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Thidiazuron and LED Lighting Enhance Taxifolin and Rutin Production in *Rhododendron mucronulatum* Turcz. Microshoot Culture

Yulianna Zaytseva[1] · **Anastasia Petruk**[1] · **Tatyana Novikova**[1]

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Abstract

Rhododendron mucronulatum Turcz., distributed throughout the northern region of East Asia has been considered to be an alternative natural source of taxifolin (dihydroquercetin) and rutin. The present study was conducted based on a biotechnological approach to develop an environment friendly and efficient system to produce taxifolin and rutin *in R. mucronulatum* microshoots, using different thidiazuron (TDZ) treatments (0.1; 0.5; 2.5 µM) in combination with various types of lighting including fluorescent (FL) and light-emitting diode (LED) (R/B− 80% red + 20% blue; 5LED-20% red + 20% blue + 20% green + 20% yellow + 20% white). The highest number of shoots per explant was obtained under 0.5 µM TDZ combined with 5LED in comparison with FL lighting. Among shoot clusters obtained under different lighting types and TDZ concentrations, a considerable increase in fresh and dry weight was observed in ones cultivated on medium, supplemented with 2.5 µM TDZ under FL and 0.5 µM TDZ at R/B or 5LED. The content of total chlorophylls in *R. mucronulatum* microshoots increased on TDZ-free medium under FL lighting, whereas, the TDZ treatment decreased chlorophylls concentration at FL and 5LED. The use of 0.1 µM TDZ at 5LED decreased the ratio of chlorophylls a + b to carotenoids and led to the highest accumulation of taxifolin and rutin, quercetin, hyperoside, and avicularin. Thus, it has been demonstrated that the application of combined action of LED and TDZ has great potential in terms of propagation efficiency, biomass accumulation, and taxifolin and rutin production in *R. mucronulatum* microshoots.

Keywords Biomass accumulation · HPLC analysis · In vitro culture · Phenolic compounds · Photosynthetic pigments

Introduction

Taxifolin (dihydroquercetin) and rutin have a broad range of physiological activities (Pharmacopoeia of people's republic of China 2005). Taxifolin has angioprotective, antioxidant, detoxification, hepatoprotective (antitoxic), radioprotective, anti-edema, antitumor effects, and stimulates the processes of regeneration of the gastric mucosa. Rutin is of strongly pronounced angioprotective effect, improves vascular microcirculation, prevents premature aging, and protects against allergic reactions (Gene et al. 1996; Hasan and Ahmad 1996; Reynolds and Martindale 1996; Zhong and Ben 1999). Moreover, recent studies have shown that taxifolin and rutin can be natural sources of potential anti-COVID-19 drug candidates (Di Pierro et al. 2021; Prasansuklab et al. 2021). Industrial production of taxifolin and rutin is based on the use of the plant raw materials. Taxifolin is obtained from Siberian larch (*Larix sibirica* Ledeb.) or Dahurian larch (*L. dahurica* Turcz.). The process of extracting a target substance from larch wood is very laborious and requires the use of a large amount of toxic organic extractants, such as ethyl acetate, acetone, hexane, or gasoline, which is incompatible with such a concept as eco-friendly production. In addition, the raw material base for obtaining taxifolin of plant origin is shrinking from year to year due to the massive harvesting of larch and forest fires. The choice of raw materials for the production of rutin is especially important. The buds of Japanese Sophora currently used for this purpose are not promising because of scarcity, and moreover, the use of buckwheat green mass for the production of rutin requires extensive areas of fertile soils for sowing.

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✉ Yulianna Zaytseva
ulianna_zaitseva@mail.ru

[1] Central Siberian Botanical Garden, Siberian Branch of the Russian Academy of Sciences, st. Zolotodolinskaya, 101, Novosibirsk 630090, Russian Federation

R. mucronulatum Turcz. (Ericaceae), a winter-hardy species native to the Russian Far East, Northern China, Mongolia, Japan, and Korea, can be an alternative source of taxifolin and rutin of natural origin. This shrub is highly valued as an herb in folk and traditional medicine (Pharmacopoeia of People's Republic of China 2005; Mok and Lee 2013). Essential oils, phenolic acids, and flavonoids have been isolated and identified in different part of *R. mucronulatum* among them taxifolin and rutin is found to be the main active constituents (Kim et al. 1996; Fu et al. 2012). Moreover, flavonoids identified in *R. mucronulatum* exhibited high aldose reductase inhibitory activity and can be a useful natural source in the development of a novel agent against diabetic complications (Mok and Lee 2013). Antioxidant and tyrosinase inhibitory effects of *R. mucronulatum* leave extracts were shown (An et al. 2005). The inhibitory activity of taxifolin glycoside from *R. mucronulatum* roots on dendritic cell responses and the effect on anti-atopic dermatitis in mice ware reported (Kim et al. 2008; Ahn et al. 2010). Moreover, flavonoids, including taxifolin, isolated from *R. mucronulatum* roots are considered as new antioxidative and anti-inflammatory agents (Choi et al. 2011).

A biotechnological method of taxifolin and rutin production based on plant tissue culture can solve the problems associated with the renewability of raw materials and ecofriendliness of production, and serve as an efficient substitute system to obtain desired natural products. In addition, i*n vitro* produced plants are independent of different external factors like geographical and seasonal variations; they provide a continuous and standardized supply of metabolites with homogenous quality and yield in comparison to the traditional production (Nadeem and Ahmad, 2019). Suspension or hairy root culture is usually used for secondary metabolite production, but these methods have shown their ineffectiveness concerning secondary metabolites of rhododendrons (Taura et al. 2018). The microshoot culture of rhododendrons can be an alternative system for the production of secondary metabolites. For instance, the essential oil accumulation in *R. tomentosum* Harmaja bioreactor-grown microshoots have been established; however, the study on the elicitation of biosynthesis with biotic and abiotic elicitors tested revealed their inability to enhance the production of target substances (Jesionek et al. 2018).

Current research is focused on developing a system based on the application of thidiazuron (TDZ) and light-emitting diodes (LED) as stimulators of phenolic production. In previous work, the stationary microshoot cultures of *R. mucronulatum* based on TDZ treatment as a trigger of shoot morphogenesis have been established (Novikova et al. 2020). TDZ, phenyl urea, is the most suitable plant growth regulator (PGR) for rapid and effective in vitro propagation (Kundu and Gantait 2018; Novikova and Zaytseva 2018). Moreover, TDZ was shown to affect not only endogenous cytokinin and auxin production but the level of secondary metabolites and essential oils in many medicinally important plants (Liu et al. 2007; Wannakrairoj and Tefera 2012; Ali et al. 2018). In addition to the effects of plant growth regulators, changes in physical factors such as lighting conditions can modulate in vitro plant metabolism. The application of LEDs allows obtaining different light spectra regulating the photosynthesis and metabolic activities, as well as optimal morphogenesis pathways for in vitro cultivation of each species (Gupta and Jatothu 2013; Kim et al. 2004a, b, c). The present research aims to study the effect of TDZ and light sources of the different spectrum (fluorescent and LED) on plant growth parameters, biomass accumulation, contents of photosynthetic pigments, and secondary metabolites, including taxifolin and rutin in *R. mucronulatum* shoot culture.

Materials and Methods

Plant Material

Microclones of *R. mucronulatum* were maintained in a collection of the Laboratory of Biotechnology (CSBG RAS, Novosibirsk, Russia) according to our previous study (Zaytseva and Novikova 2018) on Anderson's medium (AM) (Anderson 1984) containing 0.6% Bacto® agar (PanReac®, Barcelona, Spain), 3% sucrose (Shostka Chemical Reagent Factory, Shostka, Ukraine) supplemented with 1.0 µM zeatin (plant cell culture tested, BioReagent, Sigma-Aldrich®, St. Louis, MO). The isolated explants consisting of shoot apex and two nodes with axillary buds were transferred to plant growth regulator-free AM (AM0) and cultured on fresh AM0 during two passages of 4 weeks each, and these shoots were utilized for the experiments. The pH of the medium was adjusted to 5.0 before autoclaving (121 °C; 1.05 kg cm^{-2}). Plant growth regulators (PGRs) were added to the medium post-autoclaving. The cultures were maintained in culture jars (15 mL medium per vessel) at 23 ± 2 °C under cool white fluorescent light (Philips, Pila, Poland) at an intensity of 40 µmol m^{-2} s^{-1} with a 16-h photoperiod.

TDZ Treatments

Single-node microcuttings isolated from *R. mucronulatum* microclones were used as explants for current experiments. The explants were cultured on AM0 (control) and AM supplemented with various TDZ (plant cell culture tested, BioReagent, Sigma-Aldrich®) concentrations (0.1, 0.5, 2.5 µM) for 8 weeks according to the previous study without elongation stage (Novikova et al. 2020).

Lighting Treatments

The explants cultured with use of different TDZ treatments were maintained at uniformly controlled 42 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) with a 16-h photoperiod under cool-white fluorescent lamps (Philips, Pila, Poland) or LEDs of two variants (LED-SIB, Russia): 80% red (660 nm) + 20% blue (450 nm) (R/B) or 20% R + 20% B + 20% green (530 nm) + 20% yellow (590 nm) + 20% white (460, 560 nm) (5LED). Spectral distributions of LEDs were measured by a spectroradiometer (LI-250A, LI-COR®, USA). TDZ-free AM and cool-white fluorescent light (FL) served as the control.

Determination of Growth Parameters

After 8 weeks of TDZ treatments under different light quality, conglomerates of *R. mucronulatum* microshoot were collected and the number of shoots and leaves per explant, shoot length, as well as fresh weight (FW), dry weight (DW), and their ratio was evaluated. FW was estimated by weighing the plant material immediately after harvesting. DW was determined by standard drying method (Thamkaew et al. 2021).

Estimation of Chlorophyll and Carotenoid Pigments

Chlorophyll (Chl) a, Chl b, and carotenoids were analyzed and calculated, as described by Lichtenthaler and Welburn (1983). A batch of 100 mg of de novo conglomerates cultivated for 8 weeks was ground to a fine powder and transferred to 2-mL Eppendorf tube with 1 mL of 100% acetone, and then homogenized for 10 min at 4 °C. The absorbance was measured by a spectrophotometer (SF-56, OKB-Spectr, Russia) at 440, 644, and 662 nm.

Identification of Phenolic Compounds by High-Performance Liquid Chromatography (HPLC)

To study the content phenolic compounds, microshoot conglomerates obtained under TDZ and light treatments were analyzed by HPLC. The collected samples were air-dried in a shadow. For extraction, 100 mg triturated sample was placed in a closed glass vial with 15 ml 50% water–ethanol solution (Chimmed, Russia) and put on a water bath. The extracts were filtered through a 0.45 µm cellulose filter (Interlab®, New Zealand) and were diluted up to the required concentration for further analysis.

Phenolic compounds were determined by comparing the retention times and absorption spectra (250–370 nm) of unknown peaks with the reference standards using Agilent 1200 Series HPLC with a diode detector and equipped with Zorbax SB-C18 column (5 µm, 150 × 4,6 mm) (all from Agilent Technologies, Palo Alto, CA, USA). Mobile phase A was orthophosphoric acid in water (0.1%) and mobile phase B was methanol (Chimmed, Russia). The injection volume was 10 µL and flow rate 1.0 mL/min with gradient program (0–56 min 0–100% B). Stop time of the analysis was 59 min. The investigated samples were analyzed in triplicate. As analytical standards, taxifolin (Austrowaren; Austria), chlorogenic acid, quercetin (Sigma-Aldrich, Germany), rutin, avicularin, quercitrin, and hyperoside (Fluka Chemie AG, Switzerland) were used. The quantitative determination of individual components in the samples was carried out using an external standard according to van Beek (2002).

Data Collection and Statistical Analysis

For experiments with TDZ and types of lighting, tree replications per treatment were taken into account for all experiments. Each replication consisted of 15 explants. To determine the growth parameters, a minimum of 30 measurements of each parameter was taken. For estimating pigments and phenolic compound, all measurements were repeated three times. Collected data were analyzed using two-way analysis of variance (ANOVA) in STATISTICA 8 software StatSoft Inc., Tulsa, OK). Data were presented as means and standard errors (M ± SE). The significance between means was tested by Duncan's means separation test ($P = 0.05$).

Results and Discussion

Effects TDZ and LED on Axillary Shoot Proliferation

Currently, the influence of either the lighting type or plant growth regulators on the morphogenesis and growth parameters of a wide range of plants is being actively studied (Alrifai et al, 2019). In the present study, the combined action of TDZ treatments and types of lighting (FL, R/B, and 5LED) on axillary shoot development of one-node *R. mucronulatum* explants has been examined. The shoot length and the leaf number significantly depended on the type of illumination when explants were cultivated on TDZ-free media. LEDs negatively influenced the shoot length and the leaf number in *R. mucronulatum*. Thus, under R/B and 5LED lighting, the length of newly formed shoots decreased by 2 and 2.5 fold, respectively, compared with the same conditions under FL lighting. This effect could be associated with R and B ratio in tested LEDs, since R and B LEDs have been found to modulate morphogenesis and the grow in different plant taxa at that R LEDs promote elongation but B LEDs inhibit it (Gupta and Jatothu 2013). Moreover, the effect of light quality is likely to depend on plant species, developmental

stage of the plant, and environmental conditions, such as photosynthetic photon flux (Kurilcik et al. 2008).

The number of shoots per explant on the TDZ-free medium did not conversely depend on the type of lighting. However, the addition of TDZ in the nutrition medium and 5LED lighting with LEDs of the yellow and green spectrum along with the R and B has significantly increased the proliferative effect of TDZ, contrary to R/B LED. The maximum number of shoots per explant (24.05 ± 4.14) was obtained under 0.5 µM TDZ and lighting by 5LED with different spectra (Table 1). Thus, TDZ and 5LED had a synergistic influence on axillary shoot proliferation.

It is generally known that the use of R and B LEDs are the most efficient lighting conditions in micropropagation systems for plants; however, their ratio varies among different plant species. The R/B LED ratio of 80:20 is considered to be one of the optimal for plantlet growth (Nhut and Nam 2010; Gupta and Jatothu 2013). To our best knowledge, such a LED ratio has not been found for rhododendrons. However, the proliferation of blueberry (*Vaccinium corymbosum* L.) axillary buds, also a representative of *Ericaceae*, was stimulated by LED lighting with a ratio of R and B as 80:20. (Hung et al. 2016). On the contrary, in this study, the same ratio of R/B LEDs has significantly decreased the proliferative effect of TDZ leading to suppression of *R. mucronulatum* microshoot proliferation.

In the previous research, the efficient micropropagation system of *R. mucronulatum* with the use of TDZ has been developed (Novikova et al. 2020), but the current study showed that the additional application of 5LED made it more productive in terms of scale propagation. The green light plays a special role in plant growth and can penetrate more easily into plant tissues than blue or red light (Klein 1992). Gnasekaran et al. (2021) found the irradiation of monochromic green LED to show the same effect on the propagation rate of *Zingiber officinale* Rosc. as irradiation by FL. It has been reported that the addition of green LED to red and blue promoted the growth of potato seedlings (Ma et al. 2015). Moreover, the use of the green spectrum was also efficient for in vitro propagation of *Gerbera jamesonii* Bolus ex. Hooker F. and *Lamprocapnos spectabilis* L., and it provided a high number of shoots per explant (Miler et al. 2019). In the present study, the addition of white, green, and yellow LEDs to R and B significantly increased the number of shoots per explant of *R. mucronulatum*, but did not eliminate the shortening caused by TDZ. Thus, the treatment with 0.5 µM TDZ and cultivation under 5LED resulted in the maximum realization of the morphogenic potential of one-node explants of *R. mucronulatum* and a fivefold increase in the number of shoots per explant compared with the same treatment under FL lighting.

Trends in Biomass Accumulation

On TDZ-free medium, the types of lighting influenced biomass accumulation. The maximum FW and DW on AM0 medium was noted under FL lighting. In contrast to FL, LEDs reduced the biomass accumulation and FW/DW ratio (Table 2). The presence of TDZ in the culture medium has significantly increased the biomass accumulation and FW/

Table 1 Effect of lighting type and TDZ on axillary shoot proliferation of *R. mucronulatum*

Lighting type	TDZ, µM	Shoot number per explant	Shoot length, mm	Leaves number
FL	0	1.36 ± 0.23 d	16.63 ± 1.55 a	9.63 ± 0.49 a
	0.1	9.30 ± 2.16 c	9.09 ± 1.797 b	8.25 ± 0.74 ab
	0.5	5.80 ± 2.41 cd	6.66 ± 1.67 bcd	7.30 ± 1.11 bc
	2.5	19.10 ± 2.03 b	5.54 ± 0.87 bcd	5.69 ± 0.14 cde
R/B	0	1.84 ± 0.25 d	8.00 ± 0.47 bc	8.32 ± 0.35 ab
	0.1	3.45 ± 0.37 d	5.19 ± 0.49 cd	5.94 ± 0.45 cde
	0.5	3.73 ± 0.32 d	6.87 ± 0.581 bcd	6.10 ± 0.38 b cd
	2.5	0.00	–	–
5LED	0	1.60 ± 0.21 d	6.52 ± 0.46 bcd	8.40 ± 0.32 ab
	0.1	18.65 ± 3.95 b	4.15 ± 0.55 d	4.30 ± 0.39 e
	0.5	24.05 ± 4.14 a	6.85 ± 0.99 bcd	5.35 ± 0.66 de
	2.5	6.72 ± 1.03 cd	3.17 ± 0.34 d	4.22 ± 0.45 e
Significance of two-way ANOVA				
'lighting type'		+ +[x]	+ +	+ +
'TDZ'		+ +	+ +	+ +
'TDZ' ´ 'lighting type'		+ +	+ +	ns [y]

[x]Significant at $p < 0.05$

[y]Not significant

Table 2 Effect of lighting type and TDZ on biomass accumulation in *R. mucronulatum* shoot culture

Lighting type	TDZ, µM	FW, mg	DW, mg	FW/DW
FL	0	0.062 ± 0.008 bcd	0.015 ± 0.004 abc	4.33 ± 0.19 cd
	0.1	0.026 ± 0.009 d	0.004 ± 0.001 cd	6.5 ± 0.27 bc
	0.5	0.091 ± 0.027 bcd	0.013 ± 0.004 abc	7.0 ± 0.32 bc
	2.5	0.344 ± 0.106 a	0.026 ± 0.008 a	13.2 ± 0.47 a
R/B	0	0.010 ± 0.002 d	0.003 ± 0.001 d	3.3 ± 0.16 d
	0.1	0.098 ± 0.014 bcd	0.023 ± 0.005 ab	4.3 ± 0.22 cd
	0.5	0.149 ± 0.025 b	0.026 ± 0.003 a	5.7 ± 0.26 bcd
	2.5	0.033 ± 0.012 d	0.004 ± 0.001 cd	8.25 ± 0.43 b
5LED	0	0.006 ± 0.001 d	0.002 ± 0.000 d	3.0 ± 0.14 d
	0.1	0.054 ± 0.016 cd	0.008 ± 0.002 cd	6.75 ± 0.35 bc
	0.5	0.125 ± 0.034 bc	0.024 ± 0.007 ab	5.2 ± 0.24 bcd
	2.5	0.015 ± 0.004 d	0.002 ± 0.001 d	7.5 ± 0.39 b
Significance of two-way ANOVA				
'lighting type'		+ +[x]	ns [y]	Ns
'TDZ'		+ +	+ +	+ +
'TDZ' ´ ' lighting type'		+ +	+ +	+ +

[x]Significant at $p < 0.05$

[y]Not significant

DW ratio under all types of lighting. The highest FW, DW, and FW/DW were obtained under FL lighting and adding 2.5 µM TDZ in the nutrient medium. At the same time, a high level of FW and DW were observed when explants were cultivated under R/B and 5LED and in the presence of 0.5 µM TDZ, but the FW/DW ratio was 2.5-fold lower than at FL.

An increase in FW and DW under high TDZ concentration has also been shown in TDZ-induced shoot cultures of *Scutellaria alpine* L. (Grzegorczyk-Karolak et al. 2015). This trend of the TDZ effect is associated with the well-known property of this PGR at high concentrations to result in hyperhydricity of regenerants (Dewir et al., 2018; Novikova and Zaytseva 2018). In the present study, LEDs were found to decrease FW/DW ratio and that is possibly correlated with in vitro hyperhydricity of *R. mucronulatum* shoots. This effect has been also observed by Muneer et al. (2018) when R and B LEDs have been shown to play a significant role in alleviating damage, caused by hyperhydricity formed under FL in carnation genotypes. At the same time, the influence of the lighting type on FW, but not on DW of *R. mucronulatum* was detected in the recent study. Miler et al. (2019) have also shown that lighting conditions did not affect the dry matter of *Chrysanthemum grandiflorum*, *Heuchera hybrida*, *Ficus benjamina* L., and *Lamprocapnos spectabilis* L. Moreover, it was found that a significant increase in FW and DW of in vitro-grown *Vaccinium corymbosum* L. cultured under 80R/20B LEDs was achieved only when cytokinin (zeatin) was added to the medium (Hung et al. 2016). The same synergistic effect of optimal TDZ concentration and LED lighting was noted in *R. mucronulatum* biomass accumulation. Since LEDs and TDZ modulate the endogenous cytokinin biosynthesis, as well as plant metabolism generally (Mok et al. 1987; Ruzic and Vujovic 2008), these changes can result in biomass accumulation. Thus, to achieve maximum *R. mucronulatum* biomass harvest, the use of 0.5 µM TDZ and R/B or 5LED lighting is recommended.

Changes in Photosynthetic Pigment Content

The PGRs and light quality directly affect the endogenic plant metabolism promoting the synthesis of numerous important primary and secondary metabolites with the participation of photosynthetic pigments. In the present study, the type of lighting and the addition of TDZ in various concentrations had a significant effect on the content of the photosynthetic pigments. The highest content of chlorophylls was observed in *R. mucronulatum* microshoots cultured on AM0 under FL lighting in contrast to R/B and 5 LED (Table 3). The spectrum of the lighting used had a great influence on the content of photosynthetic pigments. It was reported that white LED lighting promoted higher chlorophyll content than R/B LEDs in the shoot culture of *Moluccella laevis* L. grown on the medium with PGRs (Zielińska et al. 2020). In *Zingiber officinale* var. *rubrum*, total chlorophyll and carotenoid were higher under white, blue, green, and purple (400–660 nm) LEDs than the red only (Gnasekaran et al. 2021).

In spite of the fact that TDZ has been reported to increase the chlorophyll content through in vitro culture of *Dianthus caryophyllus* L. (Genkov et al, 1997) and *Bryum argenteum* Hedw. (Sabovljevic et al. 2010), in current

Table 3 Effect of lighting type and TDZ on photosynthetic pigment content in FW of *R. mucronulatum* in vitro shoot culture

Lighting type	TDZ, µM	Chlorophyll, mg g^{-1}			Carotenoids, mg g^{-1}	(a + b)/carotation
		a	b	a + b		
Fl	0	0.71 ± 0.03 a	1.07 ± 0.05 a	1.78 ± 0.08 a	0.19 ± 0.01 b	9.36
	0.1	0.20 ± 0.01 d	0.31 ± 0.02 e	0.51 ± 0.03 de	0.11 ± 0.01 d	4.63
	0.5	0.32 ± 0.02 bc	0.44 ± 0.01 d	0.75 ± 0.03 c	0.10 ± 0.01 d	7.50
	2.5	0.09 ± 0.01 e	0.14 ± 0.00 g	0.23 ± 0.01 g	0.02 ± 0.00 f	11.50
R/B	0	0.37 ± 0.02 b	0.30 ± 0.01 e	0.59 ± 0.03 d	0.06 ± 0.00 e	9.83
	0.1	0.41 ± 0.02 b	0.59 ± 0.03 bc	1.01 ± 0.05 b	0.26 ± 0.01 a	3.88
	0.5	0.27 ± 0.01 c	0.31 ± 0.02 e	0.58 ± 0.03 d	0.10 ± 0.01 d	5.50
	2.5	0.38 ± 0.02 b	0.52 ± 0.03 c	0.90 ± 0.05 b	0.15 ± 0.01 b	6.00
5LED	0	0.45 ± 0.02 b	0.65 ± 0.03 b	1.10 ± 0.05 b	0.17 ± 0.01 b	6.47
	0.1	0.14 ± 0.01 e	0.17 ± 0.01 g	0.31 ± 0.02 f	0.08 ± 0.00 d	3.88
	0.5	0.28 ± 0.01 bc	0.47 ± 0.02 cd	0.75 ± 0.03 c	0.09 ± 0.00 d	8.33
	2.5	0.16 ± 0.01 e	0.26 ± 0.01 f	0.43 ± 0.02 e	0.10 ± 0.00 d	4.30
Significance of two-way ANOVA						
'lighting type'		++ [x]	++	++	++	
'TDZ'		++	++	++	++	
'TDZ'´ 'lighting type'		++	++	++	ns [y]	

[x]Significant at $p < 0.05$

[y]Not significant

study, TDZ-induced increase of chlorophyll content in *R. mucronulatum* shoot culture was noted under R/B LED lighting only. Moreover, the presence of TDZ in the nutrient medium reduced the chlorophyll content both at FL and at 5LED lighting. (Table 3). On the other hand, under R/B LED lighting, TDZ increased the total chlorophyll content, mainly due to an increase in the chlorophyll b content. The maximum content of carotenoids was noted in the presence of 0.1 µM TDZ under R/B LED lighting (Table 3). The same effect of LED-induced growth and carotenoids production has been noted in *Digitalis purpurea* L. leaf tissue (Kumar Verma et al. 2018).

The value of the ratio of the chlorophyll sum to carotenoids is one of the indicators of plants stress, which activates the defense response of the plant triggering the gene expression modulating the biosynthesis and accumulation of the secondary metabolites. In *R. mucronulatum*, this parameter did not significantly change during cultivation on AM0 at FL and R/B lighting but decreased under 5LED lighting (Table 3). For the most part, the presence of TDZ decreased the (a + b)/carotenoids ratio, shifting it towards an increase in the carotenoid content. The minimum (a + b)/carotenoids ratio was noted during cultivation on AM supplemented with 0.1 µM TDZ under R/B and 5LED lighting. Thus, this type of treatment is assumed to cause the most pronounced stress in *R. mucronulatum* microshoots and can enhance secondary metabolite production.

Response of Phenolic Compounds to TDZ and LEDs

The production of a wide range of flavonoids is possible by controlling the type and concentration of exogenic PGRs, as well as lighting conditions (Ibrahim and Jaafar 2012). The content of the main phenolic compounds including chlorogenic acid, dihydroquercetin, quercetin, hyperoside, rutin, avicularin, and quercitrin was analyzed by HPLC in extracts of *R. mucronulatum* microshoots.

The highest levels of the phenolic compounds tested were noted in microshoots cultivated under 5LED lighting, except chlorogenic acid. At the same time the maximum levels of taxifolin, rutin, quercetin, hyperoside, and avicularin were detected in the extracts of microshoots treated by 0.1 µM TDZ, and maximum quercitrin content was observed in the extracts of microshoots from TDZ-free medium. However, the trend of TDZ action was found to be different in FL and LEDs lighting conditions. The phenolic compounds content in extracts of microshoots treated by FL only was at a low level, except for chlorogenic acid (Table 4). Under FL lighting, the presence of 0.5 µM TDZ more than doubled the flavonoid content, and taxifolin, quercetin, and rutin—threefold compared with the control variant. In contrast, the content of chlorogenic acid decreased in the presence of TDZ or under FL and both LED types. Under R/B LED lighting, the content of phenolics in microshoot extracts cultivated on AM0 increased,

Table 4 Effect of lighting types and TDZ on content of phenolic compounds in extracts of *R. mucronulatum* in vitro shoot culture

Lighting type	TDZ, µM	Chlor acid, mg g^{-1} of DW	Ta, mg g^{-1} of DW	Q, mg g^{-1} of DW	Hy, mg g^{-1} of DW	Rutin, mg g^{-1} of DW	Avi, mg g^{-1} of DW	Qtr, mg g^{-1} of DW
FL	0	4.91 ± 0.01 a	6.41 ± 0.02 j	0.83 ± 0.02 l	4.51 ± 0.02 j	4.87 ± 0.01 j	11.90 ± 0.01 h	5.26 ± 0.04 g
	0.1	1.91 ± 0.02 g	8.46 ± 0.02 f	1.15 ± 0.02 k	4.72 ± 0.02 i	4.97 ± 0.03 i	11.64 ± 0.03 i	7.54 ± 0.06 f
	0.5	4.87 ± 0.02 a	19.16 ± 0.03 b	3.38 ± 0.02 f	11.77 ± 0.05 g	11.21 ± 0.02 e	11.85 ± 0.04 hi	6.96 ± 0.03 f
	2.5	1.47 ± 0.02 h	9.16 ± 0.02 d	2.70 ± 0.01 g	4.34 ± 0.03 k	3.93 ± 0.03 k	11.73 ± 0.03 ij	4.51 ± 0.07 h
R/B	0	4.49 ± 0.01 b	8.84 ± 0.03 e	5.87 ± 0.09 c	14.95 ± 0.05d	14.01 ± 0.05 d	31.11 ± 0.05 b	23.76 ± 0.07 c
	0.1	2.46 ± 0.01 e	7.63 ± 0.05 g	3.92 ± 0.01 e	12.29 ± 0.03f	8.02 ± 0.04 g	26.15 ± 0.04 e	10.61 ± 0.03 e
	0.5	2.42 ± 0.01 e	6.89 ± 0.02 i	2.24 ± 0.03 h	8.43 ± 0.02 h	6.17 ± 0.03 h	14.59 ± 0.09 g	5.33 ± 0.04 g
	2.5	2.75 ± 0.02 d	3.49 ± 0.03 l	2.06 ± 0.03 i	14.68 ± 0.03 e	14.26 ± 0.04 c	17.28 ± 0.04 f	3.69 ± 0.05 i
5LED	0	2.24 ± 0.01 f	5.81 ± 0.02 k	7.64 ± 0.04 b	15.19 ± 0.02 c	16.61 ± 0.01 b	28.88 ± 0.03 c	37.31 ± 0.70 a
	0.1	3.02 ± 0.03 c	23.40 ± 0.02 a	8.11 ± 0.01 a	28.66 ± 0.05 a	23.58 ± 0.04 a	49.45 ± 0.07 a	24.47 ± 0.03 b
	0.5	2.26 ± 0.02 f	11.51 ± 0.02 c	5.25 ± 0.02 d	16.44 ± 0.03 b	10.9 ± 0.03 f	27.24 ± 0.04 d	22.74 ± 0.03 d
	2.5	1.07 ± 0.03 i	1.04 ± 0.02 m	1.33 ± 0.01 j	4.64 ± 0.03 i	2.76 ± 0.04 l	3.71 ± 0.06 k	1.08 ± 0.4 j
Significance of two-way ANOVA								
'lighting type'		+ + [x]	+ +	+ +	+ +	+ +	+ +	+ +
'TDZ concentration'		+ +	+ +	+ +	+ +	+ +	+ +	+ +
'TDZ concentration * 'lighting type'		ns [y]	+ +	+ +	+ +	+ +	+ +	+ +

Chlor acid chlorogenic acid, *Ta* taxifolin, *Q* quercetin, *Hy* hyperoside, *Avi* avicularin, *Qtr* quercitrin

[x]Significant at $p < 0.05$

[y]Not significant

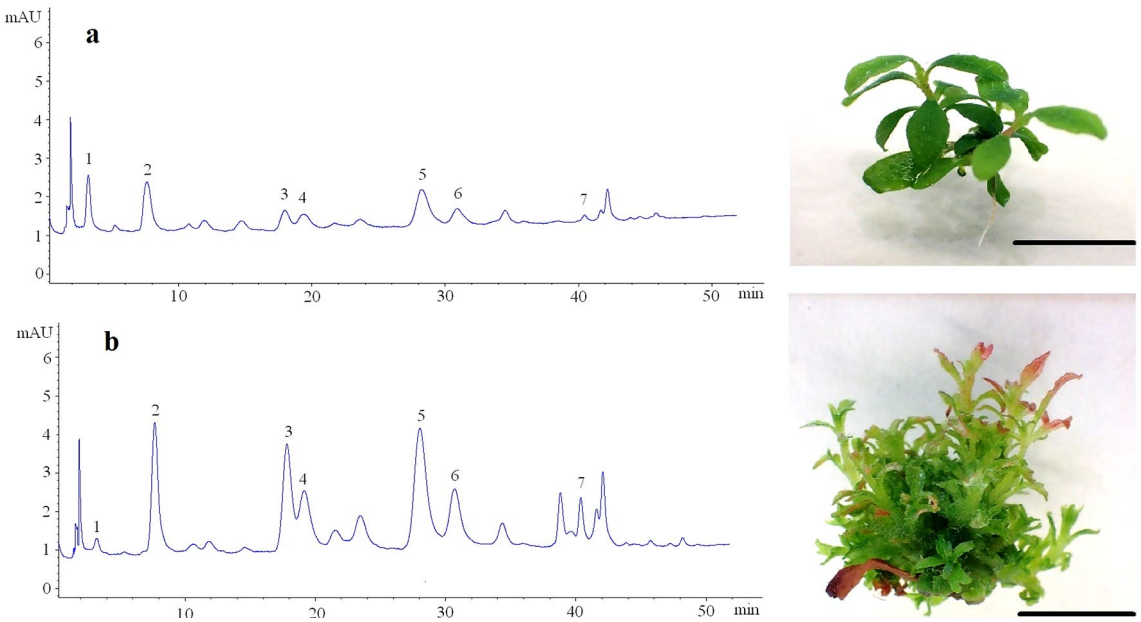

Fig. 1 Chromatogram of a water–ethanol extract of *R. mucronulatum* microshoots cultivated on TDZ-free AM and FL lighting (**a**) and 0.1 µM TDZ and 5LED (**b**). X-axis—retention time, min; on Y-axis—detector signal, in units of optical density. The peak number: (1) Chlorogenic acid; (2) Taxifolin; (3) Hyperoside; (4) Rutin; (5) Avicularin; (6) Quercitrin; (7) Quercetin. Bar—10 mm

compared with FL lighting, except for chlorogenic acid. However, the addition of TDZ in the tested concentrations reduced the content of all investigated flavonoids in the extracts (Table 4). Under 5LED lighting, the content of quercetin, hyperoside, rutin, and quercitrin increased at 0.1 µM TDZ and decreased under higher TDZ concentrations (Fig. 1).

It is known that light quality significantly affects the production of secondary metabolites, especially, those with strong antioxidant activities, such as phenolics (Alrifai et al. 2019). The modulating effect of LEDs has been studied both in vivo and in vitro. For example, when growing broccoli (*Brassica oleracea* L.), it was demonstrated that the exposure by R LED enhanced quercetin production, compared with supplementary B or white LED (Steindal et al. 2016). In contrast, *Cyclocarya paliurus* Batal. grown in vivo under B (456 nm) LED accumulated higher total phenolic compounds, specifically kaempferol, isoquercitrin, and quercetin, compared with white LED light. It was shown that B (456 nm) LED induced a significant increase in the accumulation of phenolic metabolites in vitro (Kawka et al. 2017). The same trend was noticed in *Schisandra chinensis* Turcz., *Aronia melanocarpa* Michx, and *Verbena officinalis* L. tissue culture (Szopa and Ekiert 2016; Kubica et al. 2017; Szopa et al. 2017, 2018). The lighting *R. mucronulatum* shoot culture 5LED significantly increased taxifolin, quercetin, and rutin, as well as quercitrin, hyperoside, and avicularin, production in microshoot extracts.

The flavonoid biosynthesis in in vitro cultures has been found to be modulated significantly by exogenous auxins and cytokinins (Abbas et al. 2021). It was reported that auxins decrease quercetin production in the cell culture of *Astragalus missouriensis* Nutt., by contrast, cytokinins positively affect quercetin production (Ionkova 2009). Moreover, optimal combination of PGRs enhances accumulation of quercetin in *Citrullus colocynthis* (L.) Schrad. culture (Tanveer et al. 2012). TDZ is known to induce notable changes in the metabolism of endogenous cytokinins and auxins; therefore, it could affect phenolic content. However, currently, there are only a few reports on TDZ-induced accumulation of phenolic compounds. TDZ-induced maximum phenolic and flavonoid content was detected in shoot cultures of *Ajuga bracteosa* Wall. ex Benth. and *Linum usitatissimum* L. (Ali et al. 2018; Khan et al. 2020). In the present study, the treatment by low TDZ concentrations enhanced the flavonoid production in *R. mucronulatum*. Moreover, the synergistic effect of TDZ and LED on flavonoid production was demonstrated. These results are consistent with the reported significant increase in total phenolic content after culturing callus of *Cynara cardunculus* L. subsp. *scolymus* (L.) Hegi Fiori. on a medium containing the lowest of tested TDZ concentrations (Abbas et al. 2021).

Conclusions

The present study has revealed the regulating potential of the light quality (FL, R/B, and 5LED) in combination with TDZ during the process of *R. mucronulatum* in vitro shoot culture to enhance the synthesis of secondary metabolites, including taxifolin and rutin. It was found that light quality and TDZ possessed a synergetic effect on key parameters, such as plant growth, biomass accumulation, contents of photosynthetic pigments, and flavonoids. The combination of 0.1 µM TDZ and 5LED was found to be optimal to promoting maximum taxifolin and rutin accumulation, as well as quercetin, hyperoside, and avicularin content. This approach makes it possible to achieve 2.34% of the taxifolin yield from the dry mass. To our best knowledge, this is the first report on taxifolin and rutin production based on TDZ-derived *R. mucronulatum* microshoot culture.

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Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

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