Botanica SERBICA 33 (1): 83-88 (2009)

Original Scientific Paper

Effects of day length on photosynthetic pigments and antioxidative metabolism of *in vitro* cultured moss *Atrichum undulatum* (Hedw.) P. Beauv. (*Bryophyta*)

Tijana Cvetić[1]*, Aneta Sabovljević[1], Jelena Bogdanović Pristov[2], Marko Sabovljević[1]

1 Institute of Botany and Garden Jevremovac, Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia

2 Institute for Multidisciplinary Research, Kneza Višeslava 1, 11030 Belgrade, Serbia

ABSTRACT Photoperiod is known to regulate many essential processes in plants, but physiological effects of photoperiod in vegetative stage of plant life have seldom been studied. This paper deals with effects of day length on Catherine's moss grown in aseptic culture. Photosynthetic pigments did not show significant variations as a consequence of growth in different photoperiods. Protein content and malate dehydrogenase activity were higher in long day (16h light/8h dark) than in short day (8h light/16h dark) grown plants. Total phenolic compounds contents, as well as total antioxidative capacity were shown to be higher in plants grown in long day conditions. Peroxidase activity was also higher in long day than in short day grown plants. Regulation of components of antioxidative metabolism in a moss species grown in different photoperiods are discussed in relation to same parameters in higher plants.

Key words: moss, *Atrichum undulatum*, photoperiod, vegetative phase, antioxidative metabolism

Abbreviations: ABTS - 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); DW – dry weight; FW – fresh weight; MDH – malate dehydrogenase; LD – long day; SD-short day; POD – peroxidase; RWC – relative water content

Received: 30 April 2009 Revision accepted: 30 June 2009

UDK 582.32:581.132

INTRODUCTION

Light is one of the major environmental factors affecting plant growth and development. Both light intensity and duration are essential for these processes, but they often have various effects on different species. Photoperiod effects have mostly been studied in relation to flowering induction. It is now widely accepted that photoperiod may affect flowering, dormancy, tuberization etc., as well as vegetative growth, and that the effectiveness of photoperiod varies among species (Adams & Langton 2005). However, physiological effects of photoperiod have rarely been investigated in vegetative development. Becker *et al.* (2006) have shown that photoperiod affects a number of vegetative processes in *Arabidopsis* by complex up- and down-regulation of the various compartment specific genes long before flowering or senescence starts.

In mosses, light is not the unique factor governing production of gametangia, and these cryptogams are often day-neutral (Knoop 1984; Chopra & Kumra 1988). Growth in most bryophytes is limited by water availability, with light, nutrients and temperature playing lesser roles (Glime 2007). However, as in higher plants, photoperiod should have strong influence on vegetative development,

*correspondence: tcvetic@bio.bg.ac.rs

though literature sources on this issue are quite sparse. According to Kallio & Saarnio (1986) photoperiod can play a role in development, productivity, acclimation, and other aspects in the bryophyte life span. Long days and high light intensity, combined with temperatures above 25°C, lead to anabiosis in many moss species (Schwabe 1976; Glime 2007). Photoperiod also affects protonemal growth and branching in *Ceratodon purpureus* (Larpent-Gourgaud & Aumaitre 1980). However, no data on the effects of photoperiod on physiological aspects of moss development was found in literature.

Atrichum undulatum is a robust, widespread moss. Due to its large size and high frequency of occurrence in all types of forest habitats, it has often been used as a model system in moss biology research (Bequerel 1906; Gemmell 1953; Lindemann *et al.* 1998; Beckett *et al.* 2000; and many more). Since it has been successfully introduced in axenic conditions (Bijelović *et al.* 2004; Lin *et al.* 2005; Sabovljević *et al.* 2006), *Atrichum undulatum* is suitable model for analyses of specific effects of a single environmental factor, such as day length.

Regardless of the importance of photoperiod for plants, basic physiological consequences of growth under different photoperiods on vegetative growth have never been studied in mosses. In this work we have analysed some physiological parameters under different day lengths and provided evidence of different overall and antioxidative metabolism states under these conditions.

MATHERIAL AND METHODS

Plant material and sample preparation. *Atrichum undulatum* plants were cultured in growth chamber at 25±2°C. Apical 10 mm fragments were placed on nutrient medium containing half strength Murashige-Skoog (1962) mineral salts and vitamins, 1.5% sucrose and 0.7% agar, and kept for 10 weeks under LD (16 h light/8 h dark) or SD (8 h light/16 h dark) conditions. Light was provided by cold fluorescent tubes (5-7.2 W/m^2).

For biochemical analyses only green plant fragments that grew above media were used. Material was harvested, immediately frozen in liquid nitrogen and stored at -70°C until use.

Relative water content was determined as:

RWC = (FW - DW) / ((WSW -DW) x 100,

FW is obtained by measuring tissue immediately after removal from nutrient medium, as quickly as possible in order to prevent weight loss caused by evaporation. WSW is weight of the same tissue used for FW determination, after 24h incubation in deionised water, and DW is weight after 24h drying of water saturated tissue at 100 °C.

Plant tissue was homogenized in liquid nitrogen. Photosynthetic pigments were extracted in 80% acetone for 24h. Samples were then centrifuged for 10 min at 13000 x *g* and supernatant was used for pigment determination.

For total phenolic content and antioxidative capacity, powdered tissue was extracted in 80% methanol at 60°C for 3 h, centrifuged (10 min at 13 000 x *g*) and the supernatant was used for subsequent analyses, while the pellet was dried (24 h at 100°C) and used for dry weight determination.

Total proteins were extracted from powdered tissue in isolation buffer consisting of 50 mM potassium phosphate, pH 6.5, 2 mM EDTA, 2 mM dithiothreitol, 1% polyvinylpyrrolidone, and 0.05% Triton X-100. After centrifugation (10 min 13000 x *g*) supernatant was gel-filtered (NAP-5 column, Amersham Biosciences), lyophilised and resuspended to ½ of the original volume.

Biochemical analyses. *Photosynthetic pigments* content was determined spectrophotometrically in 80% acetone, according to Lichtenthaler (1987).

Total phenolic content was estimated essentially by the method of Singleton & Rossi (1965) with gallic acid as a standard (0.1-1 mg/ml). Samples (50 µl) were incubated with 0.475 ml 5% Na_2CO_3 for 3 min and 0.475 ml 1N Folin-Chiocalte reagent was added. Mixture was placed in dark at room temperature and absorbance at 724 nm was measured after 1h incubation.

Total antioxidative capacity was determined by ABTS test. Assay medium (3 ml) contained 2 mM ABTS, 15 µM hydrogen peroxide, 0.25 µM horseradish peroxidase, 50 mM K-phosphate buffer and 50 µl extract.

Enzyme activity assays. *Peroxidase* activity was detec-ted spectrophotometrically, by monitoring the production of purpurogallin at 420 nm with extinction coefficient 12 $M^{-1}cm^{-1}$. Assay mixture contained 50 mM potassium-phosphate buffer pH 6, 42 mM pyrogallol and 8 mM hydrogen peroxide, in 1ml volume.

Activity of NAD-MDH was measured in a reaction mixture containing 10 mM HEPES-KOH pH 7.8, 0.15 mM oxaloacetate and 0.3 mM NADH, by following oxidation of NADH at 340 nm. Extinction coefficient of NADH used for calculation was 6.22 $mM^{-1}cm^{-1}$.

One unit of POD and MDH was defined as the amount of enzyme that converts one micromole of substrate into product per minute.

Protein concentration. Protein content was estimated according to Bradford (1976), modified for microtiter plate, with BSA as standard.

Statistic analyses. All data were tested by one-way ANOVA. Statistical analyses were performed using Microcal Origin 6.1

RESULTS AND DISCUSSION

Day length promoted gametophyte development, biomass production being approximately doubled (Fig 1.). No morphological or anatomical differences were visible. Plants had RWC around 77% and FW/DW ratio around 11.5, regardless of the photoperiod. Phylloid anatomy was not altered due to day length, phylloids being undulate, lanceolate, with 4-7 lamellae in both SD and LD grown plants.

With plants grown in different light regimes but with constant light intensity and other environmental factors, pigment content showed no statistically significant alterations due to day length (Fig 2). Gametophytes grown in SD had 97.0±11.3 mg chlorophyll *a* and 43.5±5.0 mg chlorophyll *b* per gram DW, while LD grown plants contained 94.8±7.3 and 39.3±3.0 mg chlorophyll *a* and *b*, respectively, per gram DW. Carotenoid content was 28.1±3.4 mg/g_{DW} in SD and 30.4 ±2.7 mg/g_{DW} in LD grown plants. Although differences in pigment contents were not statistically significant, it can be noted that SD plants had a bit higher chlorophyll content and a bit lower carotenoid content.

Protein content of plants grown under LD conditions was 1.5 times higher on the DW basis than in SD (Tab 1.). Specific activity of NAD-MDH, as a general marker of cellular activities, was in LD plants also 1.5 times higher calculated on protein basis (Tab 1.), and around 2 when calculated on DW basis.

Plants grown under long day conditions had higher phenolic contents and higher antioxidative capacity than SD grown plants (Fig 3.). Antioxidative capacity was 37.6 ascorbic acid (AA) equivalents in SD and 50.1 AA equivalents in LD plants (1.3 times more than in SD). Total phenolic compounds content, quantified as gallic acid equivalents, was also 1.3 times higher in LD than in SD grown plants. Peroxidase activity was increased in LD compared to SD-grown plants (Fig 4.).

Gametophytes of Cathrine's moss, *Atrichum undulatum*, were grown in axenic culture in SD (8h light/16h dark) and LD (16h light/8h dark) photoperiods, which affected almost all investigated parameters. Phylloid anatomy (data not presented) did not change in response to day length applied, and all phylloids were normal, although a bit more fragile compared with field-collected specimens, as in Sabovljević *et al.* (2006). Plants were taller and showed larger multiplication index (data not presented) when grown in LD. Light regime affected biomass production and overall metabolic activities. With unaltered RWC, LD plants contained 1.5 times more protein per g DW than SD grown plants.

It has been shown that chlorophyll content is regulated by day length and that this is a photoperiodic response (Sironval 1958), and in *Arabidopsis* was chlorophyll

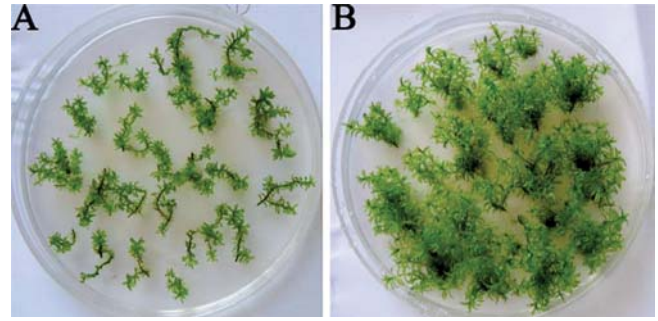

Fig 1. Shoots of in vitro cultivated *Atrichum undulatum*, grown for 10 weeks under short day (A) and long day (B) conditions.

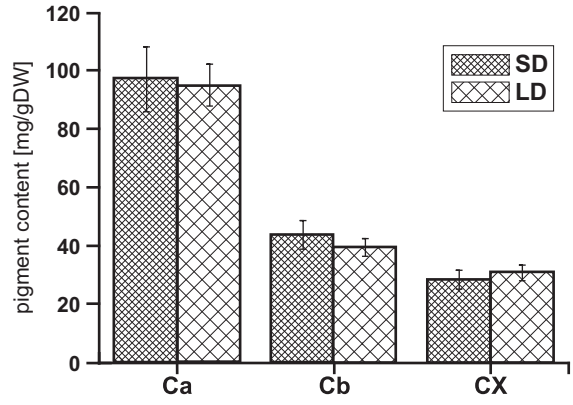

Fig 2. Chlorophyll a (Ca) chlorophyll b (Cb) and total carotenoid (CX) content of *Atrichum undulatum* shoots grown in vitro in short (SD) or long (LD) day. Each value ± SE is mean of four replicates of four samples in two independent experiments (n=32).

content higher in SD than in LD plants (Becker *at al.*, 2006). In our work, SD grown plants had somewhat higher chlorophyll and lower carotenoid content then LD grown plants. Carotenoids protect plants from oxidative damage, primarily induced by high light intensities (Block *et al.* 2001, 2007) and probably have a role in abscisic acid synthesis (Block *et al.* 2001). In our work, carotenoid content was higher in LD than in SD plants, reflecting higher need for antioxidants in conditions where plants are exposed to high light intensities for a longer period, and may also point to increased abscisic acid production. However, pigment contents did not differ at a statistically significant level. Since mosses are generally shade adapted plants (Glime 2007), lack of statistically significant differences in pigment contents probably points to saturation of photosynthesis in both SD and LD plants.

Malate dehydrogenase (MDH) is one of the enzymes that regulate synthesis and oxidation of malic acid, cellular buffer and a mobile cellular storage of electrons and CO_2 (Gietl 1992). Thus, MDH is an essential part of malate

Table 1. Total protein content and malate dehydrogenase activity in *Atrichum undulatum* gametophytes grown under short or long day conditions. Protein contents are expressed as milligram per gram dry weight, and malate dehydrogenase activity as units per milligram protein. Values with different superscripts within a row of data are significantly different ($p < 0.05$). All assays were performed in quadruplicate, in two independent experiments, with 2 repetitions for each sample (n=16).

	SD	LD
Protein content	10.60±0.86[a]	15.28±0.84[b]
MDH /mg $_{protein}$	0.78±0.07[c]	1.17±0.09[d]

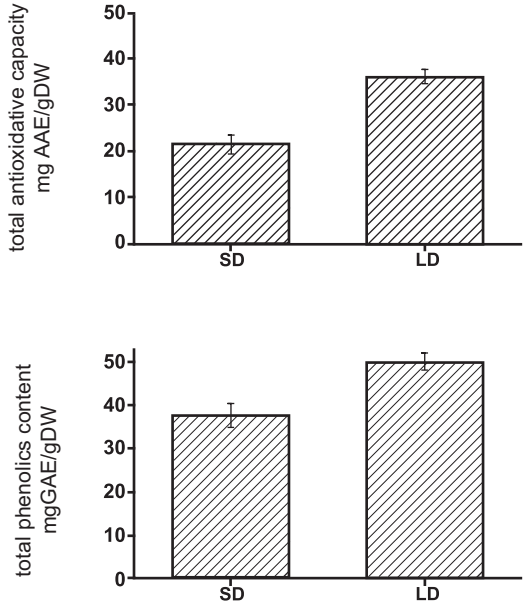

Fig 3. Total antioxidative capacity and total phenolic content of *Atrichum undulatum* shoots. Antioxidative capacity is expressed as milligram ascorbic acid equivalents (mg AAE) per gram dry weight, total phenolics as gallic acid equivalents (mg GAE) per gram dry weight. Each value ± SE is mean of four replicates of four samples in two independent experiments (n=32.)

valve, a process which balances ratio of ATP and NAD(P)H and regulates redox homeostasis (Scheibe 2004, 2005) and as such can be employed as a general metabolism marker enzyme. Higher MDH activity in *Arabidopsis* plants grown in LD when compared to SD, points to higher overall cellular metabolism (Becker *et al.* 2006). Similarly, LD grown *Atrichum* gametophytes had elevated MDH activity compared to SD grown plants. Specific MDH activity was 1.5 times higher in LD than in SD grown gametophytes and, when calculated on FW basis, this ratio reached almost 2 (Tab 1.).

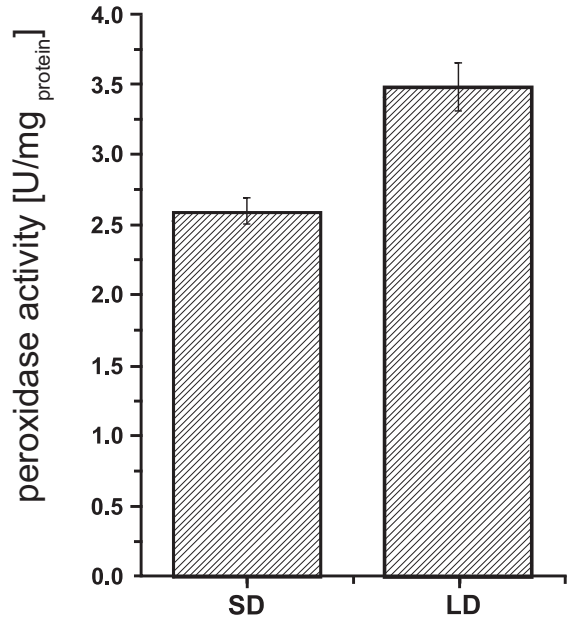

Fig 4. Peroxidase (POD) specific activitiy in *Atrichum* shoots grown in short (SD) or long day (LD). Unit definition is given under Material and methods. Each value ± SE is mean of two to four replicates of four samples (n=8-16).

We have found that *Atrichum* plants grown in LD have higher antioxidative capacity and phenolic contents than SD plants. It has been suggested that increased antioxidative capacity is related to flowering as well as increased longevity in *Arabidopsis* (Kurepa *et al.* 1998), but is also induced under different stress conditions (eg. Gillespie *et al.* 2007; Saleh & Plieth 2009). In our work, LD plants that have shown higher antioxidative capacity might be preparing for summer, high light intensities and water deficit that it is connected to. Total phenolics content values were in agreement with antioxidative capacity, both being ca 25% higher in LD than in SD grown plants. Effect of day length on flavones was even greater, having 40% higher content in LD than in SD plants, while anthocyanins were higher in SD than in LD plants (data not presented). This implies different regulation of non-enzymatic antioxidant components content, which should be studied more thoroughly.

Interrelation between photoperiod, flowering, and activities of antioxidative enzymes has been reported (Mitrović & Bogdanović 2008), but photoperiod has influence on antioxidative enzymes also during vegetative development (Becker *et al.* 2006). Investigated POD activity was higher in LD than in SD plants (Fig 4.). Peroxidases are a large family of enzymes that are included in essential developmental processes as well as stress conditions and defence (for review see Hiraga *et al.* 2001), and thus react to stress by increased activity

of specific isoforms. Our results confirm that change of conditions, day length in this case, is reflected in elevated POD activity.

CONCLUSION

Effects of photoperiod on vegetative plant development are not well understood. Cultivation of *Atrichum* gametophytes in long day conditions led to elevated metabolic activity compared to short day grown gametophytes. Photoperiod also affected antioxidative metabolism, leading to increased antioxidative capacity and higher phenolic content in LD grown plants, and activity of peroxidases. It is concluded that response of bryophytes to photoperiod is, at least in part, different than in higher plants. These differences are primarily related to regulation of components of antioxidative metabolism.

Acknowledgement - This work was supported by Serbian Ministry of Science grant No. 143016B.

REFERENCES

Adams SR & Langton FA. 2005. Photoperiod and plant growth: a review. *J. Hort. Sci. Biotech.* **80**: 2-10.

Becker B, Holtgrefe S, Jung S, Wunrau C, Kandlbinder A, Baier M, Dietz K-J, Backhausen JE & Scheibe R. 2006. Influence of the photoperiod on redox regulation and stress responses in *Arabidopsis thaliana* L. (Heynh.) plants under long- and short-day conditions. *Planta* **224**: 380-393.

Beckett RP, Csintalan Z & Tuba Z. 2000. ABA treatment increases both the desiccation tolerance of photosynthesis and nonphotochemical quenching in the moss *Atrichum undulatum*. *Plant Ecology* **151**: 65-71.

Bequerel P. 1906. Germination des spores d'*Atrichum undulatum* et d'*Hypnum velutinum*. Nutrition et developpement dans des milieux stérilisés. *Revue Gén. Botanique* **18**: 49-67.

Bijelović A., Sabovljević M., Grubišić D. & Konjević R. 2004. Phytohormone influence on the morphogenesis of two mosses (*Bryum argenteum* Hedw. and *Atrichum undulatum* (Hedw.) P. Beauv.). *Israel J. Plant Sci.* **52**: 31-36.

Block MA, Douce R, Joyard J & Rolland N. 2007. Chloroplast envelope membranes: a dynamic interface between plastids and the cytosol. *Photosynth. Res.* **92**: 225-244.

Block MA, Maréchal E & Joyard J. 2001. Role of the plastid envelope in the biogenesis of chloroplast lipids. In: Aro E-M and Andersson B (eds): Regulation of photosynthesis. Kluwer Academic Publishers, Netherlands. Pp. 195–218,

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.

Chopra RN & Kumra PK. 1988. Biology of bryophytes. Wiley, New York

Gemmell AR. 1953. Regeneration from the leaf of *Atrichum undulatum* (Hedw.) P. Beauv. *Transactions of the British Bryological Society* **2**: 203-213.

Gietl C. 1992. Malate dehydrogenase isoenzymes: Cellular locations and role in the flow of metabolites between the cytoplasm and cell organelles. *Biochim. Biophys. Acta* **1100**: 217-234.

Gillespie KM, Chae JM & Ainsworth EA. 2007. Rapid measurement of total antioxidant capacity in plants. *Nature Protocols* **2**: 867-870.

Glime JM. 2007. *Bryophyte Ecology.* Volume 1. Physiological Ecology. Ebook sponsored by Michigan Technological University and the International Association of Bryologists. Accessed on 22.04.2009. at **http://www.bryoecol.mtu.edu**

Hiraga S, Sasaki K, Ito H, Ohashi Y, & Matsui H. 2001. A large family of class III plant peroxidases. *Plant Cell Physiol.* **42**: 462-468.

Kallio P & Saarnio E. 1986. The effect on mosses of transplantation to different latitudes. *J. Bryol.* **14**: 159-178.

Knoop B. 1984. Development in bryophytes. In: Dyer A. F. & Duckett J. G. (eds.). The Experimental Biology of Bryophytes. Acad. Press, New York pp. 143-176.

Kurepa J, Smalle J, Van Montagu M & Inzé D. 1998. Oxidative stress tolerance and longevity in Arabidopsis: the late-flowering mutant gigantea is tolerant to paraquat. *Plant J.* **14**: 759-764.

Larpent-Gourgaud M. & Aumaitre MP. 1980. Photoperiodism and morphogenesis of the protonema of *Ceratodon purpureus* (Hedw.) Brid. *Experientia* **36**: 1366-1367.

Lichtenthaler HK. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Meth. Enzymol.* **148**: 350-382.

Lin L-H, Wang X-P, Hou W-R, Liu X-L & He Y-K. 2005. An efficient protocol for plant regeneration from protoplasts of the moss *Atrichum undulatum* P. Beauv in vitro. *Plant Cell Tiss. Org.* **82**: 281-288.

Lindemann P, Braslavsky SE, Hartmann E & Schaffner K. 1989. Partial purification and initial characterization of phytochrome from the moss *Atrichum undulatum* P. Beauv. grown in the light. *Planta* **178**: 436-442.

Mitrović A & Bogdanović J. 2008. Activities of antioxidative enzymes during *Chenopodium rubrum* L. ontogenesis *in vitro*. *Arch. Biol. Sci.* **60**: 223-231.

Murashige T & Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plantarum* **15**: 473-497.

Sabovljević A, Cvetić T. & Sabovljević M. 2006. The establishment and development of the catherine's moss

Atrichum undulatum (Hedw.) P.Beauv. (*Polytrichaceae*) in *in vitro* conditions. *Arch. Biol. Sci.* **58**: 87-93.

Saleh L & Plieth C. 2009. Fingerprinting antioxidative activities in plants. *Plant Methods* 5: 2.

Scheibe R. 2004. Malate valves to balance cellular energy supply. *Physiol. Plantarum* **120**: 21-26.

Scheibe R, Backhausen JE, Emmerlich V & Holtgrefe S. 2005. Strategies to maintain redox homeostasis during photosynthesis under changing conditions**.** *J. Exp. Bot.* **56**: 1481-1489.

Schwabe WW. 1976. Photoperiodism in liverworts. In: Smith, H. (ed.). Light and Plant Development. Butterworths, Boston. Pp. 371-382

Singleton VL & Rossi JA. 1965. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid "reagents". *Am. J. Enol. Vitic.* **16**: 144-158.

Sironval C. 1958. Day-length and Haematin Compounds in Plants. *Nature* **182**: 1170-1171.

REZIME

Uticaj dužine dana na fotosintetske pigmente i antioksidativni metabolizam *in vitro* gajene mahovine *Atrichum undulatum* (Hedw.) P. Beauv. (*Bryophyta*)

Tijana Cvetić, Aneta Sabovljević, Jelena Bogdanović Pristov i Marko Sabovljević

Fotoperiod utiče na mnoge procese u biljkama, ali su fiziološki efekti dužine fotoperioda na vegetativni razvoj biljaka veoma loše ispitani. U ovom radu praćen je efekat dužine dana na fiziološke karakteristike gametofita mahovine *Atrichum undulatum* u sterilnim uslovima. Analiziran je sadržaj pigmenata, proteina i fenolinih jedinjenja, kao i ukupni antioksidativni kapacitet i aktivnosti pojedinih enzima. Nije uočena statistički značajna razlika u sadržaju fotosintetskih pigmenata između biljaka gajenih na režimu dugog (16h svetlo/8h mrak) i kratkog (8h svetlo/16h mrak) dana. Sadržaj proteina, kao i aktivnost malatne dehidrogenaze, su bili veći kod biljaka gajenih na dugom danu. Ukupni sadržaj fenola, kao i ukupni antioksidativni kapacitet su takođe bili povećani na dugom danu u odnosu na kratak dan.. Aktivnost peroksidaza je na dugom danu bila povećana u odnosu na gajenje u uslovima kratkog dana. Ovi rezultati pokazuju da gajenje mahovine *Atrichum undulatum* na dugom danu dovodi do povećanja ukupnog antioksidativnog kapaciteta u odnosu na biljke gajene na kratkom danu.

Ključne reči: mahovine, *Atrichum undulatum*, fotoperiod, vegetativna faza, antioksidativni metabolizam.