<tr>
<td>Fluorescent Lamp</td>
<td>9.6 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.7</td>
</tr>
<tr>
<td>Red LED</td>
<td>10.0 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.2</td>
</tr>
<tr>
<td>Blue LED</td>
<td>13.2 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92.9</td>
</tr>
<tr>
<td>Green LED</td>
<td>10.2 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91.1</td>
</tr>
<tr>
<td>Fluorescent Lamp + Green LED</td>
<td>7.8 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>88.7</td>
</tr>
<tr>
<td>Red + Green LED</td>
<td>14.1 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93.1</td>
</tr>
<tr>
<td>Blue + Green LED</td>
<td>11.3 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91.8</td>
</tr>
</tbody>
</table>

Values represent means ± SE followed by the different superscript letters show significant differences by Turkey HSD test (*P* ≤ 0.05).
<sup>a</sup> Average number = Number of cultured explants with new PLB/shoot
<sup>b</sup> Total number of culture explant
<sup>c</sup> Percentage of PLB/shoot formation = Number of cultured explants with new PLB/shoot × 100
Total number of culture explants

In addition, the fresh weight of PLBs was recorded. The maximum fresh weight of PLBs under all seven different light treatments was 0.582 g under fluorescent lamp, and minimum fresh weight was 0.412 g under green LED.

**Effect of different light conditions on superoxide dismutase activity**

The change in total SOD activity in *C*. Waltz ‘Idol’ PLBs was observed after 42 days of *in vitro* culture under different light conditions. Of all the different light treatments, blue + green LED lighting induced the highest SOD activity with 0.35 units, followed by fluorescent + green LED with 0.32 units. Red LED-treated PLBs showed the lowest activity with 0.18 units, which was about 2 times lower than that of blue + green LED lighting. Except for red LED + green LED
lighting treatment, the total increase in SOD activity in PLBs treated under lighting subjected with green LED was about 1–2 times greater than in treatments without green LED. SOD activity of PLBs under continuous lighting of fluorescent lamp, red LED and green LED had small effect on increase in SOD activities (Fig. 2).

DISCUSSION

Light is one of the most important factors regulating plant development through photoreceptors active under specific wavelength of light (Lee et al., 2007). 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 D. 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). In our results, we found that red LED lighting with interval green LED lighting enhances PLBs growth and development more than under red LED alone. Moreover, plant growth as defined by shoot number and PLBs fresh weight was maximized under a mixture of red + blue light and blue LED (Moon et al., 2006; Shin et al., 2008; Lin et al., 2011). Our results indicated that lighting using blue + green LED and fluorescent lamp + green LED were more efficient in promoting shooting of PLBs than any other light sources (Table 1).

The use of green light in combination with red and blue LED may promote increased plant growth, since green light can penetrate into the plant canopy better than red or blue light (Klein, 1992; Smith, 1993). In lettuce plants grown in combinatorial red, blue and green (RB+G) light treatments displayed leaves with larger specific leaf area, less thickness and plant dry mass compared with RB alone (Kim et al., 2004). In Cattleya seedlings, under green light shoots length and the number of leaves per plant increased, but fresh weight and dry weight were smaller when compared with red and yellow lights sources (Islam et al., 1999). Our findings suggest, in accordance to the above mentioned studies, that green supplemental lighting could also offer benefits, when subjected with red, blue and fluorescent light and potentially increase plant growth and development by enhancing rates of photosynthesis.

In plant, SOD is one of several important antioxidant enzymes with the ability to repair oxi-

![Fig. 2](image_url) Changes in SOD activity in crude extract obtained from PLB of Cymbidium Waltz ‘Idol’ grown under different light conditions for 42 days of culture. Different letters indicate significant differences between means at $P \leq 0.05$ (Turkey’s HSD). Bars = SE.
dation damage caused by reactive oxygen species (ROS). Thus, SOD is considered a key enzyme for maintaining normal physiological conditions and coping with oxidative stress in the regulation of intracellular levels of ROS (Mittler, 2002). SOD activity increase due to various environmental factors and chemical stimuli (Fridovich, 1986; Perl et al., 1993) such as light, drought, temperature stress, salinity, heavy metals, pathogens and air pollutants (Bartosz, 1997; Mittler, 2002). In our study, the activity of SOD in PLBs increased significantly with different light conditions (Fig. 2). During the photosynthesis light reactions, the oxygen generated in the chloroplast can accept electrons passing through the photosystems, thus forming superoxide anion radicals (O$_2^\cdot-$) (Asada and Takahashi, 1987). In addition to this, when absorbed light energy exceeds the capacity of the photosystems to direct it through photosynthetic electro transport, photosynthesis is inhibited and pigments and membranes become damaged (Powles, 1984). To protect themselves, cells rapidly generate the enzyme superoxide dismutase (SOD) to converts O$_2^\cdot-$ very efficiently to H$_2$O$_2$ and O$_2$: (Pang et al., 2005). Therefore, strong light stress may reflect to the increasing of SOD activity.

In our study, the SOD activity in PLBs increased higher under blue + green LED than under red LED may reflect a lower O$_2^\cdot-$ production or a higher capacity for elimination of O$_2^\cdot-$ This indicated that under different light stress conditions such as blue + green LED, high activity of SOD is important for plant to tolerate stresses. Meanwhile, SOD activity in Oncidium PLBs and leaves were found the maximum values under blue LED and blue + green LED, respectively (Mangxi et al., 2011). In Phalaenopsis and Oncidium plantlet, SOD activity was found the maximum values under red + blue (1:3) LED and red + blue + yellow (5:3:2) LED, respectively. Even minimum values of SOD activity in both orchids species were found under red LED (Chen, 2002). In contrast, in Eleutherococcus senticosus somatic embryos, light-induced antioxidative enzymes, the highest SOD activity was found under red light, and lowest under blue + far-red light (Shohael et al., 2006). Baque et al. (2010) reported that in Morinda citrifolia, the highest levels of SOD activity were observed under red, red + blue and far-red light, but lowest under fluorescent and blue light. Therefore, increased SOD activity scavenges O$_2^\cdot-$ radicals to protect cellular components form oxidative damage, and it is important in determining the ability of plants to survive. Nevertheless, the regulation of SOD must be influenced by light sources and plant specific-species.

In addition to the relationship between SOD activity of PLBs and PLBs formation of C. Waltz ‘Idol’, SOD activity of PLBs under red + green LED were increased higher than under red LED alone, and it was observed that the percentage of PLBs formation was greater under red + green LED than red LED alone. In contrast, under fluorescent + green LED and blue + green LED were found SOD activity increased higher than those under fluorescent lamp and blue LED alone, whereas it was observed that the percentage of PLBs formation was lower under fluorescent + green LED and blue + green LED than fluorescent lamp and blue LED alone (Table 1, Fig. 2). This result implies that the composite spectra of red and blue LED has excellent energy efficiency when used for the growth of PLBs in vitro, while green LED has inhibited growth of PLBs in vitro (Kozai et al., 1997). Additionally, changes in SOD activity could affect endogenous hormone content, which consequently affect cell proliferation (Quiseng et al., 2005). The events underlying the regulation of cell division and further differentiation process are under tight control of both plant intrinsic and environmental parameters.

CONCLUSION

In conclusion, for in vitro micropropagation of hybrid Cymbidium, the effect of different light conditions on in vitro growth and activity of antioxidant enzymes via SOD activity of PLBs, red + green LED and blue + green LED were found to be best for promoting PLB formation and shooting of PLBs of C. Waltz ‘Idol’, respectively. Meanwhile, the interval illumination of green light with other light sources increased SOD activity in PLBs most efficiently. The
importance of lights as regulating factors is also observed by the high SOD activities observed in high light energy. The increased SOD activity in the presence of light leads to successful scavenging the ROS and provides evidence for occurrence of oxidative stress in different light conditions-treated PLBs of C. Waltz ‘Idol’.

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