

Research Article

Algae 2022, 37(2): 163-174

<https://doi.org/10.4490/algae.2022.37.6.7>

Open Access



Effects of light-emitting diodes on protoplast regeneration from gametophytic cells of the commercial kelp *Undaria pinnatifida* (Laminariales, Phaeophyceae)

Jose Avila-Peltroche^{1,2}, Boo Yeon Won¹ and Tae Oh Cho^{1,2,*}

¹Department of Life Science, Chosun University, Gwangju 61452, Korea

²Department of Integrative Biological Sciences & BK21 FOUR Educational Research Group for Age-associated Disorder Control Technology, Chosun University, Gwangju 61452, Korea

Light-emitting-diodes (LEDs) are a lighting source useful for the precise evaluation of light quality effect on biological systems. Despite the importance of light spectra on the regeneration of land plant protoplasts (“naked cells”), this factor has not been tested yet on protoplasts from multicellular algae. This study reports on the effects of pure primary colors (red, blue, and green), dichromatic (red plus blue, RB, 1 : 2) and white LEDs on protoplast regeneration from male and female *Undaria pinnatifida* gametophytes. We also evaluated the effect of different light spectra on pigment composition (chlorophyll *a*, chlorophyll *c*, and fucoxanthine), and the light intensities under the best condition on the regeneration process. In the early stages, blue or RB LEDs increased the percentage of dividing female protoplasts, whereas red, blue, and RB LEDs enhanced that of dividing male protoplasts. In the later stages, RB LEDs showed a positive effect only on the percentage of multiple rhizoid-like protrusions (male gametophyte). They also increased the final area of both regenerated gametophytes. The LEDs did not affect pigment composition in female gametophytes. In male gametophytes, in contrast, they reduced chlorophyll *c*, while blue, RB, and green LEDs decreased fucoxanthin. Under RB LEDs, the optimal light intensity was 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for female gametophytes and 40 to 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for male gametophytes. Our results suggest that dichromatic LED illumination (red–blue) improves regeneration of *U. pinnatifida* gametophyte-isolated protoplasts. Thus, dichromatic LEDs might a suitable light source for enhancing protoplast regeneration in brown seaweeds.

Keywords: brown algae; gametophytes; light-emitting diodes (LEDs); pigment composition; protoplast regeneration; *Undaria pinnatifida*

Abbreviations: BF, branched filament; Fx, fucoxanthin; LED, light-emitting diode; MRLP, multiple rhizoid-like protrusions; PRE, proportional reduction of error; RB, mixed red and blue

INTRODUCTION

Protoplast regeneration is a process in which a protoplast (“naked” plant cell) resynthesizes its cell wall and undergoes cell division, elongation (growth), and differentiation, resulting in a new plant (Warren 1992, Wang

and Ruan 2013, Goldman 2014). This ability is the ultimate test of protoplast viability and is essential for establishing protoplast systems (Bhojwani and Razdan 1996, Chawla 2009). Several factors affect protoplast regenera-



This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Received April 19, 2022, Accepted June 7, 2022

*Corresponding Author

E-mail: tocho@chosun.ac.kr

Tel: +82-62-230-7161, Fax: +82-62-230-7467

tion ability, such as osmoticum, plating density, culture conditions, and light (Bhojwani and Razdan 1996). Light spectral quality plays an important role in regeneration, and photosynthetic organisms respond to different wavelengths. For example, exposure to white light is often the best way to induce shoot formation in protoplasts of land plants (Compton et al. 2000). Blue and red light increase the number of asymmetric cells in protoplasts of the moss *Physcomitrella* (Jenkins and Cove 1983), while red light promotes protoplast germination in the unicellular marine green alga *Boergesenia forbesii* (Ishizawa et al. 1979).

In seaweeds, protoplast regeneration has been accomplished in 40 species (Matsumura et al. 2001, Reddy et al. 2008, Yeong et al. 2008, Lafontaine et al. 2011, Wang et al. 2014, Huddy et al. 2015, Avila-Peltroche et al. 2019, Avila-Peltroche and Won 2020). Although different factors affecting protoplast regeneration in seaweeds have been studied (Reddy et al. 2008), the effect of light spectral quality has never been analyzed.

In marine environments, different light qualities occur within the water column by absorption and scattering of light, depending on the depth, turbidity, time of the day, and other physical parameters. For example, blue-green waveband usually penetrates deepest into the water body due to its shorter and longer wavelength (Hanelt and Figueroa 2012, Falcón et al. 2020). These differences in light colors play an important role in marine macroalgal physiology, especially in kelp gametophytes, with several studies showing that spectral quality can control sexual reproduction and vegetative growth (Edwards 2022).

Light-emitting diodes (LEDs) are a rapidly developing technology consisting of electronic diodes that produce light when an electric current passes through them. They come in the three primary colors and as dichromatic, trichromatic, or white LEDs. LEDs have numerous advantages over conventional lights; for example, the light emission at specific wavelengths allows to precisely evaluate the effect of light quality on biological systems, which is especially important in photosynthesis research (Gupta and Jatothu 2013, Dayani et al. 2016). In seaweeds, the use of LEDs is still limited to a few species and have been focused on growth studies (Murase et al. 2014, Kim et al. 2015, Miki et al. 2017, Le et al. 2018).

In this study, we analyzed the regenerative effect of pure primary color (red, blue, and green), dichromatic (red-blue; hereafter RB), and white LEDs on *Undaria pinnatifida* protoplasts during early (cell division) and late (cell elongation / growth) development. We isolated the protoplasts from male and female gametophytes because of their high potential to produce bioactive com-

pounds (Dwiranti et al. 2012, Seppic 2022) and ability to be cultured in bioreactors (Rorrer and Cheney 2004, Gao et al. 2005). These features make them suitable for cellular biotechnology techniques, many of which rely on protoplasts (Reddy et al. 2008). We also investigated the effect of light spectral quality on photosynthetic pigments and light intensities under the most favorable wavelengths for regeneration.

MATERIALS AND METHODS

Establishment of gametophyte clones

Undaria pinnatifida gametophyte clones were established from mature sporophytes sampled at Geoje and Jindo Islands, Korea on Mar 1, 2013 and May 5, 2016, respectively. Sporulation, isolation, and culture were performed as previously described (Avila-Peltroche et al. 2020). Female (from Jindo Island) and male (from Geoje Island) vegetative gametophyte cultures in the early (4-day-old) and mid-exponential phase (6-day-old), respectively, were used for subsequent experiments.

Protoplast isolation

Protoplast isolation was performed following the protocols described by Benet et al. (1997) and Coelho et al. (2012) with some modifications (Avila-Peltroche et al. 2020). Approximately 100 to 200 mg of *U. pinnatifida* gametophytes from 1-L round flasks were incubated in a 0.22 µm filter-sterilized enzymatic solution (640 mM NaCl, 208 mM MgCl₂·6H₂O, 35.2 mM MgSO₄, 256 mM KCl, 3.2 mM CaCl₂, 16 mM MES [pH 6], 2,512 mOsm L⁻¹ H₂O) at 20°C with shaking at 70 rpm for 4 h (female) or 6 h (male) in the dark. The solution was supplemented with 1% cellulase RS (Yakult Co. Ltd., Tokyo, Japan), 4 U mL⁻¹ alginate lyase, and 1% driselase from *Basidiomycetes* sp. (Sigma-Aldrich, St. Louis, MO, USA). Chelation pre-treatment was conducted with a calcium-chelating solution (665 mM NaCl, 30 mM MgCl₂·6H₂O, 30 mM MgSO₄, 20 mM KCl, and 20 mM ethylene glycol-bis (β-amino-ethyl ether)-N,N,N',N'-tetraacetic acid tetrasodium salt (EGTA-Na₄) [pH 5.5] as calcium chelator) for 20 min prior to enzymatic digestion (Coelho et al. 2012). Protoplasts were filtered using a 25-µm nylon mesh to remove undigested filaments and further washed three times by centrifugation at 100 ×g for 10 min. The pellets were resuspended in an enzymatic solution. The average number of gametophyte-isolated protoplasts was calculated with a hemo-

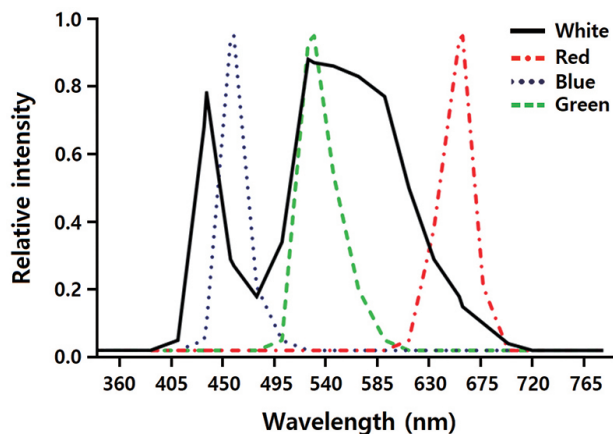


Fig. 1. Relative emission spectra of red, blue, green, and white light-emitting diodes (LEDs). Dichromatic LED corresponds to red and blue light at a 1 : 2 ratio.

cytometer. For male gametophytes, the fresh weight was approximately $(3.12 \pm 0.51) \times 10^6$ protoplasts g^{-1} and for female gametophytes, $(2.11 \pm 0.08) \times 10^6$ protoplasts g^{-1} . The viability of protoplasts and cell wall regeneration were assessed by red chlorophyll autofluorescence and staining with calcofluor white M2R (Sigma-Aldrich), respectively, as previously described (Avila-Peltroche et al. 2019). Calcofluor white was chosen for monitoring cell wall formation because it binds to β -linked fibrillar polymers such as cellulose but does not block its synthesis (Murgui et al. 1985).

LEDs and growth conditions

The effects of LED wavelengths on regeneration of the gametophyte-isolated protoplasts were investigated using a DYLED 44V system (DyneBio Co., Seongnam, Korea). Five different LEDs were installed in the growth chambers: (1) white (all wavelengths), (2) red (peak wavelength at 660 nm), (3) blue (peak wavelength at 460 nm), (4) mixed red and blue (1 : 2; RB), and (5) green (peak wavelength at 530 nm). Fig. 1 shows the emission spectra of the LEDs. Spectral wavelengths were measured using the USB2000 UV-VIS spectrometer (Ocean Optics Inc., Dunedin, FL, USA), while the LED irradiation intensities were measured using a quantum meter (MQ-500; Apogee Instruments, Logan, UT, USA). The protoplasts were grown under LEDs on a 14 : 10 h light : dark cycle.

Protoplast regeneration under different LEDs

Protoplasts were dispensed into at least 4 wells of a 12-well tissue culture plate containing 2 mL of regeneration

medium (PES with 285 mM NaCl and 5 mM $CaCl_2$). The medium was supplemented with an antibiotic mix (50 mg L^{-1} penicillin G, 25 mg L^{-1} streptomycin, and 5 mg L^{-1} chloramphenicol). The plates were incubated at 20°C in the dark and with an initial protoplast density of 9×10^3 protoplasts. Previous experiments showed that 20°C was suitable for successful protoplast regeneration. Also, this temperature promotes vegetative growth of *U. pinnatifida* gametophytes (Morita et al. 2003). After 4 days in the dark, osmotic pressure was reduced slowly using a PES medium, and cultures were gradually exposed to a final intensity of 40 μmol photons $m^{-2} s^{-1}$. The well plates were separately irradiated using white (control), red, blue, RB, or green LEDs. The culture medium was replaced weekly. The responses of cultured protoplasts were assessed during the early and late stages of regeneration. In the early stages, the percentage of dividing cells was calculated at 8 (female) and 11 (male) days in culture. In addition, the percentage of asymmetric cells formed by cell divisions was determined for all conditions because LED lighting promotes the formation of asymmetric cells in protoplasts of the moss *Physcomitrella* (Jenkins and Cove 1983). The percentage of branched filaments (BFs) or multiple rhizoid-like protrusions (MRLPs) was determined at 15 or 18 days in culture, respectively. Final growth was represented as the gametophyte area at the end of the experiment (female = 22 days in culture; male = 26 days in culture) and analyzed using image analysis software (ImageJ; US National Institutes of Health, Bethesda, MD, USA). For percentage calculations, at least 100 cells per well were counted. For area measurements, at least 30 gametophytes per well were analyzed.

Pigment extraction and quantification

Chlorophyll *a* (Chl *a*), chlorophyll *c* (Chl *c*), and fucoxanthin (Fx) were extracted from regenerated female gametophytes (10–20 mg fresh weight) at the end of the experiments using the protocol and equations of Seely et al. (1972) with dimethyl sulfoxide as the solvent. Regenerated male gametophytes were further cultured under the same conditions for 3 months to obtain sufficient biomass for reliable measurements. A microplate reader (SpectraMax Paradigm; Molecular Devices, San Jose, CA, USA) was used to quantify the pigments from at least three wells per LED treatment. Pathlength correction was performed according to the method described by Warren (2008).

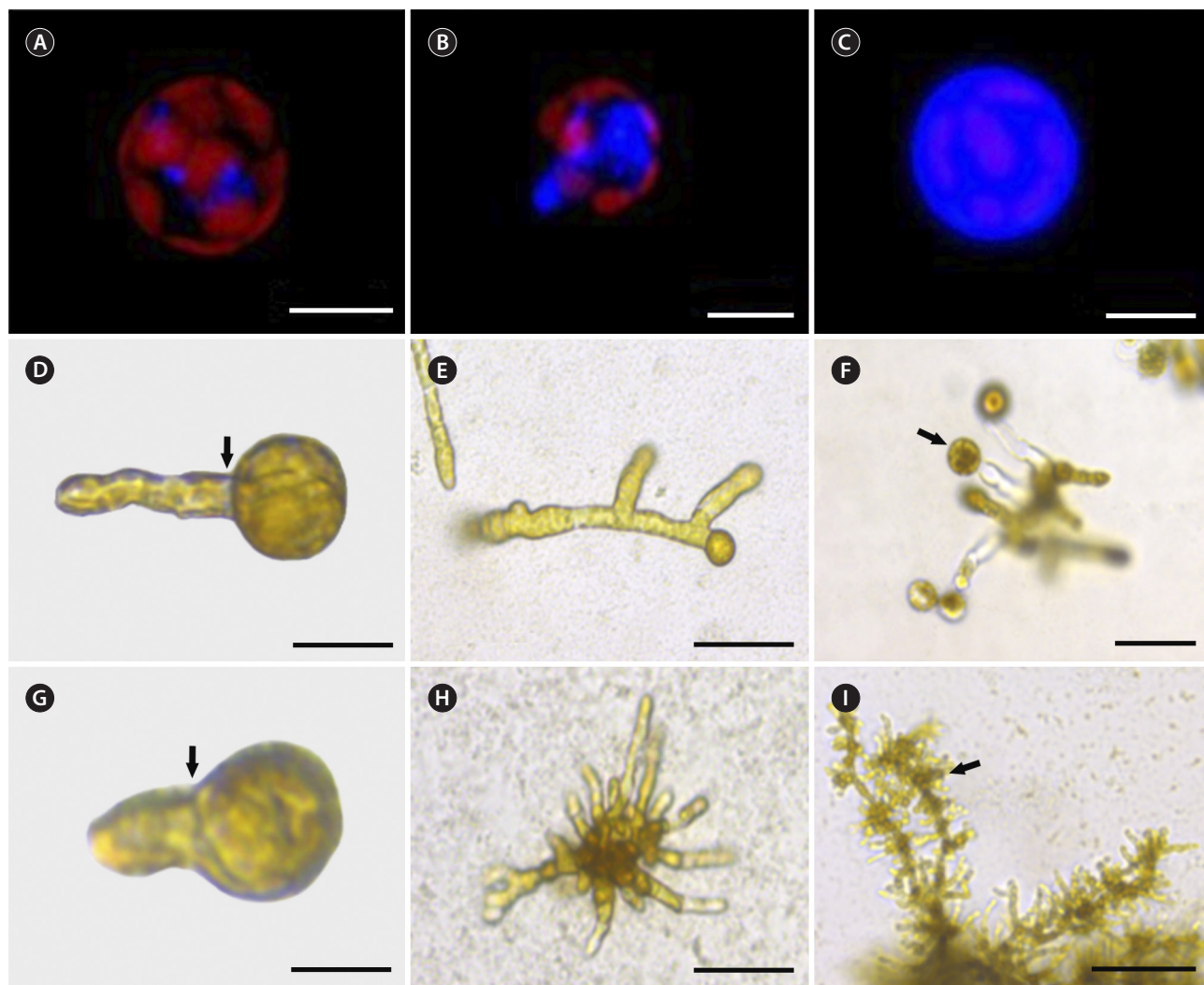


Fig. 2. Regeneration of *Undaria pinnatifida* gametophyte-isolated protoplasts under white light-emitting diode. (A) Fluorescence image of cell wall formation from protoplast initial stage after 3 h of culture. (B) Fluorescence image of cell wall formation after 16 h of culture. (C) Fluorescence image of cell wall formation after 48 h of culture. (D) First asymmetric cell division (arrow) in the female gametophyte. (E) Branched filament (female gametophyte) after 15 d in culture. (F) Regenerated female gametophyte producing oogonia (arrow) after 22 d in culture. (G) First asymmetric cell division (arrow) in the male gametophyte. (H) Multiple rhizoid-like protrusions (male gametophyte) after 18 d in culture. (I) Regenerated male gametophyte producing spermatangia (arrow) after 26 d in culture. Areas showing bright blue fluorescence indicate cellulose deposition. The red autofluorescence of the chlorophyll reveals areas without the cell wall. Scale bars represent: A–C & G, 10 μm ; D, 20 μm ; E & F, 50 μm ; H, 40 μm ; I, 100 μm .

Protoplast regeneration under different LED intensities

The effect of LED light intensity on protoplast regeneration was evaluated under RB LED treatment. The gametophyte-isolated protoplasts were cultured as described above in at least 4 wells of a 12-well plate. They were placed at different distances from the LED unit to achieve different intensities: 20, 40, 60, and 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The light intensities were measured using a quantum

meter. The regenerative responses of the cultured protoplasts were assessed as previously described.

Statistical analysis

The percentage of dividing cells, asymmetric cells, BFs, or MRLPs, were analyzed as proportions with beta regressions since beta distribution provides a flexible model for continuous variables restricted to the interval (0, 1) (Ferrari and Cribari-Neto 2004). The analyses were per-

formed using the R package betareg (Cribari-Neto and Zeileis 2010). The proportional reduction of error (PRE) statistic was used as the overall model effect size (Smithson and Verkuilen 2006).

Two separated one-way ANOVA tests were used to evaluate the differences in gametophyte area and pigment composition. Normality and homoscedasticity were checked using the Shapiro-Wilk and Levene test, respectively, before conducting ANOVA. Kruskal-Wallis or Welch's ANOVA tests were used when data did not meet normality or homoscedasticity assumptions, respectively. Effect sizes (Sullivan and Feinn 2012) were presented as ω^2 (Field 2009, Lakens 2013) in the case of significant results. The formula for ω^2 calculation was as follows:

$$\omega^2 = \frac{SS_M - (df_M) \times MS_R}{SS_T + MS_R}$$

, where SS_M is the between-group effect, df_M is the degrees of freedom for the effect, MS_R is the regression mean square, and SS_T is the total amount of variance in the data. All the analyses were performed using the car (Fox and Weisberg 2019) and userfriendlyscience packages (Peters 2018) in R.

Tukey's *post hoc* test was used when the results were significant. When data were heteroscedastic, the Games-Howell test was performed instead. The *post hoc* comparisons were conducted using the multcomp (Hothorn et al. 2008) or userfriendlyscience package (Peters 2018) in R.

The significance threshold was set at $p = 0.01$ to reduce the true type I error rate (at least 7% but typically close to 15%) (Sellke et al. 2001). It is worth mentioning that this precautionary approach also reduces the chances to detect false negatives (type II error). All graphs were created using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Protoplast regeneration under white LEDs

Freshly isolated protoplasts showed 90 to 95% viability according to red chlorophyll autofluorescence. They also had no cell wall, as shown by the absence of a blue fluorescent signal after calcofluor white staining. After 3 hours of culture, cell wall formation started as blue fluorescent spots (positive staining) on the protoplast surface (Fig. 2A). These spots grew and connected each other forming an extensive area of cell wall deposition after 16

h of culture (Fig. 2B). The entire surface of the protoplast was covered by a uniform cell wall after 2 to 6 d of culture (Fig. 2C).

Protoplast regeneration started as a single bud on one pole before the first asymmetric cell division (Fig. 2D & G). Symmetric cell division was also observed among the regenerated protoplasts but at lower percentages (14–20%). After 15 d in culture, most regenerated protoplasts isolated from female gametophytes followed unipolar germination (81%). The 15-day-old female gametophytes were visible as uniseriate BFs (Fig. 2E). New thalli were distinguished after 22 d of culture. Protoplasts isolated from male gametophytes produced MRLPs after 18 d in culture (Fig. 2H). New thalli were visible after 26 d of culture. Both regenerated gametophytes developed morphologically normal reproductive tissues (Fig. 2F & I).

Comparison of the effects of different LEDs on protoplasts isolated from female gametophytes

Overall, all analyzed LED conditions induced protoplast cell divisions. In the early stages, LEDs affected the percentage of dividing cells ($p < 0.001$, PRE = 0.133). Moreover, blue and RB LEDs significantly increased these values by 1.5 and 1.7 times, respectively, compared with the control (Fig. 3A). LED treatments influenced the number of asymmetric cells ($p < 0.001$, PRE = 0.088). More asymmetric cells formed under the blue and RB conditions; however, they represented a small increase (1.1–1.2 times) compared with the control (Fig. 3B). In the late stages, LED treatments did not affect the percentage of BFs ($p < 0.01$) (Fig. 3C), but RB LEDs promoted a significant increase in the final gametophyte area by three times compared with white LEDs ($p < 0.001$, $\omega^2 = 0.78$) (Fig. 3D). LEDs also affected the final gametophyte morphology. Under red LEDs, gametophytes showed longer filaments and more branches than those under the blue, which only presented one or none. The RB treatment produced gametophytes with longer filaments and more branches than those under the blue treatment. They were also slightly shorter than gametophytes under the red condition. Gametophytes under green LEDs showed a morphology resembling white LED conditions (Fig. 5A). Chl *a*, Chl *c*, and Fx contents were not affected by the treatments ($p > 0.01$) (Fig. 6A–C).

Comparison of the effects of different LEDs on protoplasts isolated from male gametophytes

All LED conditions allowed protoplast cell divisions.

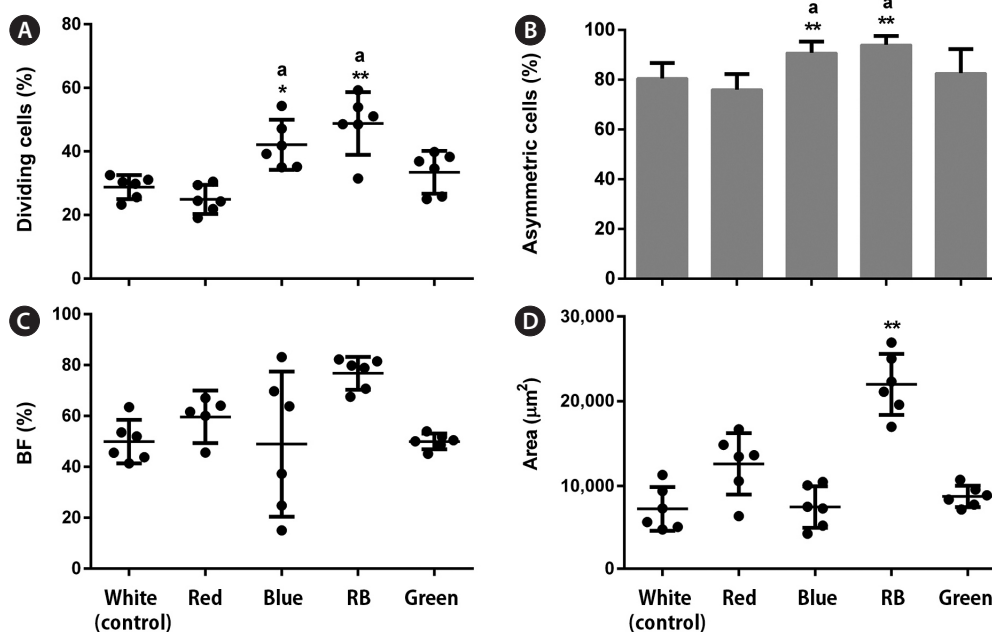


Fig. 3. Effects of light-emitting diodes (LEDs) on early (A & B) and late (C & D) stages of regeneration from female gametophytes of *Undaria pinnatifida*. RB represents dichromatic LEDs (red–blue at 1 : 2 ratio). BFs represent branched filaments in (C). Independent data points and averages (horizontal lines) are shown (n ≥ 5) in (A), (C), and (D). Bars in (B) represent averages (n = 6). Error bars indicate 95% confidence intervals. Asterisks (*) and double asterisks (**) depict significant differences between treatments and control (white) at p < 0.01 and p < 0.001, respectively. Different letters indicate significant differences (p < 0.01) between treatments that showed higher values than control.

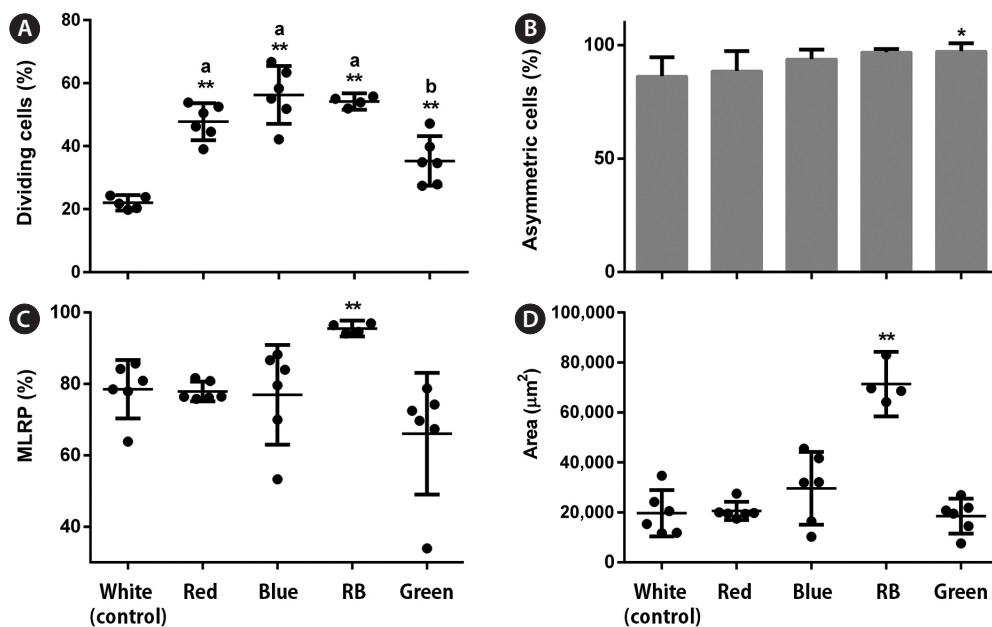


Fig. 4. Effects of light-emitting diodes (LEDs) on early (A & B) and late (C & D) stages of protoplast regeneration from male gametophytes of *Undaria pinnatifida*. RB represents dichromatic LEDs (red–blue at 1 : 2 ratio). MLRP represents multiple rhizoid-like protrusions in (C). Independent data points and averages (horizontal lines) are shown (n ≥ 4) in (A), (C), and (D). Bars in (B) represent averages (n ≥ 4). Error bars indicate 95% confidence intervals. Asterisks (*) and double asterisks (**) mark significant differences between treatments and control (white) at p < 0.01 and p < 0.001, respectively. Different letters indicate significant differences (p < 0.01) between treatments that showed higher values than control.

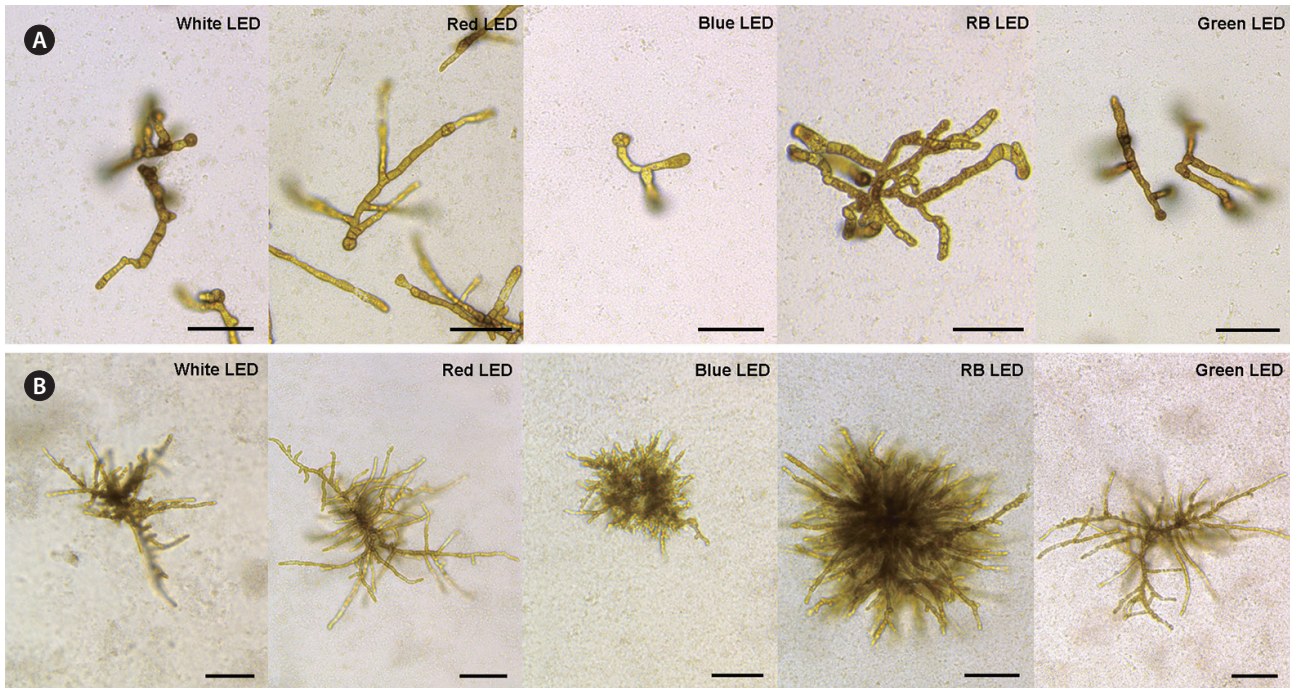


Fig. 5. Comparison of the final morphology of regenerated *Undaria pinnatifida* gametophytes cultured for 22 days (female) (A) and 26 days (male) (B) under different light-emitting diode (LED) conditions. RB represents dichromatic LEDs (red–blue at 1 : 2 ratio). Scale bars represent: A & B, 100 μm .

In the early stages, LEDs affected the percentage of dividing cells ($p < 0.001$, $\text{PRE} = 0.255$). Red, blue, and RB LEDs significantly increased these values by 2–2.5 times, respectively, compared with the control (Fig. 4A). LED treatments also influenced the number of asymmetric cells ($p < 0.001$, $\text{PRE} = 0.041$). More asymmetric cells were formed under the green condition; however, this represented a small increase (1.1 times) compared with the control (Fig. 4B). In the late stages, only RB LEDs increased the percentage of MRLP ($p < 0.001$; $\text{PRE} = 0.138$) and final gametophyte area ($p < 0.001$, $\omega^2 = 0.87$) by 1.2 and 3.6 times, respectively, compared with white LEDs (Fig. 4C & D). LEDs also affected the final gametophyte morphology. Under red LEDs, gametophytes showed longer filaments with less profuse branching than those under the blue. Gametophytes grown under the RB treatment had an “intermediate” morphology. They had longer filaments than the gametophytes under the blue and more profuse branching than those under the red treatment. Gametophytes under green LEDs showed a morphology resembling white and red conditions (Fig. 5B). The Chl *a* content was not affected by LED treatments ($p = 0.293$). The Chl *c* content dropped under all conditions compared with the white ($p < 0.001$, $\omega^2 = 0.46$), whereas the Fx content decreased under blue, RB, and green con-

ditions compared with the control ($p = 0.001$, $\omega^2 = 0.48$) (Fig. 6D–F).

Comparison of the effects of different LED light intensities on protoplast regeneration

RB LEDs were chosen for testing different light intensities on protoplast regeneration because they improved it in both protoplast types. In the early stages, the percentage of dividing cells was higher under 40 and 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ than at 20 or 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for both gametophytes ($p < 0.001$; PRE [female] = 0.134; PRE [male] = 0.236) (Fig. 7A & D). In the late stages, the percentage of BFs increased at 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ compared with the other light intensities ($p = 0.002$, $\text{PRE} = 0.118$) (Fig. 7B), while the percentage of MLRPs was higher at 40 and 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ than at 20 or 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ($p < 0.001$, $\text{PRE} = 0.814$) (Fig. 7E). The final female gametophyte area peaked at 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ($p = 0.002$, $\omega^2 = 0.46$), representing 1.8 times increase compared with the lowest value at 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 7C). The final male gametophyte area showed the highest value at 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ($p < 0.001$, $\omega^2 = 0.87$), representing 2.9 times increase compared with the lowest value at 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 7F).

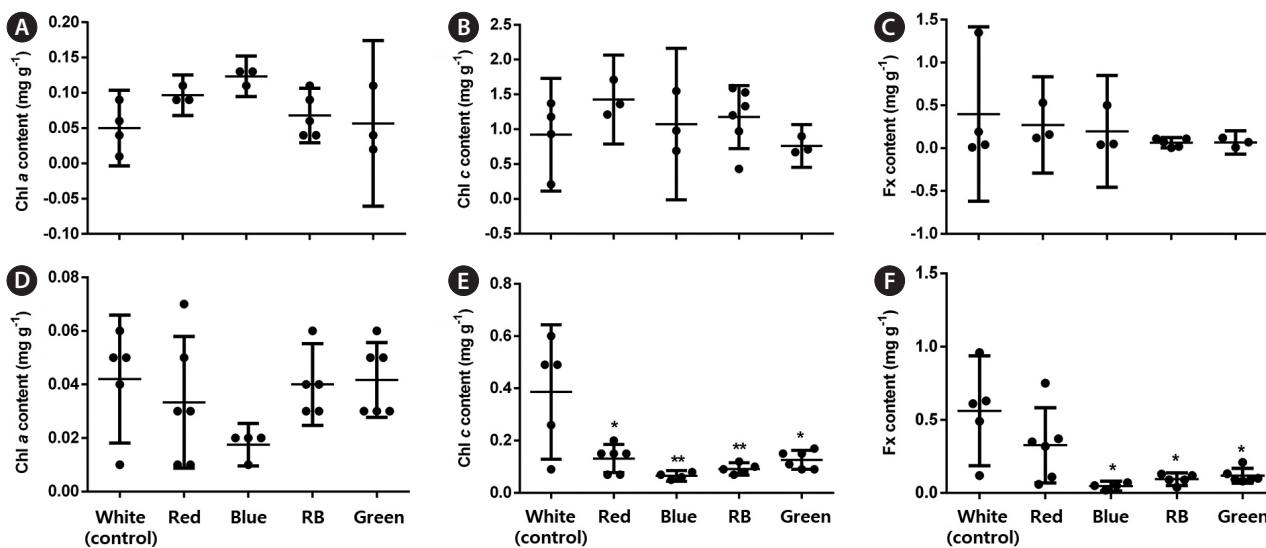


Fig. 6. Chlorophyll a (Chl a), chlorophyll c (Chl c), and fucoxanthin (Fx) concentrations in regenerated *Undaria pinnatifida* female (A–C) and male (D–F) gametophytes after exposure to different light-emitting diode conditions. RB represents dichromatic light-emitting diodes (red–blue at 1 : 2 ratio). Independent data points and averages (horizontal lines) are shown (n ≥ 3). Error bars indicate 95% confidence intervals. Asterisks (*) and double asterisks (**) show significant differences between treatments and control (white) at p < 0.01 and p < 0.001, respectively.

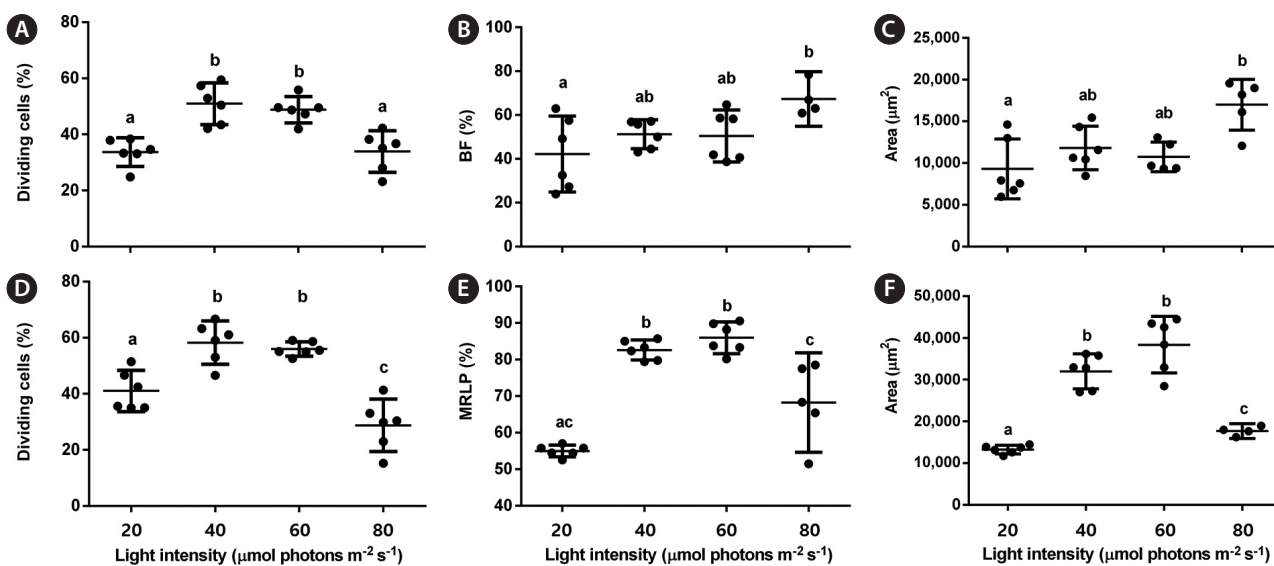


Fig. 7. Effect of different dichromatic light-emitting diode intensities (red–blue at 1 : 2 ratio) on regeneration of protoplasts isolated from *Undaria pinnatifida* female (A–C) and male (D–F) gametophytes. BFs represent branched filaments in (B). MRLPs represent multiple rhizoid-like protrusions in (E). Independent data points and averages (horizontal lines) are shown (n ≥ 4). Error bars indicate 95% confidence intervals. Different letters indicate significant differences at p < 0.01.

DISCUSSION

Protoplast regeneration in Laminariales usually exhibits symmetric or asymmetric cell division, followed by uni- or multipolar development (Benet et al. 1997). Our results show that most protoplasts from *Undaria pinnatifida* gametophytes undergo simple regeneration with the

first asymmetric cell division. Although symmetric cells were also present, their percentage was low. Gametophytes differed in their regeneration pathways. Female protoplasts regenerated through unipolar development, whereas the male through multipolar. These differences were not pointed out in previous studies (Zha and Kloreg 1996, Benet et al. 1997).

Visible light has been tested to enhance the growth of different seaweed species. In brown algae, Xu et al. (2005) and Miki et al. (2017) showed that blue LEDs improved growth in *U. pinnatifida* female gametophytes and *Sargassum horneri* germlines and immature stages. Moreover, Asensi et al. (2001) reported that blue light favors sporophyte regeneration from callus-like cell suspension cultures. Although our experiments also showed a positive effect of blue LEDs on protoplast regeneration, especially in the early stages, dichromatic LEDs were more effective on protoplast regeneration than white or the pure primary color LEDs. This effect was also evident in the final gametophyte morphology. A similar response was observed in the red alga *Gracilaria tikvahiae*, which showed higher growth under dichromatic (red + green or green + blue) or trichromatic (red + green + blue) LEDs versus the blue (Kim et al. 2015).

Our results contrast with the morphologies reported by Sato et al. (2020) in *U. pinnatifida* female gametophytes. For instance, they showed that gametophytes under red LED presented short filaments and few branches, a morphology similar to the one obtained in our blue LED treatment. These differences might be attributed to the fact that gametophytes originated from protoplasts in our work, while Sato et al. (2020) obtained female gametophytes from zoospores. Also, in Sato's work, gametophytes were initially cultured under white fluorescent light and then exposed to LEDs. Thalli morphology and growth in algae can be affected by changes in light color, even during short periods (Kuwano et al. 2014).

Blue light is efficiently absorbed by the accessory pigment Fx and thus has a crucial role in brown algal growth. It upregulates the genes encoding photosystem components: F-type H⁺-ATPase, cytochrome b6/f complex, and ferredoxin (Foster and Dring 1994, Wang et al. 2013), which stimulates photosynthesis. Blue light perception in Phaeophyceae occurs via cryptochromes and aureochromes (Takahashi et al. 2007, Deng et al. 2012, Wang et al. 2013). Interestingly, metabolism and growth in kelps may depend on the proportion of blue light illumination versus red (Wang et al. 2013), namely the increase in blue and reduction in red. This effect of the light ratio could explain our results with RB LEDs, where the red light was present at a lower proportion than the blue.

The amounts of Chl *a*, Chl *c*, and Fx should change under different light conditions because of their specific wavelength absorption patterns. Indeed, blue light enhances Chl *a* content in *U. pinnatifida* female gametophytes and the immature stage of *S. horneri* (Miki et al. 2017). Furthermore, white light has shown a positive

effect on Chl *c* and carotenoid content in both male and female *U. pinnatifida* gametophytes (Xu et al. 2005). In our experiments, LED treatments did not affect the pigment composition of regenerated female gametophytes. The lack of variation in pigment composition has been previously reported in the marine microalga *Tetraselmis suecica* and lettuce *Lactuca sativa* var. *capitata* under different color LEDs. This finding may be because the light intensity has reached a minimal value allowing sufficient synthesis and activity of photosynthetic pigments and electron carriers (Lin et al. 2013, Abiusi et al. 2014). Another explanation is that the pigment changes were transitory and thus not registered in the final pigment content. We also showed that Chl *c* and Fx concentrations were reduced under all light treatments compared with the control, but only in regenerated male gametophytes. The differences in pigment composition between male and female gametophytes can arise from different pigment and protein complexes (Xu et al. 2005).

Studies on *Ulva* and *Pyropia* protoplast cultures showed light intensity has little effect on regeneration (Reddy and Fujita 1991, Gall et al. 1993). In our experiments, whole plants regenerated under all RB LED light intensities, confirming the stronger resistance of protoplasts isolated from Laminariales gametophytes towards high illumination (Benet et al. 1997). Although this effect was similar for both gametophytes in the early stages, the optimum light intensity varied in the late stages. Protoplasts from female gametophytes showed improved regeneration at 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, while their male counterparts showed better results at lower intensities (40–60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Additionally, the light intensity had a higher size effect on male gametophytes than on the female. Ideal light intensities for *U. pinnatifida* gametophyte growth are between 40 and 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Kim and Nam 1997, Choi et al. 2005, Xu et al. 2005, Deng et al. 2009), which is similar to our findings for male gametophytes. Xu et al. (2005) found no difference in the optimal light intensities for male and female *U. pinnatifida* gametophytes based on the photosynthetic rate. Our results, nonetheless, showed that optimal light intensities depended on gametophyte sex. Destombe and Oppliger (2011) found that sex strongly influenced the growth rate of the *Laminaria digitata* gametophytes due to different reproductive strategies. It should also be noted that previous studies have not assessed growth by calculating the gametophyte area and that all used white or monochromatic light.

In conclusion, this study is the first to report on the effect of LEDs on protoplast regeneration in multicellular

marine algae. Our results suggest that dichromatic LEDs (red–blue at 1 : 2 ratio) are the most favorable for cell division and elongation of *U. pinnatifida* gametophyte-isolated protoplasts. LEDs affected only Chl *c* and Fx content in male gametophytes. Under the dichromatic light, the optimal light intensity for female gametophytes was 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and 40 to 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for the male. These results suggest that dichromatic LEDs can be used as a suitable light source for enhancing protoplast regeneration in brown algal species. Despite the well-known effects of blue and red light in kelp gametophytes, e.g., development and sexual reproduction (Sato et al. 2020, Edwards 2022), this work advances the understanding of light spectral quality in kelp gametophytes by showing, for the first time, the importance of simultaneous red and blue illumination in gametophyte development. Further studies with different light ratios are needed to clarify the effect of dichromatic light on brown seaweed growth and development.

ACKNOWLEDGEMENTS

This study was supported by research fund from Chosun University, 2021 to T. O. Cho.

CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

REFERENCES

- Abiusi, F., Sampietro, G., Marturano, G., Biondi, N., Rodolfi, L., D'Ottavio, M. & Tredici, M. R. 2014. Growth, photosynthetic efficiency, and biochemical composition of *Tetraselmis suecica* F&M-M33 grown with LEDs of different colors. *Biotechnol. Bioeng.* 111:956–964.
- Asensi, A., Gall, E. A., Marie, D., Billot, C., Dion, P. & Kloareg, B. 2001. Clonal propagation of *Laminaria digitata* (Phaeophyceae) sporophytes through a diploid cell-filament suspension. *J. Phycol.* 37:411–417.
- Avila-Peltroche, J. & Won, B. Y. 2020. Protoplast production from *Sphacelaria fusca* (Sphacelariales, Phaeophyceae) using commercial enzymes. *J. Mar. Biosci. Biotechnol.* 12:50–58.
- Avila-Peltroche, J., Won, B. Y. & Cho, T. O. 2019. Protoplast isolation and regeneration from *Hecatonema terminale* (Ectocarpales, Phaeophyceae) using a simple mixture of commercial enzymes. *J. Appl. Phycol.* 31:1873–1881.
- Avila-Peltroche, J., Won, B. Y. & Cho, T. O. 2020. Optimization of protoplast isolation from the gametophytes of brown alga *Undaria pinnatifida* using response surface methodology. *J. Appl. Phycol.* 32:2233–2244.
- Benet, H., Gall, E. A., Asensi, A. & Kloareg, B. 1997. Protoplast regeneration from gametophytes and sporophytes of some species in the order Laminariales (Phaeophyceae). *Protoplasma* 199:39–48.
- Bhojwani, S. S. & Razdan, M. K. 1996. *Plant tissue culture: theory and practice, a revised edition*. Elsevier, Amsterdam, 338 pp.
- Chawla, H. S. 2009. *Introduction to plant biotechnology*. 3rd ed. Science Publishers, Enfield, 109 pp.
- Choi, H. G., Kim, Y. S., Lee, S. J., Park, E. J. & Nam, K. W. 2005. Effects of daylength, irradiance and settlement density on the growth and reproduction of *Undaria pinnatifida* gametophytes. *J. Appl. Phycol.* 17:423–430.
- Coelho, S. M., Scornet, D., Rousvoal, S., Peters, N., Dartevielle, L., Peters, A. F. & Cock, J. M. 2012. Isolation and regeneration of protoplast from *Ectocarpus*. *Cold Spring Harb. Protoc.* 2012:361–364.
- Compton, M. E., Saunders, J. A. & Veilleux, R. E. 2000. Use of protoplasts for plant improvement. In Trigiano, R. N. & Gray, D. J. (Eds.) *Plant Tissue Culture Concepts and Laboratory Exercises*. CRC Press, Boca Raton, FL, pp. 249–261.
- Cribari-Neto, F. & Zeileis, A. 2010. Beta regression in R. *J. Stat. Softw.* 34:1–24.
- Dayani, S., Heydarizadeh, P. & Sabzalian, M. R. 2016. Efficiency of light-emitting-diodes for future photosynthesis. In Pessarakli, M. (Ed.) *Handbook of Photosynthesis*. 3rd ed. CRC Press, Boca Raton, FL, pp. 761–783.
- Deng, X., Qin, S., Zhang, Y. & Jiang, P. 2009. Comparison of photobioreactors and optimal light regime for rapid vegetative propagation of transgenic *Undaria pinnatifida* gametophytes. *J. Chem. Technol. Biotechnol.* 84:1486–1492.
- Deng, Y., Yao, J., Wang, X., Guo, H. & Duan, D. 2012. Transcriptome sequencing and comparative analysis of *Saccharina japonica* (Laminariales, Phaeophyceae) under blue light induction. *PLoS ONE* 7:e39704.
- Destombe, C. & Oppliger, L. V. 2011. Male gametophyte fragmentation in *Laminaria digitata*: a life history strategy to enhance reproductive success. *Cah. Biol. Mar.* 52:385–394.
- Dwiranti, F., Hiraoka, M., Taguchi, T., Konishi, Y., Tominaga, M. & Tominaga, A. 2012. Effects of gametophytes of *Ecklonia kurome* on the levels of glucose and triacylglycerol

- in db/db, prediabetic C57BL/6J and IFN- γ KO mice. *Int. J. Biomed. Sci.* 8:64–75.
- Edwards, M. S. 2022. It's the little things: the role of microscopic life stages in maintaining kelp populations. *Front. Mar. Sci.* 9:871204.
- Falcón, J., Torriglia, A., Attia, D., Viénot, F., Gronfier, C., Behar-Cohen, F., Martinsons, C. & Hicks, D. 2020. Exposure to artificial light at night and the consequences for flora, fauna, and ecosystems. *Front. Neurosci.* 14:602796.
- Ferrari, S. L. P. & Cribari-Neto, F. 2004. Beta regression for modeling rates and proportions. *J. Appl. Stat.* 31:799–815.
- Field, A. 2009. *Discovering statistics using SPSS. 3rd ed.* Sage Publication, Los Angeles, CA, 389 pp.
- Foster, R. M. & Dring, M. J. 1994. Influence of blue light on the photosynthetic capacity of marine plants from different taxonomic, ecological and morphological groups. *Eur. J. Phycol.* 29:21–27.
- Fox, J. & Weisberg, S. 2019. *An R Companion to Applied Regression. 3rd ed.* Available from: <https://socialsciences.mcmaster.ca/jfox/Books/Companion/>. Accessed Feb 15, 2022.
- Gall, E. A., Chiang, Y. -M. & Kloareg, B. 1993. Isolation and regeneration of protoplasts from *Porphyra dentata* and *Porphyra crispata*. *Eur. J. Phycol.* 28:277–283.
- Gao, J., Zhang, Y., Wang, H. & Qin, S. 2005. Suspension culture of gametophytes of transgenic kelp in a photobioreactor. *Biotechnol. Lett.* 27:1025–1028.
- Goldman, D. 2014. Regeneration, morphogenesis and self-organization. *Development* 141:2745–2749.
- Gupta, S. D. & Jatothu, B. 2013. Fundamentals and applications of light-emitting diodes (LEDs) in in vitro plant growth and morphogenesis. *Plant Biotechnol. Rep.* 7:211–220.
- Hanelt, D. & Figueroa, F. L. 2012. Physiological and photomorphogenic effects of light on marine macrophytes. *In* Wiencke, C. & Bischof, K. (Eds.) *Seaweed Biology: Novel Insights into Ecophysiology, Ecology and Utilization*. Springer, Berlin, pp. 3–23.
- Hothorn, T., Bretz, F. & Westfall, P. 2008. Simultaneous inference in general parametric models. *Biom. J.* 50:346–363.
- Huddy, S. M., Meyers, A. E. & Coyne, V. E. 2015. Regeneration of whole plants from protoplasts of *Gracilaria gracilis* (Gracilariales, Rhodophyta). *J. Appl. Phycol.* 27:427–435.
- Ishizawa, K., Enomoto, S. & Wanda, S. 1979. Germination and photo-induction of polarity in the spherical cells regenerated from protoplasm fragments of *Boergesenia Forbesii*. *Bot. Mag. Tokyo.* 92:173–186.
- Jenkins, G. I. & Cove, D. J. 1983. Light requirements for regeneration of protoplasts of the moss *Physcomitrella patens*. *Planta* 157:39–45.
- Kim, J. K., Mao, Y., Kraemer, G. & Yarish, C. 2015. Growth and pigment content of *Gracilaria tikvahiae* McLachlan under fluorescent and LED lighting. *Aquaculture* 436:52–57.
- Kim, Y. S. & Nam, K. W. 1997. Temperature and light responses on the growth and maturation of gametophytes of *Undaria pinnatifida* (Harvey) Suringar in Korea. *J. Korean. Fish. Soc.* 30:505–510 (in Korean with English abstract).
- Kuwano, K., Abe, N., Nishi, Y., Seno, H., Nishihara, G. N., Iima, M. & Zachleder, V. 2014. Growth and cell cycle of *Ulva compressa* (Ulvophyceae) under LED illumination. *J. Phycol.* 50:744–752.
- Lafontaine, N., Mussio, I. & Rusig, A. -M. 2011. Production and regeneration of protoplasts from *Grateloupia turururu* Yamada (Rhodophyta). *J. Appl. Phycol.* 23:17–24.
- Lakens, D. 2013. Calculating and reporting effect sizes to facilitate cumulative science: a practical primer for t-tests and ANOVA. *Front. Psychol.* 4:863.
- Le, B., Shin, J. -A., Kang, M. -G., Sun, S., Yang, S. H. & Chung, G. 2018. Enhanced growth rate and ulvan yield of *Ulva pertusa* using light-emitting diodes (LEDs). *Aquac. Int.* 26:937–946.
- Lin, K. -H., Huang, M. -Y., Huang, W. -D., Hsu, M. -H., Yang, Z. -W. & Yang, C. -M. 2013. The effects of red, blue, and white light-emitting diodes on the growth, development, and edible quality of hydroponically grown lettuce (*Lactuca sativa* L. var. *capitata*). *Sci. Hort.* 150:86–91.
- Matsumura, W., Yasui, H. & Yamamoto, H. 2001. Successful sporophyte regeneration from protoplasts of *Undaria pinnatifida* (Laminariales, Phaeophyceae). *Phycologia* 40:10–20.
- Miki, O., Okumura, C., Marzuki, M., Tujimura, Y., Fujii, T., Kosugi, C. & Kato, T. 2017. Contrasting effects of blue and red LED irradiations on the growth of *Sargassum horneri* during the germling and immature stages. *J. Appl. Phycol.* 29:1461–1469.
- Morita, T., Kurashima, A. & Maegawa, M. 2003. Temperature requirements for the growth and maturation of the gametophytes of *Undaria pinnatifida* and *U. undarioides* (Laminariales, Phaeophyceae). *Phycol. Res.* 51:154–160.
- Murase, N., Abe, M., Noda, M. & Suda, Y. 2014. Growth and maturation of gametophyte in *Eisenia bicyclis* under different light quality from light emitting diodes (LEDs). *J. Nat. Fish. Univ.* 62:147–152 (in Japanese with English abstract).
- Murgui, A., Elorza, M. V. & Sentandreu, R. 1985. Effect of papulacandin B and calcofluor white on the incorporation of mannoproteins in the wall of *Candida albicans* blastospores. *Biochim. Biophys. Acta Gen. Subj.*

- 841:215–222.
- Peters, G. 2018. userfriendlyscience: Quantitative analysis made accessible. R package version 0.7.2. Available from: <https://userfriendlyscience.com>. Accessed Feb 15, 2022.
- Reddy, C. R. K. & Fujita, Y. 1991. Regeneration of plantlets from *Enteromorpha* (Ulvales, Chlorophyta) protoplasts in axenic culture. *J. Appl. Phycol.* 3:265–275.
- Reddy, C. R. K., Gupta, M. K., Mantri, V. A. & Jha, B. 2008. Seaweed protoplast: status, biotechnological perspectives and needs. *J. Appl. Phycol.* 20:619–632.
- Rorrer, G. L. & Cheney, D. P. 2004. Bioprocess engineering of cell and tissue cultures for marine seaweeds. *Aquac. Eng.* 32:11–41.
- Sato, Y., Endo, H., Oikawa, H., Kanematsu, K., Naka, H., Mogamiya, M., Kawano, S. & Kazama, Y. 2020. Sexual difference in the optimum environmental conditions for growth and maturation of the brown alga *Undaria pinnatifida* in the gametophyte stage. *Genes* 11:944.
- Seely, G. R., Duncan, M. J. & Vidaver, W. E. 1972. Preparative and analytical extraction pigments from brown algae with dimethyl sulfoxide. *Mar. Biol.* 12:184–188.
- Sellke, T., Bayarri, M. J. & Berger, J. O. 2001. Calibration of p values for testing precise null hypotheses. *Am. Stat.* 55:62–71.
- Seppic. 2022. Ephemer. The trusted materials and ingredients global search engine. Available from: <https://www.seppic.com/en/ephemer>. Accessed Feb 15, 2022.
- Smithson, M. & Verkuilen, J. 2006. A better lemon squeezer? Maximum-likelihood regression with beta-distributed dependent variables. *Psychol. Methods* 11:54–71.
- Sullivan, G. M. & Feinn, R. 2012. Using effect size—or why the P value is not enough. *J. Grad. Med. Educ.* 4:279–282.
- Takahashi, F., Yamagata, D., Ishikawa, M., Fukamatsu, Y., Ogura, Y., Kasahara, M., Kiyosue, T., Kikuyama, M., Wada, M. & Kataoka, H. 2007. AUREOCHROME, a photoreceptor required for photomorphogenesis in stramenopiles. *Proc. Natl. Acad. Sci. U. S. A.* 104:19625–19630.
- Wang, L. & Ruan, Y. -L. 2013. Regulation of cell division and expansion by sugar and auxin signaling. *Front. Plant Sci.* 4:163.
- Wang, W. -J., Wang, F. -J., Sun, X. -T., Liu, F. -L. & Liang, Z. -R. 2013. Comparison of transcriptome under red and blue light culture of *Saccharina japonica* (Phaeophyceae). *Planta* 237:1123–1133.
- Wang, Z., Sui, Z., Hu, Y., Zhang, S., Pan, Y. & Ju, H. 2014. A comparison of different *Gracilariopsis lemaneiformis* (Rhodophyta) parts in biochemical characteristics, protoplast formation and regeneration. *J. Ocean. Univ. China* 13:671–676.
- Warren, C. R. 2008. Rapid measurement of chlorophylls with a microplate reader. *J. Plant. Nutr.* 31:1321–1332.
- Warren, G. S. 1992. The cell biology of plant cell culture systems. In Fowler, M. W., Warren, G. S. & Murray, M. -Y. (Eds.) *Plant Biotechnology: Comprehensive Biotechnology (2nd Supplement)*. Pergamon Press, New York, pp. 99–127.
- Xu, Z., Dapeng, L., Hanhua, H. & Tianwei, T. 2005. Growth promotion of vegetative gametophytes of *Undaria pinnatifida* by blue light. *Biotechnol. Lett.* 27:1467–1475.
- Yeong, H. -Y., Khalid, N. & Phang, S. -M. 2008. Protoplast isolation and regeneration from *Gracilaria changii* (Gracilariiales, Rhodophyta). *J. Appl. Phycol.* 20:641–651.
- Zha, X. & Kloareg, B. 1996. Gametophyte protoplast culture of an edible alga *Undaria pinnatifida*. *J. Anhui Agric. Univ.* 23:602–605 (in Chinese).