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The use of different LEDs wavelength and light intensities on in vitro proliferation of cherry rootstock: influence on photosynthetic pigments, photosystem II photochemistry and leaf anatomy

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Research Article

Keywords: Gisela 6, light-emitting diodes, chlorophyll content, chlorophyll fluorescence parameters, light microscopy, micropropagation

Posted Date: June 30th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1789324/v1

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Abstract

Plant growth and development are strongly influenced by light quality conditions in their environment. In this study, shoot proliferation, photosynthetic pigments, leaf anatomy and photosystem II photochemistry of Gisela 6 cherry rootstock (*Prunus cerasus* x *Prunus canescens*) were investigated. Initially, three light wavelength absorption spectra including 400–700 nm (mainly blue-green, White Fluorescent Light, WFL), 450–550 nm (mainly blue-red: BR, LED2) and 450–660 nm (blue-green-yellow-orange-red, LED3) under 40 μmol m^{-2} s^{-1} intensity were studied. Following that, three Light Emitting Diodes (LED) intensities including 20 (LED1), 40 (LED2), and 120 μmol m^{-2} s^{-1} (LED4) were investigated under BR spectrum. The culture medium used was the MS (Murashige and Skoog) containing 4.4 μM 6-benzyladenine (BA), 0.05 μM α-naphthalene acetic acid (NAA) and 0.3 μM gibberellic acid (GA_3) (30 days, 16h photoperiod, 22 ± 2 ^oC). WFL exhibited non-significant differences with LED2 (shoot number 4.25, proliferation rate 4.30, shoot height 0.91 cm). LED2 displayed higher fresh and dry shoot biomass, better PSII functionality, increased Chl a/b, Chl b and carotenoids content, avoiding oxidative damage as evidenced by the decreased stressed explants (5.36%). Leaf anatomy under LED2 and WFL was similar. LED4 presented the highest rate of stressed explants, with their leaf anatomy being severely affected, as leaves had a reduced mesophyll cell density. In LED4, the proportion of the absorbed energy used in photochemistry (Φ_{PSII}) was decreased whereas the increased quantum yield of non-regulated energy loss in PSII (Φ_{NO}) could not be compensated by the increased photoprotective energy dissipation (Φ_{NPQ}). Therefore, LED2 was the most beneficial for growing Gisela 6 plantlets *in vitro*.

Key Message

Higher proliferation rate, shoot biomass, photosystem II functionality, Chl a/b and carotenoids under LED2 [450-550 nm, mainly blue-red, 40 μmol/m^2/s] whereas high light stress (120 μmol/m^2/s) adversely affected leaf anatomy.

Introduction

Currently, sweet cherry dwarf and semi-dwarf rootstocks are used in orchards for intensive production, in order to increase culture efficiency and fruit quality (Drkenda et al. 2012). The *Prunus cerasus* x *Prunus canescens* hybrid (Gisela 6) is a semi-dwarf rootstock, compatible with most of the sweet cherry varieties, commonly used in cherry tree nurseries, because it grows in a broad range of soils, mainly the heavy ones (Andersen et al. 1999). Trees grafted on Gisela 6 do not seem to be prone to suckering, while the dwarfing pattern of this rootstock favors rapid entry to production, a feature especially advantageous for the founding and advance of super-intensive cherry cultivations (Long and Kaiser 2010). Cutting and layering (conventional production methods) of Gisela 6 are difficult and do not produce high quality, homogeneous, disease-free, planting material in large quantities. However, the use of micropropagation methodologies, could assist to overcome any of the problems encountered (Aka-Kacar et al. 2010).

Light, being simultaneously both a signal and an energy source, is an environmental factor exceptionally important in commercial micropropagation laboratories, for establishing optimal plant growth and development. In order to increase photosynthetic photon flux (PPF), fluorescent lamps (FLs) are generally used for *in vitro* culture. Alas, light emitted by FLs, contains unwanted, low quality wavelengths (350 to 750 nm) that do not favor plant growth and phytomorphogenesis (Gupta and Jatothu 2013). A lighting source that has attracted considerable interest, during the recent years, are the light-emitting diodes (LEDs). LEDs as an illumination source, show numerous unique advantages, such as a customizable spectra, a relative decreased mass and volume, low heat release, photon output linear with the electrical input current, adaptable light intensity, longer life spans and high energy conversion effectiveness, favoring reduced production expenses (Gupta and Jatothu 2013; Massa et al. 2008). When *in vitro* cultures are being illuminated with FLs, power consumption used for lighting represents the 65% of the total energy used (Bornwaver and Tantau 2012). LED lighting sources require 10 to 100 lower energy when compared with conventional lighting e.g. FLS (Dutta Gupta 2017). Highly increased light intensities hamper normal photomorphogenesis, and lead to capital forfeit due to augmented power use (Dobránszki and Mendler-Drienyovszki 2014). Researchers so far, have studied the relation between light and growth, photosynthetic

efficiency features, carbon and nitrogen consumption, volatile secretion and leaf anatomy in many plants of increased agricultural/horticultural value (Ma et al. 2021). However, it is difficult to reach consensus on which light spectrum is the most suitable for each plant species (Ma et al. 2021).

Toning LED wavelengths to plant photoreceptors could present a solution for the best possible crop production (Massa et al. 2008). Sun emits light spectrum that varies from 300 to 2600 nm. The two absorption peaks of chlorophyll in the sunlights spectrum are at wavelengths from 430–450 nm and 640–660 nm (Long et al. 2018) so, the wavelengths which directly influence plant photosynthesis are between 400 and 700 nm, in which blue (B) light (420–500 nm) and red (R) light (620–700 nm) play important roles, while yellow and green are minimally absorbed (Pan 2008). Red light, governs chloroplast function and flowering, stimulates stem and petiole growth (Naznin et al. 2019) favoring biomass accumulation (Kreslavski et al. 2013). Blue light on the other hand, favors, stomatal opening, photosynthesis and pigment accumulation, while, playing an important role in photo-morphogenesis, (Naznin et al. 2019), by influencing leaf expansion, growth phototropism and inhibiting plant stem elongation (Long et al. 2018). Blue light also reduces *in vitro* plantlet growth (Hunter and Burritt 2004). Yellow light also seemed to positively influence shoot elongation and leaf development (Glowacka 2004). Since, the relation between light quality and quantity with the endogenous hormonal levels influences plant growth (Ouyang et al. 2015), it has been noted that B and R LEDs, stimulated the growth of several horticultural plants (Naznin et al. 2019), while studies also revealed that different LED lighting combinations caused a biomass increase (Idrees et al., 2018), affected shoot formation and *ex vitro* survival rate (Dutta Gupta and Sahoo 2015).

Leaves grown under FLs (a light source traditionally used in *in vitro* cultures) show different anatomical features, than those of plants *in vitro* grown under LED lighting (Gonçalves et al. 2008). This indicates that LEDs do not alter leaf anatomy like traditional light conditions do, being at the same time energy efficient. However, the internal structure of leaves could be affected by different light qualities (Slattery et al. 2018), so leaf specific anatomical traits could appear altered by differential LED illumination.

An approach to understand the optimal light intensity is to estimate light energy use efficiency (Montgomery and Givnish 2008). In this context, chlorophyll fluorescence is a sensitive method that could be applied to calculate and study any alteration in photosynthetic efficiency, detect how numerous external factors affect plant growth (Ouzounis et al. 2015), and generally monitor plant health status (Moustakas et al. 2020). As already established, *in vitro* phytomorphogenesis, is influenced by the use of monochromatic (alone or in combination) red and blue LEDs (Gupta and Jatothu 2013; Massa et al. 2008), however, the effects of different and combined LED light qualities during *in vitro* culture of Gisela 6 rootstock have not yet being studied. Therefore, the purpose of this research was to evaluate the effects of light environment i.e. different LED light wavelength absorption spectra and light intensities on shoot proliferation, photosynthetic pigments, photosystem II photochemistry and leaf anatomy in order to determine the optimal light environment and provide some reference data to optimize the industrial approach for the *in vitro* culture of Gisela 6.

Materials And Methods

Plant material

Shoot tips explants sub-cultured every 4 weeks on MS (Murashige and Skoog 1962) medium fortified with 4.4 μM BA, 0.05 μM NAA, 0.3 μM GA_3, 20 g L^{-1} sucrose (Duchefa, The Netherlands) and 6 g L^{-1} Plant Agar (Duchefa, The Netherlands) were used as the stock plant material for further experimentation. The culture medium was autoclaved at 121°C for 20 min after adjusting the pH value to 5.8 and inserting the solidifying agent. The explants were then transferred into Magenta vessels (62 mm × 95 mm, 200 mL, Sigma-Aldrich), each containing 35 mL of medium. Following that, the explants were incubated in a growth chamber under controlled conditions of temperature (22 ± 2°C), photoperiod (16h light/ 8h darkness), and light intensity (40 μmol m^{-2} s^{-1}) provided by cool white fluorescent lamps (WFLs).

Different light absorption wavelength spectra and intensities on in vitro shoot proliferation

The experimental material used was shoot node explants 1-1.5 cm long with 2–3 buds obtained from previous stock *in vitro* cultures. The environmental conditions related to temperature, photoperiod, light intensity and illumination type (WFLs) were similar as previously stated.

Initially, three light wavelength absorption spectra; 400–700 nm (mainly blue-green, BG), 450–550 nm (mainly blue-red, BR) and 450–660 nm (blue-green-yellow-orange-red, BGYOR) under constant light intensity of 40 μmol m^{-2} s^{-1} were used for *in vitro* shoot proliferation of Gisela 6. The cool white fluorescent light (WFL) (PHILIPS, 36W/830 G13 1214mm) served as the control treatment (BG, 400–700 nm), thus, three in total treatments were included: WFL (BG, 40 μmol m^{-2} s^{-1}), LED2 (BR, 40 μmol m^{-2} s^{-1}), and LED3 (BGYOR, 40 μmol m^{-2} s^{-1}). Following that, the effect of three LED light intensities (20, 40 and 120 μmol m^{-2} s^{-1}) under the same wavelength absorption spectrum of 450–550 nm (mainly blue-red, BR) on *in vitro* shoot proliferation of Gisela 6 was tested, thus, three in total treatments were included: LED1 (BR, 20 μmol m^{-2} s^{-1}), LED2 (BR, 40 μmol m^{-2} s^{-1}), and LED4 (BR, 120 μmol m^{-2} s^{-1}). After a 30-day culture period, the following data were recorded related to various shoot proliferation attributes: percentage (%) of explants with new shoots induction, number of new shoots ≥ 0.3 cm per explant, shoot length (cm), shoot proliferation rate, fresh weight (FW) and dry weight (DW) of explants, FW/DW ratio, and percentage (%) of abiotic-stressed explants.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured in 20min dark-adapted Gisela 6 leaves with the use of an Imaging-PAM fluorometer M-series MINI-version (Walz, Effeltrich, Germany), as described by Moustaka et al. (2018). Five leaves from five different plants were measured from each treatment (LED1, LED2, LED3, LED4 and White Fluorescent Light) at the actinic light intensities of 20 μmol m^{-2} s^{-1} for LED1, 40 μmol m^{-2} s^{-1} for LED2, LED3 and WFL and 120 μmol m^{-2} s^{-1} for LED4, similar to their growth light. Via Win software (Heinz Walz GmbH, Effeltrich, Germany), the maximum efficiency of photosystem II (PSII) photochemistry (F_v/F_m), the efficiency of excitation energy capture by open PSII reaction centers ($F_{v'}/F_{m'}$), the allocation of absorbed light energy at photosystem II (PSII) that is the effective quantum yield of PSII photochemistry (Φ_{PSII}), the quantum yield of regulated non-photochemical energy loss in PSII (Φ_{NPQ}), the quantum yield of non-regulated energy loss in PSII (Φ_{NO}), the non-photochemical quenching that reflects heat dissipation of excitation energy (NPQ), the photochemical quenching that is the redox state of the plastoquinone pool (q_P) that represents the fraction of open PSII reaction centers and the excess excitation energy (EXC) were assessed (Demmig-Adams et al. 1996).

Photosynthetic pigment analysis

The extraction of the photosynthetic pigments was performed as described by Gao et al. (2020). The optical density (OD) was measured using a PharmaSpec UV-1700 spectrophotometer (Shimadzu, Japan) at 470 nm (OD_{470}) for carotenoids, 663 nm (OD_{663}) for Chl a, and 645 nm (OD_{645}) for Chl b. The content of each pigment in the leaves was calculated using the following formulas (Gao et al. 2020):

Chla (mg g^{-1}) = (12.72 OD_{663} – 2.59 OD_{645}) V/ 1000 W

Chlb (mg g^{-1}) = (22.88 OD_{645} – 4.67 OD_{663}) V/ 1000 W

Carotenoids (mg g^{-1}) = (1000 OD_{470} – 3.27 Chla – 104 Chlb) V/ (229 x 1000 W),

where V is the total volume of the extract (ml) and W is the FW (g) of the sample.

Light microscopy

Pieces of leaves from microshoots exposed to WFL-control, LED1, LED2, LED3 and LED4 were prepared for chemical fixation, as previously reported (Dobrikova et al. 2021). In short, leaf segments were firstly immersed in a fixation solution containing 3% paraformaldehyde + 3% glutaraldehyde buffered with 0.05 M sodium cacodylate at pH 7.0 at room temperature for 6 h. Subsequently, the leaf pieces were post-fixed in a 2% osmium tetroxide solution similarly buffered for another 3 h. Following dehydration in an acetone series, leaf pieces were treated with propylene oxide and were finally embedded in Durcupan ACM resin. Semi-thin sections (0.5–2 μm) that were obtained with the use an ultramicrotome (LKB 8801A, Stockholm, Sweden) outfitted with a glass knife; were stained with a 0.5% (*w/v*) toluidine blue O solution, and observed under a Zeiss Axioplan light microscope equipped with a AxioCam MRc 5 digital camera (Zeiss, Berlin, Germany).

Statistical analysis

Data related to shoot proliferation parameters were analyzed by using the statistical package SPSS 17.0 (SPSS Inc., Illinois, New York, USA) and Analysis of Variance (one-way ANOVA). Descriptive data were compared using the Duncan's multiple range test at a level of $P \leq 0.05$. Results are expressed as mean ± standard error (SE). The first experiment included 3 treatments (WFL - control, LED2, LED3) with 56 repetitions/ treatment (7 explants/ vessel x 8 vessels/ treatment) corresponding to three light wavelength absorption spectra of 40 μmol m^{-2} s^{-1} intensity. The second experiment included 3 treatments (LED1, LED2, LED4) with 56 repetitions/ treatment (7 explants/ vessel x 8 vessels/ treatment) corresponding to three light intensities of the same wavelength absorption spectrum (BR, 450–550 nm).

Mean values obtained by two independent experiments with five leaf samples per treatment per experiment were considered regarding chlorophyll fluorescent parameters, whereas three samples per treatment per experiment were considered for the analysis of photosynthetic pigments. Results are expressed as mean ± standard error (SE). For chlorophyll fluorescence and photosynthetic pigment analysis, the Student's t-test (StatView computer package, Abacus Concepts, Inc Berkley, CA, USA) was used at a level of $P < 0.05$.

Results

In vitro shoot proliferation

Under constant 40 μmol m^{-2} s^{-1} light intensity, multiple shoot formation was 100% irrespective of spectrum and light intensity in all three treatments (WFL, LED2, LED3). WFL and LED2 gave similar shoot numbers (3.8–4.25), shoot heights (0.90–0.91 cm), proliferation rates (3.86–4.30) and percentages of stressed explants (5.36–10.72%). However, there was a noticeable increase in the FW (0.365 g) and DW (0.0.046 g) of explants as well as in the FW/DW ratio (9.24) in LED2, differing significantly from WFL and LED3. LED3 exhibited the lowest shoot number (3.32), shoot height (0.79 cm) and proliferation rate (3.36) compared to WFL. The lowest percentage of explants with stress symptoms (5.36%) was recorded in LED2 and the highest (21.43%) in LED3. Intermediate stress symptom percentages were recorded to the 10.72% of the explants in WFL, without significant differences to the other treatments. There was a 4-fold increase in the percentage of stressed explants between LED2 and LED3 - with the latter - to be x4 times higher (21.43%) than the former one (5.36%). Therefore, WFL and LED2 proved to be the most effective treatments for shoot proliferation of Gisela 6, exhibiting similar results (Table 1; Fig. 1a).

Table 1
Effect of three wavelength spectra 400–700 nm (mainly blue-green, BG), 450–550 nm (mainly blue-red, BR) and 450–660 nm (blue-green-yellow-orange-red, BGYOR) under light intensity of 40 μmol m^{-2} s^{-1} on *in vitro* shoot proliferation parameters in the Gisela 6 rootstock after 30 days of culture in MS medium enriched with 4.4 μM BA, 0.05 μM NAA, 0.3 μM GA_3, 20 g L^{-1} sucrose (pH 5.8) and 6 g L^{-1} Plant Agar. Means ± SE with different letters in a column are statistically different according to Duncan's multiple range test at $P \leq 0.05$.

Treatment code	Absorption wavelength spectrum	Light intensity (μmol m^{-2} s^{-1})	Number of new shoots/ explant	Shoot height (cm)	Proliferation rate	FW (g)	DW (g)	FW/DW ratio	Stressed explants (%)
WFL	BG	40	4.25 ± 0.18 a	0.91 ± 0.04 a	4.30 ± 0.19 a	0.308 ± 0.023 b	0.037 ± 0.003 b	8.34 ± 0.02 b	10.72 ± 5.23 ab
LED 2	BR	40	3.80 ± 0.18 ab	0.90 ± 0.04 a	3.86 ± 0.18 ab	0.365 ± 0.025 a	0.046 ± 0.004 a	9.24 ± 0.50 a	5.36 ± 3.76 b
LED 3	BGYOR	40	3.32 ± 0.18 b	0.79 ± 0.04 b	3.36 ± 0.19 b	0.310 ± 0.022 b	0.037 ± 0.003 b	8.37 ± 0.02 b	21.43 ± 6.04 a
P-values (one-way ANOVA)			*0.002***	*0.049**	*0.002***	*0.043**	*0.047**	*0.048**	*0.032**
* $P \leq 0.05$, ** $P \leq 0.01$									

Under the same wavelength absorption spectrum (BR, 450–550 nm), multiple shoot formation to the 100% of the explants was achieved irrespective of light intensity (20, 40 and 120 μmol m^{-2} s^{-1}) (LED1, LED2, LED4). LED2 of the intermediate light intensity of 40 μmol m^{-2} s^{-1} gave better results related to shoot number (3.8), shoot height (0.90 cm), proliferation rate (3.86), FW/DW ratio (9.24), FW (0.365 g) and DW (0.046 g) of the final explants. In addition, the lowest percentage of explants with stress symptoms (5.36%) and the highest FW/DW ratio (9.24) was recorded under LED2. Even though, LED1 and LED2 did not lead on a noticeable change of shoot height (0.83–0.90 cm), LED2 gave substantially better results in the other proliferation parameters measured attributes than LED1. Mild chlorosis, light brown coloration and bleaching of the leaves of the initial explant, reduced vigor and robustness of the explants, i.e. smaller number and size of leaves, less intense green color of the leaves and slight twisting inwards of the leaves of the base were the main symptoms of abiotic stress imposed under LED4. These stress symptoms in LED4 were more severe, intense, in higher number of initial explants (60.71%), being considerably increased compared to LED1 (17.86%) and LED2 (5.36%) (Table 2; Fig. 1b-1c).

Table 2

Effect of three light intensities (20, 40 and 120 µmol m^{-2} s^{-1}) with the same wavelength absorption spectrum of 450–550 nm (mainly blue-red, BR) on *in vitro* shoot proliferation parameters in the Gisela 6 rootstock after 30 days of culture in MS medium enriched with 4.4 µM BA, 0.05 µM NAA, 0.3 µM GA_3, 20 g L^{-1} sucrose (pH 5.8) and 6 g L^{-1} Plant Agar. Means ± SE with different letters in a column are statistically different according to Duncan's multiple range test at $P \leq 0.05$.

Treatment code	Light intensity (µmol m^{-2} s^{-1})	Absorption wavelength spectrum	Number of new shoots/ explant	Shoot height (cm)	Proliferation rate	FW (g)	DW (g)	FW/DW ratio	Stressed explants (%)
LED 1	20	BR	3.14 ± 0.18 b	0.83 ± 0.04 a	3.18 ± 0.18 b	0.295 ± 0.014 b	0.034 ± 0.002 b	8.62 ± 0.01 b	17.86 ± 5.88 b
LED 2	40	BR	3.80 ± 0.18 a	0.90 ± 0.04 a	3.86 ± 0.18 a	0.365 ± 0.025 a	0.046 ± 0.004 a	9.24 ± 0.50 a	5.36 ± 3.76 c
LED 4	120	BR	3.43 ± 0.17 ab	0.65 ± 0.03 b	3.43 ± 0.17 ab	0.271 ± 0.027 b	0.036 ± 0.004 b	7.55 ± 0.01 c	60.71 ± 14.48 a
P-values (one-way ANOVA)			*0.029**	*0.000****	*0.025**	*0.010**	*0.020**	*0.000****	*0.001***
* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$									

Changes in chlorophyll fluorescence parameters under different light conditions

Under constant light intensity (40 µmol m^{-2} s^{-1}), all chlorophyll fluorescence parameters (Φ_{PSII}, F_v/F_m, Φ_{NPQ}, Φ_{NO}, NPQ, q_P, $F_{v'}/F_{m'}$, EXC) showed no significant difference under any treatment [WFL (BG, 40 µmol m^{-2} s^{-1}), LED2 (BR, 40 µmol m^{-2} s^{-1}), LED3 (BGYOR, 40 µmol m^{-2} s^{-1})] irrespective of absorption wavelength spectrum (Table 3).

Table 3

Chlorophyll fluorescence parameters of Gisela 6 leaves, under light intensity of 40 μmol m^{-2} s^{-1} and three different light wavelength absorption spectra provided by White Fluorescent Light (WFL) and two LED lamps (LED2 and LED3). Means ± SE denoted by different letters are statistically different ($p < 0.05$).

Treatment code	Absorption wavelength spectrum	Light intensity (μmol m^{-2} s^{-1})	Φ_{PSII}	F_v/F_m	Φ_{NPQ}	Φ_{NO}	NPQ	q_P	$F_{v'}/F_{m'}$	EXC
WFL	BG	40	0.389 ± 0.091 a	0.636 ± 0.136 a	0.136 ± 0.034 a	0.476 ± 0.094 a	0.075 ± 0.024 a	0.667 ± 0.052 a	0.583 ± 0.128 a	0.647 ± 0.130 a
LED 2	BR	40	0.431 ± 0.131 a	0.683 ± 0.103 a	0.114 ± 0.024 a	0.456 ± 0.125 a	0.068 ± 0.025 a	0.668 ± 0.133 a	0.638 ± 0.094 a	0.653 ± 0.129 a
LED 3	BGYOR	40	0.346 ± 0.082 a	0.576 ± 0.043 a	0.124 ± 0.006 a	0.531 ± 0.084 a	0.065 ± 0.010 a	0.619 ± 0.110 a	0.548 ± 0.037 a	0.799 ± 0.141 a
p-values (one-way ANOVA)			*0.389 ns*	*0.222 ns*	*0.313 ns*	*0.442 ns*	*0.678 ns*	*0.656 ns*	*0.282 ns*	*0.578 ns*
ns: > 0.05										

Taking into account the statistically increased FW and DW and decreased percentage of stressed explants of LED2 compared to WFL and LED3, chlorophyll fluorescence parameters under the same wavelength absorption spectrum and different LED intensities (20, 40, 120 μmol m^{-2} s^{-1}) were estimated. Under LED4, Φ_{PSII} decreased significantly compared to the other treatments while its highest value was observed under LED1, though not being significantly different compared to LED2. Φ_{NPQ} did not change under LED1 and LED2, but increased significantly under LED4 and the same pattern was also observed for Φ_{NO} (Fig. 2).

F_v/F_m was significantly decreased under LED4 but it did not differ under the other treatments, having a slight, non-significant, increase under LED1 (Fig. 3a). q_P did not differ under LED1 and LED2 and was only significantly decreased under LED4 (Fig. 3b). Under LED1, $F_{v'}/F_{m'}$ was significantly increased compared to LED4 and slightly, non-significantly increased compared to LED2 (Fig. 3c). NPQ showed no significant difference under any treatment (Fig. 4a) and EXC was significantly increased under LED4 (Fig. 4b).

Photosynthetic pigment content under different light conditions

Under 40 μmol m^{-2} s^{-1} constant light intensity and irrespective of wavelength absorption spectrum, photosynthetic pigments content including Chl a, Chl b, total chlorophyll Chl (a + b), Chl a/ Chl b and carotenoids showed no significant difference under any treatment (WFL, LED2, LED3) (Table 4).

Table 4

Photosynthetic pigment content in Gisela 6 leaves, under light intensity of 40 μmol m^{-2} s^{-1} and three different light wavelength absorption spectra provided by White Fluorescent Light (WFL) and two LED lamps (LED2 and LED3). Means ± SE denoted by different letters are statistically different ($p < 0.05$).

Treatment code	Absorption wavelength spectrum	Light intensity (μmol m^{-2} s^{-1})	Chl a (mg g^{-1})	Chl b (mg g^{-1})	Total Chl (a + b) (mg g^{-1})	Chl a/Chl b	Carotenoids (mg g^{-1})
WFL	BG	40	0.013 ± 0.001 a	0.010 ± 0.001 a	0.024 ± 0.001 a	1.249 ± 0.108 a	0.136 ± 0.005 a
LED 2	BR	40	0.014 ± 0.001 a	0.012 ± 0.003 a	0.026 ± 0.003 a	1.173 ± 0.344 a	0.143 ± 0.008 a
LED 3	BGYOR	40	0.013 ± 0.001 a	0.012 ± 0.004 a	0.024 ± 0.005 a	1.221 ± 0.393 a	0.129 ± 0.012 a
p-values			*0.319 ns*	*0.619 ns*	*0.463 ns*	*0.940 ns*	*0.137 ns*
ns: > 0.05							

In the case of the same wavelength spectrum (BR, 450–550 nm) and different LED light intensities (20, 40, 120 μmol m^{-2} s^{-1}), Chl a and total chlorophyll content Chl (a + b) did not differ under any treatment (LED1, LED2, LED4). Under LED1, Chl b increased significantly compared to LED2 that presented the lowest value, whereas under LED4, Chl b did not differ significantly compared to either LED1 or LED2. The ratio Chl a/ Chl b increased significantly under LED2 compared to LED1 that presented the lowest value, while under LED4, Chl a/ Chl b had no significant difference compared to either LED1 or LED2. Carotenoids reached the highest content under LED2, but without any significant difference compared to LED1 and they were found significantly decreased under LED4 having the lowest value (Table 5).

Table 5

Photosynthetic pigment content in Gisela 6 leaves, under the same light wavelength absorption spectrum (BR, 450–550 nm) and three different LED intensities (20, 40, 120 μmol m^{-2} s^{-1}) (LED1, LED2, LED4). Means ± SE denoted by different letters are statistically different ($p < 0.05$).

Treatment code	Absorption wavelength spectrum	Light intensity (μmol m^{-2} s^{-1})	Chl a (mg g^{-1})	Chl b (mg g^{-1})	Total Chl (a + b) (mg g^{-1})	Chl a/Chl b	Carotenoids (mg g^{-1})
LED 1	BR	20	0.013 ± 0.001 a	0.016 ± 0.001 a	0.029 ± 0.001 a	0.777 ± 0.038 b	0.138 ± 0.006 ab
LED 2	BR	40	0.014 ± 0.001 a	0.012 ± 0.003 b	0.026 ± 0.003 a	1.173 ± 0.344 a	0.143 ± 0.008 a
LED 4	BR	120	0.013 ± 0.001 a	0.014 ± 0.005 ab	0.026 ± 0.005 a	1.083 ± 0.462 ab	0.128 ± 0.008 b
p-values			*0.265 ns*	*0.019**	*0.553 ns*	*0.046**	*0.037**
ns: > 0.05, * P ≤ 0.05							

Leaf anatomy under different light conditions

Gisela 6 leaves are dorsiventral with the leaf parenchyma comprising of both palisade and spongy mesophyll. Leaf anatomy appeared to differ from the WFL-control (Fig. 5a) in the microshoots growing under LED1 (Fig. 5b), LED3 (Fig. 5d) and LED4 (Fig. 5e), while under LED2 illumination leaf anatomy was similar to that of observed in WFL illuminated plants (Fig. 5c *cf.*

5a). A general trait of LED1, LED3 and LED4 effects on leaf anatomy was the increase of the spongy mesophyll intracellular space i.e. a reduction in spongy mesophyll cell density (Fig. 5b,d,e *cf.* Figure 5a,c). The reduction of the spongy mesophyll cell density was more obvious under LED1 lighting (Fig. 5b) followed by LED3 and LED4 (Fig. 5d and 5e; respectively). Under LED4 lighting, the upper epidermal cells also increased in size, (and by this the whole upper epidermis appeared thicker) and were filled with a material which was intensively stained (arrows in Fig. 5e). Palisade mesophyll did not appear to be affected under LED1, LED3 and LED4 (Fig. 5b,d,e), however under LED1 and LED3 lighting intensively stained material seemed to fill the palisade mesophyll cells (Fig. 5b,d).

Discussion

Different absorption wavelength spectra under the same light intensity on shoot proliferation

Effect on shoot number and proliferation rate

In the present study with Gisela 6, when explants cultured under three different absorption wavelength spectra of the same light intensity 40 µmol $m^{-2}s^{-1}$, shoot number and proliferation rate were best stimulated, being non-significant different, in the case of WFL (BG) treatment and in LED2 (BR). Similarly, FLs and white LED increased propagation ratio in *Gerbera jamesonii* 'Rosalin' (Gök et al. 2016). Bello-Bello et al. (2016) illustrated that red-blue LED mixtures resulted in higher shoot proliferation rates in comparison to the FLs. Martinez-Estrada et al. (2016) (mixed red-blue light) and Azmi et al. (2016) (blue light) found out that other light absorption wavelength spectra than FL were more effective in raising the number of microshoots. The absence or complete absence of the far red color from the common fluorescent lamps, which plays an important role in the growth and development of plants mainly in the stem elongation and phytochrome activity, while they are in abundance in green and yellow light which are of lower efficacy for plants function (Shin et al. 2008). Thus, it is clearly depicted that the reactivity of plants to different illumination conditions depends to a large extent on the genotype of the plant species, and the mechanisms governing these morphogenetic responses of plants should be thoroughly investigated.

Effect on shoot elongation

Enhancement or inhibition of stem elongation is species-dependent and could be driven by synergistic interactions between phytochrome and receptors of blue or red light (Kim et al. 2004). Cherry plantlets illuminated with red LEDs had longer internodes compared to the affected plants by WFLs and blue LEDs (Muleo and Thomas 1997). Plant development is most affected by red and blue lights (Shukla et al. 2017) owing to the fact that they actively participate in photosynthetic CO_2 assimilation as the predominant energy sources (Lin et al. 2013). In Gisela 6, the height of microshoots was higher under WFL (mainly BG) and LED2 (mainly BR) whereas a significant decrease was recorded under LED3 (BGYOR). The positive effect of blue-red LED and/ or blue-green WFLs on height of Gisela 6 microshoots could be attributed to (1) red light stimulation of endogenous gibberellins involved in mitosis and cell proliferation (Manivannan et al. 2015) by regulating gibberellic acid biosynthesis (Ouyang et al. 2015), (2) induced expression of a gene that inhibits auxin action and stimulates stem elongation (Li et al. 2017), or to (3) the high effectiveness of the inclusion of the green light to the spectrum in boosting stem lengthening at an early stage (Folta et al. 2005). Faster plantlet growth under mixed red – blue or blue-green-red-far red LED light than white light and FLs were reported in the recalcitrant pear (*Pyrus communis* L.) (Lotfi et al. 2019), beech (*Fagus sylvatica* L.), holm oak (*Quercus ilex* L.) and wild cherry (*Prunus avium* L.) (Astolfi et al. 2012), as well as in *Eucalyptus*, *Musa*, and *Spathiphyllum* (Nhut and Nam 2010). A possible explanation for the stimulation of Gisela 6 shoots height could be explained by the fact that red and blue lights contribute to faster growth of the plant compared to white light, while the absorption of green light by phytochromes and cryptochromes leads to reduced vegetative development (Folta and Maruhnich 2007).

Effect on shoot biomass

In several plant species, different red-blue LED mixtures and high light intensities of *in vitro* cultures were revealed to be connected with higher fresh and dry weight yields than the cultures grown under FLs (Bello-Bello et al. 2016; Gupta and Jatothu 2013; Kim et al. 2004). In the studied Gisela 6, the fresh and dry biomass yield of shoots was higher under LED2 treatment (BR). In consistency with findings of the present study, shoot cluster weight, shoot number, and shoot height in *P. communis* L. were maximum under blue + red LEDs combinations (Lotfi et al. 2019). These results for maximum biomass performance under BR LED are in agreement with those reported in three Mediterranean forest plant species, including *F. sylvatica*, *Q. ilex* and *P. avium* illuminated by blue - green- red -far red LED mixture (Astolfi et al. 2012). Based on the observations of Zheng et al. (2008), the increase in the endogenous level of carbohydrates is favored more under the combined effect of red and blue light, thus more photosynthetic products for plants and higher fresh weights.

Different light intensities under the same absorption wavelength spectrum (BR) on shoot proliferation

In Gisela 6, shoot number, proliferation rate and biomass were highest in BR spectrum under the intermediate light intensity of 40 µmol m^{-2} s^{-1} (LED2) and lowest under the marginal intensities of 20 and 120 µmol m^{-2} s^{-1} (LED1 and LED4). In line with our findings, Sáez et al. (2012) revealed that proliferation rate of *Castanea sativa* Mill. microshoots was increased after a 3-fold increase in intensity from 50 to 150 µmol m^{-2} s^{-1}. The highest light intensity of 120 µmol m^{-2} s^{-1} had an inhibitory effect on shoot proliferation especially shoot height in Gisela 6, raising drastically by 3.5–11 times the percentage of explants showing abiotic stress symptoms at the same time. Lin (2008) pointed out that bud differentiation is adversely affected perhaps because of high light-induced stress. Indeed, there is an inverse correlation between increasing light availability and decreasing plant height attributed on the one hand to apical dominance due to increased levels of endogenous auxin and on the other hand on the syndrome of shade avoidance and the process of etiolation induced by other PGRs such as gibberellic acid and brassinosteroids (Vanneste and Friml 2009). The leaves are the most sensitive and adjustable organ of a plant that perceive faster several stresses and perform a better adaptive mechanism, thus the monitored changes are more easily to be observed in the leaves than in other plant organs including the shoots or the roots (Guerfel et al. 2009), for example in this study with mild chlorosis, light brown coloration and bleaching of initial leaves, smaller number and size of leaves, less intense green color of leaves, and base leaves twisted inwards in Gisela 6 microshoots grown under LED4. Plants cultured *in vitro* when undergo high irradiation have the tendency to form larger leaves as a countermeasure to ensure a natural autotrophic mode of operation to cope with stress (Yao et al. 2017), extending the leaf surface for better photosynthesis and higher light absorption by the plant (Lambers et al. 2008). It is clearly pointed out that the stress imposed by the high light intensity neither had a strong inhibiting effect on shoot proliferation of Gisela 6 nor succeeded to have a stimulating action at the same time.

Photosynthetic pigment content under different light conditions

Key role in plant growth and development play the quality of light in which the plants are exposed through photosynthesis and photosynthetic pigments synthesis (Gao et al. 2020). In accordance with previous results in *Gerbera jamesonii* grown *in vitro*, obtained by Pawlowska et al. (2018), in Gisela 6 plantlets, the photosynthetic pigments were found slightly increased under BR (LED2) (Table 4). Nevertheless, they were not significantly different compared to BG (WFL) and BGYOR (LED3) (Table 4). In the present study, under different light intensities perceived by the same BR spectrum, chl a and total chl (a + b) content in Gisela 6 did not have any significant difference between the different light intensities, however there was a significant decline in chl b content under the intensity of 40 µmol m^{-2} s^{-1} (LED2) (Table 5), due to the further increase in light intensity that resulted in the degradation of photosynthetic pigments depicted by the reduced chlorophyll content in tissues (Zhou et al. 2008). Any alterations in the photochemistry of photosystem, especially those concerning the content of PSII:PSI ratio and the size of PSII light-harvesting antenna, can be mirrored by the modifications in the ratio of chl a/ chl b (Xu et al. 2020). In our study, the Chl a/b ratio was the highest under LED2 and differed compared to LED1 (Table 5), suggesting more profound adaptability of light, greater electron transportation capability of chlorophyll and stronger activity of the enzyme involved in the Calvin cycle (Xu et al. 2020). An up-rising trend in total carotenoids content was observed under 40 µmol m^{-2} s^{-1}, whereas a further 3-fold increase in light intensity from 40 to 120 µmol m^{-2} s^{-1} caused a

significant decrease (Table 5). Carotenoids function as photo-sensitizers exhibiting a scavenging action against the increased production of reactive oxygen species (ROS) (Lin et al., 2020). The augmentation of carotenoids in the xanthophyll cycle is imperative for the diffusion of the surplus energy created as a result of the high light intensity (Junior et al. 2020). On one hand, plants with lower chlorophyll content can use photosynthetic centers more efficiently than plants with excessive chlorophyll, thus higher content of these pigments does not necessarily indicate higher photosynthetic efficiency (Saebo et al. 1995), while on the other hand, high light intensity can contribute to ROS emancipation and cell damage (Moustakas 2022). Thus, it is evident that the most appropriate selection and optimization of lighting parameters in tissue culture systems is different for each plant species and is of the utmost.

Changes in chlorophyll fluorescence parameters under different light conditions

Any observed modifications in the values of chlorophyll fluorescence parameters depict the evolution of several photosystem metabolic procedures including light energy absorption, utilization, transfer and dissipation of PSII (Schreiber et al. 1995). Xu et al. (2020) showed that chlorophyll fluorescence parameters, namely Φ_{PSII} and q_P, differed in *Cunninghamia lanceolata* plantlets under different light qualities. However, in our results, under the three different light wavelength absorption spectra (BG, BR and BGYOR), there was not any significant difference in any chlorophyll fluorescence parameter (Table 3). Our data showed that Φ_{PSII} was substantially higher in LED1 and LED2 corresponding to 20 and 40 μmol m^{-2} s^{-1} intensities, respectively compared to the highest 120 μmol m^{-2} s^{-1} intensity (LED4) (Fig. 2). The considerable higher Φ_{NO} under LED4 could not be offset by the augmented photoprotective energy diffusion (Φ_{NPQ}) (Fig. 2). Moustaka et al. (2020) state that Φ_{NO} is composed of internal conversions and intersystem crossing in chlorophyll fluorescent parameters that lead to the production of the singlet oxygen ($^1O_2$) through the triplet state of chlorophyll ($^3chl^*$). PSII can lead to the creation of $^1O_2$, which is a ROS exerting a very strong damaging effect (Telfer 2014) that cause programmed cell death activated when produced at high levels (Roach et al. 2020). LED1 and LED2 gave significantly higher Φ_{PSII} (Fig. 2) and this fact can be ascribed based on the PSII function model either to the fraction of open PSII reaction centers (q_P) or to the efficiency of these centers ($F_{v'}/F_{m'}$) (Genty et al. 1989). In our experiment, q_P (Fig. 3b) and $F_{v'}/F_{m'}$ (Fig. 3c), were significantly higher under LED1 and LED2 compared to LED4. This could also be the reason that Gisela 6 plantlets had an increased biomass under LED1 and LED2 (Table 2). In addition, under LED4, Gisela 6 plantlets exhibited a significant higher excess excitation energy (EXC) (Fig. 4b). Over production of EXC may result in the proportional raise in the production of $^1O_2$ which is known to possess a detrimental effect provoking concrete damage to cells (Sperdouli et al. 2021). There is a close relationship between avoidance of excess energy in chloroplasts and satisfactory activation of photoprotective mechanisms (Moustakas et al. 2020) as it was observed under LED1 and LED2, implying that lower light intensities contribute to impeding of surplus stimulation energy at PSII (Fig. 4b).

Changes in leaf anatomy under different light conditions

Both light intensity and light quality significantly affected leaf anatomical traits (Fig. 5). The leaf mesophyll structure changes observed, could be due to the fluctuation of both palisade and spongy mesophyll tissues, the relative cell density of which is directly controlled by the light spectrum (Borsuk et al. 2022), since, palisade mesophyll cells allow light diffusion inside the chloroplasts, while spongy mesophyll cells, enhance light scattering and by this, increase light capture (Ustin and Jacquemoud 2020). Commonly, it has been stated that during *in vitro* culture of many plants, mesophyll structural changes occur due to the specific light spectrum used (Ma et al. 2021). For example Macedo et al. (2011) showed that in *in vitro* cultured *Alternanthera brasiliana* plants, blue LED light amplified the thickness of palisade *vs*. spongy mesophyll tissue, while, red LED lighting reduced total leaf thickness, with the most severe effects to be observed in the spongy mesophyll tissue. In accordance, leaf blades of Gisela 6 microshoots developed under LED light that contained either high intensity (LED4) or low intensity (LED1) Blue-Red spectrum produced suppressed spongy mesophyll, while LED2 (40 μmol m^{-2} s^{-1} intensity) had the same effect to the leaf anatomy as WFL did. The noted spongy mesophyll suppression could be mirroring the microshoot leaf response to radiation stress (Wellman 1983). The manifested protective mechanism could be mirrored

by the low numbers of spongy mesophyll cells, and the leaves structural adaptations to radiation (Ustin and Jacquemoud 2020) could explain the observed anatomical changes. However, this reduction can be also due to the lowered photosynthetic activity (Li et al. 2008), as observed in LED4 lighting conditions (Fig. 5). Moreover, during micropropagation, epidermal tissue thickness is depended on the light quality and of the concentration of the different growth regulators which the culture medium contains (Cioć and Pawłowska 2020). Controlling these external factors, one could counterbalance the harmful effects of the increased moisture occurring inside the culture vessels i.e. the epidermal thinning (Hazarika 2006). LED4 lighting applied during multiplication of microshoots of Gisela 6, increased the thickness of the adaxial epidermis of the leaves, which is somehow something desirable, however LED4 light stressed the microshoots (Table 2).

It has been stated that blue light addition to the LED spectrum is important for normal anatomical leaf development (Zheng and Van Labeke 2017). However these results indicate that light intensity is also of importance judging by the differences in the anatomy observed between LED1, LED2 and LED4 (Fig. 5b, c and e; respectively). Gisela 6 leaf anatomy was also altered by the addition to the LED spectrum of green, yellow and orange light (LED3), similar spectrum is observed under tree canopy (Krueger 1981) and light spectrum under canopy shading conditions affect leaf anatomy (Wu et al. 2017).

Conclusions

Cultivar responses to the same or similar light spectrums seem to be plant specific, and therefore further research is vital in order to distinguish the best ratio of blue/red LED combinations to produce optimally grown Gisela 6 plants. Among the three absorption wavelength spectra under 40 μmol m^{-2} s^{-1} light intensity, WFL (400–700 nm, mainly BG) and LED2 (450–550 nm, mainly BR) exhibited higher shoot numbers, proliferation rates and shoot heights whereas fresh and dry biomass yields were significantly higher under LED2. Among the three light intensities of blue-red color, best proliferation results (shoot number, shoot height, proliferation rate, biomass) were obtained under 40 μmol m^{-2} s^{-1}. Photosynthetic pigments and chlorophyll fluorescence parameters of Gisela 6 *in vitro* plantlets were not found to be significantly affected by the three different light qualities. When the plantlets were subjected to the three different light intensities of blue-red color, the ones under 40 μmol m^{-2} s^{-1} (LED2) displayed a better PSII functionality and an increased Chl a/b ratio and carotenoids content, avoiding oxidative damage as evidenced by the significantly decreased stressed explants. Moreover, leaf anatomy under LED2 illumination showed similar features with WFL-lighted Gisela 6 plants. Therefore, LED2 proved to be the most appropriate treatment for growing Gisela 6 plantlets *in vitro*. The spectral energy distribution of LEDs could fit the needs of various plant tissue culture aspects including commercial micropropagation providing lower-cost, more energy- and higher-yield efficient production systems, compared to high electricity consumption/ low output efficiency of the WFLs.

Declarations

Authors Contribution Statement

Conceptualization, experimental design, validation, supervision, project administration, funding acquisition, writing - review & editing were provided by K.G. Investigation, implementation of the experiments, data processing, formal analysis, data curation, writing–original draft, review & editing were performed by V.S., I.S., and I.D.A. In particular, *in vitro* shoot proliferation was performed by V.S., photosynthetic pigments content and chlorophyll fluorescence parameters were estimated by I.S., and scanning light microscopy images of leaf anatomy was obtained by I.D.A. All authors read and approved the final manuscript.”

Acknowledgments

This research has been co-financed by the European Regional Development Fund of the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH – CREATE – INNOVATE (project code: T1EDK – 00281) entitled “Evaluation - Improvement - Promotion of Important Traditional Greek Fruit Varieties” (Acronym: GrEatFruit).

Funding: *This work was supported by the European Regional Development Fund of the European Union and Greek national funds (grant number: T1EDK – 00281).*

Competing Interests: *The authors have no relevant financial or non-financial interests to disclose.*

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Figures

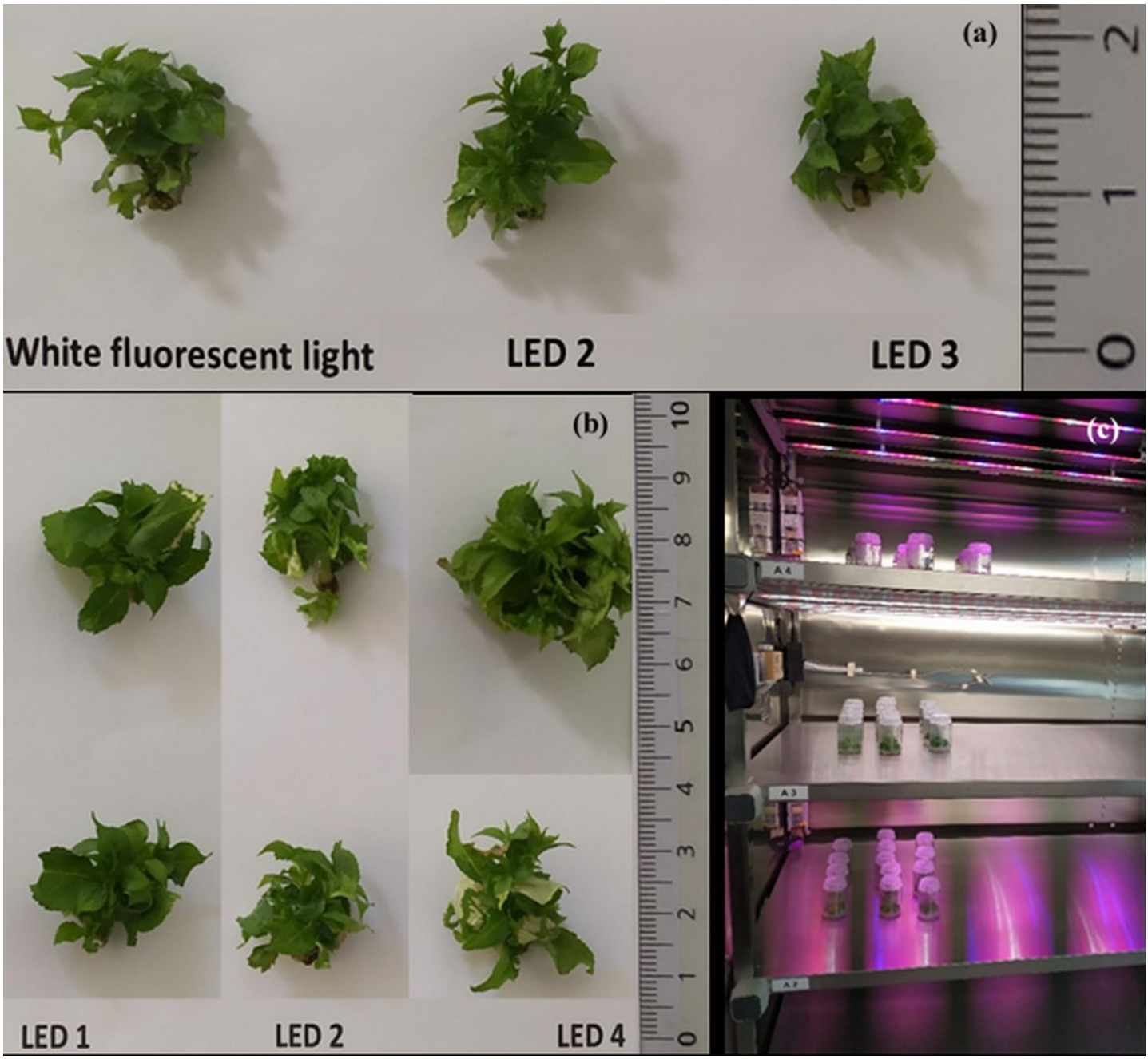


Figure 1

In vitro proliferation of Gisela 6 microshoots under the effect of **(a)** three absorption wavelength spectra including 400-700 nm (mainly blue-green, BG)/White Fluorescent Light (WFL), 450-550 nm (mainly blue-red, BR)/LED 2, and 450-660 nm (blue-green-yellow-orange-red, BGYOR)/LED3 under 40 µmol m^{-2} s^{-1} light intensity, **(b, c)** blue-red (BR) absorption wavelength spectrum and three light intensities (20, 40, 120 µmol m^{-2} s^{-1}) including three LED lamps (LED1, LED2 and LED4)

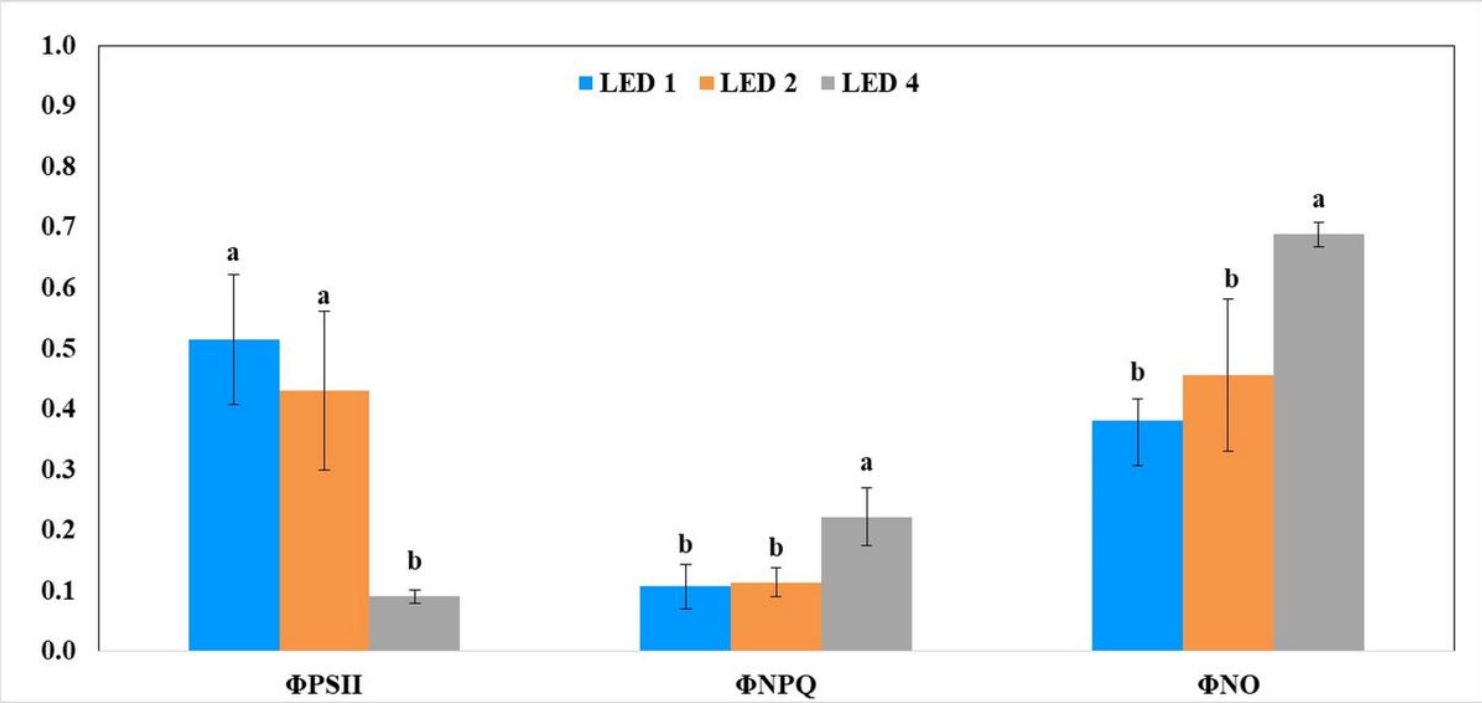


Figure 2

Changes in the effective quantum yield of PSII photochemistry (Φ_{PSII}), the quantum yield of regulated non-photochemical energy loss in PSII (Φ_{NPQ}) and the quantum yield of non-regulated energy loss (Φ_{NO}), in Gisela 6 leaves under blue-red (BR) absorption wavelength spectrum and three light intensities (20 µmol m^{-2} s^{-1}/ LED1, 40 µmol m^{-2} s^{-1}/LED2 and 120 µmol m^{-2} s^{-1}/LED4). Error bars are standard errors. Columns denoted by different letters indicate statistically significant differences ($p< 0.05$)

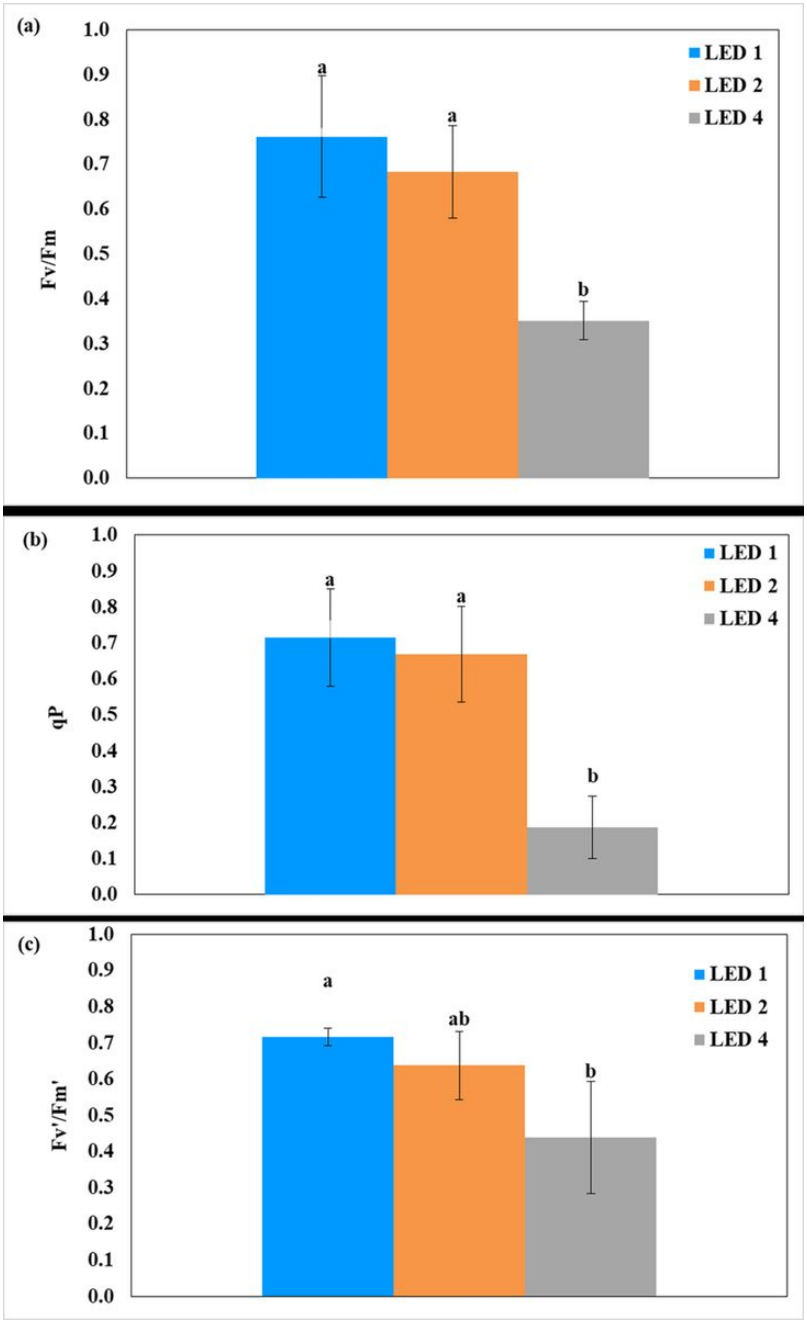


Figure 3

Changes in **(a)** the maximum efficiency of PSII photochemistry (F_v/F_m), **(b)** the fraction of open PSII reaction centers pool (q_P) and **(c)** the efficiency of open PSII reaction centers ($F_{v'}/F_{m'}$), in Gisela 6 leaves under blue-red (BR) absorption wavelength spectrum and three light intensities (20 µmol m^{-2} sec^{-1}/ LED1, 40 µmol m^{-2} s^{-1}/LED2 and 120 µmol m^{-2} s^{-1}/LED4). Error bars are standard errors. Columns denoted by different letters indicate statistically significant differences ($p< 0.05$)

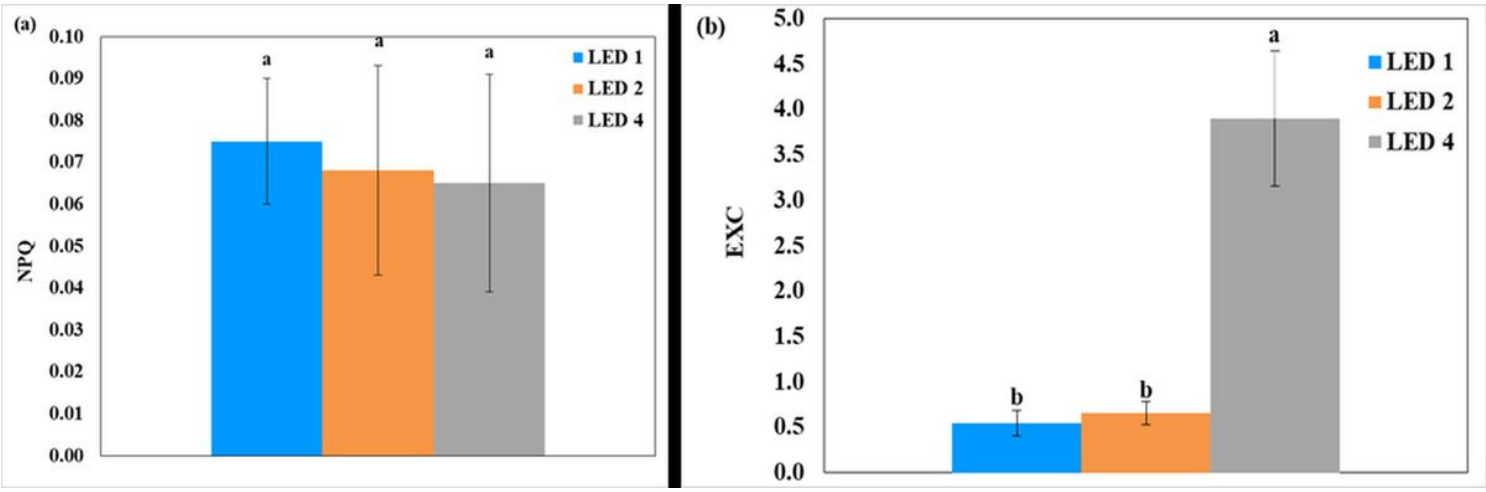


Figure 4

Changes in **(a)** the non-photochemical quenching (NPQ) and **(b)** the excess excitation energy (EXC), in Gisela 6 leaves under blue-red (BR) absorption wavelength spectrum and three light intensities (20 µmol m^{-2} s^{-1}/ LED1, 40 µmol m^{-2} s^{-1}/LED2 and 120 µmol m^{-2} s^{-1}/LED4). Error bars are standard errors. Columns denoted by different letters indicate statistically significant differences ($p < 0.05$)

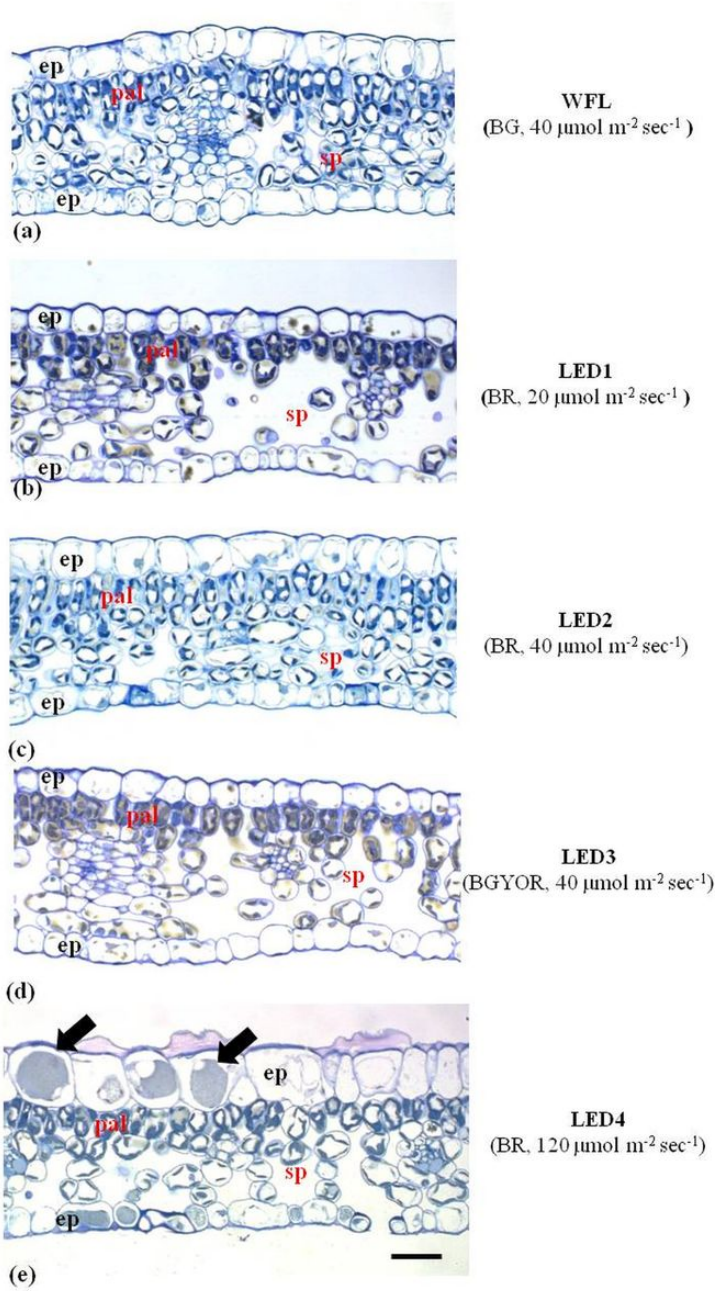


Figure 5

Leaf cross sections of microshoot leaves grown under WFL (a), LED1 (b), LED2 (c), LED3 (d) and LED4 (e), lighting conditions, stained with toluidine O. Arrows in e, indicate intensively stained material. Ep: epidermis, pal: palisade mesophyll, sp: spongy mesophyll. Scale bar: 20µm