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Responses of Micropropagated Rhubarb (*Rheum rhaponticum*) Plantlets to Different Growing Media and Light Conditions in the Greenhouse

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Abstract: Cultivating red-stalked rhubarb plants is an important source of raw materials for producing health-promoting foods. The quality and quantity of rhubarb crops are significantly dependent on planting material. To obtain high-quality planting material for the value selection of the rhubarb 'Raspberry', we evaluated the morphological and physiological responses of micropropagated plantlets to different growth substrates and light quality during early growth ex vitro in the greenhouse. The plantlets were grown in high-EC (GM1) and low-EC (GM2) peat substrates under four light-emitting diodes (LED) light treatments as supplementary lighting (SL) in the wintertime: 100% red (R), 100% blue (B), white light [44.4% green (G), 24.4% B, 28.9% R; 2.2% far red (FR)] and R+B+G+FR (49.4/16.3/10.3/23.8%) light. Compared to the control (natural sunlight), applied LED lighting significantly increased all growth parameters, but only in plantlets grown in GM1 substrate. Among LED treatments, R+B+G+FR light had the most stimulative effect on all growth parameters (length of leaf petioles, leaf area, biomass) and soluble sugar production. Still, it decreased the levels of phenolic compounds in the leaf petioles. Phenolic synthesis, mainly anthocyanins, was the highest under white light (622.8 mg·100 g^{-1} dry mass), followed by red (601.8 mg·100 g^{-1}), blue (464.4 mg·100 g^{-1}), and R+B+G+FR light (416.4 mg·100 g^{-1}). High anthocyanin accumulation under R-LED light was associated with high antioxidant activity and growth cessation. Hence, for optimal effects related to plant growth and anthocyanin biosynthesis, the use of W-LED lighting is recommended for the early growth ex vitro of micropropagated rhubarb plantlets.

Keywords: antioxidant activity; anthocyanins; ex vitro growth; LEDs; soluble sugars

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1. Introduction

Garden rhubarb (syn. culinary rhubarb) is a well-known horticultural plant valued for its stalks (leaf petioles), which are a rich source of biologically active compounds, such as polyphenols, including anthocyanins, and organic acids and fibre [1,2]. It is commonly called *Rheum rhaponticum* (European rhubarb), *R. rhabarbarum*, *R.* × *hybridum* or *R.* × *cultorum* [3,4]. The first culinary rhubarb cultivars were believed to be hybrids between *R. rhaponticum* L., *R. undulatum* L. (syn. *R. rhabarbarum* L.), species initially brought to Europe for medicinal purposes, and unknown *R. hybridum* [5–7]. Red-stalked rhubarb cultivars are of great commercial value due to their visually attractive colour, use in the food industry as natural colourants, and pro-health value [6,7]. Rhubarb stalks are used in making marmalades, juices, and marinades. Adding rhubarb to some products, including strawberries, apples, or pumpkins, may result in acidity, stability of polyphenolic compounds, reduction of unfavourable colour, and increased antioxidant properties [8].

The profitability of growing rhubarb as a source of bioactive substances is largely determined by the planting material, its health, and genetic identity. Due to the low efficiency of rhubarb propagation by crown division, the common method is propagation by seeds. The cultivation of seedlings very often provides for low yield size and quality

(green leaf petioles). Tissue cultures are helpful for the vegetative propagation of virus-free rhubarb plants and the rapid multiplication of valuable selections with the highest possible content of bioactive ingredients [9]. However, many garden rhubarb genotypes are recalcitrant in vitro. It is due to its ploidy level ($2n$ = 44), which determines the size of leaf petioles. On the other hand, tetraploids show lower regeneration potency and adaptation to ex vitro conditions [10]. Moreover, micropropagated plantlets differed from those propagated by traditional methods. The specific in vitro conditions (high humidity, low irradiance, high sugar, and high mineral contents) result in abnormal morphology, anatomy, and physiology of the plantlets. Transitioning from in vitro to ex vitro conditions is very stressful for delicate microplants [11,12]. Our previous study showed that the increased temperature during greenhouse growth in the spring resulted in rapid growth cessation, leaf senescence, and endodormancy development of micropropagated rhubarb plantlets. A similar response was noticed for rhubarb plantlets grown in the phytotron under a 10-h photoperiod [13]. To date, no studies have attempted to investigate the effect of light quality on the growth of young rhubarb plantlets.

Light, as a primary energy source, governs the main processes of nutrition and development, including carbohydrate fixation, nitrogen assimilation, and amino acid biosynthesis. Moreover, it plays a crucial signalling and regulatory role in developmental and metabolic processes. Plants sense changes in photon flux density (PFD between 380 and 750 nm), daily light integral (DLI), quality (wavelength and spectral ratios), and timing (photoperiod) by specific photoreceptors. These photoreceptors translate light energy into biological signals and activate downstream pathways to regulate plant growth, morphogenesis, and primary and secondary metabolism [14]. Light-emitting diodes (LEDs) light sources provide an opportunity for directly intervening in plant growth, development, and metabolism [15,16]. Numerous studies reported the successful applications of LEDs in promoting in vitro growth and morphogenesis. However, the optimal light spectra significantly differ between plant species, cultivars, and developmental stages [17]. Moreover, only a few studies have evaluated the effect of LED lighting at the acclimatisation stage and early growth ex vitro. For example, Chuang et al. [18] obtained better acclimatisation of *Malus* rootstock plantlets to ex vitro conditions using mixed LEDs (particularly 80% red, 10% blue and 10% green light) compared to monochromatic red, blue, or green light. Similarly, Tarakanov et al. [19] reported a beneficial effect of mixed LEDs (46% red, 30% green, 23% blue) on the survival rate, photosynthesis, and growth of *Rubus idaeus* 'Orange Miracle' plantlets. There is no information on the effect of LED lighting on the ex vitro growth of micropropagated rhubarb plantlets.

In addition, the type of growth substrate has been shown to affect the survival rate of micropropagated plantlets under ex vitro conditions [20,21]. It is increasingly evident that the mineral nutrition status of plants greatly affects their ability to adapt to adverse environmental conditions [22].

The study aimed to determine the influence of the growing substrate and the supplementary LED light spectrum on the early ex vitro growth in the greenhouse of micropropagated rhubarb 'Raspberry' plantlets. Identification and quantification of phenolic compounds and soluble sugars by the HPLC method were performed to evaluate the rhubarb response plantlets' light quality.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

In vitro propagated plantlets of the selected genotype of rhubarb 'Raspberry' (Polish name 'Malinowy') were used for the study. The plants were propagated in vitro by the method developed by Wojtania and Mieszczakowska-Frąc [9]. For acclimatisation, in vitro rooted shoots were planted in multicellular trays of 30 mm diameter in substrates based on sphagnum peat with the addition of perlite under high humidity conditions. Acclimatisation occurred in a phytotron (25 ± 2 °C; PPFD—50–60 µmol m^{-2} s^{-1}). After 10 days, the shoots were fed 0.1% Kristalon Green (Yara Vlaardingen B.V., Vlaardingen,

The Netherlands), containing 18:18:18 (*w*/*w*/*w*) NPK. When microplants formed a well-developed root system (6 weeks), they were used for further ex vitro adaptation in the greenhouse with various light and substrate treatments.

The experiments were conducted in a high-tech greenhouse at the National Institute of Horticultural Research in Skierniewice, Poland, from 2 January to 25 February 2020. Micro-cuttings were transplanted to 77 × 75 × 70 mm multicellular trays with two growing media: GM1—Floragard peat substrate (Vertriebs, Germany) + perlite (4:1) and GM2—neutralised sphagnum peat (SIA "Florabalt SIA"; Latvia) + perlite (4:1). The chemical and physical properties of the components are presented in Table 1. Plants were manually watered as needed to maintain adequate soil moisture. Fertigation was carried out once a week with 0.1% Kristalon Green. The temperature during the day/night was set to 21/18 °C, and the relative humidity was set to 60–65%.

Table 1. Initial nutrient concentrations and selected physical and chemical properties of the substrates.

Parameter	Floragard Substrate (GM1)	Neutralised Sphagnum Peat (GM2)
pH (H_2O; 1:2)	6.2	6.2
EC (mS cm^{-1})	1.14	0.47
N-NO_3 (mg·dm^{-3})	93.0	19.0
N-NH_4 (mg·dm^{-3})	87.5	<1.0
P-PO_4 (mg·dm^{-3})	85.0	39.0
K (mg·dm^{-3})	193.0	68.0
Mg (mg·dm^{-3})	111.0	111.0
Ca (mg·dm^{-3})	1151.0	1915.0
Na (mg·dm^{-3})	10.0	10.0
Cl^- (mg·dm^{-3})	38.0	44.0
S-SO_4 (mg·dm^{-3})	84.0	27.0
Fe (mg·dm^{-3})	8.05	10.1
Mn(mg·dm^{-3})	4.7	2.24
Cu (mg·dm^{-3})	1.2	0.83
Zn (mg·dm^{-3})	2.2	1.9
Organic matter (%)	91.9	90.0
Total pore space (%)	94.7	95.3
Bulk density (g·cm^{-3})	85.7	76.7
Water volume at −10 cm H_2O (%)	68.3	60.0
Air volume at −10 cm H_2O (%)	26.4	35.3

2.2. Light Treatments

The plantlets were grown in a greenhouse chamber divided into five areas, each isolated from any external light source. The zones were lit up by different spectra of LED light (Osram, Munich, Germany): R—red (660 nm); W—white (cool-white diodes, 5000 Kelvin); colour-mixtures of R, B, G, and FR—far-red (730 nm), and B—blue (450 nm) with a 16-h photoperiod and photosynthetic flux density (PPFD) at 125 µmol m^{-2} s^{-1}. The relative spectra of the light treatments are shown in Table 2 and Figure 1. Without supplemental lighting, the control plants were grown only in daylight (natural day length: 8–9 h). The average DLI inside the greenhouse (natural sunlight) during the experimental period was 1.4 mol m^2 d^{-1}. The PPFD measurements and spectral quantification were performed using a spectrometer, GL Spectrolux (GL Optic, Puszczykowo, Poland).

Table 2. The percentage share of individual colours in light-emitting diodes LED light treatments and control (natural sunlight in a greenhouse). The fraction of the integral photon flux ranged from 340 to 780 nm in ultraviolet, blue, green-yellow, red, and far-red). Spectra were recorded and averaged at six locations.

LED Light Treatments	UV-A 340–400 nm	Blue 400–500 nm	Green-Yellow 500–600 nm	Red 600–700 nm	Far-Red 700–780 nm	R:FR Ratio
Control	3.9	22.1	28.0	27.4	16.8	1.6
R-Red	-	-	-	100.0	-	-
W-White	0.1	24.4	44.4	28.9	2.2	11.3
R+B+G+FR	0.2	16.3	10.3	49.4	23.8	2.1
B-Blue	-	100.0	-	-	-	-

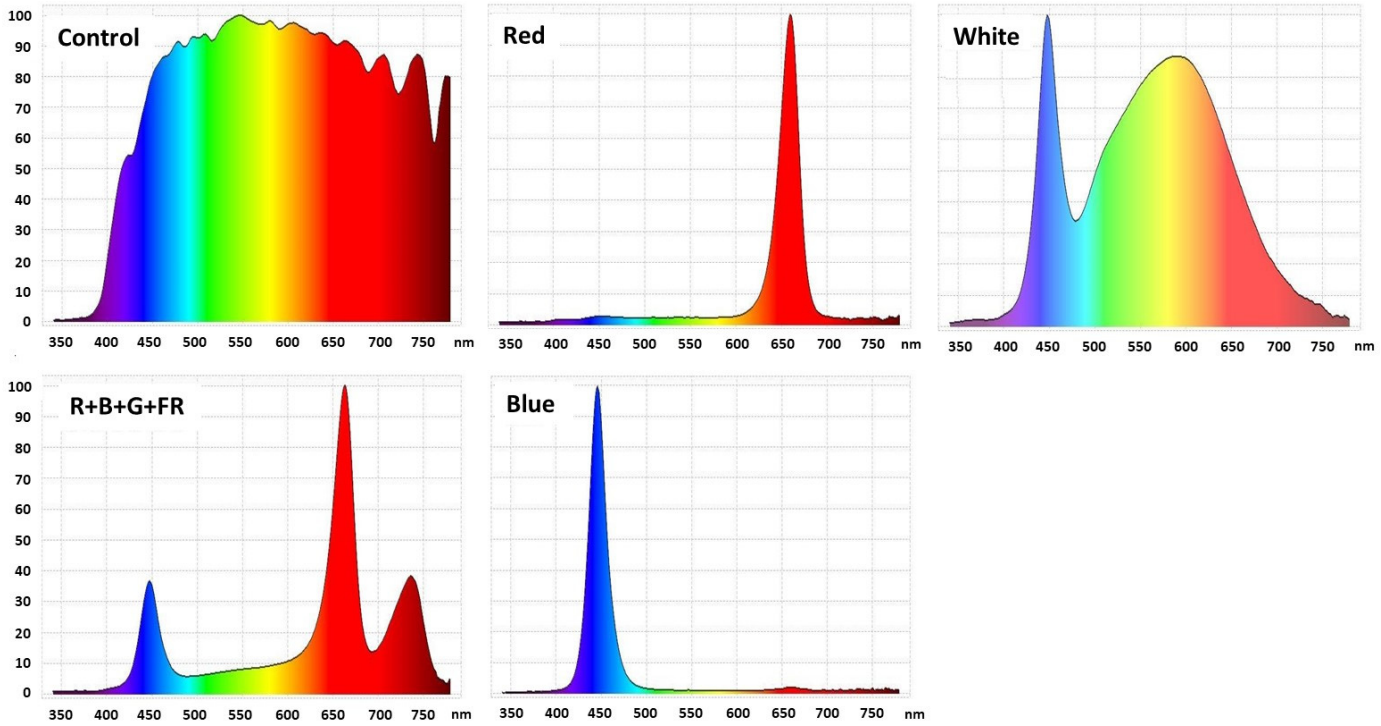

Figure 1. Spectral characteristics of lighting treatments: control—natural light inside the greenhouse in the winter, R—red, W—white, G—green, FR—far red, B—blue R+B+G+FR.

2.3. Plant Growth Parameters

The plant survival rate, petiole length, leaf area, and visual estimation of the roots were determined 6 weeks after the transfer of rhubarb plantlets to greenhouse conditions. The root system assessment was conducted on a three-point scale: 1—no visible roots or single roots, 2—a poorly developed root system, and 3—a well-developed root system. For plantlets growing in GM1, the growth parameters of the underground parts (the length of leaf petioles, leaf area, and mass) were determined after 1, 6, and 10 weeks of growth in the greenhouse. Additionally, ten plantlets were selected randomly and pooled to determine the contents and types of soluble sugars and phenolic compounds (anthocyanins, flavonols, flavan-3-ols, and phenolic acids) in the leaf petioles and the antioxidant activity.

2.4. Phenolic Compound Analysis

An HPLC analysis of phenolic compounds was performed based on the method represented by Nielsen et al. [23], with some modifications. Approximately 50 mg of lyophilised rhubarb powder was extracted in 2 mL of 60% methanol acidified with formic acid (1%) for 20 min in the ultrasonic bath. The mixture was centrifuged for 10 min (7000× *g*). All extracts before HPLC injection were filtered using a 0.45 µm PTFE filter.

The polyphenols separation was performed using a Phenomenex® Fusion RP column (250 mm × 4.6 mm; particle size 4 µm) on an Agilent 1200 HPLC system equipped with a DAD detector. The elution conditions were as follows: 1 mL min^{-1}, temperature 25 °C, wavelength: 520 nm (anthocyanins), 360 nm (flavonol), 320 nm (phenolic acids), and 280 nm (flavan-3-ols); mobile phase consisting of water: formic acid (95:5 (*v*/*v*)) (A), and acetonitrile (B) in gradient flow. The calculations of polyphenols were quantified by calibration curves with the standards of cyanidin-3-glucoside and cyanidin-3-rutinoside, rutin, chlorogenic acid, and (+)-catechin. The polyphenol content was expressed in mg·100 g^{-1} DM.

2.5. Soluble Sugar Analysis

A HPLC analysis of sugars (sucrose, glucose, and fructose) was carried out with an HP 1200 system (Waldbronn, Germany) equipped with a RI detector, according to European Standard EN 12630. The sugars were separated on a Bio Rad Aminex-87C column (300 × 7.5 mm) by water purified by MiliQ System (Milipore, Burlington, MA, USA) as a mobile phase. The isocratic flow was 0.6 mL·min^{-1}, and the column temperature was 80 °C. The lyophilised powder (100 mg) was extracted in 4 mL of redistilled water for 20 min in an ultrasonic bath, and the obtained suspension was centrifuged (7000× *g*, 10 min). Before HPLC determination, the resulting sugar extract was filtered through a Sep-Pak® PLUS C18 filter (Waters). The sugars were quantified by calibration curves for sucrose, glucose, and fructose, and the results were expressed as mg·100 g^{-1} dry mass (DM).

2.6. Antioxidant Activity Measurement

Free-radical scavenging activity was determined using the $ABTS^{\bullet+}$ radical cation [24,25]. The sample extract (500 µL) was mixed with an $ABTS^{\bullet+}$ solution (5 mL). Then, for 6 min, the reaction was carried out, and the mixture was kept at room temperature in a dark place. Absorbance was measured at 734 nm using a spectrophotometer, the Cary 3E UV-Visible (Varian). At least four measurements were made for each sample at different concentrations to reduce the initial $ABTS^{\bullet+}$ solution absorbance from 20% to 80%. The linear regression method was applied to calculate the content of the sample, leading to a 50% decrease in the $ABTS^{\bullet+}$ solution absorbance, and recalculated to Trolox equivalents as mmol per 100 g of freeze-dried rhubarb. The extract was prepared as follows: 100 mg of freeze-dried rhubarb was mixed with 10 mL of 70% methanol and then extracted for 20 min in an ultrasonic bath. The suspension was filtered through a Whatman No. 3 filter paper.

2.7. Statistical Analyses

The experiment comprised 240 plantlets (10 multicellular trays) and 48 plantlets (2 multicellular trays) for each of the 5 light treatments. Data were subjected to a one-factor and two-factor analysis of variance with STATISTICA software, version 13.1 (StatSoft Inc., Tulsa, OK, USA). The significance of the differences between means was evaluated by Duncan's test at $p = 0.05$. Also, Pearson's linear correlation was calculated for the chosen parameters.

3. Results and Discussion

3.1. Growth and Morphological Characteristics of Plants

The early growth ex vitro of micropropagated rhubarb plantlets was significantly affected by growing media (Figure 2). The plantlets showed significantly higher survival rates, and better growth and development of the leaves and secondary root system when they were grown in the GM1 (the higher EC-Floragard substrate) than those in the GM2 (the lower EC-neutralised sphagnum peat) (Figure 2). In general, sphagnum peat is a widely used substrate for acclimatisation and ex vitro growth of micropropagated plantlets. It offers many advantages, such as low bulk density, pathogen-freeness, high water retention capacity, and easy root penetration [26,27]. Sphagnum peat moss does not naturally have a high mineral content because it forms in nutrient-poor areas. Many plant species, including *Lonicera caerulea* [28], *Helleborus niger* [29], *Fragaria* × *ananassa* [20], and *Ananas*

comosus [30] showed successful ex vitro growth in sphagnum peat when only fed with commercially available fertiliser. There are various types of peat, which may be classified based on the plant origin of the peat, its degree of decomposition, and its nutrient content [27,31]. Both substrates used in this study are based on Latvian white peat moss of botanical composition: *Sphagnum fuscum* (75%), *Sphagnum angustifolium* (5%) and *Eriophorum vaginatum* (15%). As was shown in Table 1, the main differences in substrate composition were in NPK and S levels. This suggests that micropropagated rhubarb plantlets need high levels of nutrients to initiate growth and leaf formation at the stage of early growth ex vitro.

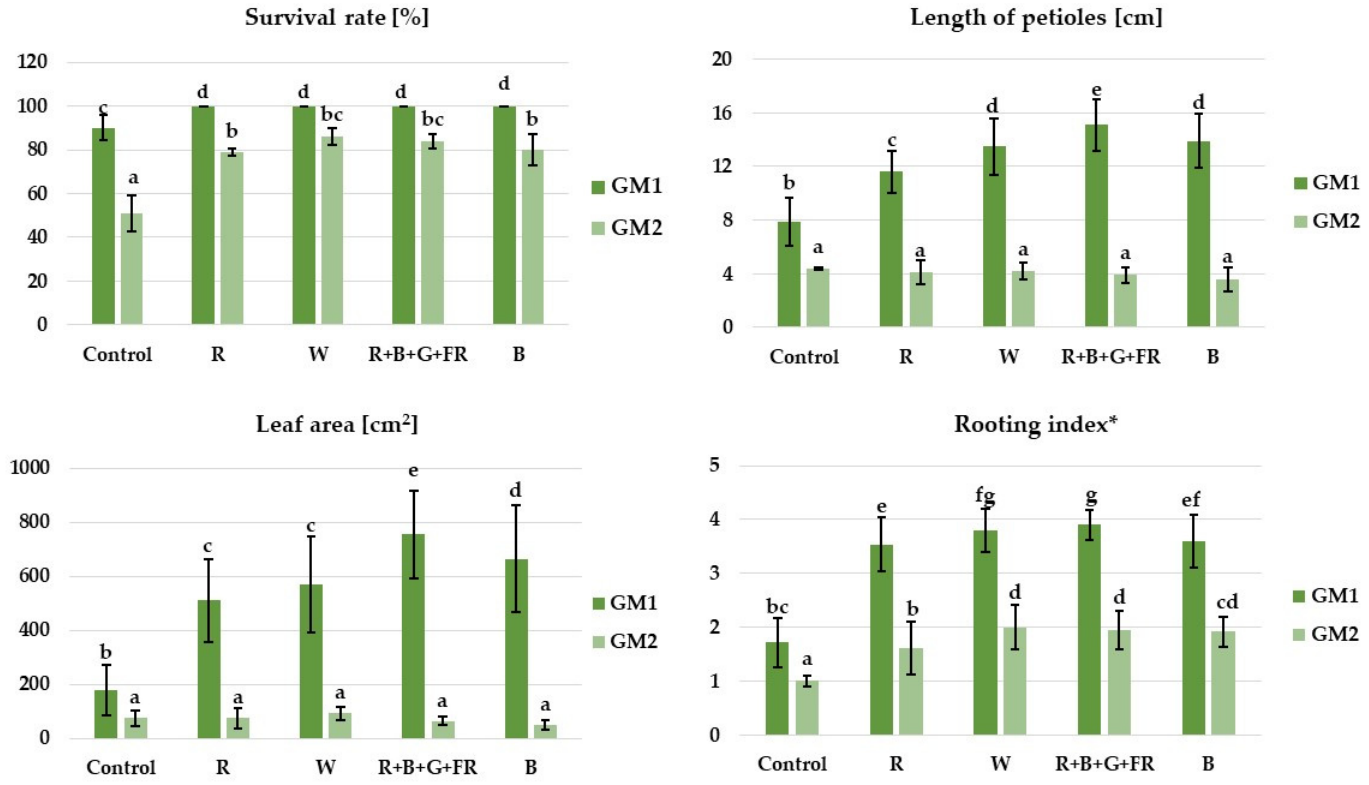

Figure 2. The effect of growth substrates (GM1—Floragard substrate; GM2—neutralised sphagnum peat) and supplementary lighting by different LEDs (control—natural light, R—red, W—white, B—blue, R+B+G+FR—far red) on the growth rate responses of *Rheum rhaponticum* 'Raspberry' after 6 weeks of growth under a 16-h photoperiod in the greenhouse. Bars represent means ± SE. According to Duncan's test, the means of each growth parameter indicated with the same letter do not differ significantly. * root system assessment was conducted on a three-point scale: 1—no visible or single roots, 2—a poorly developed root, and 3—a well-developed root system.

In addition, a significant interaction between substrate type and light treatment on the growth of the leaves and root system has been observed (Table 3). The micropropagated rhubarb plantlets grown in GM2 and under limited light conditions (control) characterised by the lowest growth rate and 51% survival frequency (Figure 2). Supplemental lighting with different light spectra generated by LEDs enhanced the root system development and survival rate of plantlets growing in low EC-GM2 but had little effect on the growth of rhubarb leaves (Figure 3). In the case of plantlets grown in GM1, LED lighting increased total leaf length by 91.0%, total leaf area by 318.5%, rooting rate by 129.4%, survival by 10% (Figure 2), and the mass of petioles by 290% (Figures 2 and 4). Moreover, all growth parameters were significantly affected by light quality.

Table 3. F-statistic from a two-way analysis of variance for light treatment and substrate type during the ex vitro growth of rhubarb 'Raspberry' in the greenhouse.

Trait/Interaction	Leaf Petiole Length		Leaf Area [cm^2]		Rooting Index	
	F	*p*	*F*	*p*	*F*	*p*
Light	45.8	<0.000 ***	37.0	<0.000 ***	134.2	<0.000 ***
Substrate	1352.0	<0.000 ***	845.1	<0.000 ***	998.0	<0.000 ***
Light × Substrate	27.2	<0.000 ***	42.2	<0.000 ***	20.3	<0.000 ***

*** $p \leq 0.001$.

Figure 3. The morphology of the rhubarb 'Raspberry' plantlets: (**A**) After 6 weeks of growing under lighting by different LED treatments (R—red, W—white, B—blue, R+B+G+FR—far red) in various growth media (on the left—in the neutralised sphagnum peat (GM2); on the right—Floragard substrate (GM1); (**B**) after 10 weeks of growing under W-LEDs and in GM2 (on the left) and GM1 (on the right).

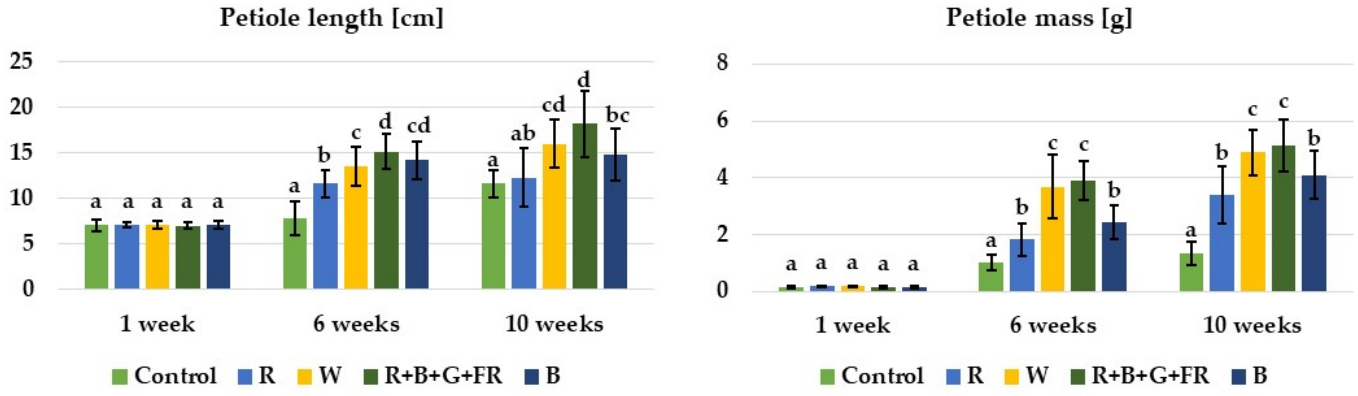

Figure 4. The effect of light quality on the growth rate responses of *Rheum rhaponticum* 'Raspberry' after 1, 6, and 10 weeks of growth in Floragard substrate and under different LED light treatments (control—natural light, R—red, W—white, B—blue, R+B+G+FR—far red). Bars represent means ± SE. According to Duncan's test ($p = 0.05$), the mean values indicated with the same letter within each term do not differ significantly.

Among the LED treatments used in this study, R+B+G+FR light resulted in higher rhubarb plantlet formation (longer leaf petioles and larger leaf blades) (Figures 2 and 5). Supplemental lighting with wide-spectrum light also resulted in better growth of the root system, which is favourable for the production of planting material. On the other hand, monochromatic red and blue LEDs inhibited the growth of plantlets and biomass accumulation (Figure 4).

Figure 5. Phenotypic characteristics of the rhubarb 'Raspberry' plantlets after 10 weeks of growth in the greenhouse: on the left—the leaf petioles of plantlets grown in Floragard substrate under different light conditions (control—natural light, R—red, W—white, B—blue, R+B+G+FR—far red); on the right, the rhubarb plantlets grown in Floragard substrate (GM1) under R+B+G+FR-LEDs.

Greenhouse lighting is an important factor in the vegetable crop and seedling production. In our study, the beneficial effects of LED lighting on the micropropagated rhubarb plantlets were observed, but only for those grown in high-EC substrate. Our previous study showed that young rhubarb plants very easily develop dormancy during the acclimatisation and early growth in ex vitro. Its induction was observed in response to a short day and was correlated with the enhanced expression levels of heat shock proteins (*HSP22* and *HSP70.1*) and heat stress transcription factor (*HSFA2*) genes. This suggested light deficiency is a stress factor during ex vitro growth of rhubarb plantlets [13]. This study showed nutrient and light deficiency resulted in the deaths of 49% of the plantlets after 6 weeks of growth in the greenhouse.

Among the treatments with supplementary lighting, the monochromatic red light created the worst conditions for rhubarb growth in the greenhouse (Figures 2 and 4). The inhibitory effect of red light on the plant height and leaf area has been previously observed in many plant species, including raspberry plantlets during early growth ex vitro [19]. We previously found that the growth cessation of rhubarb plantlets was related to dormancy induction and was associated with significant changes in ABA and auxin levels [13,32]. It is known that multiple hormonal pathways are often modulated by light to mediate developmental changes [33,34]. For example, Kondo et al. [35] reported that red light enhanced gene expression levels involved in ABA biosynthesis, 9-cis-epoxycarotenoid dioxygenase (*VvNCED*), and ABA concentration in grape berries. We suggest that inhibition of the growth of rhubarb plantlets by monochromatic red light might partly result from changes in hormone levels and signalling.

Among the wide-spectrum lights used in this study, R+B+G+FR-LEDs created better conditions for rhubarb growth in the greenhouse than W-LEDs. It can be due to differences in the R:FR ratio between the two spectra (Table 2). It has been observed that the increase of the R:FR ratio from 2.1 to 11.3 decreased the length of leaf petioles and leaf area by 10.6% and 24.4%, respectively (Figure 2). Promotion of stem elongation and foliar expansion by low R:FR is known as the shade-avoidance response that can capture more photosynthetic radiation and enable the plantlets to better compete with neighbouring plants [36]. Similar to rhubarb plantlets, the reaction has been previously observed for many shade-avoiding species, including petunia, geranium [37], and lettuce [38]. The rhubarb plantlets that were grown under R+B+G+FR-LED also showed the most active growth after 10 weeks of

growth in the greenhouse (Figure 4). This suggests that multi-wavelength irradiation with a relatively high share of red light (49.4%) and far-red (23.8%) in combination with blue light (12.9%) and a green-yellow fraction of visible light (14.5%) was most conducive to efficient growth and quality of rhubarb planting material. A similar light spectrum used as the sole light source was also beneficial for the ex vitro growth of hazelnut plantlets [39].

3.2. Soluble Sugars in the Leaf Petioles

Soluble sugar production in the leaf petioles was determined to show the physiological responses to the different light treatments. After 10 weeks, the supplementary LED lighting enhanced the total soluble sugar content in the leaf petioles by 3–4.6 fold compared to the control (natural light). Among the three soluble sugars tested, fructose was dominant (an average of 46%) in LED treatments, followed by glucose (37%), and sucrose (17%) (Figure 6). The plantlets without supplemental lighting do not produce sucrose. The highest level of soluble sugars was found in the rhubarb plantlets exposed to R+B+G+FR-LEDs. It was coincident with the highest growth rate responses of rhubarb plantlets. In this combination, the leaf petioles contained more soluble sugars by 49%, 39%, and 35% than those taken from plantlets grown under R-, B-, and W-LEDs, respectively. In addition, the supplementary lighting with R+B+G+FR-LEDs enhanced glucose accumulation by 49–65% compared to other LED treatments.

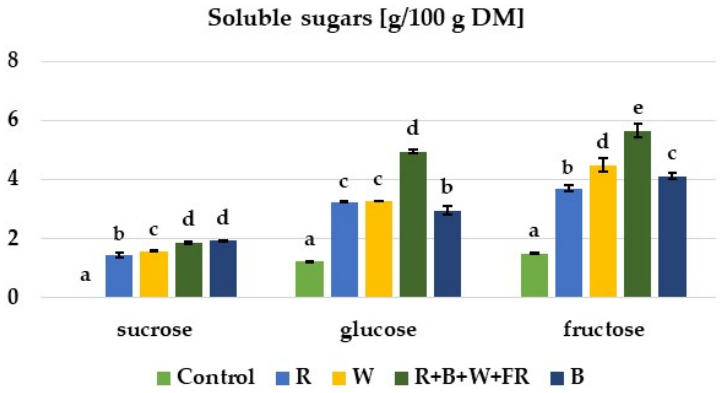

Figure 6. The soluble sugar levels in the leaf petioles of *Rheum rhaponticum* 'Raspberry' after 10 weeks of growth in GM1 and under different LED light treatments (control—natural light, R—red, W—white, B—blue, R+B+G+FR—far red). Bars represent means ± SE. Values marked with the same letter within each sugar type do not differ significantly according to the Duncan's test ($p = 0.05$).

Soluble sugars are photosynthesis products that regulate plants' growth and development [40]. Many studies showed that enhanced monosaccharide content correlated with more efficient photosynthetic apparatus [41]. The quality of light affects photosynthesis by its chloroplast structure, leaf size regulation, stomata opening, or photosystem II [42–44]. Monosaccharides are the basic building blocks and a common energy source for metabolism. They also play an important role as nutrient and metabolite molecules that activate specific or hormone-crosstalk pathways, thus resulting in an important modification of gene expression [45,46]. This study showed a positive relationship between all growth parameters (petiole mass and length, leaf area) and the soluble sugar contents in the leaf petioles of the rhubarb plantlets.

3.3. Phenolic Compounds in the Leaf Petioles

We assessed the effect of light quality on the levels of some major groups of phenolic compounds generally present in garden rhubarb leaf petioles. As shown in Table 4, supplementary LED lighting enhanced the levels of total phenolic compounds in the rhubarb petioles by 271% compared to the control. Anthocyanins were the richest group of polyphenolic compounds in rhubarb 'Raspberry' plantlets during early growth ex vitro in the greenhouse (Table 4). The highest anthocyanin production was recorded

under white light (585 mg·100 g^{-1} DM), followed by red (561 mg·100 g^{-1} DM), blue (435 mg·100 g^{-1} DM), and R+B+G+FR light (374 mg·100 g^{-1} DM). In all combinations, the dominant was cyanidin-3-O-rutinoside (86.0–93.0%), followed by cyanidin-3-O-glucoside (8.8–11.8%) and delphinidin-3-O-rutinoside (2.1–2.4%).

Table 4. Phenolic compounds of rhubarb 'Raspberry' plantlets during ex vitro growth in the greenhouse. Means indicated with the same letter within individual polyphenolic type are not significantly different (p = 0.05) according to Duncan's test; the lowest value is marked with "a".

Phenolic Compounds [mg/100 g DM]	Light Treatments				
	Control	Red	White	R+B+G+FR	Blue
Anthocyanins:	216.4 ± 0.79 a	561.0 ± 6.3 d	585.0 ± 0.69 e	374.1 ± 0.42 b	435.4 ± 4.0 c
Cyanidin-3-O-rutinoside	200.8 ± 6.3 a	488.4 ± 6.7 d	503.2 ± 3.4 e	332.2 ± 2.3 b	387.7 ± 3.6 c
Cyanidin-3-O-glucoside	10.8 ± 0.11 a	59.9 ± 0.17 d	68.8 ± 0.63 e	33.0 ± 0.11 b	38.4 ± 0.17 c
Delphinidin-3-O-rutonoside	4.8 ± 0.04 a	12.7 ± 0.25 c	13.0 ± 0.28 c	8.9 ± 0.08 b	9.2 ± 0.22 b
Flavonols	3.1 ± 0.17 a	4.4 ± 0.20 d	4.1 ± 0.13 c	3.6 ± 0.02 b	3.4 ± 0.03 b
Flavan-3-ols (Catechin)	12.7 ± 1.3 a	32.3 ± 0.57 c	30.8 ± 0.03 c	36.5 ± 0.60 d	23.4 ± 0.06 b
Phenolic acids	1.9 ± 0.03 a	4.1 ± 0.09 d	3.0 ± 0.05 c	2.1 ± 0.12 b	2.1 ± 0.05 b
Total phenolic compounds	234.0 ± 19.2 a	601.8 ± 6.8 d	622.8 ± 0.58 e	416.4 ± 11.8 b	464.4 ± 3.9 c

Anthocyanins are water-soluble flavonoid pigments widely found in plant petals, fruits, and vegetables, acting as photoprotectors and antioxidants [47]. In rhubarb stalks, anthocyanins can be produced only in the skin, or skin and flesh, along the entire length of the stalks or only at their bases. Of the twenty-nine red-stalked rhubarb genotypes analysed by Takeoka et al. [1], the total anthocyanin content ranged from 19.8 to 341.1 mg·100 g^{-1} DM. In this study, only the plantlets grown in the greenhouse's natural light conditions corresponded to those values. Our study confirmed that cyanidin-3-O-ritinoside and cyanidin-3-O-glucoside are the main anthocyanins responsible for the red pigment of rhubarb stalks. However, the anthocyanin levels in the LEDlit 'Raspberry' plantlets were 83.6% higher than those obtained by Kalisz et al. [7] for field-grown 'Red Malinowy' (English name 'Red Raspberry').

Anthocyanin synthesis is controlled by external and internal factors such as light, temperature, nutrient deficiency, phytohormones, and sugar concentration [48,49]. Different light spectra were effective in stimulating anthocyanin synthesis in horticultural crops. Very often, controversial results have been obtained. For example, in contrast to our study, blue light significantly increased the biosynthesis of anthocyanins in some fruit species [50], lettuce [51], and purple peeper fruit [52]. In other plant species, including different microgreens, broccoli, kale, amaranth, tatsoi, parsley, perilla, pea [53,54], and apple trees, [55] anthocyanin synthesis was induced under R-LED light. In rhubarb, the red light also significantly enhanced anthocyanin biosynthesis, but at the same time, inhibited growth of the plantlets was observed. Moreover, the leaf petioles derived from plantlets grown under R-LEDs had low levels of soluble sugars. Secondary metabolites (such as anthocyanins) are often accumulated under stressful conditions. Their biosynthesis requires an extensive energy input, which plants derive from accumulated sugars in the cells [56]. It is suggested that the growth inhibition of rhubarb plantlets under R-LEDs is a stress response mediated by anthocyanins. On the other hand, anthocyanin production in the rhubarb was the highest in the plantlets grown under W-LEDs light, with a relatively high share of green/yellow light (44.4%) in combination with red (28.9%), blue (24.4%), and far-red (2.2%) light. In this case, high anthocyanin levels in the rhubarb stalks coincide with the active growth of the plants. A recent study showed that green light positively effects plant biomass production and their better environmental acclimatisation [57]. The stimulatory effect of supplemental green light combined with red and blue LEDs or high-pressure sodium lamps on anthocyanin production was previously observed in different baby leaf lettuce cultivars [54,58].

3.4. Antioxidant Activity

As was shown in Figure 7, the LED lighting of micropropagated rhubarb plantlets during the early growth ex vitro stage significantly affected their antioxidant activity. Supplementary lighting with R-LEDs resulted in the highest antioxidant activity value in the ABTS test (16.1 mmol Trolox·100 g^{-1} DM), which was higher by 242% than for control plantlets grown under natural light, and by 41.4%, 30.3%, and 29% than those grown under B-, W-, and R+W+B+FR-LEDs, respectively (Figure 7). Similar antioxidant activity that we found for R-LDEDs-grown plantlets characterised field-grown rhubarb 'Raspberry' plants, but only those harvested in spring [7]. In contrast to micropropagated plantlets, mature rhubarb 'Raspberry' plants dominated flavon-3-ols, mainly catechin, which probably was responsible for the antioxidant response. We found strong (r = 0.832) positive correlations between ABTS antioxidant activity and light-stimulated anthocyanin levels (Table 5).

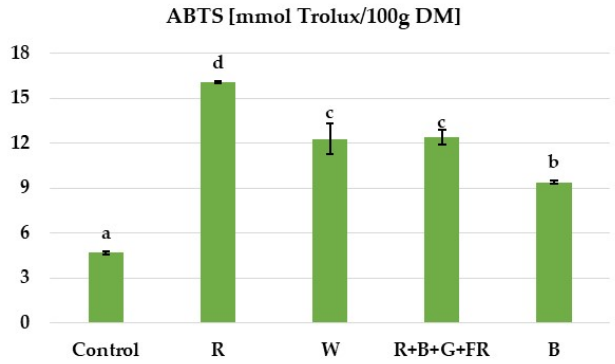

Figure 7. Antioxidant activity of rhubarb 'Raspberry' plantlets after 10 weeks of growth in GM1 and under different LED light treatments (control—natural light, R—red, W—white, B—blue, R+B+G+FR—far red). Bars represent means ± SE. Values marked with the same letter within each sugar type do not differ significantly according to the Duncan's test (p = 0.05).

Table 5. Pearson's linear correlation matrix between growth parameters (petiole mass, petiole length, leaf area), total soluble sugars, total anthocyanins, and total phenolic content, and antioxidant activity of rhubarb petiole extracts. The differences in correlation are shown in distinct colours. Dark magenta indicates a high correlation, and light magenta shows a low correlation.

	Petiole Mass	Petiole Length	Leaf Area	Sugars	Anthocyanins	Phenolic Compounds
Petiole length	0.800					
Leaf area	0.872	0.666				
Sugars	0.891	0.718	0.934			
Anthocyanins	0.605	0.195	0.520	0.528		
Phenolic compounds	0.625	0.219	0.543	0.560	0.999	
ABTS	0.607	0.257	0.616	0.708	0.832	0.852

It has been shown that anthocyanins have potent antioxidant activity to scavenge free radicals and reactive oxygen species (ROS) due to the number of hydroxyl groups and glycosylation in the rings [59]. They protect plants by absorbing excess UV light, preventing lipid peroxidation, and suppressing the activity of ROS [60,61]. In this study, a significant increase in anthocyanin synthesis and antioxidant activity induced by red LEDs was related to growth inhibition and biomass reduction in rhubarb plantlets. We previously found that the growth cessation is a sign of dormancy induction being an adaptation strategy to stress conditions [13,32]. This indicated that anthocyanin participates in protecting rhubarb plantlets from suboptimal environmental conditions developed by red light. It is thought that anthocyanins may also protect young rhubarb plants from other abiotic and biotic stresses, e.g., grey mould. Further studies are needed to understand the mechanism and interaction between antioxidants and the light signal transduction pathways.

4. Conclusions

Our study for the first time showed the relevance of high-EC substrate and supplemental LED lighting on the early ex vitro growth of micropropagated rhubarb plantlets. The quality of light generated by LEDs significantly affected the growth rate and primary and secondary metabolism in rhubarb plantlets. An optimal effect related to plant growth and anthocyanin biosynthesis can be obtained using W-LEDs lighting. It is essential for the efficient year-round production of rhubarb planting material. The regulatory role of the LED light on anthocyanin biosynthesis and antioxidant activity has great potential in the organic production of rhubarb planting material.

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