Effects of Ultraviolet A Supplemented with Red Light Irradiation on Vinblastine Production in Catharanthus roseus

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We investigated whether it is possible to produce vinblastine by irradiation of blue light (B, 440 nm) and ultraviolet A light (UVA, 370 nm) to Catharanthus roseus for domestic production of vinblastine using an environmentally controlled room with artificial lighting, such as a plant factory. Catharanthus roseus plants were cultivated under red light (R, 660 nm) for 28 d and then were cultivated under 3 light quality treatments: UVA supplemented with R, B, or R for 7 d. At 3 d after treatments, vinblastine content in the leaves increased sharply under UVA supplemented with R compared with R alone. The vinblastine content under B was 1/6 of that under UVA supplemented with R. Vinblastine content increased as the UVA intensity was increased from 0 to 10 W m⁻². UVA irradiation to the leaf discs made from the younger leaves raised the vinblastine content in the leaf discs more than those from the aged leaves. Therefore, UVA light should be irradiated to the young plants at early vegetable stage. For the domestic production of vinblastine, we proposed that the cultivation method of irradiating young plants with 10 W m⁻² UVA for more than 3 d.

Keywords : anti-cancer drug, light emitting diode, medicinal plant, monoterpenoid indole alkaloid, plant factory, secondary metabolite

INTRODUCTION

Catharanthus roseus produces monoterpenoid indole alkaloids which are a well-known source of drugs (Carter and Livingston, 1976). Especially, vinblastine and vincristine are made from dimeric monoterpenoid indole alkaloids (DIAs) for various cancer chemotherapies. In addition, vindesine and vinorelbine of semi-synthetic alkaloids are used in the same applications as vinblastine and vincristine. DIAs are synthesized via 3’, 4’-anhydrovinblastine, which is synthesized from the coupling of the monomeric precursors, vindoline and catharanthine. Unfortunately, these drugs are very expensive as C. roseus accumulates very low amounts of DIAs in leaves. A coupling reaction of vindoline and catharanthine rarely occurs in nature. Although many researchers have studied total or semi-synthetic techniques for DIAs production by chemical and enzymatic methods (Kutney et al., 1988; Misawa et al., 1988; Kuehne et al., 1991; Yokoshima et al., 2002; Shirahama et al., 2006; Ishikawa et al., 2009), these techniques have not resulted in sufficient benefit. These drugs, derived from DIAs, can still be extracted and purified from large amounts of C. roseus plants, which are cultivated in large fields (Roepke et al., 2010).

C. roseus is a perennial plant that is native to subtropical and tropical regions. Optimal temperature for growth of C. roseus plants is between 21 and 27°C (Blazich et al., 1995). In Japan, C. roseus cannot be cultivated continuously throughout the year in outdoor conditions, because the temperature is less than 18°C between November and March. Hence, Japan imports the drugs derived from DIAs. The supply of these drugs might become unstable by weather fluctuation and competition with foreign countries. For the stable supply of these drugs in Japan, it is desirable to produce DIAs domestically. In addition, the regulation system of alkaloid content in C. roseus is influenced by environmental conditions, such as light intensity (Liu et al., 2011; Fukuyama et al., 2015) and nitrogen content in fertilizer (Gholamhoss et al., 2011; Guo et al., 2014). Since it is necessary to control environmental conditions of C. roseus cultivation strictly for stable DIAs production, this cultivation would be preferred to operate in an enclosed environmentally controlled room with artificial lighting, such as a plant factory. We investigate the optimal environmental conditions and cultivation methods to achieve high yield of DIAs using an enclosed environmentally controlled room with artificial lighting.

Blue light (B, peak wavelength was 450 nm) and UVA (peak wavelength was 370 nm) light irradiation to multiple shoot cultures or soil-cultured C. roseus plants induced the increase of vinblastine content and the decrease of vindoline and catharanthine content (Hirata et al., 1991; 1992; 1993). On the other hand, the growth of C. roseus grown under monochromatic red light (peak wavelength was 660 nm) irradiation increased compared with light irra-
diation with the other colors (Fukuyama et al., 2013). Based on these reports, to obtain both the high biomass and high vinblastine content in leaves, we designed a cultivation method where *C. roseus* was grown under red light, and then these plants were irradiated with B or UVA light from one week before harvest. We confirmed whether it is possible to increase vinblastine content in the leaves of *C. roseus* cultivated using the method which was designed by us in an environmentally controlled room with artificial lighting. Moreover, for improvement of vinblastine content, we investigated optimum light intensity and irradiation time of UVA light, and the effect of leaf age on vinblastine production in leaves. In addition, we investigated the availability of B light on vinblastine content in leaves. Finally, we propose a cultivation method developed for vinblastine production.

**MATERIALS AND METHODS**

*Plant material and seedling culture*

The seeds of *C. roseus* ‘Titan Dark Red’ (M&B Flora Inc., Hokuto, Yamanashi, Japan) were germinated on a urethane mat, which fully absorbed deionized water, in the dark, at a room temperature of 23°C for 3 d. The seedlings were grown in an environmentally controlled room with a 16 h light period and a room temperature of 23°C. Irradiance was provided by white fluorescent lamps and maintained at a photosynthetic photon flux density (PPFD) of 100 μmol m⁻² s⁻¹. PPFD was measured using a LI-190 sensor (LI-COR Inc., Lincoln, Nebraska, USA) on the urethane mats. At 5 d after germination, 100 seedlings which had expanded cotyledons, were transplanted to a plastic container (400 mm × 600 mm × 80 mm) containing a 6 L nutrient solution, and grown hydroponically. The nutrient solution used was OAT-house A solution (Otsuka AgriTechno Co., Ltd., Tokyo, Japan) described by Hanyu and Shoji (2002), and electrical conductivity (EC) and pH were set at 1.5 dS m⁻¹ and 5.6, respectively. The nutrient solution was renewed once every 7 d and was continuously aerated.

**Experiment 1 Changes in vinblastine content during B or UVA light irradiation**

At 35 d after the germination, 48 seedlings were transplanted to 3 plastic containers containing a 10 L nutrient solution, and then were grown under R light irradiation of 150 μmol m⁻² s⁻¹ using R LEDs. PPFD was measured using a LI-190 sensor (LI-COR Inc., Lincoln, Nebraska, USA) and the light period, room temperature, EC, and pH were the same as during seedling culture. The 4 plants were harvested at 1, 3, 5, and 7 d after starting the light quality treatments. Four plants were harvested before the light quality treatments as 0 d treatment samples. Whole leaves were stored in plastic packs at −50°C. The frozen leaves were lyophilized for 48 h, and dried leaves were weighed and used for the measurement of vinblastine content.

**Experiment 2 Effects of different UVA intensities on vinblastine content in leaves**

At 35 d after germination, 10 seedlings were transplanted to a plastic container containing 10 L nutrient solution and then were grown under red light irradiation of 150 μmol m⁻² s⁻¹ using R LEDs.

The fourth leaves, which were numbered after the primary leaf followed by cotyledon, were harvested at 63 d after germination. Five hundred leaf discs of 7 mm diameter were made from the fourth leaves of 10 plants, and then 25 leaf discs were floated on a 86 mm diameter dish filled with distilled water. Five dishes were exposed to each UVA light intensity treatment, which were 0, 1, 5, and 10 W m⁻². UVA light intensity was adjusted by the distance between the dishes and UV fluorescent lamps. In all UVA treatments, 150 μmol m⁻² s⁻¹ R light was irradiated as background light for driving photosynthesis. Exposure time was 16 h/d. At 5 d after starting UVA light treatments, 25 leaf disks were collected in a 2 mL microtube and immediately frozen in liquid nitrogen. These microtubes were stored at −50°C. The frozen leaf disks were lyophilized, and dried leaf disks were used for the measurement of alkaloid contents.

**Experiment 3 Effects of UVA irradiation on vinblastine content in leaves at different leaf positions**

At 35 d after the germination, 5 seedlings were transplanted to a plastic container containing 10 L nutrient solution and then these were grown under red light irradiation of 150 μmol m⁻² s⁻¹ using R LEDs.

The second, third, and fourth leaves were harvested at 63 d after germination. A total of 250 leaf discs of 7 mm diameter were made at each leaf position from the 5 plants, and then 25 leaf discs were floated on a 86 mm diameter dish filled with distilled water. Five dishes per treatment were exposed to 5 W m⁻² UVA light supplemented 150 μmol m⁻² s⁻¹ R light (UVA⁺) and 150 μmol m⁻² s⁻¹ R light (UVA⁻) as control. At 5 d after starting UVA light treatments, each of 25 leaf disks were collected in a 2 mL microtube and immediately frozen in liquid nitrogen. These microtubes were stored at −50°C. The frozen leaf disks were lyophilized, and dried leaf disks were used for the measurement of vinblastine contents.

**Measurement of alkaloid contents**

Extraction of alkaloids was performed according to Asano et al. (2010), with some modifications. The dried leaf was ground into fine powder with a mortar and pestle. Leaf powder was transferred to 2 mL microtube and added to 10 times volume methanol. The mixture was sonicated for 30 min and then centrifuged at 15,000 g for 3 min at 4°C. The supernatant was collected. These extraction steps were repeated 3 times. All the supernatants were
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gathered and filtered through a 0.45 μm membrane filter.

For measurement of vindoline and catharanthine, 10 μL of extract were analyzed by high-performance liquid chromatography (Chromaster, Hitachi High-Technologies Co., Tokyo, Japan) using a CAPCELL PAK MG II column (4.6 mm inner diameter × 150 mm, 5 μm, Shiseido Co., Ltd., Tokyo, Japan) with 10 mM sodium-phosphate buffer pH 7.0 - acetonitrile mixture at a flow rate of 1 mL min⁻¹. The following gradient step was used: 30% (v/v) acetonitrile between 0 and 3 min, 60% (v/v) acetonitrile between 8 and 20 min. These alkaloids were detected at 250 nm by diode array detector. The standards of vindoline and catharanthine were purchased from LKT Laboratories, Inc. (USA). This standard was also analyzed by using this method and obtained a calibration curve of vindoline and catharanthine.

For vinblastine measurement, 5 μL of extract were analyzed by ultra-performance liquid chromatography (ACQUITY UPLC systems, Waters, Milford, Massachusetts, USA) and tandem mass spectrometer (SYNAPT HDMS, Waters, Milford, Massachusetts, USA) using a ACQUITY UPLC BEH C18 column (2.1 mm inner diameter × 150 mm, 1.7 μm, Waters, Milford, Massachusetts, USA) with 0.1% formic acid in water — acetonitrile mixture at a flow rate of 0.3 mL min⁻¹. The following gradient step was used: 20% (v/v) acetonitrile at 0 min, 30% (v/v) at 7 min, 95% (v/v) between 9 and 14 min, and 20% (v/v) between 14.5 and 19.8 min. The mass spectrometer was operated with a capillary voltage of 4.5 kV, cone voltage of 72 V. Vinblastine was detected from product ions 811 m/z by mass spectrometer and identified retention time compared with authentic standard. The vinblastine reference standard was purchased from Wako Pure Chemical Industries, Ltd. (Japan). This standard was also analyzed by using this methods and a calibration curve of vinblastine was obtained.

**Statistical analysis**

Means and standard error were calculated from 4 plants (Experiment 1) or 5 dishes (Experiments 2 and 3). In all experiments, using less than 5% as the level of significance, statistical analysis was subject to the Tukey-Kramer honestly significant difference (HSD) test.

**RESULTS**

**Experiment 1 Changes in vinblastine content during B or UVA light irradiation**

Total leaf dry weight in all of the treatments were observed the trend to increase during the light quality treatment period (Fig. 1). On the other hand, the significant differences of total leaf dry weight between light qualities were not observed.

In the R-treatment plants, vinblastine content was less than 3.01 μg g⁻¹ DW through the light treatment period (Fig. 1). Vindoline content in the UVA-treatment plants was higher than that in the R and B-treatment plants after 3 d. At 7 d, the vinblastine content in the leaves of the plants irradiated with UVA light was 12 times higher than those irradiated with R light. The vinblastine yield in the UVA-treatment plants was 16 times higher than in the R-treatment plants at 7 d (Fig. 1). According to the result of Tukey-Kramer HSD test between UVA-treatment plant, vinblastine yield at 7 d was significant greater than that at 1 d. The vinblastine content in the leaves of the plants irradiated with B light tended to be slightly higher those with R light at 5 and 7 d.

**Experiment 2 Effects of different UVA intensities on vinblastine content in leaves**

Vindoline and catharanthine content in the leaf discs were decreased by increasing UVA intensity (Fig. 2). In contrast, vinblastine content was increased with increasing UVA intensity. Vinblastine content of the leaf discs incubated under 10 W m⁻² UVA light was 54 times higher than that of the leaf discs incubated under 0 W m⁻² UVA light.
Experiment 3 Effects of UVA irradiation on vinblastine content in leaves at different leaf positions

Figure 3 shows vinblastine content at different leaf positions. At 63 d after germination, the fourth leaves were the youngest fully expanded leaves on the plants. Vinblastine content in the leaf disks irradiated with UVA light was higher as the leaf position was higher, while its content in the leaf disks irradiated without UVA light was low irrespective of leaf position.

DISCUSSION

The increase of vinblastine content in C. roseus leaves was possible by irradiation of UVA to the plants grown under R light (Fig. 1). Our results indicated that irradiation with UVA light for more than 3 d was preferred to produce vinblastine in leaves of the plants grown under R light. Previous study showed that the biomass production of the multiple shoot culture grown under R and UVA light for 4 weeks was smaller than that of the multiple shoot culture grown under R light for 4 weeks (Hirata et al., 1992). Therefore, the irradiation time of UVA light would be superior from 3 to 7 d before harvesting than continuous UVA lighting throughout the cultivation period.

Through in vitro experiment, 3', 4'-anhydrovinblastine was generated non-enzymatically by not only UVA light but also B light irradiation to the test tube containing flavin mononucleotide (FMN), Mn²⁺, vindoline, and catharanthine (Hirata et al., 1993). This non-enzymatic reaction was caused by FMN-mediated catharanthine oxidation via the excitation of FMN by B or UVA light irradiation. 3', 4'-anhydrovinblastine concentration in this test tube under UVA light was higher than that under B light. These results might be caused by the excitation of FMN which was more easily achieved under UVA light than B light. We anticipated that the same reaction of in vitro experimentation would be caused in the leaves. UVA light irradiation compared with B light irradiation was more efficient in vinblastine production in the leaves of C. roseus.

The maximal vinblastine production in this study was observed under 10 W m⁻² UVA light (Fig. 2). We anticipate that when more than 10 W m⁻² UVA light is irradiated to leaves, more vinblastine content will be produced. We need to research the vinblastine content in leaves of plants grown under more than 10 W m⁻² UVA light. Previous study indicated the vinblastine precursors in multiple shoot culture were decreased for using vinblastine synthesis after UVA irradiation (Hirata et al., 1992). In in vitro experimentation, catharanthine was decreased with increasing UVA light intensity (Hirata et al., 1999) and it may be degraded by FMN-mediated light oxidation. Since lower leaves in a plant receive little vertical light because of upper leaves that shield lower leaves from light, lower leaves of the plant grown under UVA light hardly synthesized vinblastine.

The ability of vinblastine synthesis using UVA light irradiation was greater in the leaf discs made from upper leaves than in those from lower leaves (Fig. 3), which should be related to leaf senescence. Vindoline and catharanthine were accumulated in different leaf tissues which were idioblast/laticifer and epidermal cells, respectively (Mersey and Cutler, 1985; Roepke et al., 2010).
These compounds have to be transported to same cell at the time of vinblastine synthesis. The concentration and/or activity of these transporters would be decreased as leaf senescence was advanced. On the other hand, in vitro experimentation, the conversion rate from 3', 4'-anhydrovinblastine to vinblastine was decreased by decreasing NAD(P)H concentration (Hirata et al., 1997). Therefore, NAD(P)H are used for reducing the compounds including 3', 4'-anhydrovinblastine. This conversion rate would be lower in an old leaf than in a young leaf.

The young and upper leaves of the plants grown under R light were the best materials for producing vinblastine using UVA light irradiation. For efficient production of vinblastine, we proposed the plant cultivation with high planting density under R light until the plants are at a young stage, such as when the first leaves have just expanded. Then these young plants were irradiated with UVA light supplemented with R light for more than 3 d. Since the multistage cultivation beds are installed to improve production efficiency per land area, a plant factory with artificial lighting is suitable to produce small height plants, such as young plants. The plant height when the first leaves had just expanded was approximately 4 cm. There is a possibility to achieve the domestic production of vinblastine by more than 10 W m⁻² UVA light irradiation to the young plant cultivation with high planting density under R light using a plant factory with artificial lighting and the multistage cultivation beds.

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REFERENCES


