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Research article

The effect of 24-epibrassinolide on the green alga *Acutodesmus obliquus* (Chlorophyceae)

Marta Talarek-Karwel, Andrzej Bajguz*, Alicja Piotrowska-Niczyporuk, Iwona Rajewska

University of Bialystok, Faculty of Biology and Chemistry, Institute of Biology, Department of Plant Biochemistry and Toxicology, 1J Konstantego Ciolkowskiego St., 15-245 Bialystok, Poland

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ABSTRACT

Brassinosteroids play an important role in the plant growth and development as well as in the adaptation of plants to environmental stresses. Studies have shown the effect of 24-epibrassinolide (EBL) in the range of concentrations 0.0001–10 μM on the green unicellular alga *Acutodesmus obliquus* (Chlorophyceae) during 7 days of cultivation. EBL is an effective stimulator of algal growth as it causes an increase in the number of cells and the contents of selected metabolites such as proteins, monosaccharides, and photosynthetic pigments (chlorophylls, carotenes, and xanthophylls). Furthermore, EBL inhibits the formation of reactive oxygen species such as hydrogen peroxide and oxidative damage as evidenced by a decrease of the lipid peroxidation. The positive effect of EBL resulting from the cellular oxidative state can be alleviated by antioxidants such as ascorbate peroxidase, catalase, superoxide dismutase, and ascorbate.

1. Introduction

Algae are a specific group of pioneering plants due to not only their prevalence in very different climatic and habitat conditions but also high resistance to adverse environmental factors. They are the base in the aquatic food chain as they are the main producer of oxygen and are essential in circulation of elements in nature. Algae are ecologically important organisms, which produce up to 70% organic matter and cooperate in the mineralization of many organic substances (Borowitzka et al., 2016). Microalgae play a crucial role in sourcing new therapeutic products, cosmetics, food, industrial materials, and even biofuel biodiesels (Yu et al., 2015). A large number of extensive studies have confirmed many biological functionalities of natural algal products, which include anti-inflammatory, immunomodulatory, anticoagulant, antioxidant, antidiabetic, tyrosinase inhibitory and UV-protective, and antimicrobial effects (Borowitzka et al., 2016). Large-scale application of algae is restricted by very low productivity of microalgae-based processes. One of the ways to improve accumulation of high-value bioproducts and cell growth in cultivation of microalgae can be using various stimulators, such as phytohormones and their analogs which regulate multiple aspects of microalgal metabolism (Yu et al., 2015).

BRs are plant polyhydroxy steroids and are classed as phytohormones. Since the discovery of brasssinolide (BL) – the first BR, 62 naturally occurring compounds have been found. They are widely distributed in many plants and have been detected in all plant organs, i.e. pollen, anthers, seeds, leaves, stems, roots, flowers, and grain. Although endogenous BRs have been found in plants at very low concentrations on the microgram or nanogram levels of BRs per kilogram of fresh weight, they exhibit high activity (Bajguz, 2009; Kanwar et al., 2017). BRs have been isolated from 90 plant species including one bryophyte (*Marchantia polymorpha*), one pteridophyte (*Equisetum arvense*), 26 algae (Chlorophyceae, Trebouxiophyceae, Ulvophyceae, and Charophyceae strains), six gymnosperms and 53 angiosperms (12 monocotyledons and 41 dicotyledons) (Bajguz, 2009; Kanwar et al., 2017; Stirk et al., 2013). BRs are structurally very similar to animal (i.e. androgens, estrogens, corticoids) and insect steroid hormones (i.e. ecdysteroids). They have also a significant role in the development and growth of vascular plants and green algae. They elicit a broad spectrum of morphological and physiological responses, i.e. cell division and elongation, biosynthesis of cell wall components, synthesis of DNA, RNA, and proteins, photosynthesis, enzyme activity, ethylene biosynthesis. Furthermore, BRs can act autonomously with other endogenous plant hormones (Bajguz and Piotrowska-Niczyporuk, 2014). BRs also play a major role in plant stress alleviation. Thus they have the ability to boost the antioxidant system of plants used to confer resistance in plants against a variety of abiotic stresses, such as thermal, drought, heavy metal, pesticides, and salinity (Rajewska et al., 2016). The main factor influencing the biological activity of BRs is a different structure of the 5α-cholestane skeleton where 7-oxalactone type, 6-

* Corresponding author.
E-mail address: abajguz@uwb.edu.pl (A. Bajguz).

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ketone type, and non-oxidized form of ring B can be distinguished. All the BRs of 7-oxalactone type, e.g. 24-epibrassinolide (EBL) are biologically more active than 6-ketone compounds, e.g. castasterone (Bajguz and Czerpak, 1998).

EBL is one of the most effective BR in algae and vascular plants. The increase in both cell number and content of primary metabolites, such as protein, monosaccharides, photosynthetic pigments, and antioxidants activity was observed in the green alga *Chlorella vulgaris* treated with EBL (Bajguz, 2000, 2011; Bajguz and Czerpak, 1998). Furthermore, EBL stimulates the growth of a freshwater diatom, *Asterionella formosa* and increases the activity of key metabolic enzymes (Mekhalfi et al., 2012). It also stimulates the growth and development of higher plants, e.g. garden strawberry (*Fragaria* × *ananassa*), tomato (*Lycopersicon esculentum*), geranium (*Pelargonium graveolens*), soybean (*Glycine max*) (Asghari and Zahedipour, 2016; Hayat et al., 2011; Swamy and Rao, 2009).

Oxygen is one of the most substantial elements required by plants to their cell organelles, such as the chloroplast and mitochondria, to produce satisfactory amounts of energy to continue their development. Reactive oxygen species (ROS), e.g. hydrogen peroxide (H_2O_2), are natural products of plant metabolism, which participate actively in signal transduction and are formed in different cell compartments, such as the mitochondria, apoplast, endoplasmatic reticulum, chloroplasts, and peroxisomes (Gill and Tuteja, 2010). Plants growing in natural environmental conditions are exposed to biotic and abiotic stresses. These factors can lead to molecular, biochemical, physiological, and morphological changes. One of the consequences of environmental stresses is an enhanced production of ROS, such as H_2O_2, which can lead to oxidative stress causing degradational changes to cell membranes, proteins, nucleic acids, and lipids (Bajguz and Hayat, 2009). Plants have developed many biochemicals and strategies to take control over the physiological ROS production or the boosted level of ROS under stress factors (Apel and Hirt, 2004). Substances known as antioxidants are functionally divided into enzymatic and nonenzymatic components. They work in a well-organized scheme and occur in almost all cell organelles (Gill and Tuteja, 2010; Szőllősi, 2014). Plant hormones like BRs can alleviate stress by streamlining the antioxidant systems, including the enzymatic ones and thus scavenging the free radicals (Rajewska et al., 2016; Soares et al., 2016).

BRs applied to microalgae improve their growth, accumulation of high-value bioproducts, and make them more resistant to various stresses (Bajguz, 2011; Bajguz and Czerpak, 1998; Yu et al., 2015). The effect of BRs on physiology of green algae has been previously reported. Although, most of the previous investigations were done using *C. vulgaris* as the model species (Bajguz, 2000, 2011; Bajguz and Czerpak, 1998), another unicellular green alga, i.e. *Acutodesmus obliquus* might also be a good bioindicator, sequester of metal ions, and can be used as a model system for studies of algal physiology and their biochemical responses to the presence of different stressors (Bajguz, 2011; Borowitzka et al., 2016; Lourie et al., 2010; Piotrowska-Niczyporuk et al., 2017). Due to the lack of data on the effects of BRs on the *A. obliquus* cultures, this study aimed to verify if the application of EBL has any impact on the growth rate, oxidative stress, antioxidant response, and the content of primary metabolites (proteins, monosaccharides, photosynthetic pigments). Thus, the hypothesis that EBL is an effective growth stimulator of *A. obliquus* and its effect is concentration-dependent was examined.

2. Material and methods

2.1. Algal material and culture conditions

The wild-type *A. obliquus* (Turpin) Hegewald and Hanagata 2000, SAG strain no. 276-6 was obtained from the SAG Culture of Algae Collection (Germany). The homogenous population of the young synchronous algal cells was collected by centrifugation (10000 × *g*, 10 min) and used for the experiments. Complete synchronization was obtained by a regular change of light and dark periods (Piotrowska-Niczyporuk et al., 2017). Every day at the beginning of the light period the synchronization of the culture was examined, using a microscope, by studying the cell division and the diagrams of cell size distribution. When the pre-cultures reached the exponential growth phase, cultures were initiated by the introduction of inoculums containing about 1.5×10^6 (about 22 mL) algal cells. The culture medium was the pure mineral autoclaved Bold Basal Medium (BBM). The total concentrations of the nutrients and trace elements in the growth medium were following: 2.94 mM $NaNO_3$; 0.17 mM $CaCl_2{\cdot}2H_2O$; 0.304 mM $MgSO_4{\cdot}7H_2O$; 0.431 mM K_2HPO_4; 1.29 mM KH_2PO_4; 0.428 mM NaCl; 0.428 mM Na_2EDTA; 1.38 mM KOH; 44.8 μM $FeSO_4{\cdot}7H_2O$; 0.462 mM H_3BO_3; 76.7 μM $ZnSO_4{\cdot}7H_2O$; 18.2 μM $MnCl_2{\cdot}4H_2O$; 15.7 μM $CuSO_4{\cdot}5H_2O$; 42.1 μM $Co(NO_3)_2{\cdot}6H_2O$; 16.1 μM $Na_2MoO_4{\cdot}2H_2O$; and 1 mL H_2SO_4. The pH of the medium was adjusted to 7.0 with 1 M NaOH prior to autoclaving. *A. obliquus* cells were cultured in an Erlenmeyer flask (250 mL) containing 100 mL medium. The axenic cultures were cultivated for 7 days under controlled sterile conditions at 25 ± 0.5 °C in a phytotron owned by Department of Plant Biochemistry and Toxicology, Institute of Biology, University of Bialystok. Illumination was supplied during a 16-h photoperiod (8-h dark period) by a bank of fluorescent lights yielding a photon flux 50 μmol m^{-2} s^{-1} at the surface of the tubes. The cell suspension was bubbled by atmospheric air at 1 L min^{-1}. EBL (Sigma-Aldrich Co., USA) was dissolved in 96% ethanol. The strongest solution (10 μM EBL) was transferred directly into the culture medium and weaker solutions (0.0001–1 μM EBL) were prepared by serial dilution. Equal amounts of ethanol were added to the control.

In preliminary experiments, a series of concentrations 1 pM-1 mM of EBL were applied to the algal cell culture (data not shown). Studied parameters, i.e. the number of *A. obliquus* cells; the content of protein, chlorophylls, carotenes, xanthophylls, monosaccharides, malondialdehyde (MDA) and H_2O_2; and activity of ascorbate peroxidase (APX), superoxide dismutase (SOD) and catalase (CAT) not only have been measured in six different concentrations of EBL (0.0001 μM, 0.001 μM, 0.01 μM, 0.1 μM, 1 μM and 10 μM) but also in four periods of time (on the first, third, fifth and seventh day of the cultivation).

2.2. Determination of cell number

The number of cells was estimated by direct counting of cells using a Bürker chamber (Piotrowska-Niczyporuk et al., 2017). After thorough mixing of each flasks, 100 μL of the algal suspension was collected in each culture on the 1st, 3rd, 5th, and 7th day of cultivation. Then 100 μL algal sample was applied under the cover glass of the Bürker chamber. The number of cells in 80 small squares was counted under the microscope. It was calculated using the formula: $CN = a \times v \times 5 \times 10^4$ (CN – cell number; a – amount of cells in small squares; v – volume of algal suspension).

2.3. Determination of total soluble protein

To determine the content of protein in algal cells, 10 mL of the algal culture was collected by centrifugation at 10000 × *g* for 10 min and bovine serum albumin (Sigma-Aldrich Co., USA) was used as the standard, following the Bradford (1976) method.

2.4. Determination of photosynthetic pigments

For high-performance liquid chromatography (HPLC) analysis, all samples were prepared under subdued light. Algal culture (1 mL) was collected on glass fiber filters and extracted in 99.9% methanol. Cells were disrupted for 5 min using bead mill (TissueLyser LT, Qiagen) and next centrifuged at 10000 × *g* for 10 min. Extracts were then filtered through 0.45 μm, PTFE, HPLC syringe cartridge filters fitted with glass fiber prefilters (Scientific Resources Inc., Eatontown, NJ, USA) to remove cell and filter debris (Zapata et al., 2000).