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CALLUS INDUCTION AND ORGANOGENESIS IN VITRO OF CATTLEYA FROM PROTOCORM-LIKE BODIES (PLBs) UNDER DIFFERENT LIGHT CONDITIONS

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Abstract. In this paper we report the research on the effect of the different light *in vitro* conditions on growth and development of interspecific hybrid *Cattleya intermedia* × *C. aurantiaca* (Orchidaceae) maintained as stock in vitro shoot culture. Callus was obtained from shoots explants on initiation medium which was composed of MS mineral salts and vitamin set, supplemented with 30 g l^{-1} sucrose, 2.0 mg dm^{-3} adenine sulphate, 9.7 mg dm^{-3} ascorbic acid, with addition of 1.0 µM TDZ, whereas callus maintenance medium was instead supplemented with 4.95 µM BA, and 1 µM NAA. Proliferating protocorm-like bodies (PLBs) of that precious plant material were exposed to the irradiation with monochromatic light characterized by different wavelength. Some interesting lines have been obtained on white, blue, red, far red, and ultraviolet light respectively, which proved to be diversified in the proliferation rates as well as in the morphological and anatomical features. The light treatment also significantly affected regenerative potential of studied culture. Blue light applied during two subsequent culture passages was proved to be the best option in order to regenerate shoots *via* PLBs. Nevertheless blue, red and far red irradiation of cultures led to distinctive reduction in the content of chlorophyll and carotenoid pigments, compared with culture irradiated with either white or ultraviolet light.

Key words: *in vitro* culture, Orchidaceae, tissue proliferation, photomorphogenesis, micropropagation

INTRODUCTION

The plant morphogenesis is influenced mainly by environmental factors such as light, temperature regime, the equilibrium of gaseous substances in air or its humidity. However, both nutrient and regulatory molecules level in plant tissues could be perceived as significant factor, indispensable to attain elevated level of horticultural crop production [Cerny-Koenig et al. 2004, Ilias and Rajapaske 2005, Kumar et al. 2007,

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Woźny 2011, Yeach et al. 2012]. That could be the *modus vivendi* of enormous efforts constantly directed by breeders to improve numerous characters associated with plant aesthetics, resistance to stresses of biotic or abiotic origin, and their productivity. The availability of plant material characterized as having resistance to pests, diseases, drought, salinity, or different noxious substances is still a matter of utmost importance. The great potential of green biotechnology to improve yield characteristics is nowadays certainly true [Altman 1999, Mudalige and Kuehnle 2004, Nishimura and Dangl 2010, Karban 2011, Halford 2012, Petchthai et al. 2015]. Techniques of green technology are applicable to numerous species which belong to Orchidaceae, one of the most recently evolved among plant families. Divisions among orchids species and genera are not distinct, and thus numerous interspecies, and even intergenetic hybrids can be easily obtained.

The species from the genus *Cattleya* are tropical epiphytes, with sympodial growth and rather large pseudobulbs. They grow in warm conditions, and are flowering at light brighter than preferred by most orchid genera. For cut-flower growers *Cattleya* is extremely important crop. Clones of disease-free, fotoperiodically controllable cultivars are desirable, because in cultivation they proved very profitable. As far as *in vitro* plant and tissue culture is concerned, an simple and independent from external weather conditions experimental system − it is whole set of techniques to propagate such ornamentals or even to produce new cultivars [Pindel and Miczyński 1996, Aldelberg et al. 1997, Peres and Kerbauy 1999, Lin et al. 2000, Pražak 2001, Batygina et al. 2003, Cybularz-Urban and Hanus-Fajerska 2006, 2008, Arditti 2008, Petchthai et al. 2015]. Year after year there are published numerous reports on cell and tissue culture of different species representing epiphytic orchids, as they are frequently chosen as model plant material for plant development, and even for some kind of metabolic studies. In *in vitro* conditions morphogenesis can be controlled by: physiological stage of both explants donor, and cultures, components of nutrient medium, osmotic potential of the medium, humidity of culture vessel, composition of its gaseous phase, and especially by light conditions [Islam et al. 2000, Shin et al. 2008, de Arajoújo et al. 2009]. Nowadays the important goal of experiments is to elaborate innovative, and at the same time economically justified technologies. For this reason, we herewith present the study undertaken with the aim to improve the control of morphogenetic potential during *in vitro* cultivation of particular precious *Cattleya* interspecific hybrid.

MATERIALS AND METHODS

The source material was interspecific hybrid *Cattleya intermedia* × *C. aurantiaca*, cultivated in *in vitro* conditions. The plant material was obtained from Orchid Collection maintained in Botanical Garden of Jagiellonian University. In order to start tissue culture explanted buds with fragments of pseudobulbs tissue were thoroughly washed. After short immersion in 70 % v/v ethanol alcohol, explants were surface sterilized in mercuric chloride solution (0.1 % v/v) for 60 s, and afterwards rinsed several times with sterile water. Meristems were excised under stereoscopic microscope and put into MS medium (pH 5.5) [Murashige and Skoog 1962]. Shoots regenerated with 72% frequency from meristem culture served as the primary source of callus, which was obtained in

four week on MS medium modified by Kozak [1991] with 30 g l^{-1} sucrose, 2.0 mg dm^{-3} adenine sulphate, 9.7 mg dm^{-3} ascorbic acid, additionally supplemented with 1 µM TDZ, and solidified with 8 g l^{-1} Difco Agar. Proliferating callus pieces were put into same medium but without TDZ, whereas supplemented with 4.95 µM BA, and 1.0 µM NAA. Sub-culturing was done in eight weeks intervals. Cultures were maintained at the day temperature of 25°C, and the night temperature of 16°C, with 16/8 photoperiod under light of different spectra. Following light treatments were applied: white – W; (390–760 nm, Tungsram F33 40W lamp) assumed as the first control treatment, far red – Fr, (770–800 nm, 100 W incandescent light and filters: standard filter no. 420 and no. 420, Compact light B.V. Amsterdam), red – R, (647–770 nm, Philips TLD, 36 W), blue – B, (450–492 nm, Philips TLD, 36 W). Radiometric measurements were made in the horizontal plane in the height of 5 cm above culture vessels, and quantum irradiance amounted to 30 µmol m^{-2} s^{-1} with exception of far red treatment (15 m^{-2} s^{-1}). Spectral characteristic are according data published elsewhere by Bach and Świderski [2000]. Thermal radiation was controlled by the temperature regime fixed in the climate chamber. Additionally cultures were also exposed to UV-B radiation – UV, and the other control treatment were maintained in full darkness − D. As a result during the experiment a double control treatment was used: (1) illumination of culture with white light and (2) absolute darkness. Each experimental treatment was done three times, with 10 replicates per each treatment. Single replicate was Erlenmayer flask with 10 pieces of callus (250 mg). The culture was regularly observed macroscopically from its beginning, whereas biometrical data and anatomical features were for the first time recorded after eight weeks of respective light treatment. For dry weight determination the material was dried in 105°C for 30 min, and then kept in 60°C to constant weight. Chlorophylls and carotenoids content was determined by light absorption spectroscopy in acetone extracts of 200 mg samples of regenerated shoots according to the method of Wellburn [1994] and expressed as mg·100 g^{-1} fresh weight of sample. Absorbance values at 662, 645, and 470 nm were recorded with Jasco V-530 spectrophotometer. Plant material taken for microscopy studies have been fixed in 3% glutaraldehyde. Sections 1 µm thick were cut from randomly selected Epon 812 blocks with the Tesla 490A ultramicrotome, and afterwards were studied in Olympus CX40 microscope. Culture has also been documented photographically with the use of stereoscopic microscope. Assessed biometrical parameters concerning the efficiency of micropropagation were fresh and dry weight, number of regenerated shoots, content of chlorophylls and carotenoids. Experiments were organized according to randomized design with 100 explants per treatment, and three repetitions of experimental block. Chosen biometrical data was subjected to analysis of variance with mean separation by Tukey's multiple range test. Significant differences between means were presented at the level of $p = 0.05$.

RESULTS AND DISCUSSION

The culture medium used in reported experiments was entirely appropriate to obtain proliferating *Cattleya intermedia* × *C. aurantiaca* callus lines. The effect of light conditions on either tissue proliferation of explanted callus or on its organogenetic potential

after 16 weeks of cultivation is shown in Table 1, whereas Figure 1 presents the influence of illumination with different types of lamps on studied *in vitro* culture either after eight (fig. 1 b, c) or after sixteen weeks of cultivation (fig. 1a). The morphological features of callus, and proliferating protocorm-like bodies were definitely different, and dependent on particular light treatment applied to the respective culture line. We observed that in control treatments uniform nodular callus proliferated, green on white light (Wa), and white in darkness (Da). Ultraviolet (UVa) brought about the local necrosis of about forty percent of explants, whereas in far red (Fra) treatment the majority of explants (80%) have been necrotized step by step. Blue (Ba) and red (Ra) treatments were comparable as far as callus proliferation is concerned. The callus line obtained in blue light was green. It could even be treated as comparable to white control treatment, but paler. Culture illuminated with red light were rather yellow-green, with sporadically occurring necrotic tissue damages. We have identified one specific callus line that proliferated with high growth rate when irradiated with blue light. Additionally in this particular line we have noted the tendency to differentiation into tracheids (fig. 1Bc). Shoots readily regenerated from PLBs regenerated on callus clumps (fig. 1b). Blue light applied to the culture was proved to be appropriate to regenerate as a result of caulogenesis quite numerous shoots. As we can see on Table 1 in that case the attainted mean number of shoot regenerated during one passage was 94.2 (in the same homogenous group as in white control treatment − 87.7). This kind of illumination (blue), was also clearly advantageous on number of formed PLBs on *Cattleya intermedia* × *C. aurantiaca* callus tissue (fig. 1 Bb). To quantify the data characterizing proliferative potential of cultures mean fresh and dry tissue weight values are presented in Table 1. White control treatment was proved the best option (279.9 mg) in that respect, following ultraviolet and blue treatments, where 246.6 mg versus 233.4 mg of tissue have been obtained. The results of total chlorophyll (Chl. *a* + *b*), chlorophyll *a*, chlorophyll *b*, and total carotenoids analysis are presented in Figure 2. As shown (fig. 2), the concentration of evaluated pigments were remarkably different under each particular light treatment. Blue, red and far red treatments led to significant reduction in content of photosynthetically active pigments compared with culture irradiation with either white or even ultraviolet light. *Cattleya intermedia* × *C. aurantiaca* culture which was irradiated with white light contained approximately fivefold higher content of total chlorophylls than those illuminated with blue light, and even more in red and far red treatments, yet the proportion of chlorophyll *a* to chlorophyll *b* content was more similar in all applied light treatments, and ranged from 4 (W) to 2.5 (B). Excluding darkness, the lowest level of carotenoids was detected under red and far red illumination.

In the case of ornamental plants which are cultivated *in vitro*, the manipulation of physical culture conditions can drastically ameliorate the final effect of propagation protocols [Bach and Pawłowska 2006, Aldelberg et al. 2007, de Arajoújo et al. 2009, Halford 2012]. After exposure of proliferating *Cattleya intermedia* × *C. aurantiaca* callus cultivated *in vitro* to different spectra of monochromatic light different types of tissue have been obtained. Individual lines in a noticeable way varied in proliferation rate, and organogenetic potential. Some publications reported similar phenomena [Sarbia-Ochoa et al. 2010, Wiszniewska et al. 2015], moreover, as proliferation is derivative of fresh (or dry) matter tissue content it strongly depends on level of nutrient uptake. The photo-

Table 1. The effect of light treatment on proliferation and caulogenesis in callus originated from *Cattleya intermedia* × *C. aurantiaca* hybrid, after 16 weeks of tissue cultivation

Light treatmen	Fresh matter (mg)	Dry matter (mg)	Callus morphology	Shoot number (second passage)	Remarks
White (control)	279.9 ±5.8	85.0	green tissue, nodulated in structure	87.7 ±6.2 d*	culture in proper condition
UV-B	246.6 ±5.2	52.5	green tissue with approximately 40% of explants with necrotized lesions	49.9 ±5.3 c	almost 10% of shoots have died
Blue	233.4 ±6.2	39.8	85% of explants green, the rest yellowish	94.2 ±7.6 d	almost 10 % of malformed shoots
Red	199.4 ±7.9	28.9	90% of explants lightly greenish, 10% yellow with sporadic necrotizing lesions	77.9 ±5.7 cd	about 60% of highly elongated shoots
Far-red	32.13 ±1.3	7.1	20% of explants greenish, 80 % yellowish with necrotizing lesions	25.6 ±2.4 b	about 70% of regenerated shoots extremely short
Darkness (additional control)	110.0 ±3.4	13.7	white-yellow tissue with nodulated structure	9.8 ±2.1 a	the etiolated shoots (lacking chloroplasts)

*Mean from 100 explants obtained after 16 weeks of cultivation, values within column followed by the same letter are not significantly different

synthetic pigments analysis are usually aimed at the quick and easy estimation of some physiological features during cultivation. Similar to the presented results the lowest accumulation level of photosynthetically active pigments were obtained not only in orchids, but also in some other taxonomically distant plant species grown under red or far red light sources, and results proved better in blue light [Tibbitts et al. 1983, Tanaka et al. 1998, Islam et al. 1999, 2000, Shin et al. 2008]. Da Silva and Debergh [1997] underlined that the blue to red light or red to far red ratios could be among critical factors affecting photomorphogenesis. Results obtained in herewith presented experiment indicated that in the case of *Cattleya intermedia* × *C. aurantiaca* callus blue light stimulated proliferation, and shoot regeneration through protocorm-like bodies (PLBs). It is consistent with findings of Chakravaty and Sopory [1998] with *Amaranthus paniculatus*, and this shift in morphogenetic potential seem to be quite typical for callus culture of *Cymbidium* and several other genera classified to Orchidaceae [Bach and Świderski 2000, Batygina et al. 2003, Huan et al. 2004, Bach i Pawłowska 2006, Lavrentyeva and Ivanikov 2007, Sarbia-Ochoa et al. 2010, Bach et al. 2015]. Due to the above, even if the frequently studied physiological features of callus considerably differ from that of the regenerated shoots, we have concluded that light treatments characterized by different wavelength can significantly affect both proliferative and regenerative potential of ornamental plant material cultivated *in vitro*.

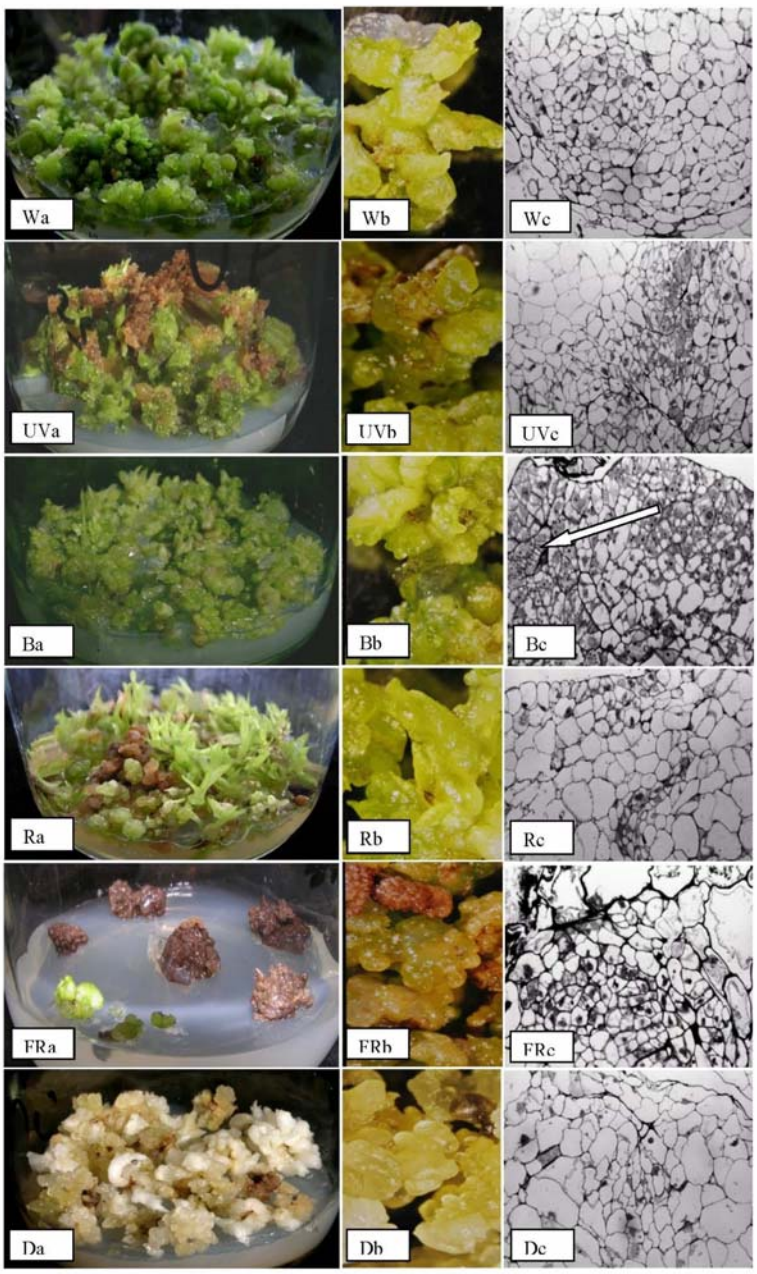

Fig. 1. **a:** The influence of white (W), ultraviolet (UV-B), blue (B), red (R), Far-red (Fr) light and in continuous darkness (D) on *Cattleya intermedia* × *C. aurantiaca* callogenesis and caulogenesis after 16 week of culture; **b**: Enlarged tissue pieces with shoots regenerating from PLBs after 8 weeks of cultivation; bar represents 3 mm, **c**: Anatomical features of callus with regenerated shoots after 8 weeks of cultivation; bar represents 200 µm

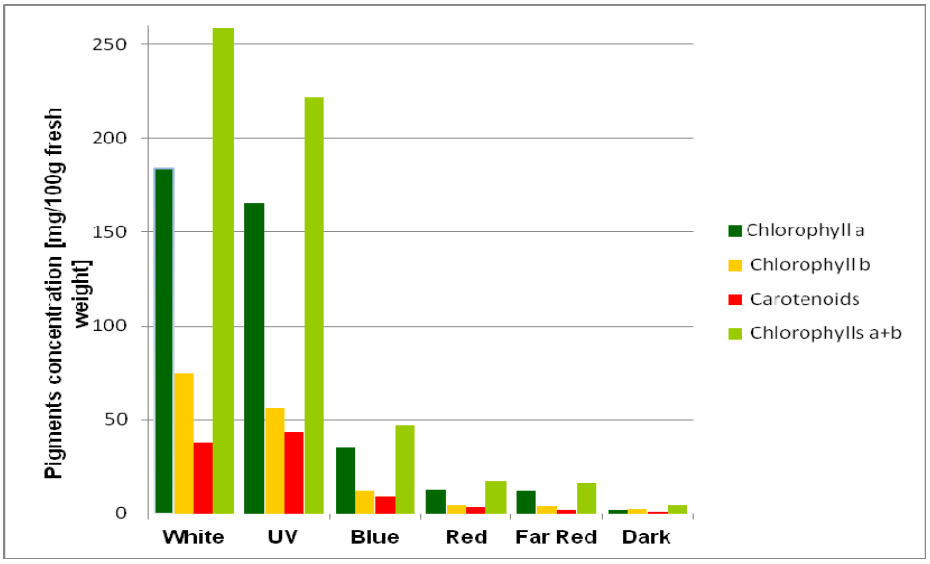

Fig. 2. Chlorophyll and carotenoid content in shoots of *Cattleya intermedia* × *C. aurantiaca* cultivated under white (W), ultraviolet (UV-B), blue (B), red (R), Far-red (Fr) light, and in continuous darkness (D)

CONCLUSIONS

1. Growth, morphology of *Cattleya intermedia* × *C. aurantiaca* callus and its organogenetic potential was differently affected by light characterized by different wavelenght.
2. Blue light has stimulated the number of shoots regenerated from PLBs.
3. White and UVB irradiation led to increase in content of chlorophyll a and b in regenerated shoots.
4. Tissue clumps or shoots of studied *Cattleya* interspecific hybrid displayed distinct morphological and physiological features when obtained in the presence of different monochromatic light treatments.

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INDUKCJA KALUSA I ORGANOGENEZA *IN VITRO* CATTLEYA Z CIAŁ POTOKORMOPODOBNYCH (PLBs) W RÓŻNYCH WARUNKACH ŚWIETLNYCH

Streszczenie. W pracy opisano badania nad wpływem różnych warunków świetlnych *in vitro* na wzrost i rozwój międzygatunkowego mieszańca *Cattleya intermedia* × *C. aurantiaca* (Orchidaceae), którego zasób stanowiły kultury pędowe. Kalus indukowano z eksplantatów pędowych na zestalonej pożywce składającej się z zestawu soli mineralnych i witamin według MS, wzbogaconej 30 g l^{-1} sacharozy, 2,0 mg dm^{-3} siarczanu adeniny, 9,7 mg dm^{-3} kwasu askorbinowego i z dodatkiem 1,0 µM TDZ, podczas gdy w pożywce proliferacyjnej zastosowano 4,95 µM BA, i 1,0 µM NAA. Badany materiał roślinny w formie ciał protokormopodobnych (PLBs) eksponowano na światło monochromatyczne o zróżnicowanej długości fali świetlnej. Na świetle białym, niebieskim, czerwonym, dalekiej czerwieni i ultrafiolecie uzyskano wartościowe linie, które różniły się tempem proliferacji, morfologią i cechami anatomicznymi. Poszczególne traktowania wywarły również znaczący wpływ na potencjał regeneracyjny badanej kultury. Światło niebieskie okazało się najbardziej korzystne w regeneracji pędów za pośrednictwem PLBs. Naświetlanie kultur światłem niebieskim, czerwonym lub daleką czerwienią doprowadziło do znaczącej redukcji zawartości chlorofili i karotenoidów w porównaniu z zawartością tych barwników w materiale eksponowanym na światło białe lub ultrafioletowe.

Słowa kluczowe: kultura *in vitro*, Orchidaceae, proliferacja tkanki, fotomorfogeneza, mikrorozmnażanie

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