Effect of Wavelength and Light Intensity from Ultraviolet and Violet Light-emitting Diodes for Optimizing Vinblastine Production in the Leaves of Madagascar Periwinkle

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Abstract. Vinblastine accumulation in Madagascar periwinkle leaves is rapidly induced by ultraviolet A irradiation. This study aimed to elucidate the effects of light quality and intensity (wavelengths between 280 and 405 nm) on vinblastine accumulation. In the first experiment, leaves were incubated for 5 days under light-emitting diodes (LEDs) with five distinct peak wavelengths: 280, 310, 365, 385, and 405 nm. Vinblastine accumulation was observed only in leaves treated with 365- and 385-nm LEDs. The leaves were irradiated for 5 days in the second experiment with eight light intensities between 1 and 150 W·m^{-2} from 365- and 385-nm LEDs. The vinblastine concentration was maximized at 25 or 50 W·m^{-2} using 385-nm LEDs. These findings suggest that the optimal light conditions for vinblastine production in periwinkle leaves using LEDs are irradiation with 385-nm LEDs at an intensity of 25 W·m^{-2} for 5 days.

Madagascar periwinkle (*Catharanthus roseus*) accumulates more than 100 monoterpenoid indole alkaloids (MIAs) distributed throughout the plant (Carter and Livingston 1976). Some MIAs, such as vinblastine and vincristine, are clinically used as anticancer therapies. The concentrations of these compounds in the dried plant leaves are ~0.01% and 0.0003%, respectively (Guéritte and Fahy 2005), because the coupling of catharanthine and vindoline to form vinblastine and vincristine is unlikely to occur naturally. One possible reason for the unlikely coupling reaction of catharanthine and vindoline in nature is the physical distance between these two compounds in leaf tissue (Roepke et al. 2010). Catharanthine is localized in epidermal cells, whereas vindoline is localized in idioblast cells (Magnotta et al. 2006; Murata and De Luca 2005; Murata et al. 2008; Roepke et al. 2010; Yamamoto et al. 2019). In addition, an industrial method for synthesizing these alkaloids has not yet been established. Therefore, an efficient method for producing vinblastine and vincristine is required.

The reaction is interpreted as proceeding nonenzymatically and starts with the excitation of flavin mononucleotide (FMN) after absorption of ultraviolet-A light. The excited FMN oxidizes catharanthine, which combines with vindoline (Duangteraprecha et al. 1997; Hirata et al. 1999). These reactions appear to cause the living periwinkle leaves (Asano et al. 2010; Hirata et al. 1991, 1993). The coupling reaction of catharanthine with vindoline produces 3,4-anhydrovinblastine (AVBL), which is then converted to vinblastine via reduction by NAD(P)H in in vitro assays (Hirata et al. 1997). Fig. 1 shows the schematic representation of this reaction.

Our group developed a method to efficiently produce vinblastine by controlling the ultraviolet-A light irradiation in a plant factory using artificial light (PFAL). Because periwinkle plants grown under monochromatic red light grow faster than those exposed to blue light, mixtures of red and blue light, or white light (Fukuyama et al. 2013), they were first grown under monochromatic red light and then exposed to ultraviolet-A light from ultraviolet fluorescent lamps with a peak wavelength of 380 nm. Exposure to ultraviolet-A light for 3 to 5 d increased vinblastine concentrations in attached and detached periwinkle leaves (Fukuyama et al. 2017, 2023). These findings suggest that controlling the light environment using monochromatic red and ultraviolet-A light in PFAL is a promising technique for vinblastine production.

FMN in water exhibits four absorption peaks at 220, 265, 370, and 450 nm (Copeland and Spiro 1986). To increase the efficiency of vinblastine in PFAL, we predicted that irradiating the absorption spectra of FMN with ultraviolet light would be preferable. Additionally, the optimal light intensity for efficient vinblastine production remains unclear.

In this study, we aimed to elucidate the influence of light quality and intensity from the light-emitting diodes (LEDs) emitting light within the FMN absorption bands on vinblastine accumulation in the periwinkle. We evaluated the concentrations of vinblastine and its precursors in the periwinkle leaves incubated under LEDs at five distinct peak wavelengths (280, 310, 365, 385, and 405 nm), and eight light intensities (1 to 150 W·m^{-2}) using LEDs with wavelengths of 365 and 385 nm. This study provides the optimal light conditions for vinblastine production in periwinkle leaves using LEDs.

Materials and methods

Plant culture

The seeds of periwinkle 'Titan Pure White' (M&B Flora Inc., Hokuto, Yamanashi, Japan) were sown on a urethane mat, which fully absorbed deionized water in the dark at a room temperature of 23 °C for 3 d. These seedlings were placed in an environmentally controlled room with a 16-h light period, and a room temperature of 23 °C. Irradiation was provided

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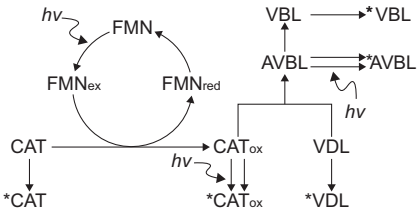

Fig. 1. Vinblastine synthesis scheme via the light-driven nonenzymatic coupling reaction (modified from Hirata et al. 1999). AVBL, 3, 4-anhydrovinblastine; CAT, catharanthine; CATox, oxidated catharanthine; FMN, flavin mononucleotide; FMNex, excited FMN; FMNred, reduced FMN; VBL, vinblastine; VDL, vindoline. The asterisk indicates a degradation form.

using white fluorescent lamps and maintained at a photosynthetic photon flux density (PPFD) of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The PPFD was measured using a PPFD sensor (LI-190, LI-COR Inc., Lincoln, NE, USA) on a cultivation board. Five days after sowing, 100 seedlings with developing cotyledons were transplanted into a plastic container (400 × 600 × 80 mm) containing 6 L of nutrient solution and grown hydroponically. The nutrient solution was an OAT-house A recipe (OAT Agrio Co., Ltd., Chiyoda, Tokyo, Japan) described by Hanyu and Shoji (2002), and electrical conductivity (EC) and pH were maintained at 1.0 $dS \cdot m^{-1}$ and 5.6, respectively. The EC and pH were controlled using a fertilizer-management machine (CEM Corporation, Bunkyo-ku, Tokyo, Japan).

The third and fourth leaves were collected 45 and 52 d after sowing, respectively. The leaves were floated on pure water in a plastic pot and incubated under LED light treatments in Expts. 1 and 2. When the leaf blades were large, the tips and bases were cut off to make 40 × 30-mm rectangles such that the leaves would not overlap when floating in water.

Samples were collected before and after the LED light treatments to measure the concentration of catharanthine, vindoline, and vinblastine. Samples were stored in a –20 °C freezer. All experiments were performed in triplicate.

Expt. 1. LED irradiation at five distinct peak wavelengths between 280 and 405 nm. Each pot containing leaves floating in the water was irradiated with LEDs (Nichia Corporation, Tokushima, Japan) at five distinct peak wavelengths (280, 310, 365, 385, and 405 nm) for 5 d (Fig. 2).

Simultaneously, the background light was irradiated with white fluorescent light for 24 h over 5 d. The spectra of the light sources were measured using a spectrum analyzer (LR-1; ASEQ Instruments, Vancouver, Canada). The ultraviolet-LEDs and white fluorescent lamps had intensities of 5 $W \cdot m^{-2}$ and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, respectively. The LED light intensity and PPFD were measured using a ultraviolet radiometer (X9-6, Gigahertz-Optik GmbH, Bavaria, Germany) and a PPFD sensor installed on the surface of the plastic pot. The LED light intensities were corrected by multiplying by the corrected coefficients specific to each LED. These corrected coefficients were provided by the Nichia Corporation and were based on measurements made with a spectrometer that had been calibrated for light intensity. We sampled after the LED treatments for 5 d and before the LED treatment (0 d). Additionally, the dark controls had no light sources, whereas the controls only had white fluorescent lamps for 5 d.

Expt. 2. LED irradiation at different intensities between 365 and 385 nm. Rectangular leaf samples measuring 40 × 30 mm were prepared by trimming the top and base of the leaves to ensure consistency of the irradiated areas. No effects of this processing on alkaloid concentrations in leaves were observed (Supplemental Fig. 1). These rectangular leaves were exposed to eight light intensities between 365- and 385-nm LEDs along with white fluorescent lamps at 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The light intensities in the LED treatments were 1, 5, 25, 50, 75, 100, 125, and 150 $W \cdot m^{-2}$. The treatment using white fluorescent lamps without LEDs was 0 $W \cdot m^{-2}$.

Measurement of alkaloid concentrations. Alkaloid extraction from leaves was performed based on the method by Asano et al. (2010), with modifications. The frozen leaves were lyophilized for 48 h. The dried leaves were ground into fine powder at 20 Hz for 60 s using the Tissue Lyser II (Qiagen, Hilden, Germany). We transferred 30 mg of leaf powder to a 2-mL microtube and added 300 µL of methanol. The mixture was sonicated for 30 min and centrifuged at 15,000 g_n for 3 min at 4 °C; subsequently, the supernatant was collected. These extraction steps were repeated three times. All the supernatants were collected and filtered through a 0.45-µm membrane filter.

For the analysis of high concentrations of catharanthine and vindoline in leaf extracts using a diode array detector, 10 µL of extract was analyzed using high-performance liquid chromatography (HPLC; Chromaster, Hitachi High-Technologies Co., Japan). A CAPCELL PAK MG II column (4.6 mm i.d. × 150 mm, 5 µm, Osaka Soda Co., Ltd., Japan) was used with a mobile phase of 10 mM sodium phosphate buffer (pH 7.0):acetonitrile (ratio not specified) at a flow rate of 1 mL/min. The following gradient was employed: 30% acetonitrile (v/v) from 0 to 3 min and 60% (v/v) from 8 to 20 min. These alkaloids were detected at 250 nm. Vindoline and catharanthine standards were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). These standards were also analyzed using the aforementioned method to generate calibration curves for vindoline and catharanthine.

To analyze low alkaloid concentrations in leaf extracts, 1 µL of extract was analyzed using ultra-performance liquid

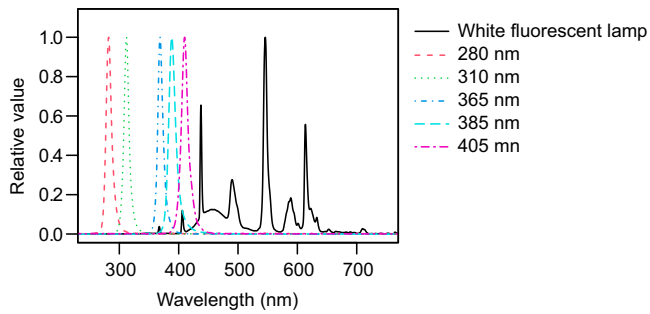

Fig. 2. Spectrum of a white fluorescent lamp and five distinct wavelength light-emitting diodes.

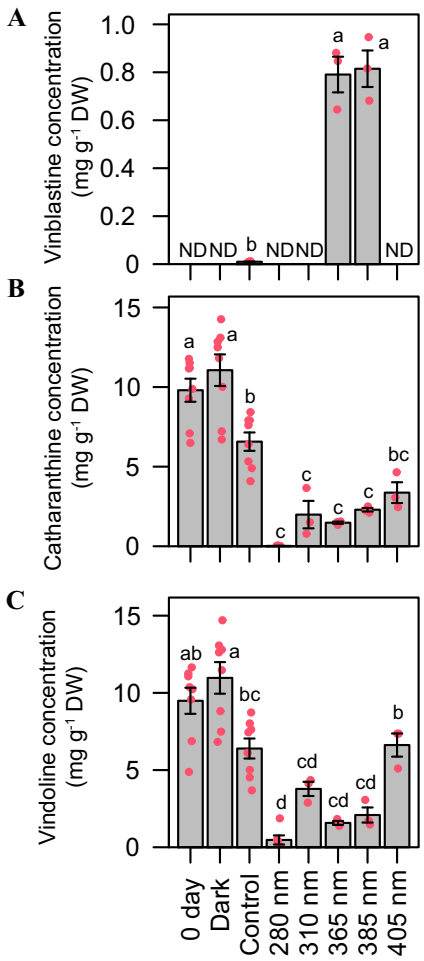

Fig. 3. Vinblastine (A), catharanthine (B), and vindoline (C) concentrations in the leaves incubated under different wavelength light-emitting diodes for 5 d. Gray and error bars represent mean and standard error, respectively. Red dots represent each actual value of the sample. ND indicates no alkaloid detected. Means with different letters are significantly different for each treatment at $P < 0.05$ for Tukey's honestly significant difference test (n = 3–10).

chromatography (UPLC; ACQUITY UPLC system, Waters, Milford, MA, USA) coupled with a tandem mass spectrometer (Xevo G3 QTof, Waters, USA). An ACQUITY UPLC BEH C18 column (2.1 mm i.d. × 100 mm, 1.7 µm, Waters, USA) was used with a mobile phase of 0.1% formic acid in the water:acetonitrile (80:20, v/v) mixture at a flow rate of 0.3 mL/min. The following gradient was employed: 20% acetonitrile (v/v) at 0 min; 30% (v/v) at 7.5 min; 95% (v/v) from 9.5 to 15.0 min; and 20% (v/v) from 15.1 to 20.5 min. Leaf extract samples were diluted 100-fold with 80:20 (v/v) 0.1% formic acid in the water:acetonitrile mixture before injection. The column oven and autosampler temperatures were maintained at 40 and 4 °C, respectively. Alkaloid concentrations were quantified using multiple reaction monitoring (MRM). The MRM transitions are listed in Supplemental Table 1. Retention times and mass spectra of the identified alkaloids were compared with those of authentic standards purchased from Selleck Chemicals (Houston, TX, USA).

Measurement of the absorption spectrum of FMN. The standard FMN reagent (riboflavin sodium phosphate, Wako Special grade, 065-00171) was purchased from FUJIFILM Wako Pure Chemical Corporation (Chuo-ku, Osaka, Japan). This was dissolved in pure water and the concentration adjusted to 1.0 mmol·L^{-1}. Subsequently, the 1.0·mmol·L^{-1} FMN solution was diluted to 20 µmol·L^{-1} using a 100 mmol·L^{-1} sodium phosphate buffer at five pH levels: 5.0, 6.0, 6.5, 7.0, and 7.5. We measured the absorption spectrum of this solution between 200 and 550 nm (1-nm increments) at 20 °C using a spectrometer (UV-1800, SHIMADZU CORPORATION, Nakagyo-ku, Kyoto, Japan) equipped with a temperature-controlled cell holder (TCC-100, SHIMADZU CORPORATION, Nakagyo-ku, Kyoto, Japan).

Statistical analysis. In Expts. 1 and 2, we calculated the mean and standard error for each alkaloid concentration from three rectangular leaves. We then performed multiple comparisons using Tukey's honestly significant difference (HSD) test. The significance level was set at $P < 0.05$ for Tukey's HSD test. However, these analyses were excluded for treatments in which alkaloids were detected in only one of the three replicates. In Expt. 2, a two-way analysis of variance (ANOVA) was used to determine whether the effects of wavelength and intensity were statistically significant at $P < 0.05$. Statistical analyses were performed using the statistical software R version 4.4.1 (R Core Team 2024).

Results

In Expt. 1, LED irradiation of leaves with distinct peak wavelengths revealed that vinblastine was detected only in the 365- and 385-nm LED treatments (Fig. 3A). Catharanthine concentrations in the LED irradiation treatments tended to be lower than in the control (Fig. 3B). Notably, catharanthine was undetectable in the 280-nm LED treatment. Vindoline concentrations showed a trend similar to that of catharanthine (Fig. 3C),

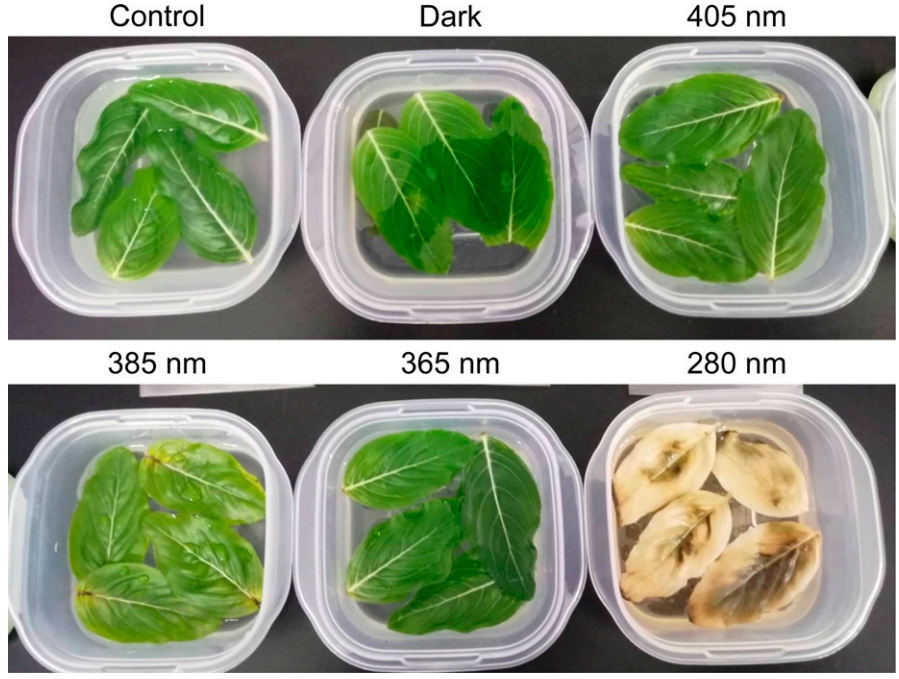

Fig. 4. Periwinkle leaves incubated under light-emitting diodes with different wavelengths for 5 d.

but the decrease in concentration was not as pronounced as that of catharanthine compared with that of the control. All treated leaves were bleached in the 280-nm LED treatment (Fig. 4).

In Expt. 2, irradiation with different light intensities from a 365-nm LED resulted in a light intensity-dependent increase in vinblastine concentration up to 50 $W{\cdot}m^{-2}$, followed by a decrease (Fig. 5A). The catharanthine concentration tended to decrease in a light intensity–dependent manner up to 25 $W{\cdot}m^{-2}$ (Fig. 5B). Catharanthine was scarcely detectable in treatments with light intensities of ≥50 $W{\cdot}m^{-2}$. Vindoline showed a trend similar to that of catharanthine (Fig. 5C). Some leaves in treatments with light intensities of ≥75 $W{\cdot}m^{-2}$ were also bleached (Fig. 6). Alkaloids in the bleached leaves were either undetectable or their concentrations were extremely low compared with those in the nonbleached leaves.

Irradiation with different light intensities from the 385-nm LED resulted in a light intensity-dependent increase in vinblastine concentration up to 50 $W{\cdot}m^{-2}$, similar to the 365-nm peak wavelength LED treatments (Fig. 7A). However, the vinblastine concentrations in the 50 $W{\cdot}m^{-2}$ treatment showed high variability. These treatments with light intensities >75 $W{\cdot}m^{-2}$ tended to decrease in a light intensity-dependent manner. Vinblastine precursor concentrations in the 385-nm LED treatments were similar to those in the 365-nm LED treatments (Fig. 7B and C). Some leaves in treatments with light intensities of ≥100 $W{\cdot}m^{-2}$ were bleached (Fig. 6). The alkaloid concentrations in these bleached leaves were similar to those in the 365-nm LED treatments. The ANOVA results for the three alkaloids showed that only intensity significantly differed among the Expt. 2 treatments.

We measured the absorption spectrum of the FMN, which overlapped with the emission bands of the LEDs at five distinct wavelengths (Fig. 8). FMN exhibited four peaks at 220, 266, 373, and 444 nm and three valleys at 240, 306, and 401 nm. No significant shifts in the peaks or valleys were observed under different pH buffers. The FMN spectrum in the sodium phosphate buffer was not significantly different from that in the water (Copeland and Spiro 1986).

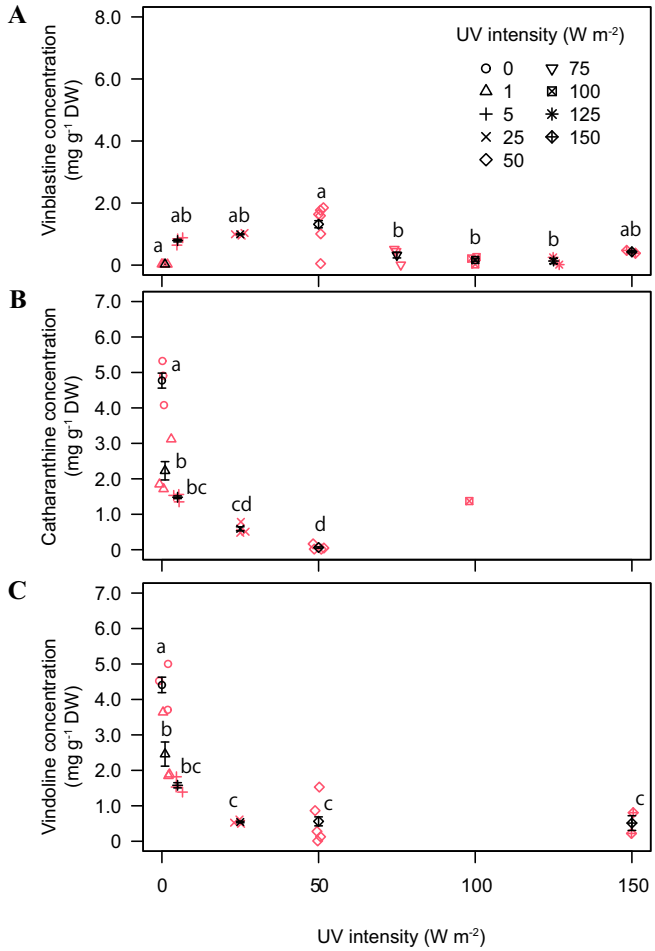

Fig. 5. Vinblastine (A), catharanthine (B), and vindoline (C) concentrations in the rectangular leaves incubated under different intensities of 365-nm light-emitting diodes for 5 d. Black dots and error bars represent mean and standard error, respectively. Red dots represent each actual value of the sample. Without mean value descriptions (black dots), alkaloids were detected from only one of three samples. Without actual value descriptions (red dots), alkaloids were not detected in the three samples. Different letters above each mean value are significantly different for each treatment at $P < 0.05$ for Tukey's honestly significant difference test (n = 3–5).

Discussion

We speculated that vinblastine accumulates at 365- and 385-nm owing to three factors: 1) the absorption spectrum of FMN, 2) competition between FMN and other light absorbers, and 3) the degree of cell damage caused by ultraviolet light exposure.

The third peak corresponding to the shortest wavelength among the four FMN absorption peaks overlapped with the emission spectra of the 365- and 385-nm LEDs. This overlap was associated with increased vinblastine accumulation following irradiation with the 365- and 385-nm LEDs (Fig. 3C). However, our previous study reported that vinblastine accumulation in periwinkle leaves was caused by irradiation with 440-nm LEDs at ~40 $W{\cdot}m^{-2}$. The emission spectrum of the 440-nm LEDs also overlapped with that of the FMN molecule. However, no vinblastine accumulation was observed in the leaf rectangle irradiated with 405-nm LEDs at 5 $W{\cdot}m^{-2}$. We believe that it was difficult to sufficiently excite FMN by irradiation with low-intensity blue light because of light absorption by chlorophyll a and b and carotenoids. Chlorophyll a and b and carotenoids function as photosynthetic and accessory pigments, respectively, and are abundant in the leaves. These pigments strongly absorb blue light. If we had irradiated

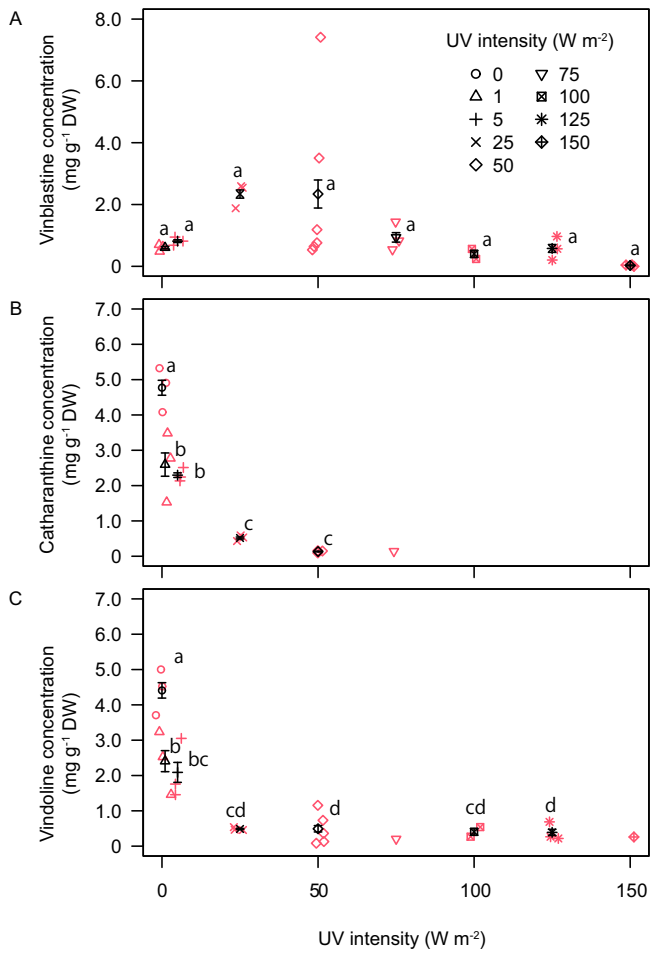

Fig. 6. Vinblastine (A), catharanthine (B), and vindoline (C) concentrations in the rectangular leaves incubated under different intensities of 385-nm light-emitting diodes for 5 d. Black dots and error bars indicate mean and standard error, respectively. Red dots represent each actual value of the sample. Without mean value descriptions (black dots), alkaloids were detected from only one of three samples. Without actual value descriptions (red dots), alkaloids were not detected in the three samples. Different letters above each mean value are significantly different for each treatment at $P < 0.05$ for Tukey's honestly significant difference test (n = 3–5).

periwinkle leaves with over 5 W·m^{-2} light from 405-nm LEDs to a periwinkle leaf, vinblastine accumulation might have occurred. Although the distance in the direction of leaf thickness required to reach light varies with wavelength, vinblastine accumulation is likely caused by the excitation of FMN, as free FMN is ubiquitously present in plant cells (Deng et al. 2011).

Irradiation with 280-nm LEDs caused the leaves to turn white and were evidently damaged, probably owing to cell death caused by DNA damage. The synthesis of the vinblastine precursors vindoline and catharanthine is dependent on multiple enzymes involved in the alkaloid synthesis pathway, and their biosynthesis is inhibited. For the coupling reaction to occur, vinblastine precursors must be transported from their respective cells to the leaf tissue. Alkaloid transport is mediated by several transporters, similar to CrTPT2 (Yu and De Luca 2013). However, alkaloids may have leaked from the bleached leaves. Catharanthine and vindoline were identified in the water in which the leaf rectangles had been floated, regardless of the treatments (Supplemental Fig. 2). However, we conclude that the leakage of alkaloids did not influence the above discussion. This is because the maximum percentages of leakage of catharanthine and vindoline, relative to the amounts in the leaf rectangles before ultraviolet treatments (set as 100%), were 0.53% and 2.3%, respectively. These values were negligible in comparison with the total alkaloid content in the leaves.

Therefore, leaves with extensive cell death, such as bleached leaves, likely showed lower accumulation of vinblastine and its precursor, owing to the disruption of these essential processes for synthesis (Figs. 3 and 4). By reducing the irradiation time or intensity compared with the conditions used in this study, 280-nm LED irradiation may result in vinblastine accumulation.

Another possibility is that ultraviolet-A light may generate reactive oxygen species (ROS), which could potentially activate the AVBL synthase enzyme CrPRX1 (Costa et al. 2008). CrPRX1, a peroxidase, requires hydrogen peroxide (H_2O_2), a type of ROS, as a substrate for its activity. Considering that ultraviolet-A photons, including those from these LEDs, can generate ROS (reviewed by Verdaguer et al. 2017), vinblastine synthesis may be stimulated by CrPRX1 using H_2O_2 produced through this mechanism.

In conclusion, the optimal light wavelength and intensity for light-driven vinblastine accumulation in periwinkle leaves likely depends on three factors: overlap of the irradiated light spectrum and the absorption spectrum of FMN, excitation of a sufficient number of FMN molecules, and maintenance of essential biological processes for the synthesis and transport of vinblastine precursors. Based on these findings and previous studies, a suitable method for vinblastine production using PFAL might be irradiation at 25 W·m^{-2} with a 385-nm LED for 5 d after growing the plants under monochromatic red light. This suitable method was based on the vinblastine concentration in young leaves, however, and therefore, the vinblastine concentration must be considered using the entire plant. The vinblastine accumulation ability of senescent leaves by ultraviolet irradiation was lower than that of young leaves (Fukuyama et al. 2017). The optimal wavelength and intensity for ultraviolet irradiation of all leaves of the entire plant are expected to be determined in the future.

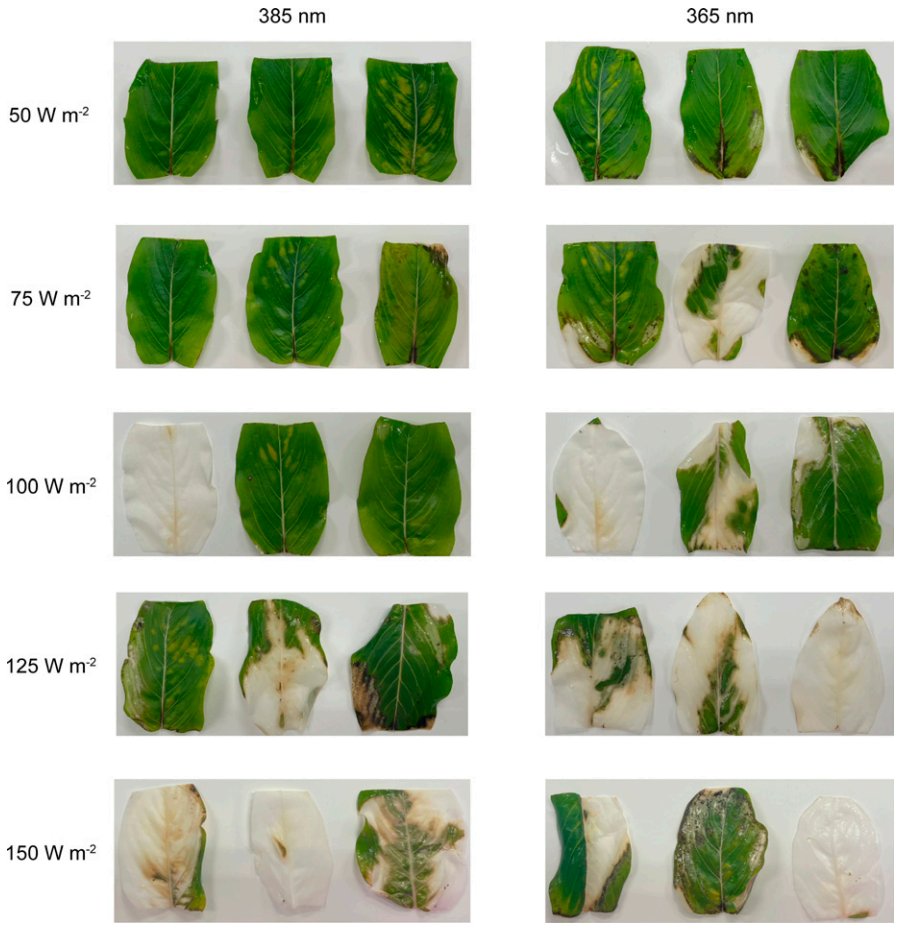

Fig. 7. Periwinkle leaves incubated under different light intensities from 365- and 385-nm light-emitting diodes for 5 d.

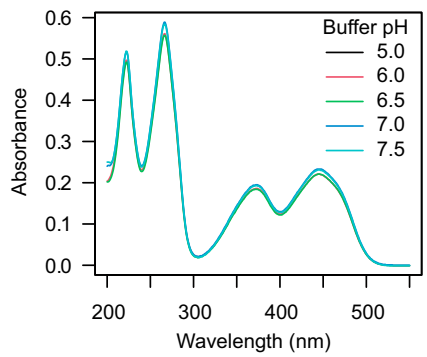

Fig. 8. Absorption spectrum of 20 µmol·L^{-1} flavin mononucleotide dissolved in 100 mmol·L^{-1} sodium phosphate buffer at five pH levels at 20 °C.

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