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Growth, Metabolite Profile, Oxidative Status, and Phytohormone Levels in the Green Alga *Acutodesmus obliquus* Exposed to Exogenous Auxins and Cytokinins

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Abstract
The effects of auxins and cytokinins at the range of concentrations 0.0001–100 µM on *Acutodesmus obliquus* (Chlorophyceae) cultures were studied. Microalga exhibited sensitivity to cytokinins in the following order: 0.01 µM *t*Z > 0.1 µM Kin > 1 µM DPU, whereas the hierarchy of auxin activity was: 0.01 µM IAA > 0.1 µM IBA > 0.1 µM PAA. Cytokinins possessed higher stimulating properties on the cell number, whereas auxins increased the size of cells. Differences in the metabolite profiles of the cultures treated with phytohormones were observed. Auxins and cytokinins had a positive effect on the photosynthetic apparatus enhancing the level of chlorophylls, carotenes, and xanthophylls. In comparison with auxins, cytokinins more effectively delayed oxidative damage by increasing the level of non-enzymatic antioxidants (ascorbate, glutathione) and the activity of enzymes scavenging reactive oxidative species (catalase, glutathione reductase, ascorbate peroxidase). On the other hand, auxins stimulated superoxide dismutase activity and provoked hydrogen peroxide generation, which may be involved in cell enlargement. All phytohormones reduced the content of abscisic acid and controlled the level of endogenous auxin and cytokinins suggesting complex interactions. Different dynamics of *A. obliquus* responses to auxins and cytokinins clearly demonstrated their diverse roles in algal growth and metabolism.

Keywords Antioxidants · Auxin · Cytokinin · Metabolome · Phytohormone homeostasis

Introduction

Microalgae such as *Acutodesmus* (formerly *Scenedesmus*) *obliquus* (Chlorophyceae) are single cell photosynthetic organisms that are known for rapid growth and high energy content. They have a close evolutional relationship with vascular plants. The presence of auxins and cytokinins in green algae and their effects on growth to enhance the potential viability of commercial applications of alga-based renewable biomass production were confirmed (Stirk et al. 2009, 2013, 2014). Green algae contain mainly adenine cytokinins with an isoprene side chain at the N^6-position, for example, N^6-(Δ^2-isopentenyl)adenine (iP) and *trans*-zeatin (*t*Z) as well as their ribosides and ribotides (Stirk et al. 2011, 2013, 2014). The best-known adenine-type cytokinin with a heterocyclic group at N^6 is kinetin (Kin), which for a long time was recognized as a synthetic product (Barciszewski et al. 2000). In addition, there is a class of structurally unrelated synthetic phenylurea-type cytokinins (for example, *N,N′*-diphenylurea; DPU) with high activity in algal cultures (Piotrowska and Czerpak 2009). Natural auxins such as indole-3-acetic acid (IAA), phenylacetic acid (PAA), and indole-3-butyric acid (IBA) were identified in green algae from the *Chlorella* and *Scenedesmus* genus (Mazur et al. 2001; Stirk et al. 2013, 2014; Žižková et al. 2017). A diverse role for cytokinins and auxins during green algal cell growth was confirmed in green algae and cyanobacteria (Žižková et al. 2017). Auxins regulate cytokinin levels in plants and *vice versa*. For example, cytokinin-overproducing *Arabidopsis thaliana* had lower IAA levels (Nordström et al. 2004), whereas other studies indicated that cytokinins can both

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down- and up-regulate auxin concentrations. Treatment of *A. thaliana* with various cytokinins led to increased auxin synthesis in young leaves, shoot apex, and roots (Jones et al. 2010). Moreover, both phytohormones are also known to antagonize the effects and level of abscisic acid (ABA) in the microalga *Nannochloropsis oceanica* (Lu et al. 2014). However, the effect of exogenous auxins and cytokinins on the endogenous phytohormone level was not studied in green alga *A. obliquus*.

Positive effects of auxins and cytokinins have been reported for improvement of algal growth rate, biomass production, and synthesis of valuable compounds, such as antioxidants, carotenoids, chlorophylls, carbohydrates, and proteins (Czerpak and Bajguz 1997; Piotrowska and Czerpak 2009; Bajguz and Piotrowska-Niczyporuk 2013, 2014; Piotrowska-Niczyporuk and Bajguz 2014; Kozlova et al. 2017). However, there are several gaps in the understanding of the impact of phytohormones on algal biochemistry including the effect on the level of low molecular weight metabolites. Metabolomics has provided new insights into understanding intracellular metabolites, discovering potential biomarkers, and can be used to reveal the global response of biochemical network reactions to phytohormones (Piotrowska-Niczyporuk et al. 2017).

Plant growth and developmental processes as well as environmental responses require the action and cross-talk of phytohormones and reactive oxygen species (ROS). For example, ROS are involved in typical auxin-mediated phenomena like root gravitropism in maize primary root (Joo et al. 2001). Auxins increase the activity of antioxidant enzymes regulating ROS levels which could be associated with plant development (Szechyńska-Hebda et al. 2007) and algal viability (Piotrowska-Niczyporuk and Bajguz 2014), whereas cytokinins are known as potent active oxygen scavengers (Barciszewski et al. 2000). Cytokinins delay senescence by slowing down decomposition of macromolecules, principally those that are components of photosynthetic apparatus, through increasing activities of antioxidant enzymes scavenging ROS from the cell (Zavaleta-Mancera et al. 2007).

The aim of the present study was to compare the effect of auxins (IAA, IBA, PAA) and cytokinins (*t*Z, Kin, DPU) at a range of concentrations 0.0001–100 µM on *A. obliquus* growth and the contents of soluble proteins as well as monosaccharides. Gas chromatography–mass spectrometry (GC–MS) was applied to investigate the metabolic profile of algal cells exposed to auxins and cytokinins. To gain a better insight into the relation between functionality of the photosynthetic apparatus and phytohormones, the level of chlorophylls, carotenes, and xanthophylls was studied in detail. The hypothesis that phytohormone-induced changes in the growth and metabolism may be connected with their influence on the hydrogen superoxide (H_2O_2) generation, the content of antioxidants (ascorbate and glutathione), and the activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase, and ascorbate peroxidase) involved in ROS scavenging was also tested. Finally, the effects of exogenous phytohormones on the levels of endogenous auxin (IAA), cytokinins (*t*Z, iP), and ABA involved in algal growth responses were evaluated.

Materials and Methods

Test Organism and Growth Conditions

The wild-type *A. obliquus* 276-6 (formerly named: *Scenedesmus obliquus* (Turpin) Hegewald and Hanagata 2000, SAG Strain No. 276-6) was purchased from the SAG Culture of Algae Collection (Germany). The homogenous population of young synchronous algal cells was collected by centrifugation and used for subsequent experiments. Complete synchronization has been obtained by regular changes of light and dark periods as previously described (Piotrowska-Niczyporuk et al. 2017). Growth of cultures was initiated by introduction of inoculums containing about 1.5×10^6 (about 21 mL) algal cells when the pre-cultures grown for 7 days reached the exponential growth phase. Initial cell density was 6.5×10^6 mL^{-1}. *A. obliquus* cells were cultured in an Erlenmeyer flasks containing 100 mL autoclaved pure mineral Bold Basal Medium (BBM, pH 7.0). The axenic cultures were incubated for 7 days at 25 ± 0.5 °C, in a 16:8 (light:dark) cycle and illuminated from below with a light intensity of 50 µmol m^{-2} s^{-1} at the surface of the tubes. Using air pumps cell suspension was bubbled by atmospheric air at 1 L min^{-1} to provide necessary CO_2.

Determination of Optimum Auxin and Cytokinin Concentrations for Algal Growth

To determine the optimum concentrations of auxins (IAA, IBA, PAA) and cytokinins (*t*Z, Kin, DPU) for *A. obliquus* growth expressed as the number of cells, all phytohormones were applied at the following concentrations: 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 µM. To achieve those concentrations, appropriate amounts of phytohormones were dissolved in 50% ethanol and then added to Erlenmeyer flasks with BBM medium (100 mL). An equal amount of 50% ethanol was added to the control. The final ethanol concentration in the culture media did not exceed 1% (v/v) and this amount did not affect algal growth. Each treatment consisted of four replicates and each experiment was repeated at least twice at different times. Algal growth was determined by measuring cell number, using a Bürker chamber, in each culture every 24 h during 7 days of cultivation.

Cell Size Determination

To verify the biovolume of *A. obliquus* cells (μm^3), a light microscope (Leica, Germany) and a MultiScanBase v.14 (Computer Scanning System CSS) for imaging analysis were employed. The size of the cells was studied on the 1st, 3rd, 5th, and 7th day of cultivation.

GC–MS Analysis of Intracellular Metabolite Profile

The metabolite profile was analyzed on the 5th day of algal culture when the differences between the control cultures and the algae treated with phytohormones were the most visible. Cell suspensions (50 mL) were harvested by centrifugation, disrupted by sonication (Vibra Cells, Sonics and Materials Inc., USA) and dried at 40 °C. The dry material was extracted by three portions of methanol (10 mL). After methanol evaporation under a nitrogen stream, pyridine and *bis*(trimethylsilyl)trifluoroacetamide (BSTFA) (Sigma-Aldrich Co., USA) were added to the residue. The reaction mixture was heated for 0.5 h at 60 °C to obtain trimethylsilyl derivatives which were separated and analyzed using a gas chromatograph (7890B GC System) with a mass selective detector MSD 5977A (Agilent Technologies, USA). This device was fitted with an HP-5MS-fused silica column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The helium flow rate through the column was 1 mL min^{-1} in constant flow mode. The injector (250 °C) worked in splitless mode and the injection volume was 1 µL. There was a 4.5 min solvent delay at the start of each run and the total run time was about 100 min per sample. The initial column temperature was 50 °C rising to 300 °C at 5 °C min^{-1}. The MSD 5977A detector acquisition parameters were as follows: transfer line temperature equaled 250 °C and the detector was held at 290 °C. Detection of spectra was performed in a full scan mode from 39 to 600 a.m.u. at 70 eV of ionization energy. Compounds were identified with the aid of an automatic system of processing data (Agilent MassHunter Workstation software Qualitative Analysis) from GC–MS supplied by the National Institute of Standards and Technology (NIST) database (Piotrowska-Niczyporuk et al. 2017).

Determination of Soluble Proteins and Monosaccharides

The content of soluble protein in algal cells was determined following the Bradford (1976) method, using bovine serum albumin as the standard. The monosaccharide content was estimated according to the Somogyi (1952) method. The contents of soluble proteins and monosaccharides were calculated on cell number and normalized on cell biovolume. All parameters were studied on the 1st, 3rd, 5th, and 7th day of cultivation.

Determination of the Contents of Algal Photosynthetic Pigments

Algal monocultures were collected on glass fiber filters and extracted in methanol (99.9%) for pigment isolations. Cells were disrupted by sonication (Vibra Cells, Sonics and Materials Inc., USA) for 3 min. Extracts were clarified using 0.45 µm PTFE filters (A&A Biotechnology, Poland). An Agilent 1260 Infinity Series, USA HPLC system with an autoinjector (500 µL sample loop), refrigerated autosampler compartment, thermostatic column compartment, quaternary pump with in-line vacuum degasser, and photo-diode array detector set to monitor visible absorbance at 450 and 665 nm was used for pigment studies. An Eclipse XDB-C8 column (150 × 4.6), kept at 25 °C with a column oven was used for pigment separation and analysis. Eluent A was a mixture of methanol/acetonitrile/0.25 M aqueous pyridine (pH 5.0) solution (50/25/25, v/v/v), whereas eluent B was methanol/acetonitrile/acetone (20/60/20, v/v/v). The gradient was linear from the specified initial percent solvent A to 100% from the 1st to 40th minute and the flow rate was 1 mL min^{-1} (Zapata et al. 2000). The analytical data were integrated using the Agilent OpenLAB software. The contents of individual photosynthetic pigments were analyzed on the 1st, 3rd, 5th, and 7th day of cultivation, calculated on cell number, and normalized on cell biovolume.

Malondialdehyde and Hydrogen Peroxide Determination

Lipid peroxidation was determined by measuring the amount of total malondialdehyde (MDA) (Heath and Packer 1968). Algal cells (10 mL) were harvested by centrifugation at 10,000×*g* for 10 min and the resulting pellet was treated with 0.25% (w/v) thiobarbituric acid (TBA) in 10% (w/v) trichloroacetic acid (TCA). After heating at 95 °C (30 min), the mixture was cooled and centrifuged. The absorbance of the supernatant at 532 nm was recorded and corrected for unspecific turbidity by subtracting the value at 600 nm. The level of H_2O_2 in *A. obliquus* cells was measured spectrophotometrically at 390 nm by reaction with 1 M KI. The results were calculated using a standard curve prepared with fresh H_2O_2 solutions (Alexieva et al. 2001). The contents of MDA and H_2O_2 were determined on the 1st, 3rd, 5th, and 7th day of cultivation, calculated on cell number, and normalized on cell biovolume.

Determination of Ascorbate and Glutathione Content

For extraction of total ascorbate, *A. obliquus* cells were harvested by filtration and quickly homogenized in liquid N_2 and 5% (w/v) TCA (Kampfenkel et al. 1995). The

homogenate was centrifuged for 5 min at 15,600×*g* (4 °C) and the supernatant was assayed for ascorbate content in a reaction mixture with 10 mM dithiothreitol (DTT), 0.2 M phosphate buffer (pH 7.4), 0.5% *N*-ethylmaleimide (NEM), 10% TCA, 42% H_3PO_4, 4% 2,2′-dipyridyl, and 3% $FeCl_3$. For glutathione analysis, the suspension of algal cells was harvested by centrifugation and resuspended in 0.1% trifluoroacetic acid (TFA) with 6.3 mM diethylenetriaminepentaacetic acid to allow protein precipitation (Le Faucheur et al. 2006). Algal cells were homogenized in a bead mill using the TissueLyser LT (QIAGEN, Germany). The homogenate was centrifuged, and supernatant was used for the analysis of glutathione content after derivatization with monobromobimane using HPLC (Agilent 1260 Infinity Series, USA). Glutathione was separated on a COSMOSIL Packed Column 5C-18-MS-II (4.6 µm × 250 mm) (Nacalai, USA) kept at 37 °C with a column oven. The mobile phase was methanol and 0.1% TFA. The sample (25 µL) was injected and run in a slightly concave gradient from 12 to 35% (v/v) methanol during 29 min (flow rate 0.5 mL min^{-1}). Fluorescence was monitored by an Agilent 1260 Infinity Fluorescence Light Detector (Agilent Technologies, USA) (380 nm excitation and 470 nm emission wavelength). The analytical data were integrated using the Agilent OpenLAB software. The ascorbate and glutathione contents were determined on the 1st, 3rd, 5th, and 7th day of cultivation, calculated on cell number, and normalized on cell biovolume.

Determination of the Antioxidant Enzymes Activities

The antioxidant enzymes were extracted in 50 mM phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.05% Triton X-100, 2% polyvinylpyrrolidone, and 1 mM ascorbic acid. Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined by measuring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich 1971). One unit of SOD (per mg protein) was defined as the amount causing 50% inhibition of the photochemical reduction of NBT. Catalase (CAT) (EC 1.11.1.6) activity was estimated by recording the decrease in the absorbance of H_2O_2 at 240 nm (Aebi 1984). One unit of CAT activity was assumed as the amount of enzyme that decomposes 1 µmol of H_2O_2 per mg of soluble protein per minute at 30 °C. The method given by Nakano and Asada (1981) was followed for determining ascorbate peroxidase (APX) (EC 1.11.1.1) activity. The enzyme activity was calculated as the amount of the enzyme that oxidizes 1 µmol of ascorbate consumed per mg of soluble protein per min at 30 °C. Glutathione reductase (GR) (EC 1.8.1.7) activity was determined from the rate of NADPH oxidation as measured by the decrease of absorbance at 340 nm (extinction coefficient 6.2 mmol L^{-1} cm^{-1}) at 25 °C (Schaedle and Bassham 1977). The activity of antioxidant enzymes in *A. obliquus* cells was analyzed on the 1st, 3rd, 5th, and 7th day of cultivation.

Phytohormone Analysis

Algal samples were collected on the 1st, 3rd, 5th, and 7th day of cultivation by centrifugation. Next, the algal residue was homogenized using a bead mill (The TissueLyser LT QIAGEN, Germany) for cell disruption and extracted with 500 µL modified *Bieleski* solvent (methanol/H_2O, 80/20, v/v) at 4 °C for 12 h. [$^2H_5$] IAA (15.0 ng g^{-1}), [$^2H_6$] ABA (50 ng g^{-1}), [$^2H_5$] iP (50 ng g^{-1}), and [$^2H_5$] *t*Z (30 ng g^{-1}) were added to algal samples as internal standards. The extract was separated by centrifugation and the residue was re-extracted three times. The combined extracts were evaporated to near dryness. The purification method was employed as described by Dobrev and Kaminek (2002) with minor modifications. The extracted supernatants or the standard solutions were redissolved in 2.0 mL of 1 M formic acid (FA) and then loaded on Oasis MAX cartridges (Waters Corp., Milford, MA, USA) pre-conditioned with 1 M FA and methanol. IAA, and ABA were eluted with 2 mL of methanol containing 1% FA, whereas iP and *t*Z were eluted with 0.1 M NH_4OH in 60% methanol. The obtained elutes were dried, then reconstituted in 50 µL of 5% methanol, and filtered through a 0.45 µm PTFE filter for analysis. An Agilent 1260 Infinity series HPLC system consisting of a degasser, binary pump, autosampler, and column oven coupled to an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS mass spectrometer with Dual AJS ESI source was employed for the separation and quantification of the phytohormones. The analytical column Poroshell 120 EC-C18 (3.0 × 50 mm, 2.7 µm) (Agilent Technologies, USA) was equilibrated at 25 °C. The injection volume was 5 µL. The mobile phase consisted of water with 0.1% FA (A)/methanol (B) at a flow rate of 0.5 mL min^{-1}. The gradient profile started with 5% of eluent B for 5.0 min and then increased linearly to 95% within 8.0 min, and returned to 5% of eluent B, followed by a re-equilibration time of 3.0 min. The MS parameters were optimized with mixtures of standard solutions ESI Tuning Mix (Agilent Technologies, USA). Nitrogen was used as the nebulizer, drying, and collision gases. The nebulizer gas pressure, drying gas pressure, curtain gas pressure, source voltage, and source temperature were set at 60 psi, 50 psi, 30 psi, 3.5 kV, and 350 °C, respectively. Positive-ion mode was used to analyze cytokinins, whereas ABA and IAA were determined using negative-ion mode. The phytohormone contents were calculated on cell number and normalized on cell biovolume.

Replication and Statistical Analysis

Each measurement consisted of four replicates and each experiment was carried out at least twice at different times. Before selecting the appropriate statistical analysis method, the data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). The normality of data and homogeneity of variances were reported. Further, no significant outliers were found in data. Therefore, the data were analyzed using one-way ANOVA and the *F*-test established that there are statistically significant differences between calculated arithmetic means. The means were grouped using Tukey's Post hoc test (IBM SPSS Statistics 21, USA). The level of significance in all statistical tests and comparisons was $p < 0.05$.

Results

Exogenous Auxins and Cytokinins Stimulated Algal Growth in Different Manner

Auxins and cytokinins are effective phytohormones in stimulating *A. obliquus* growth during 7 days of cultivation (Fig. 1). The highest activity of IAA and *t*Z occured at concentration of 0.01 µM; IBA, PAA, and Kin at 0.1 µM; as well as DPU at 1 µM. The number of cells was stimulated effectively by cytokinins, whereas cell biovolume was higher in auxin-treated cultures (Fig. 2a). For example, 0.01 µM *t*Z stimulated cell number by 155.4%, 0.1 µM Kin by 126.2%, and 1 µM DPU by 110.7%, whereas auxins enhanced cell number by 63.7–89.5% on the 5th day of cultivation. In contrast, cell biovolume increased after 0.01 µM IAA, 0.1 µM IBA, and 0.1 µM PAA application by 39.1, 27.6, and 22.2%, respectively on the 5th day of cultivation. The decrease in cell size by 5.2–7.8% was observed in cytokinin-treated cultures. The auxin hierarchy regarding their stimulatory effects on cell number and biovolume was: 0.01 µM IAA > 0.1 µM IBA > 0.1 µM PAA, whereas *A. obliquus* exhibited sensitivity to cytokinins in the following order: 0.01 µM *t*Z > 0.1 µM Kin > 1 µM DPU indicating that natural phytohormones are characterized by higher activity in algal cultures at the lower concentrations used in the experiments.

Exogenous Auxins and Cytokinins Induced Changes in Metabolite Profile

Metabolite profile studies of *A. obliquus* cells on the 5th day of cultivation indicated a clear distinction between phytohormone-treated and control cultures (Table 1). Among primary amino acids, the contents of alanine, glycine, homocysteine, isoleucine, leucine, phenylalanine, proline, tyrosine, and valine significantly decreased in response to auxins and cytokinins. In contrast, the glutamate content increased after 5 days of phytohormone treatment. Statistically significant accumulation of free fatty acids (arachidonic acid, hexadecanoic acid, heptadecanoic acid, linoleic acid, linolenic acid, octadecanoic acid, and oleic acid) and decrease in both organic (citrate, fumarate, gluconate, malate) and phosphoric acid contents was observed under auxin and cytokinin exposure. Moreover, the level of glucose was reduced, while the contents of alcohols increased in response to phytohormones.

Auxins and Cytokinins Increased Soluble Protein and Photosynthetic Pigments and Decreased Monosaccharide Contents

Auxin- and cytokinin-treated *A. obliquus* cells showed increases in the contents of soluble proteins (Fig. 2b) and photosynthetic pigments (Tables 2, 3) as well as a decrease in monosaccharide level. The highest increase in the soluble protein level was observed in cultures treated with cytokinins by 101.8% (0.01 µM *t*Z), 67.6% (0.1 µM Kin), and 58.6% (1 µM DPU), whereas auxins induced their accumulation by 96.4% (0.01 µM IAA), 48.6% (0.1 µM IBA), and 34.2% (0.1 µM PAA) on the 5th day of cultivation (Fig. 2b). The contents of monosaccharides were the most effectively inhibited by 15.2–26.4% in cultures treated with cytokinins and by 12.8–30.4% in response to auxins on the 3rd day of algal cultivation (Fig. 2c).

Among auxins, 0.01 µM IAA induced the highest increase in the contents of carotenes (8.0% α-carotene, 7.3% β-carotene), chlorophylls (59.7% chlorophyll *a*, 38.5% chlorophyll *b*), and xanthophylls (19.8% antheraxanthin, 32.0% astaxanthin, 19.8% cryptoxanthin, 11.2% lutein, 18.3% neoxanthin, 46.1% violaxanthin, 14.2% zeaxanthin) on the 5th day of cultivation (Tables 2, 3). Cytokinins were more effective in stimulation of photosynthetic pigments. For example, 0.01 µM *t*Z induced increase in α-carotene and β-carotene by 12.2 and 18.9%, chlorophyll *a* and *b* by 83.7 and 202.6%, as well as in xanthophyll pigments (34.8% antheraxanthin, 67.1% astaxanthin, 24.3% cryptoxanthin, 23.6% lutein, 22.8% neoxanthin, 54.0% violaxanthin, 14.2% zeaxanthin) on the 5th day of experiment (Tables 2, 3).

Auxins Opposite to Cytokinins Induced ROS Generation in Algal Cells

The presence of auxins in *A. obliquus* cultures leads to production of H_2O_2 as evidenced by the increase in its content in response to 0.01 µM IAA (60.1%), 0.1 µM IBA (50.9%), 0.1 µM and PAA (23.9%) on the 5th day of cultivation (Fig. 3a). In contrast, cytokinin supplementation had an inhibitory effect on H_2O_2 levels. The content of H_2O_2 decreased gradually by 24.2, 16.5, and 13.2% after

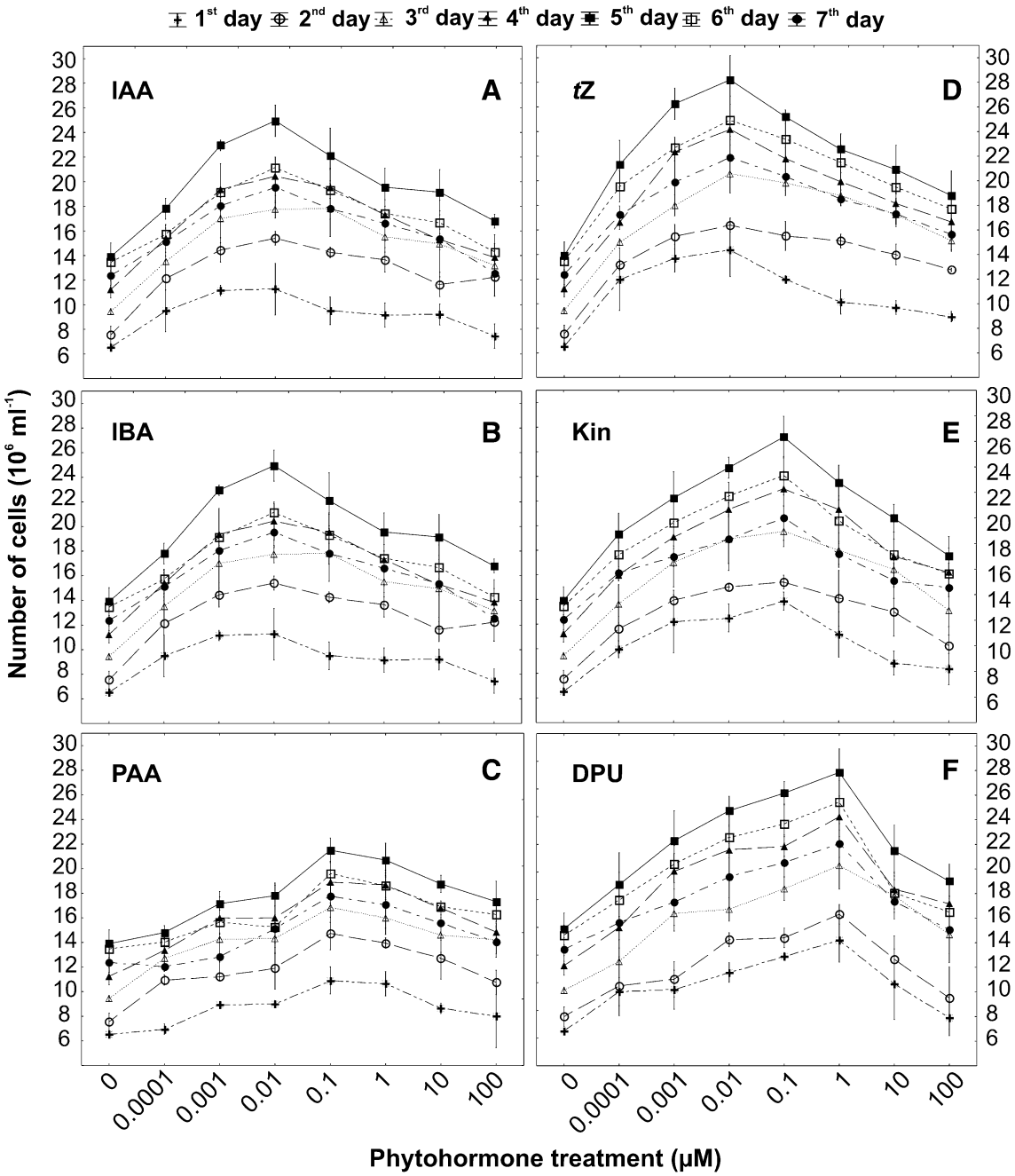

Fig. 1 The number of cells in *Acutodesmus obliquus* cultures under the influence of auxins (IAA, IBA, PAA) and cytokinins (*t*Z, Kin, DPU) at range of concentrations 0.0001–100 μM in relation to control (0) during the 7 days of algal cultivation. Data are the means of four independent experiments ± SD

exposure to 0.01 μM *t*Z, 0.1 μM Kin, and 1 μM DPU, respectively, during the whole 7-day period of cultivation. The increase in MDA content by 12.8, 8.1, and 5.4% was observed in cultures treated with 0.01 μM IAA, 0.1 μM IBA, and 0.1 μM PAA, respectively on the 3rd day of cultivation (Fig. 3b). However, this parameter decreased in cells exposed to auxins, especially to 0.1 μM PAA by 12.2% on the 7th day of experiment. In contrast, cytokinins inhibited MDA accumulation hence this marker of oxidative stress decreased by 32.5, 24.6, and 22.1% in cells treated with 0.01 μM *t*Z, 0.1 μM Kin, and 1 μM DPU, respectively on the 7th day of cultivation.

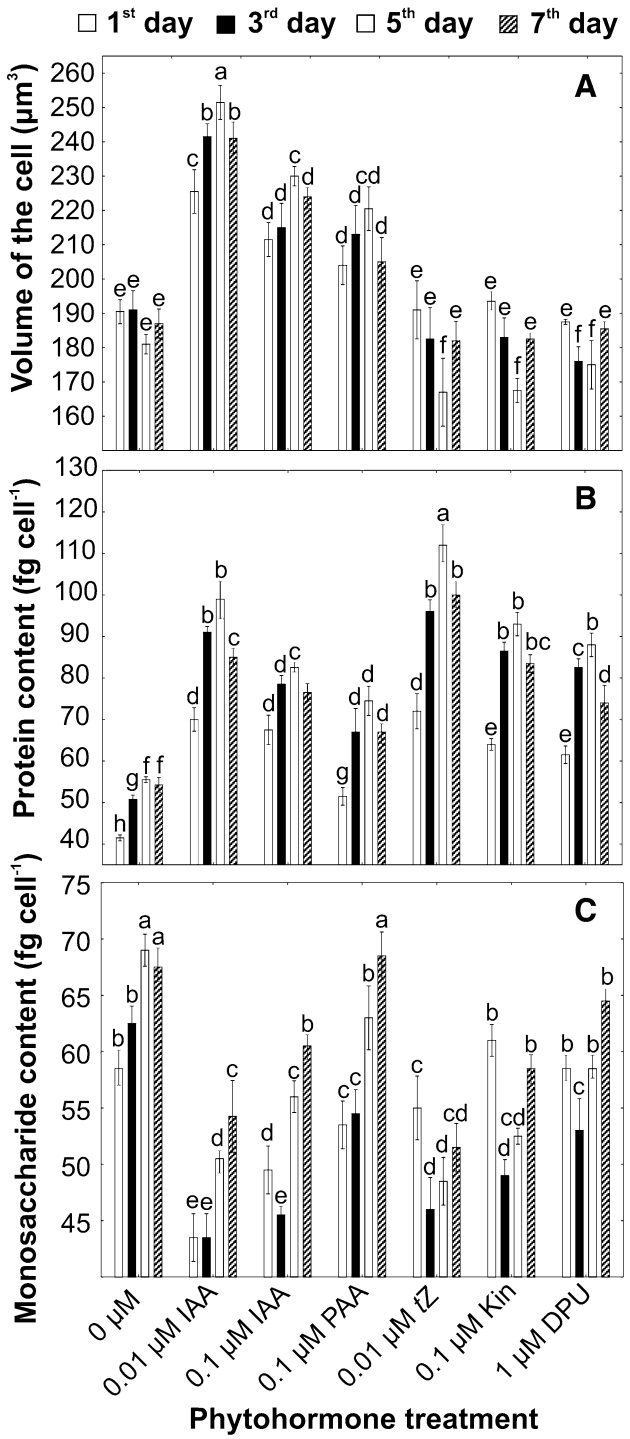

Fig. 2 The effect of auxins (IAA, IBA, PAA) and cytokinins (*t*Z, Kin, DPU) at optimal concentrations on the size of the cells (**a**) and the contents of proteins (**b**) and monosaccharides (**c**) in *Acutodesmus obliquus* in relation to control (0 μM) on the 1st, 3rd, 5th, and 7th day of cultivation. Data are the means of four independent experiments ± SD. Treatments with at least one letter the same are not significantly different according to Tukey's Post hoc test

Auxins and Cytokinins Induced Increased Levels of Non-enzymatic Antioxidants

All tested phytohormones stimulated ascorbate and glutathione accumulation in *A. obliquus* cells (Fig. 4a, b). For example, treatment with 0.01 μM IAA induced 16.5 and 28.9% increase in ascorbate and glutathione level, respectively, on the 5th day of cultivation. IBA and PAA at 0.1 μM were less effective in stimulation of the level of both antioxidants. A significant increase in the antioxidant contents (28.1% in the case of ascorbate on the 5th day and 45.2% in the case of glutathione on the 7th day of cultivation) was also observed after 0.01 μM *t*Z application. In addition, exposure of *A. obliquus* to 0.1 μM Kin and 1 μM DPU caused weaker, but a statistically significant increase in ascorbate (7.1–20.3%) and glutathione (14.9–26.6%) levels on the 7th day of cultivation.

Auxins and Cytokinins Enhanced The Activities of Antioxidant Enzymes

Similarly, auxins and cytokinins influenced the activity of antioxidant enzymes involved in the scavenging of ROS (Fig. 5a–d). The highest enhancement of the activity of SOD was observed in cultures treated with auxins; among them 0.01 μM IAA induced 59.5% increase in this enzyme activity. In contrast, the efficiency of H_2O_2 production catalyzed by SOD was lower in cells exposed to cytokinins, for example, 0.01 μM *t*Z stimulated its activity by 53.7%. The activities of other antioxidant enzymes were more effectively stimulated by cytokinins in comparison with auxins. For example, 30.4, 30.2, and 107.7% increase in CAT, APX, and GR activity, respectively, appeared as a consequence of algal exposure to 0.01 μM *t*Z. Among auxins, 0.01 μM IAA enhanced the activity of CAT by 22.9%, APX by 18.9%, and GR by 60.6% on the 5th day of cultivation. Other phytohormones possessed lower stimulating properties on the activity of enzymes involved in ROS scavenging.

Phytohormone Cross-Talk Under Exogenous Auxins and Cytokinins

Cytokinins in comparison with auxins inhibited ABA synthesis in *A. obliquus* cells more effectively (Fig. 6a). For example, the decrease in the ABA content by 42.9, 39.2, and 31% was noted in cultures treated with 0.01 μM *tZ*, 0.1 μM Kin, and 1 μM DPU, respectively, on the 7th day of cultivation. Among auxins, 0.01 μM IAA decreased ABA content the most effectively (by 35.0%). The endogenous IAA level was stimulated in algal cells in the presence of 0.1 μM IBA (11.5%) on the 7th day of cultivation (Fig. 6b). Exogenous application of cytokinins induced an increase in endogenous IAA content by 13.2% in case of 0.01 μM *t*Z treatment, and

Table 1 List of those metabolites whose levels significantly changed after exposure of *A. obliquus* to auxins and cytokinins on the 5th day of cultivation using GC–MS method

Metabolite	Phytohormone treatment					
	0.01 µM IAA	0.1 µM IBA	0.1 µM PAA	0.01 µM *tZ*	0.1 µM Kin	1 µM DPU
Alanine	−7.9	−6.0	nd	−9.8	−9.4	−10.2
Glutamate	10.8	9.6	8.5	12.9	12.5	7.9
Glycine	−35.7	−25.3	−18.1	−39.6	−37.4	−37.3
Homocysteine	−39.6	−41.2	−30.3	−57.2	−54.5	−26.1
Isoleucine	−37.2	−37.6	−9.9	−38.5	−40.2	−27.5
Leucine	−9.1	−8.9	nd	−16.7	−20.4	nd
Phenylalanine	−10.5	nd	nd	−22.3	−12.8	−12.9
Proline	−11.7	−8.5	nd	−7.8	nd	−5.4
Tyrosine	−7.4	nd	nd	−6.9	−7.9	nd
Valine	−6.6	−5.9	−7.4	−7.8	−6.3	nd
Glucose	−14.0	−12.0	−5.9	−13.6	nd	−6.9
Glycerol	17.9	8.1	nd	11.8	7.3	nd
myo-Inositol	9.8	8.5	8.2	10.2	7.6	8.1
Citric acid	−10.3	−6.1	nd	−6.2	−7.2	nd
Fumaric acid	−9.6	nd	−6.5	−17.4	−6.1	nd
Gluconic acid	8.9	8.5	nd	15.1	11.5	nd
Phosphoric acid	−7.8	nd	nd	−6.3	−7.0	nd
Malic acid	nd	−7.9	−15.8	−17.7	−7.4	−6.3
Arachidonic acid	53.2	43.8	21.7	51.5	32.4	27.9
Hexadecanoic acid	32.5	16.4	10.3	28.5	23.3	15.7
Heptadecanoic acid	25.5	26.3	12.4	30.6	24.2	8.8
Linoleic acid	47.6	45.1	36.4	50.9	44.2	21.0
Linolenic acid	32.1	21.6	15.5	35.8	22.7	15.5
Octadecanoic acid	27.4	28.2	10.5	19.6	16.5	12.7
Oleic acid	46.8	40.3	22.7	47.9	38.8	20.1

The numbers indicate the percentage increase or decrease in metabolite level compared to the level in the control culture. Negative numbers indicate that the compound's occurrence decreased in the exposed culture; nd means no differences between the control and phytohormone-treated cultures

by 8.5 and 8.9% under exposure to 0.1 µM Kin and 1 µM DPU, respectively, on the 7th day of cultivation. The addition of all auxins to algal cultures repressed biosynthesis of *tZ* because its level was decreased by 15.1–25.3%, whereas the contents of iP were stimulated by 5.2–12.5% on the 3rd day of cultivation (Fig. 6c, d). Synthetic urea-type cytokinin (1 µM DPU) enhanced endogenous levels of *tZ* by 25.4% on the 1st day and iP by 13.7% on the 5th day of cultivation. The addition of other cytokinins to culture media did not affect the endogenous level of *tZ* and iP.

Discussion

Auxins and cytokinins can simultaneously promote both *A. obliquus* growth and biosynthesis of targeted molecules. Although the range of concentrations of all phytohormones demonstrated a stimulatory effect on the cell number, the degree of influence and most effective concentrations varied with the hormone type indicating that IAA and *tZ* are the most active at concentration of 0.01 µM; IBA, PAA, and Kin at 0.1 µM; whereas DPU at 1 µM, IAA is the most biologically active auxin, whereas *tZ* possesses the highest activity among cytokinins in *A. obliquus* cells. Moreover, natural auxins and cytokinins more actively stimulated algal growth expressed as cell number at lower concentrations compared to a synthetic phytohormone (DPU). This corresponds to previous results where cytokinins stimulated cell proliferation in green algae such as *C. vulgaris, S. quadricauda*, and *Dictyosphaerium pulchellum* but had no effect on cell enlargement (Burkiewicz 1987; Piotrowska and Czerpak 2009; Hunt et al. 2011; Bajguz and Piotrowska 2013, 2014; Piotrowska-Niczyporuk and Bajguz 2014). Thus, cytokinins may act as signaling molecules during cell-cycle progression in *A. obliquus*. The stimulating effect of auxins on the number of algal cells was also confirmed. For example, the application of IAA to microalga *S. armatus* cultures resulted in the stimulation of cell division, growth, and formation of four-celled colonies (Mazur et al. 2001). Exogenous auxins induced also algal growth and biomass yield in *C. vulgaris*

Table 2 Carotene and chlorophyll contents in cells grown at the presence of auxins and cytokinins at optimal concentrations in relation to control (0 µM) on the 1st, 3rd, 5th, and 7th day of *A. obliquus* cultivation

Pigment	Contents of carotenes and chlorophylls (fg cell^{-1})							
	Day of culture	Phytohormone treatment						
		0 µM	0.01 µM IAA	0.1 µM IBA	0.1 µM PAA	0.01 µM *t*Z	0.1 µM Kin	1 µM DPU
α-Carotene	1st	4.128 ± 0.023^{g}	4.309 ± 0.048^{e}	4.311 ± 0.062^{e}	4.235 ± 0.101^{f}	4.367 ± 0.007^{e}	4.318 ± 0.111^{e}	4.233 ± 0.085^{f}
	3rd	4.126 ± 0.011^{g}	4.414 ± 0.085^{d}	4.407 ± 0.028^{d}	4.359 ± 0.047^{d}	4.622 ± 0.105^{d}	4.571 ± 0.097^{c}	4.505 ± 0.116^{c}
	5th	4.306 ± 0.033^{e}	4.652 ± 0.063^{b}	4.588 ± 0.014^{c}	4.512 ± 0.078^{c}	4.831 ± 0.087^{a}	4.709 ± 0.044^{b}	4.690 ± 0.058^{b}
	7th	4.357 ± 0.052^{e}	4.418 ± 0.061^{d}	4.421 ± 0.026^{d}	4.395 ± 0.073^{e}	4.666 ± 0.055^{b}	4.482 ± 0.069^{d}	4.486 ± 0.017^{d}
β-Carotene	1st	6.108 ± 0.061^{f}	6.215 ± 0.132^{e}	6.208 ± 0.077^{e}	6.113 ± 0.085^{f}	6.311 ± 0.109^{e}	6.288 ± 0.076^{e}	6.288 ± 0.076^{e}
	3rd	6.089 ± 0.100^{f}	6.374 ± 0.127^{e}	6.322 ± 0.118^{e}	6.262 ± 0.120^{e}	6.909 ± 0.131^{b}	7.199 ± 0.155^{a}	6.499 ± 0.155^{d}
	5th	6.234 ± 0.065^{e}	6.688 ± 0.103^{c}	6.549 ± 0.026^{d}	6.407 ± 0.057^{d}	7.415 ± 0.213^{a}	7.358 ± 0.076^{a}	6.858 ± 0.076^{b}
	7th	6.335 ± 0.118^{e}	6.639 ± 0.096^{c}	6.501 ± 0.084^{c}	6.433 ± 0.029^{c}	6.825 ± 0.147^{b}	6.829 ± 0.094^{b}	6.829 ± 0.094^{b}
Chlorophyll *a*	1st	70.145 ± 1.388^{k}	79.687 ± 2.074^{h}	78.266 ± 1.578^{h}	75.314 ± 12.025^{i}	95.552 ± 1.451^{f}	85.102 ± 2.007^{g}	80.776 ± 1.660^{h}
	3rd	70.346 ± 2.145^{k}	84.579 ± 3.166^{g}	84.608 ± 2.721^{g}	80.409 ± 1.677^{a}	110.473 ± 2.332^{d}	99.348 ± 1.462^{e}	90.008 ± 2.113^{a}
	5th	72.449 ± 0.772^{j}	115.668 ± 3.461^{c}	110.588 ± 1.689^{d}	103.002 ± 2.193^{e}	133.099 ± 4.168^{a}	124.567 ± 3.843^{b}	103.244 ± 2.579^{e}
	7th	74.581 ± 1.336^{i}	101.084 ± 4.007^{e}	98.116 ± 2.364^{a}	90.531 ± 3.007^{a}	129.773 ± 5.011^{b}	110.009 ± 1.906^{d}	100.605 ± 3.227^{e}
Chlorophyll *b*	1st	22.508 ± 0.095^{i}	28.663 ± 1.311^{h}	27.055 ± 0.844^{h}	23.494 ± 1.599^{i}	31.258 ± 2.008^{g}	30.661 ± 1.416^{g}	27.504 ± 1.072^{h}
	3rd	22.315 ± 0.141^{j}	30.915 ± 1.007^{g}	30.213 ± 1.573^{g}	27.115 ± 2.038^{h}	45.206 ± 1.665^{b}	40.778 ± 2.304^{d}	30.912 ± 1.541^{g}
	5th	26.707 ± 0.822^{h}	35.354 ± 1.903^{e}	33.668 ± 2.256^{f}	30.001 ± 1.435^{d}	47.763 ± 2.117^{a}	43.911 ± 1.766^{c}	33.378 ± 2.005^{f}
	7th	28.005 ± 1.116^{h}	33.377 ± 2.065^{f}	32.459 ± 0.798^{f}	30.132 ± 0.913^{d}	43.088 ± 1.752^{c}	38.581 ± 0.998^{c}	32.318 ± 1.212^{f}

Results show the mean values of four independent pigment determinations with the respective standard deviations. Treatment with at least one letter the same are not significantly different according to Tukey's Post hoc test

(Piotrowska-Niczyporuk and Bajguz 2014; Liu et al. 2017), *S. quadricauda* and *C. pyrenoidosa* (Liu et al. 2016; Kozlova et al. 2017), *C. sorokiniana* and *Scenedesmus* sp. SDEC-8 (Yu et al. 2017), *C. protothecoides* (Parsaeimehr et al. 2017) as well as *Scenedesmus* sp. LX1 (Dao et al. 2018).

Cytokinins supported a higher specific growth rate which corresponds well with the higher cell number in *A. obliquus* cultures, whereas auxins are primarily responsible for cell enlargement. The present results correspond well with literature data showing that auxins increased the diameter of the algal cells such as *C. sorokiniana, Scenedesmus* sp. SDEC-8 (Yu et al. 2017), and *S. quadricauda* (Kozlova et al. 2017). Auxins may promote cell enlargement through ROS production (Kawano 2003). The present results are consistent with this theory because auxin-induced increase in cell size was positively correlated with higher SOD activity involved in H_2O_2 generation in *A. obliquus* cultures treated with auxins.

GC–MS screening of *A. obliquus* cells revealed a variation in the composition of low molecular metabolites due to the type of auxins and cytokinins, and the variations were more visible for the *t*Z and IAA treatments. For example, supplementation of *A obliquus* cells with *t*Z and IAA induced the highest increase in the level of fatty acids. The existing evidence suggested that exogenous auxins and cytokinins effectively regulate fatty acid biosynthesis and profile in green algae, which might be directly or indirectly related with lipid biosynthesis for biodiesel production (Liu et al. 2017; Kozlova et al. 2017; Parsaeimehr et al. 2017; Yu et al. 2017; Dao et al. 2018). This is the first report that fatty acid accumulation in *A. obliquus* may be promoted by auxins (IAA, IBA, PAA) and cytokinins (*t*Z, Kin, DPU). The content of glucose decreased in response to auxins and cytokinins, indicating that the rate of utilization of glucose may be accelerated for the production of other metabolites. Auxin and cytokinin treatment caused also a decrease in organic acids, which are intermediates of the tricarboxylic acid cycle suggesting that they may be utilized in fatty acid synthesis. The increase in *myo*-inositol in *A. obliquus* cells treated with phytohormones may contribute to stress acclimation as it has been proposed for *Schizochytrium* sp. strains (Ren et al. 2013). In this study, the decrease in the level of most amino acids suggests their utilization in the synthesis of peptides such as glutathione and proteins, whose contents increased in *A. obliquus* cells exposed to auxins and cytokinins.

Sugars and proteins are both major contributors to the cellular composition of microalgae that are affected by various endogenous and environmental factors. Exogenous auxins and cytokinins induced increased soluble protein contents and decreased monosaccharide levels in *A. obliquus* cells. The accumulation of soluble proteins occurred when the microalgal cells displayed the maximal algal metabolic and mitotic activity under phytohormone treatment as shown in previous experiments performed on *C. pyrenoidosa* (Czerpak and Bajguz 1997) or *C. vulgaris* (Piotrowska and Czerpak 2009; Bajguz and Piotrowska-Niczyporuk 2013, 2014; Piotrowska-Niczyporuk and Bajguz 2014). The

Table 3 Xanthophyll pigment contents in cells grown at the presence of auxins and cytokinins at optimal concentrations in relation to control (0 μM) on the 1st, 3rd, 5th, and 7th day of *A. obliquus* cultivation

Pigment	Content of xanthophylls (fg cell^{-1})							
	Day of culture	Phytohormone treatment						
		0 μM	0.01 μM IAA	0.1 μM IBA	0.1 μM PAA	0.01 μM *t*Z	0.1 μM Kin	1 μM DPU
Antheraxanthin	1st	3.046 ± 0.104^{i}	3.286 ± 0.009^{h}	3.106 ± 0.057^{i}	3.085 ± 0.009^{i}	3.889 ± 0.110^{e}	3.654 ± 0.017^{f}	3.401 ± 0.109^{g}
	3rd	3.052 ± 0.023^{i}	3.783 ± 0.121^{e}	3.283 ± 0.081^{h}	3.206 ± 0.037^{h}	4.325 ± 0.007^{c}	4.147 ± 0.205^{e}	3.989 ± 0.067^{e}
	5th	3.678 ± 0.205^{f}	4.405 ± 0.130^{c}	4.223 ± 0.065^{d}	4.191 ± 0.055^{e}	4.959 ± 0.093^{a}	4.761 ± 0.108^{b}	4.404 ± 0.205^{c}
	7th	3.754 ± 0.018^{e}	4.289 ± 0.054^{d}	4.155 ± 0.071^{e}	4.102 ± 0.108^{e}	4.604 ± 0.028$_{b}$	4.312 ± 0.032^{c}	4.001 ± 0.078^{c}
Astaxanthin	1st	2.102 ± 0.092^{h}	2.331 ± 0.045^{g}	2.247 ± 0.102^{g}	2.189 ± 0.053^{h}	2.580 ± 0.112^{f}	2.503 ± 0.067^{f}	2.223 ± 0.017^{g}
	3rd	2.155 ± 0.025^{h}	2.703 ± 0.201^{e}	2.628 ± 0.077^{f}	2.308 ± 0.048^{g}	2.962 ± 0.085^{e}	2.806 ± 0.114^{e}	2.551 ± 0.054^{f}
	5th	2.243 ± 0.108^{g}	2.961 ± 0.009^{d}	2.784 ± 0.034^{e}	2.440 ± 0.096^{c}	3.748 ± 0.024^{a}	3.631 ± 0.071^{a}	3.008 ± 0.082^{d}
	7th	2.307 ± 0.082^{g}	3.001 ± 0.101^{d}	2.511 ± 0.075^{f}	2.377 ± 0.031^{e}	3.520 ± 0.061^{b}	3.415 ± 0.032^{c}	2.845 ± 0.175^{c}
Cryptoxanthin	1st	5.309 ± 0.077^{j}	6.014 ± 0.009^{g}	6.011 ± 0.084^{g}	6.035 ± 0.033^{g}	6.176 ± 0.037^{f}	6.100 ± 0.012^{f}	6.014 ± 0.066^{g}
	3rd	5.510 ± 0.106^{i}	6.355 ± 0.087^{d}	6.230 ± 0.017^{e}	6.178 ± 0.055^{f}	6.360 ± 0.055^{d}	6.305 ± 0.043^{d}	6.248 ± 0.059^{e}
	5th	5.622 ± 0.110^{h}	6.734 ± 0.050^{b}	6.510 ± 0.062^{c}	6.476 ± 0.103^{c}	6.987 ± 0.114^{a}	6.628 ± 0.085^{b}	6.531 ± 0.076^{c}
	7th	5.538 ± 0.095^{i}	6.621 ± 0.043^{b}	6.508 ± 0.101^{c}	6.311 ± 0.025	6.808 ± 0.056^{a}	6.615 ± 0.072^{b}	6.433 ± 0.091^{c}
Lutein	1st	2.478 ± 0.083^{f}	2.521 ± 0.045^{e}	2.503 ± 0.063^{e}	2.488 ± 0.097^{f}	2.583 ± 0.066^{e}	2.504 ± 0.057^{e}	2.794 ± 0.007^{c}
	3rd	2.498 ± 0.012^{e}	2.609 ± 0.027^{d}	2.587 ± 0.068^{e}	2.502 ± 0.055^{e}	2.841 ± 0.039^{b}	2.763 ± 0.056^{c}	2.572 ± 0.091^{e}
	5th	2.505 ± 0.079^{e}	2.786 ± 0.104^{c}	2.701 ± 0.096^{c}	2.611 ± 0.043^{d}	3.096 ± 0.009^{a}	2.803 ± 0.044^{b}	2.598 ± 0.019^{e}
	7th	2.587 ± 0.102^{e}	2.633 ± 0.066^{d}	2.598 ± 0.072^{e}	2.586 ± 0.088^{e}	2.845 ± 0.039^{b}	2.658 ± 0.059^{d}	2.602 ± 0.046^{d}
Neoxanthin	1st	8.317 ± 0.012^{j}	9.356 ± 0.101^{e}	9.209 ± 0.009^{f}	9.123 ± 0.055	9.497 ± 0.048^{d}	9.433 ± 0.061^{d}	9.119 ± 0.057^{g}
	3rd	8.445 ± 0.010^{i}	9.467 ± 0.099^{d}	9.289 ± 0.108^{f}	9.261 ± 0.015^{f}	9.831 ± 0.053^{b}	9.759 ± 0.073^{b}	9.406 ± 0.092^{d}
	5th	8.232 ± 0.075^{k}	9.736 ± 0.066^{b}	9.457 ± 0.081^{d}	9.345 ± 0.022^{e}	10.112 ± 0.113^{a}	9.855 ± 0.042^{b}	9.658 ± 0.104^{c}
	7th	8.558 ± 0.038^{h}	9.722 ± 0.045^{b}	9.336 ± 0.088^{e}	9.406 ± 0.107^{d}	10.074 ± 0.098^{a}	9.703 ± 0.016^{b}	9.798 ± 0.053^{b}
Violaxanthin	1st	3.049 ± 0.046^{k}	4.226 ± 0.058^{e}	4.101 ± 0.027^{f}	4.072 ± 0.033^{g}	4.236 ± 0.090^{e}	4.208 ± 0.051^{e}	4.153 ± 0.091^{f}
	3rd	3.161 ± 0.005^{j}	4.437 ± 0.027^{d}	4.215 ± 0.046^{e}	4.183 ± 0.024^{f}	4.437 ± 0.105^{d}	4.395 ± 0.068^{d}	4.306 ± 0.027^{e}
	5th	3.235 ± 0.067^{i}	4.726 ± 0.015^{b}	4.411 ± 0.102^{d}	4.309 ± 0.055^{e}	4.983 ± 0.074^{a}	4.701 ± 0.100^{b}	4.572 ± 0.018^{c}
	7th	3.440 ± 0.018^{h}	4.630 ± 0.007^{b}	4.327 ± 0.063^{e}	4.178 ± 0.078^{f}	4.690 ± 0.089^{b}	4.570 ± 0.028^{c}	4.418 ± 0.056^{d}
Zeaxanthin	1st	11.076 ± 0.042^{l}	12.428 ± 0.066^{f}	12.226 ± 0.039^{h}	12.118 ± 0.077^{h}	12.245 ± 0.120^{h}	12.633 ± 0.107^{e}	12.517 ± 0.048^{e}
	3rd	11.273 ± 0.025^{k}	12.743 ± 0.017^{d}	12.365 ± 0.089^{g}	12.301 ± 0.055^{g}	13.247 ± 0.106^{d}	13.185 ± 0.205^{d}	13.051 ± 0.131^{d}
	5th	11.308 ± 0.073^{j}	12.909 ± 0.105^{d}	12.321 ± 0.067^{g}	12.227 ± 0.081^{g}	13.903 ± 0.202^{a}	13.766 ± 0.099^{b}	13.652 ± 0.087^{c}
	7th	11.315 ± 0.091^{i}	12.618 ± 0.088^{e}	12.402 ± 0.095^{f}	12.343 ± 0.103^{g}	12.914 ± 0.119^{d}	12.658 ± 0.154^{e}	12.446 ± 0.118^{f}

Results show the mean values of four independent pigment determinations with the respective standard deviations. Treatment with at least one letter the same are not significantly different according to Tukey's Post hoc test

monosaccharide contents in *A. obliquus* cells treated with phytohormones distinctly decreased suggesting their utilization for biosynthesis of other organic compound such as fatty acids.

Cytokinins in comparison with auxins were more effective in the stimulation of photosynthetic pigment (chlorophylls, carotenes, xanthophylls) accumulation in *A. obliquus* cells. It is likely that phytohormones may reduce the chlorophyll destruction and/or increase chlorophyll synthesis. Previous results demonstrated that chlorophyll biosynthesis in green alga is accelerated by cytokinins (Piotrowska and Czerpak 2009) and auxins (Hunt et al. 2011; Piotrowska-Niczyporuk and Bajguz 2014). Cytokinins have been also reported to have an inhibitory effect on chlorophyll breakdown by chlorophyllase, Mg-dechelatase, and peroxidase-linked chlorophyll bleaching in vascular plants (Costa et al. 2005). Probably, similar mechanisms occur in *A. obliquus* due to the significant increases in the chlorophyll *a* and *b* levels in the presence of auxins and cytokinins. Furthermore, treatment of algal cells with phytohormones causes the accumulation of carotenoids which are a class of important pigments absorbing visible light and protecting the chloroplasts against ROS. Results are confirmed by previous experiments showing that auxins and cytokinins stimulated α- and β-carotene as well as xanthophylls in green alga *C. pyrenoidosa* (Czerpak and Bajguz 1997). The higher content of xanthophylls in *A. obliquus* cells suggests also the activation of the xanthophyll cycle in response to auxins and cytokinins. This is supported by a study from Synková et al. (2004, 2006) showing that transgenic tobacco with overproduction of cytokinins was characterized by increased activities of APX, SOD, CAT, and glutathione-*S*-transferase, which play an important role in the detoxification of ROS responsible for photosynthetic apparatus degradation. Higher content of carotenoids is positively correlated with the increase in the level of non-enzymatic antioxidants and

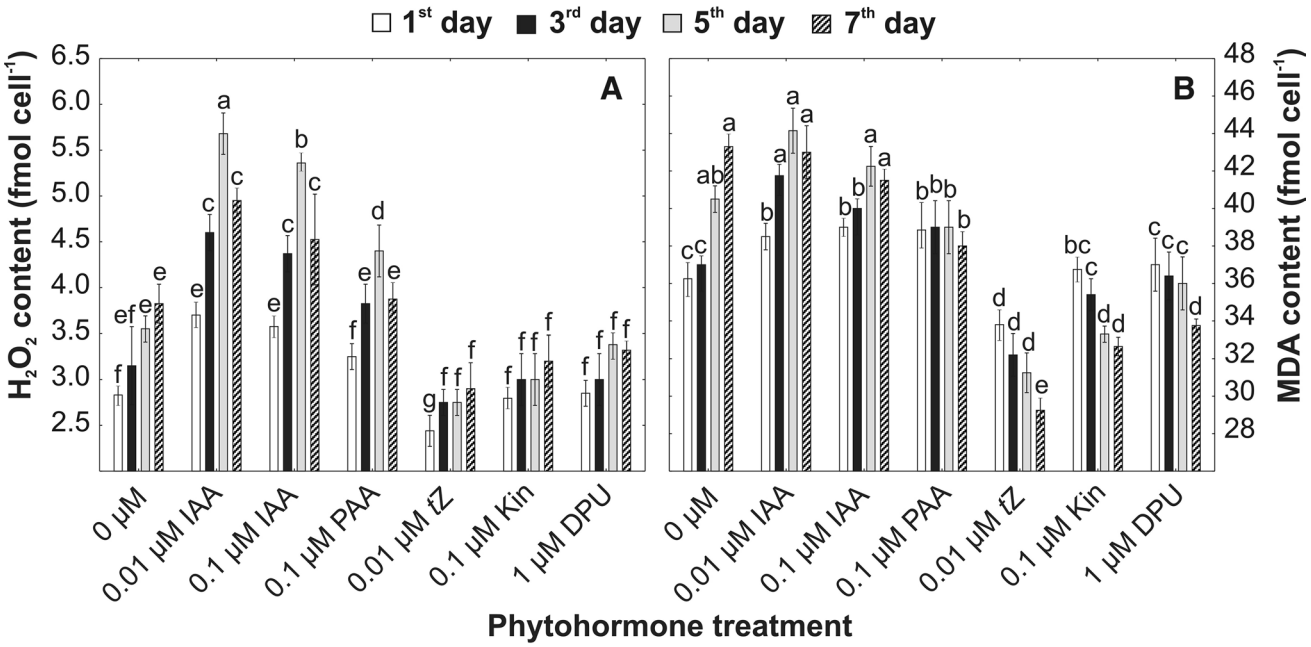

Fig. 3 The effect of auxins (IAA, IBA, PAA) and cytokinins (*t*Z, Kin, DPU) at optimal concentrations on the hydrogen peroxide (**a**) and malondialdehyde (MDA) (**b**) content in *Acutodesmus obliquus* in relation to control (0 µM) on the 1st, 3rd, 5th, and 7th day of cultivation. Data are the means of four independent experiments ± SD. Treatments with at least one letter the same are not significantly different according to Tukey's Post hoc test

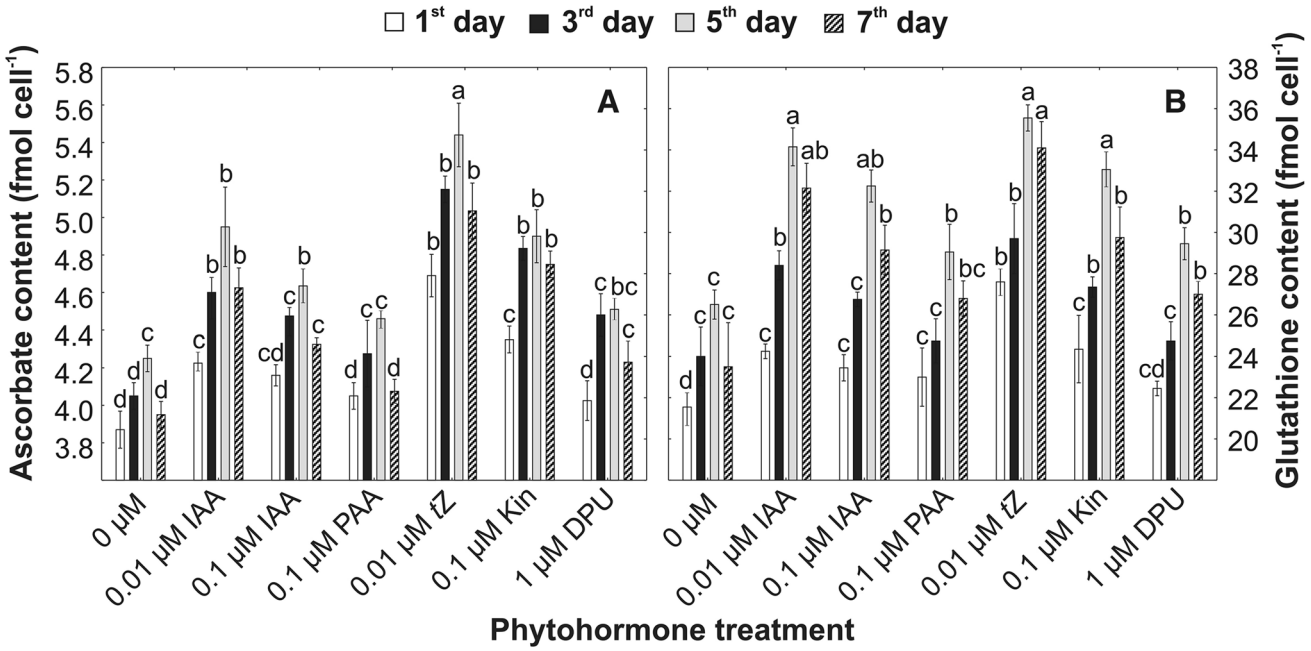

Fig. 4 The effect of auxins (IAA, IBA, PAA) and cytokinins (*t*Z, Kin, DPU) at optimal concentrations on the ascorbate (**a**) and glutathione (**b**) content in *Acutodesmus obliquus* in relation to control (0 µM) on the 1st, 3rd, 5th, and 7th day of cultivation. Data are the means of four independent experiments ± SD. Treatments with at least one letter the same are not significantly different according to Tukey's Post hoc test

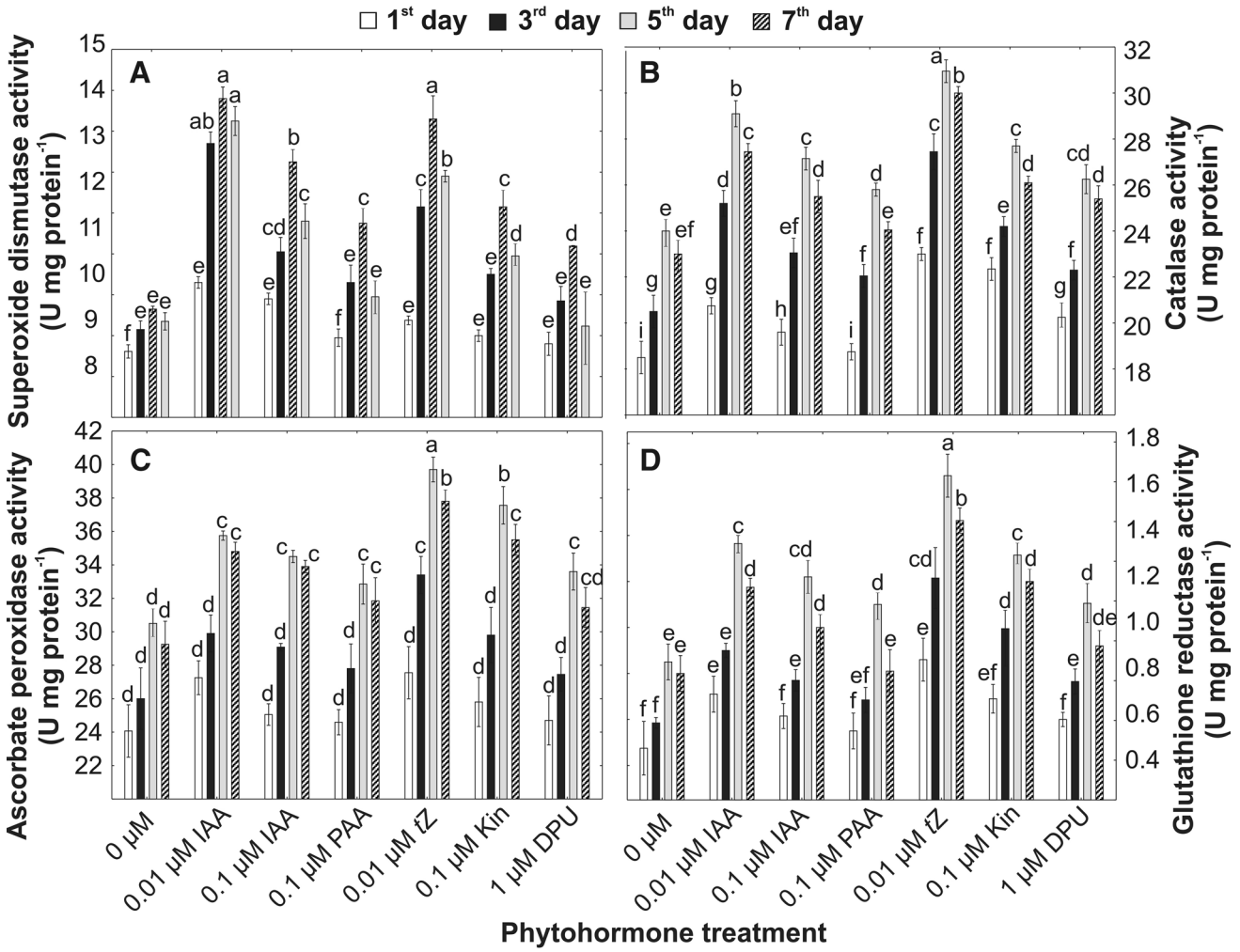

Fig. 5 The effect of auxins (IAA, IBA, PAA) and cytokinins (*t*Z, Kin, DPU) at optimal concentrations on the activity of antioxidant enzymes: superoxide dismutase (**a**), catalase (**b**), ascorbate peroxidase (**c**), and glutathione reductase (**d**) in *Acutodesmus obliquus* in relation to control (0 µM) on the 1st, 3rd, 5th, and 7th day of cultivation. Data are the means of four independent experiments ± SD. Treatments with at least one letter the same are not significantly different according to Tukey's Post hoc test

activity of antioxidant enzymes in *A. obliquus* cells treated with auxins and cytokinins.

ROS, such as H_2O_2, are produced in response to many endogenous and/or environmental stimuli in many cell types. The rapid increase in H_2O_2 content in *A. obliquus* cultures was recorded under the influence of exogenous auxins (IAA, IBA, and PAA), which corresponds well with higher activity of SOD, a main enzyme involved in H_2O_2 production. Although high levels of ROS result in an oxidative burst and hypersensitive cell death, low levels of ROS have been reported to act as signaling molecules promoting many cellular processes including cell proliferation and differentiation. For example, auxins promoted cell growth and physiological responses through ROS production (Kawano 2003). A compilation of evidence indicates that $HO^{\cdot}$, probably derived from $O_2^{\cdot-}$ and H_2O_2 in a Haber–Weiss-type reaction in the cell wall, may be an essential component of the biochemical mechanism engaged in cell-wall loosening during IAA-induced cell growth (Schopfer 2001). Based on the similarities between responses of vascular plants and *A. obliquus* cells, it is hypothesized that, the production of H_2O_2 as a result of higher SOD activity may mediate the auxin-induced enlargement of algal cells. In contrast to auxins, cytokinins decreased ROS levels and reduced the size of *A. obliquus* cells. Moreover, cytokinins more effectively in comparison with auxins inhibited lipid peroxide generation which was obvious from lower MDA contents. Probably, cytokinins diminished lipid peroxidation through the stimulation of non-enzymatic (ascorbate, glutathione) and enzymatic (SOD, CAT, APX, GR) antioxidants. The protection against oxidative stress by cytokinins seemed

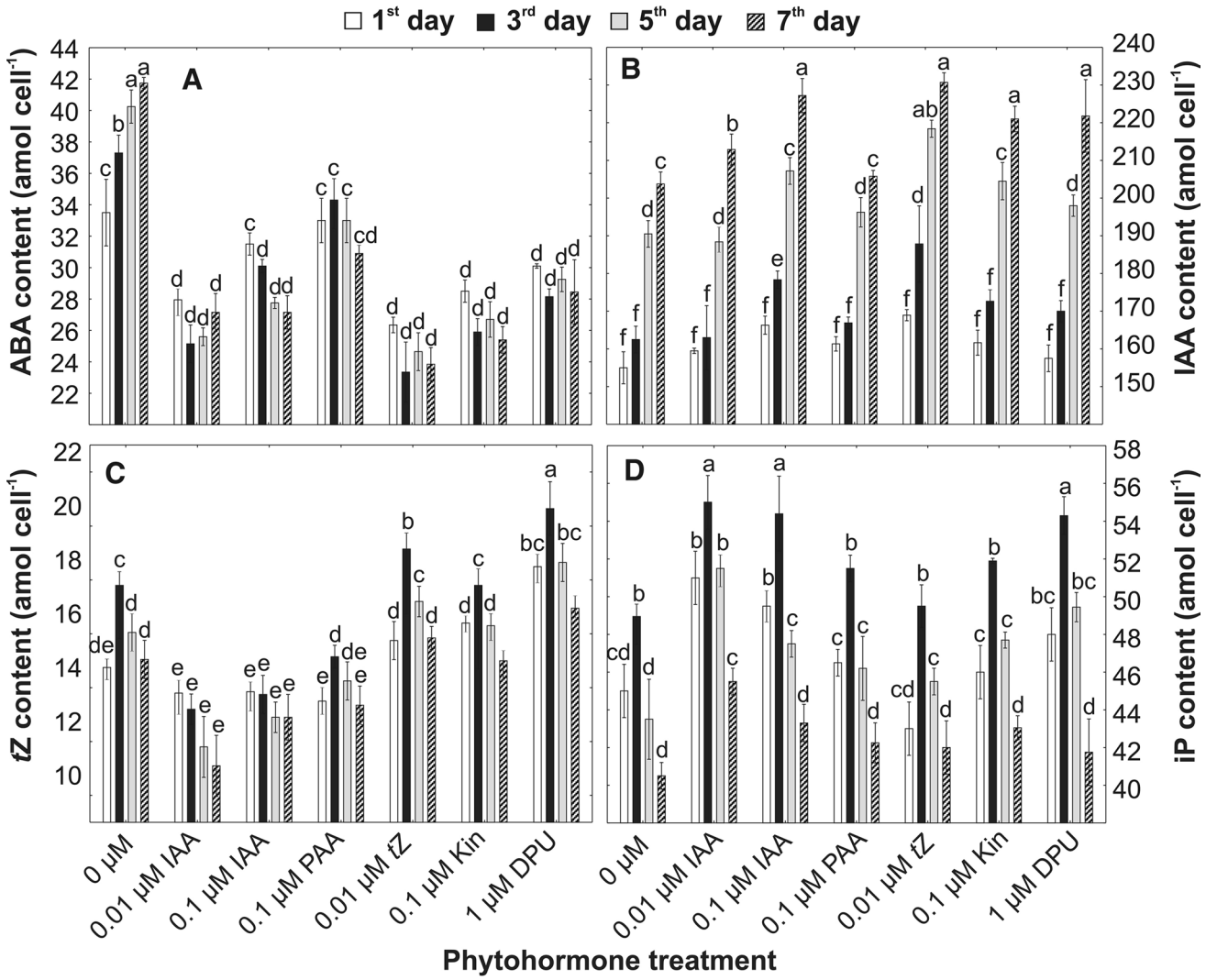

Fig. 6 The effect of auxins (IAA, IBA, PAA) and cytokinins (*t*Z, Kin, DPU) at optimal concentrations on endogenous levels of abscisic acid, ABA (**a**), indole-3-acetic acid, IAA (**b**), *trans*-zeatin, *t*Z (**c**), and N^6-(Δ^2-isopentenyl)adenine, iP (**d**) in *Acutodesmus obliquus* in relation to control (0 µM) on the 1st, 3rd, 5th, and 7th day of cultivation. Data are the means of four independent experiments ± SD. Treatments with at least one letter the same are not significantly different according to Tukey's Post hoc test

to be a general phenomenon. For example, Kin and zeatin riboside were found to scavenge ROS which may help to maintain plant viability (Barciszewski et al. 2000). In comparison with cytokinins, the activities of antioxidant enzymes were less effectively stimulated in the presence of auxins. Previous results obtained on *C. vulgaris* cultures confirmed that the activity of antioxidant enzymes regulating ROS levels was enhanced by auxins. This effect was associated with the stimulation of algal growth and viability (Piotrowska-Niczyporuk and Bajguz 2014). Moreover, results obtained by Szechyńska-Hebda et al. (2007) suggested that exogenous natural (IAA) and synthetic (2,4-D) auxins can stimulate the activities of CAT, SOD, and peroxidases leading to regeneration process of *Triticum aestivum* L. tissues. The precise control of H_2O_2 amounts in *A. obliquus* cells treated with phytohormones may be involved in regulation of cell growth and metabolism.

The increase in the content of non-enzymatic antioxidants (ascorbate, glutathione) was also observed in *A. obliquus* exposed to auxins and cytokinins. These results are supported by the data obtained by Tyburski et al. (2008) indicating that the exposure of the roots of tomato seedling to exogenous auxin inhibited the activity of ascorbate oxidase involved in ascorbate catabolism resulting in increase in the ascorbate level and growth responses. Moreover, the exogenously applied ascorbate to cultures of green alga *C. protothecoides* stimulated algal growth, biomass productivity, and fatty acid content (Parsaeimehr et al. 2017). Therefore, increased ascorbate content induced by auxins and cytokinins may also have a positive effect on *A. obliquus* growth

and metabolite accumulation. An increase in the content of glutathione in *A. obliquus* cultures induced by auxins and cytokinins was reported. Similarly, a higher glutathione level was detected in tobacco mesophyll protoplasts cultured in the presence of synthetic auxin 2,4-dichlorophenoxyacetic acid (Takahashi and Nagata 1992) and in roots of tomato seedlings after IAA treatment inducing positive growth responses (Tyburski and Tretyn 2010). Moreover, transgenic tobacco plants with a reduced pool of cytokinins demonstrated a decline in ascorbate and reduced glutathione level, lower photosynthetic activity, and photosynthetic pigment levels as well as a triggered senescence program (Synková et al. 2004, 2006). Thus, higher ascorbate and glutathione content in cells of *A. obliquus* treated with auxins and cytokinins seems to be positively correlated with metabolic activity, photosynthetic pigment accumulation, and algal growth.

Although treatment with exogenous phytohormones was shown to stimulate the growth unicellular microalgae, limited data on the effects of their endogenous levels are available. A significant decrease in ABA content was observed after application of cytokinins (*t*Z, Kin, and DPU), whereas auxins less effectively inhibited ABA accumulation. Thus, the positive effect of auxins and cytokinins on cell-cycle proliferation expressed as cell number of *A. obliquus* may be connected with depletion in ABA level widely considered as a cell-cycle inhibitor. An antagonistic synergy between ABA and cytokinin biosynthesis has been also confirmed in microalga *Nannochloropsis oceanic* indicating that cytokinins stimulated algal growth, while ABA acted as a repressor of microalgal growth (Lu et al. 2014).

The interactions between auxins and cytokinins in *A. obliquus* cells are more complicated. Compared with the control, the contents of IAA showed a moderate increase in algal cells exposed to auxin IBA and cytokinins during the whole 7-day period of cultivation. Therefore, based on available data, it could be inferred that the elevated levels of IAA in response to cytokinin treatment might be one of the contributors to higher activity of these phytohormones in comparison with auxins. The discovery that both hormones play a crucial role in the metabolic control in each other's biosynthesis further illustrates the complexity and depth of their interrelationships in *A. obliquus* cells. Moreover, an elevation of cytokinin levels in transgenic *A. thaliana* causes a rapid increase in auxin biosynthesis rates in young, developing tissues and alter auxin responses (Nordström et al. 2004). Conversely, a reduction in cytokinin levels leads to a lower content of auxins, suggesting that cytokinins are essential regulators of auxin biosynthesis (Jones et al. 2010). Although the basis for these changes is not fully understood, cytokinin-induced inhibition of enzymes that conjugate free IAA into inactive forms has been suggested as a putative mechanism (Nordström et al. 2004). Strong evidence for the role of cytokinins in maintaining appropriate levels of auxins in microalga *A. obliquus* is provided by the obtained data.

Auxins are rapid and potent regulators of biosynthesis of cytokinins in algal cells repressing the synthesis of *t*Z and stimulating iP level. Among auxins, IAA had the highest effect on endogenous cytokinin contents in *A. obliquus* which is mainly due to growth induction and metabolite accumulation. Probably, auxins may diminish the level of active cytokinins through reduction of their synthesis as well as by induction of their oxidative breakdown or conjugation. The treatment of *A. obliquus* cells with auxins induced the increase in the level of iP, which is the most common cytokinin in green microalgae regulating cell proliferation (Stirk et al. 2011; Žižková et al. 2017). Among cytokinins, DPU was also able to stimulate *t*Z and iP levels in *A. obliquus*. Urea-type cytokinins possess an inhibitory effect on cytokinin oxidase activity, which catalyze the irreversible degradation of adenine cytokinins in plants (Piotrowska and Czerpak 2009). Therefore, DPU may precisely regulate the level of endogenous cytokinins and induce indirectly the growth and biochemical responses of *A. obliquus*. In addition, conversion of nucleotides to nucleosides and promotion of adenine cytokinin synthesis is also stimulated by urea-type cytokinins (Ricci et al. 2001). The endogenous *t*Z level did not change after *t*Z feeding of algal cells indicating that it was quickly metabolized to inactive metabolites as it was previously demonstrated (Žižková et al. 2017). This evidence collectively confirms the existence of a complex web of signal interactions between auxins and cytokinins in the control of growth and metabolism of green microalga *A. obliquus*.

Conclusions

Cytokinins, in comparison with auxins, were characterized by higher activity in green alga *A. obliquus* cultures. The increases in cell number and the contents of fatty acids, photosynthetic pigments, and non-enzymatic antioxidants as well as the activity of antioxidant enzymes with a decrease in monosaccharides, amino acids, and organic acids were observed in algal cells in response to phytohormones. Cytokinins participated in the removal of ROS from the cell leading to inhibition of lipid peroxidation and delaying senescence of algal cultures. In contrast, hydrogen peroxide generation in algal cells may be involved in auxin-induced cell enlargement. Changes in the level of endogenous ABA, IAA, and *t*Z as well as iP were demonstrated after application of exogenous phytohormones. Different dynamics of responses to auxins and cytokinins clearly indicated diverse roles of both groups of phytohormones in the regulation of algal growth. Moreover, the observed effects on the growth and metabolite levels in *A. obliquus* is of great practical

importance and may lead to gains in efficiency and precision in the use of external auxins and cytokinins.

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Compliance with Ethical Standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial relationships, and that there was no potential conflict of interest.

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