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Growth light substantially affects both primary and secondary metabolic processes in *Catharanthus roseus* plants

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Abstract

Common periwinkle (*Catharanthus roseus* L.) is an important medicinal plant used by the pharmaceutical industry. The present work aimed to determine the effect of low light intensity on the primary and secondary metabolic processes, using various photosynthesis and targeted and untargeted analytical techniques. Growth light had only limited effects on the photosynthetic electron transport processes, although membrane stability seemed slightly higher in plants growing under higher light conditions. The reduced growth light caused a reduction in certain primary metabolites, including amino acids and sugars, and it also reduced the contents of most of the phenolic compounds investigated in the present experiments. Interestingly, the differences in the growth light caused a much less pronounced difference in the alkaloid contents than that found in the flavonoid contents. However, besides the growth light, genotypic differences, most evident in flower colour, also affected some metabolic processes, including primary and secondary processes.

Keywords: acclimation processes; flower colour; growth light; metabolomics; periwinkle; vinca alkaloids.

Introduction

Common periwinkle [*Catharanthus roseus* (L.) G. Don] is not only a popular perennial ornamental garden plant but also an important raw material for the pharmaceutical industry. Its shoots and sometimes its roots are also used. Up to now, more than 130 alkaloids have been detected in this plant species (van der Heijden *et al.* 2004). Among

Highlights

- Growth light may modify membrane stability in *Catharanthus roseus*
- Reduced growth light affects both primary and secondary metabolites
- Genotypic differences also affected certain metabolic processes

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Abbreviations: C_i – intercellular CO_2 concentration; E – transpiration rate; FM – fresh mass; F_v/F_m – maximum photochemical efficiency of PSII; g_s – stomatal conductance; NPQ – nonphotochemical quenching; P_N – net photosynthetic rate; SD – standard deviation; WUE – water-use efficiency; $Y_{(II)}$ – effective PSII quantum yield; $Y_{(NO)}$ – quantum yield of nonregulated energy dissipation; $Y_{(NPQ)}$ – quantum yield of regulated energy dissipation; Φ_{PSII} – actual photochemical efficiency of PSII.

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others, sedative and antihypertensive compounds can be extracted from it and used for medicines that reduce the number of white blood cells and prevent tumours. However, although the therapeutic use of the main anticancer metabolites, vinblastine and vincristine, is well established as semisynthetic dimers in various lymphomas and leukemia, serious side effects are also encountered, and therefore the focus has been shifting towards the metabolites they are synthesized from, for example, vindoline, to serve as derivatization core molecules.

Changes in environmental factors significantly influence not only plant growth and development but also the production of secondary metabolites. Light not only has a direct effect on photosynthetic processes and, through this, on the rate of biomass growth, but it can also indirectly or directly influence many acclimation processes. More reliable and reproducible cultivation methods can be developed with accurately controlled light intensity.

The influences of growth light on physiological and biochemical processes in plants have been investigated in several plant species from various aspects. Logically, since light is the driving force of photosynthesis, under optimum growth conditions, growing at greater quantities of light may lead to higher photosynthetic activity, leading to higher biomass accumulation, and normally higher yield production. Through photosynthesis, the light also affects the accumulation of primary metabolites. Elevated light may increase the level of carbohydrate accumulation, which is partly due to the increased activity of enzymes involved in carbon metabolism (Tang *et al.* 2022). However, high light intensities, especially under stress conditions, may also cause photoinhibition, leading to not only a reduction of the photosynthetic efficiency but also influencing various physiological processes, related mainly to stress acclimation. To reduce the harmful effects of high-light conditions, plants have evolved various mechanisms. Many of them are more or less similar in most photosynthetic organisms, including dissipating light energy and reducing energy uptake by reducing the photosynthetic antenna cross-section (Croce 2020, Vecchi *et al.* 2020, Levin and Schuster 2023), *via* nonphotochemical quenching (NPQ) pathways (Bassi and Dall'Osto 2021, Ruban and Wilson 2021), or by rapidly repairing the injured photosynthetic elements (Krieger-Liszkay 2005, Li *et al.* 2018, Sharma *et al.* 2023). Furthermore, there are also species-specific mechanisms, involving, among others, the accumulation of carotenoid biosynthesis-related proteins in the thylakoid membranes (Levin *et al.* 2023). Besides regulating the photosynthetic processes, light also influences certain acclimation mechanisms, especially under unfavourable environmental conditions (Gondor *et al.* 2021, Janda *et al.* 2021a, Pál *et al.* 2022, Rahman *et al.* 2023, Utasi *et al.* 2023).

The accumulation of secondary metabolites is also substantially affected by light intensity. However, most of the studies focus on metabolites with antioxidant or other protective properties, including carotenoids, anthocyanins, other flavonoids, *etc.* (Thoma *et al.* 2020). Despite its practical importance, the effect of light on alkaloid synthesis in *C. roseus* has been much less studied. Earlier works suggested that while the biosynthesis of vindoline is strongly activated by light in developing seedlings, catharanthine may accumulate without light stimulation (De Luca *et al.* 1986, Vázquez-Flota and De Luca 1998, Vázquez-Flota *et al.* 2000). Other results suggested that reduced light may also enhance the accumulation of vinblastine, in contrast to catharanthine and vindoline in *Catharanthus* seedlings (Liu *et al.* 2011). Furthermore, besides visible light, UV exposure, which may modify among others the carotenoid composition (Badmus *et al.* 2022), may also influence the alkaloid synthesis in *Catharanthus* plants (Asano *et al.* 2010, Rady *et al.* 2021).

Catharanthus plants generally show high adaptability to indoor conditions (Okazawa and Nishijima 2017). However, besides the extensive research on the light regulation of alkaloid synthesis in *Catharanthus* plants, little is known about how this plant responds to different light conditions. Therefore, in the present work, the following questions were investigated: (*1*) how does growth light affect the basic photosynthetic processes, the primary metabolic reactions, some of the regulatory factors involved in acclimation processes, and the synthesis of medically important vinca alkaloids; (*2*) whether these functions are related to the colour of the flowers of the given plant.

Materials and methods

Plant material and growth conditions: Pre-grown *Catharanthus roseus* (L.) G. Don plants (Titan hybrids 'Red', 'Rose', and 'Polka Dot' types, characterized with red, pink, and white flowers, respectively) were used in the experiments. Since they phenotypically only differed in flower colour, we refer to them by this, not by the name of the type. They were purchased from the local market at flowering 10-leaf stage, then they were transferred in pots to a *Conviron PGR-15* climate chamber (*Conviron Ltd*., Winnipeg, Canada) under controlled growth conditions, growing at 26/20°C of day/night temperatures, with a 16/8-h light/dark period either at 270 (normal light; NL) or at PPFD of 80 μmol m^{-2} s^{-1} (LL, shaded with a white mosquito net) and at 70% relative humidity. Plants were watered regularly with tap water. After 23 d, physiological measurements were carried out, and the last two fully developed leaves were collected in the middle of the 16-h light period for further biochemical and gene expression analyses.

Chlorophyll *a* fluorescence induction parameters were measured with a pulse amplitude-modulated fluorometer (*Imaging-PAM M-series*, *Walz*, Effeltrich, Germany). Detached leaves were dark adapted for 20 min, after which the F_v/F_m parameter was determined using a 1.0-s saturated pulse (PPFD = 3,000 μmol m^{-2} s^{-1}) provided by a LED-Array Illumination Unit *IMAG-MAX/L* (λ = 450 nm). Photosynthesis was then activated using 100 μmol(photon) m^{-2} s^{-1} of actinic light intensity, and the quenching analyses were performed using a 1.0-s saturation pulse at a 40 s^{-1} frequency. The measurements

were started at 25°C and after 10 min, the temperature started to increase to 55°C at a rate of 1.5°C min^{-1}. The quenching parameters, such as effective quantum yield of PSII, $Y_{(II)}$, regulated $Y_{(NPQ)}$, and nonregulated $Y_{(NO)}$ nonphotochemical quenching parameters were determined according to the nomenclature described by Klughammer and Schreiber (2008).

Gas-exchange measurements: Gas-exchange parameters, such as the net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), intercellular CO_2 concentration (C_i), and water-use efficiency (WUE = P_N/E), were determined with the use of a *Ciras-3* portable photosynthesis system instrument (*PP Systems*, Haverhill, MA, USA). The measurements were performed on the youngest fully developed leaves of four randomly selected plants per each colour of flowers. These parameters were recorded at the steady-state level of photosynthesis (after 15 min of light adaptation) at 390 µmol(CO_2) mol^{-1}, 26°C of leaf temperature, and 70% relative humidity with the use of PPFD of 270 (NL) or 80 (LL) µmol m^{-2} s^{-1}, respectively.

RNA extraction and quantitative RT-PCR (qRT-PCR) analyses: To evaluate gene expression, total RNA was extracted from leaf samples using *TRI* reagent. RNA quality and quantity were determined by gel electrophoresis and nanodrop (*NanoDrop 2000c*, *Thermo Scientific*, USA). The samples were treated with *DNase I* and cleaned with a *Direct-zol ™ RNA MiniPrep Kit* (*Zymo Research*, Irvine, CA, USA) according to the manufacturer's instructions. cDNA synthesis was carried out using *M-MLV Reverse Transcriptase* (*Promega*, Madison, WI, USA). cDNAs were used as a template using the following primer sequences: F: 5'-GGTGTCCTCAAGCCTGGTAT-3' and R: 5'-CATTGTCACCAGGAAGAGCC-3' as a reference gene (*CrEF1a*, EU007436); F: 5'-TGACAGTCC-CGAAGGTGTGG-3' and R: 5'-CGCCGGGAACAT-GTAGCTCT-3' for strictosidine synthase gene (*CrSTR*, X61932); F: 5'-TCCGAAAACAAGCCCATCGT-3' and R: 5'-AAGGAGCGGTTTCGGGGATA-3' for tryptophan decarboxylase gene (*CrTDC*, X67662), *PCRBIO SyGreen Mix* (*PCR Biosystems*, London, UK) and *BioRad CFX96 Touch™ Real-Time PCR Detection System* (*Bio-Rad*, Hercules, CA, USA) were used for quantitative real-time PCR reaction. Primer sequences were used according to Sander (2009). Three biological and three technical replicates for each sample were used for qRT-PCR analysis. For normalization, the housekeeping gene *CrEF1α*, encoding elongation factor 1-alpha, was used. The relative gene expression values were determined with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Metabolic screening using two-dimensional gas chromatography time-of-flight mass spectrometry: The sample preparation of primary metabolites was carried out according to Gondor *et al.* (2021) with some modifications. Leaves of 200 mg were extracted with 0.5 ml of 60% (v/v) methanol after the addition of 30 µl of 1 mg ml^{-1} ribitol solution as an internal standard using a *Spex SamplePrep 1600 MiniG* instrument (Metuchen, NJ, USA) for 5 min at 1,500 rpm, followed by a 5-min ultrasonic treatment at room temperature. After centrifugation (10 min at 4°C at 16,500 × *g*), the supernatant was collected, and the pellet was re-extracted again with 60% (v/v) methanol and centrifuged as written above. This extraction was repeated with 90% (v/v) methanol and after that, the supernatants were combined, and an aliquot was evaporated in a vacuum centrifuge to dryness. For derivatization, 40 µl of methoxyamine hydrochloride dissolved in pyridine (20 mg ml^{-1}) was added and incubated for 90 min at 37°C, followed by the addition of N-trimethylsilyl-N-methyl-trifluoroacetamide and kept for 30 min at the same temperature. A *Pegasus 4D GCxGC TOFMS* (*LECO*, St. Joseph, MI, USA) equipped with a 30-m primary (Rxi-5MS phase) and a 1.5-m secondary (Rxi-17Sil MS phase) column was used for the analysis. The sample of 1.0 µl was injected into the primary column in split mode, the temperature of the transfer line and ion source was 250°C, and He (*Gruppo SIAD*, Bergamo, Italy) was used as a carrier gas at a constant flow rate (1 ml min^{-1}). The thermal program started at 70°C for 3 min, then increased to 320°C at a 7°C min^{-1} rate and maintained this temperature for 5 min with a 3.25-s modulation period in the 2D GC mode. For the identification of the compounds, external standards and Kovats retention index were used. Data evaluation was carried out using the *LECO ChromaTOF* program (*LECO*, St. Joseph, MI, USA) and *Fiehn* and *NIST* databases.

Metabolic screening by targeted ultra-performance liquid chromatography–tandem mass spectrometry: Portions of 0.2 g of liquid N_2-homogenized frozen fresh mass (FM) plant material were transferred into 2-ml safety Eppendorf tubes and stored at –80°C until extraction. For extraction, HPLC-grade chemicals and for elution UPLC-MS-grade acetonitrile were used (*VWR*, Radnor, PA, USA). Nonlabelled reference materials were purchased from the *Merck-Sigma* group (Darmstadt, Germany). Before extraction, samples were spiked with 20 ng of labelled [$^2H_6$](+)-*cis*,*trans*-abscisic acid (*OlChemIm s.r.o.*, Olomouc, Czech Republic) as an internal standard. Samples were extracted with 1 ml of methanol:water (2:1), followed by 5 s of vigorous vortexing; then, samples were shaken with a *Spex Mini G 1600* in a cryo-cooled rack at 1,500 rpm for 3 min. After centrifugation at 16,500 × *g* and 4°C for 10 min, supernatants were collected, and the remaining pellets were re-extracted by repeating the extraction procedure once more. The respective supernatants were pooled to a final sample ratio of 0.1 g(FM) ml^{-1}, and the methanol–water sample solution was liquid–liquid partitioned by adding 1 ml of n-hexane to remove carotenoids and lipids. Afterward, centrifugation at 10,000 × *g* (at 4°C for 10 min) was used to collect the lower methanol:water phase that was finally filtered through a 0.22-µm PTFE syringe filter, then transferred to injection vials and submitted directly to analysis. UPLC-MS/MS analysis and elution were carried out according to Vrhovsek *et al.* (2012) and Pál *et al.* (2019) with slight modifications. Briefly, separation

was achieved on a *Waters HSS T3* column (1.8 µm, 100 mm × 2.1 mm) using an *Acquity I class UPLC* system (*Waters*, Milford, MA, USA). Gradient elution was used with 0.1% (v/v) formic acid, both in water (A) and acetonitrile (B). Tandem mass spectrometry detection was performed on a *Xevo TQ-XS* (*Waters*) equipped with a *UniSpray*™ source operated in the multiple reaction monitoring (MRM) mode. The respective MRM transitions used for the quantitation of components, details of UPLC, and mass-spectrometric conditions are listed in Table 1S (*supplement*).

High-resolution mass spectrometry-based targeted metabolomics for the screening of vinca alkaloids was carried out with a *Waters Acquity I class UPLC* system equipped with a PDA detector, which was coupled to a *Vion ESI-IMS-QTOF-MS* instrument (*Waters*). Separation was performed on a *BEH-C18* reversed phase (RP) UPLC column (100 mm × 2.1 mm × 1.7 µm; *Waters*) at 40°C. For gradient elution, water and acetonitrile containing 0.1% (v/v) formic acid were used. The injected sample volume was 1.0 µL. The applied gradient and the UPLC-ESI-MS-QTOF-MS parameters are described in Tables 2S and 3S (*supplement*). Identification of alkaloids was either based on authentic standards (vincristine, vinblastine) or reference MS/MS spectra from the *MoNA* database (for 3',4'-anhydrovinblastine, catharanthine, deacetylvindoline, loganic acid, strictosidine, vindoline, vindolinine), the study of Akhgari *et al.* (2015) (vincadifformine), Pan *et al.* (2019) (secologanin), and Eng *et al.* (2022) (19-*S*-vindolinine). For the quantification of dimeric vinca alkaloids, the standard addition method was used to set up the calibration, with authentic standards of vinblastine and vincristine in the concentration range of 0–200 µg mL^{-1}. The concentration of 3',4'-anhydrovinblastine was determined against the authentic standard of vinblastine, which can therefore be regarded as a semiquantitative approach for this compound.

Statistical analyses: Data represent mean values and standard deviations. *Student*'s *t*-test was used to determine statistically significant differences between the different light conditions. Two-way analysis of variance (*ANOVA*) was used to demonstrate the independent effects of light and flower colours together with interactions on the certain investigated parameters. One-way *ANOVA* was also used to highlight the significant differences between the treatments. The analyses for photosynthesis parameters, plant hormone contents, and certain metabolites were completed with *Duncan*'s post-hoc test performed using *SPSS 16.0*.

Results

Plant growth and gas-exchange parameters: To detect the effects of different growth light intensities on the physiological state of *C. roseus* plants, certain physiological parameters were determined. After 23 d, a slight, but statistically significant difference was found between the heights of plants grown under different light conditions, as it was 15.2 ± 3.0 cm for NL, and 18.5 ± 2.9 cm for shaded plants, respectively.

Net photosynthesis (P_N) usually depends heavily on the light intensity. Table 1 shows that reduced light substantially decreased the net photosynthetic activity (P_N) of leaves; however, stomatal conductance (g_s) did not differ significantly, and transpiration (E) was also just slightly lower under LL than NL conditions. In contrast to these, water-use efficiency (WUE) and the intercellular CO_2 concentration (C_i) were substantially lower and higher, respectively, in shaded (LL) plants than at NL conditions, indicating a slower CO_2 metabolism under similar transpiration levels at lower than at higher light conditions.

Since flowering plants were used for the experiments, the plants were sorted according to flower colour for further tests: groups with white, red, and pink flowers were created. The colour of the flower had no substantial effect on the photosynthetic activity of the plant either at NL or LL conditions: except for the slight difference in transpiration between red and pink plants, none of the measured gas-exchange parameters showed a statistically significant difference between the different colour groups (data not shown).

Chlorophyll *a* fluorescence measurements: The fluorescence induction parameters provide valuable information about the photosynthetic electron transport-related processes in plants. At normal growth temperature, the different growth light conditions did not significantly affect either the maximum (F_v/F_m) or actual [$Y_{(II)}$] quantum yield parameters (Fig. 1). Similarly, there was no difference in the nonregulated nonphotochemical quenching parameter, $Y_{(NO)}$. However, the regulated nonphotochemical quenching, $Y_{(NPQ)}$, was slightly, but statistically significantly higher in NL than in shaded (LL) plants (Fig. 1).

Table 1. Gas-exchange parameters, namely net photosynthetic rate [P_N, µmol(CO_2) m^{-2} s^{-1}], stomatal conductance [g_s, mmol(H_2O) m^{-2} s^{-1}], transpiration rate [E, mmol(H_2O) m^{-2} s^{-1}), water-use efficiency [WUE, P_N/E, µmol(CO_2) mmol(H_2O)$^{-1}$], and intercellular CO_2 concentration [C_i, µmol mol^{-1}] of *Catharanthus roseus* plants grown under NL or LL conditions. Data are given as mean ± SD values using plants with different (white, red, and pink) flowers. * and *** represent significant differences between NN and LL conditions at P<0.5 and 0.001 levels, respectively (n = 12).

	P_N	g_s	E	WUE	C_i
NL	22.3 ± 2.1	85.8 ± 7.6	1.24 ± 0.10	18.1 ± 2.5	143.6 ± 11.2
LL	7.0 ± 0.4***	82.2 ± 11.7	1.09 ± 0.21*	6.6 ± 1.3***	246.6 ± 37.8***

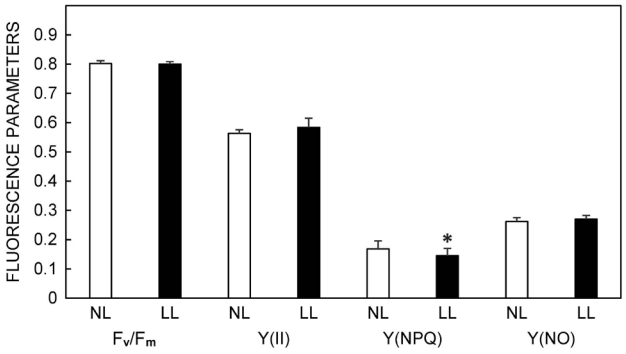

Fig. 1. Chlorophyll *a* fluorescence induction parameters in *Catharanthus roseus* plants grown either at control (NL) or reduced light (LL) conditions. Measurements were carried out at room temperature in a steady state. Mean ± SD, * represents statistically significant differences between NL and LL values at $p<0.05$ level. F_v/F_m – maximum photochemical efficiency of PSII; $Y_{(II)}$ – effective PSII quantum yield; $Y_{(NO)}$ – quantum yield of nonregulated energy dissipation; $Y_{(NPQ)}$ – quantum yield of regulated energy dissipation.

The temperature dependence of $Y_{(II)}$ showed that from 28 up to 46°C, this parameter was slightly higher in LL than in NL plants (Fig. 2). However, after that, the tendency changed, and especially from 53°C it was higher for NL than for LL plants. The opposite trend was observed in the $Y_{(NPQ)}$ parameter, while $Y_{(NO)}$ only differed at the highest measuring temperature, 55°C.

When plants with different flower colour were analysed separately, slight differences were also observed in the leaf fluorescence induction parameters, depending on the flower colour (Fig. 1S, *supplement*). Both at NL and LL, the highest and lowest F_v/F_m were measured in plants with pink and red flowers, respectively. In contrast to this, the highest $Y_{(II)}$ was detected in plants with red flowers at NL, while at LL conditions, the differences were not statistically significant. The higher $Y_{(NPQ)}$ at NL than at LL found in the mixed population was mainly due to the white-flowering plants. In the case of red or pink plants, these differences between NL and LL conditions were not pronounced. The $Y_{(NO)}$ parameter was slightly higher in red than in pink or white plants (Fig. 1S).

Analysis of the primary metabolites: For a better understanding of the biochemical responses of *C. roseus* to different light conditions, in the next step, a metabolomics analysis focusing on the primary metabolites was carried out. Organic acids, amino acids, sugars, and sugar alcohols were analysed and the list of the detected compounds is presented in Table 4S (*supplement*). The amount of certain compounds was below the limit of quantitation (LOQ), furthermore, others were measured with high uncertainty or with a large standard deviation (RSD > 30%), therefore they were not used in further evaluations. The amount of amino acids mainly decreased in the shaded plants; only the content of threonine increased significantly (Table 2).

If the flower colours are taken into consideration, the serine content also increased in white-flowering shaded plants (Fig. 2S, *supplement*).

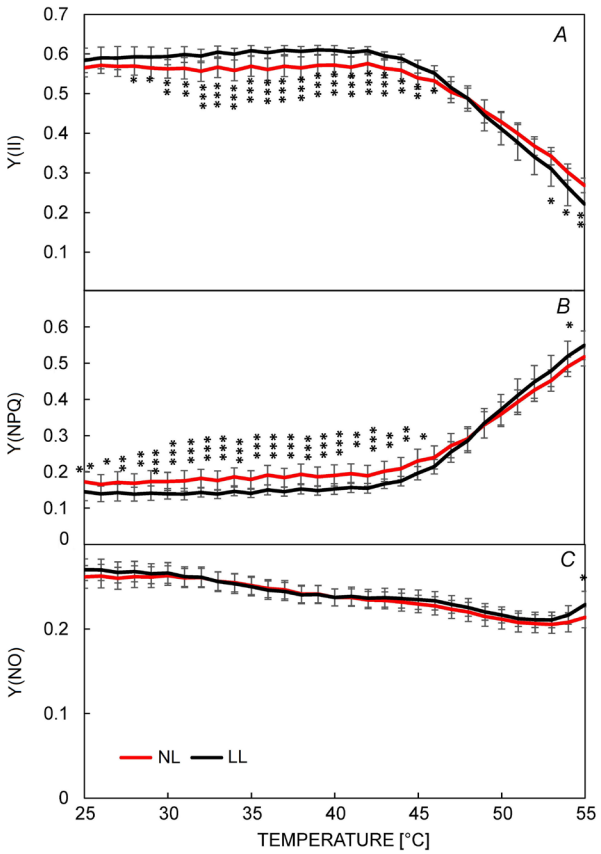

Fig. 2. Temperature dependence of the chlorophyll *a* fluorescence induction parameters $Y_{(II)}$ (*A*), $Y_{(NPQ)}$ (*B*), and $Y_{(NO)}$ (*C*) in *Catharanthus roseus* plants grown either at control (NL) or reduced light (LL) conditions. Mean ± SD, *, **, and *** represent statistically significant differences between NL (*red lines*) and LL (*black lines*) values at $p<0.05$, 0.01, and 0.001 levels, respectively. $Y_{(II)}$ – effective PSII quantum yield; $Y_{(NO)}$ – quantum yield of nonregulated energy dissipation; $Y_{(NPQ)}$ – quantum yield of regulated energy dissipation.

In the case of the tricarboxylic acid (TCA) cycle, the 2-ketoglutaric acid content decreased, the malic acid content increased in a high manner, and the citric acid content remained at the same level in shaded plants. This high increase in malic acid was a result of its elevated content in the shaded Polka Dot (white flowers) and Red plants because no changes were found in the pink-flowering Rose type at LL. The other compounds in the TCA cycle were below the LOQ. Interestingly, sugar contents did not differ significantly.

Hormone and phenolics measurements: To get deeper into the metabolite pathways, in the next step, targeted analyses focusing on certain plant hormones and phenolic compounds were carried out. Altogether, 27 compounds were tested; however, vanillic acid, indole-acetic acid (auxin), syringic acid, and naringenin were under or close to their detection limit, so these compounds were not used in the further evaluation in Table 5S (*supplement*). Among the plant hormones, salicylic acid, jasmonic

acid, its conjugated form, jasmonic-acid-LE/ILE SUM, and abscisic acid, together with its degradation product, phaseic acid, were detected using this method. While salicylic acid and jasmonic acid did not differ significantly between the two light conditions used in this experiment, the conjugated form of jasmonic acid and both abscisic acid and phaseic acid were lower in the shaded plants than under normal light conditions (Fig. 3).

Table 2. Primary metabolites [μg g^{-1}(FM)] in *Catharanthus roseus* plants grown either at control (NL) or reduced light (LL) conditions. Mean values ± SD, n = 9. Values below the limit of quantitation were considered as 0. *, **, *** – significant at 0.05, 0.01, and 0.001 levels, respectively. ISTD – amount was calculated using ribitol as the internal standard.

Compound	NL	LL
L-isoleucine	9.5 ± 2.1	5.3 ± 4.8*
L-serine	27.1 ± 21.2	43.4 ± 43.1
L-threonine	22.0 ± 0.96	30.7 ± 6.02***
Phenylalanine	11.5 ± 11.2	4.8 ± 5.4
L-aspartic acid	163.6 ± 53.6	96.0 ± 104.1
Alanylglycine (ISTD)	2.6 ± 0.57	0.2 ± 0.31***
GABA	110.0 ± 85.6	59.8 ± 57.6
α-ketoglutaric acid	2,956.5 ± 437.7	2,169.7 ± 681.9**
Fumaric acid	56.9 ± 41.4	19.9 ± 23.1*
Citric acid	104.6 ± 27.6	95.9 ± 26.6
Malic acid (ISTD)	8.2 ± 2.99	155.7 ± 176.6*
Ribonic acid (ISTD)	0.1 ± 0.04	0.02 ± 0.04***
Maltose	52.7 ± 10.6	48.2 ± 11.5
Galactose (ISTD)	1.0 ± 0.08	0.8 ± 0.22
Sucrose (ISTD)	9.1 ± 3.58	6.8 ± 8.16
D-glucose	1,118.4 ± 650.7	1,526.3 ± 764.7
Glucose-6-phosphate	31.8 ± 1.92	26.1 ± 14.8
Myo-inositol	402.5 ± 364.2	806.3 ± 394.2*
Spermidine	0.13 ± 0.01	0.0 ± 0.0***

The relatively large standard deviation values in this demonstration are due to the fact that plants with different flower colours gave different absolute values. Fig. 4 shows that the leaves of plants with red flowers grown at NL contained significantly higher contents of salicylic acid than all the other samples. A similar trend was also observed for the jasmonic acid conjugates, and in the case of jasmonic acid. In contrast to these, the abscisic acid contents were mainly affected by growth light, and much less by the colour of the flower (Fig. 4).

As expected, most of the phenolic compounds, including flavonoids, detected with the used UPLC-MS/MS method showed reduced contents in the shaded plants. Furthermore, most of them could be detected in the highest amounts in the leaves of plants with red flowers. Except quercetin, which was significantly lower in the leaves of red plants than that in white under low light conditions, but not at normal light (Fig. 5).

Investigation of vinca alkaloids: In the next step, the two main indole alkaloids, vincristine and vinblastine, together with their precursors and metabolites were screened in the samples. Besides vincristine and vinblastine, ten components of the related pathway could be tentatively identified (Table 3). As no reliable quantification could be carried out for the nondimeric compounds due to the lack of authentic standards, statistical analyses were conducted on the abundance (counts per second, cps) datasets. The effect of low light intensity on alkaloid production was less obvious than it was found for other physiological and biochemical parameters, as it was found especially in the case of photosynthetic parameters or in certain hormone and phenolic contents. From the 12 compounds, only three showed significant differences, having slightly lower level of abundance in the shaded plants (Table 3) and none of the most studied dimeric vinca alkaloids (*i.e.*, vincristine, vinblastine, and 3',4'-anhydrovinblastine) were included among these three.

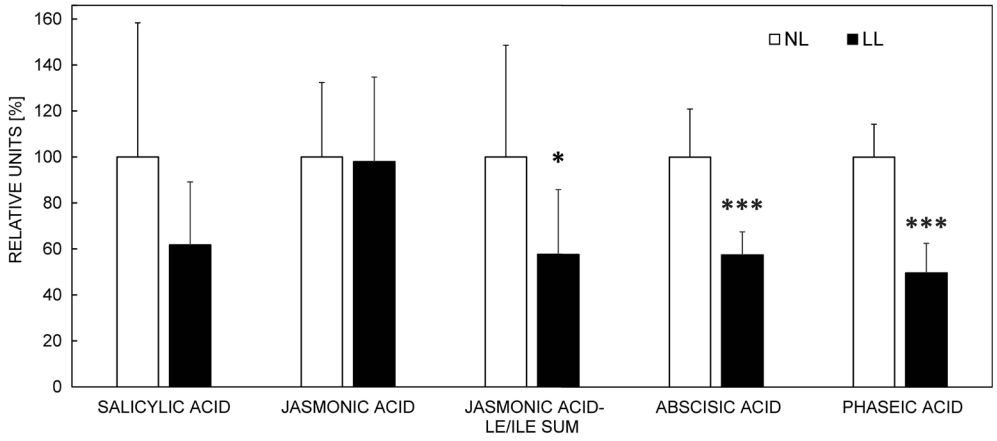

Fig. 3. Certain plant hormones and related compounds in *Catharanthus roseus* plants grown either at control (NL, *white bars*) or reduced light (LL, *black bars*) conditions. Data are given as percent of the NL values. The absolute values, expressed as ng g^{-1}(FM), for NL plants are: salicylic acid, 30.7; jasmonic acid, 569.8; jasmonic-acid-LE/ILE SUM, 20.7; abscisic acid, 9.98; phaseic acid, 1.55. Mean ± SD, * and *** represent statistically significant differences between NL and LL values at p<0.05 and 0.001 levels, respectively.

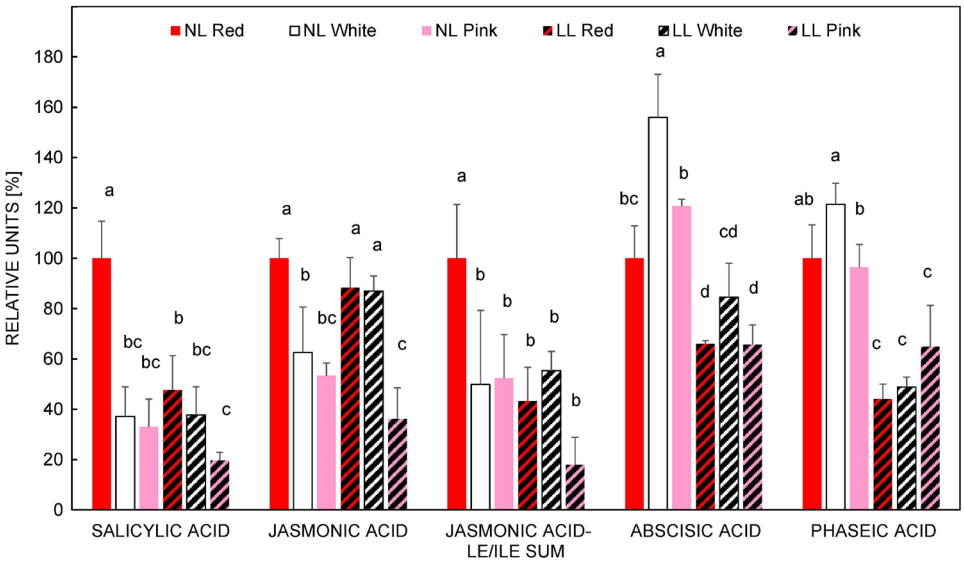

Fig. 4. Certain plant hormones and related compounds in *Catharanthus roseus* plants grown either at control (NL, *filled bars*) or reduced light (LL, *stripped bars*) conditions are separated according to the flower colour (Red: 'Red', *red bars*; Pink: 'Rose', *pink bars*; White: 'Polka Dot', *white bars*). Data are given as percent of the NL Red values ± SD. The absolute values, expressed as ng g^{-1}(FM), for NL Red plants are: salicylic acid, 87.8; jasmonic acid, 808.0; jasmonic-acid-LE/ILE SUM, 53.3; abscisic acid, 13.8; phaseic acid, 2.94. *Different letters* indicate statistically significant differences between the treatments at $p<0.05$ level.

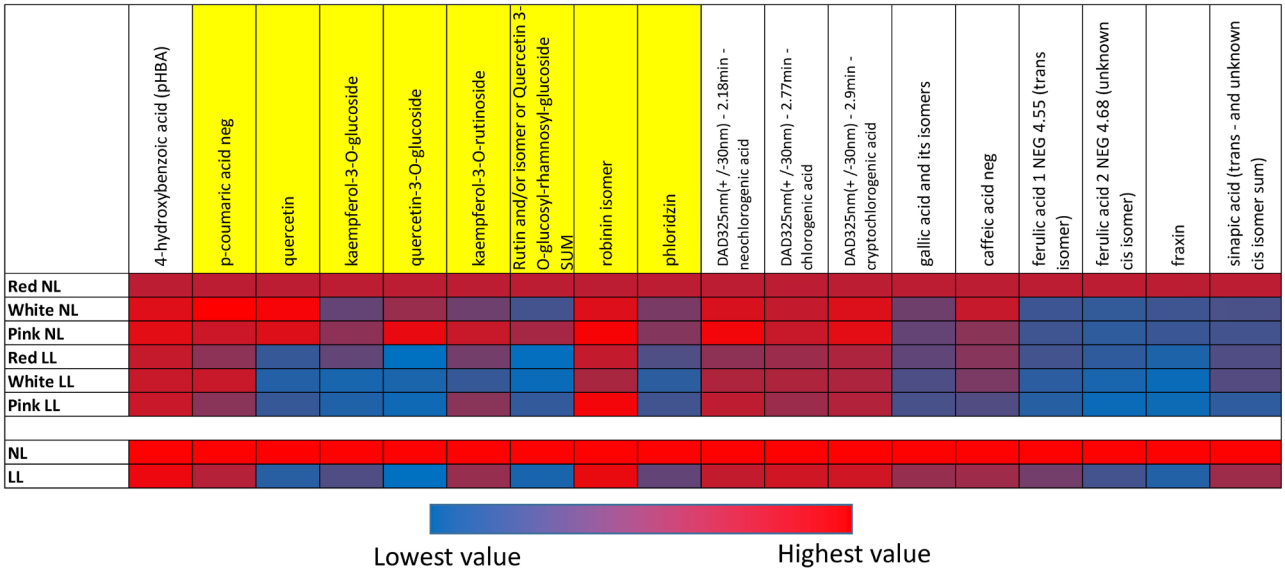

Fig. 5. Heat map of different phenolic compounds, including flavonoids (indicated with *yellow background*) in *Catharanthus roseus* plants grown either at control (NL) or reduced light (LL) conditions, separated according to the flower colour (Red: 'Red'; Pink: 'Rose'; White: 'Polka Dot'), normalised to Red NL plants. *Lower panels* marked with NL and LL indicate average values irrespective of colours, normalised to NL values.

The expression levels of two genes playing roles in the terpenoid and indole alkaloid biosynthesis pathway, tryptophan decarboxylase (*CrTDC*) and strictosidine synthase (*CrSTR*), were also examined in pooled samples, irrespective of flower colour. Results show that the expression level of *CrSTR* was significantly higher in the leaves of plants grown under low light conditions than under normal light (Fig. 6). However, the expression of the *CrTDC* gene did not differ significantly (data not shown).

The contents of certain alkaloids also differed in plants with different flower colours (Table 6S, *supplement*). Especially, 3',4'-anhydrovinblastine, vincristine and, to a lesser extent, vinblastine showed significant flower colour dependency (Fig. 3S, *supplement*). Interestingly, red flower plants usually contained lower contents of

Table 3. Abundance of vinca alkaloids and related compounds detected in *Catharanthus roseus* plants grown either at control (NL) or reduced light (LL) conditions. Mean of counts per second (cps) ± SD values, $n = 9$. ** and *** represent statistically significant differences between NL and LL values at $p<0.01$ and 0.001 levels, respectively.

Compound	NL	LL
19-*S*-vindolinine	24,797 ± 988	25,513 ± 673
3',4'-anhydrovinblastine	9,961 ± 3,023	10,263 ± 3,944
Catharanthine	9,957 ± 554	9,980 ± 1,081
Deacetylvindoline	313 ± 91	254 ± 74
Loganic acid	25,310 ± 1,664	18,085 ± 2,864***
Secologanin	48,404 ± 2,650	42,631 ± 3,123**
Strictosidine	846 ± 242	803 ± 273
Vinblastine	2,652 ± 816	2,561 ± 1,277
Vincadifformine	471 ± 206	551 ± 277
Vincristine	1,532 ± 616	1,709 ± 1,200
Vindoline	505 ± 40	468 ± 100
Vindolinine	20,308 ± 592	18,607 ± 782***

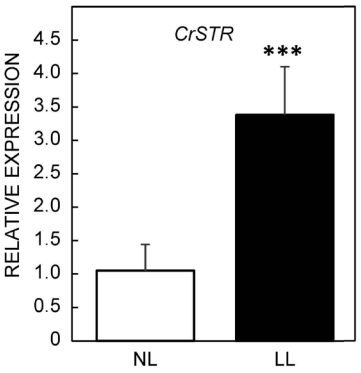

Fig. 6. Relative expression levels of the *CrSTR* gene in *Catharanthus roseus* plants grown either at control (NL, *white bar*) or reduced light (LL, *black bar*) conditions. Mean ± SD, *** represent statistically significant differences between NL and LL values at $p<0.001$ level.

alkaloids than white or pink ones, in spite of the fact that certain compounds, such as catharanthine and precursors (deacetylvindoline, secologanin, strictosidine, vincadifformine) were detected at their highest contents in red-flower plants.

Discussion

In order to better understand the metabolic differences in *C. roseus* plants under different growth light conditions, mature, flowering plants were placed in phytotron chambers, where a group of plants was grown under shaded conditions with reduced light. Since this plant species tolerates full sunlight relatively well, the main question was not what the high light intensity would cause, but how the main physiological and biochemical processes take place under shaded conditions. Two-way *ANOVA* confirmed that growth light intensity significantly affected various photosynthetic and metabolic processes. Furthermore, genotypic variations characterised with different flower colours may also affect the responses to different light conditions (Table 7S, *supplement*).

As expected, lower P_N was detected under the shade, and higher C_i also indicated reduced carboxylation activity. However, neither g_s nor transpiration rate were significantly different. These agree with other findings because, although g_s is usually higher at elevated light intensities, it rarely limits the photosynthetic processes without stress conditions inducing stomatal closures. Furthermore, above a threshold light intensity value, g_s may also decrease (Pan and Guo 2016).

Shaded plants could also be characterised by lower contents of the majority of primary metabolites, amino acids and organic acids detected with the *GCxGC* technique, which can be due to the reduced availability of CO_2 assimilation products. The only exceptions we found were threonine and malic acid, which were higher in the shaded than in the NL plants. These are partly in agreement with the idea that, besides their role as precursors of proteins, amino acids can also be catabolized to generate energy when photosynthesis does not meet the plant's energy demands (Galili *et al.* 2014).

Among the chlorophyll *a* fluorescence induction parameters, the F_v/F_m values were around 0.8, indicating that the given growth conditions did not cause severe stress in the plants. The slightly higher $Y_{(NPQ)}$ value found in the NL plants indicated a higher potential to dissipate the excessive excitation energy in the PSII antenna complex as heat (Wong *et al.* 2014, Vredenberg 2018, Nosalewicz *et al.* 2022). Only slight differences were found in the steady-state fluorescence parameters between the different types with different flower colours. Differences were found only in the case of white flower plants. These results indicated that the white-flowering plants can be more sensitive to light than the red and pink varieties. This is in accordance with previous results, where it was found that higher antioxidant activity was measured in plants with red flowers than in white ones (Bhutkar and Bhise 2011).

Light intensity also affected the temperature dependence of the fluorescence parameters, especially in $Y_{(II)}$ and $Y_{(NPQ)}$. The changes in these parameters at elevated temperatures may indicate an alteration in the thermostability of the thylakoid membranes (Darkó *et al.* 2019, Janda *et al.* 2021b). The present results indicate that NL plants can be characterised by a slightly higher thermostability than shaded plants. This supports the view that light contributes to temperature acclimation processes in higher plants. The thermostability of the thylakoid membranes may also relate to the de-epoxidation of the xanthophylls, which play a role in the stabilization and protection of thylakoids against heat-induced disorganization (Havaux *et al.* 1996). However, the exact mechanisms of the contribution of light to membrane stability are still poorly understood.

In order to follow the effects of different growth light conditions from the primary light intensity dependent processes (*i.e.*, photosynthetic processes and primary metabolites) to the secondary metabolic processes, further

detailed chromatographic investigations were carried out. The synthesis of phenolic compounds, including flavonoids in plants, is influenced by various environmental factors, especially light (Ghasemzadeh *et al.* 2010, Xu *et al.* 2020, Sutulienė *et al.* 2022). Flavonoids are well-known antioxidant compounds that play a role in photoprotection as well. Under the present experimental conditions, after shading, the most pronounced reduction in phenolic contents occurred at the levels of fraxin, quercetin, quercetin-3-O-glucoside, kaempferol-3-O-glucoside, and a compound that was identified as rutin and/or isomer or quercetin 3-O-glucosyl-rhamnosyl-glucoside sum. This supports the theory that in *C. roseus*, the flavonol pathway is also among the most light-regulated ones in the phenylpropanoid metabolism.

Vinca alkaloids, mainly vindoline, vinblastine, and vincristine, are all derived from *C. roseus*, so increasing the yields of their biosynthesis in the plant has been a common goal for many years. In the present work, the effects of light intensity were also investigated for the production of the main alkaloids. First, the effects of growth light on the expression levels of two key genes, *CrTDC* and *CrSTR*, were investigated in the leaves of plants pooled from different genotypes. Strictosidine synthase (EC 4.3.3.2) is one of the key enzymes in the biosynthesis of alkaloids. It catalyses the formation of strictosidine from tryptamine and secologanin (Stöckigt *et al.* 2008). Strictosidine serves as the central intermediate in indole alkaloid biosynthesis, including, among others, quinine, vinblastine, and vincamine (Loris *et al.* 2007, Boccia *et al.* 2022). Overexpression of these genes may lead to elevated level of alkaloid production, including vindoline, vinblastine, and catharanthine (Sharma *et al.* 2018). Earlier works have shown that short-term exposure to UV-B may increase the expression level of the *CrSTR* gene; however, with a combination of treatment with jasmonic acid, the induction was less obvious (Rady *et al.* 2021). This suggested that light quality is also an important factor in this regulation, but other mechanisms may also affect it. However, in contrast to UV exposure, shading did not significantly affect the *CrTDC* expression level. In contrast to this, a substantial upregulation was found in the case of *CrSTR* gene in the shaded plants.

In conclusion, the present results demonstrate how growth light affects photosynthetic processes, and in turn, primary metabolic composition in *C. roseus* plants. P_N was much more affected under low light conditions than stomatal conductance, leading to reduced contents of primary metabolites. Light may also affect the heat stability of the thylakoid membrane. Furthermore, although growth light has a substantial effect on various stress-related processes, including hormonal changes or antioxidant activities, the alkaloid contents were less affected under the present light conditions. The different types of Titan *C. roseus* hybrids, characterized by different flower colours, also showed different responses to the changing light conditions. However, further studies are needed to determine how the effects of individual environmental factors influence the responses of plants with different genetic backgrounds.

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