

The response of protocorm-like bodies of nine hybrid *Cymbidium* cultivars to light-emitting diodes

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Abstract

The objective of this study was to assess how the protocorm-like bodies (PLBs) of nine hybrid *Cymbidium* cultivars would respond to light-emitting diodes (LEDs) at different ratios under *in vitro* conditions. The red (R) to blue (B) LED ratio (100% R; 60% R + 40% B; 50% R + 50% B; 40% R + 60% B; 100% B; control = plant growth fluorescent light (PGF)) affected *neo*-PLB formation in a cultivar-dependent way: 100% R or 100% B had a negative effect on *neo*-PLB production while 40% R + 60% B favored *neo*-PLB formation more than under PGF, although the resulting PLBs were of poor quality, i.e., could not be used for clonal purposes. This study reinforces the fact that it is essential to test optimal conditions for the *in vitro* culture of individual hybrid *Cymbidium* cultivars and that light conditions can quantitatively influence the organogenic result. Furthermore, caution must be exercised when interpreting the results, as these are based on the timing of sampling and on the developmental stage of the material.

Key words: light-emitting diode, protocorm-like body, Teixeira *Cymbidium* medium.

Abbreviations: B, blue; LED, light-emitting diode; PLB, protocorm-like body; R, red; TC, Teixeira *Cymbidium* medium.

Introduction

Lighting is one of the most important abiotic factors affecting the success of an *in vitro* plant culture, and can in some instances constitute a form of stress, either when excessive and reaching the light saturation point (e.g., Rakić et al. 2015), or when too weak (e.g., Liu et al. 2014). Consequently, the performance of a plant *in vitro*, which depends on its physiology, can be influenced by the quality of the light source. Of two light sources that have seen expanded applications in plant research, light-emitting diodes (LEDs; Dutta Gupta, Jatothu 2013) and cold-cathode fluorescent lamps (e.g., Norikane et al. 2013), the former is the focus in this study due to its reliability (Chang et al. 2012).

The most common form of LEDs used in horticultural experiments are blue (B) and red (R), since these correspond to absorption peaks of chlorophyll *b* and chlorophyll *a*, respectively, at 455 and 660 nm, respectively (Massa et al. 2008). LEDs have been used in experimental systems to manipulate plant growth, usually under different ratios (e.g., a recent case using papaya; Teixeira da Silva 2014a and references therein). The use of R and B LEDs is not new to *Cymbidium* research and has been shown to enhance *Cymbidium* plantlet growth *in vitro* under CO₂ enrichment (Tanaka et al. 1998) and morphogenesis of *Cymbidium* protocorm-like body (PLB) segments (Nhut et al. 2005). Using half-PLB segments as explants and *Cymbidium*

Twilight Moon cultivar ‘Day Light’, Huan and Tanaka (2004) induced most callus under 75% R + 25% B, but most new PLBs (i.e., *neo*-PLBs) with a 25% R + 75% B LED ratio. Despite these studies, there has been no attempt to explore whether the response to different PLB ratios is a standard and cultivar-independent response. Thus, using this cultivar as the basis for comparison, the effect of different ratios of B : R LEDs was tested on nine hybrid *Cymbidium* cultivars. The ability to manipulate organogenesis and morphogenesis in *in vitro* conditions using methods – in this case LEDs – other than chemical means, such as the conventional application of plant growth regulators, is attractive, since it allows the abiotic environment to be strictly controlled (e.g., the R : B LED ratio). Such an experimental system is attractive for studying light-induced stress mechanisms, because the LED ratio can be fine-tuned to establish a specific ratio (Fig. 1), while the light intensity can also be perfectly programmed. Since no such developed systems exist for orchids, this makes this study unique, allowing for a similar experimental design to be established for any orchid to conduct light-induced stress studies.

Materials and methods

Chemicals and reagents

All plant growth regulators were purchased from Sigma-Aldrich (St. Louis, USA) and were of tissue culture grade. All other chemicals and reagents were of the highest analytical

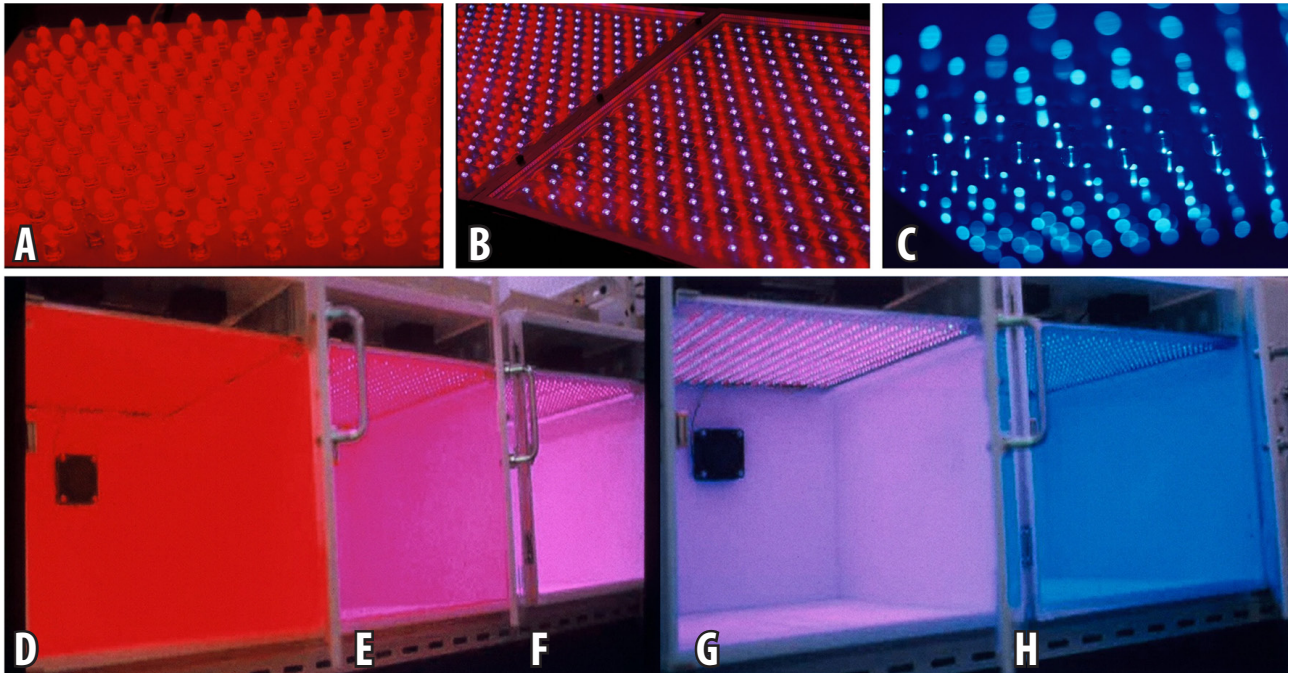


Fig. 1. Distribution of red (R) and blue (B) LED bulbs used in the present study. A, R LEDs; B, R + B LEDs; C, B LEDs; D, 100% R; E, 60% R + 40% B; F, 50% R + 50% B; G, 40% R + 60% B; H, 100% B. R and B LEDs were arranged on the ceiling of “LED PACKS”, 25 × 31 × 25 cm, described in Tanaka et al (1998). Each LED PACK contained 176 LED bulbs (5 mm diameter, Sharp Electric Ltd, Tokyo, Japan) and the R + B LED ratio was adjusted based on a uniform division of R + B LED bulbs. LED PACKS were placed in culture rooms in which CO₂ concentration was maintained at 3000 ppm.

grade available and were purchased from Wako or Nacalai Tesque (Osaka, Japan), unless specified otherwise.

Plant material: stocks, culture medium and culture conditions

Stock cultures of PLBs of eight hybrid *Cymbidium* cultivars (kindly donated by Bio-U, Tokushima, Japan; see Table 1) were maintained on Teixeira *Cymbidium* medium 1 (TC-1), whose mineral constituents can be found in Teixeira da Silva (2012). Subcultures and *in vitro* culture conditions are detailed in Teixeira da Silva (2014b), while details on the theoretical aspects related to explant sizes and timing of sampling are described in Teixeira da Silva and Dobránszki (2014) and Teixeira da Silva and Dobránszki (2013), respectively. More specifically, PLBs of all hybrids originally developed from shoot-tip culture on Vacin and Went (1949) agar medium without plant growth regulators, were induced and subcultured (PLB induction and proliferation medium) every two months on TC-1 medium (Teixeira da Silva 2012), which was supplemented with 0.1 mg L⁻¹ α-naphthaleneacetic acid (NAA) and 0.1 mg L⁻¹ kinetin (Kin), 2 g L⁻¹ tryptone and 20 g L⁻¹ sucrose, and solidified with 8 g L⁻¹ Bacto agar (Difco Labs, Becton Dickinson and Co., NJ, USA), following Teixeira da Silva et al. (2005) and Teixeira da Silva and Tanaka (2006). All media were adjusted to pH 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min. Cultures were kept on 40 mL medium in 100-mL Erlenmeyer flasks,

double-capped with aluminium foil, at 25 °C, under a 16-h photoperiod with a photosynthetic photon flux density of 45 μmol m⁻² s⁻¹ provided by 40-W plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Hiroshima, Japan). Longitudinally dissected two pieces of PLB (3 to 4 mm in diameter) segments, 10 per flask, were used as explants for *neo*-PLB induction and proliferation, and as the experimental material.

The LED irradiation source

Red (wavelength, 660 nm; material, GaN; GL5UR3K1 = 3 cd, 5 mm in diameter; Sharp Electric Ltd., Tokyo, Japan) and blue (wavelength, 450 nm; material, GaAlAs, NLPB 500 = 1 cd, 5 mm in diameter, Nichia Chemicals Ltd., Tokushima, Japan) LEDs were arranged on the ceiling of “LED PACKS”, 25 × 31 × 25 cm (Fig. 1), originally described in Tanaka et al. (1998). Each LED PACK contained 176 LED bulbs (5 mm in diameter, Sharp Electric Ltd.) and the R+B LED ratio was adjusted based on a uniform division of R+B LED bulbs, for example, 88 R : 88 B, evenly interspersed, for the 50% R : 50% B treatment. The following LED ratios were examined: 100% R; 60% R + 40% B; 50% R + 50% B; 40% R + 60% B; 100% B. LED PACKS were placed in culture rooms in which CO₂ concentration was maintained at 3000 ppm. The intensity of LEDs was maintained at 45 μmol m⁻² s⁻¹ under a 16-h photoperiod. The R : B LED ratio as well as the intensity of light were adjusted using a PA36-2A Regulated DC Power Supply (Kenwood TMI Corp., Tokyo,

Table 1. Response (number of *neo*-PLBs) of nine hybrid *Cymbidium* cultivars' half-PLBs to different combinations of red : blue LED ratios (n = 30). Cultivar crosses (BioU classification): 91-8 (Aroma Candle 'Hot Heart') = Jenteel 'Pair Look' × Seaside 'Crown Princess'; 167-1 (Pretty Poetry 'Malachite') = Mini Sarah 'Artisan' × Eastern Star 'Green Fields'; 204-1 (Alice Beauty 'No 1') = Alice Luna × Sleeping Beauty 'Mistuko'; 246-2 (Twilight Moon 'Day Light') = Lovely Bunny 'Romeo' × Hiroshima Golden Cup 'Sunny Moon'; 485-12 (Spring Night 'No 12') = Tiny Sour × Twilight Moon 'Day Light'; 536-1 (Dream City 'No 1') = Great Katy 'Tender' × Lucky Flower 'Anmitsuhime'; 553-1 (Call Me Love 'Snow Princess') = Jenteel 'Pair Look' × Great Katy 'Tender'; 649-4 (Energy Star 'No 4') = Morning Moon 'Great Tiger' × Twilight Moon 'Day Light'; 653-2 (Sweet Moon 'No 2') = Yellow Candy 'Lemon Fresh' × Twilight Moon 'Day Light'. Results from 100 red and 100% blue are not presented since almost no PLBs formed in all nine cultivars. PGF values reported in Teixeira da Silva and Dobránszki (2014) and cultivars listed based on their decreasing *neo*-PLB production under PGF. Different letters across all treatments and for each cultivar indicate significant differences at $P \leq 0.05$ (according to DMRT). LED, light-emitting diode; PGF, plant growth fluorescent light; PLB, protocorm-like body

| <i>Cymbidium</i> cultivar | Red (R) : blue (B) LED ratio | | | |
|---------------------------|------------------------------|---------------|---------------|---------------|
| | PGF | 60% R : 40% B | 50% R : 50% B | 40% R : 60% B |
| 246-2 | 8.3 b | 4.2 d | 7.4 c | 10.1 a |
| 653-2 | 7.2 b | 3.4 d | 5.9 c | 8.6 a |
| 649-4 | 6.4 b | 2.6 c | 6.1 b | 7.3 a |
| 485-12 | 4.6 b | 1.8 d | 3.4 c | 5.5 a |
| 167-1 | 3.2 b | 0.9 d | 2.7 c | 4.4 a |
| 553-1 | 3.1 b | 0.6 d | 2.2 c | 4.0 a |
| 91-8 | 2.6 a | 0.1 c | 1.1 b | 2.9 a |
| 536-1 | 2.2 a | 0 c | 1.8 b | 2.6 a |
| 204-1 | 1.8 a | 0 c | 0.9 b | 2.1 a |

Japan) and a LI-250 Light Meter (LI-COR Inc., Nebraska, USA). Plant growth fluorescent light (PGF; 40-W, Homo Lux), at the same light intensity and photoperiod, served as the control light treatment.

Growth analysis

Growth was quantified by the number of new PLBs that formed (i.e., *neo*-PLBs).

Statistical analysis

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 10 replicates per treatment. All experiments were repeated in triplicate (n = 30, total sample size per treatment). Data was subjected to analysis of variance (ANOVA) with mean separation by Duncan's multiple range test using SAS® vers. 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were assumed at $P \leq 0.05$.

Results and discussion

The objective of this study was to assess whether different ratios of red and blue LEDs could increase *neo*-PLB production in nine *Cymbidium* cultivars, and to assess if the response to LED treatments was cultivar-dependent. Only red (i.e., 100% R) or only blue (i.e., 100% B) LEDs had a detrimental effect on *neo*-PLB production (Fig. 2D) for all nine cultivars, while a lower R:B ratio favoured a greater production of *neo*-PLBs relative to the control light source, PGF (Table 1).

Huan and Tanaka (2004), using Twilight Moon 'Day Light', one of the cultivars also used in this study, showed

that callus was most prolific under a 75% R + 25% B LED regime, but that most PLBs formed with a 25% R + 75% B LED ratio. Using a different LED ratio (explained in more detail in Teixeira da Silva 2014a and Fig. 1) in this study, it was found that a 40% R + 60% B LED regime favoured *neo*-PLB formation more than under PGF (control light) (Table 1) although the PLBs formed were of a more irregular shape (Fig. 2A vs 2B), which would make them difficult to use in clonal culture. Teixeira da Silva and Tanaka (2006) demonstrated that the tiny PLBs that formed from PLB explants were *de facto* somatic embryos and that the calli that Huan and Tanaka (2004) were describing were in fact undeveloped PLBs. This may explain why Huan and Tanaka (2004) claim to have observed as many as 89.6 *neo*-PLBs per half-moon PLB segment under PGF and 73.6-98.0 *neo*-PLBs per half-moon PLB segment under different R:B LED ratios. In contrast, Teixeira da Silva and Tanaka (2006), Teixeira da Silva (2012) and the results of this study showed an almost 10-fold lower number of *neo*-PLBs per half-moon PLB segment under PGF and different R : B LED ratios, most likely because tiny, underdeveloped PLBs, which are extremely difficult to count, and in some cases are fused to one another, were not considered. This is because, in practical terms, they represented a PLB cluster to be subcultured intact, and only a limited number of fully developed *neo*-PLBs develop further into shoots. The numbers thus reported by Huan and Tanaka (2004) may have thus been grossly over exaggerated, although their study indicated that the choice of red or blue LED, and their ratio, could influence the morphogenic outcome, a conclusion confirmed in this study, but for a wide range of cultivars. The timing of sampling also strongly affects

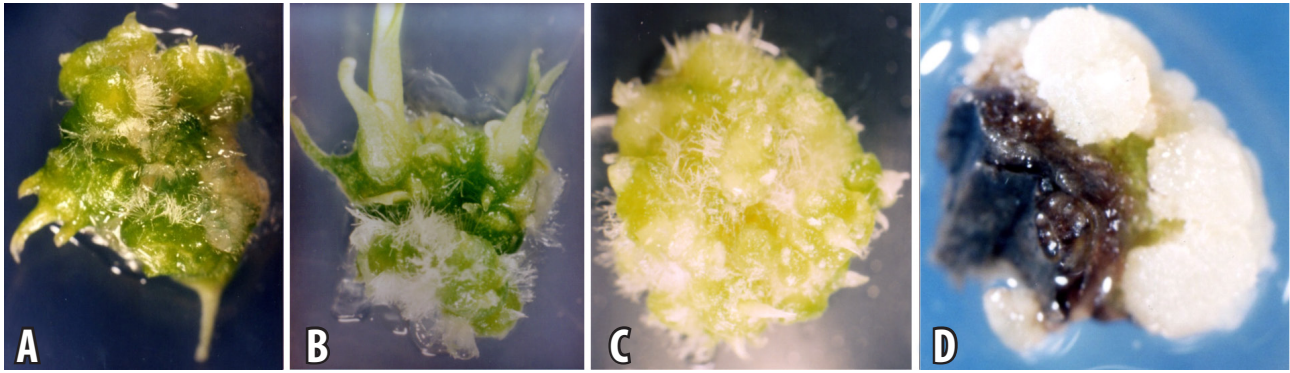


Fig. 2. Growth of hybrid *Cymbidium* Twilight Moon 'Day Light' in response to different light conditions. A, PGF; B 40% R + 60% B LED ratio; C, 60% R + 40% B LED ratio; D, 100% R.

the quantitative outcome, a very strong factor in hybrid *Cymbidium* (Teixeira da Silva, Dobránszki 2013). Thus, if the number of *neo*-PLBs were to be assessed at 20, 45, 60 or 90 days after plating on culture medium, the quantitative results would be vastly different. In this study, the "optimal" developmental stage of 45 days, in which the shoot apex has barely developed, but where the *neo*-PLB has attained maximum size optimal for sub-culture, was used.

There is an increase in endopolyploidy in response to extreme light spectral ranges in *Phalaenopsis* PLBs (Park et al. 2010), and in papaya seedling tissue (Teixeira da Silva 2014a), which is not uncommon for *Cymbidium* PLB tissues exposed to different abiotic stresses (Teixeira da Silva, Tanaka 2006). Follow-up experiments should determine the stress-related events in *neo*-PLB formation, including the expression of stress-related enzyme systems. There are currently no studies in orchids that have made such a detailed biochemical assessment, and this experimental design demonstrates that an LED system (Fig. 1) can be used as a powerful tool for examining such abiotic stress-related responses.

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