RESPONSES OF HYBRID ASPEN *IN VITRO* CULTURES TO DIFFERENT PROPORTIONS OF RED AND BLUE LIGHT

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Abstract

Light is one of the most significant environmental factors affecting the growth of plants, as it is the driving force of photosynthesis. Among others, the red and blue light are the most relevant, as these spectral regions are absorbed by chlorophyll the most. In addition, red and blue light trigger specific photomorphogenic responses that allow plants to capture the available light efficiently. Accordingly, the proportion of red and blue light (R:B ratio) is considered one of the most important characteristics of light for plants, as optimal R:B provides balanced growth and photosynthesis. The aim of this study was to evaluate how the *in vitro* cultures of hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) are affected by different R:B ratios under fixed illumination intensity. We examined the growth characteristics of plantlets under wide spectrum LED luminaires with three different R:B proportions – 1:1, 7:3 and 9:1. While photosynthesis-related variables were significantly affected by light, the effect on morphology was less pronounced. Overall, increased proportions of red light decreased the photosynthetic performance of plantlets without giving significant benefits in the form of longer shoots that could be used to facilitate propagation effectiveness. Nonetheless, the effect of light treatment remains at least partially clones-specific and should be considered in case of further application for propagation purposes.

Keywords: LED, *Populus tremula* x *P. tremuloides*, photosynthesis, photomorphogenesis, R:B ratio.

Introduction

As demand for wood-related products continues to increase, the use of highly productive and stress tolerant genotypes of forest tree species becomes more relevant (Konnert *et al*., 2015). In the Eastern Baltic region, hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) is a promising option, as it exhibits superior growth compared to local tree species and can be used for wide range of applications (Niemczyk *et al*., 2019). The superiority of hybrid aspen derives from the phenomenon of heterosis (or hybrid vigour), where offspring exhibits traits that exceed those of parent species, resulting in faster growth, higher productivity, and resilience to stress factors (Li, Howe, & Wu, 1998; Hu & Thomas, 2019). Characteristics of heterosis are expressed in F_1 generation, accordingly reproductive material of hybrid aspen clones needs to be propagated vegetatively (Stanton, Neale, & Li, 2010). For this, propagation by micro shoot cultures *in vitro* is the most relevant technique, as it provides higher productivity compared to conventional vegetative propagation methods (Winton, 1971).

Light is one of the most significant environmental factors affecting plant growth both *in vitro* and *ex vitro*, as it is used as the primary energy source for photosynthesis (Wang *et al*., 2022). Red and blue spectral regions are the main driving force of photosynthesis, as these photons are absorbed by chlorophyll the most (Lichtenthaler, 1987). Accordingly, plants have developed photoreceptors allowing them to detect light conditions and adjust their architecture to capture the available light efficiently (Wang *et al*., 2022).

Red light is absorbed by phytochromes and is associated with increased shoot elongation, accumulation of starch (Muleo & Thomas, 1997; Werbrouck *et al*., 2012; Chen *et al*., 2018; Fan, Manivannan, & Wei, 2022), and is used for photosynthesis more efficiently than blue light (Fan *et al*., 2013). Blue light is absorbed by cryptochromes and is associated with chlorophyll synthesis (Hung *et al*., 2016; Chen *et al*., 2018) as well as chloroplast movement and stomatal size and opening (Hogewoning *et al*., 2010; Fan *et al*., 2013; Chen *et al*., 2018; Moosavi-Nezhad *et al*., 2021), all of which significantly contribute to photosynthetic capacity. Therefore, a balanced spectrum is a prerequisite as a means to provide efficient photochemical reactions and morphogenesis. Furthermore, plants have evolved to perceive the wide spectrum of sunlight; accordingly, the effect of monochromatic illumination can be misleading (Kondratovičs *et al*., 2022) and the light signal should be interpreted as an interaction of several wavelengths simultaneously (Hogewoning *et al*., 2010; Wang *et al*., 2022).

The ratio of red and blue light (R:B) is considered one of the most important characteristics of light due to its significance in photosynthesis (Fan, Manivannan, & Wei, 2022). A balanced distribution between both wavelengths ensures optimal development of plants; however, it depends highly on the ecology of species and has been intensely studied in various crops (Piovene *et al*., 2015; Naznin *et al*., 2016a; Naznin *et al*., 2016b; Chen *et al*., 2018). As regards the effects of R:B ratio, trees, which are long living organisms have been little studied. The aim of this study was to evaluate how the *in vitro* cultures of hybrid aspen are affected by different R:B ratios under fixed illumination intensity. We hypothesize, that hybrid aspen clones will exhibit higher plasticity in terms of morphological changes due to more plastic adaptation mechanisms utilized by trees.

Materials and Methods

This research was carried out in the Laboratory of Plant Physiology of Latvian State Forest Research Institute 'Silava' in the summer season of 2023. The plant material used in this study was already established micro shoot cultures of locally bred hybrid aspen clones named No. 28 and No. 90 with intermediate field productivity (Zeps *et al.*, 2022). Prior to the experiment, the stock material was maintained by routinely transplanting every 4 weeks on fresh ½ concentration Murashige & Skoog (MS) medium (Murashige & Skoog, 1962), supplemented with 0.1 mg L^{-1} indole-3-butiric acid (IBA), 20g L^{-1} sucrose and solidified with 6g L^{-1} plant agar (Sigma-Aldrich, USA). Prepared culture medium was poured into 300 mL glass jars (approximately 30 mL per jar), covered with aluminium foil and sterilized in an autoclave for 15 min at 121°C under 110 kPa of pressure. The pH of the culture medium was adjusted to 5.8 prior to autoclaving. For the experiment, fully regenerated hybrid aspen micro shoots were excised into approximately 1.5 cm long explants and transferred to culture vessels (8 explants per each vessel) containing the same medium as mentioned above.

To evaluate the effects of R:B ratio on hybrid aspen clones, *in vitro* cultures were cultivated in growth chamber on shelf systems equipped with full-spectrum LED luminaires with three different R:B ratios – 9:1; 7:3 and 1:1 (Table 1). The total photon flux (wavelength range 400-750 nm) on shelf surface of all treatments was set to 70±7 µmol m^{-2} s^{-1}. Measurement of spectral composition and photon flux were carried out using AvaSpec ULS2048 spectrometer (Avantes, The Netherlands) to ensure the uniformity of illumination. Environmental conditions in the growth chamber were maintained constant during the experiment – temperature in growth chamber was set to 25ºC, relative humidity at 30-40% and photoperiod to 16/8h (day/night).

Table 1

Spectral distribution of studied light treatments (% of total photon flux in the range of photosynthetically active radiation)

Spectral regions and ratios	50% Red, 50% Blue (1:1)	70% Red, 30% Blue (7:3)	90% Red, 10% Blue (9:1)
Blue 400-500 nm	35.0	21.4	7.1
Green 500-570 nm	12.7	13.1	12.4
Yellow 570-590 nm	2.7	2.8	2.6
Orange 590-625 nm	7.0	7.4	7.2
Red 625-700 nm	35.5	52.9	62.0
Far-red 700-750 nm	1.8	1.8	1.7
Red:Blue (R:B)	1.01	2.47	8.75

After 4 weeks of cultivation in above mentioned conditions, we determined the morphological and physiological characteristics of regenerated plantlets. Stem and internodal length was measured with a caliper, and the number of internodes was determined. Leaf morphology-related parameters were determined by analysing scanned images of leaves. Newly formed leaves of each plantlet without petioles were placed on a tablet in order from youngest to oldest, covered with transparent glass and scanned with Canon LC4800P scanner (Canon Inc., Japan). The obtained high-resolution (1275 × 1752 px) grayscale images were then processed with WinFolia Pro 2019 software (Regent Instruments Inc., Canada). Minor deformations such as cracks and overlaps were graphically corrected with the software to increase the accuracy of measurements. Further analysis was conducted on edited images – the average and total leaf area as well as the number of leaves of each plant were determined.

In parallel to morphological measurements, the physiological parameters of the same plants were measured. Relative chlorophyll concentration (SPAD) was measured for each newly formed leaf (expressed as a mean value of 5 measurements for each leaf), while data representation occurred on a plant scale (each SPAD value is the mean value of all one plant's leaves). Chlorophyll a fluorescence, as an indicator of photosynthetic efficiency was measured with HandyPea (Hansatech Instruments Ltd., UK). 4th leaf from the top of each plantlet was used to represent the fluorescence of entire plant. Leaves were detached from plant and dark-acclimated in a special clip for 15 min, in order to open all excited reaction centres of photosystems. Measurements were carried out on dark-acclimated leaves by exposing to short (1s) and saturated (1500 µmol m^2 s^{-1}) pulse of red light and measuring reemitted fluorescent light. Initial assessment of data was carried out in PEA PLUS v.1.13 (Hansatech Instruments Ltd., UK) – fluorescence parameters were evaluated and exported as data points for analysis. To describe the functionality of photosynthetic apparatus, performance parameters Area, Fv/Fm, $PI_{ABS,}$ and PI_{TOTAL} as well as special energy fluxes ABS/RC, DIo/RC, TRo/RC, ETo/RC and describing absorption, dissipation, trapping, and electron transport rates, respectively, were further analysed.

The effects of R:B treatment and clone and interaction of factors were assessed using Two-way ANOVA. For the variables that exhibited the highest significant effect of light treatment or light by clone interaction based on F-values, the estimated marginal means were compared with Tukey's HSD test. Data analysis was performed with Microsoft Excel 2019. Altogether, data from 314 plants was acquired (3 light treatments on 2 clones each).

Results and Discussion

In vitro cultures of hybrid aspen exhibited sensitivity to the different R:B ratios, as indicated by a significant effect of light treatment on studied variables. However, the effects were uneven as different R:B ratios primarily affected the physiology of plantlets: functionality and effectiveness of PS II (indicated by TRo/RC and ABS/RC), as well as the overall performance of photosynthetic apparatus (Area, PI_{ABS} and PI_{TOTAL}) (Table 2). The relative chlorophyll content (SPAD) was also significantly affected by different R:B ratios; however, the effect of light treatment for this variable was clone-specific (Table 2), indicating different adaptation mechanisms of photosynthetic capacity of clones. The effect of light treatment on morphology was less pronounced, as only the number of internodes (which was also clone-specific) and number of leaves, were significantly affected by light treatment.

Table 2

Evaluation of the significance of fixed effects and interaction on variables (represented as F-values)

Variables	Clone	Light	Interaction of factors
Mean leaf area	1.497	1.176	1.728
Summary leaf area	8.393**	0.934	2.067
Number of leaves	17.162***	3.601*	0.634
Main shoot length	0.501	1.424	0.18
Number of internodes	46.72***	1.36	5.73**
Area	49.349***	5.890**	2.407
ABS/RC	105.300***	3.821*	1.205
DIo/RC	86.927***	2.287	0.955
TRo/RC	100.944***	7.465***	1.266
ETo/RC	15.898***	0.294	0.515
PI_{TOTAL}	110.447***	4.885**	0.991
PI_{ABS}	215.76***	9.16***	0.29
Relative chlorophyll content	30.53***	18.14***	4.81**

*, **, and *** represent the levels of significance at p-values 0.05; 0.01 and 0.001, respectively. ABS/RC, DIo/RC, TRo/RC, ETo/RC are specific energy fluxes in photosystems – absorption per reaction centre, dissipation per reaction centre, trapping rate per reaction centre and excitation transfer per reaction centre, respectively, while PI_{TOTAL} and PI_{ABS} represent performance of overall photosynthesis and light absorption, respectively.

When exposed to new environmental conditions, the sensory system of plants induces changes and first adjustments occur on a molecular and then on a physiological level, affecting respiratory rate, photosynthesis, stomatal opening, and other processes (Chelli-Chaabouni, 2014). Changes in morphology are more robust in this matter. Morphological traits have been reported to exhibit carry-over (or legacy) effects, meaning that current structures are the result of environmental conditions (light included) to which plants were exposed before the changes (Jiang *et al.*, 2011; Trouwborst *et al.*, 2016), and have been considered in the context of propagation process of hybrid aspen (Zeps *et al.*, 2022). Accordingly, it can be assumed that to overcome these legacy effects, significant and therefore presumably stressful stimulus is required. However, light treatment in this study did not affect maximum quantum yield (Fv/Fm) (Table 2), which is used as a sensitive indicator of the efficiency of light absorption (Maxwell & Johnson, 2000; Moosavi-Nezhad *et al.*, 2021), indicating that the treatment did not cause significant stress.

Furthermore, in crop and herb species, changes of R:B usually result in adjustments of leaf size, length of stem, and other morphological structures (Hahn, Kozai, & Paek, 2000; Piovene *et al.*, 2015; Naznin *et al.*, 2016a; Naznin *et al.*, 2016b). However, from the perspective of ecology, perennial tree species have long-lasting biological cycles and, accordingly, have developed more plastic growth strategies than annuals or biannuals in order to endure potentially stressful environment for longer periods of time (Chelli-Chaabouni, 2014). One of such strategies would include adaptation to a wider range of conditions and therefore higher phenotypic plasticity and lower sensitivity to changing environmental conditions. In the case of hybrid aspen, this is further enhanced by the effect of heterosis, where clones exhibit higher plasticity to environmental conditions compared to parent species (Li, Howe, & Wu, 1998; Hu & Thomas, 2019). Such plasticity to illumination was also linked to field performance with more productive clones being less sensitive (Kondratovičs *et al.*, 2022). Clones, used in this study were with intermediate field productivity. Accordingly, it can be assumed that the selected light treatment was not a strong enough stimulus to cause changes in morphology due to higher plasticity characteristic to tree species and further enhanced by the effect of heterosis.

Clones adjusted to changed R:B ratios primarily via adjustments of photosynthetic apparatus. Although similar relative chlorophyll concentrations (SPAD) were observed for both clones under R:B ratios 1:1 and 7:3, values of the variable of clone No.28 cultivated under 9:1 were significantly lower, indicating on negative effect of elevated amount of red light 'Figure 1A'. Additionally, with an increasing proportion of red light the efficiency of photosynthetic apparatus and overall vitality of plantlets, represented by PI_{ABS}, dropped, as indicated by a gradual linear decrease 'Figure 1B'. At the same time, specific energy fluxes, represented by TRo/RC, increased with the proportion of red light 'Figure 1C', indicating elevated energy distribution and utilization rates within the photosynthetic apparatus.

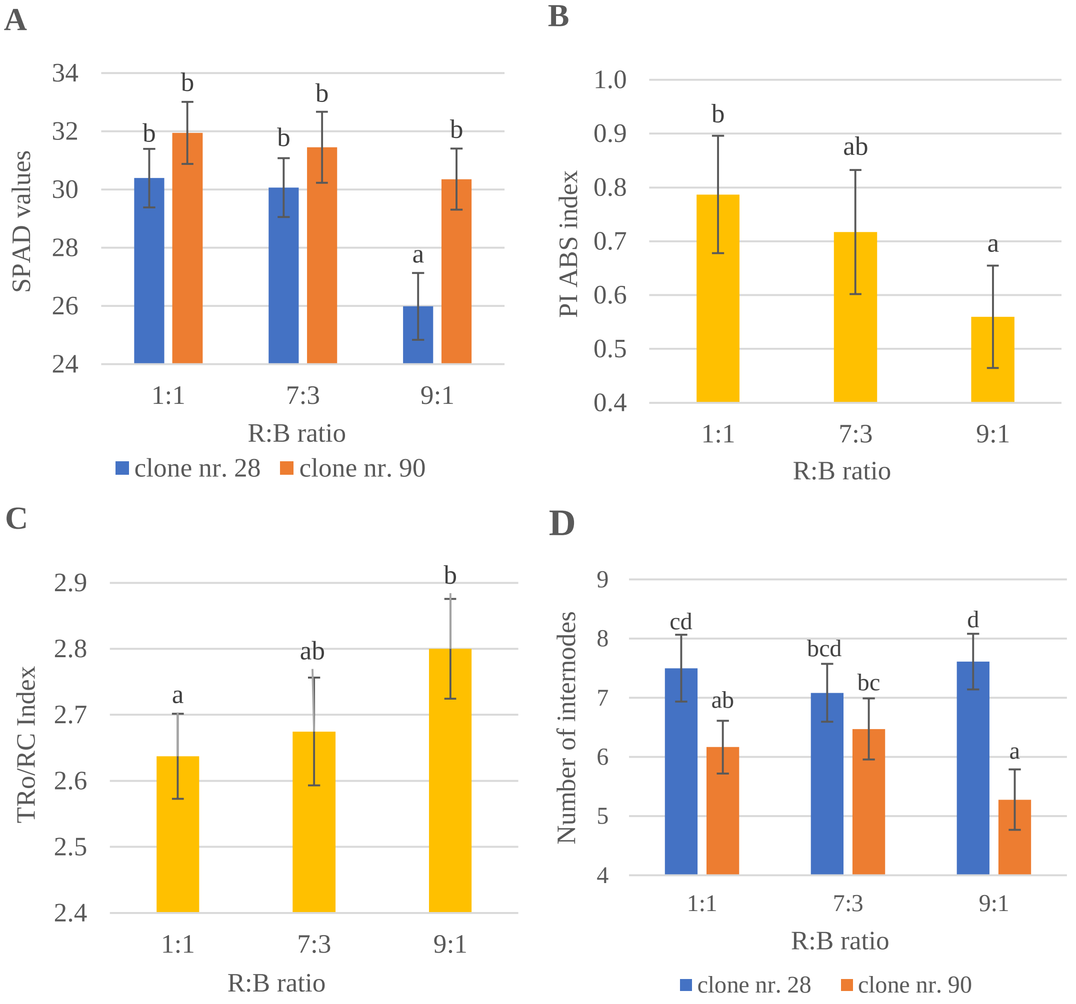

Figure 1. Responses of relative chlorophyll content (A), PI_{ABS} (B), energy trapping rate (C) and number of internodes (D) to different R:B ratios in illumination.

Increasing proportion of red light also resulted in a decrease of the number of internodes, as indicated by significant differences between 7:3 and 9:1 'Figure 1D'. However, the decrease was not linear, as the number of internodes for 9:1 was similar to those of 1:1, indicating a complex mechanism of regulation.

Although red light is actively used for photosynthesis, it has been recorded to inhibit photosynthesis. In cucumber, monochromatic red light has been observed to cause partially reversible damage to photosynthetic systems (so-called 'red syndrome') (Trouwborst *et al.*, 2016). At the same time, increasing blue light to at least 7% of total photon flux, seemed to be sufficient to overcome 'red syndrome' and increase photosynthetic capacity two times and continued to increase it until up to 50% of total photon flux was reached (Hogewoning *et al.*, 2010). Similarly, the application of blue light was reported to promote photosynthesis by increasing chlorophyll concentrations of *in vitro* cultures of potato and highbush blueberry (Hung *et al.*, 2016; Chen *et al.*, 2018), as well as the efficiency of the photosynthetic apparatus of watermelon seedlings *ex vitro* (Moosavi-Nezhad *et al.*, 2021) and mung bean (Kumar *et al.*, 2020). Blue light has also been reported to increase the number of internodes in *Prunus cerasifera* cultures *in vitro* (Muleo & Thomas, 1997). This, however, does not fully explain why significant differences between 9:1 and 1:1 R:B ratios were not detected. Accordingly, it can be assumed that the lack of differences could have resulted from marginal values of dispersion, as the effect of light treatment, indicated by the F-value, is relatively low (Table 2).

Although red light seemed to increase specific energy flux rates, in many cases this is considered either a photoprotective or compensatory mechanism. In excessive illumination, usually more light is absorbed and trapped, leading to increased ABS/RC and TRo/RC values (Moosavi-Nezhad *et al*., 2021). However, to protect photosystems, a proportionally higher amount of energy is dissipated as heat, accordingly increasing DIo/RC flux at the same time. On the contrary, in limited light conditions, electron transport within photosystems occurs more efficiently, leading to increased ETo/RC values (Maxwell & Johnson, 2000; Kumar *et al*., 2020). Accordingly, the specific photon fluxes need to be examined in the context of overall photosynthetic performance. Increased TRo/RC values, in this case, were most likely a compensatory mechanism to provide stable energy flow for photosynthesis under decreased efficiency as indicated by decreasing performance index (figure 1B). Similar increases of TRo/RC as a response to elevated amounts of red light have been observed in mung bean and grafted watermelon seedlings (Kumar *et al*., 2020; Moosavi-Nezhad *et al*., 2021). In these studies, however, red light also caused changes in Fv/Fm value, which was not true in our study, further emphasizing the lower susceptibility of trees to stress caused by illumination changes. Nonetheless, conditions *in vitro* differ significantly from those *ex vitro*, and the observed responses are a result of interaction between several independent factors, such as the composition of the culture medium. For example, due to reduced gas exchange between the culture vessel and the surrounding environment, the photosynthetic rate of *in vitro* cultures remains questionable (Hahn, Kozai, & Paek, 2000). Furthermore, both phytochromes and cryptochromes interact with auxins and cytokinins, respectively (Wang *et al*., 2022), and there is still a lack of information concerning the magnitude of these factors.

Conclusions

1. We conclude that physiological responses of hybrid aspen *in vitro* culture to changes in R:B ratio are more versatile than changes in morphology. Accordingly, plants adjust to changing light conditions first with adjustments in physiology.
2. Elevated proportions of red light are unsuitable for the propagation of hybrid aspen clones, as they decrease the photosynthetic performance of plantlets without giving significant benefits in the form of longer shoots that could be used to facilitate propagation effectiveness.
3. Nonetheless, the effect of light treatment remains at least partially clones-specific, thus requiring additional consideration in case of further application for propagation purposes.

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