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LED lighting affects the biomass accumulation and leaf stomatal characteristics of raspberry (*Rubus idaeus* L.) *in vitro*

LED светлините влияят върху натрупването на биомаса и характеристиките на устицата на малината (*Rubus idaeus* L.) в *in vitro* условия

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ABSTRACT

Light-emitting diodes (LEDs) have become an alternative light source to the fluorescent lamp (FL) for the maintenance of plant tissue cultures due to their low heat emission, low power consumption and the ability to fine-tune the light spectrum. In this study, the effect of LEDs on the growth and leaf stomata features of *in vitro* cultivated raspberries (*Rubus idaeus* L. 'Lloyd George') was examined. The plantlets were grown *in vitro* under a lighting system based on the Philips GreenPower LED research module. Four groups of LEDs emitting white (W), red (R), blue (B), and mixed (W:R:B: far red = 1:1:1:1) lights and FL (control) were used. As a second control (marked as EV), plants grown in the multiplication and rooting stage under fluorescent lamps and then acclimatized *ex vitro* in a greenhouse for 90 days in natural light were included. Relative growth rate (RGR), protein content as well as stomata morphology and density of the plantlets were analysed after three passages under corresponding light treatment. The results show that different LEDs specifically affect the growth and size of leaf stomata and density of *in vitro* cultured raspberry plants and can be applied as an effective modulator of morphogenesis during micropropagation. The combination of white, blue, red and far red LED stimulated the accumulation of biomass and proteins, as well as the formation of a higher number of stomata on the lower surface of the leaves, which would be a prerequisite for more effective control of water loss from plantlets during the *ex vitro* acclimatization.

Keywords: *ex vitro* acclimatization, light quality, micropropagation, stomata

РЕЗЮМЕ

LED-светлинните източници станаха алтернативни на флуоресцентните лампи (FL) за растителните тъканни култури поради ниската си топлинна емисия, малкото количество енергия, което консумират, и възможността за фино регулиране на спектъра на светлината. В това проучване ние изследвахме ефектът на LED-светлините върху растежа и характеристиките на устицата в листния епидермис на *in vitro* култивирани малини (*Rubus idaeus* L. 'Lloyd George'). Растенията бяха отгледани *in vitro* в система на осветяване, базираща се на Philips GreenPower LED изследователски модул. Бяха използвани четири групи LED-светлини: бяла (W); червена (R); синя (B); смесена (W:R:B:far-red=1:1:1:1) и FL (контрола). Като втора контрола (означена с EV) бяха включени растения от фазите на мултипликация и вкореняване под флуоресцентна светлина, които след това бяха аклиматизирани *ex vitro* в оранжерия за 90 дни при естествена светлина. Относителната скорост на растеж (RGR), съдържанието на общ разтворим протеин, както и морфологията и гъстотата на устицата бяха анализирани при растения,

отгледани след три пасажа при съответните светлинни въздействия. Резултатите показаха, че различните LED-светлини повлияват по специфичен начин растежа на *in vitro* микрорастенията от малина и размера и гъстотата на устицата в листния епидермис и могат да бъдат прилагани като ефективни системи на осветление за *in vitro* микроразмножаване. Комбинацията от бяла, червена, синя и далечночервена светлини стимулира натрупването на биомаса и протеини, както и образуването на по-голям брой устица по долната повърхност на листата, което може да бъде предпоставка за по-ефективен контрол на загубата на вода от експлантите в периода на *ex vitro* аклиматизацията.

Ключови думи: *ex vitro* аклиматизация, качество на светлината, микроразмножаване, устица

INTRODUCTION

Raspberries (*Rubus idaeus* L., *Rosaceae*) are a source of a wide variety of bioactive substances and are grown for their excellent taste, as well as for the healing and dietary properties of the fruits. The significant beneficial effects of their fruits on human health have been widely recognized both in antiquity and in modern times (Beekwilder et al., 2005; Simonovic et al., 2019). This increased their demand and the development of methods for their reproduction. *In vitro* micropropagation of raspberries under sterile, controlled conditions has been applied for more than 40 years to obtain large numbers of pathogen-free plants of selected genotypes. However, according to several authors, the presence of many cultivars with great differences in their requirements for recovery and propagation make the raspberry particularly unamenable to *in vitro* cultivation (Reed, 1990; Zawadzka and Orlikowska, 2006a, 2006b; Wu et al., 2009). A lot of experimental work has been done to optimize cultivation conditions, mineral composition of nutrient media (Rušič and Lazić, 2004; Poothong and Reed, 2014, 2015), growth regulators (Hunkova et al., 2016) and others.

Fluorescent lamps (FL) have long been used to grow plants *in vitro*, but in the last twenty years, they have often been replaced by light-emitting LEDs due to their low heat emission, low energy consumption and the ability to fine-tune the light spectrum. Differences in spectral quality between LEDs and fluorescent lamps directly affect photosynthesis, growth and morphogenesis of *in vitro* grown plants. Various studies have shown more vigorous plants when cultivated *in vitro* under LED lighting conditions (Hahn et al., 2000; Moon et al., 2006; Lin et al., 2011; Hung et al., 2016; Batista et al., 2016; Ferreira et al., 2017). However, many authors have noted that requirements for the spectral composition of light and photosynthetic photon flux density (PPFD) are genotype-specific (Gupta and Jatothu, 2013; Shulgina et al., 2021). Therefore, it is essential to conduct research to optimize the protocol using LED technologies to assess the impact on the morphogenesis of different plant species.

In the scientific literature, there are only a few reports on the influence of LED lighting on raspberries grown *in vitro*. Rocha et al. (2013) reported that red LEDs increased the shoot number and rooting efficiency of raspberry plantlets compared to fluorescent lights. According to Poncetta et al. (2017), the mixed LED light yielded less efficient multiplication of red raspberry in comparison to fluorescent lights, but with higher quality shoots.

A previous study (Nacheva et al., 2021) showed that raspberry plantlets had clearly distinguishable responses to lights of different wavelengths. The combination of white, red, blue, and far red LED lights stimulates the growth, biomass accumulation, and net photosynthetic rate of plantlets during *in vitro* propagation. Furthermore, raspberry shoots rooted *in vitro* under combined or white light grew better during greenhouse acclimatization and their content of photosynthetic pigments was higher (Nacheva et al., 2023b). This determines the need for more research to identify the spectral composition of light suitable for improving the *in vitro* cultivation of raspberries and their subsequent adaptation *ex vitro*.

The structural characteristics and functional state of the leaf stomata are decisive factors for the proper development of micropropagated plantlets not only during the *in vitro* period but especially during their adaptation *ex vitro*. High stomatal density and big dimensions may increase leaf gas exchange and improve photosynthesis and plant development *ex vitro*. However, the increasement of these characteristic values can cause excessive evaporation and water stress for plants, since *ex vitro* atmospheric moisture is lower, especially if the roots are not well developed (Zeps et al., 2022). The light quality is one of the main environmental signals that control the development, density, size and aperture of stomata (Kim et al., 2004; Chaerle et al., 2005; He et al., 2020). Light with the same spectral characteristics does not elicit the same stomata response in different plant species (Kim et al., 2004; Poudel et al., 2008; Wang et al., 2016; Cioć et al., 2019; Díaz-Rueda et al., 2021). The purpose of this study was to establish, compare and analyse some morphological, biochemical and anatomical features of raspberry plantlets micropropagated *in vitro* under different LED-lights in order to estimate the optimal lighting mode. These analyses could indicate the necessary steps to optimize *in vitro* conditions for more successful *ex vitro* acclimation and better raspberry propagation.

MATERIALS AND METHODS

Plant material and growth condition

Red raspberry (*Rubus idaeus* L. 'Lloyd George') *in vitro* shoot explants were grown on solid DKW (Driver and Kuniyuki, 1984) medium, supplemented with 2.5 µM 6-benzylaminopurine (BAP), 0.05 µM indol-3-butyric acid (IBA), 30 g/L sucrose, 6.5 g/L Phyto agar (Duchefa, The Netherlands) (Nacheva et al., 2021). The medium (pH 5.6) was autoclaved at 121 °C for 20 min. Explants were grown in glass jars (volume 200 mL) with transparent Magenta B-Cap lids with 25 mL nutrient medium per vessel. Plantlets were subcultured at 4-week intervals. At each subculture, shoots (10 – 15 mm) with two-three leaves were transferred to the fresh medium with five explants per vessel. Jars were placed in four cameras illuminated with LEDs emitting in white (W), red (R, 650-670 nm), blue (B, 455-485 nm), and far red (FR, 725-750 nm) spectra provided by Philips GreenPower LED research module (Philips Lighting, www.philips.com/horti) under stable temperature 22±2 °C and 16-h photoperiod (87 ± 7.5 µmol/m^{2}s photosynthetic photon flux density, PPFD). Respectively, four treatments marked W, R, B and mixed (W:R:B:FR = 1:1:1:1) were studied. As a control were used plantlets grown in the same way under fluorescent lamps (FL). As a second control (marked as EV), plants grown in the multiplication and rooting stage under fluorescent lamps and then acclimatized *ex vitro* in a greenhouse for 90 days in natural light were included. In three passages of four weeks, some physiological, biochemical and morphological parameters of plantlets grown under different light were evaluated.

Relative Growth Rate

Relative Growth Rate (RGR, $mg_{DW}/mg_{DW}day$) was calculated using the equation $RGR = (\ln W_2 - \ln W_1) / (t_2 - t_1)$, where W_1 and W_2 are plantlet dry weight at time t_1 and t_2, respectively. The RGR of *ex vitro* acclimatized plants was calculated similarly on day 90 after planting the plants for acclimatization in soil substrate.

Soluble protein content

The Bradford method (1976) was used to determine total soluble protein content. Fresh leaves (300 mg) were homogenized with 5 mL cold phosphate buffer. The mixture was centrifuged at 10000 rpm for 15 min. For the protein assays 1 mL supernatant was mixed with 5 mL cooled Bradford reagent. The extinction was measured at 595 nm. The bovine serum albumin is used as a standard.

Light microscopy study of the leaf stomata

To study stomatal distribution and morphology the first fully developed leaves were collected from five plantlets for each treatment. Small segments (4-5 mm^2) were taken from the central part of the leaf lamina and were fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12h at 4 °C. Then the fragments were

washed with distilled water, bleached and washed again. Microscopic slides with glycerol were prepared. The samples were observed under Amplival 4 light microscope (Carl Zeiss, Jena, Germany). For each LED light treatment, thirty measurements of the stomatal length and width (in µm) as well as the stomatal density (number/mm^2) were measured. Microphotographs (2560x1960 pixels) at 400x magnification were taken with an EcoBlue digital microscope (EC.1657) with an integrated 5.0 MP USB-2 camera (Euromex, The Netherlands).

Statistical analysis

The results were subjected to a one-way analysis of variance (ANOVA), followed by the comparison of group means (Dunn's test) with the program Past 4.01 (Hammer et al., 2001).

RESULTS AND DISCUSSION

Treatment with lights with different spectra significantly affected the growth of *Rubus idaeus* L. 'Lloyd George' plantlets (Figures 1, 2).

Figure 1. Philips GreenPower LED research module in the growth chamber (left). Red raspberry (*Rubus idaeus* L. 'Lloyd George') grown at different light conditions (center). FL - Fluorescent lamps (control), B – blue LEDs, R – red LEDs, WBR – mixed LEDs, W - white LEDs. EV-acclimatized plant (right).

Compared with control (FL) and monochromatic LED light, the combined WBR LED light increased the RGR of the raspberry plantlets *in vitro*. The RGR under mixed WBR were the highest at 1.38 times that of the control. The lowest RGR was reported in plants grown under red light (0.0149 mg_{DW}/mg_{DW}day), which is 2.5 times less than those grown under mixed LED light (WBR). Surprisingly low, comparable to that in red light, was the RGR in plants cultivated under white LED light. Therefore, the application of red, white or blue LED light separately was not favorable for biomass accumulation in *in vitro* raspberry plants.

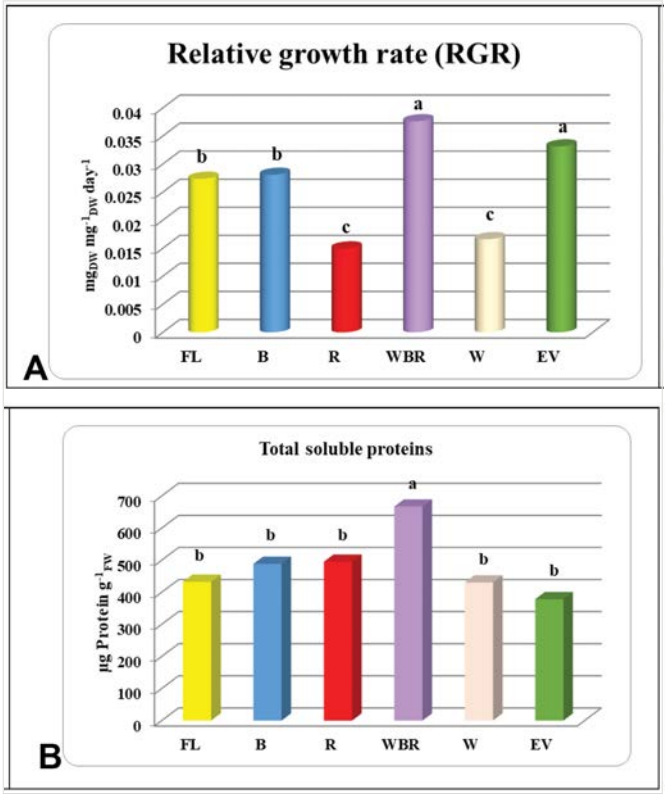

Figure 2. Effect of different light treatments on the RGR (A), protein content (B) of raspberry (*Rubus idaeus* L. 'Lloyd George') plantlets. FL - Fluorescent lamps (control), W - white LEDs, R – red LEDs, B – blue LEDs, WBR – mixed LEDs, EV-acclimatized plant. Means in the column, followed by different letters are significantly different at $P \leq 0.05$.

In contrast to the lowest RGR found in raspberry plantlets grown under red light, studies by various authors have shown that red light promotes the development of the photosynthetic apparatus of plants and the accumulation of starch, while blue light is associated with the synthesis of chlorophyll, the development of the stomata and the

accumulation of secondary metabolites (Kobayashi et al., 2013; Bian et al., 2018). Many studies have indicated that red light can increase plant height, whereas blue light has the reverse effect, but aids in healthy growth (Manivannan et al., 2015; Bantis et al., 2016; Sun et al., 2017; Lobiuc et al., 2017; Xie et al., 2018; Wang et al., 2018). In contrast to monochromatic light, combined red-blue light is more beneficial for plant growth and biomass accumulation (Guo et al., 2015a, 2015b; Sun et al., 2017; Ye et al. 2017; Lobuic et al., 2017; Xie et al., 2018).

More and more authors have been considering mixed LEDs rather than monochromatic blue or red LEDs for plant growth optimization (Gupta and Jatothu, 2013). Similar to these results, several authors pointed out that the combination of red and blue LEDs enhances *Mentha* and *Fragaria* growth as compared to other monochromatic spectra (Nhut et al., 2003; Gupta and Jatothu, 2013; Sabzalian et al., 2014).

Also, the results obtained by Pawlowska et al. (2018) and Cioć et al. (2019) noted that a combination of red and blue LEDs (7:3) improved *Gerbera jamesonii* Bolus multiplication and photosynthetic pigment content.

Similar results were reported by Nhut and Nam (2010). Combining LEDs with a 4:1 red/blue ratio improves *in vitro* growth of eucalyptus, banana and spathiphyllum plants compared to white light.

The highest total soluble protein content was measured in WBR, which was more than 50% higher than in control (FL) raspberry plants (Figure 2 B). The lowest soluble protein content, almost as much as in the control, was reported in plants grown under white (W) light.

The raspberry leaves differentiated *in vitro* under different light treatments were hypostomatic and the stomata were fully developed (Figure 3). The largest-sized stomata were observed in the leaves of the plantlets grown under FL light (control ones) (Table 1). The stomata in the W and WBR light variants were slightly smaller than the control ones and bigger than the stomata in the leaves differentiated under monochrome B and R light. There were minor differences in the values of the ratio of the stomatal length to the stomatal width. The most rounded (1.07) were the stomata developed under mixed LED-light treatment (WBR). The treatment with WBR light resulted in the highest stomatal density which was considerably higher than in the other treatments and almost twice as much as the control one. The lowest stomatal density was found in raspberry plants grown under blue light. According to Mihovilović et al. (2020), the combination of red and blue LED also induced a significantly higher stomatal number of *Amelanchier alnifolia* plants *in vitro* compared to those cultured under FL.

Comparing *ex vitro* (EV) to *in vitro* control plants (FL) the stomata were significantly different in size and density. The stomata in the *ex vitro* plants (EV) were more oval in shape (1.32 ratio of stomatal length to width), smaller in size but with higher density (Table 1, Figure 3).

The light quality plays a crucial role in morphogenetic responses during leaf development (Silvestri et al., 2019). Variations of stomatal density in the leaves of raspberry plantlets propagated *in vitro* under different LED light treatments were observed. Monochrome B or R light-induced low stomatal density, while significantly higher stomatal density was recorded for mixed WBR, W or FL light. Similar was the effect during micropropagation

Table 1. Effect of different light treatments on the stomatal characteristics of raspberry (*Rubus idaeus* L. 'Lloyd George') plantlets

Light treatment	FL	B	R	WBR	W	EV
Stomatal lenght	$24.7^{a} \pm 3.8$	$19.3^{bc} \pm 3.3$	$20.2^{bc} \pm 3.2$	$21.0^{b} \pm 3.5$	$21.2^{ab} \pm 4.5$	$17.4^{c} \pm 2.6$
Stomatal width	$22.0^{a} \pm 3.5$	$16.2^{bc} \pm 3.4$	$17.6^{b} \pm 2.2$	$19.5^{ab} \pm 3.5$	$18.0^{b} \pm 4.0$	$13.1^{c} \pm 2.5$
Stomatal density	$172.1^{de} \pm 18.4$	$130.7^{e} \pm 19.1$	$205.4^{cd} \pm 28.4$	$361.9^{a} \pm 44.6$	$302.6^{ab} \pm 43.0$	$245.1^{bc} \pm 42.6$

Means followed by a common letter are not significantly different by the Dunn's test at $P < 0.05$

FL - Fluorescent lamps (control), W - white LEDs, R – red LEDs, B – blue LEDs, WBR – mixed LEDs, EV-acclimatized plant

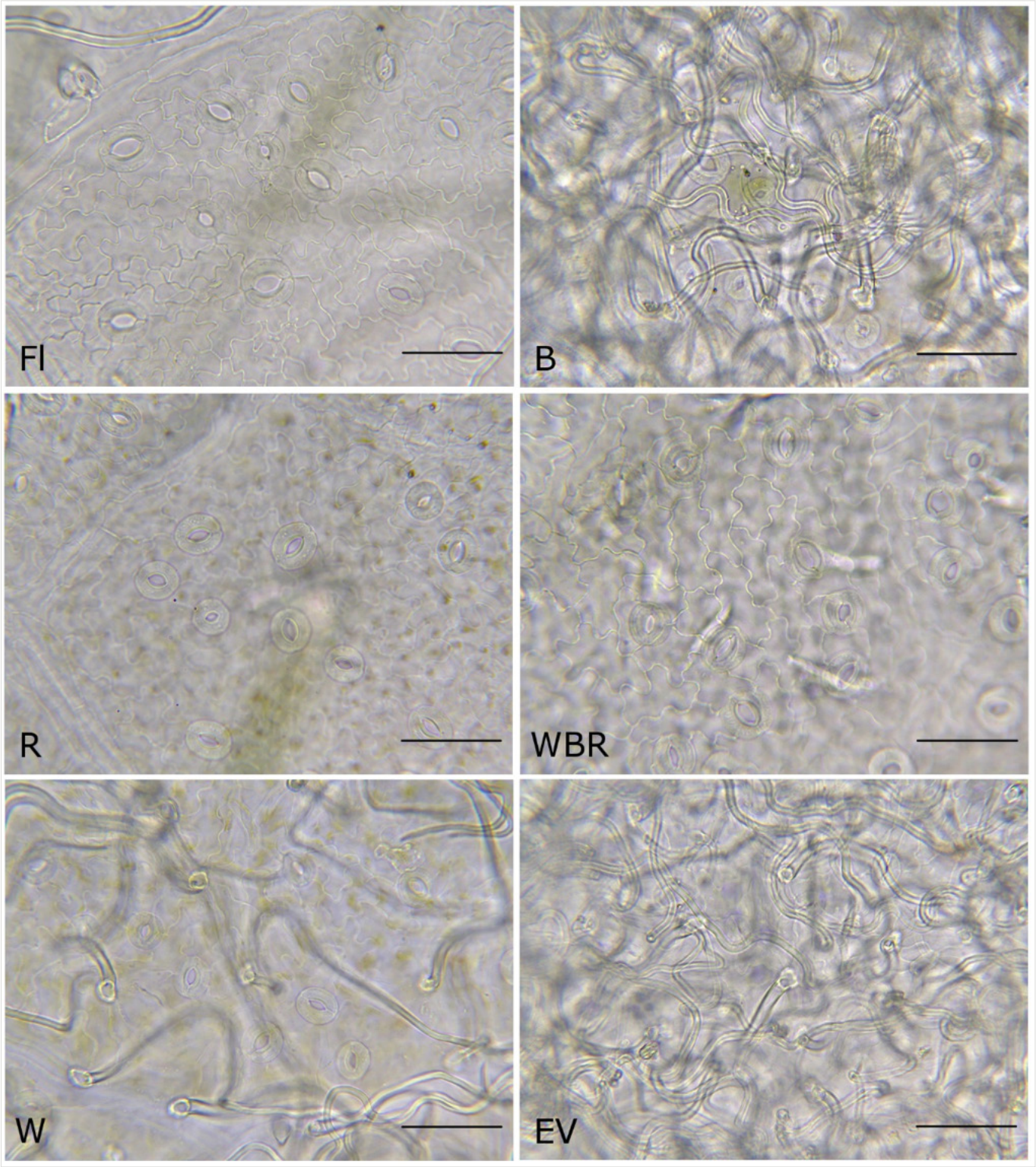

Figure 3. The abaxial (lower) epidermis of leaves of raspberry plantlets grown under different light treatments (FL - Fluorescent lamps (control), W - white LEDs, R – red LEDs, B – blue LEDs, WBR – mixed LEDs, EV-acclimatized plant. Scale bar = 50 µm)

of *Withania somnifera* (Lee et al., 2007), *Alternanthera brasiliana* (Macedo et al., 2011), gerbera (Cioć and Pawlovska, 2020), *Camelia oleifera* (He et al., 2020), plum (Nacheva et al., 2023). On the contrary, in chrysanthemum and grapes under blue (B) light the number of stomata was the highest (Kim et al., 2004; Poudel et al., 2008).

Regarding the length and width of the stomata in this experiment, some significant differences in the dimensions occurred. The smallest stomata were observed in plantlets propagated under monochrome B and R regimes. The values were bigger for mixed light and were the highest for FL control. In plums, the values for length and width were lower for FL and monochrome B and R lights and higher for mixed lights (Nacheva et al., 2023a). In *Lactuca sativa*, the stomatal length was small for R and B light and high for mixed light while the different light regimes did not affect the stomatal width (Wang et al., 2016). In grapes (Poudel et al., 2008) and olives (Díaz-Rueda et al., 2021) light quality during *in vitro* cultivation did not clearly affect the stomatal size. These varied results confirmed that the stomatal characteristics were affected by the light spectrum, but in species-dependent way.

Stomata are known to play a major role in maintaining plant water balance and CO_2 uptake for photosynthesis, enabling the plant to optimize the processes of photosynthesis and transpiration (Chaerle et al., 2005). A change in stomatal density alters the number of gas exchange sites per unit leaf area (Hill et al., 2014). Plants are forced by environmental conditions to continuously adjust stomatal openings to optimize the rate at which water or CO_2 is exchanged. Also, plants counteract the changing environmental conditions by modulating stomatal development in newly formed organs (Lake et al., 2001). It was found that stomatal development and their density could be affected by light intensity and quality (Kim et al., 2004; Vieira et al., 2015; Zheng and Van Labeke, 2017; Qi and Torii, 2018).

It is known that stomata vary not only in terms of their number but also in their size, shape and degree of opening depending on environmental conditions (Tichá et al., 1999). All these characteristics influence the stomatal conductance for CO_2 diffusion (Camargo and Marenco, 2011). In *in vitro* plants, the stomata are most often round and wide open (Tichá et al., 1999). Also, intensively growing plants develop fewer and larger stomata (Kim et al., 2004; Gupta et al., 2013).

However, in the current study, the RGR of plantlets cultivated under mixed light is the highest and corresponds to the highest stomata density. Similar to our results, Cioć and Pawłowska (2020) found that the multiplication factor in gerbera was highest under RB light, and stomatal density was similar to other light regimes with a lower multiplication factor. Similarly, relative to our raspberry results, the stomata in gerbera leaves developed under RB light did not have a larger area.

According to Wang et al. (2007), higher transpiration rate allows better nutrient absorption, which could be a possible explanation for the high relative growth rate (RGR) and protein of raspberry plants grown under mixed light (WBR), as well as increased intensity of photosynthesis found in previous study (Nacheva et al., 2021). A similar assumption was made by Cioć and Pawlovska (2020) - a greater number of stomata would probably improve gas exchange and lead to more intensive photosynthesis, which in turn would facilitate acclimatization.

Environmental conditions for *ex vitro* growth are quite different from those used for *in vitro* cultivation (Kozai et al., 1992; Hazarika, 2003, 2006). The advantages of the *in vitro* micropropagation of raspberry could be fully realized only if the plants propagated *in vitro* in the sterile culture were successfully acclimatized to *ex vitro* conditions. The change of environment from *in vitro* to *ex vitro* conditions is considered one of the strongest environmental stresses, which greatly reduces the success of micropropagation (Hazarika, 2006; Pospíšilová et al., 2007). It is believed that the main cause of plant mortality during *ex vitro* acclimatization is water stress (Hazarika, 2003; Teixeira da Silva et al., 2017). For that reason, to study the adaptive capacity of the plantlets grown *in vitro*, it is necessary to take into account the changes in the anatomical and morphological structure and features of the antioxidant system (Hasanuzzaman et al., 2020).

Cultivation of plants in *in vitro* conditions at very high humidity (over 95%), limited gas exchange, low light, as well as the presence of carbohydrates in the nutrient medium lead to deformations in the structure of leaves and stomata. After being removed from the culture vessels, plants cannot control their water balance well, which is one of the main reasons for large plant losses (sometimes up to 100%) during greenhouse acclimatization (Hazarika, 2003, 2006; Pospišilová et al., 2007). According to these authors, low survival rates during *ex vitro* acclimation are often due to inappropriate conditions during micropropagation.

CONCLUSION

The results presented in this study confirmed the need for permanent anatomical diagnostics to adjust the cultivation conditions in order to improve the *ex vitro* survival rate and the quality of the micropropagated plants.

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