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The effect of osmotic stress, lighting spectrum and temperature on growth and gene expression related to anthocyanin biosynthetic pathway in wild strawberry (*Fragaria vesca* L.) *in vitro*

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ABSTRACT

The goal of this research was to evaluate the effect of light, temperature, sucrose and PEG on the growth of *Fragaria vesca in vitro* and the expression of regulatory *Myb10, WD40* and enzyme-coding genes *CHI, CHS, DFR, EGL, F3H* and *UFGT*, which are essential for anthocyanin biosynthesis. We observed plants' response to osmotic stress, the decrease in growth and microshoot weight. A change in the expression of the investigated genes was evident under the suboptimal concentration of sucrose. The addition of PEG to the medium caused a decrease in microshoot weight and gene expression. Blue + red lights of the LED lighting system significantly affected microshoot growth *in vitro*. Red and blue + red + UV lights slightly reduced microshoot weight and caused a reddish colour of petioles, which indicate increased anthocyanin synthesis. Moreover, most of the studied genes' expression tended to increase when shoots were exposed to blue, blue + red and blue + red + UV lights. A temperature of 15°C (vs 22°C) significantly reduced the mean fresh weight of microshoots while increasing *CHI* and *CHS* gene expression and decreasing *WD40* gene expression. Exposure to a higher temperature (30°C) induced the vitrification of microshoots, although the fresh weight did not differ from that of the control. Gene expression also depended on the duration of exposure. In the case of *CHS*, gene expression remained the same or increased after exposure for 1 week and then decreased after exposure for 4 weeks.

Keywords: controlled conditions, flavonoids, growth, metabolism, pigmentation, stress

Abbreviations: ABA, abscisic acid; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; EGL, enhancer of glabra; F3H, flavanone 3-hydroxylase; LED, light-emitting diode; MS, Murashige and Skoog; Myb10, MYB domain protein 10; MYB-bHLH-WD40, a complex of MYB transcription factors (TFs), basic helix–loop–helix (bHLH) TFs and WD-repeat proteins; PEG, polyethylene glycol; qPCR, quantitative real-time PCR; UFGT, UDP glucose: flavonol 3-O-glucosyltransferase; WD40, approximately 40 amino acid repeat terminating in a tryptophan-aspartic acid (W-D) dipeptide.

INTRODUCTION

Anthocyanins are a class of flavonoids that are important in plant–environment interactions and reactions to biotic or abiotic stresses (Carbone et al., 2009; Gould and Lister, 2006; Khan and Abbas, 2023). Stress induces a number of processes in plants, including the accumulation of secondary metabolites and an increase in reactive oxygen species and free radicals. The synthesis of various antioxidants is a plant defence

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mechanism. Phenolic compounds, such as anthocyanins, are very important in neutralising free radicals. In this case, metabolites of the anthocyanin biosynthesis pathway such as proanthocyanidins, catechins, quercetins and others are powerful antioxidants and key molecules in plant molecular stress responses (Hijaz et al., 2018; Loberant and Altman, 2010; Matkowski, 2008). Regulation of anthocyanin biosynthesis genes occurs at the transcription level in plants. According to the results of species that have already been studied, the main regulator of anthocyanin biosynthesis is the 'MBW' complex (MYB-bHLH-WD40), a complex of MYB transcription factors (TFs), basic helix–loop–helix (bHLH) TFs and WD-repeat proteins (Jaakola, 2013; Lin-Wang et al., 2014; Starkevič et al., 2015; Khan and Abbas, 2023). It has been shown that by using gene silencing or transformation techniques it is possible to manipulate or control the accumulation of anthocyanins and other flavonoid compounds in *Fragaria* (Fischer et al., 2014; Griesser et al., 2008; Lin-Wang et al., 2014). Polyphenol compounds in plants have been reported to vary as a function of developmental and environmental factors and play an important role in genetic variation (Carbone et al., 2009; Tulipani et al., 2011). Environmental and biological factors such as light, temperature and signalling biomolecules including sugars and hormones alter the function of regulatory proteins that control anthocyanin biosynthesis by activating different structural genes at both transcriptional and post-transcriptional levels (Das et al., 2012; Okutsu et al., 2018; Zhang et al., 2018).

Isolated plant cell and tissue cultures can be successfully used to produce high-value secondary metabolites and have been extensively studied in recent decades. The ability to control physical and chemical conditions allows the development of methodologies that increase the production of plant metabolites or even the synthesis of new compounds (Appelhagen et al., 2018; Simões et al., 2012). Plant tissues after excision and during cultivation are exposed to stress factors and their combinations, which they did not experience under natural conditions during evolution. Therefore, *in vitro*, culture provides opportunities and tools to study anthocyanin gene expression (Cassells et al., 2003). It has been shown that anthocyanin production is greatly influenced by growing conditions that cause metabolic or osmotic stress (Do and Cormier, 1990). Among the many factors influencing anthocyanin production in plant cell and tissue culture are phytohormones, medium pH, exposure to light, physical parameters such as temperature, etc. (Appelhagen et al., 2018; Deroles, 2009; Kissimon et al., 1999).

The aim of this work was to investigate how different light conditions, temperature and osmotic compounds, such as sucrose and polyethylene glycol (PEG), might affect the growth of *Fragaria vesca in vitro* and the expression of several genes involved in anthocyanin biosynthesis.

MATERIALS AND METHODS

Plant propagation in vitro

Microshoots of wild strawberry (*Fragaria vesca* subsp. *vesca* L.) *in vitro* culture was derived from seedlings of the everbearing alpine variety 'Rügen'. Ripe achenes of *F. vesca* were germinated *in vitro* as described by Miller et al. (1992). The achenes were dried and kept in the refrigerator for 3 months. They were then sterilised by rinsing with 96% ethyl alcohol and then with sterile water, incubated for 10 min in 6% sodium hypochlorite solution (Carl Roth, Karlsruhe, Germany) and again rinsed (4×) with sterile distilled water. Sterilised achenes were cut transversely by using a scalpel. The cut achenes (embryo axis part) were then placed in 200-mL glass flasks containing 30 mL of agarised Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 3% sucrose and without phytohormones. Germinated microshoots were cultivated at 22 ± 3°C under 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluorescent lamp illumination and a 16-hr photoperiod in flasks with MS medium supplemented with 1 $mg \cdot L^{-1}$ benzylaminopurine, 3% sucrose and 0.8% plant agar. The medium was adjusted to pH 5.8 and autoclaved at 120°C for 30 min. The microshoots were subjected to three different treatments. For the determination of the light spectrum effect, the microshoots were transferred to a growth chamber where they were illuminated with a custom-made light-emitting diode (LED) lighting system containing four separate modules for parallel growth runs: blue, red, red + blue and red + blue + UV. The main photosynthetic photon flux (PPF) was provided by blue 452 nm (B; LedEngin LZ1-00B200, Osram Sylvania, Wilmington, MA, USA) and deep red 662 nm (R; Luxeon Rebel LXM3-PD01-0300, Philips Lumileds Lighting Co. San Jose, CA, USA) light supplemented with high-power UV-A 386 nm (LedEngin LZ440UB00-U4, Osram Sylvania, Wilmington, MA, USA) light. Photosynthetic photon flux density (PPFD) – 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in total was measured and regulated at the plant level using the light spectrometer FLAME-S-UV-VIS-ES (Ocean optics, Dunedin, FL, USA). Standard fluorescent lamps (broad-spectrum 400–800 nm, Philips Lighting, Eindhoven, Netherlands) were used as a control.

For the determination of the temperature effect, microshoots in flasks were grown in a climatic chamber at 15°C, 22°C (standard), 30°C and a 16-hr photoperiod. For the evaluation of the osmotic compounds (sucrose and PEG 6,000 MW) effect, the plants were transferred to MS medium supplemented with 1.5%, 3%, 6% and 9% sucrose and to MS supplemented with 3% sucrose and 5%, 10% and 12% PEG. Five explants per flask were grown in three repeats for every experiment separately. Microshoot weight in grams and colour changes were evaluated after 30 days, and the mean and the standard error of the mean were calculated. Samples of each treatment's microshoots (0.1–0.2 g) were frozen in liquid nitrogen and kept at –70°C until RNA extraction.

Gene expression analysis: RNA isolation and cDNA synthesis

Frozen samples were homogenised using a Retsch Mixer Mill 400 (Retsch GmbH, Haan, Germany). RNA was isolated using the GeneJET Plant RNA Purification Mini Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) in accordance with the manufacturer's recommendations, from three biological replicates. The quantity and quality of RNA were measured spectrophotometrically with an Implen P330 nanophotometer (Implen GmBH, München, Germany). Samples were treated with DNase I (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) at 37°C for 30 min.

cDNA was synthesised using the RevertAid First Strand cDNA Synthesis Kit and oligo $(dT)_{18}$ primer (Thermo Fisher Scientific Baltics) following the manufacturer's recommendations. The absence of genomic DNA was confirmed by PCR, and primers for elongation factor-1α subunit intron were used (Bonasera et al., 2006). PCRs were carried out using *Taq* DNA polymerase (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) with the following cycling parameters: 2 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 56°C and 30 s at 72°C; 10 min at 72°C.

Quantitative real-time PCR (qPCR) amplification was carried out using an Eppendorf EP Gradient S Thermocycler. Specific primers were designed for anthocyanin biosynthesis pathway genes in wild strawberry (Supplementary Table S1 in Supplementary Materials).

A reference gene *β*-actin was used as an internal reaction control. All reactions were performed in a 20 μL volume with *DreamTaq* polymerase (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) and EvaGreen dye (Biotum Ltd., Vladimir, Russia) using the following cycling parameters: 2 min at 95°C; 40 cycles of 30 s at 95°C, 45 s at 60°C and 45 s at 72°C. Later, a melting curve detection gradient from 60°C to 95°C was used. No template control reactions (NTCs) were included in all experiments. An MCE-202MultiNa system (Shimadzu Ltd, Tokyo, Japan) and a DNA-1000 Kit (Shimadzu Ltd, Tokyo, Japan) were used to measure the size and amount of PCR products.

Relative gene expression was estimated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). The expression data were analysed using Microsoft Excel 2010.

Statistical analysis

Statistically significant differences between treatments were evaluated using one-way analysis of variance (ANOVA). Variation within treatments was determined by calculating the values of the standard error of the mean (SEM). Significant differences were assessed by using Fisher's test at 5% and 1% probability levels.

RESULTS AND DISCUSSION

Effect of sucrose and PEG

Sucrose is important in the regulation of plant metabolism and enhances anthocyanin synthesis and accumulation in plants (Hijaz et al., 2018; Solfanelli et al., 2006). In the current study, wild strawberry microshoots grown on MS growth medium with 3% sucrose reached the average weight of 0.3 g (Table 1). Similar results were

Table 1. Average weight of wild strawberry microshoots, affected by osmotic components in MS medium, light and growth temperature.

Trait	Parameter	Average weight of microshoot (g)
Osmotic components	Sucrose 15 g · L^{-1}	0.27 ± 0.03
	Sucrose 30 g · L^{-1}	**0.30 ± 0.03**
	Sucrose 60 g · L^{-1}	0.13 ± 0.04*
	Sucrose 90 g · L^{-1}	0.10 ± 0.04*
	Sucrose 30 g · L^{-1} + PEG 50 g · L^{-1}	0.12 ± 0.10*
	Sucrose 30 g · L^{-1} + PEG 100 g · L^{-1}	0.11 ± 0.11*
	Sucrose 30 g · L^{-1} + PEG 120 g · L^{-1}	0.44 ± 0.60*
Light	Fluorescent	0.28 ± 0.01
	Blue	0.31 ± 0.02
	Red	0.27 ± 0.02
	BR	0.34 ± 0.03
	BRUV	0.24 ± 0.01
Temperature	15ºC	0.13 ± 0.03*
	22ºC	0.34 ± 0.06
	30ºC	0.30 ± 0.15

Standard (control) conditions are marked in bold. Values are mean ± SEM.
*Significant differences compared to the control assessed by Fisher's test ($p < 0.05$).
BR, Blue + Red; BRUV, Blue + Red + UV; MS, Murashige and Skoog; PEG, polyethylene glycol.

established in cultivated strawberry (Abdullah et al., 2013). However, differences in microshoot weight were not significant among strawberry plants grown on MS medium with 1.5% and 3% sucrose. Microshoots were healthy and did not show stress signs, and the colour of petioles was very rarely reddish. The weight of the microshoot significantly decreased up to one-third, reaching 0.13 g and 0.10 g in plants grown on MS medium with 6% and 9% sucrose, respectively. Microshoots with reddish petioles were more common, especially when the concentration of sucrose in the medium was the highest. We propose that the reduction in microshoot weight and petiole colouring was influenced by osmotic stress, thus reducing or blocking plant metabolism, except in the case of adaptational pathways, which have been activated. Since increased amounts of sucrose (4%–8%) in the medium caused enzyme-like superoxide dismutase (SOD) activation and protein and polyamine accumulation in potato microshoots *in vitro*, Sajid and Aftab (2022) suggest that this indicates a response to the oxidative stress induced by osmotic stress.

The expression of the phenylpropanoid pathway, specifically anthocyanin biosynthesis genes in wild strawberry, was evaluated. Our study showed that the expression of regulatory *Myb10, WD40* genes was significantly lower in microshoots grown on MS medium with 1.5% sucrose than in those grown on standard MS medium with 3% sucrose (Figure 1). The decrease in the expression of regulatory genes was possibly related to the decrease in growth due to an insufficient amount of sucrose.

Compared to the control, the expression of the *CHI* (chalcone isomerase) and *CHS* (chalcone synthase) genes was significantly lower in the plants grown on MS medium with 6% sucrose but higher in the microshoots grown on medium with 9% sucrose. These results suggest that sucrose levels of 6% and lower in the growth medium are possibly too low to induce osmotic stress in microshoots. The reduction in gene expression under 6% sucrose was more likely to be related to the regulatory properties of sucrose than to osmotic stress. Sucrose is known to regulate the expression of many gene systems, including ABA synthesis. In turn, an increase in the ABA level during stress increases the expression of *WD40* and other anthocyanin synthesis genes and anthocyanin accumulation (Li et al., 2014; Liu et al., 2018; Mattioli et al., 2020).

UFGT (UDP glucose: flavonol 3-O-glucosyl-transferase) could be distinguished from other studied genes because its expression decreased at the peak of

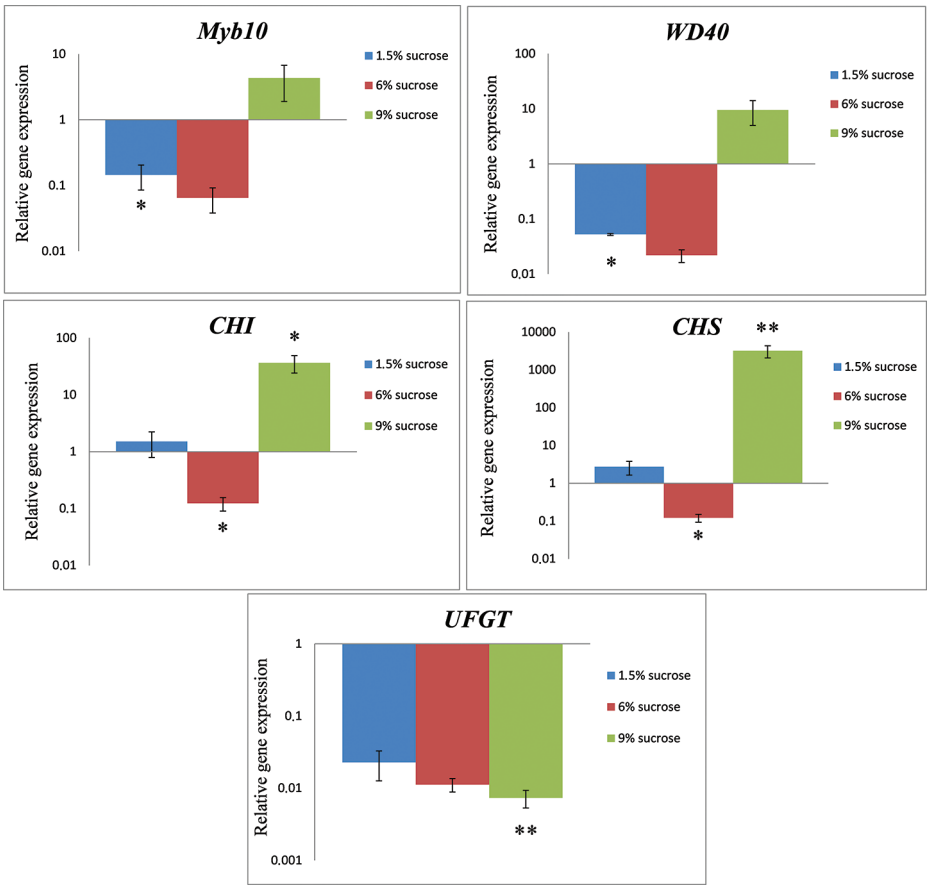

Figure 1. Gene expression in wild strawberry microshoots grown on MS medium with different concentrations of sucrose. MS medium with 3% sucrose was used as a control. Bars denote the standard error of the mean. Asterisks indicate significant differences compared to the control assessed by Fisher's test (*$p < 0.05$, **$p < 0.01$), $n = 3$. CHI, chalcone isomerase; CHS, chalcone synthase; MS, Murashige and Skoog; Myb10, MYB domain protein 10; UFGT, UDP glucose: flavonol 3-O-glucosyltransferase; WD40, a tryptophan-aspartic acid (W-D) dipeptide.

osmotic stress induced by 9% sucrose. The expression of other genes (*Myb10, WD40*) under the same conditions increased or tended to increase. Increased expression of most genes in the microshoots grown on MS medium with 9% sucrose remained intense due to osmotic stress and might be related to anthocyanin synthesis.

The increase in the expression of most studied genes (except *UFGT*) as well as the reddish colour of petioles in the medium with 9% sucrose indicated that anthocyanin biosynthesis starts when osmotic stress reaches a certain threshold. The decrease in *UFGT* expression during sucrose stress shows that catechin and proanthocyanidin synthesis was not induced at the same time. Because flavonoid biosynthesis in *Fragaria* plants is tissue-specific, anthocyanins are mostly synthesised in generative organs, but not in leaves or stems. Several genes that regulate anthocyanin biosynthesis have been identified in *Arabidopsis* (Jeong et al., 2018). Therefore, we do not yet know the whole picture of anthocyanin biosynthesis regulation in response to sucrose.

PEG is widely used in modelling drought-induced stress in an *in vitro* system (Sakthivelu et al., 2008; Verma et al., 2013). In our study, the lowest concentration of PEG (5%) in the MS medium caused a significant reduction in the weight of wild strawberry microshoots to 0.12 g, which was 2.5 times lower than in control microshoots grown on the MS medium with 3% sucrose (Table 1). According to Yosefi et al. (2022), PEG-induced water stress negatively affected shoot fresh and dry weight and physiological and biochemical traits. However, an increase in the PEG amount in the growth medium to 12% resulted in larger microshoots (0.44 g). Therefore, the variation was also larger. Microshoots were pale and watery, but no reddish petioles were observed. In this case, osmotic stress was different from stress caused by sucrose. The microshoots were not growing, they were soaked with PEG and their weight increased due to PEG concentration in the tissues.

The addition of 5%–12% of PEG to the MS medium resulted in reduced expression of all studied genes (Figure 2). PEG concentrations of 5% and 10% in the growth medium were the most inhibiting for the expression of *WD40* and *CHS* genes, while the expression reduction of *DFR* (dihydroflavonol reductase),

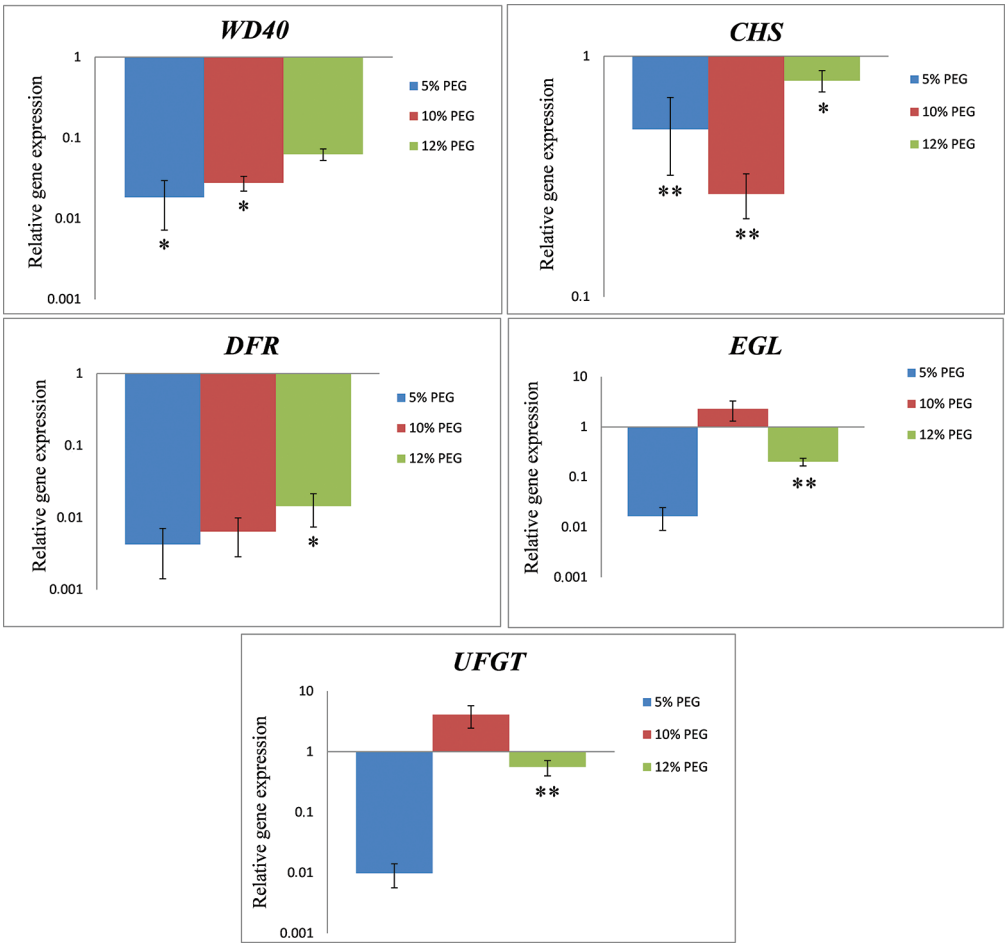

Figure 2. Gene expression in wild strawberry microshoots grown on MS medium with different concentrations of PEG. MS medium with 3% sucrose was used as a control. Bars denote the standard error of the mean. Asterisks indicate significant differences compared to the control assessed by Fisher's test (*$p < 0.05$, **$p < 0.01$), $n = 3$. CHS, chalcone synthase; DFR, dihydroflavonol reductase; EGL, enhancer of glabra; MS, Murashige and Skoog; PEG, polyethylene glycol; UFGT, UDP glucose: flavonol 3-O-glucosyltransferase; WD40, a tryptophan-aspartic acid (W-D) dipeptide.

EGL (enhancer of glabra) and *UFGT* genes was significant when 12% of PEG was applied. It is possible that a lower concentration of PEG blocks single metabolic pathways and induces some adaptation reactions, while higher concentrations inhibit the whole metabolism. In *Arabidopsis*, it was demonstrated that *MYB12* or *PAP1* overexpression and flavonoid over-accumulation were key to enhanced tolerance to drought stress (Nakabayashi et al., 2014). Cui et al. (2017) noticed that drought stress has a synergistic effect with other stresses that induce gene (*UFGT* and *MYBA1*) expression and anthocyanin accumulation in grapevine, even though drought stress alone does not have an effect.

The expression of the anthocyanin pathway genes in microshoots varied under different osmotic conditions, possibly due to peculiarities of the plant metabolism and the nature of osmotic compounds. Plants cannot use PEG in the same way as sucrose – although PEG enters the plant, it is not metabolised.

Effect of light

Our experiments with wild strawberry microshoots showed that their weight depends on the light spectrum during growth *in vitro*. The highest average weight (0.34 g) of wild strawberry microshoots was observed under blue + red lights (452 + 662 nm) (Table 1), and it was significantly higher than that of plants grown under red (662 nm) (0.27 g) and blue + red + UV (452 + 662 + 386 nm) (0.24 g) lights, but did not differ significantly from the plants grown under blue (452 nm) (0.31 g) and fluorescent lights (0.28 g). The average weight of microshoots grown under red light (0.27 g) was close to that of the control (0.28 g). This could imply that red light predominates in fluorescent light. Microshoots grown under fluorescent, red and blue + red + UV lights had partially reddish petioles that show increased anthocyanin synthesis (Figure 3). Lower plant weight and accumulation of anthocyanins under blue + red + UV lights in our experiment suggest that microshoots experience stress, which may be related to UV damage. It is known that UV light causes stress and induces the accumulation of anthocyanins in plants (Deroles, 2009; Guo et al., 2009).

Although no reliable differences in the expression of flavonoid biosynthetic pathway genes were found, most genes' expression tended to increase when shoots were exposed to blue and blue + red lights for 3 days and to blue + red + UV lights for 9 days (Supplementary Figure S1 in Supplementary Materials). The increase in *CHS* and *UFGT* genes' expression correlated with the increase in the anthocyanin content in lettuce under UV light (Goto et al., 2016). UV light damages cells and increases the concentration of free radicals. Therefore, it intensifies antioxidant flavonoid synthesis.

Anthocyanin accumulation might also be associated with stress conditions in plants caused by an improper light spectrum, or it may occur as a consequence of the change in the content of endogenic carbohydrates. According to Miranda and Williams (2007), blue and yellow light increased the level of sucrose in plants developed *in vitro* compared to white light. The photochemical efficiency of photosystem II (PSII) is usually low under blue light, but the rate of photochemical reaction is high. The morphogenetic effect, fresh and dry weight, and chlorophyll and nitrogen content of microshoots depend on the proportion of those two parameters (Miranda and Williams, 2007).

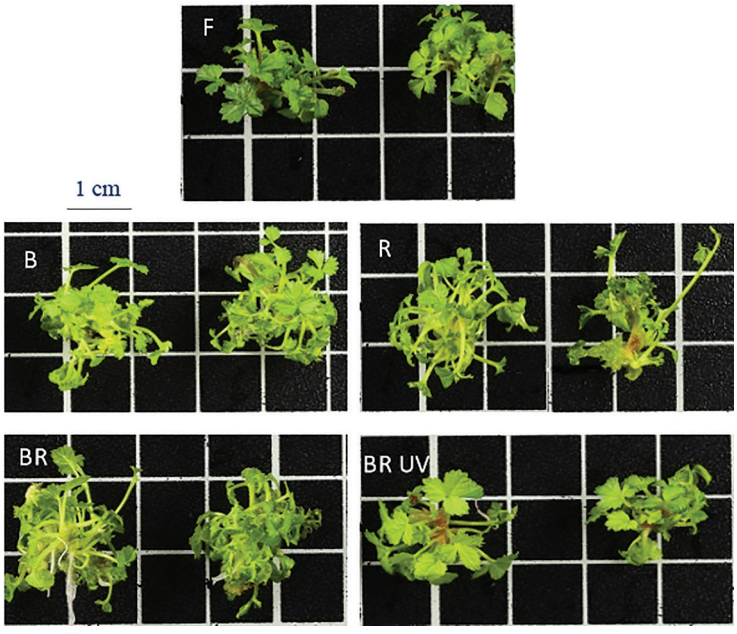

Figure 3. Wild strawberry microshoots grown under different light spectra: fluorescent light (F), LED lighting system: blue light (B), red light (R), blue + red lights (BR), and blue + red + UV lights (BR UV). LED, light-emitting diode.

Blue and red lights are necessary for photosynthesis, but they also have regulatory properties and influence plant metabolism and morphogenesis (Samuolienė et al., 2013). Tripathy and Brown (1995) noticed the root-perceived photomorphogenic inhibition of the shoot and decreased chlorophyll content in wheat seedlings induced by red light. In our experiment, microshoots were almost without roots, but under red and fluorescent lights, we noticed signs of stress that appeared in the red petioles of some of the microshoots and slightly less intensive growth and proliferation compared with microshoots grown under blue and blue + red lights. According to Zhang et al. (2018), blue light causes an increase in *CHI*, *DFR*, *ANS* (anthocyanidin synthase) and *FLS* (flavonol synthase) expression and a decrease in *CHS* and *F3H* (flavanone 3-hydroxylase) expression. Red light not only increases the amount of anthocyanins but also potentially contributes to the synthesis of proanthocyanidins by inducing leucocyanidin reductase and anthocyanin reductase.

Effect of temperature

Microshoots grown at 22°C were found to have the highest average fresh weight (0.34 g) (Table 1). This is the standard temperature for cultivating wild strawberry plants *in vitro*. The mean fresh weight of microshoots (0.3 g) grown at 30°C did not differ significantly from the mean fresh weight of microshoots grown under standard conditions. Vitrification and necrosis were observed in some microshoots grown at 30°C, indicating the stress condition of the plants. The mean fresh weight of wild strawberry microshoots grown at 15°C was significantly lower than the weight of microshoots grown under standard conditions and reached only 0.13 g. Despite their reduced size, these microshoots looked quite healthy. However, the colour of the petioles was more reddish than the colour of the microshoots grown at 22°C. This shows anthocyanin accumulation in the microshoots.

The anthocyanin biosynthesis is strongly affected by temperature as some genes regulating cold resistance are involved in this pathway (Christie et al., 1994). During winter, anthocyanins protect the leaves of evergreen plant species by reducing damage caused by low-temperature stress. Anthocyanin accumulation at low temperatures and degradation at high temperatures have been noticed in plants' fruits and tissue cultures (Deroles, 2009; Gaiotti et al., 2018). In this study, the influence of lower (15°C) and higher (30°C) temperatures on the gene expression of the anthocyanin biosynthesis pathway was evaluated (Figures 4 and 5). At 15°C, the expression of *CHI* and *CHS* genes was higher than that of the control (in the case of *CHS*, for at least 2 weeks); only *WD40* expression was lower (significantly lower after 3 weeks). Okutsu et al. (2018) indicated that the level of gene expression of the anthocyanin biosynthesis pathway is affected by the timing of plant exposure to high-temperature stress. Here, the duration of exposure at 15°C had no discernible effect on the expression of the genes tested (Figure 4). The expression of the regulatory *WD40* gene, as well as *CHI* and *DFR*, was almost the same as that in the control, when microshoots were grown at 30°C for 1 week. On the other hand, the expression of the *CHS* gene increased and may be explained by the primary reaction to stress (Figure 5). After 4 weeks at the same temperature, the expression of *WD40*, *CHI*, *CHS* and *DFR* genes decreased substantially. It is in line with the findings of most researchers, and it could be a sign that gene expression decreases under long-term high-temperature stress. It was shown that high ambient temperatures repress anthocyanin biosynthesis through a COP1-HY5 signalling module (Kim et al., 2017). However, transcript levels and activity of *PsANS* and *PsUFGT* remained stable or increased when a high temperature (35°C) was applied to plums (Niu et al., 2017). Exposure to light and high temperature (32°C) induced the expression of *MYB16* in apple callus, resulting in inhibition of anthocyanin biosynthesis (Wang et al., 2016). The authors suggest that the light-induced change in anthocyanin biosynthesis is primarily caused by altered MYB10 transcript levels, while temperature affects the expression of *bHLH3/33, MYB16, MYB17, MYB111* and other repressors (Lightbourn et al., 2007).

CONCLUSIONS

From this study, we can conclude that the expression of genes from the anthocyanin biosynthesis pathway is dynamic and depends on a particular organ, synergism of environmental conditions, timing and duration of stress and adaptivity to stress.

Induction of osmotic stress by addition of PEG to MS medium, lighting spectrum and exposure to 15 and 30°C temperatures had an evident impact on phenotype changes of microshoots, their weight and, in many cases, the expression of anthocyanin genes.

The regulatory significance of sucrose (carbohydrate) and temperature should be addressed in future studies of anthocyanin pathway gene expression and anthocyanin accumulation. Our study showed that despite the negative effect of increased osmotic pressure, higher sucrose concentration increased the expression of anthocyanin pathway genes but decreased growth. Wild strawberry microshoots are similarly affected by lower ambient temperatures.

The results of our experiments also show that to achieve maximum anthocyanin production from biomass in controlled conditions, the medium composition, temperature conditions and exposure duration must be precise. Conditions that are suitable for maximum biomass production are not appropriate for maximum anthocyanin production. As a result, we believe that this

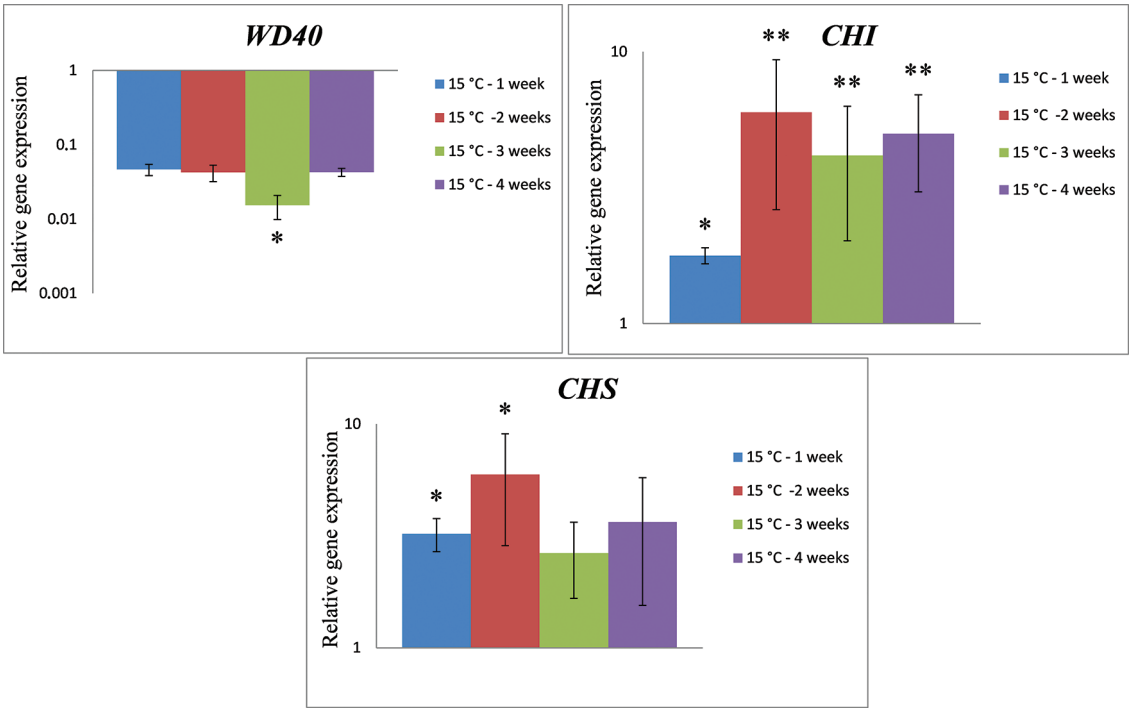

Figure 4. Gene expression in wild strawberry microshoots grown at 15°C for 1–4 weeks. Microshoots grown at 22°C were used as a control. Bars denote the standard error of the mean. Asterisks indicate significant differences compared to the control assessed by Fisher's test (*$p < 0.05$, **$p < 0.01$), $n = 3$. CHI, chalcone isomerase; CHS, chalcone synthase; WD40, a tryptophan-aspartic acid (W-D) dipeptide.

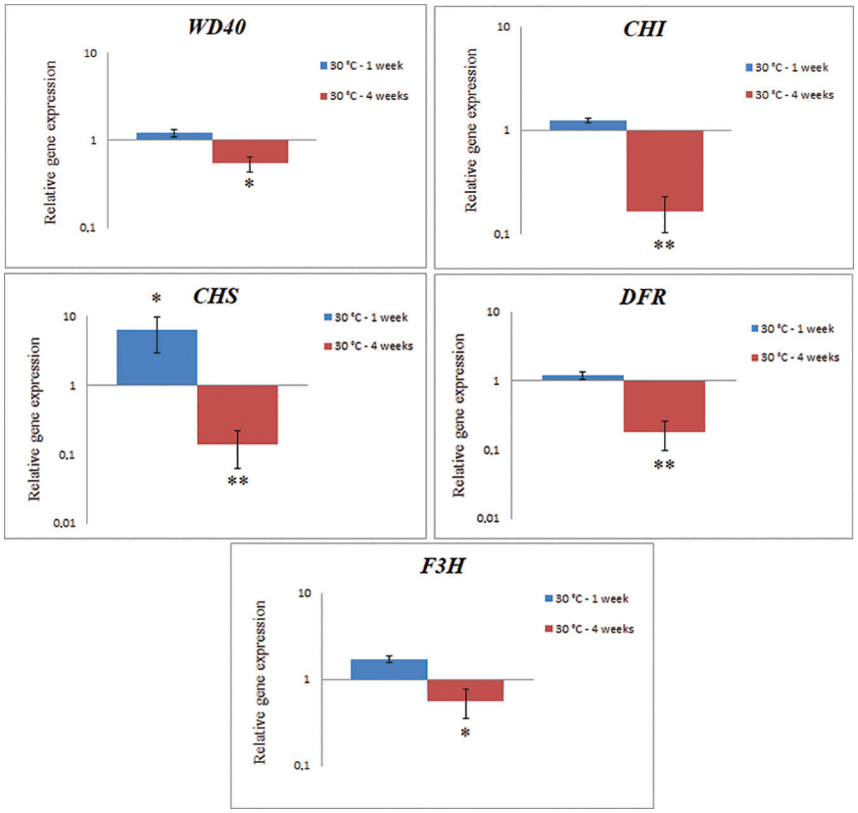

Figure 5. Gene expression in wild strawberry microshoots grown at 30°C for 1–4weeks. Microshoots grown at 22°C were used as a control. Bars denote the standard error of the mean. Asterisks indicate significant differences compared to the control assessed by Fisher's test (*$p < 0.05$, **$p < 0.01$), $n = 3$. CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3H, flavanone 3-hydroxylase; WD40, a tryptophan-aspartic acid (W-D) dipeptide.

kind of harmonisation is possible. We also believe that future research will help us get closer to this goal.

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AUTHOR CONTRIBUTIONS

R.R. and V.B. – conceptualisation. R.R and J.V. – methodology. V.B. – software. A.S. and V.S. – validation. V.B. – formal analysis. J.V. and R.R. – investigation. A.S. – resources. J.V. – data curation. R.R. – writing – original draft preparation. J.V. – writing – review and editing. J.V. – visualisation. V.S. – supervision. R.R. – project administration. R.R. – funding acquisition. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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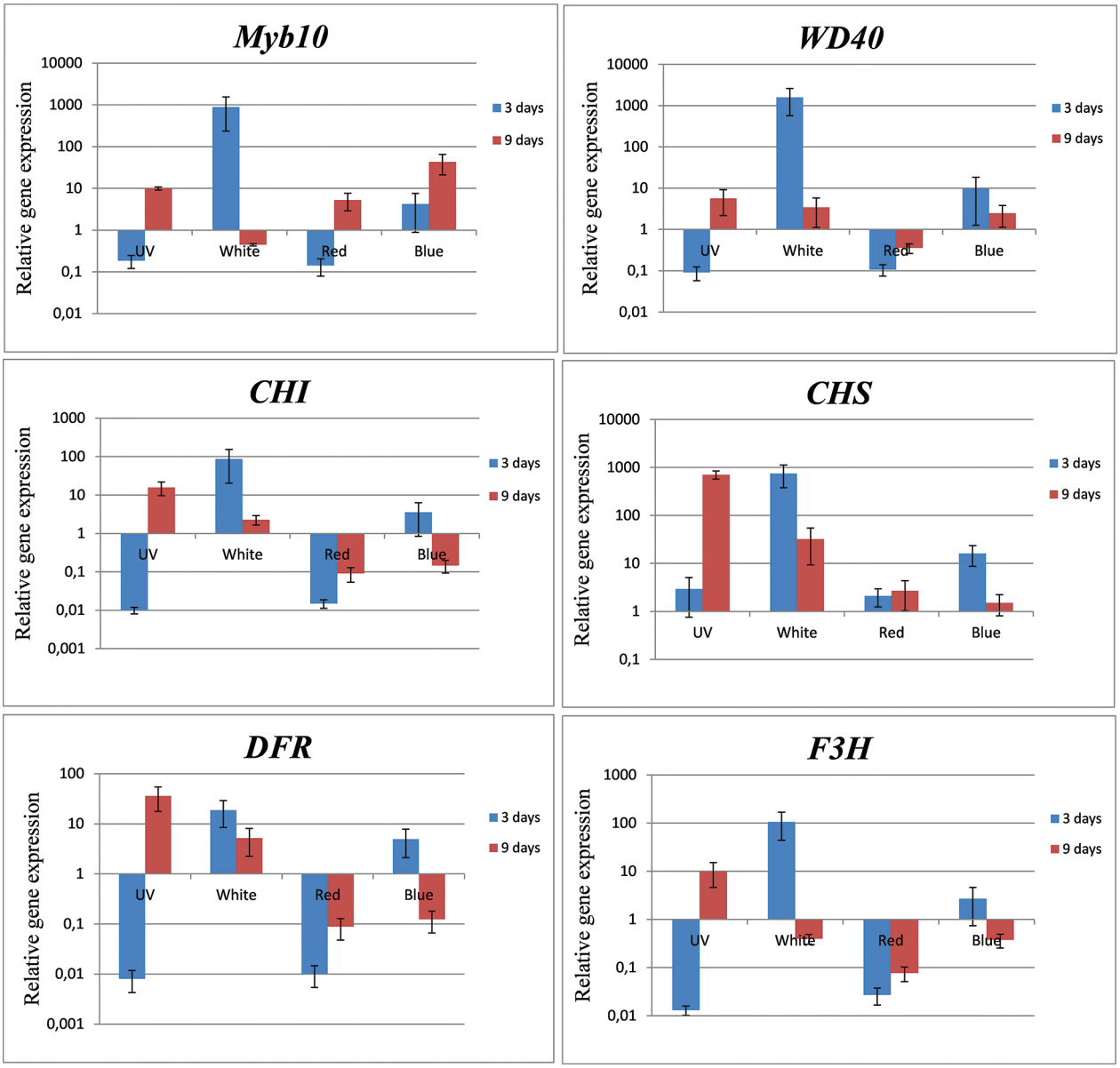
SUPPLEMENTARY MATERIALS

Supplementary Table S1. Primers used for anthocyanin biosynthesis gene expression analysis

Gene	Accession number	Primer sequence	Annealing temperature, °C	Product size, bp
Myb10	EU155163	F: 5′-CGGAAGATTGCCAGGAAGAAC-3′ R: 5′-ATGAAGGTTCGTGGTCGAGG-3′	62.4 63.1	165
WD40-TTG1	XM_004307863	F: 5′-AGCAGGACTTGAGGTACATGG-3′ R: 5′-ACGCAATCGCATTCACACTC-3′	63 62.8	129
EGL	XM_004308329	F: 5′-GCCTTCGATAAACAAGCGGAAG-3′ R: 5′-TCTCTATCAGAACCTCCTGCTC-3′	63.1 61.9	131
UFGT	XM_004307828	F: 5′-GCGCATGGTTCAGTTGGAG-3′ R: 5′-GACCAATCTTCCACACATCCTC-3′	62.8 62	151
DFR	KC894052	F: 5′-GTCTCATTACCGGACTTTCGC-3′ R: 5′-CTCTGCTTTCGGATGCTCG-3′	62.1 61.9	131
F3H	AB201760	F: 5′-CACAGCAGGTTGTCCATAGC-3′ R: 5′-AGTGTAAGTCATCGGCTCCTC-3′	62.2 62.6	114
CHI	XM_004307403	5′-AAAGATCAGACCTTCCCACCC-3′ R: 5′-TCAATCACCGCATTCCCAAC-3′	63 62.4	119
CHS	AB250913	F: 5′-ACTTTTCTGGATTGCACACCC-3′ R: 5′-GTCTTGTGCCCATTAGCTGC-3′	62.6 62.8	189
β-actin*	XM_004306544	F: 5′-TCAACTATGTTCCCTGGTATTGC-3′ R: 5′-CTCCCTTGGAAATCCACATCTG-3′	62 62.1	175

CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; EGL, enhancer of glabra; F3H, flavanone 3-hydroxylase; Myb10, MYB domain protein 10, member of myeloblastosis family transcription factors; UFGT, UDP glucose: flavonol 3-O-glucosyltransferase; WD40-TTG1, approximately 40 amino acid repeat terminating in a tryptophan-aspartic acid (W-D) dipeptide-transparent testa glabra 1.

*β-actin was used as a reference gene.



Supplementary Figure S1. Gene expression in wild strawberry microshoots grown on MS medium under different light spectra. Microshoots grown under fluorescent lamps were used as a control. Bars denote the standard error of the mean. CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3H, flavanone 3-hydroxylase; MS, Murashige and Skoog; Myb10, MYB domain protein 10; WD40, a tryptophan-aspartic acid (W-D) dipeptide.