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Note

Modification of light quality improves the growth and medicinal quality of clonal plantlets derived from the herbal plant *Gentiana*

Hideyuki Takahashi*, Hidetoshi Yamada, Chiharu Yoshida, Tomohiro Imamura

Iwate Biotechnology Research Center, Kitakami, Iwate 024-0003, Japan
*E-mail: h-takahashi@ibrc.or.jp Tel: +81-197-68-2911 Fax: +81-197-68-3881

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Abstract Gentians are medicinal plants whose roots and rhizomes have been used as natural medicines for gastrointestinal problems. The active components are bitter compounds known as secoiridoid glycosides such as gentiopicrin and swertiamarin. Previously, procedures for the *in vitro* clonal culture of gentian plantlets were established. In this study, the effect of different wavelengths of light emitted by LED on plantlet growth and the concentrations of active components was investigated. We found that plantlet growth was promoted after irradiation with red light. Because a similar trend was observed in all gentian cultivars tested, the improved effect of red light on plantlet growth seemed to be a common characteristic of *Gentiana* species. We also found that both leaves and roots of the plantlets contained gentiopicrin and swertiamarin. *Gentiana triflora* 'Maciry' had the highest concentration of both compounds compared with several gentian lines and cultivars. Furthermore, the concentrations of roots were increased by far-red and blue light irradiation. These results suggest that modification of light quality by LED is an effective strategy for improving the growth and concentration of active components in gentian plantlets that can be used as a novel ingredient in natural medicine.

Key words: Clonal culture, gentian, light emitting diode, medicinal plant.

Gentians (*Gentiana* spp.) are annual, biennial, and perennial flowering plants in the family Gentianaceae native to alpine regions in the world. Most perennial species form underground rhizomes that enlarge with each passing year. The roots and rhizomes have been used as an herbal medicine for several gastrointestinal disorders. Furthermore, gentian roots also have anti-inflammatory and anti-neoplastic activities (Yu et al. 2004; Matsukawa et al. 2006). Previous reports show that gentian roots contain bitter secoiridoid glycosides such as gentiopicroside (gentiopicrin) and swertiamarin (Schaufelberger et al. 1984; Jiang et al. 2005). These compounds were reported to be the active components of gentian roots that can benefit hepatitis and dyslipidaemia patients (Yamahara et al. 1978; Vaidya et al. 2009).

In Japan, *G. triflora*, *G. scabra*, and their hybrids are cultivated as important ornamental plants (Yoshiike 1992). Although numerous cultivars have been developed, gentians are almost never used medically probably because Japanese cultivars contain lower amounts of active components than gentians grown in China and Europe. Furthermore, the high cost of sterilizing field-grown gentian roots is prohibitive. Therefore, a novel method for cultivating gentian and for increasing the concentrations of active components is necessary in order to use Japanese cultivars as herbal medicines. In this study, we propose that *in vitro* clonal cultures of gentian are suitable for use as herbal medicines. Previously, a method for the *in vitro* culture of gentian plantlets was established (Hosokawa et al. 2000), enabling the mass propagation of clones with identical genetic backgrounds. The clones displayed consistent qualities, including growth rate, flower color, and metabolite contents (Nakatsuka et al. 2010; Takahashi et al. 2012). Optimum culture conditions were investigated (Takahashi et al. 2012), however, the conditions are insufficient to sustain maximal growth and biosynthesis of sufficient quantities of active components.

Light quality is one of the most important factors regulating plant growth and development (Kendrick and Kronenberg 1994; Ma et al. 2001); therefore, we examined the effect of light quality on gentian plantlet growth and accumulation of secoiridoid gycosides. Gentian plantlets were cultured on solid MS medium containing 3% (w/v) sucrose and 0.25% (w/v) gellan gum at 22°C with a 16/8 h light/dark photoperiod as described previously (Takahashi et al. 2012). To determine which wavelength would be appropriate for growth, plantlets of

Abbreviations: LED, light emitting diode; FW, fresh weight; DW, dry weight.
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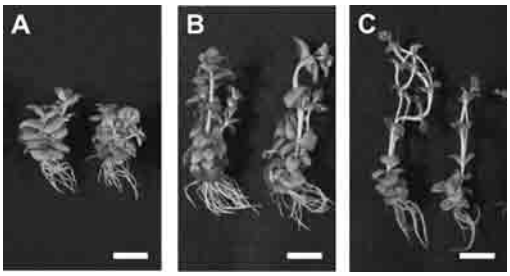

Figure 1. Effect of LED light on growth of plantlets derived from *G. scabra* 'Alta'. The plantlets were cultured under white light (A), red light (B), and far-red light conditions (C) at 22°C with a 16/8 h light/dark photoperiod. The light intensity of white, red (660 nm), and far-red (735 nm) light was set at 40 μmol m^{-2} s^{-1}. Scale bar = 2 cm.

G. scabra 'Alta' were cultured with blue light at 470 nm, red light at 660 nm, and far-red light at 735 nm of a peak wavelength irradiated by the LED lights (CCS Inc.). A fluorescent lamp (FL40SS/W/37, Panasonic) was used as the white light source. All light intensities were measured with the use of a SUN ILLUMIND SLX-1330 (Sanyo) and set at 40 μmol m^{-2} s^{-1}. After growth in a 16/8 h light/dark photoperiod for 4 weeks, the phenotype of the plantlets was evaluated. Red light increased internode length and leaf number, whereas far-red light increased only internode growth (Figure 1). Blue light showed almost no effect (data not shown). These results suggest that red light may improve plantlet growth. Furthermore, we investigated the effect of red light on the growth of *G. triflora* lines (AZH20, EW, Ma1, and Y5-1-4), *G. scabra* 'Alta' and hybrids 'Polano white' and 'Albireo' to evaluate varietal differences (Figure 2). Red light significantly improved the growth of most cultivars, except for Albireo and Ma1, but a trend for increased growth was observed in Albreo and Ma1.

To reveal the effect of light quality on the accumulation of active components, we quantified the concentrations of gentiopicrin and swertiamarin. Extraction of the compounds from gentian plantlets was performed according to the method of Takahashi et al. (2009) with minor modifications. Samples were powdered with a Micro Smash M100 (TOMY) and homogenized with ice-cold 50% (v/v) methanol for 10 min. Homogenates were then centrifuged at 20,000×***g*** for 5 min, the supernatant was filtered through a Millipore 3-kDa-cutoff filter (Amicon), and the filtrates were used for analysis. Gentiopicrin and swertiamarin were separated on a ZORBAX Eclipse Plus C18 column (4.6×250 mm, 5 μm; Agilent Technologies) using a water–methanol gradient (methanol 50% to 70% in 15 min) at flow rate of 0.25 ml/min. Compounds were identified and quantified by liquid chromatography-time of flight mass spectrometry (LC-TOFMS) (Agilent Technologies) using Agilent Mass Hunter Workstation Software (Agilent Technologies). Quantitative accuracy

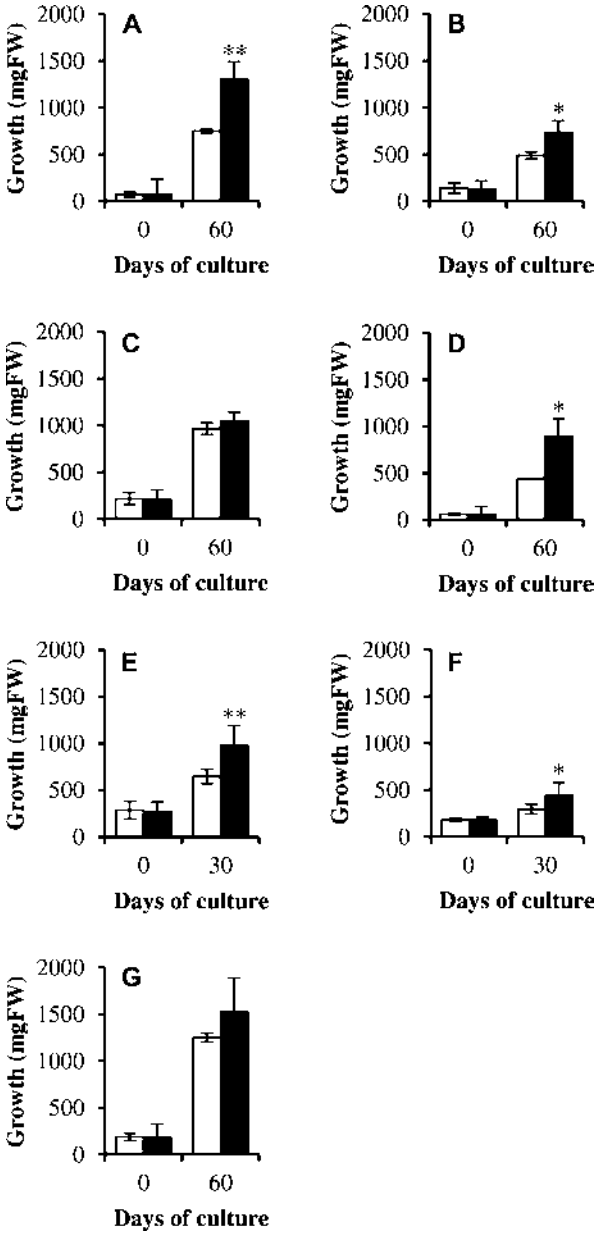

Figure 2. Effect of red light on the growth of gentian plantlets. Growth of AZH20 (A), EW (B), Ma1 (C), Y5-1-4 (D), Alta (E), Polano white (F), and Albireo (G). *G. triflora* (A–D), *G. scabra* (E), and hybrids (F–G) cultured under white (white column) or red (black column) light conditions for 30 days (E, F) or 60 days (A–D, G). Data are means±SD (n=4); significant differences from plantlets cultured under white light were evaluated by Student's *t*-test and are represented by asterisks (* $p<0.1$ or ** $p<0.05$).

was verified with known concentrations of reference standard compounds. The concentrations were expressed as μg/gDW. First, we compared the concentrations of gentiopicrin and swertiamarin in plantlets of several gentian lines and cultivars 2 months after subculture (Figure 3A). The concentration of gentiopicrin was higher than that of swertiamarin in all the plantlets. The highest level of gentiopicrin was observed in *G. triflora* 'Maciry' with a concentration of 677 μg/gDW.

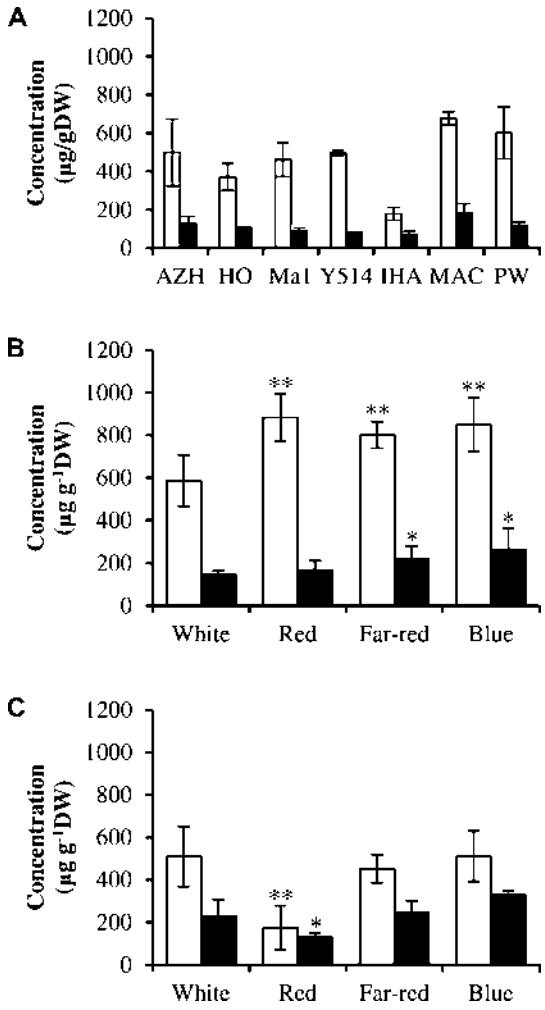

Figure 3. Concentration of active components extracted from gentian plantlets. White columns represent gentiopicrin and black columns represent swertiamarin. The plantlets were cultured with either white, red, far-red, or blue lights. (A) Concentration of gentiopicrin and swertiamarin in roots of gentian lines such as AZH20 (AZH), HO, Ma1, and Y5-1-4 (Y514) and cultivars such as Ihatovo (IHA), Maciry (MAC), and Polano white (PW) cultured with white light for 2 months after subculture. (B) Effect of LED lights on the concentration of gentiopicrin and swertiamarin in roots of the plantlets of *G. triflora* 'Maciry'. (C) Effect of LED lights on the concentration of gentiopicrin and swertiamarin in leaves *G. triflora* 'Maciry' plantlets. Data are means±SD calculated from three independent experiments and significant differences from plantlets cultured under white light were evaluated by Student's *t*-test and are represented by asterisks (* $p<0.1$ or ** $p<0.05$).

The highest level of swertiamarin was also observed in *G. triflora* 'Maciry' with a concentration of 183 μg/gDW. Therefore, the effect of LED lights on the concentrations of gentiopicrin and swertiamarin was determined using this cultivar. The plantlets were cultured with white, red, far-red, and blue light for 2 weeks, and average DWs (mg) were 58.9, 70.7, 51.2, and 58.9, respectively. Comparison of the gentiopicrin concentration in roots of each LED light treated plantlet revealed that the concentration was significantly increased by all LED light irradiation treatments. Red, far-red, and blue lights induced increases of 1.51-, 1.37, and 1.45-fold relative to white light, respectively. The concentration of swertiamarin increased by far-red and blue light treatments, and the increased levels were 1.50- and 1.78-fold higher relative to white light. We also quantified the concentration of these compounds in leaves (Figure 3C). The gentiopicrin concentration was lower in leaves than in roots, whereas the swertiamarin concentration was higher in leaves than in roots. Red light irradiation decreased the concentrations of gentiopicrin and swertiamarin, but far-red and blue lights had no effect. These results suggest that far-red and blue light irradiation is suitable for increasing the concentration of both gentiopicrin and swertiamarin.

The most commonly used gentians as a medicine are European and Chinese varieties, including *G. lutea*. Dried roots of *G. lutea* contain high concentrations of gentiopicrin (2.5 to 3.5%) and swertiamarin (0.15 to 0.20%) (Ariño et al. 1997). Although the concentrations of these compounds in *G. triflora*, *G. scabra*, and their hybrids were apparently lower, *in vitro* clonal plantlets can be re-cultured semi-permanently by shoot tip culture. In this study, we found that active components were present not only in roots but also in the leaves of gentian plantlets. Furthermore, our results highlight the possibility that irradiation with specific wavelengths of LED light can be an effective technique for increasing gentian growth and levels of active components. In further work, elucidating the molecular mechanism of the light response in active compound biosynthesis should lead to significant improvement in the accumulation of these compounds in gentian plantlets.

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