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Light Source and Cytokinin Type Affect Multiplication Rate, Chlorophyll Content and Stomata Formation of *Amelanchier alnifolia* Shoots *in Vitro*

Izvor osvjetljenja i tip citokinina utječu na stopu multiplikacije, sadržaj klorofila i formiranje puči kod izdanaka *Amelanchier alnifolia* u *in vitro* uvjetima

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ABSTRACT

Amelanchier alnifolia, a deciduous shrub or small tree with edible berry-like fruit, is gaining importance as a commercial fruit crop. The application of micropropagation could complement conventional propagation methods of *A. alnifolia*. The present study aimed to investigate how two light treatments—a mix of red and blue LED lights with dominant wavelengths of 457 and 658 nm (RB LED) and conventional fluorescent lamps emitting light at broad wavelengths of 400-700 nm (FL)—in combination with three cytokinins (CK) belonging to either substituted adenines (6-benzylaminopurine, BAP; meta-Topolin, mT) or to phenylurea cytokinins (thidiazurone, TDZ) affected the multiplication rate, chlorophyll content, and stomata formation in *in vitro* culture of *A. alnifolia* axillary shoots. The two light sources proved equally effective in producing axillary shoots, but FL favorably influenced the elongation and chlorophyll content. On the other hand, RB LED lights triggered a significantly higher stomata number of *A. alnifolia* plantlets *in vitro* compared with those developed under FL. Presented results reveal a negative ratio between the stomata number and chlorophyll content in response to different cytokinins: TDZ induced the highest number of stomata while BAP significantly increased the chlorophyll content. As the largest number of axillary shoots per explant was achieved on the medium with addition of TDZ, it could be beneficial to use TDZ for multiplication, whereas the use of BAP could be advantageous in the last passage of multiplication before rooting.

Keywords: LED light, fluorescent light, cytokinin, BAP, mT, TDZ, in vitro propagation, chlorophyll content, stomata formation

SAŽETAK

Amelanchier alnifolia je listopadni grm ili niže stablo s jestivim bobičastim plodovima radi kojih se sve više komercijalno uzgaja. Primjena mikrorazmnožavanja mogla bi nadopuniti uobičajene metode razmnožavanja *A. alnifolia*. Ovo istraživanje imalo je za cilj istražiti utjecaj dva tretmana svjetla - mješavine crvenih i plavih LED svjetiljki s dominantnim valnim duljinama od 457 i 658 nm (RB LED) i konvencionalnih fluorescentnih lampi koje emitiraju svjetlost u širokom rasponu valnih duljina od 400 do 700 nm (FL) - u kombinaciji s tri citokinina koji pripadaju bilo supstituiranim adeninima (6-benzilaminopurin, BAP i meta-topolin, mT) ili fenilurea citokininima (tidiazuron, TDZ) na stopu umnažanja, sadržaj klorofila i formiranje puči u *in vitro* kulturi aksilarnih izdanaka *A. alnifolia*. Dva izvora svjetlosti pokazala su se jednako učinkovita u stvaranju aksilarnih izdanaka, ali FL su povoljno utjecale na izduživanje izdanaka i sadržaj klorofila. S druge strane, pod RB LED svjetlima formirao se značajno veći broj puči kod *A. alnifolia* izdanaka *in vitro* u usporedbi s onima razvijenim pod FL. Ovdje prikazani rezultati otkrivaju negativan omjer između broja puči i sadržaja klorofila pod utjecajem

različitih citokinina: TDZ je inducirao stvaranje najvećeg broja pući, dok je BAP značajno povećao sadržaj klorofila. S obzirom da je najveći broj aksilarnih izdanaka po eksplantatu formiran na mediju s dodatkom TDZ-a, TDZ je pogodan u fazi multiplikacije, dok bi se pozitivan utjecaj BAP-a mogao iskoristiti u posljednjoj supkultivaciji prije ukorjenjivanja.

Ključne riječi: LED svjetlo, fluorescentno svjetlo, citokinin, BAP, mT, TDZ, in vitro raznmožavanje, sadržaj klorofila, formiranje pući

INTRODUCTION

Amelanchier alnifolia Nutt. (Saskatoon berry) is a fruit-bearing shrub, native to the Prairie Provinces of Canada, that is becoming increasingly important as a commercial fruit crop (Remphrey et al., 2006). In Europe, Saskatoon Berry is still little known and not widespread in cultivation, but it seems that there is a large potential for the development of Saskatoon Berry as a new crop for dessert fruit and processing. Recently, Saskatoon berries were introduced for commercial cultivation in Finland (Lavola et al., 2012), while in the Czech Republic, new cultivars have been bred (Rop et al., 2012).

Saskatoon fruit contains a desirable complement of anthocyanin compounds that confer health-related benefits upon consumption (Ozga et al., 2006). Moreover, the whole Saskatoon plant may be used as a potential source of valuable ingredients, and different phenolics occur in the whole Saskatoon plant (in the berries, leaves, and stems) in relatively high concentrations (Lavola et al., 2012). Because *A. alnifolia* has excellent dietary properties and there is an increased market demand for this species, the application of micropropagation could complement conventional propagation methods to supply the market with quality seedlings.

The most important factors that affect the success of micropropagation are the environmental conditions, light, and temperature, as well as nutrient media and/or plant growth regulator composition. The traditional light source used for *in vitro* propagation is fluorescent lamps (Bello-Bello et al., 2016). They have been a popular source of plant lighting because of the white light output that mimics daylight (Gupta and Agarwal, 2017). In the commercial micropropagation of a particular plant species, cost-effectiveness is of great importance. In the longer term, Light Emitting Diode (LED) lights certainly have an advantage over fluorescent lights because of their lower power consumption and long life, although initial investment in LED lights is much higher than that in fluorescent lights (Gupta and Jatothu, 2013). Besides that, the spectral output of fluorescent lamps cannot be regulated, whereas the flexibility of matching LED wavelengths to photoreceptors may enable more optimal production, influencing plant morphology and chlorophyll content (Bello-Bello et al., 2016). It has been reported that red light is important for shoot and stem elongation, phytochrome responses, and changes in plant anatomy (Bello-Bello et al., 2016), while blue light influences chloroplast development, chlorophyll formation, and stomata opening (Senger, 1982).

LED colors or combinations commonly used for *in vitro* culture are white, red, blue, and mixture rates of blue and red. Several authors reported better *in vitro* responses of different species when a combination of red and blue LED light was used (Nhut et al., 2003; Azmi et al., 2014; Ferreira et al., 2017). Increased plant growth (except stem elongation), the smallest number of stomata, and the highest photosynthetic rate were observed in Chrysanthemum under mixed red/blue LED treatments; this finding has been attributed to the similarities of the spectral energy distribution of red/blue light to chlorophyll absorption (Kim et al., 2004). Vieira et al. (2015) compared the effects of LED lights (white and deep red/white) and fluorescent lights on the stomata formation and chlorophyll levels in micropropagated banana plantlets. They found increased chlorophyll content and the greatest number of stomata under LED treatments, and this corresponded with higher plantlets. However, some studies have shown that fluorescent light can result in better micropropagation, as proved by Poncetta et al. (2017) for raspberries.

Considering plant growth regulators, previous studies on the micropropagation of *Amelanchier alnifolia* have used only BAP for shoot proliferation (Pruski et al., 1990), and more recently, the effects of different classes of cytokinins (CKs) on *in vitro* proliferation (Yang and Du, 2017) and on endogenous concentrations of CK metabolites in *A. alnifolia in vitro* cultures were investigated (Moyo et al., 2018). Cytokinins are a group of naturally occurring N6-substituted adenine compounds that are essential in regulating many physiological processes in plants (Stirk et al., 2010). They control apical dominance in the shoot, branching, leaf senescence, root growth, and chlorophyll production (Mok, 1994). Cytokinins have also been proven to regulate stomatal behavior *in vivo*, including the opening and conductance of stomata, transpiration, photorespiration, and thereby, also photosynthesis (Aremu et al., 2012; Dobránszki and Mendler-Drienyovszki, 2014). CKs are indispensable in plant micropropagation techniques, as they promote cell division and secure normal and proportional organ development (Plíhal et al., 2013).

6-Benzylaminopurine (BAP) is currently the most widely used aromatic cytokinin in the micropropagation industry because of its effectiveness and affordability (Bairu et al., 2007; Aremu et al., 2012). Pruski et al. (1990) used BAP (8.88-13.3 µM) for the successful mass production of shoots and for the subsequent rooting of *Amelanchier alnifoila*. On the other hand, the hydroxylated analogue of BAP named N6-(3-hydroxybenzyl) adenine (meta-Topolin, mT) was found to be more active than benzyladenine in the promotion of shoot proliferation in *Amelanchier alnifoila* (Moyo et al., 2018). According to the review of Dobránszki and Teixeira da Silva (2014), meta-Topolin was also found to be a successful alternative to BAP for avoiding shoot growth suppression in apple shoot multiplication.

While most of the natural (like mT) and synthetic cytokinins (like BAP) are substituted adenines, there are also phenylurea cytokinins, such as thidiazuron. As a synthetic cytokinin, thidiazuron elicits a physiological responses similar to natural purine cytokinins, with activity being similar or exceeding that of natural cytokinins (Karanov et al., 1992). Because synthetic cytokinins are effective in low concentrations and are highly stable, being resistant to oxidases (Karanov et al., 1992; Mok and Mok, 2001), they are very useful in agriculture (Stirk and van Staden, 2010). Thidiazuron is being selected for the micropropagation of a wide array of woody species because of its ability to stimulate shoot proliferation more efficiently than purine adenine derivatives, but it is ineffective for proliferation in some species (Huetteman and Preece, 1993).

Since *A. alnifolia* is considered "difficult-to-root", and the conventional propagation via cuttings has failed to produce adequate numbers of plants (Pruski et al., 1990), successful efforts have already been made for the micropropagation of this species (Pruski et al. 1990; Yang and Du, 2017; Hunková et al., 2017; Moyo et al. 2018). However, to our knowledge, no studies have been reported on the effect of different light sources on the development of *A. alnifolia* axillary shoots *in vitro*. Apart from a growth effect, in this study, for the first time, the effect of different light sources and different cytokinin types on chlorophyll content and the number of stomata in micropropagated *A. alnifolia* shoots was determined. Stomata provide plants with the ability to adjust to environmental changes (Vieira et al., 2015), while photosynthetic pigments such as chlorophyll content can enhance the *ex vitro* survival of plantlets (Aremu et al., 2012). Therefore, these parameters are important when evaluating the quality of shoots produced *in vitro*. The purpose of this work was to establish whether the quality of light and different cytokinins affect the growth and development of *A. alnifolia* plantlets. The effects of two light treatments - a red and blue LED light mixture (with dominant wavelengths of 457 and 658 nm) and conventional fluorescent lamps (emitting light at broad wavelengths of 400 - 700 nm) - and three different cytokinins (6-benzylaminopurine, meta-Topolin, and thidiazuron) on the multiplication rate, stomata development, and chlorophyll content in micropropagated *A. alnifolia* shoots were studied.

MATERIALS AND METHODS

Plant material and treatments for shoot proliferation

Four-week-old in vitro shoots of *Amelanchier alnifolia* (cultivar Smokey) grown previously on MS (Murashige and Skoog, 1962) proliferation medium supplemented with 3% sucrose, 0.8% Bacto™ Agar, 100 mg/L myo-inositol, and 1 mg/L BAP were used for the first experiment setting. To test the effect of three cytokinin types on axillary shoot proliferation, three different media compositions were used (Table 1). Hormone-free MS medium (MS HFM) was used as a control. Shoots, 0.5 cm long, were placed vertically in the medium. In further subcultivations, shoots were multiplied and subcultured, always in the same media composition, at 5- to 6-week intervals. Cultures were maintained in a plant growth chamber under 16 h light conditions at 23 °C. The effect of two different light sources was examined: mixture of blue and red LEDs (Philips GreenPower LED top lighting module DR/B HB) with dominant wavelengths of 457 and 658 nm, respectively and fluorescent lamps (FL) (Osram L 36W/77 FLUORA) emitting light at broad wavelengths of 400 - 700 nm (Figure 1, Figure 2 and Table 2). The light spectrum for each lamp was measured with a spectrometer Spectrometer S (3B Scientific, Germany). The light intensity of both LED and fluorescent light was 40 µE m^{-2} s^{-1}.

Table 1. Media composition for micropropagation of *A. alnifolia* shoots *in vitro*

	Media composition
MS HFM	MS macro and micro elements (Murashige and Skoog, 1962), MS vitamins, 100 mg/L inosytol, 3% sucrose, 0.8% Bacto™ Agar, pH 5.8
MS mT 0.6	MS HFM + 0.6 mg/L meta-Topolin (mT)
MS BAP 0.6	MS HFM + 0.6 mg/L 6-benzylaminopurine (BAP)
MS TDZ 0.1	MS HFM + 0.1 mg/L thidiazurone (TDZ)

Experiments were conducted in a completely randomized design. Data on the shoot number and shoot length were recorded from 72 explants: each combination of light source and cytokinin treatment (or MS HFM) consisted of 18 explants placed in two Magenta boxes; the experiment was replicated four times (four successive subcultivations).

Table 2. Applied fluorescent and LED lights spectrum data

Color	Length (nm)	Light spectrum of two light sources (%)	
		FL	RB LED
Ultraviolet	< 400	0.00	0.00
Blue	400-499	33.77	47.53
Green	500-599	18.15	0.00
Red	600-699	48.08	52.47
Far-red	700-800	0.00	0.00

FL fluorescent lamps; *RB LED* red and blue LED light mixture with dominant wavelengths of 457 and 658 nm, respectively

Determination of photosynthetic pigment content

Randomly, three plantlets from each combination of light source and cytokinin treatment were collected for photosynthetic pigment content analysis. Chlorophylls *a* and *b* were quantified as outlined by Inskeep and Bloom (1985). In brief, 25 mg of apical leaves of *Amelanchier alnifolia* was extracted in 3 mL of N,N-dimethylformamide (Sigma-Aldrich) in a glass tube. The samples were kept in a refrigerator in darkness for 5 days. Thereafter, 1 mL of the extracted sample was transferred to a disposable cuvette, and the absorbance was measured using a spectrophotometer (Specord 40 Analytik Jena AG) at 647 and 664.5 nm. Chlorophyll a, chlorophyll b, and total chlorophyll (mg/L) contents were calculated as Chl *a* = $12.70A_{664.5} - 2.79A_{647}$; Chl *b* = $20.70\,A_{647} - 4.62\,A_{664.5}$; total Chl = $17.90\,A_{647} + 8.08\,A_{664.5}$, where A is the absorbance of the specified wavelength Inskeep and Bloom, 1985). This was converted to mg/g fresh weight.

Determination of stomatal index

Stomata were analyzed using a nail polish imprint method (Hilu and Randall, 1984). For each combination of light source and cytokinin treatment, transparent nail polish imprints were prepared from at least eight of the most developed (the largest) leaves taken from three randomly chosen plantlets. Samples were prepared by applying a thin layer of nail polish onto the middle region

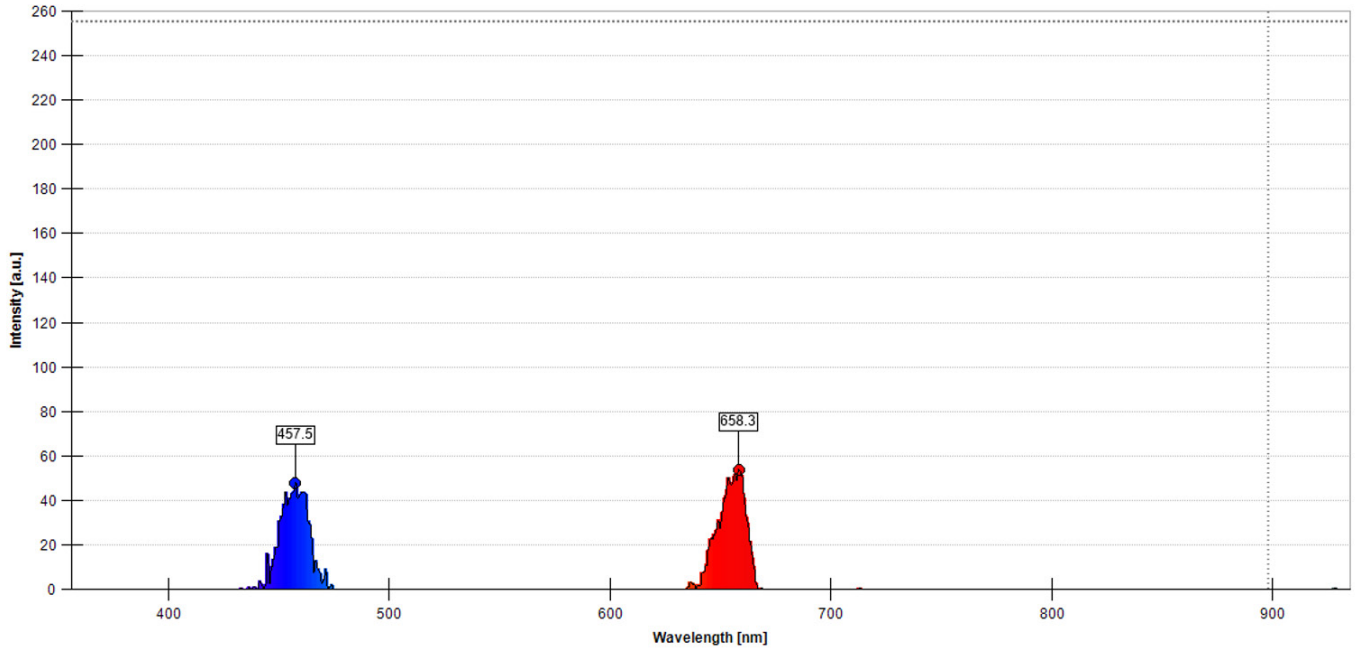

Figure 1. Spectral characteristics of LED lamps used in the experiment, generated with a spectrometer Spectrometer S (3B Scientific, Germany)

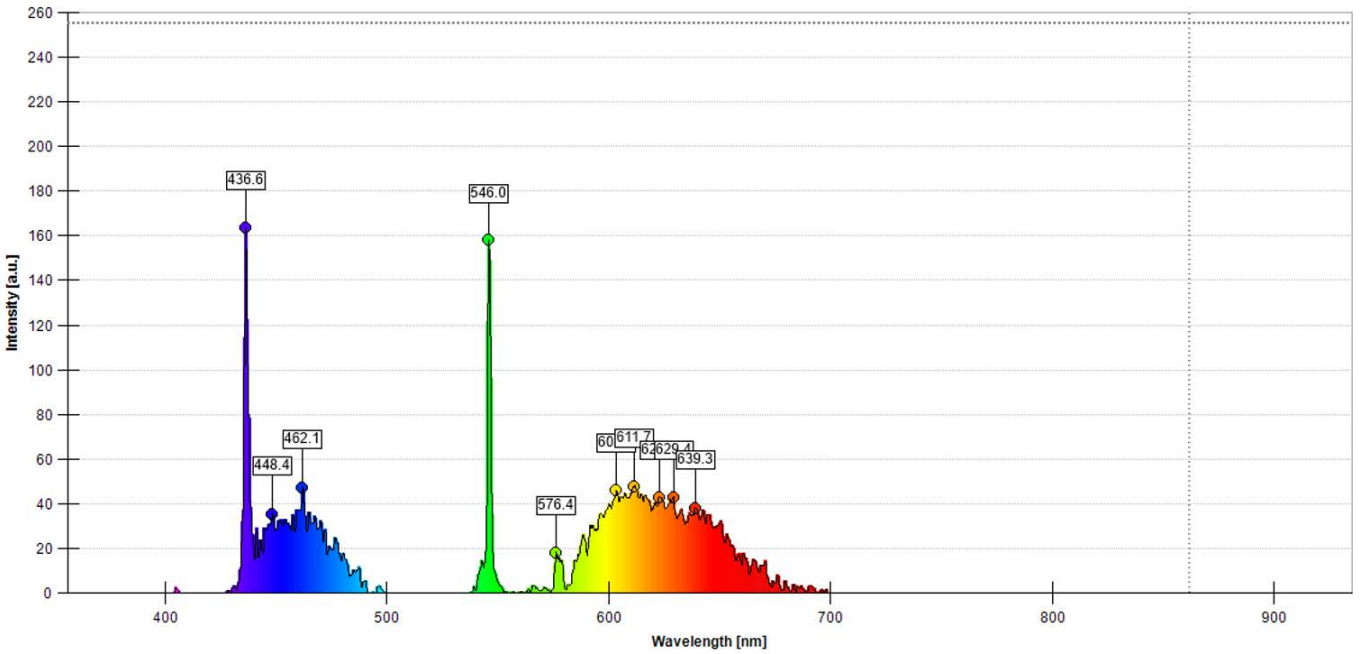

Figure 2. Spectral characteristics of fluorescent lamps used in the experiment, generated with a spectrometer Spectrometer S (3B Scientific, Germany)

of the leaf blade. After drying, the nail polish was peeled off using transparent adhesive tape and placed on the slide. The imprints were observed using an Optika B-500 Ti (Optika, Italy) light microscope (objective 40x) equipped with an Optikam Pro 3LT digital camera (Optika, Italy). The stomata were analyzed only on the abaxial leaf epidermis, as no stomata were found on the adaxial epidermis. The number of stomata and epidermal cells was counted on the microscopic images obtained from at least three different points in each imprint. The stomatal index was calculated as number of stomata/ (number of epidermal cells + number of stomata) × 100 (Kubinova, 1994).

Data analysis

Data on the number of axillary shoots developed per explant, mean shoot length, chlorophyll content, and stomatal index were subjected to ANOVA. For means comparison, a Bonferroni Post-Hoc test at P<0.05 was used. The statistical analysis of the data was carried out by the SAS/STAT® (SAS/STAT 2010) program package.

RESULTS AND DISCUSSION

Effect of different light sources and different cytokinins on shoot proliferation of Amelanchier alnifolia

In the present study, both fluorescent and LED light proved equally effective in producing axillary shoots, but fluorescent light favorably influenced elongation (Table 3). These results are consistent with those of some other authors. Bello-Bello et al. (2016) reported an equal number of proliferated shoots per explant in fluorescent light with dominant peak wavelengths from 545 to 610 nm and LED light (blue + red, ratio 1:1, with dominant wavelengths of 460 and 660 nm, respectively) during micropropagation of *Vanilla planifolia* Andrews. However, shoots were significantly longer when cultured under combined blue + red LED light. Poncetta et al. (2017) reported that micropropagating raspberries produced more shoots per explant under fluorescent than under LED light. Plantlets grown under fluorescent lights also had longer shoots, which is consistent with the findings of this study. On the contrary, Nhut et al. (2003) and Moon et al. (2006) found that the growth of strawberry plantlets and *Tripterospermum japonicum* was the best at red and blue LED light with dominant wavelengths of 450 and 660 nm, at a ratio of 7:3, respectively.

Three types of cytokinins were used in this experiment: two adenine-type cytokinins (BAP and mT) and a phenylurea cytokinin (TDZ). Hormone-free MS medium (HFM) was used as a control. The cytokinin type significantly influenced the mean number of shoots per explant and the mean shoot length of *A. alnifolia* axillary shoots (Table 3, Figure 3). The greatest mean number of axillary shoots developed per explant (8.3) was achieved on medium supplemented with TDZ (Table 3).

Table 3. Effect of light source and cytokinin type on *A. alnifolia* micropropagation efficiency

Source of variation		
	Mean number of shoots per explant	Average length of the shoots (mm)
Light source	ns	**
Cytokinin	***	***
Light source x Cytokinin	ns	***

Mean number of shoots per explant	
Cytokinin	*Means for Cytokinin*
BAP 0.6	3.5 B
mT 0.6	3.3 B
TDZ 0.1	8.3 A
HFM	1.0 C

Average length of the shoots (mm)		
Light source	Cytokinin	Average length of the shoots (mm)
FL	BAP 0.6	6.0 b
	mT 0.6	9.7 a
	TDZ 0.1	4.4 b
	HFM	6.1 b
RB LED	BAP 0.6	5.8 b
	mT 0.6	7.2 a
	TDZ 0.1	4.1 c
	HFM	7.1 a
Light source	*Means for light source*	
FL	6.6 A	
RB LED	6.1 B	
Cytokinin	*Means for Cytokinin*	
BAP 0.6	5.9 B	
mT 0.6	8.5 A	
TDZ 0.1	4.3 C	
HFM	6.6 B	

Significant effect: **P<0.01; ***P<0.001; n.s. not significant. Values followed by the same letters within the column do not differ significantly by Bonferroni test at P<0.05; FL, fluorescent lamps; RB LED, red and blue LED light mixture

Figure 3. Micropropagation of *A. alnifolia* under fluorescent light on (a) BAP, (b) mT, and (c) TDZ. (Scale bar = 1 cm)

This multiplication rate is higher than those obtained in *A. alnifolia* cv. Nelson when the same concentration of TDZ was used (Yang and Du, 2017). TDZ is a synthetic cytokinin that is less sensitive to enzymatic degradation, and therefore, at low concentrations, it can induce larger number of axillary shoots relative to other types of cytokinins (Magyar-Tábori et al., 2010). This is consistent with the results of this study which showed that TDZ applied at six times lower concentration (0.1 mg/L) than mT and BAP (0.6 mg/L) still resulted in better shoot proliferation. Although TDZ sometimes has undesirable effects, such as the appearance of aberrant shoots, as was the case with the micropropagation of apples (Kereša et al., 2012), this did not occur in *A. alnifolia*. Media supplemented with 0.6 mg/L BAP or mT produced a similar mean number of shoots per explant (Table 3), and this is comparable to the results of Moyo et al. (2018), who found a similar shoot proliferation efficiency of *A. alnifolia* cv. Prince Williams in the media supplemented with 1µM BAP or mT. The results of this study are also in line with the results from Wojtania and Gabryszewska on *Pelargonium x hederaefolium*; the authors used 0.5 mg/L BAP or mT and found no significant difference in the number of shoots. On the other hand, a higher multiplication rate of *Aloe polyphylla in vitro* shoots was found on medium supplemented with 2.5 µM BAP than on an equimolar concentration of mT (Bairu et al., 2007).

In the present study, the greatest mean shoot length (9.7 mm) was observed on medium supplemented with 0.6 mg/L mT (under fluorescent light) (Table 3). The length of shoots produced on mT has been studied also by other authors who suggested that mT could increase multiplication rate and have lower reducing effect on shoot length compared to BAP (Wojtania and Gabryszewska, 2001; Dobránszki and da Silva, 2010; Moyo et al., 2018). Investigating the micropropagation of Prunus rootstocks, Gentile et al. (2014) found that mT did not produce a larger number of shoots compared with BAP but had a positive effect on the growth and quality of shoots which is consistent with results of this study. On the other hand, the shortest shoots (4.1 mm) were produced on medium supplemented with TDZ (under LED light), on which the greatest micropropagation rate was achieved. A negative relationship between the number and length of shoots was reported by other authors in the micropropagation of apple (Welander, 1985; Marin et al., 1993; Dobránszki and da Silva, 2010) and *Pelargonium × hortorum* and *Pelargonium × hederaefolium* cultivars (Wojtania, 2010). In this study, TDZ inhibited shoot elongation which is in corroboration with the findings of Huetteman and Preece (1993) and van Nieuwkerk et al. (1985). However, the interaction of TDZ in the nutrient medium and fluorescent lights had a positive effect on shoot length. Under fluorescent lights, the average length of shoots developed on medium with TDZ did not differ significantly from the average length of shoots developed on medium with BAP (Table 3). Therefore, it may be useful for *A. alnifolia* to micropropagate on medium with TDZ in order to produce a higher number of shoots and then use mT or BAP for the last subculture before rooting. Although mT resulted in higher shoots, it seems that BAP is a favorable CK for *A. alnifolia* because of more successful rooting on media containing BAP, as shown by Moyo et al. (2018). This, however, remains to be tested

on a specific genotype.

On the control medium, without the addition of cytokinins, there was no multiplication, and the leaves were yellowing and falling apart. Shoots from HFM were therefore not used for chlorophyll content and stomata formation studies.

Effect of different light sources and different cytokinins on chlorophyll content, and stomata formation of Amelanchier alnifolia

In the present study, type of lighting significantly affected chlorophyll content and stomata formation during micropropagation of *Amelanchier alnifolia* axillary shoots (Figure 4, Table 4). *A. alnifolia* plantlets grown under fluorescent light had significantly higher Chl *a*, Chl *b* and total Chl contents than plantlets grown under RB LED light. Similarly, higher chlorophyll content in planlets cultured under conventional fluorescent lighting compared to LED lighting (red, blue or red + blue mixture) has been found in *Myrtus communis* (Cioć et al., 2018), *Vanilla planifolia* (Bello-Bello et al., 2016) and *Zantedeschia jucunda* (Jao et al., 2005).

The three cytokinins studied resulted in significant differences ($P<0.05$) in chlorophyll content (Table 4). The addition of BAP significantly increased chlorophyll a, chlorophyll b, and total chlorophyll content when compared with mT and TDZ (Table 4). This is in agreement with the findings of Dobránszki and Mendler-Drienyovszki (2014), who found that chlorophyll content in the leaves of *in vitro* grown apple plantlets was the highest when benzyl-adenine (BA) was applied as the single source of cytokinin in the medium when compared with benzyladenine riboside and meta-Topolin alone or in combination. An important function of cytokinins is the delay of senescence by accumulating and maintaining photosynthetic pigments in plants (Zwack and Rashotte, 2013; Cortleven and Schmülling, 2015). *In vitro* studies have, however, demonstrated both stimulatory and inhibitory effects of different cytokinins on photosynthetic pigment content in micropropagated species (Aremu et al., 2012, Genkov et al., 1997; Muniz de Oliveira et al., 2008). For instance, in *in vitro* culture of axillary buds of *Dianthus caryophyllus*, the effect of TDZ on photosynthetic pigment content depended on the concentration applied: at concentration equimolar to that of BAP (0.4 mM), TDZ was found to decrease the pigment content, but at 10-fold or 100-fold lower concentration TDZ had stimulatory effect, similar to that caused by BAP (Genkov et al., 1997). In the leaves of *Annona glabra*, presence of 1 mg/L BAP in the culture medium was found to significantly increase the concentration of Chl *a*, while the same concentration of TDZ had opposite effect, i.e. significantly reduced its content (Muniz de Oliveira et al., 2008). For this reason, authors (Muniz de Oliveira et al., 2008) suggested lower concentration of TDZ to be used. In the present study, 10x lower concentration of TDZ was used (0.1 mg/L). Still, chlorophyll content was significantly reduced on medium supplemented with TDZ when compared to media supplemented with BAP and mT. However, in the case of *chlorophyll b* content, there was a positive interaction of FL light and TDZ. On the other hand, the interaction of mT in the nutrient medium and RB LED lighting had a positive effect on total Chl content and Chl *a* content (Table 4). LED lighting has been also reported to enhance chlorophyll content in other species. Red + blue LED light mixture with dominant wavelengths of 660 and 450 nm, respectively elevated chlorophyll content in *Doritaenopsis* plants grown *in vitro* compared to red or blue LED and fluorescent light treatments (Shin et al., 2008) and, in *Brassica napus*, Chl *a* concentration were greater when plantlets were grown under red + blue (1:3) LED light than under fluorescent light and other LED treatments (Li et al., 2013). On the other hand, Jao et al. (2005) and Kurilčik et al. (2008) found that the blue component (dominant wavelengths of 460 and 450 nm, respectively) in the illumination spectrum increased the content of photosynthetic pigments and inhibited plantlet extension in *in vitro* plantlets of *Z. jucunda* and chrysanthemum, respectively.

Stomata play a fundamental role in regulating plant water use and CO_2-uptake for photosynthesis, allowing the plant to optimize and balance the photosynthetic performance (Chaerle et al., 2005). The plants must

therefore constantly regulate stomata in coordination with various environmental stimuli. This regulation is achieved by adjusting stomatal aperture to optimize the rate by which water or CO_2 is exchanged. However, plants can also respond to prevailing environmental conditions by modulating the frequency of stomata development in new organs (Lake et al., 2001). It was found that the development of stomata and stomatal density can be affected by light intensity (Qi et al., 2018) and light quality (Kim et al., 2004; Vieira et al., 2015; Zheng and Van Labeke, 2017).

Table 4. Effect of different light sources and cytokinin treatments on chlorophyll content (mg/L), stomata index and stomatal density (number of stomata per mm^2) of *A. alnifolia* shoots

Source of variation						
		Chlorophyll content			Stomata index	Stomatal density
		Chl *a*	Chl *b*	Total Chl		
Light source		*	***	**	***	**
Cytokinin		***	***	***	***	***
Light source x Cytokinin		***	**	***	***	ns
Light source	Cytokinin	Chlorophyll content				
		Chl *a*	Chl *b*	Total Chl	Stomata index	
FL	BAP 0.6	2.53 a	0.55 a	3.07 a	15.37 b	
	mT 0.6	1.57 c	0.35 b	1.92 c	14.69 b	
	TDZ 0.1	1.18 d	0.34 b	1.52 d	15.65 b	
RB LED	BAP 0.6	1.68 c	0.33 b	2.01 bc	15.78 b	
	mT 0.6	2.00 b	0.33 b	2.34 b	17.66 a	
	TDZ 0.1	0.92 d	0.18 c	1.10 e	18.45 a	
Means for light source						
		Chlorophyll content			Stomata index	Stomatal density
		Chl *a*	Chl *b*	Total Chl		
FL		1.76 A	0.41 A	2.17 A	15.24 B	211.23 B
RB LED		1.53 B	0.28 B	1.82 B	17.3 A	237.13 A
Means for Cytokinins						
		Chlorophyll content			Stomata index	Stomatal density
		Chl *a*	Chl *b*	Total Chl		
BAP 0.6		2.11 A	0.44 A	2.54 A	15.58 B	188.95 C
mT 0.6		1.79 B	0.34 B	2.13 B	16.18 B	216.57 B
TDZ 0.1		1.05 C	0.26 C	1.31 C	17.05 A	267.02 A

Significant effect: *P<0.05; **P<0.01; ***P<0.001; n.s. not significant. Values followed by the same letters within the column do not differ significantly by Bonferroni test P<0.05; FL, Fluorescent light; RB LED, red and blue LED light mixture; Chl a, chlorophyll a content; Chl b, chlorophyll b content; Total Chl, total chlorophyll content

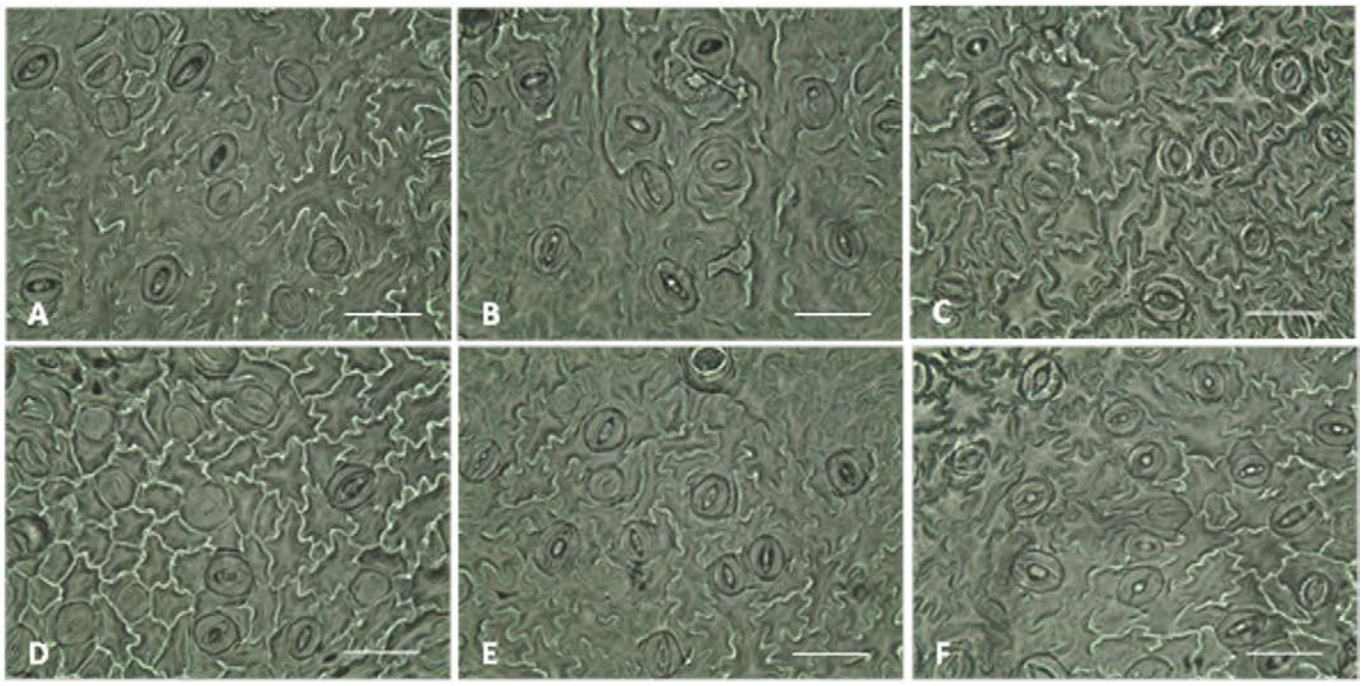

Figure 3. Stomatal fingerprints of adaxial leaf surfaces of *A. alnifolia* plantlets under different light sources and different cytokinin types: (A) fluorescent light, mT; (B) fluorescent light, BAP; (C) fluorescent light, TDZ; (D) RB LED light, mT; (E) RB LED light, BAP; (F) RB LED light, TDZ (scale bar = 50 μm)

In *Amelanchier alnifolia* plantlets grown in RB LED lights stomata number was significantly higher compared to those developed under fluorescent lamps (Figure 4, Table 4). The plantlets grown under RB LED lights also showed a tendency of lower growth rate (Table 3) and had lower chlorophyll content (Table 4). Similar results were obtained in a study on the effects of a continuous LED spectrum on *Quercus ithaburensis* var. *macrolepis* at the seedling developmental stage (Smirnakou et al. 2017). Kim et al. (2004) found a negative ratio between the stomata number and the net photosynthetic rate of chrysanthemum plantlets developed under fluorescent and mixed red/blue LED light (1:1), while Li et al. (2010) found a negative ratio between the stomata number and the net photosynthetic rate of cotton plantlets grown under fluorescent and mixed red/blue LED light (3:1). Moreover, Baroli et al. (2008) suggested that the systemic developmental signal that determines stomatal density in expanding leaves is not directly linked to photosynthetic capacity.

Studying the effect of cytokinins on stomata morphology in *in vitro* culture of pistachio, Namli and Tilkat (2007) found abnormal stomata development using TDZ and kinetin, while stomata obtained from 1 mg/L BAP in the medium were similar to those developed *in vivo*. In the present study, plantlets that developed on medium with the addition of BAP had the greatest chlorophyll content and the lowest stomatal density. Since the development of photosynthetic tissue under *in vitro* conditions is a major factor determining the survival of plants when they are transferred *ex vitro* (Yang and Yeh, 2008), this could be an additional reason to use BAP in the last passage of micropropagation before rooting. Plantlets developed on the medium with the addition of TDZ had the lowest chlorophyll content (Table 4) and the multiplication rate on this medium was the highest (Table 3). Since the stomatal index and stomatal density were the highest on the medium supplemented with TDZ (Table 4), it can be assumed that the addition of TDZ in the growth medium promoted the transpiration rate which could be disadvantage during acclimatization. A change in stomatal density modifies the number of sites available for gas exchange per unit leaf area (Hill et al., 2014). A higher transpiration rate is correlated with

better absorption of nutrients (Wang et al., 2007), which could be a possible explanation for the high multiplication rate of *A. alnifolia* shoots on medium supplemented with TDZ. It can be concluded that chlorophyll content was not crucial for shoot proliferation efficiency in *in vitro* conditions because a great proportion of carbon is of heterotrophic origin (Wolf et al., 1998).

Thus, results of this study reveal a negative relationship between stomata number and chlorophyll content in response to different cytokinins. Farber et al. (2016) explained the effect of CK on stomatal density in tomato (*Solanum lycopersicum*) plants. They compared wild-type tomato and transgenic tomato overexpressing a CK-degrading enzyme. Wild-type plants with higher CK activity showed a higher number of stomata per unit area, a higher transpiration rate, and faster water loss when exposed to drought. However, the stomatal index was similar in both types of plants, meaning that plants with lower CK activity (transgenic) had fewer and larger pavement cells and fewer stomata. This suggests that CK deficiency affects epidermal cell division but not stomatal patterning (Farber et al., 2016). In this study, however, the stomata index was significantly higher in shoots grown on medium containing TDZ, especially under the LED light. As discussed earlier, different species respond differently to similar light sources, and it is not easy to identify in advance which light will be better for *in vitro* cultivation of a particular species. Additionally, the chlorophyll content of *in vitro* plantlets grown under different light qualities varies according to plant species (Li et al., 2013). Therefore, other combinations of LED lights could have different effects on the chlorophyll content and stomata formation of *A. alnifolia* axillary shoots grown in vitro.

CONCLUSIONS

Recently, LED lights are frequently used as an alternative light source in plant tissue culture. In the present study, the effect of a red + blue LED light mixture with the dominant peaks at 457 and 658 nm, respectively) and conventional fluorescent lamps (emitting light at broad wavelengths of 400-700 nm) on the development of *A. alnifolia* shoots was determined for the first time. Presented results show a stimulating effect of conventional fluorescent lamps on shoot height and chlorophyll content. Stomatal density was lower under fluorescent light. The use of a phenylurea derivate (TDZ) in the medium produced better results in terms of an increased multiplication rate. Moreover, the interaction of TDZ in the nutrient medium and fluorescent lighting had a positive effect on shoot length. But, the highest chlorophyll content and the lowest stomatal density were achieved on medium with the addition of BAP. Therefore, to achieve the highest proliferation rate and produce shoots with higher chlorophyll content, TDZ could be used for multiplication, while the use of BAP could be advantageous in the last passage of multiplication.

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