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Effects of low light on terpenoid indole alkaloid accumulation and related biosynthetic pathway gene expression in leaves of *Catharanthus roseus* seedlings

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ABSTRACT. *Catharanthus roseus* (L.) G. Don, an important medicinal plant, produces the powerful alkaloids vinblastine (VBL) and vincristine (VCR), which are known for their remarkable inhibitory activity against tumor cells. This paper is the result of a study on the effects of low light/UV light on the contents of chlorophyll and alkaloids, and on the expression of terpenoid indole alkaloids (TIAs) biosynthesis pathway genes in the leaves of aseptic *C. roseus* seedlings. A plastic film treatment first induced a slight increase and then a significant decrease ($p<0.05$) in the total chlorophyll content, and a lower chlorophyll *a/b* ratio. The amounts of vindoline (VIN) and catharanthine (CAT) followed the identical pattern under plastic film but the content of VBL, generated by its precursors VIN and CAT, increased gradually until reaching a maximum (0.028 mg g^{-1}±0.0051) on the 15th day of treatment. The plastic film also induced the expression changes of TIA biosynthesis pathway genes. The further correlation analysis between alkaloid content and these genes transcriptional levels indicated that VIN accumulation was significantly correlated with the gene expression of desacetoxyvindoline-4-hydroxylase (*D4h*) and deacetylvindoline-4-*O*-acetyl transferase (*Dat*), and CAT accumulation was significantly correlated with strictosidine synthase (*Str*) gene expression ($p<0.05$). Therefore, the low light treatment with plastic film accelerated the accumulation of VBL in the leaves of *C. roseus* seedlings, which may have practical significance in the production of VBL.

Keywords: *Catharanthus roseus*; Gene expression; Low light; Plastic film; TIAs.

INTRODUCTION

Catharanthus roseus (L.) G. Don (Madagascar periwinkle), an important medicinal plant that produces more than 120 kinds of terpenoid indole alkaloids (TIAs) during secondary metabolites. The leaves of this species contain the alkaloids vinblastine (VBL) and vincristine (VCR), used in anticancer treatments. The VBL biosynthetic pathway is proposed by Kutney (1990), as a coupling product of vindoline (VIN) and catharanthine (CAT). *C. roseus* produces only very small amounts of VBL, however, and despite concentrated efforts to increase this amount, only minimal improvements have been made.

Terpenoid indole alkaloids (TIAs) metabolites biosynthesis must accommodate the primary metabolic pathways in plants; the primary metabolites are regulated by strict expression of biosynthetic pathways genes. The two early and two late TIAs biosynthetic pathway genes, tryptophan decarboxylase (*Tdc*), strictosidine synthase (*Str*), desacetoxyvindoline-4-hydroxylase (*D4h*) and deacetylvindoline 4-*O*-acetyl transferase (*Dat*) have been isolated, partially characterized, and over-expressed to improve the biosynthesis (El-Sayed and Verpoort, 2007), but the expression of these genes (*Tdc*, *Str*, *D4h* and *Dat*) in *C. roseus* seedlings under low light are little reported.

Regulation of TIAs metabolites biosynthesis can be influenced by developmental hormones and light. Tang et al. (2007) have reported the presence of low light-affected alkaloids in the leaves of *C. roseus* seedlings. Although the use of low UV light has increased the production of VIN and CAL in *C. roseus* tissue and cell cultures, this technique has not been effective in producing the important anticancer compound VBL (Ramani and Jayabaskara,

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2008). In our study, the quantities of VIN, CAT and VBL were measured in the leaves of aseptic *C. roseus* seedlings under plastic film as compared to fluorescent light, and the expression levels of 4 genes (*Tdc*, *Str*, *Dat* and *D4h*) related to TIAs biosynthetic pathway were examined by semi-quantitative RT-PCR (SQ-RT-PCR) under low light/UV light. Furthermore, we analyzed the pattern of correlation between gene expression and alkaloid accumulation and discussed the potential of four gene expressions in screening TIAs accumulation.

MATERIALS AND METHODS

Plant material and treatment

Catharanthus roseus seeds were sterilized with 75% alcohol for 20 sec, sterilized with 6% NaClO for 20-30 sec, washed 3-5 times with aseptic water and soaked in 0.1% plant agar cultivation solution. Seeds were then cultured on Murashige Skoog (MS) medium with pH 5.8 at 25°C-28°C under 2000 lx irradiance (provided by fluorescent lamp, 40 W, Shanghai, China) with 16/8 h day/night photoperiod. The cultures were subcultivated weekly. Aseptic seedling on MS medium were maintained in 200 mL flake 1/2 MS medium (Chen and Li, 2009), supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar without auxins, each flake containing 1 seedling. Seedlings at the 40-day-old stage were shaded by plastic film and kept in the shade for 30 days. The plastic film was replaced with new film twice weekly to avoid any degradation. Radiant energy at different regions of the spectrum was measured by a portable spectroradiometer (AvaSpec-2048-2-USB2, Amsterdam, Holland). The wavelengths under fluorescent light and plastic film were scanned at intervals of 1 nm, respectively. Leaves were collected and their alkaloid and chlorophyll contents measured at the same time of day on 0 d, 3 d, 7 d, 15 d and 30 d. For the SQ-RT-PCR experiment, the leaf samples were collected during the shading treatment at 0 h, 3 h, 6 h, 12 h, 24 h and 72 h, The gene expressions were determined between the control and treatment.

Alkaloid extraction and HPLC analysis

The analysis of alkaloids, including leaf content of VIN, CAT and VBL, was performed according to Luo et al. (2005). Fresh leaf powder (0.3 g) was dissolved in 10 mL absolute methanol (analytical grade). Low frequency ultrasonic technology (250 W, 40 kHz) was used to extract the alkaloids for 20 min. The methanol extract was centrifuged at 21,000 *g* for 10 min and concentrated to 1 mL for high performance liquid chromatography (HPLC, Jasco, VG, Tokyo, Japan). The HPLC system was equipped with a Waters ODS C18 reversed-phase column (250×4.6 mm, 5 μm) and a photodiode-array detector set up at 220 nm. The sample injection volume was 10 μL at a flow-rate of 1.5 mL min^{-1}.

Chlorophyll content analysis

The contents of chlorophyll *a* and *b* in the samples under plastic film and fluorescent light treatment were determined according to Arnon (1949). Total chlorophyll, chlorophyll *a* and chlorophyll *b* contents were calculated from the absorbance at 665 and 649 nm.

RNA extraction and semi-quantitative RT-PCR (SQ-RT-PCR)

Total RNA was extracted from the leaves under plastic film using Trizol reagent (Shanghai Sangon, Shanghai China). *Tdc*, *Str*, *D4h* and *Dat* SQ-RT-PCR analysis were performed according to Marone et al. (2001) and (Oliveira and Lucas, 2004). cDNA was prepared from total RNA (2 μg) using RevertAid H Minus First Strand cDNA Synthesis Kit (MBI Fermentas, Burlington, Canada), following manufacturer's instructions. Multiplex PCR reactions were performed with 2 μl of cDNA as a template, 50 mM $MgCl_2$, 10 mM dNTP mix, 10 μmol of each primer, 4 μl of *Actin* primer, 2 units of Platinum Taq DNA polymerase (which enables a ''hot-start'' method to improve specificity of annealing), 20 mM Tris-HCl (pH 8.4) and 50 mM KCl in a total volume of 50 μl. The primers used in this work were as follow:

-*Tdc*: 5′-ACACCACTAGCGAGTCCATT-3′ and
5′- CCAACAGCCAGTCTTAGCAT-3′ (GenBank Acc. No. X67662)

-*Str*: 5′- CCTTCCTATGCTCCGAATGC-3′ and
5′- CCATCGTGCTCTTGAATCTG-3′ (GenBank Acc. No. X61932)

-*D4h*: 5′- ACTATCAGAAGCTTTGGGGC-3′ and
5′- GGGGTACCTCTAATGTTCACCG-3′ (GenBank Acc. No. U71604)

-*Dat*: 5′- GAAGTTCGAATTCGTTGCCG-3′ and
5′- GCTCTAGAAGTAGCTGTTCGTTC-3′ (GenBank Acc. No. AF053307)

-*Actin*: 5′- GGCTGGATTTGCTGGAGATGAT-3′
5′- TAGATCCTCCGATCCAGACACTG-3′ (GenBank Acc. No. AK322149)

Actin gene was used as an internal control. Amplification was conducted with 28-35 cycles of PCR reaction of 30 sec denaturation at 94°C, 30 sec annealing at 45-50°C, and 1 min extension at 72°C. PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide, and were viewed under UV illumination. Pictures of the resultant PCR bands were digitized and quantified by densitometric analysis using Quantity One software (Bio-Rad, Hercules, CA, USA). Results presented were mean values of at least three independent experiments.

Data analysis

All data were subjected to one-way analysis of variance (ANOVA) and correlation analysis in the SPSS statistical package (IBM, Chicago, USA). The experiments were repeated three times and the mean and standard deviations were calculated.

RESULTS

Spectra and radiation of the plastic film

The plastic film induced a low light/UV light environment compared to the fluorescent light (300 nm-1,100 nm). As shown in Table 1, UV radiation under plastic film only reached 6% of that under fluorescent light, and the total radiation under fluorescent light was higher (8.31 W m^{-2}) than that under plastic film (0.769 W m^{-2}).

Alkaloid content analysis under low light

The amount of VIN and CAT present in leaves initially showed a slight increase, reaching its peak (0.32 ±0.07 mg g^{-1} FW and 0.63 ±0.18 mg g^{-1} FW) on the 3rd day of the treatment. This was followed by a significant decrease ($p<0.05$) over 7 days of treatment, (Figure 1a and b). The change of dimeric alkaloid VBL content in treatment showed significant difference from that under fluorescent light ($p<0.05$), the VBL content became higher than that of control, with its peak value around 0.028 ±0.0051 mg g^{-1} FW on the 15th day (Figure 1c).

Chlorophyll content analysis under low light

The total chlorophyll content in control leaves gradually increased on the first 7 days and then decreased, whereas plastic film treatment resulted in a little increase of the chlorophyll content on the first 3rd day, and significant decrease ($p<0.05$) after 3 day of treatment, the lowest value (1.71±0. 35 mg g^{-1} FW) was recorded on the 7th day (Figure 2a). The ratio of chlorophyll *a* and chlorophyll *b* in treatment leaves was consistently lower than that under fluorescent light (Figure 2b).

Gene expression analysis under low light

The mRNA levels of *Tdc*, *Str*, *D4h* and *Dat* were measured in leaves under fluorescent light and plastic film, As shown in Table 2, significant differences in gene expression were observed between the treatment and the control. There were significant increases in *Tdc* mRNA levels between 3 h and 6 h during shading treatment. *Str* expression was significantly up-regulated at 72 h during shading culture. Furthermore, the transcription levels of *D4h* and *Dat* were consistently higher in the treatment than in the control, and mRNA levels of *D4h* and *Dat* were signifi-

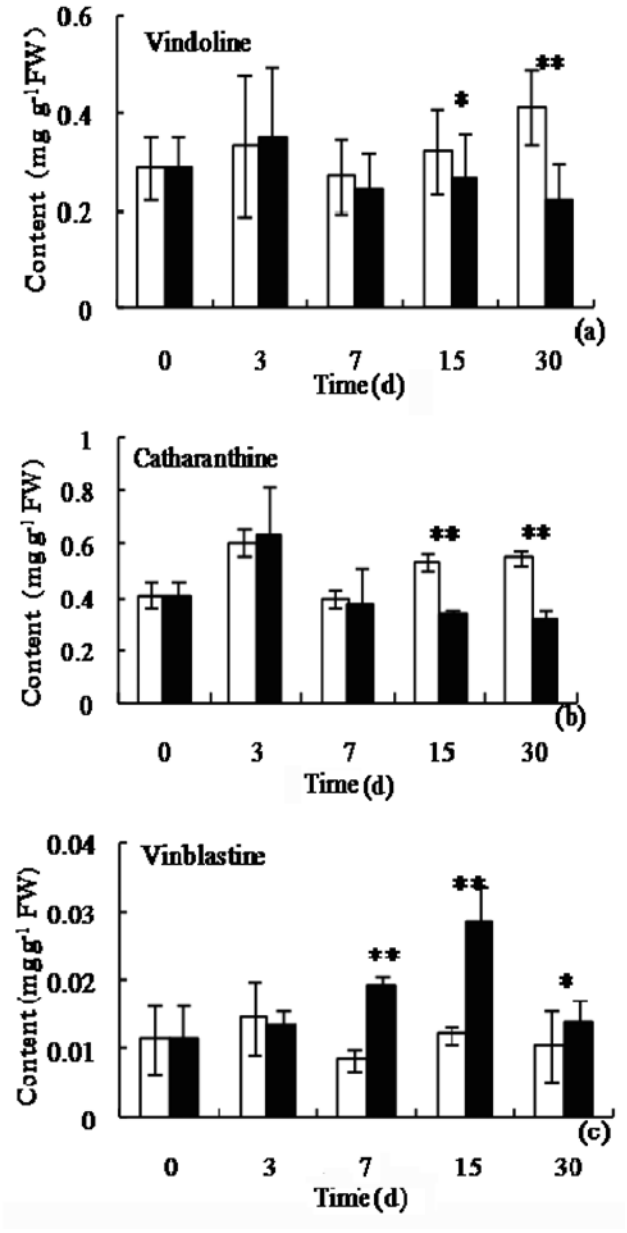

Figure 1. Effect of plastic film on the amount of vindoline (VIN), catharanthine (CAT) and vinblastine (VBL) in the leaves of aseptic *C. roseus* seedlings. Data shown are the mean and standard deviation (*$p<0.05$, **$p<0.01$; □, control; ■, red film).

Table 1. Spectra and radiant energy of control and the plastic film (W m^{-2}).

Light Spectra	Ultraviolet 300-400 nm	Blue 400-510 nm	Green 510-610 nm	Red 610-720 nm	Near infrared 720-1,100 nm	Total radiation 300-1,100 nm
The plastic film	0.0173[b] (6.0)	0.0483[b] (1.66)	0.101[b] (2.97)	0.432[b] (39.6)	0.203[b] (34.1)	0.769[b] (9.3)
Control	0.288[a]	2.914[a]	3.405[a]	1.106[a]	0.596[a]	8.31[a]

Values in parenthesis are the percents of radiant energy of different light wave bands in overall radiant energy. Means within a column followed by the same letters are not significantly different by SPSS analysis $p<0.05$. W m^{-2} is light energy irradiation per meter square.

Table 2. Expression of *Tdc*, *Str*, *D4h* and *Dat* genes in leaves cultivated and collected between treatment and control at different hours (SQ-RT-PCR arbitrary units ± standard deviation). Means were compared to the 0 h value, which was set as 1.

Genes	Control (fluorescent light)						Treatment (plastic film)				
	0 h	3 h	6 h	12 h	24 h	72 h	3 h	6 h	12 h	24 h	72 h
Tdc	1.00±0.23	3.05±0.25	3.81±0.12	6.32±0.34	15.90±0.43	3.28±0.18	13.74±0.89	14.20±0.68	4.56±0.56	13.32±1.23	5.90±0.45
Str	1.00±0.20	3.55±0.28	3.67±0.32	2.27±0.25	1.30±0.11	0.42±0.4	3.99±0.22	1.54±0.12	0.39±0.07	1.54±0.21	4.69±0.53
D4h	1.00±0.36	8.98±0.68	5.07±0.45	0.78±0.11	2.40±0.19	1.99±0.29	9.54±0.87	5.24±0.25	3.91±0.19	2.79±0.28	2.51±0.23
Dat	1.00±0.19	0.77±0.14	0.83±0.33	0.82±0.9	0.70±0.11	0.39±0.15	7.00±0.69	3.46±0.42	2.82±0.30	3.20±0.38	8.73±0.33

cantly up-regulated at 12 h and 72 h during shading culture ($p<0.01$). Thus, it is clear that low light/UV light activated the high expression of TIA pathway genes (*Tdc*, *Str*, *D4h* and *Dat*) at different hours.

Correlation analysis

Correlation analyses of alkaloid accumulation and gene expression (Table 3) show that there were significant ($p<0.05$) correlations between *Str* gene expression and CAT content, and between *D4h* and *Dat* gene expression and VIN content under plastic film treatment. No significant correlation was observed, however, between gene expression and the related alkaloid accumulation.

DISCUSSION

Light spectrum, intensity and quality play important roles in plants' development and metabolism processes. Dai et al. (2004) reported that plastic film could alter not only light intensity but also light spectrum and quality, and that low light induced by plastic film could increase camptothecin content in the leaves of *Camptotheca acuminata* seedlings. Qin et al. (2005) found that plastic film was conducive to strawberry growth and development in explant culture. In addition, low UV lighting was reported to increase the levels of dimeric alkaloids while concurrently decreasing those of VIN and CAT (Hirata et al., 1993). In the study, lower UV radiation may accelerate plant growth and improve different alkaloids accumulation, because film's emission of different spectra lights (300 nm-1,100 nm) may overshadow the hazardous effects of UV radiation (Qin et al., 2005). On the other hand, plastic films as shade material could improve the temperature and humidity (Zhang and Xu, 2008), which may activate the VBL synthesis coupling VIN with CAT and increase VBL content.

Light controls the development of plant chloroplasts and the biosynthesis of many important precursors for TIAs in developed chloroplasts (Zhao et al., 2001). The decrease of chlorophyll *a/b* ratio in the treatment is similar to what has been described for strawberry plants under plastic film (Qin et al., 2005). This may be due to an increase of chlorophyll degradation or to a decrease of chlorophyll synthesis, which affected the development of plant chloroplasts and the biosynthesis of important precursors,

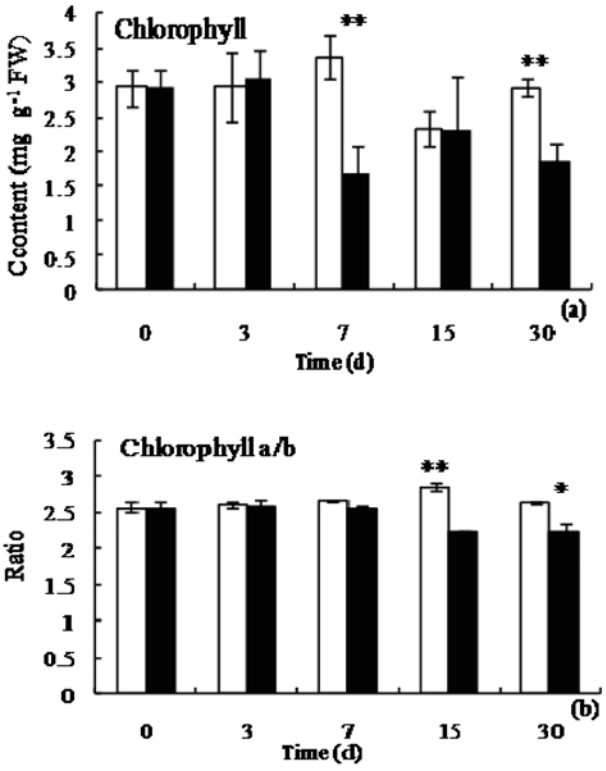

Figure 2. Effect of plastic film on the total amount of chlorophyll and the chlorophyll *a/b* ratio in the leaves of aseptic *C. roseus* seedlings. Data shown are the mean and standard deviation (*$p<0.05$, **$p<0.01$; □, control; ■, red film).

Table 3. Correlation coefficient (r) between gene expression and alkaloid accumulation between control and treatment

Genes	Control (fluorescent light)		Treatment (plastic film)	
	VIN	CAT	VIN	CAT
Tdc		0.264		0.264
Str		0.461		0.934*
D4h	-0.157		0.933*	
Dat	-0.084		0.903*	

*Is significant at $p<0.05$ in ANOVA analysis.

thus limiting the amount of substrate necessary for VBL biosynthesis. This may explain the initial increase in VBL content and its later decrease under shaded conditons (Figure 1c).

De Luca and Laflamme (2001) reported that alkaloid biosynthesis is regulated primarily at the level of gene expression. *Tdc* expression pattern is development-specific (Fernandez et al., 1989). In the experiment, *Tdc* expression was earlier up-regulated under the film. The effect of UV irradiation on expression of *Tdc* and *Str*, and CAT accumulation has been reported previously in *C. roseus* leaves (Ouwerkerk et al., 1999). Low UV irradiation has activated the transcription factors of the signaling pathway and improved *Tdc* and *Str* genes expression (Ramani and Chelliah, 2007). In the experiment, *Str* gene expression were up-regulated, and there were correlation of *Str* gene expression with CAT accumulation. On the other hand, light induction of *D4h* and *Dat* genes expression is mediated by phytochrome, and phytochrome is involved in the regulation of VIN biosynthesis (Aerts and De Luca, 1992). In the study, low light may change phytochrome, thus, *D4h* and *Dat* genes expression were activated and up-regulated, a positive correlation between *D4h* and *Dat* gene transcript abundance and VIN accumulation has been observed (Table 3). Therefore, we were able to provide a rapid method for estimating periwinkle alkaloids by analyzing their expression of TIAs biosynthetic pathway genes. The key to successful metabolite production in cultivated *C. roseus* is the establishment of an efficient culture system. Further studies need to be carried out that explore the mechanism of the culture system under the film.

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低光輻射對長春花葉片中吲哚生物鹼含量積累及相關基因表達的影響

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長春花 (*Catharanthus roseus*) 作為重要的藥用植物，以其生產的次生代謝物質長春鹼 (Vinblastine, VBL) 和長春新鹼 (Vincristine, VCR) 具有重要的抗癌作用而聞名。在塑料膜誘導的一個低光 / 低紫外光環境下，研究長春花幼苗葉片中葉綠素含量，生物鹼含量以及萜類吲哚生物鹼 (Terpenoid indole alkaloids, TIAs) 合成路徑相關基因表達的變化，結果表明：葉綠素 *a/b* 比率一直低於日光燈下葉綠素的 *a/b* 比率。與對照相比，葉綠素含量先升高然後明顯下降 ($p<0.05$)。同時，處理的葉片中文多靈 (Vindoline, VIN) 和長春質鹼 (Catharanthine, CAT) 的含量稍有升高後低於對照，但是，耦合 VIN 和 CAT 的產物 VBL 的含量逐漸升高，在處理第 15 天達到最高 (0.028 mg g^{-1} FW±0.0051)。此外，塑料遮光膜誘導了 VBL 的合成路徑相關基因的表達變化，相關性分析表明：VIN 的積累與脫乙酰文多靈 -4- 羥化酶（*D4h*）和脫乙酰文多靈 -4-*O*- 乙酰轉移酶 (*DAT*) 的基因表達均呈顯著相關性 ($p<0.05$)，而 CAT 的積累與異胡豆苷合成酶 (*Str*) 的基因表達呈顯著相關。因此，本研究採用濾光膜遮光處理促進 VBL 在幼苗葉片中的積累，對 VBL 的生產具有重要實踐意義。

關鍵詞：萜類吲哚生物鹼；長春花；基因表達；低光；塑膠膜。